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Catheter-associated urinary tract infection by *Pseudomonas aeruginosa* progresses through acute and chronic phases of infection

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Healthcare-associated infections are major causes of complications that lead to extended hospital stays and significant medical costs. The use of medical devices, including catheters, increases the risk of bacterial colonization and infection through the presence of a foreign surface. Two outcomes are observed for catheterized patients: catheter-associated asymptomatic bacteriuria and catheter-associated urinary tract infection (CAUTI). However, the relationship between these two events remains unclear. To understand this relationship, we studied a murine model of *Pseudomonas aeruginosa* CAUTI. In this model, we also observe two outcomes in infected animals: acute symptoms that is associated with CAUTI and chronic colonization that is associated with asymptomatic bacteriuria. The timing of the acute outcome takes place in the first week of infection, whereas chronic colonization occurs in the second week of infection. We further showed that mutants lacking genes encoding type III secretion system (T3SS), T3SS effector proteins, T3SS injection pore, or T3SS transcriptional activation all fail to cause acute symptoms of CAUTI. Nonetheless, all mutants defective for T3SS colonized the catheter and bladders at levels similar to the parental strain. In contrast, through induction of the T3SS master regulator ExsA, all infected animals showed acute phenotypes with bacteremia. Our results demonstrated that the acute symptoms, which are analogous to CAUTI, and chronic colonization, which is analogous to asymptomatic bacteriuria, are independent events that require distinct bacterial virulence factors. Experimental delineation of asymptomatic bacteriuria and CAUTI informs different strategies for the treatment and intervention of device-associated infections.

CAUTI | type III secretion system (T3SS) | healthcare-associated infection | asymptomatic bacteriuria

Significance

Medical devices, such as urinary catheters, introduced during healthcare procedures can lead to infections that extend hospital stays and add significant costs. While the use of catheters can lead to asymptomatic bacteriuria and catheter-associated urinary tract infection (CAUTI), the relationship between these two events is unclear. Using a murine model of CAUTI, we show that experimental CAUTI with *Pseudomonas aeruginosa* demonstrates two phases: an initial acute infection associated with symptoms including weight loss and sepsis, and a chronic colonization phase which lacks acute symptoms. The acute phase requires type III secretion system (T3SS) delivery of effector proteins, while the chronic phase occurs in the absence of T3SS. These results support that asymptomatic bacteriuria is not a requisite precursor to CAUTI.

Healthcare-associated infections (HAIs) are a major burden on the healthcare system with catheter-associated urinary tract infection (CAUTI) accounting for 30% of all HAIs (1). Hospitalized patients will have an estimated 9 to 16% risk of receiving an indwelling catheter (2) with a 3 to 7% daily risk of developing asymptomatic bacteriuria or CAUTI (3). Complications from CAUTI can lead to prolonged hospital stays and cost US hospitals $340 to $370 million, which represent 10% of the direct costs attributable to hospital-acquired infections (4). CAUTI is caused by several microbial pathogens and each pathogen has specific factors that contribute to infection. We have chosen to study CAUTI caused by *P. aeruginosa* which comprises over 10% of CAUTIs (1). Using an established murine model of CAUTI based on an earlier rat model (5), animals were catheterized and deposited with an implant in the bladder (6). Urine from the animals was voided through the catheter and an infection was initiated by the introduction of bacteria into the bladder through the catheter. After 2 wk, the animals were assessed for bacterial burden in various organs, tissues, and catheter implants (6–8). These studies revealed several important findings. First, the catheter implant is required for chronic colonization by *P. aeruginosa* (6). Second, *P. aeruginosa* forms a biofilm in the lumen of the catheter, but not the exterior surface (6). Third, *P. aeruginosa* can form biofilm in the urinary tract independently of exopolysaccharides (Pel, Psl, and alginate) (6). Fourth, urine and urea can alter *P. aeruginosa* biofilm formation and gene expression by suppressing quorum signaling of acyl-homoserine lactones (8). Together, the findings suggest that CAUTI caused by *P. aeruginosa* proceeds in a manner that is unique to this route of infection and is independent of a number of known biofilm and virulence factors. However, the bacterial factors that contribute to infection remained unclear.

P. aeruginosa utilizes several secretion systems to cause acute and chronic infections including type III secretion system (T3SS) and type II secretion system (T2SS) (9–20). T3SS injects effector proteins directly into the cytoplasm of contacting host cells. As a species, *P. aeruginosa* encode four known effectors with enzymatic activity including ExoS (21), ExoT (22), ExoU (20, 23), and ExoY (24); however, individual strains typically

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encode three effectors since ExoU and ExoS are mostly mutually exclusive (25–27). ExoU is a potent patatin-like phospholipase A2 that is activated by host ubiquitin and phosphatidylinositol 4,5-bisphosphate (28–30). ExoY is a mononucleotide cyclase that synthesizes cyclic AMP (24), but appears to have a preference for synthesizing cyclic GMP and cyclic UMP in mammalian cells (31–33). ExoS and ExoT are bifunctional proteins with an N-terminal GTP activating protein activity (34) and a C-terminal ADP-ribosylation activity (35, 36). Despite sharing over 70% identity (37), the two proteins target distinct host proteins and mediate different outcomes for the affected host cell (38–44). In contrast to T3SS, T2SS mediates the secretion of many proteins, including exotoxin, proteases, lipases, and nucleases (45), which can target cells and tissues that are in contact with the bacteria as well as those more distally located. Whether these secretion systems participate in chronic infections such as CAUTI has not yet been investigated.

To further understand the progression of infection, we infected several cohorts of outbred mice with *P. aeruginosa* and monitored them daily over the 14-d infection. The results demonstrated two phases during CAUTI. There is an acute phase during the first week in which animals lost weight and succumbed to infection. There is a chronic phase during the second week in which the majority of the animals that survived the infection were colonized with *P. aeruginosa*, but gained weight. We tested whether T3SS and T2SS participated in the acute phase of infection by performing infections with Δ*pscD*, Δ*xcp*, and Δ*pscD* Δ*xcp* mutants lacking either or both secretion systems. The results indicated that T3SS is the primary virulence factor of *P. aeruginosa* leading to acute infections during CAUTI. Interestingly, bacterial mutants lacking T3SS were able to colonize the urinary tract without causing acute symptoms, which indicates that *P. aeruginosa* utilizes distinct virulence factors to cause the acute and chronic phases of infection.

Results

***P. aeruginosa* Shows a Biphasic Progression in CAUTI.** To investigate the pathogenesis mechanism of *P. aeruginosa* during infection, we utilized a murine model of CAUTI with outbred CF-1 mice (6). After 1 wk of acclimation, mice were infected with the *P. aeruginosa* PA14 strain, or PBS with tryptone (PBS-T) as a mock control infection, and monitored for the survival rates and body weight changes for 14 d (Fig. 1*A*). Mice were sacrificed at the first signs of morbidity or at the end of the infection on day 14. All mock-infected animals survived the 14-d infection (Fig. 1*B*, solid gray line). For seven cohorts infected on independent occasions, five to seventeen animals in each cohort retained catheters. The majority of the cohorts had a two-phase response in terms of survival: 1. an acute phase in which 25 to 70% of the animals succumbed in the first 7 d of infection and 2. a chronic phase in which few animals succumbed to infection after day 7 (Fig. 1*B*, dashed lines). Since outbred mice were infected, the variation of infection outcomes for each of the cohorts is expected due to host genetic differences. Overall, about 40% of mice infected with *P. aeruginosa* survived beyond 7 d post-infection. Animals that survived past day 7 rarely succumbed to infection in the second week of the infection with greater than 91% survival (Fig. 1*B*, solid black line).

Mock-infected mice lost about 7% of body weight during the first 2 d and quickly regained weight (Fig. 1*C*, solid gray line). The infected mice lost ~10 to 15% weight in the first 2 d after infection (Fig. 1*C*). The weight loss of surviving animals remained steady until day 7 and increased over time in the second week

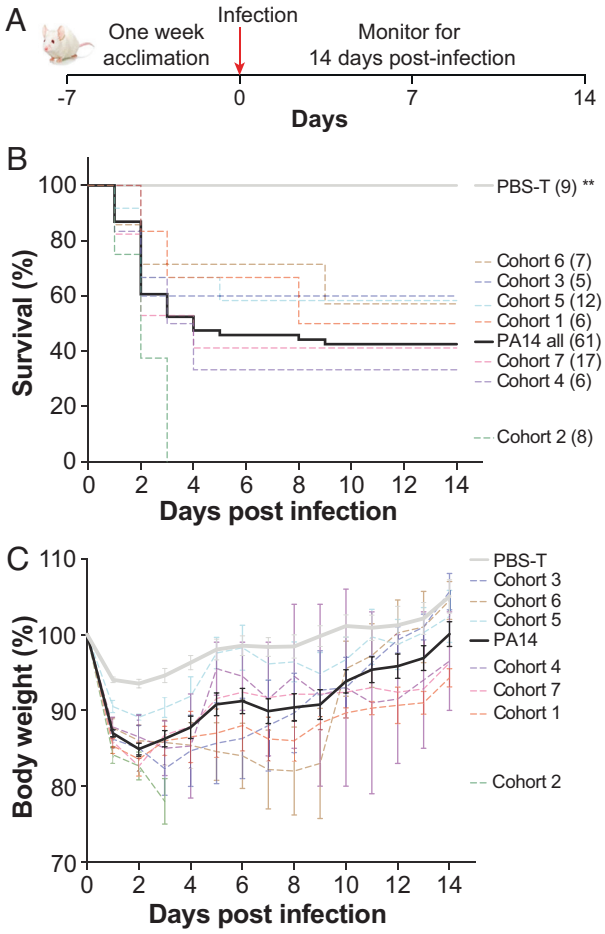

Fig. 1. *P. aeruginosa* shows a biphasic infection progression in murine during CAUTI. (*A*) Time course of murine CAUTI infection. Mice were infected with the parental PA14 strain and monitored over 14 d for (*B*) survival rates and (*C*) percent body weight of surviving animal. Data were obtained for seven independent cohorts of 5 to 17 mice; each cohort is indicated with dotted lines. The solid line represents data combined for all cohorts. Animals with catheter implant that are mock-infected are indicated in gray. Data are presented as mean ± SEM. Survival curves were analyzed between mice infected with PA14 and PBS-T mock-infected mice using a stratified log-rank test (Mantel–Cox) statistical analysis. ***P* < 0.01.

(days 8 to 14) (Fig. 1*C*). There was one cohort in which all animals succumbed to the infection on day 3, and this cohort had the largest weight loss of all groups (Fig. 1*C*). Based on these data, this murine model of CAUTI has a biphasic infection which we define as acute in the first week (days 0 to 7) characterized by high mortality and chronic in the second week (days 8 to 14) with low mortality and weight gain.

***P. aeruginosa* T3SS is Responsible for Murine Mortality.** Acute virulence factors of *P. aeruginosa* include the T3SS and the T2SS (18). To determine whether T3SS and T2SS are responsible for the acute phase in *P. aeruginosa* CAUTI, we performed the murine model of CAUTI with either PA14, isogenic mutant strains lacking T3SS (Δ*pscD*), T2SS (Δ*xcp*) or both T3SS and T2SS (Δ*pscD* Δ*xcp*). Mice infected with Δ*xcp* strain had a similar mortality rate as the parental PA14 with a biphasic infection (Fig. 2*A*). The weight of the mice infected with Δ*xcp* had a similar decrease as the parental PA14 strain (Fig. 2*B*), but animals infected with Δ*xcp* recovered weight faster after day 2. Surprisingly, 100% of mice infected with Δ*pscD* or Δ*xcp*Δ*pscD* survived during the 14-d infection resulting in a single infection phase without any

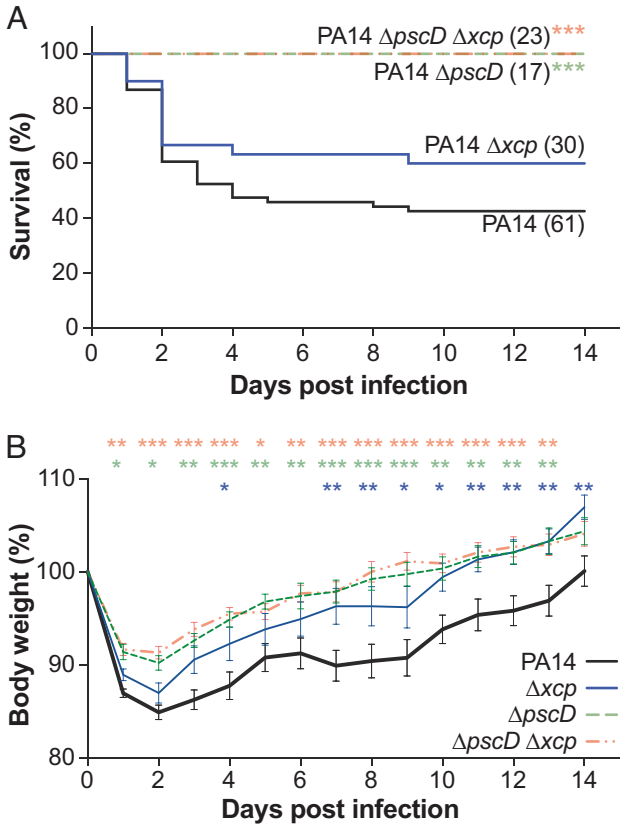

Fig. 2. *P. aeruginosa* T3SS is responsible for murine mortality during CAUTI. Mice were infected either with PA14 and isogenic mutants lacking T3SS (Δ*pscD*), T2SS (Δ*xcp*), or both secretion systems (Δ*pscD* Δ*xcp*) mutant strains. Mice were monitored over a period of 14 d for (*A*) survival rates and (*B*) percent body weight of surviving animal. PA14 data from Fig. 1 is shown for comparison. Survival curves were analyzed between mice infected with PA14 and mutant strains using a stratified log-rank test (Mantel–Cox) statistical analysis. Percent body weight change data is presented as mean ± SEM. Statistical analyses were performed using two-way ANOVA with Dunnett's multiple comparison test using the PA14 strain as a reference. **P* <0.05, ***P* <0.01, ****P* <0.001.

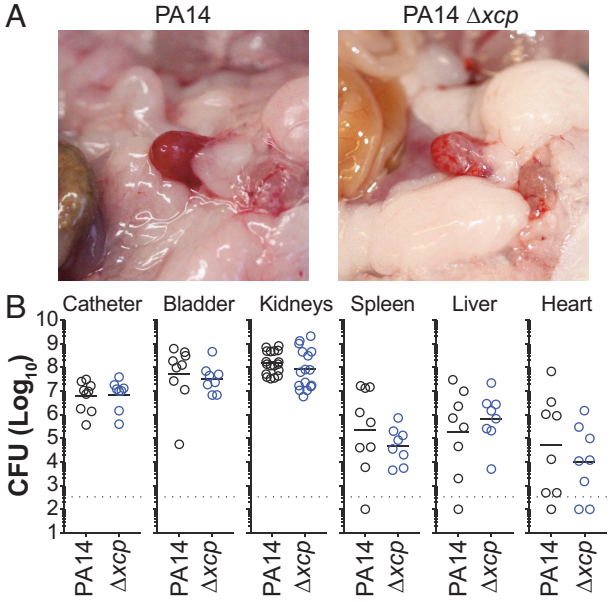

Fig. 3. Murine mortality during acute CAUTI driven by *P. aeruginosa* T3SS is accompanied by bladder hematoma and bacteremia. Data were collected during the acute phase for mice killed during 0 to 7 d post-infection as a humane endpoint. Only mice infected with PA14 or its isogenic Δ*xcp* mutant strain showed symptoms in the first 7 d. (*A*) Representative bladder images of mice killed 1-d post-infection. (*B*) CFU counts in catheter or indicated organ of killed mice. Each symbol represents a particular catheter or organ from an individual animal. Solid line indicates the geometric mean of the data. Horizontal dotted lines indicate CFU detection limit. Mann–Whitney U test revealed no difference in CFU between PA14 and Δ*xcp*.

mortality. The animals infected with mutants lacking *pscD* or both *xcp* and *pscD* had improved infection outcomes that were statistically significant when compared to PA14 ($P < 0.001$) (Fig. 2*A*). The mice infected with Δ*pscD* or Δ*xcp*Δ*pscD* lost less weight and recovered body weight faster than animals infected with PA14 starting day 2 post-infection, which resulted in statistically significant differences throughout the remainder of the infection (Fig. 2*B*). These data demonstrate that T3SS is responsible for the acute phase (week 1) infection. Because these experiments were performed on outbred mice, the acute outcomes associated with T3SS during CAUTI are independent of host genetics.

Acute Infection Driven by *P. aeruginosa* T3SS is Accompanied by Bladder Inflammation and Bacteremia. To further examine the involvement of T3SS in acute CAUTI, mice were assessed for bladder appearance and bacterial burden. Bladders of uninfected mice have a normal appearance in which the bladder is small and clear in color (*SI Appendix*, Fig. S1*A*). Bladders of animals with mock CAUTI (PBS-T) were fibrotic, but lacked hematoma (*SI Appendix*, Fig. S1*B*). Mice infected with PA14 or Δ*xcp* that were sacrificed as a humane endpoint in first 7 d after infection have enlarged bladders with profuse hematoma (Fig. 3*A*). The profuse hematomas of infected animals were not due to the inoculation procedure since on rare occasions when the catheterization procedure resulted in tissue damage, there was blood observed in the urine and the killed animal has a pinpoint hematoma. These results indicate that bladder damage by *P. aeruginosa* during CAUTI is independent of T2SS. None of the mice infected with Δ*pscD* or Δ*xcp*Δ*pscD* succumbed to infection throughout the 14 d of infection and were not evaluated for bladder damage and bacterial burden in the acute phase of infection. Organs within the urinary tract, which includes the catheter, bladder, and kidneys, had high bacterial burden for both PA14 and Δ*xcp* mutant (Fig. 3*B*). Of note, mice infected either with PA14 or Δ*xcp* that exhibited acute symptoms in the first 7 d had an elevated bacterial burden in their spleen, liver, lung, and heart (Fig. 3*B*). This suggests that mice that were infected with either PA14 or Δ*xcp* suffered symptoms including bladder hematoma and bacteremia during acute phase infection (days 0 to 7).

T2SS and T3SS are Dispensable during Chronic CAUTI. To investigate the contribution of T2SS or T3SS during chronic CAUTI, mice infected with either PA14, Δ*xcp,* Δ*pscD, or* Δ*xcp*Δ*pscD* were killed 7 to 14 d post-infection either as a humane end point or at the end of the 2-wk infection protocol. Animals were dissected, assessed for any sign of bladder damage, and assessed for bacterial burden in different organs and catheter implants. Unlike the acute phase, mice infected with PA14 or its isogenic Δ*xcp*, Δ*pscD,* or Δ*xcp*Δ*pscD* mutants showed bladders that were fibrotic, but lacked hematoma 7 to 14 d post-infection (Fig. 4*A*). Animals surviving infection beyond 7 d with PA14 had similar bacterial burden as the Δ*xcp* in the catheter, bladder, and kidneys (Fig. 4*B*). Importantly, while Δ*pscD or* Δ*xcp* Δ*pscD* mutant strains did not cause lethality in the acute phase, the bacterial burdens in the chronic phase were not statistically different from the parental WT strain (Fig. 4*B*). The data shows that *P. aeruginosa* lacking T3SS can colonize the bladder and the kidneys at day 14 post-infection. In contrast to the acute phase, there were no

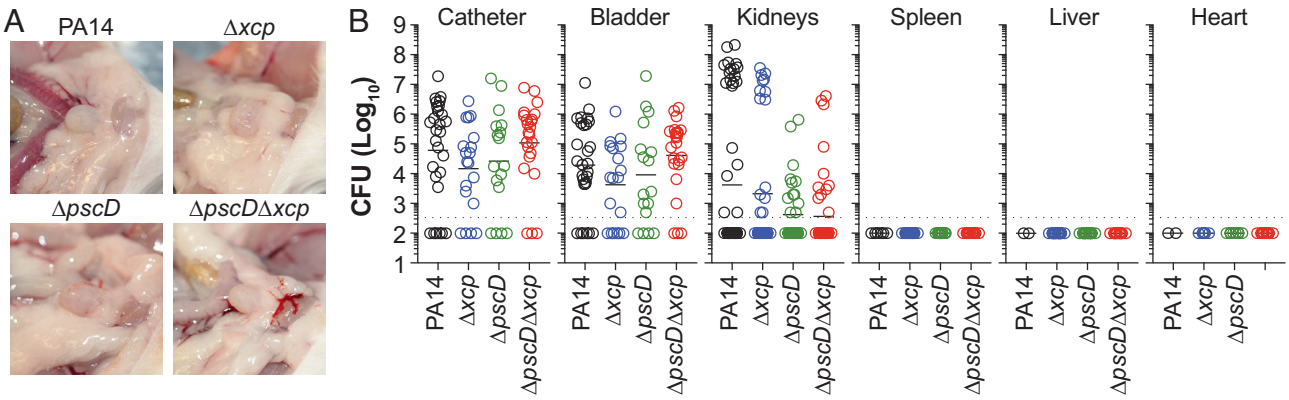

Fig. 4. T2SS and T3SS are dispensable during chronic CAUTI. Data were collected during the chronic phase for mice killed on day 7 to 14 post-infection as a humane endpoint or the completion of the experimental time course. Mice infected either with parental strain PA14 or its isogenic Δ*xcp,* Δ*pscD or* Δ*xcp*Δ*pscD* mutant strain. (*A*) Representative bladder images of mice killed on day 14 post-infection. (*B*) CFU counts in catheter or indicated organ of killed mice. Each symbol represents a particular catheter or organ from an individual animal. Solid line indicates the geometric mean of the data. Horizontal dotted lines indicate CFU detection limit. Mann–Whitney U test revealed no difference in CFU between PA14 and the mutant strains.

detectable bacteria in the spleen, liver, or heart of mice infected with PA14, Δ*xcp,* Δ*pscD, or* Δ*xcp*Δ*pscD* mutants strains implying that the mice did not suffer from bacteremia in the second week of infection (Fig. 4*B*). Since all strains were able to colonize the catheter and bladder, these findings suggest that neither T3SS nor T2SS is required for colonization during the chronic phase of CAUTI.

Histological Analysis of Bladders. To confirm our findings from the visual analysis, an independent set of infections was performed with four experimental groups and a control group that was not catheterized and not infected. Histological analysis of the bladders of uninfected animals showed folded mucosa with a single layer of continuous urothelium (Fig. 5*E*). In contrast, bladders from the animals indicated that catheterization, including mock-infected animals, showed bladder involvement (Fig. 5 *A–D* and *F*) (see *SI Appendix* for the description of histological assessment). For animals experiencing acute infection from PA14 in the first 7 d, the bladders showed significant levels of bacteria, which was correlated with hemorrhaging, loss of urothelium, and necrosis in the mucosa (Fig. 5 *G–I*). For animals infected with PA14 that survived to day 14 or those infected with Δ*pscD* Δ*xcp*, no bacteria, hemorrhage, loss of urothelium, nor necrosis was observed (Fig. 5 *G–I*). Instead, the bladders of chronically colonized animals showed hyperplasia, submucosa fibrosis and edema, and submucosa angiogenesis (Fig. 5 *K–M*). Overall, the bladders for all catheterized animals increased in length whether or not they are infected (Fig. 5*M*). Similarly, catheterized animals have increased bladder weights which increase with time (Fig. 5*O*). Animals with acute infection had a shorter duration after infection and a corresponding smaller increase in bladder weight (Fig. 5*O*). This is likely due to the absence of hyperplasia, submucosal fibrosis, and submucosal angiogenesis (Fig. 5 *K–M*). These histological results support the idea that PA14 can damage the urothelium to access deeper tissues and cause systemic infections. If the host can control bacterial dissemination in the initial phase of infection, the bladder tissues can be thickened resulting in the containment of the bacteria to the urinary tract.

***P. aeruginosa* Triggers ExoU-Dependent Host Mortality.** To determine whether T3SS injection of effector proteins is required for the acute phase symptoms during CAUTI, a Δ*pcrV* mutant was generated that is lacking the translocation pore (46, 47). All mice infected with Δ*pcrV* survived during the 14-d infection, resulting in a single infection phase without any mortality similar to the Δ*pscD* mutant lacking T3SS (Fig. 6*A*). The animals quickly gained weight after infection (*SI Appendix*, Fig. S2*A*). There was no difference in bacterial burden in the catheter, bladder, and kidneys of mice infected with PA14 and Δ*pcrV* mutants during the chronic phase of infection (Fig. 6*B*). These results indicate that injection of T3SS effectors through the PcrV pore is required for the acute phase of murine CAUTI.

As a species, *P. aeruginosa* encodes four known effector proteins, namely ExoS, ExoT, ExoU, and ExoY; individual strains typically encode only three of these effectors (25–27). PA14 encode only ExoU, ExoT, and ExoY (48). Isogenic mutants were generated lacking individual or combinations of effector genes (Δ*exoT*, Δ*exoU*, Δ*exoU* Δ*exoT* (Δ*exoUT*), and Δ*exoU* Δ*exoT* Δ*exoY* (Δ*exoUTY*) mutants) and tested in the CAUTI model. The majority (91.3%) of mice infected with Δ*exoUTY* survived for the 14-d infection, which is a statistically significant higher survival rate as compared to the PA14 (Fig. 6*A*). Mice infected with Δ*exoUT* and Δ*exoU* had a similar survival rate as the Δ*exoUTY* strain (80.6% and 72.7%, respectively) (Fig. 6*A*). In contrast, mice infected with Δ*exoT* had a survival rate similar to PA14 (Fig. 5*A*). These results indicate that ExoU expression correlates with severe CAUTI outcomes. Only mice infected with Δ*exoUTY* had a significantly higher body weight than PA14 (*SI Appendix*, Fig. S2*A*). In contrast, mice infected with Δ*exoT,* Δ*exoU,* or Δ*exoUT* lost weight that is indistinguishable from PA14 (*SI Appendix*, Fig. S2*A*). Mice surviving 14 d post-infection showed no differences in bacterial burdens in the catheters, bladders, and kidneys of mice infected with PA14 and Δ*exoT*, Δ*exoU*, Δ*exoUT,* and Δ*exoUTY* mutants (Fig. 6*B*). No bacteria were detected outside the urinary tract for animals surviving to the chronic phase (Fig. 6*B*). These data suggest that effector proteins do not alter bacterial burden or dissemination to the kidneys. Taken together, the T3SS injection of ExoU is the primary driver of acute outcomes during CAUTI.

RetS and ExsA Regulate T3SS-Mediated *P. aeruginosa* Pathogenesis during CAUTI. The expression of T3SS genes is controlled by many regulatory pathways (49). RetS (regulator of exopolysaccharide and Type III secretion) (50, 51) is an upstream regulator required for activation of ExsA, the transcriptional regulator of T3SS genes, and downstream expression of T3SS genes (52, 53). To determine whether RetS regulation of T3SS is required in pathogenesis during CAUTI, a Δ*retS* mutant was tested in the CAUTI model. All mice infected with Δ*retS* survived the 14 d of infection (Fig. 7*A*). Mice infected with Δ*retS* had a significantly higher body weight than the PA14 over

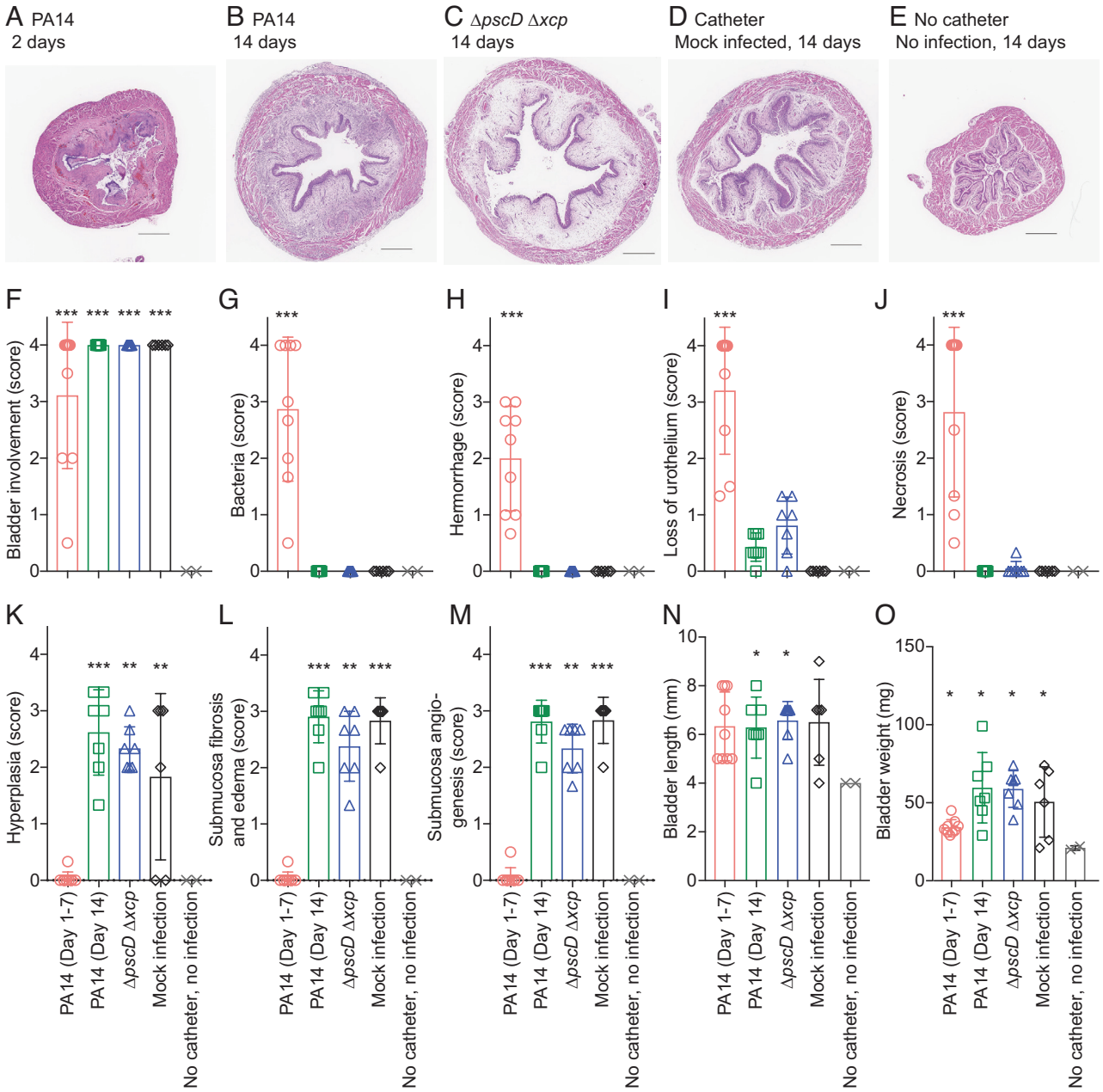

Fig. 5. Histological analysis of bladders after CAUTI and control procedures. Mice were infected either with parental strain PA14 or Δ*xcp*Δ*pscD* mutant strain. Mice were either analyzed if they succumbed to acute infection in the first 7 d post-infection or killed on day 14 post-infection. (*A–E*) Representative histological images of bladder stained with H&E. Blinded histological analyses of bladders were assessed for: (*F*) bladder involvement, (*G*) bacteria, (*H*) hemorrhage, (*I*) loss of urothelium, (*J*) necrosis, (*K*) hyperplasia, (*L*) submucosa fibrosis and edema, and (*M*) submucosa angiogenesis. Scores are indicative of the following: 0 is 0%, 1 is 1 to 25%, 2 is 26 to 50%, 3 is 51 to 75%, and 4 is 75 to 100% for each above category. (*N*) Length and (*O*) weight of bladders were measured. Columns in panels *F–O* are indicative of the mean value. Linear regression test were performed for *F–O*. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

the course of infection (*SI Appendix*, Fig. S2*B*), implying that *retS* is required for T3SS expression during CAUTI. Though mice infected with Δ*retS* remained asymptomatic over the 14-d infection, they had bacterial burden in the catheters, bladders, and kidneys comparable to animals infected with PA14 in a manner that is similar to Δ*pscD* mutants (Fig. 7*C*).

To test whether the requirement of *retS* is solely due to its regulatory effect of *exsA*, an IPTG-inducible ptac-*exsA* was integrated at a neutral *att* site on the chromosome in PA14 or the PA14 Δ*retS* mutant. These strains were induced with IPTG and tested in CAUTI. The majority (88.2%) of mice infected with PA14-*exsA* succumbed to infection within the first 3 d of post-infection (Fig. 7*A*). Similarly, 100% of mice infected with Δ*retS att*::*exsA* succumbed within the first 5 d post-infection (Fig. 7*A*). The animals that succumbed to infection had similar bacterial burden on the catheters, bladders, kidneys, and spleens as the parental PA14 strain (Fig. 7*B*). The results for the strains induced with *exsA* phenocopies the infection outcomes of animals infected with the parental PA14 strain. These data suggest that T3SS regulation during CAUTI requires *retS* and *exsA*.

***P. aeruginosa* from Chronic Colonization during CAUTI Retains T3SS Activity.** These data demonstrate that the T3SS is required for acute virulence and mortality during murine CAUTI, but chronic colonization is independent of T3SS. Expression of T3SS genes was assessed from chronically infected animals at 14 d post-infection. After isolation of the catheter, the luminal contents were eluted with RNAlater. Total RNA was isolated, depleted of

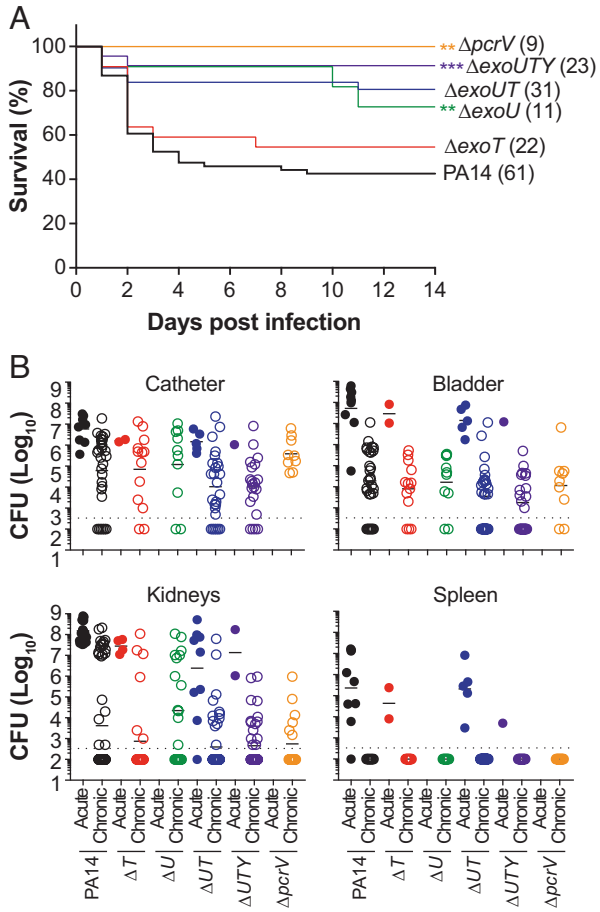

Fig. 6. ExoU is required for T3SS mediated host mortality. (*A*) Mice were infected with PA14 isogenic Δ*exoT*, Δ*exoU*, Δ*exoUT*, Δ*exoUTY*, or Δ*pcrV* mutant strain, and monitored over a period of 14 d for survival rates. PA14 data from Fig. 1 is shown here for comparison. Numbers in brackets indicate the total number of mice. (*B*) CFU counts for the catheter or indicated organ of killed mice. Organs from animals killed before day 7 are acute (closed circles) and after day 7 are chronic (open circles). Each symbol represents a particular catheter or organ from an individual animal. Solid line indicates the geometric mean of the data. Horizontal dotted lines indicate CFU detection limit. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

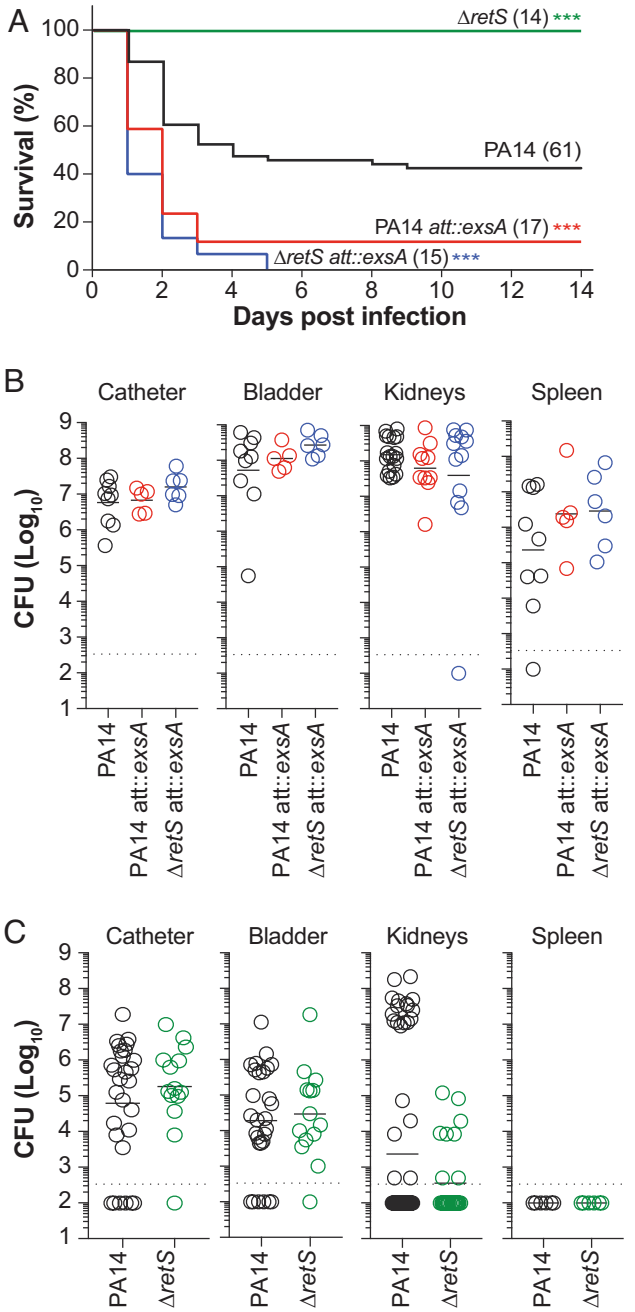

Fig. 7. A functional T3SS is required for *P. aeruginosa* pathogenesis during CAUTI. (*A*) Survival curve of mice infected either with PA14 isogenic Δ*retS* mutant or PA14 overexpressing *exsA* or Δ*retS* overexpressing *exsA* and monitored over a period of 14 d. PA14 data from Fig. 1 is shown here for comparison. Statistical analyses were performed using two-way ANOVA with Dunnett's multiple comparison test using the PA14 strain as a reference. There is no statistical difference between PA14 *att::exsA* and Δ*retS att::exsA*. CFU counts in catheter, bladder, kidneys and spleen of mice infected (*B*) with strains expressing *exsA* were killed 1 to 6 d post-infection or (*C*) with Δ*retS* killed at day 14 post-infection. Each symbol represents a particular catheter or organ. Horizontal dotted lines indicate CFU detection limit. CFU data in (*B*) and (*C*) do not show any statistical significant difference between groups. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

ribosomal RNA, and the remaining RNAs were used to prepare libraries for RNA-seq. The majority of the reads from these samples (>99.7%) mapped to the mouse genome. The remainder of the RNA were mapped to *P. aeruginosa* and reads per kilobase of gene per million reads (RPKM) were determined and compared to RNA isolated from the same strain grown in lysogeny broth (LB), which does not induce T3SS expression (54). When comparing the expression of housekeeping genes, the RPKM of these genes for RNA from catheter compared similarly to in vitro expression (*SI Appendix*, Fig. S2). Similarly, T3SS genes had similar RPKM as compared to non-T3SS growth conditions in vitro (*SI Appendix*, Fig. S3). These results suggest that there is only basal expression of T3SS genes and suggests that T3SS is not induced. Results for the Δ*pscD* Δ*xcp* strain showed similar expression further supporting this interpretation.

To determine whether T3SS remains functional after chronic CAUTI, PA14 isolates were recovered 14 d post-infection from chronically colonized mice and assayed for T3SS-mediated cytotoxicity. After infecting HeLa cells for 3.5 h, the amount of HeLa cell lysis was determined using a lactate dehydrogenase (LDH) assay. PA14 and Δ*pscD* were tested as controls in the LDH assay prior to animal infections. PA14 demonstrated 20 to 40% cytotoxicity, while Δ*pscD* isogenic mutants demonstrated less than 10% cytotoxicity as previously reported (20). All murine CAUTI isolates of PA14 recovered on day 14 of infection demonstrated cytotoxicity above the Δ*pscD* mutant in a manner similar to the parental PA14 strain (Fig. 8*A*). The results showed that these isolates retained the ability to utilize T3SS to intoxicate mammalian cells. Whether PA14 isolates from chronically colonized mice can

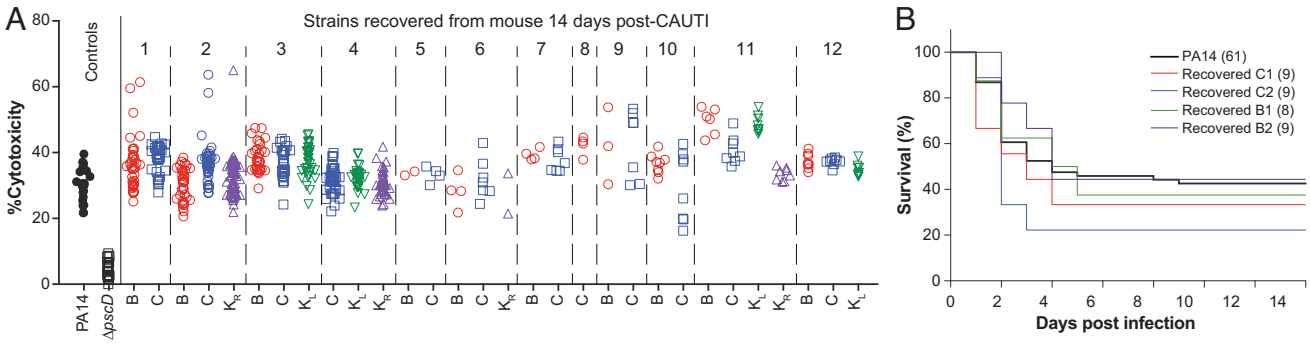

Fig. 8. CAUTI isolates recovered day-14 post-infection retain T3SS-mediated activity. (*A*) PA14 was isolated from the bladder (B), catheter (C), or the left (K_L) or right kidney (K_R) of 12 different mice (M1 to M12) 14-d post-infection and tested in triplicate for cytotoxicity toward HeLa cells (4×10^4) at an MOI of 10 for 3 to 3.5 h. As controls, PA14 and Δ*pscD* were tested prior to animal infections. CAUTI isolates were compared to Δ*pscD* by Mann–Whitney U test and all isolates are statistically different from Δ*pscD* with a *P*-value < 0.01. (*B*) PA14 isolates were recovered from four different mice killed on day 14 post-infection including two from implants or two from bladders. Mice were infected with these isolates and monitored over a period of 14 d for survival rates. See *SI Appendix*, Fig. S4 for CFU data.

recapitulate CAUTI with a similar infection outcome as the laboratory-grown PA14 remained unknown. To answer this question, four isolates recovered from the catheter and bladder of chronically colonized animals were tested in murine CAUTI. The results from these experiments indicate that all isolates recapitulated the same infection progression as the laboratory grown PA14 with distinct acute and chronic phases of infection (Fig. 8*B*). The bacterial burden on the catheter implant and tissues from mice infected with the recovered isolates are similar to the parental PA14 strain (*SI Appendix*, Fig. S4). These results indicate that the absence of acute symptoms in the chronic phase of infection is likely due to changes in the host and *P. aeruginosa*.

Discussion

HAIs involving medical devices that introduce foreign surfaces can lead to bacterial colonization and infection. The chronology of these infections in clinical settings has been challenging to ascertain. The use of animal models aids in the understanding of how these infections progress as well as for the development of treatment and intervention. In this study, we used a murine model of CAUTI to assess the infection outcome of *P. aeruginosa* over a 2-wk infection time course. Infection with the parental PA14 strain led to an acute phase within the first week of infection with animals demonstrating acute symptoms including weight loss and systemic spread of bacteria. In the second week of infection, animals enter a chronic phase of infection in which they rarely suffered acute symptoms, gained weight, and restricted bacteria to the urinary tract. Our results suggest that infection by *P. aeruginosa* in a CAUTI model progresses from an initial acute infection and transitions to chronic colonization of the catheter. Results from these animal experiments reflect certain aspects of clinical CAUTI and catheter-associated asymptomatic bacteriuria. Catheter-associated asymptomatic bacteriuria is defined as the presence of $>10^5$ CFU/mL of an organism without symptoms of UTI, whereas CAUTI has $>10^3$ CFU/mL of bacteria and symptoms of UTI (55). The relationship between catheter-associated asymptomatic bacteriuria and CAUTI has been difficult to establish (55). The prevailing view asserts that catheter-associated asymptomatic bacteriuria is a precursor of CAUTI. However, the limited available clinical evidence does not seem to support this. Our results suggest that bacterial colonization and CAUTI are independent events. If these results apply in the clinical setting, the interpretation would be that catheter-associated asymptomatic bacteriuria does not have a direct causal relationship with CAUTI. CAUTI would likely be due to the introduction of a UTI pathogen. Our results also support the clinical guidelines that contraindicate screening for asymptomatic bacteriuria in catheterized patients and treating catheterized patients with asymptomatic bacteriuria (3).

The bacterial virulence factor that contributed to the acute phase of *P. aeruginosa* infection is T3SS injection of effector proteins, particularly ExoU which contributed most to acute mortality during murine CAUTI. The T3SS requirement was supported by results from the Δ*retS* strain in which T3SS is not expressed (50, 51) and expression of *exsA*, the master transcriptional regulator of T3SS in *P. aeruginosa* (16, 54, 56). In this CAUTI model, the absence of *retS* prevents the acute phase of infection reflecting the absence of T3SS. The requirement for *retS* can be bypassed if *exsA* is expressed from an inducible promoter suggesting that the previously characterized regulatory pathway for T3SS is required for CAUTI. These results agree with observations in several acute infection models of *P. aeruginosa* in which the absence of T3SS reduces the severity of the infection (10, 16, 18–20, 23, 57, 58). Since our experiments were performed using outbred CF-1 mice, the requirement of pseudomonal T3SS is independent of host genetics during the acute phase of CAUTI. Our experiments are performed using a strain of *P. aeruginosa* that encode *exoU*. Within *P. aeruginosa* species, the majority of strains encode *exoS* rather than *exoU* (25, 26). Whether these results will be reflected in *exoS* strains should be investigated in the future. Does chronic colonization require T3SS and the acute phase of infection? Despite the absence of T3SS and the companion acute phase of infection, *P. aeruginosa* T3SS mutants can nonetheless colonize the urinary tract at levels similar to the parental strain. These findings support the concept that acute infection and chronic bacterial colonization are distinct processes that require different bacterial factors. The implication of these results is that progression to chronic colonization does not necessitate progression through an acute phase. The pseudomonal factors required for chronic colonization remain to be determined.

Comparison of results from these CAUTI experiments with other chronic infections can yield insight whether there are common requirements for chronic colonization. One example is burn wound infections by *P. aeruginosa* (59, 60) that can persist for days (61). Studies in burn wounds show a biphasic response in which the acute phase is also driven by T3SS (10, 62, 63). Investigations into quorum regulation during burn wound infection suggest that quorum signaling may be dispensable for the acute phase of infection (64). While these two phases are present, the timing of the two phases and the severity of the acute phase are different between CAUTI and burn wound infections, indicating infection site-specific factors. Many of these studies also utilize outbred mice

(10, 60, 64), suggesting the biphasic response is a common observation for models of infections that is representative of outbred populations and likely more representative of human infections.

Another analogous example is chronic, lifelong infections in cystic fibrosis (CF) patients (65). Some commonalities between CF infections and CAUTI are observations that there are acute and chronic phases of infection. Isolates from CF patients early in infection are associated with functional T3SS injection of exoproteins, whereas isolates obtained year later from the same patients are defective in T3SS (66, 67). Longitudinal sequencing studies of CF infections revealed that these acute strains are ancestors of the phenotypically different chronic isolates (68–71). Mutations in *mutS*, a key DNA repair protein, during the lifelong infection trigger accumulation of other genetic mutations leading to phenotypic diversity of *P. aeruginosa* (68). This diversity has also been observed in *P. aeruginosa* sequences from specific location of a CF lung (72). Strains from CF that are defective in T3SS can sometimes be reactivated by expression of *exsA* suggesting that some of the mutations are due to inactivation of genes encoding the T3SS complex, while other mutations inactivate the upstream regulatory pathway (16, 56). Whether the transition from the acute phase to the chronic phase observed in CAUTI is also due to mutational inactivation of T3SS was tested. Isolates recovered from the mice chronically infected with *P. aeruginosa* displayed T3SS intoxication of cultured cells and recapitulated the CAUTI survival curve of the parental strain. Together these results suggest that *P. aeruginosa* does not undergo mutational inactivation of T3SS in the 2-wk CAUTI. While there are some similarities between CAUTI, burn wound, and CF infections, the findings for CAUTI appear to be more similar to burn wound infections in the time frame of acute to chronic transition.

The key findings that chronic *P. aeruginosa* infection is biphasic and that the acute phase of infection is dispensable for chronic colonization raises a number of important questions. The first is whether acute symptoms of CAUTI are initiated by either reactivation of the bacteria chronically colonized on the catheter or the introduction of new pathogens. The second is what the factors that restrict chronically colonized bacteria to the urinary tract. If there is reactivation, what are the bacterial and host factors that contribute to reactivation? Lastly, whether our findings that there is a transition from acute phase to chronic phase applies to other HAIs and other HAI pathogens. Determining the answers to these questions will provide additional strategies to treat CAUTI caused by *P. aeruginosa* and possibly other HAI infections.

Materials and Methods

Strains and Growth Conditions. *Pseudomonas aeruginosa* PA14 and strains with specific gene deletion used in this study are listed in *S1 Appendix*, Table S1. All strains were grown in LB media with aeration at 37 °C. For exsA induction, the strains with att::pTac-exsA were induced with 1 mM IPTG for 1 h prior to infection. See *SI Appendix* for more details.

Murine Model of CAUTI. The CAUTI model was used as previously described (3, 4). See *SI Appendix* for additional details.

Histological Analysis. Bladders were dissected from animals, fixed in neutral buffered 4% formaldehyde, pH 7.0, and embedded in paraffin and sections were placed on slides (Histoserv, Inc. Germantown, MD). A blinded histological analysis was performed by an independent histologist for the following eight categories: 1. Bladder involvement, 2. Loss of urothelium, 3. Bacteria, 4. Hemorrhage, 5. Necrosis, 6. Hyperplasia, 7. Submucosa fibrosis and edema, and 8. Submucosa angiogenesis. Scores are indicative of the following: 0 is 0%, 1 is 1 to 25%, 2 is 26 to 50%, 3 is 51 to 75%, and 4 is 75 to 100% for each above category. A detailed description of these categories can be found in the *SI Appendix*.

LDH-Release Assay of *P. aeruginosa* Infection of Cultured HeLa Cells. HeLa cells grown in Dulbecco's Minimal Essential Media supplemented with 10% fetal bovine serum were washed and infected with *P. aeruingosa*. Supernatants were collected and assessed for LDH release using the Cytotoxicity Detection Kit (Roche) using the manufacturer's protocol. See *SI Appendix* for details.

RNA Isolation, RNA Sequencing, and Determining Gene Expression. The luminal contents of the catheter were eluted with RNA Later. After removing RNAlater, total RNA was isolated using RNAzol. The total RNA was depleted of mouse and *P. aeruginosa* ribosomal RNA using Illumina total RNA library preparation kit with Ribo-Zero plus and *P. aeruginosa* specific oPools (IDT Technologies) and sequenced on NextSeq 1000 for 50 bp paired read. See *SI Appendix* for additional details.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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