Interactions between effector proteins of the *Pseudomonas aeruginosa* type III secretion system do not significantly affect several measures of disease severity in mammals

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The effector proteins of the type III secretion systems of many bacterial pathogens act in a coordinated manner to subvert host cells and facilitate the development and progression of disease. It is unclear whether interactions between the type-III-secreted proteins of *Pseudomonas aeruginosa* result in similar effects on the disease process. We have previously characterized the contributions to pathogenesis of the type-III-secreted proteins ExoS, ExoT and ExoU when secreted individually. In this study, we extend our prior work to determine whether these proteins have greater than expected effects on virulence when secreted in combination. *In vitro* cytotoxicity and anti-internalization activities were not enhanced when effector proteins were secreted in combinations rather than alone. Likewise in a mouse model of pneumonia, bacterial burden in the lungs, dissemination and mortality attributable to ExoS, ExoT and ExoU were not synergistically increased when combinations of these effector proteins were secreted. Because of the absence of an appreciable synergistic increase in virulence when multiple effector proteins were secreted in combination, we conclude that any cooperation between ExoS, ExoT and ExoU does not translate into a synergistically significant enhancement of disease severity as measured by these assays.

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INTRODUCTION

An emerging theme in bacterial type III secretion is that the multiple effector proteins transported by these systems cooperate to subvert specific host-cell processes, resulting in enhanced virulence. Each factor alone has a minor effect on the overall virulence process, but together they cooperate to dramatically enhance virulence. Often, individual effector proteins target different points within a single host-cell pathway to facilitate an essential step in pathogenesis. For example YopE, YopH, YopT and YopO, four type III effector proteins of *Yersinia*, impede neutrophil and macrophage phagocytosis by targeting the actin cytoskeleton of these cells in a coordinated manner (Cornelis, 2002). The net result is that *Yersinia* bacteria are able to persist in the presence of a robust host immune response. Similar cooperation occurs between *Salmonella* type III effector proteins to orchestrate bacterial entry into intestinal epithelial cells, an essential step in the development of enteritis. A large number of type III effector proteins working in concert are required to achieve maximal levels of *Salmonella* bacterial uptake (Raffatellu *et al.*, 2005). These proteins cause a complex

series of actin cytoskeletal changes, which result in the transient formation of localized host-cell surface membrane ruffles that engulf the invading bacteria. SopB, SopE and SopE2 activate the Rho family GTPases Cdc42 and Rac1, thereby facilitating actin recruitment and polymerization at the site of bacterial entry (Hardt *et al.*, 1998; Zhou *et al.*, 2001). Two other effector proteins, SipA and SipC, further enhance actin nucleation, polymerization and cross-linking by directly binding to actin (Zhou *et al.*, 1999a,b). Once bacterial entry is complete, another effector protein, SptP, reverses actin restructuring to re-establish normal host-cell morphology (Fu & Galan, 1999). Similar to *Salmonella* spp., bacteria of the genus *Shigella* also utilize type III effector proteins to gain access to the intracellular environment of intestinal epithelial cells. IpaC and IpaA coordinately modify the host-cell actin cytoskeleton to modulate entry of this bacterium (Tran Van Nhieu *et al.*, 2000). In each of these cases, type-III-secreted effector proteins work in a coordinated fashion to bring about an important pathogenic consequence.

Experimental evidence for the existence of a cooperative relationship between virulence factors is that each factor alone has a minor effect on measures of virulence, but that together they dramatically enhance disease severity. This is

Abbreviations: ADPRT, ADP-ribosyltransferase; GAP, GTPase-activating protein; LDH, lactate dehydrogenase.

indeed the case for the effector proteins secreted by the *Yersinia* and *Salmonella* type III systems (Grosdent *et al.*, 2002; Raffatellu *et al.*, 2005). Regardless of the specific molecular interactions, the true magnitude of the contribution of an individual effector protein to pathogenesis becomes apparent only when the cooperating effector proteins are also present in virulence assays.

It is unclear whether effector proteins of the type III secretion system of *Pseudomonas aeruginosa* act in a coordinated fashion to facilitate the development and progression of disease. This opportunistic pathogen secretes four effector proteins: ExoS, ExoT, ExoU and ExoY (Frank, 1997). The enzymic and cell biological activities of these proteins are well defined. ExoS is a bifunctional toxin containing a GTPase-activating protein (GAP) domain and an ADP-ribosyltransferase (ADPRT) domain. The amino-terminal GAP activity acts on Rho family GTPases while the carboxyl-terminal ADPRT activity is directed towards Ras and other host-cell proteins (Fraylick *et al.*, 2001; Goehring *et al.*, 1999; Henriksson *et al.*, 2000, 2002; Krall *et al.*, 2002; McGuffie *et al.*, 1999; Olson *et al.*, 1999; Rocha *et al.*, 2003; Vincent *et al.*, 1999). As a result of these enzymic activities, intoxication with ExoS is associated with several observable phenotypes, including cytotoxicity and inhibition of bacterial internalization by both phagocytic and non-phagocytic mammalian cells (Cowell *et al.*, 2000; Fleiszig *et al.*, 1997; Frithz-Lindsten *et al.*, 1997; Henriksson *et al.*, 2000; Kaufman *et al.*, 2000; Olson *et al.*, 1997, 1999; Pederson & Barbieri, 1998; Vincent *et al.*, 1999). (Throughout this discussion, the term 'cytotoxicity' will be used to refer to cytolytic cell death.) Like ExoS, ExoT also has GAP activity for Rho GTPases and contains an ADPRT domain. The ADPRT activity of ExoT, however, targets the cellular Crk-I and Crk-II kinases rather than Ras (Sun & Barbieri, 2003). As a result, ExoT intoxication is associated with inhibition of bacterial internalization but not cytotoxicity (Cowell *et al.*, 2000; Garrity-Ryan *et al.*, 2000; Krall *et al.*, 2000). ExoU possesses phospholipase A₂ and lysophospholipase activities (Phillips *et al.*, 2003; Rabin & Hauser, 2005; Sato *et al.*, 2003; Tamura *et al.*, 2004) that lead to rapid lysis of mammalian cells (Coburn & Frank, 1999; Finck-Barbançon *et al.*, 1997; Fleiszig *et al.*, 1997; Hauser *et al.*, 1998a; Hauser & Engel, 1999; Vallis *et al.*, 1999). ExoY is an adenylate cyclase that increases intracellular levels of cAMP (Yahr *et al.*, 1998). Several studies have clearly demonstrated that the enzymic activities of ExoS, ExoT and ExoU contribute to pathogenesis by affecting bacterial persistence in infected tissues, bacterial dissemination and host survival (Finck-Barbançon *et al.*, 1997; Garrity-Ryan *et al.*, 2000; Hauser *et al.*, 1998a; Pankhaniya *et al.*, 2004; Shaver & Hauser, 2004). ExoY, in contrast, has not been shown to play an appreciable role in disease progression in animal models (Holder *et al.*, 2001; Lee *et al.*, 2005).

Recent observations suggest that cooperation between specific *P. aeruginosa* effector proteins does occur at the cellular level. Expression profiling of an infected pneumocyte cell

line indicated that intoxication with ExoS, ExoT and ExoY together altered transcription of a distinct set of genes relative to intoxication with each effector protein alone (Ichikawa *et al.*, 2005). Furthermore, the type III secretion phenotypes of *P. aeruginosa* clinical isolates are also consistent with cooperation between specific effector proteins. For example, clinical isolates rarely secrete ExoS, ExoT and ExoU individually. Rather, these proteins are usually secreted in specific combinations. The majority of secreting isolates produce ExoS and ExoT, whereas most of the remaining strains secrete ExoU and ExoT (Berthelot *et al.*, 2003; Feltman *et al.*, 2001; Hauser *et al.*, 2002; Jain *et al.*, 2004; Lomholt *et al.*, 2001; Roy-Burman *et al.*, 2001). One explanation for these secretion patterns is that selective pressures have favoured the evolution of strains that secrete specific combinations of cooperative effector proteins. However, the true consequences of interactions between effector proteins to overall virulence are currently unclear.

In an effort to better understand the roles of the *P. aeruginosa* type III effector proteins in pathogenesis, we have recently determined the relative virulence associated with ExoS, ExoT and ExoU when these proteins are secreted individually (Shaver & Hauser, 2004). Isogenic bacterial mutants that individually secreted ExoS, ExoT or ExoU were constructed in clinical isolate PA99 (Feltman *et al.*, 2001; Shaver & Hauser, 2004). This parent strain was chosen because it naturally secretes all three of these effector proteins. Analysis of ExoY was not performed because this protein does not play a major role in the pathogenesis of *P. aeruginosa* and because PA99 does not contain the gene encoding ExoY (Feltman *et al.*, 2001; Holder *et al.*, 2001; Lee *et al.*, 2005; Shaver & Hauser, 2004). Comparison of strains secreting ExoS, ExoT or ExoU revealed that ExoU and ExoS each individually increased bacterial persistence in the lung, spread to extrapulmonary sites and mortality, with ExoU having a more pronounced effect than ExoS (Shaver & Hauser, 2004). In this system, ExoT had no detectable effect on mortality or bacterial persistence in the lung and made only a minimal contribution to dissemination. From these results, we concluded that when secreted individually, ExoU had the greatest impact on virulence, ExoS had an intermediate effect and ExoT had a minor effect.

In the work described here, we extend our previous studies to examine whether ExoS, ExoT or ExoU act coordinately during pathogenesis. In other words, we wished to determine whether the contribution to virulence of each individual effector protein was enhanced when secreted in the context of the other effector proteins. If two or more effector proteins act in a coordinated manner to subvert host cells, then secretion of multiple effector proteins would be associated with synergistically enhanced virulence compared to secretion of individual effector proteins. Thus, for these studies, the virulence of isogenic mutants that secreted multiple effector proteins was compared to those secreting individual proteins using a variety of assays, both *in vitro* and *in vivo*. PA99 allowed us to systematically compare for

the first time the effects of combinations of ExoS, ExoT and ExoU when secreted by a strain that naturally produced all three toxins.

METHODS

Bacterial strains. A panel of *P. aeruginosa* isogenic mutants secreting one or more of the effector proteins ExoS, ExoT and ExoU was constructed using the parental strain PA99 (Table 1) (Feltman *et al.*, 2001; Shaver & Hauser, 2004). This clinical isolate naturally secretes ExoS, ExoT and ExoU, and therefore is an ideal parent strain for the construction of type III secretion mutants. PA99 does not contain the *exoY* gene and therefore does not secrete ExoY (Shaver & Hauser, 2004). Mutant strains were named according to the effector proteins secreted. For example, deletion of the *exoS* gene resulted in a strain that secreted ExoT and ExoU and was designated PA99TU. Detailed descriptions of the construction of these strains have been published previously (Shaver & Hauser, 2004), with the exception of PA99SU. Briefly, chromosomal effector genes were sequentially deleted from the parent strain by allelic replacement using plasmid constructs containing in-frame deletion alleles of each gene. To produce PA99SU, pCM104, an allelic exchange vector containing an in-frame deletion allele of the *exoT* gene (Shaver & Hauser, 2004), was conjugated into wild-type PA99. Exconjugants in which the chromosomal wild-type *exoT* allele was replaced with the deleted allele were then selected, as described previously (Shaver & Hauser, 2004). In addition to the effector mutants, two non-secreting strains, PA99null and PA99sec[−], were generated previously (Shaver & Hauser, 2004). PA99null lacks secretion of all known effector proteins, but assembles a competent secretion and translocation apparatus, while PA99sec[−] fails to build a functional secretion apparatus due to a mutation in a required structural gene, *pscJ*. Appropriate deletion of each gene was confirmed by PCR amplification and the expected secretion phenotype was verified by immunoblot analysis, as described previously (Shaver & Hauser, 2004). Other than the absence of the appropriate deleted proteins, there were no obvious changes in the amounts of the other secreted proteins by immunoblot analysis (data not shown). All mutants had equivalent growth rates in Luria–Bertani (LB) and MINS (Nicas & Iglewski, 1984) media (data not shown). Furthermore, secretion of each deleted gene was restored by complementation with the wild-type allele at a neutral chromosomal locus (data not shown). An advantage of this collection of mutants is that each effector protein is expressed from a single-copy chromosomal gene under control of its endogenous promoter.

Cytotoxicity assays. Cytotoxicity of type III secreted effector proteins in eukaryotic cells was measured using a lactate dehydrogenase (LDH) release assay (Promega). A549 bronchial epithelial-like cells (ATCC) were seeded into 24-well tissue culture dishes (Corning) and grown to 70–80% confluence in Waymouth's medium supplemented with 10% fetal bovine serum (Invitrogen). Bacteria were grown overnight in LB medium at 37 °C with shaking (250 r.p.m.). Bacterial concentrations were estimated using optical density measurement and diluted to an OD₆₀₀ of 0.1, which corresponds to 1.5×10^8 c.f.u. ml^{−1}. A549 cells were washed and placed in 1 ml medium. Bacteria were added in 150 µl Waymouth's medium (m.o.i. approximately 45) and were incubated at 37 °C in 5% CO₂. LDH release was monitored spectrophotometrically at various times post-infection. As a control for 100% lysis, several wells of cells were mixed vigorously with 0.9% Triton X-100 (Fisher Scientific) prior to LDH measurement. Uninfected cells were used as a control for spontaneous cell lysis. Percentage cytotoxicity was calculated as follows: (experimental value – cells only background)/(Triton lysis – cells only background) × 100. All strains were tested in triplicate on each of several different days. Results shown were pooled from all assays.

Aminoglycoside exclusion assays of bacterial internalization.

Bacterial internalization was quantified using antibiotic protection assays. All ExoU-secreting strains were excluded from these assays to avoid confounding effects of rapid ExoU-mediated cytotoxicity, which allows the antibiotic access to intracellular bacteria. In addition, all assays were conducted prior to the onset of ExoS-dependent cell lysis, as indicated by the results of LDH release assays. A549 cells were seeded onto transwell filters in 12-well plates (Corning) and grown to 70–80% confluence in Waymouth's medium supplemented with 10% FBS. Bacteria were grown overnight in LB medium at 37 °C with shaking (250 r.p.m.). Bacterial concentrations were estimated using optical density measurement and diluted to an OD₆₀₀ of 0.1, which corresponds to 1.5×10^8 c.f.u. ml^{−1}. A549 cells on the filter were washed and placed in 0.5 ml of the appropriate medium. The bottom well was filled with 1.5 ml medium. Bacteria were added to the top well in 150 µl medium (m.o.i. approximately 45) and were incubated at 37 °C in 5% CO₂. Following 3 h of infection, cells were washed with Waymouth's medium to remove non-adherent bacteria and then incubated in fresh Waymouth's medium supplemented with 10% FBS and amikacin (400 µg ml^{−1}; Sigma) for 2 h to kill extracellular bacteria. Filters were removed from transwells using a sterile scalpel, soaked in 0.25% Triton X-100 in medium and vortexed with sterile 3 mm glass beads for 2 min to ensure complete cell disruption. The resulting solution was serially

Table 1. Bacterial strains and mutants used in this study

<i>P. aeruginosa</i> strain	Relevant characteristics	Reference
PA99 (PA99STU)	Clinical isolate; naturally secretes ExoS, ExoT and ExoU	Feltman <i>et al.</i> (2001)
PA99ST	PA99Δ <i>exoU</i>	Shaver & Hauser (2004)
PA99SU	PA99Δ <i>exoT</i>	This work
PA99TU	PA99Δ <i>exoS</i>	Shaver & Hauser (2004)
PA99S	PA99Δ <i>exoTexoU</i>	Shaver & Hauser (2004)
PA99T	PA99null complemented with <i>exoT</i> in the <i>att</i> locus	Shaver & Hauser (2004)
PA99U	PA99Δ <i>exoSexoT</i>	Shaver & Hauser (2004)
PA99null	PA99Δ <i>exoSexoTexoU</i> , gentamicin-resistant	Shaver & Hauser (2004)
PA99sec [−]	PA99Δ <i>pscJ</i>	Shaver & Hauser (2004)

diluted and plated onto LB agar to determine the number of viable internalized bacteria per well. All strains were tested in triplicate on each of several different days. Results were pooled from all assays.

Mouse model of acute pneumonia. Studies of acute pneumonia were conducted using the aspiration mouse model (Comolli *et al.*, 1999). Briefly, bacterial cultures were grown overnight in MINS medium at 37 °C with shaking (250 r.p.m.) and were then diluted and regrown to exponential phase. Bacteria were collected by centrifugation and resuspended to the appropriate concentration in PBS. Six- to eight-week-old female BALB/c mice were anaesthetized by intraperitoneal injection of a mixture of ketamine (100 mg ml⁻¹) and xylazine (20 mg ml⁻¹). Mice were inoculated with 1.2×10^6 c.f.u. bacteria in 50 µl PBS, as determined by optical density. Inocula were confirmed by plating of serial dilutions onto Vogel Bonner minimal (VBM) agar (Vogel & Bonner, 1956). Preliminary experiments using this dose indicated that severe illness in response to the wild-type PA99STU occurred between 22 and 28 h post-infection. To ensure day-to-day reproducibility, animals infected with PA99STU and PA99sec⁻ were included in each daily experiment. All experiments were performed in accordance with the guidelines of the Northwestern University Animal Care and Use Committee.

For determination of bacterial numbers in individual organs, mice were sacrificed at 18 h post-infection. Lungs, spleens and livers were aseptically removed and individually homogenized in PBS. Bacteria in each organ were enumerated following plating of serial dilutions on VBM agar. For each experiment, at least 5 mice per strain were infected for each time point.

For determination of 50% lethal dose (LD₅₀) values, mice were anaesthetized with inhaled methoxyfluorane and were intranasally infected with a range of bacterial inocula. Mice were monitored for a total of 7 days and animal mortality was recorded. In all experiments, mice were sacrificed when severe illness developed and were scored as dead. A minimum of 10 mice were used for each strain. The LD₅₀ value was calculated according to the method of Reed & Muench (1938).

Statistical methods. The significance of differences in bacterial load, cytotoxicity and bacterial internalization was determined using ANOVA followed by multiple unplanned comparisons among all strains tested using the Tukey–Kramer HSD test with an α value of 0.05. Prior to analysis, bacterial load and bacterial internalization data were natural log-transformed to fit a normal distribution. Cytotoxicity data were arcsin-transformed prior to statistical analysis. χ^2 tests were performed on frequencies of dissemination. For all statistics, $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Cytotoxicity

The *P. aeruginosa* type III secretion system causes the death of many mammalian cell types *in vitro*, and the killing of such cells has been postulated to play an important role in the pathogenesis of pneumonia caused by *P. aeruginosa*. For example, strains that are more cytotoxic *in vitro* are in general more virulent in animal models of pneumonia (Sawa *et al.*, 1998; Schulert *et al.*, 2003). Since nearly all *P. aeruginosa* isolates that secrete ExoS or ExoU also secrete ExoT, we wished to examine whether secretion of ExoT could augment the cytotoxic capacity of ExoU or ExoS. Cytotoxicity towards A549 bronchial epithelial-like cells, which are derived from a cell type encountered by *P.*

aeruginosa during respiratory infections, was measured using LDH release assays.

Markedly different levels of cytotoxicity resulted from infection with the mutant strains (Fig. 1). Non-secreting strains PA99null and PA99sec⁻ were only minimally cytotoxic after 24 h. As expected, PA99T was no more cytotoxic than the non-secreting mutants. In contrast, PA99U was associated with rapid death of the majority of cells. Fifty percent of cells were lysed by 3 h and maximal killing of more than 80% of cells had occurred by 9 h post-infection. PA99S also resulted in significant cytotoxicity, lysing 25 and 80% of cells at 9 and 24 h, respectively. In general, additional secretion of ExoS, ExoT or both proteins along with ExoU did not appreciably increase ExoU-mediated cytotoxicity. A small trend towards increased cytotoxicity at the 3 h time-point was noted when ExoS and ExoT were both secreted together with ExoU relative to ExoU secretion alone. This difference, however, was not statistically significant. It was somewhat unexpected that secretion of ExoS along with ExoU did not result in a measurable increase in cytotoxicity, since both proteins can induce cell lysis. One possible explanation for this result is that the cytotoxic properties of ExoU are so robust that they mask any contribution by ExoS. Alternately, ExoU may simply act before the cytotoxic consequences of intoxication with ExoS

Fig. 1. *In vitro* cytotoxicity following infection of A549 cells with bacterial mutants. Cytotoxicity was determined by measurement of LDH release. Percentage lysis was calculated by comparing the amount of LDH released by bacterially infected cells to that released by cells treated with the detergent Triton X-100. Data are means $\pm$ SEM from at least three assays, each performed in triplicate. Strains are labelled according to the effector proteins they secrete. * indicates a significant difference as compared to PA99null and PA99sec⁻; \$ indicates a significant difference as compared to PA99S and PA99ST. For statistical analysis, data were arcsin-transformed prior to testing with Tukey–Kramer HSD, $\alpha < 0.05$. ◆, STU; ●, TU; ■, SU; △, ST; ◇, S; ○, T; ▲, U; ×, null; |, sec⁻.

become apparent. Similarly, ExoS-dependent cytotoxicity was unaffected by secretion of ExoT in that PA99ST and PA99S had similar cytotoxicity at all time-points tested.

These findings indicate that cytotoxic phenotypes observed following infection of A549 cells were the consequence of effector proteins acting independently and not in concert. Secretion of effector proteins together did not result in markedly enhanced cytotoxicity relative to secretion of individual effector proteins. The results reported by Vallis *et al.* (1999) are consistent with our findings; they observed that after 4 h, little difference existed in membrane permeability following infection with strains secreting ExoU alone or in combination with ExoT. Similarly, Vance *et al.* (2005) noted little change in ExoS-mediated cytotoxicity towards macrophages when ExoT was secreted along with ExoS. Interestingly, Lee *et al.* (2005) reported that ExoT had a slight protective effect against ExoS- or ExoU-mediated killing of CHO cells. Our results show a small degree of ExoT-mediated protection against killing, but this did not achieve statistical significance. These quantitative differences may be due to the use of different cell lines or bacterial strains.

Bacterial internalization

ExoS and ExoT act as anti-internalization factors, inhibiting bacterial uptake by both epithelial and phagocytic cells in culture (Cowell *et al.*, 2000; Garrity-Ryan *et al.*, 2000). Thus, strains with defective type III secretion systems are internalized at relatively high frequencies compared to strains that secrete either ExoS or ExoT (Cowell *et al.*, 2000; Evans *et al.*, 1998; Frithz-Lindsten *et al.*, 1997; Garrity-Ryan *et al.*, 2000; Ha & Jin, 2001; Hauser *et al.*, 1998b; Rocha *et al.*, 2003). These observations have led to the hypothesis that type III secretion may function as a virulence determinant by allowing bacteria to escape clearance by phagocytic cells during infection. To determine whether combinations of effector proteins dramatically enhance inhibition of bacterial uptake relative to individual effector proteins, we conducted aminoglycoside protection invasion assays using several bacterial mutants and A549 cells. For these assays, all ExoU-secreting strains were excluded, since aminoglycoside exclusion assays are not interpretable in the presence of toxins that disrupt host-cell plasma membrane integrity. For the same reason, all assays were performed following only 3 h of infection, prior to the appearance of ExoS-mediated cytotoxicity (Fig. 1).

As expected, PA99null and PA99sec⁻ had the highest levels of bacterial uptake, confirming that significant internalization occurs in the absence of secretion of ExoS or ExoT (Fig. 2). PA99S and PA99T each were internalized to a lesser degree, with PA99T exhibiting the smallest amount of bacterial uptake. ExoT secretion reduced bacterial uptake 51-fold compared to fourfold by ExoS secretion. Secretion of ExoS and ExoT together resulted in an intermediate 15-fold reduction in internalization. Thus, rather than enhancing ExoT activity, the secretion of ExoS with ExoT

Fig. 2. Bacterial internalization following 3 h infection of A549 cells. Data are means $\pm$ SEM from at least three assays performed in triplicate. Strains are labelled according to the effector proteins they secrete. * indicates a significant difference as compared to PA99sec⁻; † denotes statistical difference from PA99ST; § indicates a significant difference compared to PA99null. For statistical analysis, data were natural-log-transformed prior to testing with Tukey–Kramer HSD, $\alpha < 0.05$.

actually attenuated the potent anti-internalization activity of ExoT *in vitro*. Garrity-Ryan *et al.* (2000) also observed that ExoT displayed greater anti-internalization activity than ExoS and noted intermediate levels of bacterial internalization when ExoS was secreted with ExoT. Other studies, however, did not detect differences in invasion with secretion of ExoS, ExoT or both (Cowell *et al.*, 2000; Ha & Jin, 2001). These variable results may be due to differences in the cell types or bacterial strains tested.

Bacterial burden in the lungs

To evaluate the importance of possible interactions among ExoS, ExoT and ExoU *in vivo*, the consequences of secretion of multiple effector proteins were tested using a mouse model of acute pneumonia. We previously used this model to examine the relative contributions of ExoS, ExoT and ExoU to pneumonia when secreted individually (Shaver & Hauser, 2004). Briefly, mice were infected with 1.2×10^6 c.f.u. bacteria in 50 μ l PBS. Bacterial numbers in the lung at 18 h post-infection were determined by plating homogenized lungs on VBM agar.

Secretion of multiple toxins had little additional effect on bacterial numbers in the lung as compared to secretion of ExoS, ExoT or ExoU individually (Fig. 3). As we previously reported, PA99U and PA99S, but not PA99T, were able to persist at relatively high numbers in the lung through 18 h (Shaver & Hauser, 2004). Secretion of ExoS, ExoT or both along with ExoU did not increase bacterial numbers in the lung relative to secretion of ExoU alone. In fact, a trend towards decreased bacterial persistence with PA99SU, PA99TU and PA99STU compared to PA99U was observed, although this did not achieve statistical significance. Likewise, secretion of ExoS with ExoT did not result in a

Fig. 3. Bacterial burden in the lungs at 18 h post-infection. Data are the means $\pm$ SEM from at least five mice per group. Strains are labelled according to the effector proteins they secrete. * indicates a significant difference between PA99T and all other secreting strains (Tukey–Kramer HSD, $\alpha < 0.05$). The PA99S, PA99T, PA99U, PA99null and PA99secr⁻ data points were published previously (Shaver & Hauser, 2004) and are repeated here for ease of comparison.

statistically significant increase in bacterial numbers in the lung relative to secretion of ExoS alone, although there was a small trend in this direction. This last result has been confirmed by other reports showing that secretion of ExoS with additional effector proteins did not affect the bacterial loads in the lungs compared to secretion of ExoS alone (Lee *et al.*, 2005; Vance *et al.*, 2005). We conclude that effector proteins do not have a marked synergistic effect on bacterial persistence in the lung during acute pneumonia.

Dissemination to spleen and liver

The ability of bacteria to disseminate from the lung to the spleen and liver was also determined. We assessed both the number of organs that contained detectable bacteria (frequency of dissemination) and the number of bacteria per organ (bacterial load).

Individually, ExoS, ExoT and ExoU each contributed to bacterial dissemination (Table 2). As we reported previously (Shaver & Hauser, 2004), PA99S, PA99T and PA99U each had an increased frequency of dissemination compared to PA99null and PA99secr⁻, which had minimal extrapulmonary spread. In general, secretion of multiple effector proteins resulted in an increase in dissemination, but the net effect was additive rather than synergistic. For example, PA99STU, PA99SU and PA99TU were detected in the spleen and liver more frequently than PA99U, although these differences did not reach statistical significance. Likewise, there was a trend towards increased dissemination of PA99ST to the spleen compared to PA99S or PA99T. Similar trends were evident when bacterial load in each organ was measured (Table 2). Vance *et al.* (2005) have reported results in agreement with ours. Using a strain that naturally secreted ExoS, ExoT and ExoY, they demonstrated that secretion of two or three effector proteins was associated with the greatest dissemination to the spleen, secretion of one protein with intermediate amounts of dissemination and secretion of no effector proteins with the least dissemination. In contrast, Lee *et al.* (2005) found that dissemination was not increased by secretion of ExoT and ExoY along with ExoS. This may be due to differences in inoculum sizes or in the bacterial strains used in these two

Table 2. Dissemination of bacteria to the spleen and liver at 18 h post-infection

Strain	Spleen		Liver	
	No. of culture-positive organs/total (%)	Range of log ₁₀ c.f.u. per organ (median)	No. of culture-positive organs/total (%)	Range of log ₁₀ c.f.u. per organ (median)
PA99STU	9/11 (82)*	0.0–4.0 (2.8)†‡	11/11 (100)*§	1.7–5.4 (3.4)‡
PA99TU	4/5 (80)	0.0–3.1 (2.5)‡	5/5 (100)*	2.6–4.2 (3.0)‡
PA99SU	3/4 (75)	0.0–2.9 (2.7)	4/4 (100)	2.6–3.9 (3.0)
PA99ST	4/5 (80)	0.0–2.5 (2.2)	2/5 (40)	0.0–3.0 (0.0)
PA99S¶	2/5 (40)	0.0–3.6 (0.0)	3/5 (60)	0.0–2.7 (2.2)
PA99T¶	2/7 (29)	0.0–1.7 (0.0)	4/7 (57)	0.0–2.6 (2.0)
PA99U¶	3/5 (60)	0.0–3.7 (2.9)	4/5 (80)	0.0–4.7 (3.2)
PA99null¶	0/5 (0)	0.0–0.0 (0.0)	1/5 (20)	0.0–1.7 (0.0)
PA99secr ⁻ ¶	1/10 (10)	0.0–2.0 (0.0)	0/10 (0.0)	0.0–0.0 (0.0)

*Significantly different from PA99T (χ^2 , $P < 0.05$).

†Significantly different from PA99S (Tukey–Kramer HSD, $\alpha < 0.05$).

‡Significantly different from PA99T (Tukey–Kramer HSD, $\alpha < 0.05$).

§Significantly different from PA99U (χ^2 , $P < 0.05$).

||Significantly different from PA99S (χ^2 , $P < 0.05$).

¶These data points were published previously (Shaver & Hauser, 2004) and are repeated here for ease of comparison.

studies. Some *P. aeruginosa* strains may produce additional factors not encoded by others that enhance dissemination, thus obviating the need for multiple type III effector proteins. In any case, the additive rather than synergistic increases in dissemination observed when multiple effector proteins are secreted in combination suggest that ExoS, ExoT and ExoU enhance spreading by acting independently instead of functioning in concert.

Virulence in a mouse model of acute pneumonia

To assess the effects of combinations of secreted effector proteins on overall virulence, we determined the LD₅₀ of each strain. Measurement of the LD₅₀ stratified the tested bacterial strains into three groups: ExoU-secretors, ExoS-secretors and strains lacking both cytotoxins (Fig. 4). As reported previously (Shaver & Hauser, 2004), PA99U was associated with the highest virulence (i.e. the lowest LD₅₀ value), PA99S was of intermediate virulence and PA99T was the least pathogenic. PA99SU, PA99TU or PA99STU did not increase in virulence compared to PA99U (LD₅₀= 8.2×10^5 , 9.2×10^5 , 8.6×10^5 and 5.5×10^5 c.f.u., respectively). Comparison of PA99ST with PA99S indicated that secretion of ExoS and ExoT together did not result in significantly higher levels of virulence than secretion of ExoS alone (LD₅₀= 4.4×10^6 vs 3.1×10^6 , respectively), although there was a small trend in this direction. Together, these results indicate that *P. aeruginosa* effector proteins act individually rather than synergistically to cause mortality in the mouse model of acute pneumonia.

Fig. 4. Overall virulence, as measured by LD₅₀ values, of each strain in a mouse model of acute pneumonia. Mice were infected with a range of bacterial inocula and survival was monitored for 7 days. Each value was determined using at least 10 mice. Shorter bars indicate increased virulence. Strains are labelled according to the effector proteins they secrete. The PA99S, PA99T, PA99U, PA99null and PA99secr⁻ data points were published previously (Shaver & Hauser, 2004) and are repeated here for ease of comparison.

Conclusions

A number of bacterial type III secretion systems cause disease by translocating a group of effector proteins that target specific host-cell pathways in a coordinated fashion, leading to bacterial persistence or spread (Cornelis, 2002; Guiney & Lesnick, 2005; Tran Van Nhieu *et al.*, 2000). A hallmark of such cooperation is that individual effector proteins alone are associated with little virulence, but multiple effector proteins together have a marked pathogenic consequence. In this study, we did not find evidence suggesting that cooperation between ExoS, ExoT and ExoU of the *P. aeruginosa* type III system led to enhanced disease severity. *In vitro* cytotoxicity and internalization assays and an *in vivo* acute pneumonia model did not reveal dramatically augmented effects when ExoS, ExoT and ExoU were secreted together rather than individually. Instead, each of these proteins acted independently, causing at best additive but not synergistic effects. The absence of significant synergistic interactions between ExoS, ExoT and ExoU indicates that the pathogenic effects of these proteins can be adequately characterized by using a reductionist approach of studying each when expressed individually. Thus, these findings validate the results of earlier reports looking at effector proteins expressed alone (Finck-Barbançon & Frank, 2001; Henriksson *et al.*, 2002; Pederson & Barbieri, 1998; Pederson *et al.*, 1999; Phillips *et al.*, 2003; Rabin & Hauser, 2003, 2005; Sato & Frank, 2004).

Interestingly, the results of some of our assays suggested that ExoS, ExoT and ExoU may in fact antagonize each other to a small degree. For example, ExoS and ExoT together were less efficient at preventing bacterial internalization than ExoT alone (Fig. 2). Secretion of ExoS or ExoT with ExoU resulted in slightly lower bacterial loads in the lungs of infected mice (Fig. 3). Likewise, secretion of ExoU alone was associated with slightly lower LD₅₀ values (and therefore enhanced virulence) relative to secretion of ExoU with ExoS and/or ExoT (Fig. 4). Although many of these differences were not statistically significant, the observation of similar trends in multiple assays by us and others (Lee *et al.*, 2005) suggests that there may be subtle antagonism when multiple *P. aeruginosa* effector proteins are secreted together. Whether this occurs during the secretion process or after the effector proteins are in the host cell is unclear.

Importantly, our results do not imply that ExoS, ExoT and ExoU lack interactions at the molecular level, but merely that such interactions, if they occur, do not increase virulence to a degree measurable in our assays. Additional work is necessary to determine whether these effector proteins do indeed modify or associate with each other, but if they do, it is unlikely that such interactions are of major pathogenic significance in acute pneumonia.

Given the general lack of increased pathogenicity with multiple effectors, it is unclear why secretion of more than one effector protein has been maintained in *P. aeruginosa* populations. It is conceivable that the assays we used did not

adequately represent the range of activities required for *P. aeruginosa* to cause disease. Alternatively, because *P. aeruginosa* is an environmental organism for which humans are an accidental host (Rhame, 1979), it is possible that selective pressures driving the maintenance of multiple-effector phenotypes exist in the environment rather than in humans or mice. In this regard, it would be interesting to test whether ExoS, ExoT and ExoU cooperate in other models of *P. aeruginosa* disease, such as insects or amoebae (Miyata *et al.*, 2003; Pukatzki *et al.*, 2002). Each distinct set of effector proteins may have been maintained to provide defence against a different environmental threat. In this case, each strain of *P. aeruginosa* would maintain the set of effector proteins necessary for combating the spectrum of environmental threats present in its particular niche.

There are several limitations to our analysis. First, the cytotoxicity and internalization assays were performed using a single cell type, A549. It is conceivable that *P. aeruginosa* bacteria behave differently in assays with other cell types. This was, however, the same cell line used by Ichikawa *et al.* (2005) to demonstrate synergistic effects of ExoS, ExoT and ExoY on transcriptional profiles (Ichikawa *et al.*, 2005). Second, only a single bacterial strain, PA99, was used in these experiments and this strain has the phenotype of secreting ExoS, ExoT and ExoU, which is rare among *P. aeruginosa* isolates (Feltman *et al.*, 2001). Thus it is possible that synergistic interactions between effector proteins are strain-dependent and would be apparent with other *P. aeruginosa* strains. However, the results of our virulence and cytotoxicity assays suggest that PA99 is representative of *P. aeruginosa* strains. For example, the LD₅₀ of PA99 was 9×10^5 , which is similar to that of the frequently used ExoU-secreting strain PA103 (7×10^5) (Schulert *et al.*, 2003). Likewise, the cytotoxicity of PA99 (55 %) was similar to that of PA103 (78 %) at 3 h (Schulert *et al.*, 2003). Furthermore, many of the trends observed in our experiments were also noted by other investigators using strains PAO1 or PAK, as described in the preceding paragraphs. Together, these observations suggest that our findings are reflective of *P. aeruginosa* strains in general, but additional studies with other *P. aeruginosa* strains are necessary to confirm this. Third, the assays that we used may not measure all aspects of virulence associated with effector proteins or may not be sensitive enough to detect modest synergistic contributions to virulence. In this regard, it is interesting that in measurements of both bacterial burdens in the lungs and LD₅₀ values, PA99ST was slightly more virulent than PA99S (Figs 3 and 4). Although these differences did not approach statistical significance, they may reflect small functional interactions between these two proteins, which are nearly always secreted together.

The results of these studies show that the secretion of ExoU or ExoS primarily dictates the virulence associated with the type III secretion system of *P. aeruginosa* during acute pneumonia. Secretion of ExoT has only a modest effect on pathogenesis and does not significantly augment

the toxicities associated with ExoS or ExoU. Based on these results, we propose that strains of *P. aeruginosa* causing acute pneumonia can be divided into three major groups according to their type III secretion phenotypes. The first group consists of ExoU-secreting bacteria. These strains have the potential to cause particularly severe infections in acutely ill patients (Hauser *et al.*, 2002; Roy-Burman *et al.*, 2001) due to the potent activity of ExoU. The second group includes ExoS-secreting bacteria, which may be associated with intermediate levels of virulence. Finally, the third group of strains consists of those that do not secrete type III proteins and therefore lack the toxicities of ExoS and ExoU. These strains would thus be expected to cause the least serious illnesses unless they also encode other virulence determinants that compensate for the lack of ExoU and ExoS. Stratification of patients based upon the type III secretion properties of the strains they harbour may have important clinical utility. For example, more aggressive therapies may be warranted in patients infected with ExoU-secreting strains. Additional studies are necessary to better define the prognostic significance of type III secretion phenotypes in human patients.

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