MAJOR ARTICLE

Secretion of the Toxin ExoU Is a Marker for Highly Virulent *Pseudomonas aeruginosa* Isolates Obtained from Patients with Hospital-Acquired Pneumonia

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Overall, hospital-acquired pneumonia (HAP) caused by *Pseudomonas aeruginosa* is associated with high attributable mortality. Although the intrinsic virulence of *P. aeruginosa* undoubtedly contributes to this phenomenon, it is unclear whether all strains share this property or whether only a subpopulation of strains are capable of causing such severe disease. In this study, the virulence of 35 *P. aeruginosa* isolates obtained from patients with HAP by use of a cytolytic cell-death assay, an apoptosis assay, and a mouse model of pneumonia. The virulence of individual isolates differed significantly from one to another in each of these assays. Increased virulence was associated with the secretion of ExoU, a toxin transported by the *P. aeruginosa* type III secretion system. Secretion of ExoS or ExoY, 2 other proteins transported by this system, was not consistently associated with increased virulence. Together, these findings suggest that secretion of ExoU is a marker for highly virulent strains of *P. aeruginosa*.

Pseudomonas aeruginosa has long been recognized as a frequent and serious cause of hospital-acquired pneumonia (HAP) [1–3]. The percentage of HAP cases due to this pathogen differs among hospitals [4], but several large studies indicate that *P. aeruginosa* is probably the second-most-common cause of this illness, after *Staphylococcus aureus,* and is responsible for 15%–20% of cases [5–9]. In addition to being common, pneumonia caused by *P. aeruginosa* is also especially severe. In one study, *P. aeruginosa* and other nonfermenting gram-negative bacteria accounted for only 25% of the pathogens obtained with a protected specimen brush but were responsible for 80% of deaths due to pneumonia [10]. *P. aeruginosa* is one of the few pathogens that has been associated with increased attributable mortality in patients with HAP [5, 9–13]; attributable mortality of 40% [14] and crude mortality as high as 70% [15] have been reported. Although the reasons for the high mortality associated with HAP due to *P. aeruginosa* are multifactorial, the intrinsic virulence of *P. aeruginosa* likely plays a major role.

An important and recently recognized virulence determinant of *P. aeruginosa* is the type III secretion system [16]. This system uses complex secretion and translocation machinery to inject a set of factors, called effector proteins, directly into the cytoplasm of eukaryotic cells. At present, 4 type III effector proteins have been identified in *P. aeruginosa:* ExoS, ExoT, ExoU (also called PepA), and ExoY. Each of these proteins has toxic effects on mammalian cells. ExoS is a bifunctional protein; its N-terminus possesses Rho GTPase-activating protein activity, and its C-terminus possesses ADP-ribosyltransferase activity [17]. After injection into mammalian cells, ExoS causes actin reorganization, cell rounding, inhibition of DNA synthesis, loss of cell re-

Received 13 January 2003; accepted 22 June 2003; electronically published 21 November 2003.

Presented in part: general meeting of the American Society for Microbiology, 19–23 May 2002, Salt Lake City (abstract B-123).

Financial support: Schweppe Foundation (to A.R.H.); Northwestern Memorial Hospital (to A.R.H.); National Institutes of Health (grants AI46543 and AI01524 to A.R.H.); Distincio a la Recerca Universitaria (to J.R.); Comisio Interdepartamental de Ciencia i Tecnologia (grant SGR 2001/441 to J.R.).

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The Journal of Infectious Diseases 2003; 188:1695–706

0022–1899/2003/18811-0012$15.00

adherence, and cytotoxicity [18–25]. (In this discussion, the term "cytotoxicity" will be used to refer to cytolytic activity.) In addition, ExoS plays a role in modulating bacterial phagocytosis by professional phagocytes and in invasion into nonphagocytic cells [18, 26, 27] and induces apoptosis of epithelial cells, fibroblasts, and lymphocytes [28, 29]. The effector protein ExoT also possesses both Rho GTPase-activating protein activity [30, 31] and ADP-ribosyltransferase activity [32]. It inhibits bacterial internalization by epithelial cells and macrophages [26, 33] and has been implicated in slowing wound healing [34]. ExoU mediates rapid lysis of a variety of mammalian cell types in vitro, including macrophages, epithelial cells, CHO cells, and fibroblasts [22, 27, 35–39]. ExoY is an adenylate cyclase and causes rounding of certain cell types [22, 40]. Given the many toxic effects that type III–secreted effector proteins have on mammalian cells, it is not surprising that this system contributes to the ability of *P. aeruginosa* strains to cause severe pneumonia [41, 42].

Interestingly, the type III secretion systems of clinical isolates of *P. aeruginosa* are heterogeneous on several levels. For example, 10%–25% of clinical isolates obtained from patients with HAP do not secrete type III effector proteins under inducing conditions in vitro, despite harboring the genes that encode this system [43, 44]. Among those strains that do secrete, differences exist in the type III effector proteins that are transported. Many of these differences can be traced to strain genotypes. Although nearly all clinical isolates contain the genes encoding the type III secretion machinery, the translocation apparatus, and the *exoT* gene, only 89% of clinical isolates harbor the *exoY* gene, 72% harbor the *exoS* gene, and 28% harbor the *exoU* gene [45]. Furthermore, nearly every strain harbors either the *exoS* gene or the *exoU* gene, but not both [45]. Reasons for this dichotomy are unclear, but it has made direct comparison of the contribution to virulence of ExoS and that of ExoU, in an isogenic-strain background, difficult. The heterogeneity of the type III secretion systems of *P. aeruginosa* clinical isolates suggests that they have the potential to induce different types of host tissue injury and are associated with different levels of intrinsic virulence. Comparison of the virulence of clinical isolates would suggest which of the type III effector proteins are most toxic and contribute to the development of especially severe infections.

In the present study, we examined whether *P. aeruginosa* clinical isolates obtained from patients with HAP differed in their virulence and whether these differences were associated with secretion of particular type III effector proteins. Our hypothesis was that HAP-associated isolates that secreted ExoU, an especially toxic type III effector protein, would be more virulent than other isolates.

MATERIALS AND METHODS

Bacterial strains, cell lines, and growth conditions. All *P. aeruginosa* clinical isolates were grown from frozen cultures originally obtained from patients with HAP at Sabadell Hospital, Barcelona, Spain, between May 1993 and October 1997. None of the patients had cystic fibrosis or neutropenia. Partial characterization of these isolates has been reported elsewhere [11, 43, 46]. *P. aeruginosa* strain PA103 [47]—which produces ExoU and ExoT but not ExoS or ExoY [36, 37, 40]—and strain 388 [48]—which produces ExoS, ExoT, and ExoY but not ExoU [40, 45, 49]—were used as controls. After thawing from frozen cultures, bacterial isolates were grown on Vogel-Bonner minimal (VBM) medium agar plates [50] without repassaging. For use in individual assays, bacteria were grown for 17 h at 37°C in Luria-Bertani (LB) broth without shaking, unless otherwise noted. Immediately before infection, the bacteria were diluted with the appropriate tissue culture medium, and their concentration was determined by measuring optical density at 600 nm. The appropriate number of bacteria were then used for each assay.

Escherichia coli strains XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*Z* Δ*M15*Tn*10* (Tet^{r})]; Stratagene) and TOP10 (F^{-} *mcr*A Δ[*mrr*−*hsd* RMS−*mcr*BC] Φ80*lacZ* ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ[*ara*−*leu*]7697 *gal*U *gal*K *rps*L[Str^{R}] *end*A1 *nup*G; Invitrogen) were used for cloning and propagation of plasmids. *E. coli* strain S17.1 (*thi pro hsdR recA RP4-2* [Tet::Mu] [Km::Tn*7*]) [51] was used for transfer of constructs by conjugation into *P. aeruginosa. E. coli* strain BL21(DE3)pLysS (F^{-} *ompT hsdS*$_{B}$ *[r*$_{B}$$^{-}$*m*$_{B}$$^{-}$*] gal dcm met* [DE3] pLysS [Cm^{R}]; Novagen) was used for protein-overexpression experiments. The following concentrations of antibiotics were used during growth of bacteria: carbenicillin, 500 μg/mL; ampicillin, 50 μg/mL; and gentamicin, 100 μg/mL.

All mammalian cells were grown at 37°C in 5% CO_2 in a humidified atmosphere. A549 cells were cultured and infected in Waymouth's MB 752/1 Medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL). J774 cells were cultured and infected in Dulbecco's modified Eagle medium with 4.5 g/L glucose (Gibco-BRL) supplemented with 10% heat-inactivated FCS. Madin-Darby canine kidney (MDCK) epithelial cells were cultured and infected in minimal essential medium with Earle's salts (Gibco-BRL) supplemented with 5% heat-inactivated FCS.

Construction of an* exsA *mutation in strain PA103. In strain PA103, by use of the method of Schweizer and Hoang [52], the chromosomal wild-type allele of the *exsA* gene was replaced with an allele containing an in-frame deletion, to create a nonsecreting control strain. (ExsA is the transcriptional activator essential for expression of the type III secretion regulon [53–55].) To create pSP002, a 3-kb *Sma*I-*Eco*RI fragment con-

taining the *exsA* gene from pAH831 [56] was ligated into the multiple cloning site of pUC19 previously digested with *Sma*I and *Eco*RI. To create pSP003, the *exsA* gene was transferred to the suicide vector pEX100T [52] by blunting and ligating a 1.3-kb *Bam*HI-*Bgl*I fragment from pSP002 into the *Sma*I site of pEX100T. An in-frame deletion in the *exsA* gene was constructed by digesting pSP003 with *Nco*I and *Xho*I to remove 613 bp encoding amino acids 56–261, blunting, and self-ligating to form pSP004. Sequencing confirmed the successful generation of an in-frame deletion allele of the *exsA* gene. The deleted *exsA* allele was then used to replace the wild-type *exsA* allele in PA103. *E. coli* strain S17.1 carrying pSP004 was mated with PA103, on LB agar. Exconjugants having undergone a single recombination event were selected on VBM agar supplemented with carbenicillin. Single colonies were then transferred to VBM agar supplemented with 5% sucrose to select for loss of vector sequences, which contained the *sacB* gene, through a second recombination event. Sucrose-resistant colonies were tested for presence of the in-frame *exsA* deletion allele and loss of the wild-type allele by use of polymerase chain reaction (PCR) amplification with the primers 5′-TCCAAGCTTATGCCTCT-CCGCTCGGCG-3′ (upstream) and 5′-CCCTCTAGATCAGT-TATTTTTAGCC-3′ (downstream). Immunoblot analysis confirmed the absence of type III secretion. This strain was designated PA103Δ*exsA*.

Strain typing. The clonality of *P. aeruginosa* isolates was determined by use of random amplified polymorphic DNA (RAPD) PCR typing [57, 58], as described elsewhere [45].

Southern hybridization analysis. Southern hybridization analyses were performed by use of digoxigenin-labeled probes (Roche Molecular Biochemicals), as described elsewhere [45].

Generation of ExoY antiserum. Recombinant ExoY was produced by use of a modification of the method of Yahr et al. [40]. The *exoY* gene was amplified from strain 388 by use of primers 5′-TGCATGCATGCGTATCGACGGTCATCG-3′ (upstream) and 5′-CCGCTCGAGAGCGGGCTTTGCCAACGACC-3′ (downstream). PCR was performed by use of *Pfu* proofreading polymerase (Stratagene), according to the following protocol: 94°C for 3 min, then 25 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min 30 s, then a final extension at 72°C for 5 min. The product was ligated into pCR-Blunt using the Zero-Blunt cloning approach (Invitrogen). After digestion with *Nsi*I, fragment ends were blunted with T4 DNA polymerase and ligated into *Nde*I-digested pET-16b (Novagen). The resulting plasmid (pGS041) encoded a fusion protein consisting of ExoY with 10 N-terminal histidine residues. Clones were transformed into the expression strain BL21(DES)pLysS (Novagen) and induced with isopropyl-β-D-thiogalactoside. His-tagged ExoY was purified from whole cell extracts by use of Ni-NTA Spin Columns (Qiagen). Injections of rabbits and collection of blood samples were performed by Animal Pharm Services (Healdsburg, CA).

Immunoblot analysis. For analysis of type III–secreted proteins, *P. aeruginosa* strains were grown in MINS medium [59, 60] for ~17 h at 37°C with vigorous shaking. Bacterial supernatants were obtained from 5-mL cultures by centrifugation at 6000 *g* for 20 min at 4°C. Proteins present in supernatants were precipitated by the addition of ammonium sulfate (final concentration, 55%). After incubation on ice for 2 h, the precipitated material was isolated by centrifugation at 13,000 *g* for 20 min at 4°C. The pellet was boiled in 100 μL of 1× SDS-PAGE sample buffer for 5 min, and 70 μL of each sample was electrophoresed through a 10% SDS–polyacrylamide gel [61]. Proteins were then electrotransferred to nitrocellulose (Schleicher and Schuell) and exposed to mixtures of the appropriate antisera [62]. When aberrantly migrating bands precluded definitive identification of secreted proteins on the basis of size, immunoblot analysis was repeated using individual antiserum instead of mixtures. Polyclonal ExoS, ExoT, ExoU, PopB, and PopD antisera were prepared as described elsewhere [37, 43]. Preparation of ExoY antiserum is described in the preceding section. Goat anti–rabbit IgG horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories) diluted 1:5000 was used as a secondary antibody. Proteins were visualized by incubating the membranes in 225 μmol/L coumaric acid (Sigma Chemical), 1.25 mmol/L 3-aminophthalhydrazide (Sigma), and 0.009% hydrogen peroxide (Fisher Scientific) in 100 mmol/L Tris (pH 8.5) for 1 min and then by use of a Chemi-Imager 5500 (Alpha Innotech) or by exposing the membranes to autoradiography film.

Cytotoxicity assays. Cytotoxicity was assessed by use of quantitative lactate dehydrogenase (LDH) release assays (Sigma). A549 cells were seeded into 24-well plastic trays, grown for 3 days, washed with tissue culture medium, and infected with bacteria at an MOI of ~80 in tissue culture media lacking FCS. After 3 h, LDH release into the media was determined as described in the manufacturer's instructions. Cells in several control wells were treated with 0.05% Triton X-100 in tissue culture medium for 5 min, followed by agitation to determine the total amount of LDH released when all cells were lysed. This value was used to calculate the percentage of cells lysed in sample wells as follows: [(LDH activity in sample well)/(LDH activity in Triton X-100–treated wells)] × 100. Assays were repeated using MDCK cells and J774 cells. To promote formation of polarized monolayers, MDCK cells were grown on transwell filters (Corning Costar), as described elsewhere [63]. An MOI of 100 was used with J774 cells, and an MOI of 30 was used with MDCK cells.

Assays to measure apoptosis-like cell death. J774 cells that were 70% confluent were harvested, diluted, seeded onto 96-

well plastic trays (~10^4 cells/well), and grown for 20 h. Cells were then washed with tissue culture medium and infected at 37°C for 6 h at an MOI of ~200. After infection, cells were tested for the presence of histone-associated cytoplasmic DNA fragments by use of the Cell Death Detection ELISA^{PLUS} assay (Roche), as described in the manufacturer's instructions. In brief, wells containing the cells were aspirated, lysis buffer (provided in the assay kit) was added, and cell debris was cleared by centrifugation at 200 *g*. Supernatants were tested for the presence of histone-associated DNA fragments by ELISA (provided in the assay kit).

Mouse model of acute pneumonia. Bacteria were tested in a mouse model of acute pneumonia, as described elsewhere [64]. In brief, bacteria were grown in MINS medium at 37°C in a shaker incubator, collected by centrifugation, and resuspended in PBS. Appropriate bacterial concentrations were obtained by diluting with PBS and measuring the optical density at 600 nm or by plating serial dilutions and counting colony-forming units. Next, 50 μL of the bacterial suspension was instilled into the nares of methoxyfluorane-anesthetized 6–12-week-old BALB/c mice. Each bacterial isolate was inoculated into a minimum of 8 mice. Mice were monitored for survival over the course of the subsequent 7 days. Mice that were deemed severely ill and unlikely to recover were killed and scored as dead. The LD_{50} for each strain was determined according to the method of Reed and Muench [65]. Approval from the Animal Care and Use Committee of Northwestern University was obtained before initiating these experiments.

Statistical analysis. Results are expressed as mean ± SD. Arcsine transformations were performed where indicated, to ensure normality. Cytotoxicity, apoptosis-like cell death, and in vivo virulence were analyzed by analysis of variance (ANOVA) for significant effect of the secretion profile ($\alpha = .05$ was considered to be significant). Tukey-Kramer honest significant difference tests were used to test for significant pairwise differences between all groups; all differences reported here were significant at $\alpha = .05$.

RESULTS

Characteristics of type III secretion of* P. aeruginosa *isolates obtained from patients with HAP. As a first step in examining whether secretion of specific type III effector proteins was associated with different levels of virulence, we characterized the secretion properties of 35 *P. aeruginosa* clinical isolates obtained by use of quantitative protected specimen brushes or bronchoalveolar lavages from intubated patients who met strict criteria for HAP [43]. Because *P. aeruginosa* frequently causes outbreaks in hospitals, it was necessary to first ensure that this collection of isolates represented predominantly independent, nonclonal strains. RAPD PCR typing was performed on all 35 isolates and indicated that 89% were nonclonal (data not shown). Thus this collection of isolates constitutes a set of predominantly independent strains capable of causing HAP.

The type III secretion genotypes of these 35 isolates had been partially defined and described elsewhere [43]. It was shown that each of these isolates harbored the *popB* gene [43], indicating that at least a portion of the large cluster of genes encoding the type III secretion and translocation machinery was present [66]. In addition, it was found that 23 (66%) contained the *exoS* gene and 12 (34%) contained the *exoU* gene [43]. Each isolate contained a single copy of either the *exoS* gene or the *exoU* gene, but not both [43]. In the present study, we completed characterization of the type III secretion system of these isolates by determining the prevalence of the *exoT* and *exoY* genes. Southern hybridization analysis was performed by use of *exoT*- or *exoY*-specific probes and indicated that the *exoT* gene was present in all 35 isolates (100%) and that the *exoY* gene was present in 31 (89%) of the isolates (data not shown). Together, these results indicated that the prevalence of the *exoS, exoT, exoU,* and *exoY* genes in this collection of *P. aeruginosa* isolates is consistent with those of previously reported collections of HAP-associated isolates [45]. Furthermore, these results confirm that the *exoT* gene is present in nearly all isolates from acute infections, whereas the *exoS, exoU,* and *exoY* genes are variable traits.

Previously published reports suggested that not all strains of *P. aeruginosa* harboring type III systems are capable of secreting effector proteins [40, 43, 44]. Thus, type III secretion genotypes do not necessarily predict secretion phenotypes. For this reason, each of the 35 isolates was examined for its ability to secrete type III proteins when grown under inducing conditions in vitro. It was reported elsewhere [43] which of these isolates secreted ExoU, ExoS, ExoT, as well as PopB and/or PopD, proteins involved in translocation of effector proteins into host cells. In the present study, to determine which isolates secreted ExoY, we completed characterization of the type III secretion systems of these isolates by use of immunoblot analysis. Together, the results of these assays indicated that the 35 isolates could be divided into 2 groups. Twenty-seven isolates (77%) were capable of secreting detectable amounts of at least 1 of the examined type III proteins in vitro; for convenience, these isolates will be referred to as "secreting" isolates. The remaining 8 (23%) isolates did not secrete detectable amounts of any examined type III protein in vitro, and, for convenience, these isolates will be referred to as "nonsecreting" isolates. Of the 27 secreting isolates, 10 (37%) secreted detectable amounts of ExoU, 16 (59%) secreted ExoS, 26 (96%) secreted ExoT, 21 (78%) secreted ExoY, and 27 (100%) secreted PopB and/or PopD (figure 1).

As expected, the pattern of effector-protein secretion was not random among secreting isolates. All but 1 of the secreting

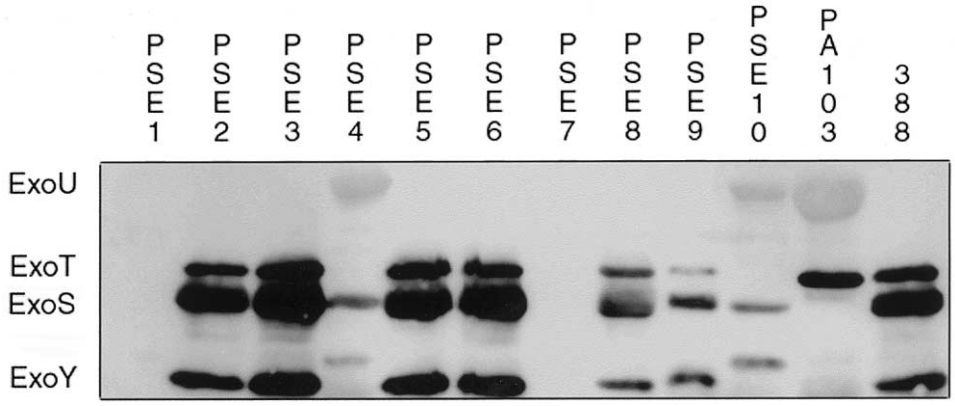

Figure 1. Secretion of type III effector proteins by representative *Pseudomonas aeruginosa* isolates obtained from patients with hospital-acquired pneumonia. Supernatants from cultures of *P. aeruginosa* clinical isolates grown under type III secretion–inducing conditions were collected, concentrated, partially purified, and subjected to immunoblot analysis. Amounts of protein in each lane were adjusted to enhance visualization of discrete bands. A mixture of polyclonal antisera against ExoS, ExoT, ExoU, and ExoY was used to detect type III effector proteins. Some isolates, such as PSE1 and PSE7, did not secrete any of the 4 effector proteins, whereas other isolates secreted 2 or 3 effector proteins. Heterogeneity existed in the sizes of some of the effector proteins. For example, on SDS–protein gels, ExoT secreted by PSE4 and PSE10 migrated faster and ExoY secreted by PSE4 and PSE10 migrated slower than did the corresponding proteins secreted by the other isolates. In these cases, protein identification was achieved by repeating the immunblot analysis with antiserum against a single protein, rather than with the mixture of antisera (data not shown). PA103—a laboratory strain that secretes ExoU and ExoT [36, 37, 40]—and 388—a laboratory strain that secretes ExoS, ExoT, and ExoY [40, 45, 49]—were used as controls.

isolates secreted ExoS or ExoU, but not both. The exception was PSE7, which had a functional type III secretion system, as evidenced by the secretion of PopB and PopD, but which did not secrete ExoS or ExoU, despite harboring the *exoU* gene. PSE7 was also the only secreting isolate that did not secrete ExoT. Secretion of ExoY, on the other hand, was not unique to either ExoS- or ExoU-secreting isolates.

Cytotoxicity of HAP-associated isolates. The ability of some *P. aeruginosa* strains to cause cytolytic cell death of mammalian cells during coculture in vitro has been proposed as a marker for bacterial virulence [41, 42, 63, 67]. For this reason, we examined the cytotoxicity of the 35 HAP-associated isolates. Bacteria were cocultured with A549 cells, a bronchial carcinoma cell line, for 3 h, at which time LDH release was measured as a marker for cell lysis. Triton X-100, a detergent that lysed all cells, and PA103, a well-characterized cytotoxic strain [63], were used as positive controls. Medium and PA103Δ*exsA*, a noncytotoxic mutant that lacks a functional type III secretion system because of disruption of the gene encoding the type III transcriptional activator ExsA [63], were used as negative controls. Clinical isolates differed markedly in their ability to lyse epithelial cells in vitro (figure 2). Some isolates, such as PSE5 and PSE7, were associated with only background levels of cytotoxicity. Others, such as PSE4 and PSE14, were associated with lysis of nearly all cells under the conditions of this assay.

As a group, ExoU-secreting isolates were significantly more cytotoxic than other isolates ($\alpha = .05$; table 1 and figure 2) and lysed an average of 88% ± 18% of infected cells. ExoS-secreting isolates lysed an average of 13% ± 14% of infected cells and were no more cytotoxic than nonsecreting isolates, which lysed an average of 10% ± 5% of infected cells (table 1 and figure 2). Individual isolates were variable in their cytotoxicity, with the least cytotoxic ExoU-secreting isolate (PSE17) lysing about the same percentage of cells as the most cytotoxic ExoS-secreting isolate (PSE13) (figure 2). Among ExoU-secreting isolates, secretion of ExoY was not associated with a statistically significant increase in cytotoxicity (table 1). Because all ExoS-secreting isolates except 1 (PSE27) also secreted ExoY, the effects of ExoY secretion in an ExoS-secreting background could not be evaluated in these assays or the assays described in the following paragraphs. Likewise, because all secreting isolates except 1 (PSE7) secreted ExoT, it was not possible to test whether cytotoxicity or other measures of virulence were associated with secretion of this effector protein. Although these results were obtained using A549 cells, similar trends were observed using macrophage-like J774 cells and polarized monolayers of MDCK epithelial cells, indicating that these results were not unique to a particular cell type (data not shown); thus, clinical isolates of *P. aeruginosa* obtained from patients with HAP were heterogeneous with respect to their ability to lyse cells in vitro. As a group, ExoU-secreting isolates were highly cytotoxic, whereas ExoS-secreting isolates and nonsecreting isolates were relatively noncytotoxic.

Induction of apoptosis-like cell death by HAP-associated isolates. It has been suggested that induction of apoptosis plays an important role in the ability of *P. aeruginosa* strains to cause

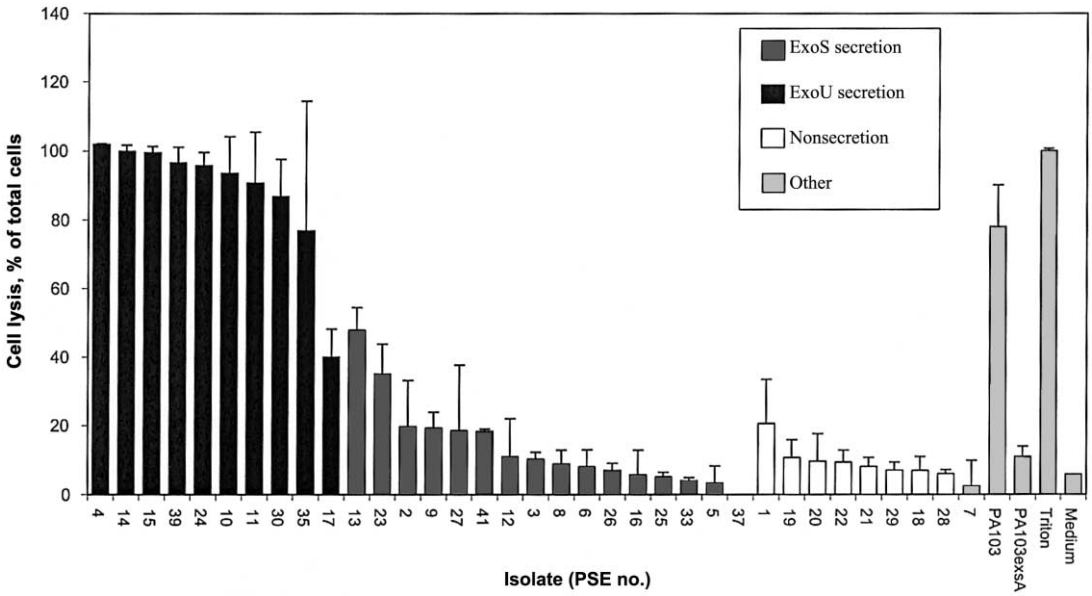

Figure 2. Cytotoxicity of *Pseudomonas aeruginosa* isolates toward A549 bronchial carcinoma cells. A549 cells were infected with each of the 35 clinical isolates for 3 h, after which the amount of LDH released into the medium was measured and used to estimate the percentage of A549 cells lysed. For purposes of clarity, secretion of ExoT and ExoY are not indicated. Triton X-100 detergent (Triton), which lysed all cells, was used as a positive control, as was the highly cytotoxic strain PA103 [63]. Medium and the noncytotoxic mutant PA103Δ*exsA* [63] were used as negative controls. Data are mean ± SD for a minimum of 3 experimental points.

severe disease [68]. Several studies indicate that type III secretion contributes to this type of cell death [28, 29, 38, 69, 70]; thus, *P. aeruginosa* clinical isolates that secrete different effector proteins may also differ in their ability to cause apoptosis-like cell death. To determine the degree of apoptosis-like cell death induced by HAP-associated isolates, we performed ELISAs that detect cytoplasmic DNA fragments bound to histones (Cell Death Detection ELISA^{PLUS}; Roche) on all 35 clinical isolates. This assay had been previously shown to accurately measure *P. aeruginosa*–induced apoptosis-like death [38]. PA103, a strain capable of inducing significant levels of apoptosis-like death [38], was used as a positive control, and PA103Δ*exsA,* which causes minimal levels of cell death [38], was used as a negative control. *P. aeruginosa* clinical isolates differed significantly in their ability to cause apoptosis-like death of J774 cells after 6 h of cocultivation (figure 3). Some isolates, such as PSE11 and PSE15, caused considerably more apoptosis-like death than did PA103, whereas other isolates, such as PSE9 and PSE22, caused only slightly more than background levels.

ExoU-secreting isolates were associated with relatively high levels of apoptosis-like cell death, corresponding to an absorbance at 405 nm (A_{405}) of 2.65 ± 0.54 (table 1 and figure 3). Intermediate levels of apoptosis-like cell death were observed in cells cocultured with ExoS-secreting isolates (A_{405}, 1.32 ± 0.71), and low levels were observed in cells cocultured with nonsecreting isolates (A_{405}, 0.56 ± 0.29). Differences between each of these groups were statistically significant (table 1). However, individual isolates within these groups were variable with regard to the degree of apoptosis-like cell death they caused. For example, some ExoS-secreting isolates (e.g., PSE6 and PSE37) were associated with more apoptosis-like cell death than were some ExoU-secreting isolates (e.g., PSE10 and PSE24) (figure 3). Among ExoU-secreting isolates, ExoY secretion was not associated with significantly more apoptosis-like cell death. Thus, type III–secreting isolates were associated with more apoptosis-like cell killing than were nonsecreting isolates, but killing was not uniquely associated with secretion of any 1 effector protein.

Virulence of HAP-associated isolates in mice. A mouse model of acute pneumonia was used to directly determine the relative virulence of the 35 *P. aeruginosa* isolates obtained from patients with HAP. The calculated LD_{50} was determined for each isolate by measuring optical density at 600 nm, to estimate bacterial concentrations (figure 4). (Repeated calculation of LD_{50} values using colony-forming unit counts obtained from plating serial dilutions of bacterial inoculums instead of optical density measurements yielded similar results [data not shown].) The laboratory strains PA103 and PA103Δ*exsA* were used as controls. PA103 is highly virulent in animal models of acute pneumonia, whereas PA103Δ*exsA* is attenuated in its virulence [36, 37, 41]. Clinical isolates varied markedly in their intrinsic virulence, with LD_{50} values ranging over nearly 2 logs. The most virulent isolate was PSE 9, with an LD_{50} value of 1.3×10^6 cfu. In contrast, the least virulent isolate was PSE 7, with an LD_{50}

Table 1. Comparison of cytotoxicity, apoptosis-like cell death, and LD_{50} values of hospital-acquired pneumonia isolates, as a function of effector protein secretion.

Parameter[a]	Secreted effector proteins					Groups with significant differences[b]
	UT (n = 4)	UTY (n = 6)	UT + UTY (n = 10)	STY (n = 15)	Nonsecreting (n = 8)	
Cytotoxicity, % of cells lysed	78 ± 27	95 ± 6	88 ± 18	13 ± 14	10 ± 5	UT > STY, UTY > STY, UT > Non, UTY > Non[c]
Apoptosis-like cell death, absorbance at 405 nm	2.48 ± 0.43	2.76 ± 0.62	2.65 ± 0.54	1.32 ± 0.71	0.56 ± 0.29	UT > STY, UTY > STY, UT > Non, UTY > Non, STY > Non
Virulence, $LD_{50} \times 10^7$ cfu	1.2 ± 0.8	1.1 ± 0.7	1.1 ± 0.7	4.6 ± 2.4	3.1 ± 1.6	STY > UTY, STY > UT

NOTE. Data are mean ± SD. Non, nonsecreting; S, ExoS; T, ExoT; U, ExoU; Y, ExoY.

[a] PSE7, which secreted PopB and PopD but no effector proteins, and PSE27, which was the only isolate that secreted ExoS and ExoT but not ExoY, were not included in the analysis.

[b] Comparisons between groups were performed by use of analysis of variance (ANOVA), followed by multiple unplanned comparisons using the Tukey-Kramer honest significant difference test, with $\alpha = .05$. All pairs of the following groups were tested: UT, UTY, STY, and Non. Nonsignificant pairings are not listed.

[c] Arcsine transformations were performed before ANOVA.

value of 8.8×10^7 cfu. Of interest, PA103 had an LD_{50} value of 4.8×10^5 cfu, which is lower than that of any of the clinical isolates examined in this study. This result suggests that this commonly used laboratory strain, which was originally isolated from human sputum [47], may not be representative of *P. aeruginosa* clinical isolates.

As a group, isolates that secreted the effector protein ExoU were more virulent. ExoU-secreting isolates had 4-fold lower LD_{50} values (and, therefore, increased virulence) than did ExoS-secreting isolates (mean, 1.1×10^7 vs. 4.6×10^7 cfu), a difference that was statistically significant (table 1). ExoU-secreting isolates also had lower LD_{50} values, compared with those of nonsecreting isolates (mean, 1.1×10^7 vs. 3.1×10^7 cfu), although this trend did not achieve statistical significance. However, within each group, the virulence of individual isolates differed markedly and overlapped with the virulence of individual isolates from other groups. For example, 1 ExoS-secreting isolate (PSE9) was more virulent than the most virulent ExoU-secreting isolate (figure 4). Among ExoU-secreting isolates, the secretion of ExoY did not significantly alter the associated LD_{50} value, suggesting that secretion of ExoY was not associated with dramatic increases in virulence. These findings indicate that secretion of ExoU is a marker for HAP-associated *P. aeruginosa* isolates that are especially lethal in a mouse model of pneumonia.

DISCUSSION

In the present study, we have used a number of assays to show that *P. aeruginosa* isolates obtained from patients with HAP differ markedly in virulence and that these differences are associated with secretion of distinct type III effector proteins. In particular, secretion of ExoU is associated with increased cytolytic activity, induction of apoptosis-like cell death, and in vivo virulence; thus, secretion of ExoU is a marker for HAP-associated isolates that are especially virulent in models of disease. It is intriguing to speculate that strains secreting this toxin may be capable of causing particularly severe disease in human patients as well.

Measurement of cytotoxicity, apoptosis-like cell death, and LD_{50} values in a mouse model of pneumonia all indicated that individual HAP-associated isolates of *P. aeruginosa* differed significantly in their virulence. Under the conditions of our assays, cytotoxicity of clinical isolates ranged from 0% to 100% of exposed cells. Levels of apoptosis-like cell death ranged from 20% to 1600% above background levels. In the mouse model of acute pneumonia, LD_{50} values ranged from 1.3×10^6 to 8.8×10^7 cfu. This degree of variation is particularly remarkable because only relatively virulent isolates were included in this study (i.e., those capable of causing HAP in humans). Such strain-to-strain differences in virulence are not unique to HAP-associated isolates. Fleiszig et al. [27, 71] showed that corneal isolates varied significantly in their cytotoxicity, and Dacheux et al. [72] and Hirakata et al. [73] showed the same for cystic fibrosis isolates. Likewise, Roy-Burman et al. [44] showed that a collection of blood and sputum isolates differed in cytotoxicity and virulence in a mouse model of pneumonia. Together, these findings support the assertion that differences in the presence, expression, and secretion of virulence determinants by individual *P. aeruginosa* strains affects their ability to injure host cells.

Our findings verify an association between secretion of ExoU and high levels of cytotoxicity [36, 37, 44, 73]. These results indicate that ExoU is a predominant cytotoxin of *P. aeruginosa*. Despite reports of ExoS-associated cytotoxic activity [23, 25], ExoS-secreting isolates were no more cytotoxic than nonsecreting isolates, under the conditions of our assays. ExoS-mediated cytotoxicity has been reported to require longer coincubation times than ExoU-mediated cytotoxicity [24, 38], and it may be that the 3-h coincubation times used in our assays were too short to detect ExoS-mediated cytotoxicity.

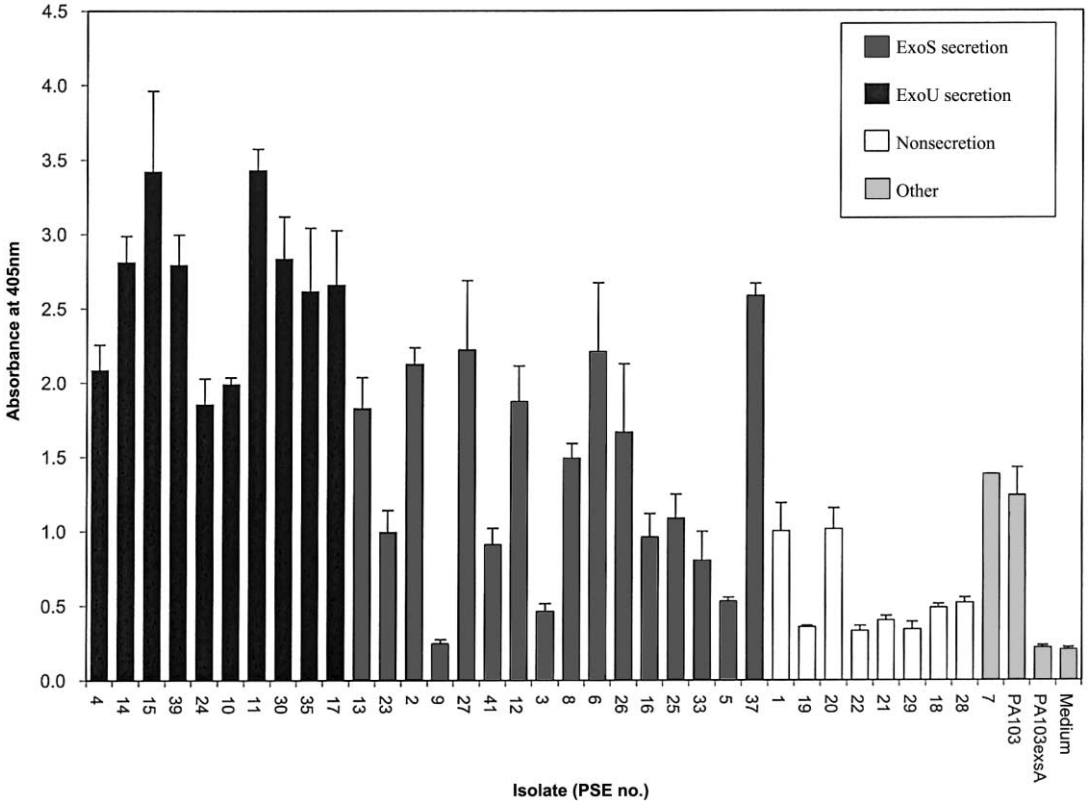

Figure 3. Apoptosis-like death of J774 cells induced by *Pseudomonas aeruginosa* isolates obtained from patients with hospital-acquired pneumonia. J774 cells were infected with each of the 35 clinical isolates for 6 h, after which cells were tested by ELISA for the presence of cytoplasmic histone-associated DNA fragments. Increased absorbance at 405 nm indicated increased levels of apoptosis-like cell death. For purposes of clarity, secretion of ExoT and ExoY are not indicated. PA103, a laboratory strain previously shown to induce high levels of apoptosis-like death in J774 cells [38], was used as a positive control. Medium and the nonapoptotic mutant PA103Δ*exsA*[38] were used as negative controls. Data are mean ± SD for a minimum of 3 experimental points.

Nonsecreting isolates were associated with minimal cytotoxicity. These observations indicate that secretion of ExoU by clinical isolates is associated with a dramatic increase in lysis of mammalian cells after relatively brief periods of coincubation.

Clinical isolates differed by how much apoptosis-like cell killing they induced in J774 cells; in general, type III–secreting isolates were associated with more apoptosis-like cell killing than were nonsecreting isolates. These results are of interest, because the role of type III secretion in apoptosis-like cell death remains controversial. A number of reports have indicated that apoptosis-like cell death requires an intact type III secretion system [29, 38, 69, 70], but it remains unclear which effector protein or proteins are responsible for this phenotype. Although Kaufman et al. [29] reported that ExoS caused apoptosis-like cell death, Hauser et al. [38] showed that strain PA103, which secreted ExoU but not ExoS, also induced apoptosis-like cell death in a type III secretion–dependent manner. Furthermore, Hauser et al. [38] also demonstrated that this killing did not require ExoU. Our present results are consistent with both of these findings. Type III–secreting isolates caused significantly more apoptosis-like cell killing than did nonsecreting isolates, but, within the group of secreting isolates, both ExoS- and ExoU-secreting isolates induced substantial apoptosis-like cell killing. A number of interpretations of these results are possible. Multiple effector proteins may be capable of inducing apoptosis-like cell death. In contrast, these findings are also consistent with a role for a novel effector protein or noneffector proteins, such as PopB or PopD, in apoptosis-like cell death. In this regard, it is interesting that PSE7, which secreted PopB and PopD but none of the known effector proteins, induced significant levels of apoptosis-like cell death (figure 3).

Because the interaction and overlap between apoptotic and cytolytic killing caused by *P. aeruginosa* are poorly understood, the results of these assays must be interpreted with caution. One possible confounding factor is that the cytotoxicity and apoptosis assays may be incapable of distinguishing these 2 types of cell death. This is unlikely, because the ELISA apoptosis assay and the LDH release assay were capable of accurately differentiating cytolytic cell death caused by streptolysin-O (a pore-forming toxin) from apoptosis caused by gliotoxin (a well-

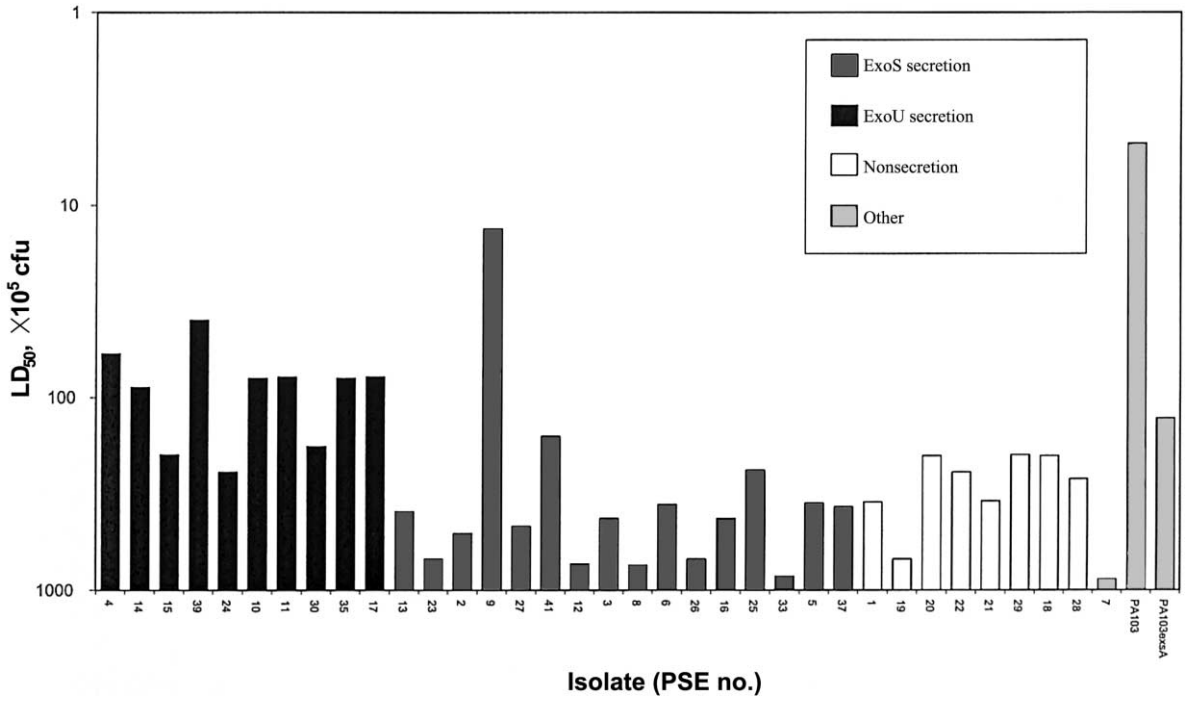

Figure 4. Virulence of *Pseudomonas aeruginosa* isolates in a mouse model of acute pneumonia. Mice were induced to aspirate a range of bacterial inoculums and monitored for mortality over the course of the subsequent 7 days. LD_{50} values were calculated using the method of Reed and Muench [65]. For ease of comparison with figures 2 and 3, the *Y*-axis has been inverted so that more-virulent isolates are represented by taller bars. The virulent laboratory strain PA103 was used as a positive control, and the less-virulent mutant PA103Δ*exsA* was used as a negative control [36, 37, 41].

characterized proapoptotic toxin) [38]. Another possibility is that 1 killing process affects the ability to measure the second process. For example, cells that are rapidly killed by lysis may be unable to show the later signs of apoptosis-like cell death. Thus, apoptosis-like cell death caused by highly cytolytic isolates may have been underestimated in our assays. However, such interference would result in the underestimation of the apoptosis-like cell death associated with ExoU-secreting isolates, and it implies that the actual association between secretion of ExoU and apoptosis-like death is even stronger than that measured here. A third possibility is that *P. aeruginosa* kills cells by a single process with features of both apoptosis and cytolysis. Mutational analysis suggests, but does not prove, that this is not the case. Disruption of the *exoU* gene eliminated much of *P. aeruginosa*–induced LDH release (a marker for cytolytic cell death) without affecting apoptosis-like cell death [38]. Because all the type III–secreted factors responsible for apoptosis-like cell death have not been definitively identified, it has not been possible to do the converse experiment (to genetically prevent apoptosis and determine whether markers of cytolytic cell death remain unaffected). Although these 2 processes are poorly understood, they have been associated with more-severe disease in animal models [63, 67, 68] and thus provide useful information on the potential virulence of clinical isolates.

We have found that secretion of ExoU was also associated with increased virulence in the mouse model of acute pneumonia. This result is consistent with those of previous reports, demonstrating that secretion of ExoU is a potent virulence determinant. Disruption of the *exoU* gene in strain PA103 resulted in attenuation of virulence in the mouse pneumonia model [36, 37], and strains that naturally lack the *exoU* gene had increased virulence after transformation with plasmids containing the *exoU* gene [74]. Moreover, in a mouse model of pneumonia, blood and sputum isolates that secreted ExoU, ExoT, and PcrV were associated with increased virulence, compared with isolates that secreted other combinations of type III proteins [44]. In contrast to secretion of ExoU, in our assays, secretion of ExoS and secretion of ExoY were not associated with increased virulence, relative to nonsecreting isolates. These results are consistent with the view that ExoU is a more-potent toxin than either ExoS or ExoY. However, the present study was not designed to show causal relationships, and other interpretations are possible. For example, secretion of ExoU may simply be a marker for strains that have acquired other important virulence determinants. Additional studies of isogenic mutants with disrupted type III effector genes will be necessary to clearly define the relative roles and potencies of ExoU, ExoS, ExoT, and ExoY in pneumonia.

An intriguing finding of the present study was that, in the mouse model of pneumonia, ExoS-secreting isolates were no

more virulent and, perhaps, were less virulent than nonsecreting isolates. At first glance, this suggests that ExoS does not contribute to the pathogenesis of pneumonia. However, it is important to note that the virulence of the nonsecreting isolates used in the present study may not be equivalent to that of *P. aeruginosa* isolates that have had their type III secretion systems disrupted in the laboratory. The nonsecreting isolates examined in the present study were not randomly selected but were all chosen on the basis of their ability to cause HAP. These isolates may have acquired or may express additional virulence determinants that compensate for the absence of type III–secreted factors and allow them to cause HAP. Nonetheless, our results suggest that a rigorous analysis of the role of ExoS in the pathogenesis of acute pneumonia is warranted.

To our knowledge, this is the first study to examine the secretion of the type III effector protein ExoY in a large collection of clinical isolates. Although it had been shown that ~90% of clinical isolates contain the *exoY* gene [45], the fraction of strains capable of secreting the ExoY protein had not been previously reported. We found that, although 89% of the HAP-associated isolates examined in the present study contained the *exoY* gene, only 60% secreted ExoY. Of type III–secreting isolates, 78% secreted detectable amounts of ExoY in vitro. In addition, secretion of ExoY was not uniquely associated with secretion of ExoU or ExoS. Thus, ExoY follows the pattern of ExoS and ExoU in that the gene encoding it is a variable trait and not all strains that harbor the gene secrete the protein in vitro. Notably, we did not observe an association between secretion of ExoY and increased cytotoxicity, induction of apoptosis-like cell death, or virulence, although the effects of ExoU may have masked a small contribution of ExoY to these phenotypes, and our small sample size may have precluded the detection of such an association.

As would be expected when examining a heterogeneous collection of clinical isolates, each of which may differ in the production of multiple virulence determinants, exceptions to the association between secretion of ExoU and increased virulence were observed. For example, PSE9, an ExoS-secreting isolate, was more virulent in the mouse model of pneumonia than were all the ExoU-secreting isolates (figure 4). This result suggests that the virulence of any particular *P. aeruginosa* isolate is the result of the cumulative effects of multiple virulence determinants, some of which vary in their presence or expression level, from strain to strain. Type III–secreted effector proteins are but one weapon in the arsenal that this organism uses to cause disease. This does not invalidate the conclusion that, in general, the secretion of ExoU by HAP-associated isolates is associated with especially high levels of virulence. It does, however, suggest that cytotoxicity and apoptosis assays, which detect only particular types of tissue damage, may not adequately measure the overall virulence potential of some *P. aeruginosa* isolates (e.g., PSE9).

Extrapolating our results to human infections requires the assumption that virulence measured in cell culture and mouse models of disease correlates with disease severity in humans. In this regard, it is encouraging to note that the isolates used in the present study were obtained from a previous investigation of type III secretion status and disease severity in humans with HAP [43]. In the previous investigation, human patients with HAP caused by type III–secreting *P. aeruginosa* isolates were more likely to have poor clinical outcomes than were patients infected with nonsecreting isolates. Infection with ExoU-secreting isolates was associated with an especially high frequency of poor clinical outcomes. Although more-definitive studies are required, the virulence of ExoU-secreting *P. aeruginosa* isolates, as defined by cell culture and animal model experiments, is consistent with their ability to cause severe disease in humans.

Together, these results invite speculation about the effect of strain differences on HAP in humans. First, *P. aeruginosa* clinical isolates may differ markedly in their ability to cause severe infections. Perhaps not all infections caused by this bacterium should be expected to progress in a similar manner. It is even conceivable that intraspecies differences in virulence may be greater than interspecies differences and may have a correspondingly greater impact on outcome. It is possible that, at least with regard to *P. aeruginosa,* identification of the bacterial genus and species is insufficient to fully characterize the disease potential of an infecting isolate. Second, because secretion of ExoU is associated with highly virulent clinical isolates of *P. aeruginosa* in model systems, patients infected with ExoU-secreting strains may be at an increased risk for especially severe disease. Such patients may benefit from early and intensive antibiotic therapy. Because tracheal colonization often precedes lower respiratory tract disease in hospitalized patients [75], preemptive strategies may be indicated for patients colonized with ExoU-secreting isolates. Third, investigational interventions, such as passive immunotherapy designed to neutralize the *P. aeruginosa* type III secretion pathway [76–78], may be most appropriate for the subset of patients infected with secreting strains and may have the best cost:benefit ratio when administered to patients infected with ExoU-secreting isolates. Although these points are speculative and require further study, it is clear that defining the pathophysiological consequences of ExoU intoxication will be instrumental in better understanding the role of this protein in progression of disease and may lead to therapeutic interventions.

Acknowledgments

We thank Herbert Schweizer, for generously supplying the vectors used for the allelic replacement experiments; Aaron

Shaver, Borko Jovanovic, and Eric Skaar, for statistical advice; Karla Fullner, for suggestions on the preparation of the manuscript; and Jordi Valles and Dolors Mariscal, for collecting the isolates.

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