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BACTERIOPHAGES ARE SYNERGISTIC WITH BACTERIAL INTERFERENCE FOR THE PREVENTION OF *PSEUDOMONAS AERUGINOSA* BIOFILM FORMATION ON URINARY CATHETERS

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Abstract

Aims—We hypothesized that pretreating urinary catheters with benign *Escherichia coli* HU2117 plus an anti-pseudomonal bacteriophage (ΦE2005-A) would prevent *P. aeruginosa* biofilm formation on catheters – a pivotal event in the pathogenesis of catheter-associated urinary tract infection (CAUTI).

Methods and Results—Silicone catheter segments were exposed to one of four pretreatments (sterile media; *E. coli* alone; phage alone; *E. coli* plus phage), inoculated with *P. aeruginosa,* then incubated up to 72 hours in human urine before rinsing and sonicating to recover adherent bacteria. *P. aeruginosa* adherence to catheters was almost 4 $\log_{10}$ units lower when pretreated with *E. coli* plus phage compared to no pretreatment ($P < 0.001$) in 24-hour experiments and more than 3 $\log_{10}$ units lower in 72-hour experiments ($P < 0.05$). Neither *E. coli* nor phage alone generated significant decreases.

Conclusions—The combination of phages with a pre-established biofilm of *E. coli* HU2117 was synergistic in preventing catheter colonization by *P. aeruginosa.*

Significance and Impact of Study—We describe a synergistic protection against colonization of urinary catheters by a common uropathogen. *E. coli*-coated catheters are in clinical trials; adding phage may offer additional benefit.

Keywords

Escherichia coli; Pseudomonads; Bacteriophages; Biofilms; Probiotics

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CONFLICT OF INTERESTS
The authors do not have any financial or other associations that might pose a conflict of interest.

INTRODUCTION

Catheter-associated urinary tract infection (CAUTI), one of the most common hospital-associated infections, is increasingly caused by highly resistant gram-negative organisms (Hidron et al., 2008). *Pseudomonas aeruginosa* is an important cause of CAUTI and is also one of the most problematic multidrug-resistant (MDR) gram-negative pathogens (Giamarellou and Poulakou, 2009, McGowan, 2006). Many strains of *P. aeruginosa* are resistant to all commonly used antimicrobial agents (McGowan, 2006), so there is a critical need for novel approaches to suppress the growth of this pathogen in the bladder. In the numerous patients who have a legitimate requirement for long-term urinary catheter use, no strategy is truly effective at preventing bladder invasion by urinary pathogens (Trautner et al., 2005).

CAUTI is also a biofilm-related infection, which contributes to the low efficacy of standard antimicrobial therapy (Donlan and Costerton, 2002, Maki and Tambyah, 2001). Organisms in biofilms are not consistently eradicated by the same therapeutically achievable levels of antimicrobial agents to which their planktonic counterparts are susceptible, and biofilms may provide a niche for antimicrobial-resistant organisms (Donlan and Costerton, 2002). Eradication of pathogenic organisms from a urinary tract with ongoing catheterization is nearly impossible; antibiotic use leads to temporary suppression followed by emergence of resistant strains (Maki and Tambyah, 2001).

Our bacterial interference approach to CAUTI treatment and prevention exploits the tenacity of biofilms by utilizing urinary catheters coated with a biofilm of *Escherichia coli* HU2117 (a derivative of *E. coli* 83972) in order to colonize the catheter and bladder with this benign, and potentially protective, strain (Prasad et al., 2009, Trautner et al., 2007). Our previous in vitro work with *E. coli* HU2117-coated catheters supports a potential role for this organism in preventing recurrent CAUTI against such diverse organisms as enterococci, *Candida*, *Providencia*, and pathogenic *E. coli* (Prasad et al., 2009, Trautner et al., 2008, Trautner et al., 2002, Trautner et al., 2003, Trautner et al., 2007). However, in clinical trials, some biofilm-forming organisms, notably *P. aeruginosa*, overgrew the *E. coli* HU2117 on urinary catheters (Prasad et al., 2009, Trautner et al., 2007). Here, we explore the potential of lytic bacteriophages (‘phages’) to improve the persistence of the benign *E. coli* on the catheter and in the bladder. The difficulty of eradicating biofilm infections from medical devices has prompted studies of the use of lytic phages for this application (Donlan, 2009). Phages are viruses that are highly specific to one or a few related target bacterial species; they are self-replicating in the presence of host cells and self-limiting in their absence; phages are frequently effective against multidrug-resistant organisms since they kill cells by different molecular mechanisms; they cannot infect eukaryotic cells and can be selected so as not to infect normal, beneficial microflora.

Many bacteriophages are known to exhibit narrow host range, and their host bacteria will develop resistance (Lenski and Levin, 1985, Mizoguchi et al., 2003), making total eradication of the target species unlikely. However, our goal is not to eradicate the pathogenic species (*P. aeruginosa*), but rather to shift the ecological balance of the urinary tract early on, such that growth of the benign biofilm is favored over growth of uropathogens. Emergence of phage-resistant populations in co-evolving host-phage systems takes some time, and can be slowed through the use of phage mixtures (Lenski and Levin, 1985, Maki and Tambyah, 2001, McGowan, 2006, Labrie et al., 2010). Additionally, in *P. aeruginosa,* phage resistance often arises through modification of bacterial surface structures whose loss reduces bacterial virulence, such as the type IV pili necessary for biofilm formation by *P. aeruginosa* (Labrie et al., 2010, Chibeu et al., 2009). Thus, development of phage resistance may come at the cost of biofilm-forming ability.

We explored the following hypotheses: (1) pretreating urinary catheters with benign *E. coli* HU2117 plus an anti-pseudomonal phage will prevent pathogen colonization of the catheter surface when challenged with *P. aeruginosa* in the host range of the phage, (2) phage-resistant variants of *P. aeruginosa* arising during these bacterial interference experiments will have diminished virulence either in terms of growth rate or catheter adherence. Our experimental design modeled an "at the bedside" treatment approach in which a cocktail of phages targeting pre-existing bladder populations of uropathogenic species would be added to the bladder at the time of insertion of a urinary catheter coated with benign *E. coli* HU2117.

MATERIALS AND METHODS

Organisms and culture conditions

E. coli HU2117 is a derivative of *E. coli* 83972, which was originally isolated in a 12-year old Swedish girl who carried it asymptomatically in her bladder (Andersson et al., 1991). *E. coli* HU2117 has a deletion in *papG* and thus cannot express functional P-fimbriae, associated with pyelonephritis (Hull et al., 2002). Our challenge pathogen was *P. aeruginosa* E2005-A, a clinical isolate from the CDC Clinical and Environmental Microbiology Branch culture collection. This organism was the host for phage ΦE2005-A, which was collected from the Snapfinger Creek Wastewater Treatment Plant in Dekalb County, Georgia, as previously described (Fu et al., 2010). Bacterial stocks were stored in Luria broth (LB) plus 10% glycerol at −80°C and propagated on Trypticase Soy Agar (TSA) plates (Becton, Dickinson and Company BBL™ Trypticase™ Soy Agar, BD211043) at 37°C, as needed. *E. coli* and *P. aeruginosa* were separately subcultured first on solid media (TSA), then in LB broth overnight (rocking, 37°C). This overnight liquid culture was diluted to an optical density at 600 nm (OD600) of 0.2–0.3 (approximately 10^8 organisms per mL) prior to use. Phage ΦE2005-A was propagated using the soft agar overlay method and stored at 4°C, shielded from light (Adams, 1959). Human urine was collected from one female donor, sterilized by filtration (0.22 μm), and stored in large-volume batches at 4°C until needed. We chose to work in real urine as our prior bacterial interference studies have all been done in human urine, both *in vitro* and *in vivo*.

Phage susceptibility of clinical *P. aeruginosa* isolates

We determined the susceptibility of 18 clinical isolates of urinary *P. aeruginosa* to the 6 phages in our library in order to choose a phage for the bacterial interference experiments. Lytic assays were performed using the spot test method (Kutter and Sulakvelidze, 2005). Briefly, bacterial lawns of each *P. aeruginosa* strain were seeded in soft agar that was allowed to solidify for at least 5 minutes; 15 μL droplets of phage suspensions (at approximately 10^8 PFU mL^{-1}) were placed on the agar and allowed to air dry for 30 minutes. Plates were incubated overnight, and each phage zone of lysis was graded on a 0–4 scale of opaque (0, no lysis) to clear (4, total lysis).

Experimental set up and protocol

24-hour exposure experiments—All-silicone urinary catheters (BARDEX Uncoated Silicone Foley Catheters, Bard Medical Division, Covington, GA, Cat. No. 165812) were each sliced into four segments (approximately 6 cm each) using sterile technique. Each catheter segment underwent one of four pretreatment conditions in an individual tube of 50 mL LB broth (Figure 1) prior to exposure to the challenge pathogen. Specifically, in step 1, catheter segments were aseptically transferred to tubes containing one of the following: i) sterile media (control); ii) 10^5 *E. coli* colony-forming units (CFU) mL^{-1}; iii) 10^8 PFU mL^{-1} phage ΦE2005-A; iv) 10^5 *E. coli* CFU mL^{-1} plus 10^8 PFU mL^{-1} phage ΦE2005-A. After a 24-hour incubation at 37°C with rocking, each catheter segment was transferred into a fresh

tube of LB broth and inoculated with *P. aeruginosa* at a concentration of 10^5 CFU mL^{-1} for a 30-minute incubation at 37°C with rocking. Catheter segments were then moved to fresh tubes of filter-sterilized human urine for the final 24-hour incubation at 37°C with rocking. Catheters were harvested by immersing each 6-cm catheter segment in 3 different tubes of 50 mL phosphate-buffered saline (PBS), followed by flushing the inner catheter lumen with 30 mL PBS. Each segment was then aseptically cut into three 1-cm sub-segments; each sub-segment was placed into a tube containing 1 mL of PBS plus 0.01% sodium dodecyl sulfate (SDS) and was sonicated for 10 min. at 50 Hz. The sonicated fluid was plated onto selective media (TSA and LB agar plus 100 μg mL^{-1} ampicillin; the LB plus ampicillin media selected for *P. aeruginosa*, which is innately resistant to ampicillin while prohibiting growth of *E. coli* HU2117) and incubated overnight to quantify the numbers of adherent *P. aeruginosa* and *E. coli* (Trautner et al., 2002, Trautner et al., 2003). The detection limit for this method was 1 CFU per 1-cm catheter segment sample. Planktonic bacteria were quantified by plating dilutions of incubated urine on the same types of selective media (TSA and LB agar plus 100 μg mL^{-1} ampicillin) and incubating overnight. At each step in the protocol, plaque assays were performed using the soft agar overlay method to enumerate phage titers (Kutter and Sulakvelidze, 2005).

Extended time course experiments: 24-, 48-, and 72-hour exposures—To investigate how the phage-enhanced interference effect would function over a longer duration of time, the interference experiments were extended to 72 hours, with daily movement of catheters to fresh filter sterilized urine. In these experiments, three (rather than one) catheter segments were placed in each 50-mL tube at the start of each experiment. At the 24-hour and 48-hour time points, one catheter segment was removed for processing as previously described, while the remaining catheter segment(s) were transferred to a fresh 50-mL tube of urine and incubated further.

Statistical analyses—Viable counts from catheter sub-segments and from spent media were log-transformed using the following algorithm: $\log_{10}$ [raw data + 1]. This transformation normalized the data and avoided the undefined function $\log_{10}$ 0. For each catheter segment in each tube, the mean of the three 1-cm catheter subsections was taken as the representative value for that catheter in that particular experiment. All experiments were repeated at least 4 times. Analyses of variance (ANOVAs) were done to compare the mean numbers of *P. aeruginosa* adherent to catheters or recovered from the urine under the four pretreatment conditions, followed by the appropriate post-hoc comparison tests (Holm-Sidak method for comparisons to the “no pretreatment” control, or Dunn’s method for all pairwise comparisons). The correlation between adherent and planktonic *P. aeruginosa* was determined using the Pearson correlation test. Numbers of *E. coli* adherent to, or in the urine surrounding, phage-treated versus untreated catheters were compared by T tests. Statistical comparisons were performed using SigmaPlot version 11.0 software (Systat Software, Inc., San Jose, California).

Characterization of potentially phage-resistant variants of *P. aeruginosa*

Biochemical testing—*P. aeruginosa* isolates recovered from catheters or from urine after 24 or more hours of exposure to ΦE2005-A were subcultured onto TSA before storing at −80°C in LB with 10% glycerol. These variants were tested for oxidase activity, ability to grow on MacConkey agar and LB agar with 100 μg mL^{-1} of ampicillin, and for ability to produce distinct colony morphology and pigments on Cetrimide agar (EMD Chemicals, Gibbstown, NJ). Strains that were oxidase positive and grew on both MacConkey and LB agars, and produced colony morphology characteristic of *P. aeruginosa* on Cetrimide agar were identified using the Vitek II automated identification system (BioMérieux, Durham, North Carolina).

Phage susceptibility—We also characterized the susceptibility of potentially phage ΦE2005-A-resistant variants of *P. aeruginosa* to seven anti-pseudomonal phages (ΦE2005-A, ΦPaer4, ΦW2005-B, ΦE2005-C, ΦPaer14, and ΦM4) (Table 1). Lytic assays were performed using the spot test method as before (Kutter and Sulakvelidze, 2005). Each phage zone of lysis was graded on a 0–4 scale of opaque (0, no lysis) to clear (4, total lysis).

Growth curve assay—Standard growth curves of all potentially phage ΦE2005-A-resistant *P. aeruginosa* variants and the parent strain *P. aeruginosa* E2005-A were performed in filter-sterilized human urine: growth curve media was sampled for plate counts every hour for 8 hours and again at 24 hours. Doubling times of 4 repetitions per isolate were compared to the parent strain of *P. aeruginosa* using the Wilcoxon Rank Sum Test.

Catheter biofilm formation assay—Two of the slowest-growing and two of the fastest-growing experimental variants (as determined by length of doubling time) were tested alongside parent strain *P. aeruginosa* E2005-A for biofilm formation on non-pretreated catheters through overnight incubation in filter-sterilized human urine. Using the biofilm growth protocol already described, at least 3 repetitions were completed for each variant, with data analysis as per the interference experiments above.

RESULTS

Phage host range in clinical *P. aeruginosa* isolates

We chose phage ΦE2005-A for our bacterial interference protocol because ΦE2005-A lysed over 50% of the clinical *P. aeruginosa* isolates tested and made clear, well-demarcated plaques, while its host *P. aeruginosa* also had distinct colonies on agar plates.

Interference experiments: after a 24-hour exposure

The *E. coli* plus phage combination was the only pretreatment condition that had a significant effect on the adherence of *P. aeruginosa* ($P < 0.001$, ANOVA followed by Holm-Sidak) (Figure 2). *P. aeruginosa* adherence to catheters was almost 4 $\log_{10}$ units lower following pretreatment with *E. coli* plus phage (0.90 $\log_{10}$ CFU cm^{-1}), in comparison to no pretreatment (4.73 $\log_{10}$ CFU cm^{-1}). In several experiments, no *P. aeruginosa* was recovered from catheters that had been pretreated with *E. coli* plus phage. The numbers of *E. coli* on catheters were not significantly affected by the presence of phage in the media ($P = 0.4$, T test).

Extended time course experiments: after exposure for 24, 48, and 72 hours

In extended time course experiments, the mean number of *P. aeruginosa* isolated from *E. coli* plus phage-pretreated catheters was more than 3 $\log_{10}$ units lower than that collected from untreated catheters at 24 hours (1.2 $\log_{10}$ vs. 4.3 $\log_{10}$ CFU cm^{-1}), 48 hours (0.5 $\log_{10}$ vs. 5.3 $\log_{10}$ CFU cm^{-1}), and 72 hours (1.6 $\log_{10}$ vs. 6.1 $\log_{10}$ CFU cm^{-1}) ($P < 0.05$ for all, ANOVA, followed by Dunn's Test) (Figure 3A). The reduction ranged from 3.6 fold to 11.7 fold. Averages of adherent *P. aeruginosa* per pretreatment condition differed significantly at 24-, 48-, and 72-hour time points (ANOVA, $P = 0.004$, $P < 0.001$, $P < 0.001$, respectively), with the significance arising from the comparison of the *E. coli* plus phage pretreatment to the control condition (no pretreatment). Of note, in the control condition, the numbers of catheter-adherent *P. aeruginosa* increased 1.8 $\log_{10}$ units over time from 24 to 72 hours (4.3 $\log_{10}$ CFU cm^{-1} vs. 6.1 $\log_{10}$ CFU cm^{-1}, $P = 0.03$, Mann-Whitney U Statistic). No such increase was seen for *P. aeruginosa* on *E. coli* alone-pretreated catheters (4.6 $\log_{10}$ vs. 5.1 $\log_{10}$ CFU cm^{-1}, $P = 0.29$, T test). However, at 72 hours, the differences between catheter-adherent *P. aeruginosa* in the control and *E. coli* pretreatment groups did not differ significantly (6.1 $\log_{10}$ vs. 5.2 $\log_{10}$ CFU cm^{-1} respectively, $P = 0.08$, T test). Overall,

neither the *E. coli*-alone nor the phage-alone pretreatment produced any significant reductions in *P. aeruginosa* recovery from catheters when compared to control catheters at any time point.

For several replicates, catheter-associated *P. aeruginosa* were not detected at all for the *E. coli* plus phage-treated catheters. Pooling results from all tubes that contained phage in all experimental replicates, we found that *P. aeruginosa* was completely eradicated from catheters in 8 of 27 (30%) experiments in which catheters had been pretreated with *E. coli* plus phage, in contrast to 0 of 14 (0%) experiments in which catheters had not been pretreated with *E. coli* plus phage ($P = 0.02$, Fisher's Exact Test). Again, the number of *E. coli* collected from *E. coli* only-pretreated catheters did not differ significantly from that recovered from *E. coli* plus phage-pretreated catheters at 24, 48, or 72 hours (T test, $P = 0.074$, $P = 0.268$, $P = 0.252$, respectively) (Figure 3B). *E. coli* biofilm on *E. coli* only-pretreated catheters increased by approximately 1 $\log_{10}$ over time, from 4.6 $\log_{10}$ at 24 hours to 5.6 $\log_{10}$ at 72 hours ($P = 0.04$, T test). The number of biofilm-associated *P. aeruginosa* correlated positively with the number of planktonic *P. aeruginosa* for all three time points (Pearson Correlation, $P \ll 0.001$) (Figure 3C).

Characterization of phage-resistant experimental variants

Thirteen potentially phage-resistant isolates of *P. aeruginosa* E2005-A (recovered from phage-treated catheters) were subcultured and stored for further characterization. Many of these variants resembled their parent strain in colony morphology but were collected because they had grown in a tube that contained the phage and thus could have become resistant to that phage. Other isolates demonstrated considerable variety in terms of colony shape, size, and color. All variants were identified as *P. aeruginosa*.

Phage susceptibility of experimental P. aeruginosa isolates—The average lytic score for the thirteen potentially phage-resistant variants of *P. aeruginosa* E2005-A when tested with ΦE2005-A was 1.4, in contrast to the lytic score of 4 for the parent strain (completely clear plaques). Two variants were fully resistant to ΦE2005-A, three showed barely any clearing in plaques, and one remained fully susceptible to ΦE2005-A. The other seven variants displayed diminished susceptibility to ΦE2005-A in the form of semi-opaque plaques.

Growth rates—The doubling time of the parent strain *P. aeruginosa* E2005-A was 33 +/− 7 minutes, while the average doubling time of experimental variants was 40 +/− 10 minutes ($P = 0.27$, Wilcoxon Rank Sum Test) (Figure 4).

Biofilm formation—The two slowest-growing (100909nF and 101127nE) and two of the faster-growing experimental variants (100806nE and 101029bF) were compared with the parent strain for ability to form biofilms on urinary catheters after overnight incubation in filter-sterilized human urine. Mean doubling times of these 4 variants were as follows (4 repetitions): 49 minutes for 100909nF, 52 minutes for 101127nE, 35 minutes for 100806nE, and 39 minutes for 1010029bF. Significantly fewer catheter-adherent organisms were recovered from slow growers 100909nF and 101127nE than from the parent *P. aeruginosa* (3.38 $\log_{10}$ and 3.13 $\log_{10}$ respectively, versus 4.8 $\log_{10}$ CFU cm^{-1} for the parent strain), ($P < 0.05$ for both comparisons to the parent strain using ANOVA followed by Holm-Sidak); fast growers 100806nE and 101029bF did not have significantly reduced biofilm formation compared to the parent strain (Figure 5).

DISCUSSION

In our previous in vitro bacterial interference studies, we found that *E. coli* 83972 (the parent strain for HU2117) itself was able to significantly impede catheter colonization by the following pathogens: uropathogenic *E. coli*, *Candida albicans*, *Enterococcus faecalis*, and *Providencia stuartii* (Trautner et al., 2002, Trautner et al., 2003). However, we did not challenge the *E. coli*-coated catheters *in vitro* with *P. aeruginosa* in those studies, and *in vivo* we found that this aggressive biofilm-forming organism could overgrow the *E. coli* HU2117 (Trautner and Darouiche, 2002, Prasad et al., 2009). The results of our current study imply that the combination of *E. coli* HU2117 and anti-pseudomonal phage could be effective at preventing catheter colonization by *P. aeruginosa*.

Our results demonstrate that *E. coli* HU2117 and phage ΦE2005-A had a strongly synergistic effect on preventing urinary catheter colonization by *P. aeruginosa*, both in overnight experiments and when the pathogen exposure time was lengthened to 72 hours. *P. aeruginosa* colonization of catheters was completely prevented in 30% of experiments in which the phage plus *E. coli* combination was used. In those experiments where colonization was not completely prevented, *P. aeruginosa* populations were several orders of magnitude lower than in all other treatments. Only the combination treatment significantly reduced *P. aeruginosa* biofilm formation. This is the first report of a synergistic effect of bacteriophage and a probiotic bacterium on prevention of pathogenic biofilm formation.

Consistent with the observations that motivated this study, *E. coli* HU2117 alone did not prevent catheter colonization by *P. aeruginosa*. However, *E. coli* HU2117 alone did have some interference effect on *P. aeruginosa*, preventing the biofilm of this organism from increasing between 24 and 72 hours (in contrast to the untreated catheters). The inability of the phage-only treatment to prevent *P. aeruginosa* growth on the catheter was unexpected though, since Fu et al. found that phage alone could significantly decrease catheter adherence by *P. aeruginosa* by over 2 logs in a flowing model using diluted tryptic soy broth (Fu et al., 2010). The difference may be related to the presence of a hydrogel coating which enabled adsorption of the phages, or the use of a phage cocktail in the Fu et al. studies. Fu et al. hypothesized that phages were associating with the hydrogel coating on the catheters used in that study, whereas the current work used uncoated silicone catheters. In several instances, the *E. coli* HU2117 biofilm increased by more than 1 log over 72 hours of incubation, as we observed in human studies (Prasad et al., 2009). Unsurprisingly, the presence of anti-pseudomonal phage did not substantially affect *E. coli* HU2117 population sizes.

The results of the present study support our original hypothesis that the combination of phage and bacterial interference will reduce biofilm formation by *P. aeruginosa* on urinary catheters to a significantly greater extent than either phage treatment or treatment by bacterial interference alone. Phage alone was clearly insufficient to kill all *P. aeruginosa* cells prior to mixed biofilm formation on the catheter, and similarly, *E. coli* was unable to out-compete *P. aeruginosa* during biofilm formation. It is likely that in the combination *E. coli* plus phage pretreatment, the phage reduced the initial *P. aeruginosa* population sufficiently to allow the *E. coli* biofilm to become further established. As a consequence, a mixed biofilm does not appear to have formed, or when it did the only *P. aeruginosa* cells recovered were phage-resistant mutants, and those in low numbers. This suggests that if the initial *P. aeruginosa* population is held in check, the *E. coli* biofilm can develop and mitigate biofilm formation by *P. aeruginosa*, possibly due to reduced competitive ability of the phage-resistant mutants. The observed correlation between numbers of *P. aeruginosa* on catheters and in urine supports this numbers-based theory, as the correlation probably results from a cyclical seeding process from catheter to urine and back. Harcombe and Bull found

that addition of a phage specific for *E. coli* in an *E. coli/Salmonella enterica* planktonic community resulted in a significant reduction in its host strain even though *E. coli* densities with phage present reached high levels in the absence of *S. enterica* (Harcombe and Bull, 2005). Kay et al. also observed a similar effect on *P. aeruginosa* in a *P. aeruginosa/E. coli* culture in the presence of a *P. aeruginosa*-specific phage (Kay et al., 2011). Both of these studies suggest that the combined effect of lytic phage infection and microbial competition can result in the predominance of one species in the biofilm, as observed in the present study.

Fu et al. addressed the issue of the rapid emergence of phage-resistant variants of *P. aeruginosa* by pretreating urinary catheters with a phage cocktail (Fu et al., 2010). We found that adding *E. coli* HU2117 to the catheter pretreatment is a highly effective alternative approach, since in 30% of the experiments in which both *E. coli* and phage had been applied, no *P. aeruginosa* was found on the catheter. A promising future direction for our interference studies will be to combine a cocktail of multiple phages with the *E. coli*-coated catheters, taking advantage of two approaches to maximize the pathogen suppression.

Despite the large reduction in *P. aeruginosa* growth following *E. coli* plus phage pretreatment, we did still observe frequent emergence of phage-resistant variants of *P. aeruginosa* in that context. Although the absolute doubling times of phage-resistant isolates of *P. aeruginosa* were all longer than for the parent strain, the wide standard deviations precluded finding a significant difference in growth rates. Nevertheless, the finding that slower-growing phage-resistant variants of *P. aeruginosa* had reduced virulence in terms of catheter adherence is a finding with potential clinical relevance, and is in accord with several other recent studies of the effect of phage exposure upon bacterial virulence. Phage resistance often arises through modification of bacterial surface structures (Labrie et al., 2010). Type IV pili and their associated twitching motility are essential for biofilm formation by *P. aeruginosa* (O'Toole and Kolter, 1998). In Chibeu et al.'s studies of another *P. aeruginosa*-phage pair, *P. aeruginosa* PAO1 and ΦKMV, the loss of type IV pili conferred resistance to phage ΦKMV but also resulted in diminished twitching motility (Chibeu et al., 2009). A mouse study of phage therapy for *Salmonella enterica* found a similar association of the acquisition of phage resistance with the loss of virulence (Capparelli et al., 2010). In our interference experiments, our goal is not to eradicate the pathogenic species, but rather to shift the ecological balance so that the benign *E. coli* can persist and protect the catheter and the bladder. Virulence modification in terms of biofilm formation suits this purpose very well.

In summary, pretreating urinary catheters with a benign *E. coli* biofilm in addition to anti-pseudomonal phages inhibits colonization by *P. aeruginosa* more effectively than either the *E. coli* biofilm or the phage pretreatment alone. This synergistic effect is promising when considering the application of this system in a clinical setting, where the phage may reinforce the ability of our benign *E. coli* biofilm to prevent uropathogen adherence and colonization.

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References

Adams, M. Bacteriophages. New York: Interscience Publishers; 1959.

Andersson P, Engberg I, Lidin-Janson G, et al. Persistence of *Escherichia coli* bacteriuria is not determined by bacterial adherence. Infect Immun. 1991; 59:2915–21. [PubMed: 1879917]

Capparelli R, Nocerino N, Iannaccone M, et al. Bacteriophage therapy of *Salmonella enterica*: a fresh appraisal of bacteriophage therapy. J Infect Dis. 2010; 201:52–61. [PubMed: 19929381]

Chibeu A, Ceyssens PJ, Hertveldt K, et al. The adsorption of *Pseudomonas aeruginosa* bacteriophage phiKMV is dependent on expression regulation of type IV pili genes. FEMS Microbiol Lett. 2009; 296:210–8. [PubMed: 19459952]

Donlan RM. Preventing biofilms of clinically relevant organisms using bacteriophage. Trends Microbiol. 2009; 17:66–72. [PubMed: 19162482]

Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002; 15:167–93. [PubMed: 11932229]

Fu W, Forster T, Mayer O, et al. Bacteriophage cocktail for the prevention of biofilm formation by Pseudomonas aeruginosa on catheters in an in vitro model system. Antimicrob Agents Chemother. 2010; 54:397–404. [PubMed: 19822702]

Giamarellou H, Poulakou G. Multidrug-resistant Gram-negative infections: what are the treatment options? Drugs. 2009; 69:1879–901. [PubMed: 19747006]

Harcombe WR, Bull JJ. Impact of phages on two-species bacterial communities. Applied and Environmental Microbiology. 2005; 71:5254–9. [PubMed: 16151111]

Hidron AI, Edwards JR, Patel J, et al. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. Infect Control Hosp Epidemiol. 2008; 29:996–1011. [PubMed: 18947320]

Hull RA, Donovan WH, Del Terzo M, et al. Role of type 1 fimbria- and P fimbria-specific adherence in colonization of the neurogenic human bladder by Escherichia coli. Infect Immun. 2002; 70:6481–4. [PubMed: 12379733]

Kay MK, Erwin TC, Mclean RJ, et al. Bacteriophage ecology in *Escherichia coli* and *Pseudomonas aeruginosa* mixed-biofilm communities. Appl Environ Microbiol. 2011; 77:821–9. [PubMed: 21131510]

Kutter, E.; Sulakvelidze, A., editors. Bacteriophages: biology and applications. Boca Raton, Florida: CRC Press; 2005.

Labrie SJ, Samson JE, Moineau S. Bacteriophage resistance mechanisms. Nat Rev Microbiol. 2010; 8:317–27. [PubMed: 20348932]

Lenski R, Levin B. Constaints on the coevolution of bacteria and virulent phage--a model, some experiments, and predictions for natural communities. Am Nat. 1985; 125:585–602.

Maki D, Tambyah P. Engineering out the risk of infection with urinary catheters. Emerging Infectious Diseases. 2001; 7:1–13. [PubMed: 11266288]

McGowan JE Jr. Resistance in nonfermenting gram-negative bacteria: multidrug resistance to the maximum. Am J Med. 2006; 119:S29–36. discussion S62–70. [PubMed: 16735148]

Mizoguchi K, Morita M, Fischer CR, et al. Coevolution of bacteriophage PP01 and *Escherichia coli* O157:H7 in continuous culture. Appl Environ Microbiol. 2003; 69:170–6. [PubMed: 12513992]

O'toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol. 1998; 30:295–304. [PubMed: 9791175]

Prasad A, Cevallos ME, Riosa S, et al. A bacterial interference strategy for prevention of UTI in persons practicing intermittent catheterization. Spinal Cord. 2009; 47:565–9. [PubMed: 19139758]

Trautner BW, Cevallos ME, Li H, et al. Increased expression of type-1 fimbriae by nonpathogenic *Escherichia coli* 83972 results in an increased capacity for catheter adherence and bacterial interference. J Infect Dis. 2008; 198:899–906. [PubMed: 18643750]

Trautner BW, Darouiche RO. Prevention of urinary tract infection in patients with spinal cord injury. J Spinal Cord Med. 2002; 25:277–83. [PubMed: 12482169]

Trautner BW, Darouiche RO, Hull RA, et al. Pre-inoculation of urinary catheters with *Escherichia coli* 83972 inhibits catheter colonization by *Enterococcus faecalis*. J Urol. 2002; 167:375–9. [PubMed: 11743359]

Trautner BW, Hull RA, Darouiche RO. *Escherichia coli* 83972 inhibits catheter adherence by a broad spectrum of uropathogens. Urology. 2003; 61:1059–62. [PubMed: 12736047]

Trautner BW, Hull RA, Darouiche RO. Prevention of catheter-associated urinary tract infection. Curr Opin Infect Dis. 2005; 18:37–41. [PubMed: 15647698]

Trautner BW, Hull RA, Thornby JI, et al. Coating urinary catheters with an avirulent strain of Escherichia coli as a means to establish asymptomatic colonization. Infect Control Hosp Epidemiol. 2007; 28:92–4. [PubMed: 17230395]

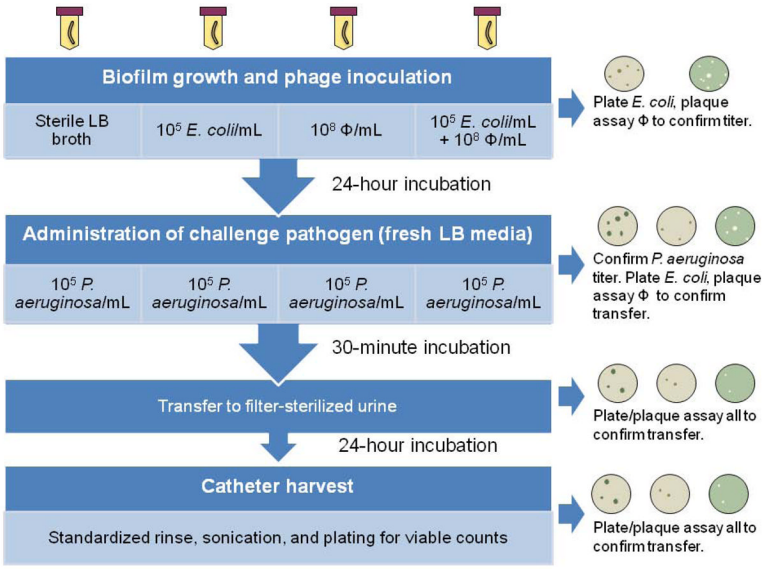

Figure 1. Experimental protocol

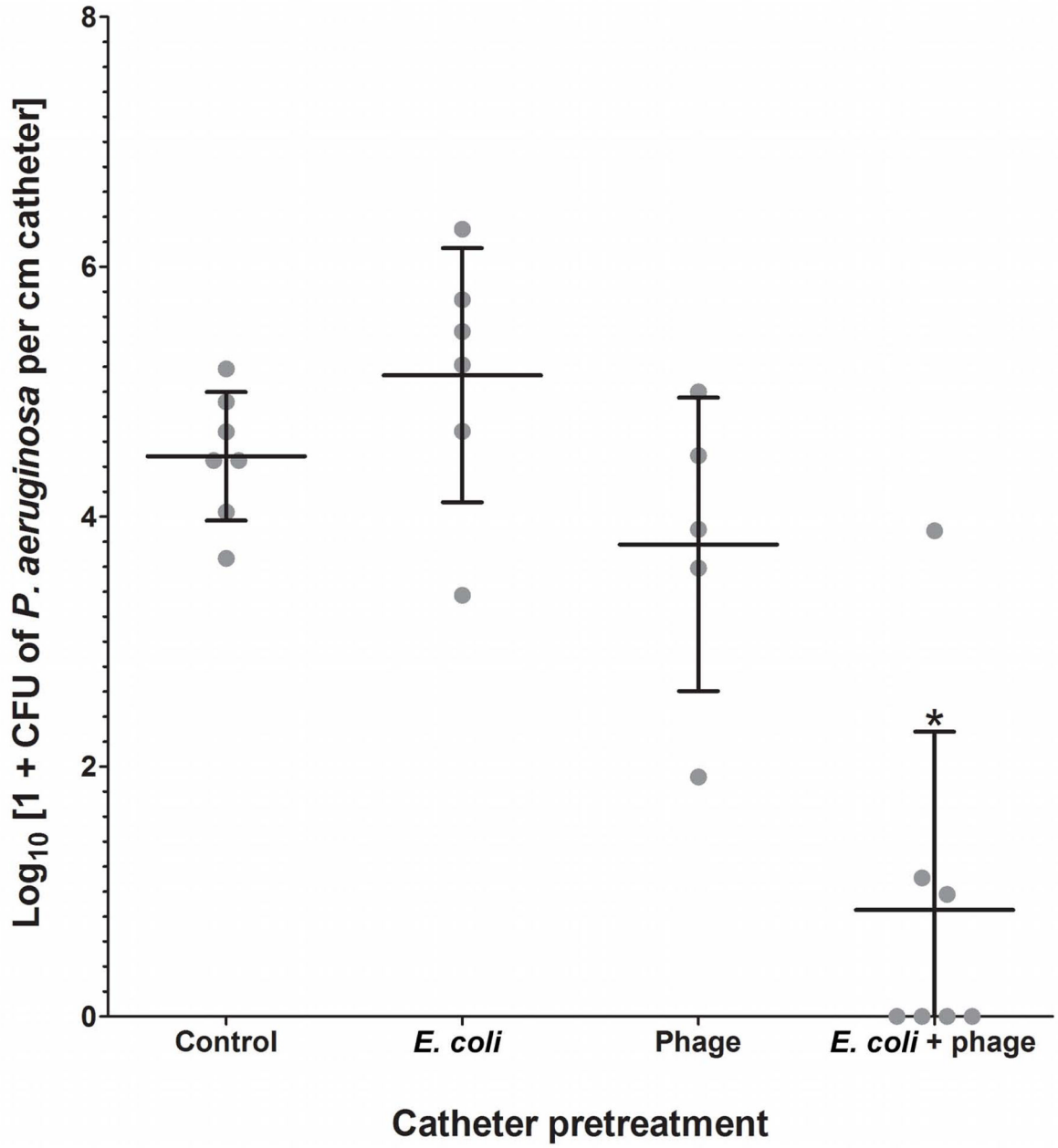

Figure 2. Effect of catheter pretreatment on biofilm formation by *P. aeruginosa* after a 24-hour exposure

Each dot represents the mean *P. aeruginosa* recovered from three catheter sub-segments in one experimental repetition, while the longer horizontal lines indicate averages of all repetitions. Error bars indicate standard deviation of the mean (n = 5 for phage-only treatment; 6 for *E. coli*-only treatment; 7 for untreated control and *E. coli* + phage treatment) (*P < 0.001, ANOVA followed by Holm-Sidak).

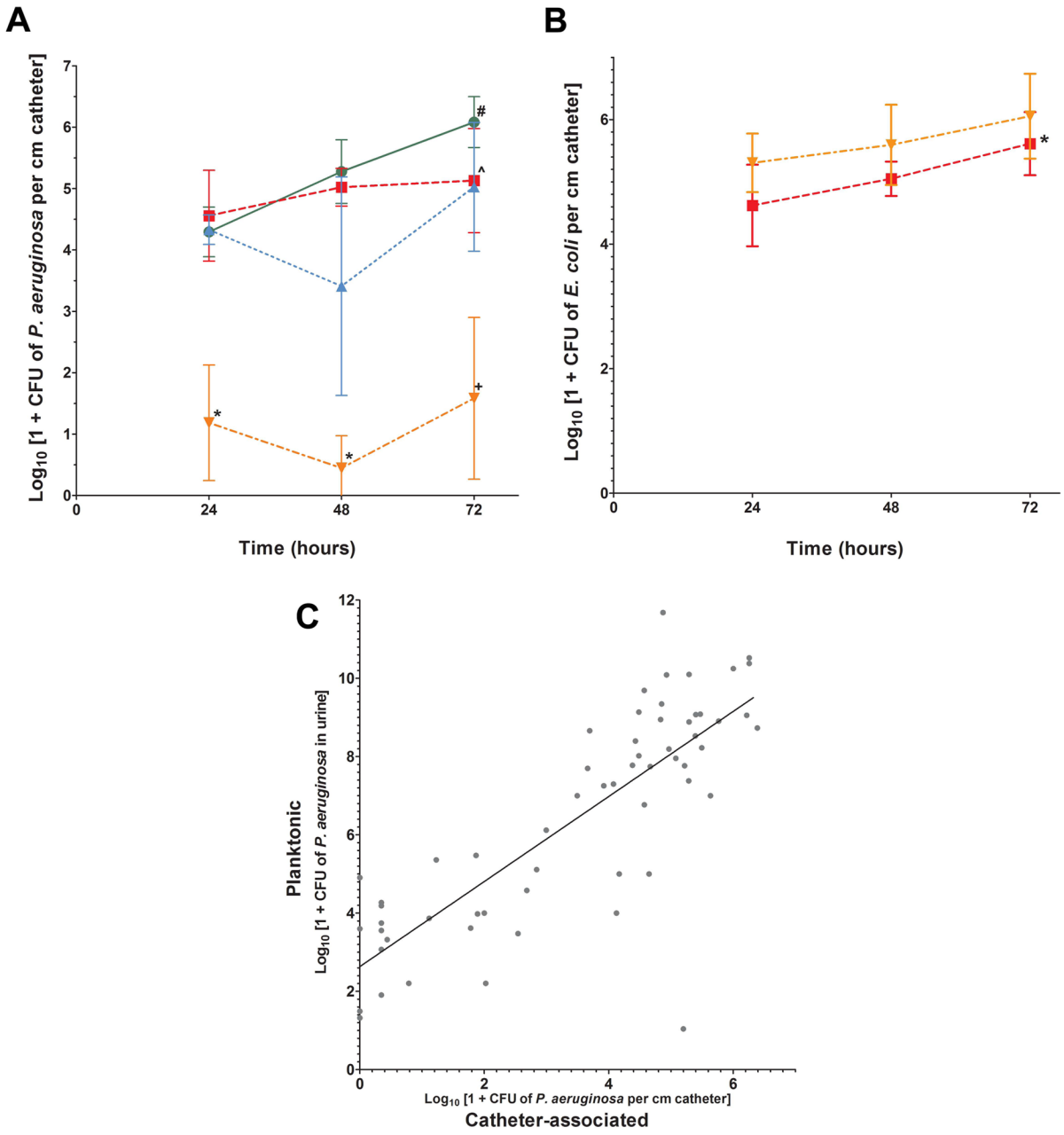

Figure 3. A) Effect of catheter pretreatment on biofilm formation by *P. aeruginosa* after exposure for 24, 48, and 72 hours

Each point represents the averages of means of *P. aeruginosa* adherent to three catheter sub-segments from at least four repetitions per pretreatment condition (*P. aeruginosa* only (green ●); *E. coli* only (red ■); Phage only (blue ▲); *E. coli* + phage (orange ▼)). Error bars indicate standard deviation of the mean. $^{\#}P < 0.03$ for the Mann-Whitney rank sum test of *P. aeruginosa* retrieved from non-pretreated 24-hour catheters compared to non-pretreated 72-hour catheters; ^P = 0.3 for the T test of *P. aeruginosa* recovered from *E. coli*-pretreated 24-hour catheters compared to 72-hour catheters; *P < 0.05, Dunn's, and $^{+}P < 0.001$, Holm-Sidak post-hoc comparison of *E. coli* plus phage pretreatment to control. **B) Recovery of *E. coli* from phage-treated and untreated catheters** Data shown are averages of mean log *E. coli* CFU cm^{-1} (error bars are standard deviations of the mean, n = 5) (No phage (red ■);

Phage (orange ▼)). *P = 0.04 for the T test of *E. coli* retrieved from non-phage-pretreated 24-hour catheters compared to 72-hour catheters. **C) Relationship between the number of planktonic and biofilm *P. aeruginosa.*** A positive correlation was identified by Pearson Product Moment Correlation (correlation coefficient = 0.810, $P = 4.776 \times 10^{-15}$, $R^2 = 0.6557$).

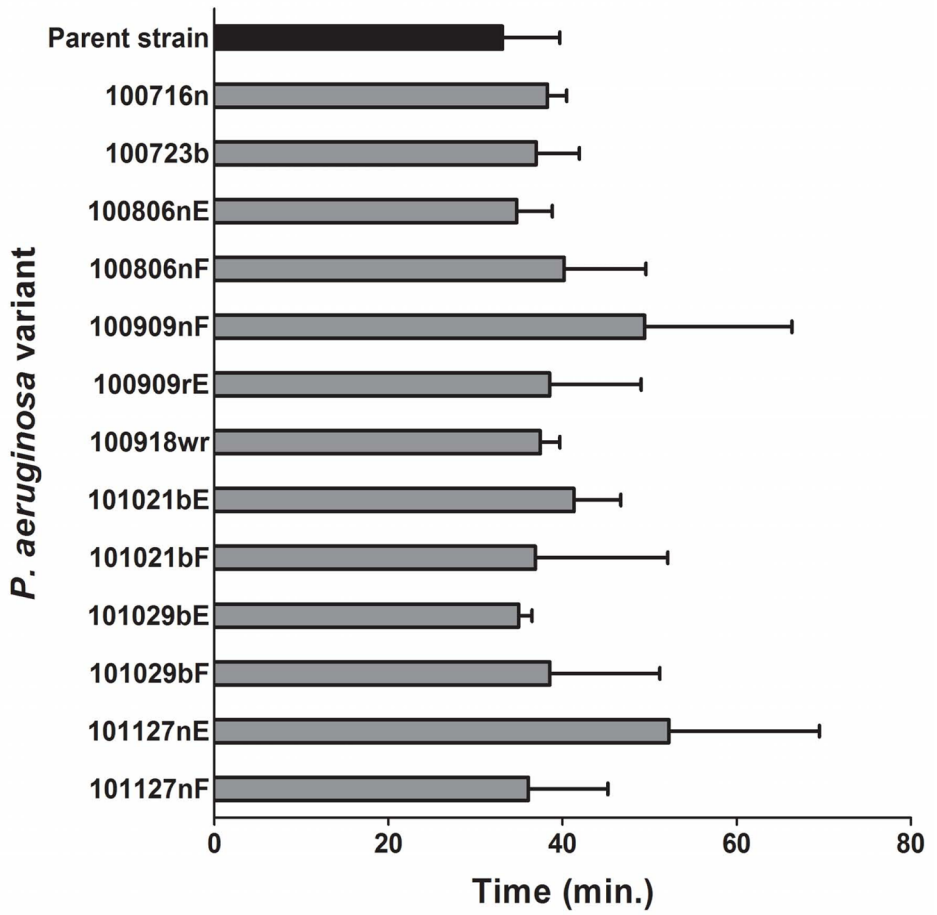

Figure 4. Doubling times of experimental *P. aeruginosa* variants

Hourly viable plate counts from each individual repetition were plotted on a logarithmic scale plot to select those data points within the exponential portion of each variant's growth. A best-fit exponential curve was fitted to those selected exponential growth data points; the equation for the curve (in the general form $y = ke^{ct}$, where y is number of organisms per mL, k and c are constants, e is Euler's number (approximately 2.718), and t is time as the independent variable) was solved for doubling time. Four repetitions were performed for each variant. No statistically significant difference between parent strain and variants' growth rates was found (P = 0.27, Wilcoxon Rank Sum Test).

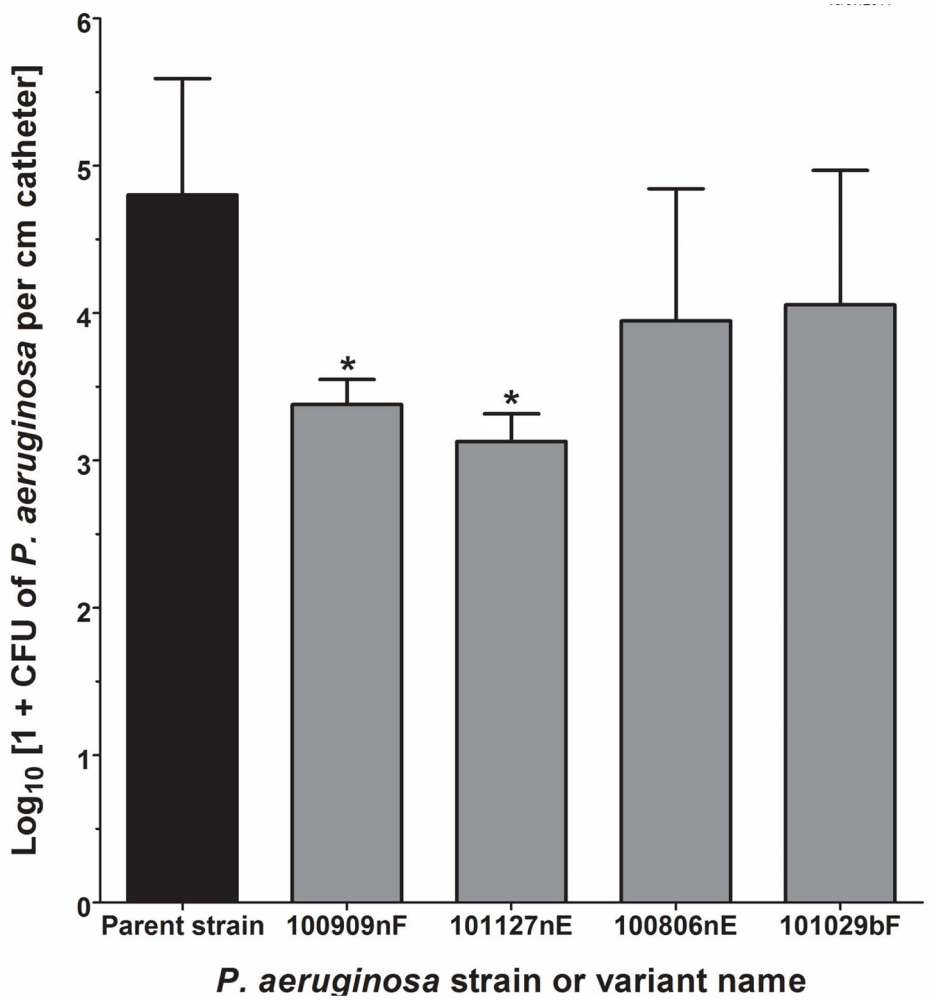

Figure 5. Catheter adherence ability of selected experimental variants

The two slowest-growing (100909nF and 101127nE) and two of the faster-growing experimental variants (100806nE and 101029bF) were tested in 3–8 repetitions for adherence to urinary catheters during overnight incubation in urine. Significantly fewer numbers of 100909nF and 101127nE) were recovered from catheter surfaces than the parent strain (*P = 0.026, ANOVA).

Table 1

Characteristics of six anti-pseudomonal phages used in this study

Phage	Bacterial host	Source
ΦE2005-A	*P. aeruginosa* EAMS2005-A *	CDC †
ΦPaer4	*P. aeruginosa* Paer4	CDC †
ΦW2005-B	*P. aeruginosa* EAMS2005-B *	CDC †
ΦE2005-C	*P. aeruginosa* EAMS2005-C *	CDC †
ΦPaer14	*P. aeruginosa* Paer14	CDC †
ΦM4	*P. aeruginosa* M4	HPA Colindale ‡

NOTE. CDC = Centers for Disease Control and Prevention.

*Clinical isolates.

†Phage and bacterial host strains received from the CDC originated in the collections of the Biofilm Laboratory in the Clinical and Environmental Microbiology Branch, CDC, Atlanta, Georgia.

‡Health Protection Agency, Colindale, United Kingdom.