Cyclic Di-GMP Signaling Contributes to *Pseudomonas aeruginosa*-Mediated Catheter-Associated Urinary Tract Infection

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ABSTRACT

Bis-(3'-5') cyclic dimeric GMP (c-di-GMP) controls the lifestyle transition between the sessile and motile states in many Gram-negative bacteria, including the opportunistic human pathogen *Pseudomonas aeruginosa*. Under laboratory conditions, high concentrations of c-di-GMP decrease motility and promote biofilm formation, while low concentrations of c-di-GMP promote motility and decrease biofilm formation. Here we sought to determine the contribution of c-di-GMP signaling to biofilm formation during *P. aeruginosa*-mediated catheter-associated urinary tract infection (CAUTI). Using a murine CAUTI model, a decrease in CFU was detected in the bladders and kidneys of mice infected with strains overexpressing the phosphodiesterases (PDEs) encoded by *PA3947* and *PA2133* compared to those infected with wild-type *P. aeruginosa*. Conversely, overexpression of the diguanylate cyclases (DGCs) encoded by *PA3702* and *PA1107* increased the number of bacteria in bladder and significantly increased dissemination of bacteria to the kidneys compared to wild-type infection. To determine which of the DGCs and PDEs contribute to c-di-GMP signaling during infection, a panel of *PA14* in-frame deletion mutants lacking DGCs and PDEs were tested in the CAUTI model. Results from these infections revealed five mutants, three containing GGDEF domains (Δ *PA14_26970*, Δ *PA14_72420*, and Δ *siaD*) and two containing dual GGDEF-EAL domains (Δ *morA* and Δ *PA14_07500*), with decreased colonization of the bladder and dissemination to the kidneys. These results indicate that c-di-GMP signaling influences *P. aeruginosa*-mediated biofilms during CAUTI.

IMPORTANCE

Biofilm-based infections are an important cause of nosocomial infections, since they resist the immune response and traditional antibiotic treatment. Cyclic di-GMP (c-di-GMP) is a second messenger that promotes biofilm formation in many Gram-negative pathogens, including *Pseudomonas aeruginosa*. Here we determined the contribution of c-di-GMP signaling to catheter-associated urinary tract infection (CAUTI), an animal model of biofilm-based infection. *P. aeruginosa* with elevated levels of c-di-GMP during the initial infection produces an increased bacterial burden in the bladder and kidneys. Conversely, low concentrations of c-di-GMP decreased the bacterial burden in the bladder and kidneys. We screened a library of mutants with mutations in genes regulating c-di-GMP signaling and found several mutants that altered colonization of the urinary tract. This study implicates c-di-GMP signaling during CAUTI.

Pseudomonas aeruginosa is an opportunistic human pathogen that is a leading cause of nosocomial infections, including catheter-associated urinary tract infection (CAUTI), central line-associated bloodstream infection (CLABSI), ventilator-associated pneumonia (VAP), and surgical site infection (SSI) (1). In CAUTI, dissemination of bacteria from the catheter can lead to pyelonephritis and bacteremia. The common theme for these infections is the ability of *P. aeruginosa* to form biofilms on the surfaces of medical devices and implants (2, 3). Once formed, *P. aeruginosa* biofilms are more resistant to antibiotics (4–6) and phagocytic immune cells (7). Thus, the mechanism whereby *P. aeruginosa* forms biofilms *in vivo* is under investigation.

P. aeruginosa is a model organism for the study of biofilm formation under laboratory conditions (2, 3, 8, 9). Numerous studies have revealed that cyclic di-GMP (c-di-GMP) is a second messenger that promotes the transition of planktonic, motile bacteria to sessile biofilms (10, 11). In *P. aeruginosa*, c-di-GMP regulates these processes through allosteric binding to many protein receptors to alter their function. c-di-GMP binds the FleQ transcriptional regulator (12), leading to the repression of the flagellum genes (13) and the activation of the *psl* and *pel* exopolysaccharide operons (14) as well as the surface adhesin CdrA (15). In addition to transcriptional regulation, c-di-GMP binds to PelD to activate

the production of the Pel exopolysaccharide (16, 17) and to FimX to repress type IV pilus-mediated twitching motility (18, 19). Together, these c-di-GMP receptor proteins act in concert to transition planktonic bacteria to biofilms in response to elevated levels of c-di-GMP.

In the cell, c-di-GMP is generated from two GTPs by diguanylate cyclases (DGCs) (20, 21) and removed through linearization by phosphodiesterases (PDEs) containing either the EAL (22, 23) or HD-GYP domain (24, 25). *P. aeruginosa* utilizes c-di-GMP signaling extensively and carries 40 genes that contain DGC or PDE domains. The functions of these genes in phenotypes regulated by c-di-GMP have been studied systematically using transposon in-

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sertion mutants (26), overexpression plasmids (26), and in-frame deletion mutants (27). Overexpression of a subset of DGCs elevated levels of c-di-GMP in the cell and the corresponding increase in biofilm formation, while overexpression of a subset of PDEs reduced biofilm formation (26). Transposon insertion mutants in general had more subtle defects in biofilm formation, suggesting the possibility of functional redundancy between two or more of the genes encoding DGC and PDE (26). Recently, a collection of in-frame deletion mutants was generated and characterized for c-di-GMP-regulated phenotypes (27). Multiple mutants had defects in flagellum-based and type IV pilus-based motility as well as biofilm formation (27). Together, these studies indicate that the regulation of cellular levels of c-di-GMP is complex.

In the present study, we sought to determine whether c-di-GMP signaling contributes to the colonization of the bladder and dissemination to the kidneys during CAUTI by utilizing strains overexpressing DGCs and PDEs. Strains overexpressing DGCs were able to colonize the bladder and significantly increased the dissemination to the kidneys compared to the wild type. Conversely, strains overexpressing PDE had reduced colonization of the bladder and dissemination into the kidneys. These results indicated that c-di-GMP signaling contributes to CAUTI. We utilized the in-frame deletion mutant library to determine if any of the DGCs or PDEs was the primary contributor to c-di-GMP signaling during CAUTI. A subset of these in-frame deletion mutants was selected based on changes in *in vitro* biofilm formation, polysaccharide production, and motility-related phenotypes and tested in the chronic CAUTI model. From these studies, 5 mutants, three containing DGC domains (Δ PA14_26970, Δ PA14_20110 [*siaD*], and Δ PA14_72420) and two containing dual DGC-PDE domains (Δ *morA* and Δ PA14_07500), showed a 1-log reduction in colonization in the bladder. Since all five of these mutants showed defects in swimming and/or twitching motility, in-frame deletion mutants of flagellin (Δ *fliC*) and type IV pili (Δ *pilA*) were tested in the CAUTI model. We found that neither flagella nor type IV pili contribute to colonization of the bladder during CAUTI. Our results suggest that c-di-GMP signaling, through the actions of multiple DGCs, contributes to CAUTI.

MATERIALS AND METHODS

Bacterial growth. All bacterial strains used in this study were grown in Luria broth (LB) medium at 37°C overnight. For the PA14 strains harboring pMMB plasmids for DGC and PDE overexpression, strains were grown in LB with 15 μ g/ml gentamicin overnight and subcultured for 4 h with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) prior to infections. Cells were centrifuged, the supernatant was removed, and cells were resuspended in phosphate-buffered saline with 1% tryptone (PBS-T). The inoculum was serially diluted and plated on LB agar plates to determine concentration. George A. O'Toole kindly provided the collection of PA14 DGC and PDE in-frame deletion mutants. A list of strains used in this study is given in Table 1.

Biofilm assays. PA14 strains containing the pMMB plasmids were grown overnight in LB with 15 μ g/ml gentamicin. Cultures were diluted 1:100 in LB with gentamicin and in the presence or absence of 1 mM IPTG. Biofilms were set up in 96-well polystyrene plates and incubated in a humidified chamber at 37°C for 24 h. Unattached cells were washed away with water three times, and the remaining biofilms were then stained with 0.1% crystal violet. Stained cells were resuspended in 30% acetic acid, and absorbance was measured 590 nm in a spectrophotometer.

Murine CAUTI model. The *P. aeruginosa*-mediated CAUTI model was as described previously (28, 29). Briefly, female CF-1 mice were anes-

TABLE 1 Strains used in this study

Strain	Relevant characteristic	Reference
PA14	Wild type	33
PA14 pMMB PA3702	<i>wspR</i> overexpression	26
PA14 pMMB PA1107	<i>roeA</i> overexpression	26
PA14 pMMB PA2133	PA2133 overexpression	26
PA14 pMMB PA3947	<i>rocR</i> overexpression	26
PA14 Δ PA14_16500 (<i>wspR</i>)	<i>wspR</i> in-frame deletion	27
PA14 Δ PA14_50600 (<i>roeA</i>)	<i>roeA</i> in-frame deletion	27
PA14 Δ PA14_49890 (<i>yfiN</i>)	<i>yfiN</i> in-frame deletion	27
PA14 Δ PA14_26970	PA14_26970 in-frame deletion	27
PA14 Δ PA14_53310	PA14_53310 in-frame deletion	27
PA14 Δ PA14_56280 (<i>sadC</i>)	<i>sadC</i> in-frame deletion	27
PA14 Δ PA14_04420	PA14_04420 in-frame deletion	27
PA14 Δ PA14_20110 (<i>siaD</i>)	<i>siaD</i> in-frame deletion	27
PA14 Δ PA14_72420	PA14_72420 in-frame deletion	27
PA14 Δ PA14_42220 (<i>mucR</i>)	<i>mucR</i> in-frame deletion	27
PA14 Δ PA14_60870 (<i>morA</i>)	<i>morA</i> in-frame deletion	27
PA14 Δ PA14_66320 (<i>dipA</i>)	<i>dipA</i> in-frame deletion	27
PA14 Δ PA14_65540 (<i>fimX</i>)	<i>fimX</i> in-frame deletion	27
PA14 Δ PA14_53140 (<i>rbdA</i>)	<i>rbdA</i> in-frame deletion	27
PA14 Δ PA14_56790 (<i>bifA</i>)	<i>bifA</i> in-frame deletion	27
PA14 Δ PA14_21190	PA14_21190 in-frame deletion	27
PA14 Δ PA14_07500	PA14_07500 in-frame deletion	27
PA14 Δ PA14_71850	PA14_71850 in-frame deletion	27
PA14 Δ PA14_15810 (<i>rocR</i>)	<i>rocR</i> in-frame deletion	27
PA14 Δ PA14_36990	PA14_36990 in-frame deletion	27
PA14 Δ PA14_36260	PA14_36260 in-frame deletion	27
PA14 Δ PA14_59790 (<i>pvrR</i>)	<i>pvrR</i> in-frame deletion	27
PA14 Δ PA14_14530	PA14_14530 in-frame deletion	27
PA14 Δ PA14_63210	PA14_63210 in-frame deletion	27
PA14 Δ <i>fliC</i>	<i>fliC</i> in-frame deletion	26
PA14 Δ <i>pilA</i>	<i>pilA</i> in-frame deletion	26

thetized and catheterized with a 0.5-cm piece of polyethylene catheter tubing in the bladder. After emptying the contents of the bladder, 3×10^7 bacteria were inoculated into the bladder through the guide catheter. Mice infected with strains containing pMMB plasmids were given 1 mM IPTG through their drinking water to induce the *tac* promoter. The mice were infected for 14 days and sacrificed. The bladders and kidneys were isolated, homogenized, serially diluted, and plated on LB plates for CFU counts. One-way analysis of variance (ANOVA) was used to determine statistical significance.

RESULTS

c-di-GMP levels during initial infection affect colonization of bladder and kidneys. We are interested in determining whether genes participating in *P. aeruginosa* biofilm formation *in vitro* contribute to *in vivo* biofilm-based infection. Previous data indicated that *P. aeruginosa* formed biofilms during the murine model of CAUTI independently of the Pel and Psl exopolysaccharides (29). Here we tested whether the c-di-GMP signaling pathway, which also regulates *P. aeruginosa* biofilm formation *in vitro*, participates during CAUTI. To determine if either increasing or decreasing the concentration of c-di-GMP at the onset of infection would affect the colonization in the bladder or dissemination to the kidneys during CAUTI, mice were inoculated with wild-type PA14, strains that overexpressed DGCs (PA14 pMMB PA3702 and PA14 pMMB PA1107), and strains that overexpressed PDEs (PA14 pMMB PA3947 and PA14 pMMB PA2133). These overexpression strains were initially tested to confirm their *in vitro* biofilm phenotypes. In the absence of IPTG, DGCs PA1107 and

FIG 1 Overexpression of DGCs and PDEs during initial infection affects colonization of the bladder and kidneys. (A) *In vitro* biofilms for DGCs and PDEs expressed on pMMB plasmids in the presence or absence of 1 mM IPTG. (B and C) Bladders (B) and kidneys (C) from mice infected with PA14 (circles), PA14 pMMB PA3702 (squares), PA14 pMMB PA1107 (diamonds), PA14 pMMB PA3947 (triangles), or PA14 pMMB PA2133 (stars). Each symbol represents an individual organ. Bars represent geometric means. Asterisks indicate P values of ≤ 0.05 . Dashed lines represent the limit of detection.

PA3702 produced 2- and 10-fold more biofilm, respectively, than the PA14 empty-vector control. Meanwhile, PDEs PA2133 and PA3047 produced slightly less biofilm than the empty vector. These results indicate that the *tac* promoter of these genes allows expression in the absence of inducer. As expected, induction of DGCs PA1107 and PA3702 with IPTG resulted in an additional 18- and 2-fold increase in biofilm formation, respectively, compared to that with uninduced PA1107 and PA3702. Conversely, induction of PDEs PA2133 and PA3947 reduced biofilm formation approximately 2-fold compared to that with uninduced PA2133 and PA3947 (Fig. 1A). Compared to wild-type PA14,

overexpression of DGCs PA1107 and PA3702 increased biofilm formation approximately 30-fold, while overexpression of PDEs PA2133 and PA3947 reduced biofilm formation 5- and 2-fold, respectively (Fig. 1A).

In CAUTI, there was a higher number of CFU detected in the bladders of mice infected with PA14 pMMB PA3702 (1.4×10^6 CFU/bladder) and PA14 pMMB PA1107 (2.6×10^6 CFU/bladder) than in mice infected with wild-type PA14. In contrast, fewer CFU were detected in the bladders of mice infected with PDE strains PA14 pMMB PA3947 (1.1×10^3 CFU/bladder) and PA14 pMMB PA2133 (2.2×10^4 CFU/bladder) than in those infected with the wild type (1.5×10^5 CFU/bladder) (Fig. 1B). In the kidneys, significantly more CFU were detected in mice infected with DGC strain PA14 pMMB PA3702 (1.2×10^6 CFU/kidney) and PA14 pMMB PA1107 (3.0×10^6 CFU/kidney) than in those infected with the wild type (4.4×10^2 CFU/kidney) (Fig. 1C). In fact, CFU were detected in all kidneys from mice infected with PA14 pMMB PA3702 and PA14 pMMB PA1107 compared to the wild type, where CFU were detected in roughly half of the kidneys (Fig. 1C). In contrast, similar numbers of CFU were detected in the kidneys of mice infected with PA14 pMMB PA3947 (2.5×10^2 CFU/kidney) and PA14 pMMB PA2133 (3.6×10^1 CFU/kidney) compared to wild-type-infected mice. These data indicate that infection with *P. aeruginosa* that initially elevated c-di-GMP signaling increased colonization in the bladder and dissemination and replication in the kidneys during CAUTI. In contrast, infection with *P. aeruginosa* that initially reduced c-di-GMP signaling decreased colonization in the bladder. Together, these results indicate that c-di-GMP signaling contributes to CAUTI.

Deletion of specific GGDEF and/or EAL domain-containing genes reduces colonization of the urinary tract during CAUTI. We sought to determine the physiologically relevant DGC and/or PDE that contributes to CAUTI. Based on sequence analysis of the PA14 strain, there are 40 predicted DGCs and/or PDEs (16 containing the GGDEF domain only, 16 containing GGDEF-EAL, 5 containing EAL only, and 3 containing HD-GYP only) (26). The O'Toole lab has previously generated a collection of 40 individual in-frame deletion mutants of each of these genes and tested these mutants for phenotypic changes, including increased or decreased biofilm formation, motility, and Pel polysaccharide production (27). In this study, 24 mutants (9 containing the GGDEF domain only, 9 containing GGDEF-EAL [dual domain], 5 containing EAL only, and one containing HD-GYP only) that have been previously reported to regulate phenotypic changes *in vitro* were tested using the CAUTI model for altered colonization of the urinary tract and dissemination. Of the mutants with mutations in genes encoding only the DGC domain, the Δ PA14_26970, Δ PA14_20110 (*siaD*), and Δ PA14_72420 mutants showed a reduction of greater than 1 log for the geometric mean of the number of CFU recovered from the bladder compared to the PA14 strain (Fig. 2A). Of the mutants with mutations in genes encoding both the GGDEF and EAL domains, the Δ PA14_60870 (*morA*) and Δ PA14_07500 mutants showed a 1-log reduction in the CFU recovered from the bladder (Fig. 2A). In addition to mutants that showed reduction in colonization of the bladder, two mutants, the Δ PA14_56280 (*sadC*) and Δ PA14_56790 (*bifA*) mutants, showed an increase in the CFU recovered from the bladder that is 1 log higher than that for the PA14 strain. The mutants with mutations in genes encoding only PDE domains (EAL or HD-GYP) all performed similarly to the wild type (Fig. 2A). In contrast to coloni-

FIG 2 Deletion of specific DGCs and PDEs affects the colonization of the bladder and dissemination to the kidneys during CAUTI. Results for bladders (A) and kidneys (B) from mice infected with PA14 with in-frame deletions in genes containing the GGDEF domain only (circles), GGDEF and EAL (squares), EAL only (triangles), or HD GYP only (diamonds) are shown. Each symbol represents an individual organ. Bars represent geometric means. Dashed lines represent the limit of detection. Deletion mutants highlighted in green are those mutants with at least a 1-log increase in CFU detected in either the bladder or the kidneys compared to that for wild-type PA14. Deletion mutants highlighted in blue are those mutants with at least a 1-log decrease in CFU detected in the bladder compared to that for wild-type PA14.

zation, dissemination from the bladder to the kidneys and replication within the kidneys appears to occur stochastically in that most mice have either a disseminating infection with a high number of CFU in the kidneys or no infection at all. We have analyzed

the data by providing the geometric mean of the CFU in all kidneys (Fig. 2, solid bars) and a ratio of the number of infected kidneys to the total number of kidneys above each data set. Several of the mutants were able to disseminate and replicate at a higher

FIG 3 Type IV pili contribute to dissemination to the kidneys. Results for bladders (A) and kidneys (B) from mice infected with wild-type PA14, PA14 Δ fliC, or PA14 Δ pilA are shown. Each symbol represents an individual organ. Bars represent geometric means. Dashed lines represent the limit of detection.

frequency than the wild type and had at least a 1-log increase in the geometric mean. Of the genes encoding DGCs, Δ PA14_56280 (*sadC*) mutant dissemination and replication occurred at a frequency of 75%, and the geometric mean was 3.7×10^4 CFU/kidney, while the wild type had a dissemination and replication frequency of 21% and a geometric mean of 1.6×10^2 CFU/kidney. Mutants with mutations in genes encoding GGDEF-EAL domains, Δ PA14_65540 (*fimX*) and Δ PA14_56790 (*bifA*), had at least a 1-log increase in the number of CFU detected in the kidneys, with geometric means of 2.1×10^4 CFU/kidney and 2.6×10^4 CFU/kidney, respectively. Of all the mutants tested, the Δ PA14_56280 (*sadC*) and Δ PA14_56790 (*bifA*) mutants were the only ones that were able to colonize the bladder and kidneys better than the wild type. Most of the mutants had a similar mean CFU/kidney as the wild type (Fig. 2B). These results suggest that there are specific DGCs and/or PDEs that can either increase or decrease the colonization of the bladder and dissemination to the kidneys during CAUTI.

Requirement of flagella and type IV pili in CAUTI. Since our results suggest that a subset of DGC and/or PDE genes contributes to CAUTI, we tested whether known c-di-GMP-regulated processes contribute to CAUTI. Previously, the Pel and Psl exopolysaccharides, which mediate biofilm formation *in vitro*, were shown to be dispensable during CAUTI. We asked whether flagella and type IV pili that contribute to acute infections also participate during CAUTI. Mutants with in-frame deletion mutations of the major flagellin (Δ fliC) and major pilus (Δ pilA) were tested in the murine model of CAUTI. The Δ fliC and Δ pilA mutants colonized the bladder with means of 1.4×10^6 CFU and 1.1×10^6 CFU, respectively (Fig. 3A). These results suggest that flagella and type IV pili are not the primary determinant for *P. aeruginosa* to colonize the bladder. The Δ fliC (3 out of 16 kidneys) and Δ pilA (3 out of 16 kidneys) mutants were able to disseminate into the kidney at a frequency similar to that for PA14 (5 out of 20 kidneys). However, the geometric mean CFU of the Δ pilA mutant in the kidneys was reduced to 2.0×10^3 CFU (Fig. 3B). These results indicate that flagella are dispensable for colonization and dissemination, while type IV pili are dispensable for colonization in the bladder but contribute to dissemination.

DISCUSSION

In this study, we demonstrate that induction of DGCs immediately prior to infection enhances colonization in the bladder, indicating that c-di-GMP contributes to biofilm formation during CAUTI. In contrast, induction of PDEs immediately prior to infection reduces colonization of the bladder. Since bacteria not attached to the catheter are likely rapidly removed by urination, these results also support the idea that the c-di-GMP signaling pathway positively regulates biofilm formation during CAUTI. Similar observations have been made in other chronic infection models for *P. aeruginosa* involving implant of foreign devices. One murine model is based on the insertion of silicone catheters into the peritoneal cavity (30). The contribution of c-di-GMP signaling was tested by utilizing a strain of *P. aeruginosa* harboring an inducible PDE, YhjH, from *E. coli* (31). Twenty-four hours post-implantation, the expression of YhjH was induced by injection of arabinose into the peritoneal cavity. Strains with YhjH had more bacteria disseminate into the peritoneal cavity and the spleen than those with the vector control, indicating that the reduction in c-di-GMP levels in the bacteria correlated with detachment of *P. aeruginosa* from the preformed biofilm (31). In a separate study, a Δ yfiR mutant, which has constitutively activated YfiN DGC activity, was subjected to a subcutaneous catheter model of chronic infection (32). Despite having a reduced growth rate associated with elevated levels of c-di-GMP, the Δ yfiR mutant was able to persist within the catheters. Taken together, the previous studies and our current studies suggest that alteration of the c-di-GMP signaling pathway can shift the outcome of the infection. Increasing the c-di-GMP concentration enhances the association with the foreign body, while decreasing the c-di-GMP concentration increases the dispersion of *P. aeruginosa*.

To determine the DGC and PDE that are relevant for CAUTI, we tested 24 in-frame deletion mutants from the 40 total in the PA14 in-frame deletion mutant library of DGCs and PDEs. These strains were selected based on their *in vitro* c-di-GMP-related phenotypes, including biofilm formation, Pel polysaccharide production, and motility (27). Of the mutants tested, we found that mutants with mutations in GGDEF-containing genes (Δ PA14_02110 [*siaD*], Δ PA14_26970, and PA14_72420) and dual GGDEF-EAL-containing genes (Δ PA14_07500 and Δ PA14_60870 [*morA*]) showed a reduction in colonization in the bladder. These mutants have previously been characterized extensively for biofilm formation, Congo red binding, swimming motility, swarming motility, and twitching motility (27). Analysis of these mutants for Congo red binding indicates that the Δ PA14_07500 and Δ PA14_60870 (*morA*) mutants failed to produce the Pel polysaccharide, whereas the Δ pa14_02110 (*siaD*) and Δ pa14_26970 mutants increased production of Pel. Analysis of these mutants for flagellum-mediated swimming and swarming motility revealed that all five of the mutants were defective in swimming and swarming motility. Analysis of these mutants for twitching motility indicated that the Δ PA14_02110 (*siaD*), Δ PA14_26970 and PA14_72420 mutants had a reduced twitch zone, whereas the Δ PA14_07500 and Δ PA14_60870 (*morA*) mutants had wild-type twitching motility. Comparison of the *in vivo* CAUTI results with the *in vitro* assays indicate that a common defect is in swimming motility. However, this is likely not the case, for two reasons. First, the Δ PA14_14530, Δ PA14_21190 and Δ PA14_66320 (*dipA*) mutants, with similar swimming and swarming defects (27), were able to colonize dur-

ing CAUTI similarly to the wild type. Second, the $\Delta fliC$ strain was able to cause CAUTI similarly to PA14. Since mutations in the exopolysaccharides (29), flagella, and type IV pili are not required for *P. aeruginosa* to cause CAUTI, the c-di-GMP-regulated process that contributes to CAUTI is likely through an unknown mechanism that is likely specific to the urinary tract. Furthermore, the moderate reduction in bacterial burden for any of the single mutants with mutations in genes encoding DGC and PDE suggests that there is functional redundancy of DGCs in the urinary tract. Future testing of combinatorial mutants that show moderate defects may resolve this apparent redundancy. Furthermore, there are an additional 16 DGC and/or PDE mutants out of the 40 total mutants that were not tested in this study and were previously shown not to have *in vitro* phenotypes that differed from those of the wild type. These mutants should be tested in future studies to determine their involvement in CAUTI.

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