Type III Secretion of ExoU Is Critical during Early *Pseudomonas aeruginosa* Pneumonia

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**ABSTRACT** The *Pseudomonas aeruginosa* type III secretion system has been associated with poor outcomes in both animal models and human patients. Despite a large number of studies exploring the regulation of type III secretion *in vitro*, little is known about the timing of secretion during mammalian infection. Here we demonstrate that the *exoU* gene, which encodes the highly cytotoxic type III effector ExoU, is induced early during acute *P. aeruginosa* pneumonia. Immunofluorescence microscopy indicated that the amount of ExoU protein in the lung also increased over time. The importance of early expression was examined using a strain of *P. aeruginosa* with inducible production of ExoU. Delays in expression as short as 3 h led to reduced bacterial burdens in the lungs of mice and improved survival. Our results demonstrate that early expression of *exoU* is critical to bacterial survival during pneumonia and suggest that therapeutic interventions that delay ExoU secretion for even short periods of time may be efficacious.

**IMPORTANCE** *Pseudomonas aeruginosa* is a major contributor to the large numbers of health care-associated infections occurring annually, particularly for immunocompromised patients. Although this organism possesses many virulence factors, the type III secretion system plays an especially important role in both animal models and humans. This system forms a needle-like apparatus that injects toxins directly into eukaryotic cells. The most toxic protein secreted by this molecular machine is ExoU, which causes rapid cell death. In this study, we demonstrated that *exoU* was expressed and ExoU was produced early during acute pneumonia in a mouse model. Delaying expression of *exoU* by as little as 3 h enhanced clearance of bacteria and survival of infected mice. Our findings highlight the importance of understanding the regulation of virulence factor expression during infection when designing therapeutic strategies to inhibit the toxic effects of these proteins.

**M**any Gram-negative bacteria possess the ability to deliver effector proteins directly into eukaryotic host cells through type III secretion systems (T3SSs). Some bacteria use T3SSs to infect plants, animals, or humans (1), while others utilize these systems to maintain symbiotic relationships with plant and animal hosts (2). Functionally, a T3SS consists of a secretion apparatus, chaperones, regulatory proteins, and secreted translocation and effector proteins. The arsenal of effector proteins wielded by bacteria varies widely across T3SSs, having evolved to fit the lifestyle requirements of a particular bacterium within its host. In contrast, the T3SS secretion apparatus is relatively well conserved. Due to the complexity of T3SSs, they are governed by intricate regulatory mechanisms. Cell culture model systems have been used to define these regulatory pathways at multiple levels—transcription, translation, and posttranslation (3). These studies have demonstrated that T3SSs are tightly controlled by both global and local regulatory networks to ensure that secretion occurs in the appropriate context.

Despite a large amount of information on the molecular mechanisms by which the type III secretion apparatus is controlled *in vitro*, relatively little is known about how type III secretion is wielded by bacteria *in vivo*. For many T3SSs, the cellular targets and the timing of injection during actual infections remain unclear. Recent work suggests that secretion *in vivo* is tightly controlled with regard to time and location (4). Other studies demonstrate that secretion characteristics observed *in vitro* may differ significantly from those seen *in vivo* (5). Despite its obvious importance, the time during the course of infection when type III secretion occurs remains largely unexplored, as does the consequences of this timing to the disease process. Such knowledge will become more critical as type III secretion inhibitors are identified and enter preclinical and clinical trials (6), and physicians require guidance regarding when and how to utilize these agents to optimize clinical responses.

The T3SS of *Pseudomonas aeruginosa* is a major virulence determinant that contributes to pathogenesis in a broad variety of model systems, including amoebae, insects, zebrafish, and mammals. In humans infected with *P. aeruginosa*, a functional T3SS is
a marker for strains associated with poor clinical outcomes in patients (7). Four effector proteins are secreted by the *P. aeruginosa* T3SS, namely, ExoU, ExoS, ExoT, and ExoY. Of these four proteins, ExoU had the most dramatic impact on outcomes in a mouse model of pneumonia (8). ExoU possesses phospholipase A2 (PLA2) activity that causes rapid lysis of mammalian cells (9). The *exoU* gene is located in the *P. aeruginosa* genome in a bicistronic operon with the *spcU* gene, which encodes a chaperone required for efficient secretion of ExoU (10). Approximately one-fourth of the strains isolated from acute infections carry the *exoU* gene (11), and these strains have been associated with especially severe disease (7, 12). As a result, considerable effort has been dedicated to developing inhibitors of ExoU and *P. aeruginosa* type III secretion (6, 13).

Previous studies have suggested a model for ExoU activity during early pneumonia. Inoculation of *P. aeruginosa* into the lungs of mice resulted in the rapid recruitment of large numbers of inflammatory cells, primarily neutrophils (14). These cells arrived in the lungs within the first 6 h of infection and, in the absence of functional T3SS, were capable of killing *P. aeruginosa* bacteria. As a result, bacterial numbers in the lungs declined by almost 3 orders of magnitude within the first 24 h, from an inoculum of 1.2 × 10⁶ CFU to approximately 5 × 10⁴ CFU (15). Bacteria capable of secreting ExoU, however, exhibited a dramatically changed course of infection. Inflammatory cells, including neutrophils, were still rapidly recruited to the lungs, but they were injected with ExoU and killed, thus allowing bacteria to persist and even increase in numbers (14, 15). As a result, differences in the numbers of CFU recovered from the lungs of mice infected with ExoU− bacteria versus ExoU+ bacteria were apparent as early as 9 h postinfection (15). These findings suggest that under the conditions of these experiments, ExoU is expressed and exerts a biologically significant effect during the first 9 h of infection.

In the present study, we characterize the temporal expression of the *exoU* gene in the mouse lung to determine whether it is consistent with the postulated role of ExoU in intoxication of phagocytes during early pneumonia. Our findings indicate that *exoU* is expressed during the first several hours of pneumonia. Furthermore, delaying toxin gene expression by as little as 3 h resulted in a significant attenuation of the infection. These observations for the first time define the temporal expression of the *P. aeruginosa* T3SS *in vivo*. They support an essential role for ExoU in allowing *P. aeruginosa* to withstand the rapid influx of inflammatory cells during very early pneumonia and suggest that therapeutic strategies aimed at delaying type III secretion for even a short period of time may be efficacious.

**RESULTS**

The *exoU* gene is expressed early in the mouse lung during acute pneumonia. To evaluate the timing of *exoU* gene expression during acute pneumonia, we infected mice by intranasal aspiration with PA99, a clinical isolate of *P. aeruginosa* that secretes the effectors ExoU, ExoS, and ExoT (8). Quantitative reverse transcription-PCR (qRT-PCR) was used to examine the expression of *exoU* transcripts in the lungs during infection. Results were normalized to expression of the rpoD housekeeping gene (16). Expression of *exoU* in infected mice began to increase as early as 3 h postinfection and was significantly higher by 4.5 h postinfection compared to broth culture (Fig. 1). Expression then remained increased through the first 12 h of infection. Expression data were repeated using a second reference gene, *proC* (16), and similar results were obtained (data not shown). These observations indicate that *exoU* gene expression is rapidly induced in the lungs of infected mice.

![Figure 1](image-url)  
**FIG 1** The *exoU* gene is expressed early in the mouse lung during acute *P. aeruginosa* pneumonia. Total RNA was isolated from the lungs of mice infected with *P. aeruginosa* PA99 for the times indicated. RNA was reverse transcribed to cDNA and used in quantitative PCR to quantify *exoU* transcripts. *exoU* transcript levels were normalized between samples using a constitutively expressed bacterial gene, *rpoD*, and are reported relative to expression of *exoU* in broth culture under growth conditions that do not induce type III secretion. Each diamond represents the value for an individual mouse, and the black bars represent medians for the group of mice. Four mice were used for each time point.

*ExoU* protein is detected in the mouse lung early during acute pneumonia. We next examined whether ExoU protein was produced in the lungs of mice infected with *P. aeruginosa*. Mice were infected with *P. aeruginosa* PA99, and their lungs were removed at time points corresponding to those examined by qRT-PCR. Lung sections were examined for bacteria and for the presence of ExoU using *P. aeruginosa*-specific and ExoU-specific primary antibodies, respectively, and fluoroescently labeled secondary antibodies. PA99null, a mutant of PA99 with disruptions in the *exoU*, *exoS*, and *exoT* genes, was used as a negative control. A low-magnification (×25) axial image of a lung section labeled in this manner is shown in Fig. 2A. Regions of the image included in analysis are outlined in blue. Background levels of staining for ExoU were observed in lung sections from uninfected mice and from mice infected with PA99null for 6 h, although large numbers of bacteria were present in the PA99null-infected samples (Fig. 2B and C). The level of ExoU present in the lungs of PA99-infected mice 10 min postinfection did not differ significantly from that seen with the negative controls despite the presence of bacteria (Fig. 2D). ExoU was readily detected by 3, 4.5, 6, and 12 h postinfection (Fig. 2E to H). To quantify ExoU and bacteria, ExoU and PA99 fluorescence was measured for each lung cross section in its entirety (Fig. 2A). The total area of ExoU fluorescence increased significantly above background at 3 h postinfection and remained elevated through 12 h postinfection (Fig. 2I). Some, but not all, of the ExoU protein colocalized with bacteria (Fig. 2H), suggesting that it had been recently secreted. A trend toward increasing numbers of PA99 bacteria in the lungs over time was observed. These results indicate that the rapid increase in *exoU* transcript levels early during acute pneumonia is also accompanied by rapid production of ExoU protein.
Expression and secretion of ExoU are tightly controlled in an ATC-inducible strain of *P. aeruginosa*. If our hypothesis that the timely secretion of ExoU is important for the ability of *P. aeruginosa* to avoid clearance by recruited phagocytes is correct, then delaying secretion of ExoU should be detrimental to bacterial survival during pneumonia. To investigate this further, we created a strain of PA99 with inducible expression of both the *exoU* and *spcU* genes. The *exoU* and *spcU* genes were placed under the control of a tetracycline-responsive promoter and inserted into the chromosome of *P. aeruginosa* PA99null, a mutant that encodes a functional T3SS but no effector proteins; the resulting strain was designated PA99null/H11001ptetU. PA99null/H11001ptetU was grown in Luria-Bertani (LB)-EGTA medium, which induces expression of the genes encoding the type III secretion apparatus. To induce the *exoU* gene, the medium was supplemented with 0, 10, 25, 50, or 100 ng/ml anhydrotetracycline (ATC) (a tetracycline analog). These concentrations of ATC had no effect on growth or survival of *P. aeruginosa* (data not shown). Expression of the *exoU* gene was quantified by qRT-PCR, with normalization to expression of the *rpoD* gene. PA99U, an isogenic strain that contains an intact allele of the *exoU* gene at its native site in the chromosome and under the control of its endogenous promoter (8), was used as a control. Induction of the *exoU* gene increased in a dose-dependent manner (see Fig. S1A in the supplemental material). Approximately 10 ng/ml ATC was sufficient to cause a level of *exoU* expression in strain PA99null + ptetU that was similar to that observed in strain PA99U.

Next, we examined the secretion of ExoU protein by the inducible strain. *P. aeruginosa* PA99null + ptetU was grown in LB-EGTA medium with various concentrations of ATC, and ExoU secretion was detected by immunoblot analysis (see Fig. S1B in the supplemental material). Quantification of the intensity of the observed bands indicated that more ExoU was secreted as the concentration of ATC was increased (Fig. S1C). The amounts of protein secreted...
from PA99null+ptetU supplemented with 25 ng/ml ATC were similar to those secreted by the control strain PA99U. To ensure that ExoU protein detected in PA99null+ptetU supernatants was indeed due to secretion and not simply released from lysed bacteria, we created the control strain PA99secr+ptetU. Like PA99null+ptetU, this strain expresses exoU-spcU under the control of a tetracycline-responsive promoter, but it contains a deletion in the pscJ gene that prevents formation of a functional type III secretion apparatus. Supernatants of PA99secr+ptetU supplemented with ATC did not contain detectable ExoU, but the protein could be detected within lysates of bacterial pellets (Fig. S2). Thus, ExoU protein in the supernatants of cultures of the inducible strain was the result of secretion, not bacterial lysis. Together, these findings indicate that ExoU is secreted from PA99null+ptetU upon stimulation with ATC.

Delayed induction of the exoU gene is associated with decreased cytotoxicity in vitro. We next sought to validate the inducible strain in a cell culture model of ExoU cytotoxicity. We infected HeLa cells with P. aeruginosa PA99null+ptetU grown at a multiplicity of infection (MOI) of 5. Bacteria were grown in the presence of 0, 10, 25, 50, or 100 ng/ml ATC to ensure that the exoU gene was stably induced at the time of infection (Fig. 3A). Culture medium was assayed at 1-h intervals for a period of 4 h after infection to determine the amount of released lactate dehydrogenase, a measure of mammalian cell lysis. Measurement were normalized to 100% lysis by Triton X-100. Error bars indicate the standard errors of the means for triplicate wells. Values that are significantly different (P value of <0.05) from the value for ATC added at 0 min are indicated by an asterisk.
only 50% cell lysis by 4 h postinfection. Bacteria grown in the presence of either 25 or 50 ng/ml ATC resulted in less cytotoxicity early during infection, but the cytotoxicity was equivalent to PA99U by 4 h postinfection. In contrast, PA99null + ptetU grown with 100 ng/ml ATC caused levels of cytotoxicity similar to those caused by PA99U at 2, 3, and 4 h of infection. The experiment was also conducted with PA99secr + ptetU grown in the presence of ATC. Under these conditions, no cytotoxicity was observed (Fig. 3A), indicating that the HeLa cell killing required a functional type III secretion apparatus. These results demonstrated that PA99null + ptetU with 100 ng/ml ATC exhibited cytotoxic activity similar to that of PA99U. It is unclear why 10 to 25 ng/ml of ATC was sufficient to cause normal levels of expression and secretion of ExoU but 100 ng/ml ATC was required for equivalent levels of cytotoxicity (Fig. 3B; see Fig. S1A and S1C in the supplemental material). It is possible that host cell contact induces higher levels of expression of exoU than low-calcium medium (e.g., LB-EGTA medium) does. Thus, higher concentrations of ATC are required for levels of expression equivalent to that of PA99U when cell contact is the type III secretion stimulus compared to LB-EGTA medium. Since cell contact is thought to be the biologically relevant inducer of type III secretion in P. aeruginosa (17), we used 100 ng/ml ATC in subsequent experiments.

Next we used the cell culture system to determine whether the inducible strain was a useful tool for artificially delaying ExoU-mediated toxicity. Unlike the preceding experiment, P. aeruginosa PA99null + ptetU was grown in LB medium without ATC so that the timing of exoU expression could be controlled by adding ATC to the HeLa cell-bacterium cocultures. HeLa cells were infected, ATC was added at 0, 30, or 60 min postinfection, and cytotoxicity was measured. Delaying exoU expression by 30 min did not have an appreciable impact on cytotoxicity at 2 or 3 h postinfection (Fig. 3B). In contrast, a 60-min delay of exoU expression resulted in significantly decreased levels of cytotoxicity at 2 and 3 h postinfection. At 4 h postinfection, approximately 90% of cells were lysed in all the samples (Fig. 3B). These findings are consistent with previous observations that a delay exists between injection of ExoU into cells and the subsequent death of these cells due to ExoU-mediated cytotoxicity. Thus, delayed induction of exoU in the inducible strain resulted in a delay in cell killing.

Delayed expression of exoU leads to decreased bacterial burden in the lungs of infected mice. While previous studies have demonstrated that secretion of ExoU can lead to more severe disease in mouse models of P. aeruginosa pneumonia (15), no study has examined the kinetics of exoU expression during infection and the importance of the timing of its expression to the course of disease. To begin to address this question, we infected mice with P. aeruginosa PA99null + ptetU grown in the absence of inducer and then injected ATC into mice intraperitoneally at 0, 3, 6, or 12 h postinfection. Bacterial numbers in the lungs of infected mice were subsequently measured at 18 h postinfection (Fig. 4). Mice that were infected with PA99null + ptetU and injected with ATC at the time of infection had similar numbers of bacteria in their lungs as mice infected with PA99U, indicating that these conditions replicated a wild-type infection in the mouse model of acute pneumonia. Quantitative RT-PCR confirmed that induction of expression at the time of infection resulted in exoU transcript levels in the lungs of mice infected with PA99null + ptetU that were increased throughout the time course of infection (see Fig. S3A in the supplemental material). Delaying exoU expression for 3, 6, or 12 h did not impede the ability of bacteria to reach wild-type levels of expression in a reasonable time frame (Fig. 3B). Delaying exoU expression by just 3 h resulted in a significant decrease in bacterial numbers compared to mice that received ATC at the time of infection (Fig. 4). Delaying exoU expression until 6 or 12 h postinfection resulted in more drastic reductions in bacterial numbers, such that at 18 h postinfection these mice had bacterial burdens in their lungs similar to mice infected with uninduced PA99null + ptetU or PA99null. These observations indicate that early expression of exoU is important for bacterial survival in the mouse model of acute pneumonia.

Delayed exoU expression is associated with improved mouse survival in the model of acute pneumonia. To further characterize the effect of delaying exoU expression on virulence in the mouse model of acute pneumonia, we measured mouse survival. Mice were infected with P. aeruginosa PA99null + ptetU grown in the absence of ATC. ATC was then administered at 0, 3, 6, or 12 h postinfection, and mice were monitored for survival for 72 h (Fig. 5). All mice that received ATC at the time of infection succumbed to disease within 60 h, similar to mice infected with...
PA99U. Delaying exoU expression by 3 h, which resulted in significantly lower bacterial numbers in the lungs at 18 h (Fig. 4), also led to a 30% improvement in survival. A delay of 6 h led to a further increase in survival, and a delay of 12 h was associated with 100% survival of infected mice, mirroring infection with PA99null or PA99null + ptetU without ATC. The results shown in Fig. 4 and 5 suggest that expression of exoU provides little benefit to the bacteria once bacterial numbers have been depleted below a particular threshold needed to overcome the immune response. Together, these observations indicate that early expression of exoU is critical for progression to severe pneumonia.

DISCUSSION

Type III secretion has been known to play a significant role in the virulence of many Gram-negative pathogens for quite some time, but to date, few studies have examined the kinetics of expression and secretion of these virulence factors during mammalian infections. In the present study, we used qRT-PCR to demonstrate that expression of exoU in P. aeruginosa is rapidly (within 4.5 h) induced in the lungs of mice during acute pneumonia. Increased gene expression is accompanied by a corresponding increase in the amounts of ExoU protein in the lungs of these mice. Furthermore, this early expression is critical for the pathogenesis of the infection; a delay in induction of as little as 3 h is associated with decreased bacterial numbers in the lungs and improved survival of infected mice. Our data suggest that early expression and secretion of ExoU are crucial for the ability of P. aeruginosa to cause severe pneumonia and that therapeutic strategies capable of causing even modest delays in type III secretion could be beneficial.

Previous work in our laboratory showed that by 6 h postinfection large numbers of neutrophils were recruited to the lungs of mice infected with P. aeruginosa (14). Here we demonstrate that P. aeruginosa is equipped to counter these inflammatory cells by inducing expression and production of ExoU during this same time frame. Expression of the exoU gene was apparent already at 3 h postinfection and remained elevated throughout the first 12 h of infection. Likewise, ExoU protein was detected in lung sections by immunofluorescence as early as 3 h postinfection. Although bacteria were localized to distinct sites within the lungs at 3 and 4.5 h postinfection, they were more widespread at 6 and 12 h postinfection, suggesting dissemination throughout the lungs (data not shown). A portion of the ExoU protein colocalized with bacteria, suggesting that it had been recently secreted. Some of the ExoU protein, however, was not associated with bacteria, perhaps because it had been secreted by bacteria that had subsequently moved or were killed, or because it had diffused following release from lysed mammalian cells.

To test whether early secretion of ExoU is truly critical for the pathogenesis of pneumonia caused by P. aeruginosa, we utilized a tetracycline-responsive promoter system to control the timing of exoU expression. This system allowed us to precisely regulate the expression and secretion of this toxin during infection in a mouse model of pneumonia. We found that a delay in expression of as little as 3 h was sufficient to reduce the bacterial burden in the lung and improve survival outcomes for the mice. Delaying exoU expression by 12 h resulted in infection severity similar to that observed with a P. aeruginosa strain lacking exoU. These results indicate that early expression of exoU is critical for bacterial survival and persistence during pneumonia. Interestingly, inducing exoU expression 12 h after infection did not result in mouse mortality, even though substantial numbers of bacteria remained in the mouse lungs at this time point (Fig. 4 and 5). These observations suggest that a delicate balance exists between the virulence of P. aeruginosa and the sterilizing activity of the early inflammatory immune response. ExoU is sufficient to tilt this balance in favor of P. aeruginosa. Once bacterial numbers have been reduced below a certain threshold, the balance may be irreversibly biased in favor of the host, and secretion of ExoU is no longer capable of “rescuing” the infection. Alternatively, it is conceivable that the type III secretion machinery is incapable of exporting effector proteins after the first several hours of infection. Thus, ExoU protein produced later is not secreted and therefore cannot impact the course of the infection. Previous results suggest that type III injection is ongoing throughout the first 24 h of infection (14), making this second scenario less likely.

Although we conclude that expression of exoU during the first hours of infection is critical for the course of pneumonia, it does not necessarily follow that reduced numbers of bacteria are immediately apparent in the lungs upon delay of exoU expression. Expression of exoU at early time points may not have an observable impact on bacterial CFU until later time points. Following production of ExoU, bacteria must bind to neutrophils and inject ExoU into these cells. ExoU must then kill the injected neutrophil. A period of time will then elapse before the absence of this neutrophil has a measurable impact on bacterial numbers (i.e., the time required for an uninjected neutrophil to find, attach to, ingest, and kill a P. aeruginosa bacterium). Each of these steps will require a finite period of time, and together these times will define the delay between exoU expression and an increase in the number of bacteria observed in the lung. This may be the explanation for why we observed equivalent numbers of CFU of P. aeruginosa PA99 and PA99null in the lungs of mice at 6 h postinfection (Fig. 21) even though expression of exoU during this time was critical for increased CFU in the lungs of mice at 18 h postinfection (Fig. 4). Additional studies are necessary to define the time required for each of these steps in the pathogenic function of ExoU.

A number of studies have demonstrated that infections with P. aeruginosa strains harboring functional T3SSs are associated...
with increased mortality, higher bacterial burdens, prolonged bacterial persistence, and more-frequent relapse (18). As a result, efforts have been made to identify inhibitors of this secretion system (19). Our results suggest that inhibitors of the *P. aeruginosa* T3SS may have prophylactic efficacy even if they are only capable of blocking activity for a relatively short period of time. For example, a coating of T3SS inhibitors applied to an endotracheal tube may prove effective by transiently blocking secretion and thus preventing *P. aeruginosa* bacteria from gaining a foothold. A slight delay in type III secretion may be sufficient to allow the host immune system to eradicate bacteria. Our findings underscore the importance of the timing of expression in optimizing the therapeutic use of virulence factor inhibitors. These results combined with previous work support a model for one aspect of ExoU’s role in *P. aeruginosa* pathogenesis. During early pneumonia, neutrophils are rapidly recruited to the lungs in response to detection of bacteria. To defend themselves, bacteria rapidly induce the exoU gene and produce ExoU protein that is injected into these inflammatory cells. Intoxicated neutrophils succumb to the lethal activity of ExoU, allowing bacteria to persist, multiply, and disseminate from the lungs into the bloodstream. In this way, ExoU contributes to poor outcomes in acute pneumonia but is vulnerable to agents that inhibit its early activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and mutants used in this study are listed in Table S1 in the supplemental material. PA99 is a *P. aeruginosa* clinical isolate that encodes the genes for ExoU, ExoS, and ExoT but lacks the gene for ExoY (11). Previously, the genes encoding ExoS and ExoT were disrupted to generate PA99U, a strain that secretes only ExoU (8). *P. aeruginosa* PA99null was previously generated by disrupting the genes encoding all three effector proteins, and PA99secr- was previously generated by disrupting *pscJ*, a gene encoding an essential component of the type III secretion apparatus (8). Bacterial cultures were streaked from frozen cultures onto either Luria-Bertani (LB) or Vogel-Bonner (VBM) agar (20). *Escherichia coli* strain TOP10 was used for cloning and propagation of plasmids. *E. coli* strain S17.1 was used for mating of constructs into *P. aeruginosa*.

Antibiotics were used when necessary at the following concentrations: ampicillin at 50 μg/ml, tetracycline at 20 μg/ml, and kanamycin at 50 μg/ml for *E. coli*; carbenicillin at 500 μg/ml and tetracycline at 100 μg/ml for *P. aeruginosa*.

Mouse model of acute pneumonia. Studies of acute pneumonia were conducted using the aspiration model of infection described previously by Comolli et al. (21). Briefly, overnight cultures of *P. aeruginosa* were grown in 5 ml of LB medium supplemented with 5 mM EGTA with shaking, diluted into fresh medium, and regrown for 2 to 3 h to achieve an exponential phase of growth. Bacteria were collected by centrifugation and resuspended to the appropriate density in phosphate-buffered saline (PBS). Six- to eight-week-old female BALB/c mice were anesthetized by intraperitoneal injection of a mixture of xylazine (20 mg/ml) and ketamine (100 mg/ml), which resulted in obtundation for 45 min. Mice were intranasally inoculated with 1.2 × 10⁶ CFU of bacteria in 50 μl PBS, as determined by optical density and confirmed by the plating of serial dilutions onto VBM agar. To monitor the effect of delayed induction of expression of exoU in survival, mice underwent intraperitoneal injection with anhydrotetracycline (ATC) at a dose of 4 mg/kg of body weight at 0, 3, 6, or 12 h following inoculation and then every 12 h for the duration of the experiment. This dose, which is higher than published doses (22), was required for reproducible results and exoU expression levels equivalent to those observed in mice infected with *P. aeruginosa* PA99U (see Fig. S3A and S3B in the supplemental material). After 18 h of infection, the mice were euthanized, and their lungs were aseptically removed. The lungs of each individual mouse were then homogenized in 5 ml PBS. Homogenates were serially diluted and plated onto VBM agar, and colonies were enumerated after incubation at 37°C for 24 h. For survival experiments, mice were infected with each strain as described above. Survival was monitored for 72 h after infection. Mice that became severely ill as defined by predetermined criteria were euthanized and scored as dead.

Animals were purchased from Harlan Laboratories, Inc. (Indianapolis, IN) and housed in the containment ward of the Center for Comparative Medicine at Northwestern University. All experiments were approved by the Northwestern University Animal Care and Use Committee.

qRT-PCR experiments. RNA was isolated from *P. aeruginosa* PA99 cultures grown to exponential phase in LB medium using the RNAeasy approach (Qiagen, Valencia, CA). Total RNA was isolated from 3 × 10⁶ CFU of *P. aeruginosa* PA99 grown in LB medium, as determined by optical density. Bacteria were incubated with RNAprotect Bacteria reagent (Qiagen) according to the manufacturer’s instructions prior to RNA isolation. Generation of cDNA from total bacterial RNA was performed in the same manner as described below. Negative controls included a sample with nuclease-free water as the template and an RNA sample without the reverse transcriptase step.

Quantitative PCR was performed using a MqyQ2 real-time PCR detection system (Bio-Rad, Hercules, CA). Primers are listed in Table S2 in the supplemental material and were designed with the aid of the Primer2 website (http://frodo.wi.mit.edu/primer3/). Each reaction mixture was composed of 1.25 μl of iQ SYBR green supermix (Bio-Rad), 1 μl of cDNA, 2 μl dimethyl sulfoxide (DMSO), 2.5 μl (50 μM) of each primer, and nuclease-free water to a final volume of 25 μl. Thermal cycling conditions were as follows: 1 cycle of 3 min at 95°C, followed by 40 cycles with 1 cycle consisting of 10 s at 95°C and 30 s at 55°C. *rpoD* and *proC* were used as reference genes, since they were previously shown to have stable expression in *P. aeruginosa* under a variety of conditions (16). Fold changes in expression relative to growth in LB medium were determined using the comparative 2^ΔΔCT method (23) for measurement of exoU expression in the mouse lung. Expression of exoU in *P. aeruginosa* PA99null+petU in response to ATC was determined similarly.

To evaluate expression of exoU during acute pneumonia, mice were infected with 3.0 × 10⁶ CFU of *P. aeruginosa*. This slightly higher inoculum (relative to the standard dose of 1.2 × 10⁶ CFU) was chosen to enhance detection of exoU transcripts. Groups of 4 mice were sacrificed at the indicated times postinfection. The lungs were removed from the mice and immediately incubated overnight in an excess of RNAAlater RNA stabilization solution (Ambion, Woodward, TX) at 4°C. The lungs were placed in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) and homogenized for 30 to 60 s. Homogenized samples were incubated for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes and then transferred to RNase-free 2-ml tubes with 0.2 ml of chloroform added. Samples were mixed by vigorous shaking and incubated for 2 to 3 min at room temperature. Phases were separated by centrifugation at 12,000 × g for 15 min at 4°C. The RNA in the aqueous phase was precipitated by the addition of 0.5 ml isopropyl alcohol and incubation at room temperature for 10 min followed by centrifugation at 12,000 × g for 10 min at 4°C. The RNA pellet was washed twice with 1 ml of cold 75% ethanol and resuspended in 100 μl of RNase-free water. The RNA was treated with Turbo DNase (Ambion) according to the manufacturer’s instructions. Bacterial RNA was further enriched by using the MICRO-BEnrich approach (Ambion) to remove the large amounts of contaminating eukaryotic RNA. A total of 1 μg of each RNA sample was converted to cDNA using the Advantage RT-for-PCR method (Clontech, Mountain View, CA).

To evaluate expression of exoU in mice infected with *P. aeruginosa* PA99null+petU following induction with ATC, mice were infected with a dose of 1.2 × 10⁶ CFU of bacteria. Expression of the exoU gene was induced by intraperitoneal injection of ATC at a dose of 4 mg/kg at 0, 3, 6, or 12 h post-inoculation. Lungs were subsequently removed and RNA was isolated as described.
**Polyclonal antibody against *P. aeruginosa*.** Strain PA99 was heat killed at 65°C for 1 h and injected into rabbits by Covance Custom Immunology Services, Denver, PA. Sera were collected, diluted, and used in immunofluorescence experiments.

**Detection of ExoU and *P. aeruginosa* in fixed lung sections.** Mice were infected intranasally with 3 × 10⁶ CFU of *P. aeruginosa*. This slightly higher inoculum (relative to the standard dose of 1.2 × 10⁸ CFU) was chosen to enhance detection of ExoU protein. Mice were sacrificed at the indicated times, and their lungs were inflated with 10% neutral buffered formalin via cannulation of the trachea. Lungs were removed and fixed in 10% formalin overnight before being embedded in paraffin. Slides mounted with 4-μm lung sections were prepared by the Mouse Histology and Phenotyping Laboratory at Northwestern University. Sections were deparaffinized and rehydrated, followed by incubation for 5 min with 0.2% Triton X-100 to permeabilize cells. Endogenous peroxidases were quenched using Peroxo-Block (Invitrogen, Camarillo, CA) according to the manufacturer’s instructions. Next, 4-μm sections of lung tissue were immunolabeled with ExoU-specific rabbit antiserum (24) followed by Alexa Fluor 546-conjugated tyramide goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. Following ExoU labeling of tissue, slides were subjected to a previously described microwave treatment (25) to prevent cross-reactivity that might occur between the antibodies against ExoU and the antibodies against *P. aeruginosa*, since both primary antibodies were generated in rabbits. The slides were then incubated with the *P. aeruginosa*-specific antisera. Labeled bacteria were visualized using Alexa Fluor 488-conjugated tyramide goat anti-rabbit secondary antibodies (Molecular Probes). The sections were then counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (Molecular Probes) to visualize the nuclei of cells. Sections were mounted with ProLong gold antifade reagent (Molecular Probes). Fluorescence microscopy images were acquired at a magnification of 200× using the TissueFAXS imaging system (TissueGnostics, Vienna, Austria). This process was repeated three separate times using adjacent lung sections. Quantification of ExoU and *P. aeruginosa* PA99 labeling was performed using total area measurement algorithms in TissueQuest 4.1 software (TissueGnostics). Positive labeling events were determined by setting the thresholds for mean intensity and area based on events counted in negative-control samples.

**Generation of strains encoding ATC-inducible exoU genes.** As a first step in constructing mutants of *P. aeruginosa* PA99null and PA99secr- that expressed ExoU under the control of an ATC-inducible promoter, a plasmid was constructed that contained the following elements: the tetR gene, which encodes an ATC-modulated repressor of the promoter P_{tet}; the spcU gene, which encodes the chaperone of ExoU; and the exoU gene. The constitutive promoter P_{525}, and the tetR gene followed by transcriptional terminators were amplified by PCR from plasmid pWL212 using the primers P_{525}Sac5' and Term 4 Bam3' (see Table S2 in the supplemental material) and ligated into the mini-CTXI plasmid previously digested with SacI and BamHI. The resulting construct was designated pHH201. The P_{tet} promoter was amplified by PCR from plasmid pWL213 using the primers P_{tet}-405'5'Pst and P_{tet} -1'3' (Table S2), and the operon containing exoU and spcU was amplified from *P. aeruginosa* PA99 using the primers P_{tet}-exoU5' and spcU Spel 3' (Table S2). P_{tet} was joined to the exoU-spCU fragment by splicing with overlap extension (SOE)-PCR (26) with the primers P_{tet}-exoU5' and P_{tet}-405'5'Pst (Table S2). The resulting fragment was ligated to pCR-Blunt (Invitrogen) to create pHH202. The primers exoU SD 5' and exoU SD 3' (Table S2) were used to eliminate two PstI restriction sites from the exoU gene within pH200 using the QuickChange II XL site-directed mutagenesis method (Stratagene, La Jolla, CA). The P_{tet}-exoU-spCU fragment was purified from pH200 followed by digestion with PstI and Spel and ligated into pH201 previously digested with PstI and BamHI, creating pH203. pH203 was transformed into *E. coli* S17.1 cells, and this strain was used to conjugate the construct into either *P. aeruginosa* PA99null or PA99secr-. The construct was integrated into the chromosomal attB site of these *P. aeruginosa* strains, and the vector backbone was excised by the method of Hoang et al. (27). Successful integration of the ATC-inducible exoU-spCU construct was confirmed by PCR amplification and nucleotide sequencing.

**Cytotoxicity assays.** HeLa cells were grown in 24-well tissue culture plates to 100% confluence. *P. aeruginosa* strains were grown overnight in LB medium with or without ATC at 37°C with shaking. On the day of infection, bacteria were diluted to 1 × 10⁶ CFU/ml in serum-free Eagle’s minimal essential medium (ATCC, Manassas, VA). A total of 100 μl of each bacterial suspension was added to 900 μl of serum-free Eagle’s medium with or without ATC at 10, 25, 50, or 100 ng/ml and dispensed into the wells containing HeLa cells to obtain an MOI of approximately 5. Centrifugation for 5 min at 750 × g was performed to synchronize infections, followed by incubation of culture plates at 37°C in the presence of 5% CO₂. Control wells were treated with 0.9% (vol/vol) Triton X-100 in serum-free Eagle’s essential medium to achieve 100% lysis of HeLa cells. Cytotoxicity was measured by quantifying the release of lactate dehydrogenase using the CytoTox 96 cytotoxicity system (Promega, Madison, WI). Briefly, medium overlying infected HeLa cells was collected at 1, 2, 3, or 4 h post-infection and cellular debris was removed by centrifugation at 180 × g. A total of 50 μl of medium was incubated with 50 μl of assay substrate for 30 min at room temperature followed by the addition of 50 μl of assay stop solution to each well. The A_{510} was then measured using an E_{max} plate reader (Molecular Devices, Sunnyvale, CA). The percentage of cell lysis was calculated using the following formula: 100 × (A_{510} of the sample − A_{510} of uninfected cells)/A_{510} of Triton X-100-treated cells − A_{510} of uninfected cells).

Experiments to determine the kinetics of exoU induction by ATC were done in the same manner, except that bacteria were not grown with ATC. Expression of exoU was induced by the addition of 100 ng/ml ATC to the wells 0, 30, or 60 min postinfection.

**Immunoblot analysis.** Bacteria were grown in LB medium for ~16 h at 37°C with vigorous shaking. Bacteria were subcultured into fresh LB medium supplemented with 5 mM EGTA to induce type III secretion, and ATC was added at appropriate concentrations. After 3 h of growth, cultures were pelleted by centrifugation. Bacterial supernatants were collected from 10-ml cultures by centrifugation at 6,000 × g for 20 min at 4°C. Proteins present in the supernatants were precipitated by the addition of ammonium sulfate (final concentration of 55%). After incubation on ice for 3 h, precipitated protein was recovered by centrifugation at 12,000 × g for 20 min at 4°C. The pellet was resuspended in 200 μl of 10 mM NaCl and boiled after the addition of 5X SDS-PAGE sample buffer for 10 min. A total of 30 μl of each sample was electrophoresed through a 10% (wt/vol) SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and exposed to polyclonal rabbit antisera against ExoU (25). Goat anti-rabbit IRDye 680 conjugate (LI-COR, Lincoln, NE) diluted 1:10,000 was used as a secondary antibody. The membranes were imaged using a LI-COR Odyssey apparatus. Boxes were manually placed around bands of interest to obtain raw intensity values of near-infrared fluorescence with background subtracted using Image Studio 1.1 software (LI-COR).

**Statistical methods.** Student’s t test was performed to compare measurements of exoU expression, fluorescence quantification, cytotoxicity, and CFUs from mouse infections. The log rank test was used to analyze differences in mouse survival. A P value of <0.05 was considered significant.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00032-13/-/DCSupplemental.

Figure S1, TIF file, 0.1 MB.
Figure S2, TIF file, 0.1 MB.
Figure S3, TIF file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.
Figure S2, TIF file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
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