Spring 4-2014

Examination of Host Range of Pseudomonas aeruginosa phages UT1, SN-T, and PEV2 for Treatment of Bacterial Biofilms in Fuels

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Abstract

Biofilms are slimy substances made up of bacteria that attach to surfaces. Biofilms can be found in humans (lung of Cystic Fibrosis patients), natural settings (rocks in streams) and man-made environments (medical devices, pipelines). Biofilms are also found in aviation fuel tanks, causing physical issues such as clogging in fuel lines and changing the chemical makeup of the fuel via bacterial metabolism. Bacterial viruses, known as bacteriophage, show potential for reducing biofilms through phage therapy. The goal is to find a phage or combination of phage with a broad host range that would be most effective in reducing the biofilms of bacteria isolated from fuel tanks. Known phages UT1, SN-T, and PEV2 were tested against these biofilms, both individually and in combination. They were tested to measure inhibition, in which phage and bacteria were incubated together, and remediation, in which biofilms were allowed to preform before the addition of phage. Biofilms were assayed for biomass (via crystal violet staining) and viable cell count in the presence of phage or combination of phages to determine the amount of biofilm inhibition and remediation. Results suggest that the host ranges of UT1, SN-T, and PEV2 were not as broad as anticipated, inhibition techniques were more effective than remediation techniques, and phage used in combination resulted in greater biofilm reduction than when used singly.

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Acknowledgments

I would like to thank Dr. Jayne Robinson for providing me with the opportunity to work in her lab, and for her continual guidance over the past three years. I would also like to thank Caitlin Bojanowski for teaching me the techniques I used and for helping put this project together.

Thank you to the Department of Biology for providing laboratory resources, and to the University Honors Program for funding and for the opportunity to present my research.

A special thank you to my parents, for their constant support and their continual encouragement to work hard, follow my dreams and do the best I can everyday.

Finally, thank you to the University of Dayton, for providing me with a well-rounded education. The environment of community, academic success, and service has allowed me to grow immensely, both as a student and as a person.

Background

Many types of bacteria are capable of forming a biofilm, which is a community of microorganisms attached to a surface[1]. The formation of a biofilm begins when a free-floating planktonic cell attaches to a surface. This cell continually divides as others gradually attach to this surface, eventually forming microcolonies. These microcolonies can be made up of either single bacterial species or mixed species. The structure of the biofilm is maintained by the extracellular polymeric substance, or EPS[2]. Comprised of DNA, carbohydrates, polysaccharides, and protein, the EPS protects the bacterial cells from environmental factors such as changes in pH, dehydration, and antimicrobial agents[3]. Since biofilms exhibit such a high resistance to antimicrobial agents such as antibiotics as well as to the immune system, they may become a source of deep-seated and persistent infections[4].

Common infections that typically involve biofilms include urinary tract infections (*Escherichia coli*), septicemia (*Streptococcus pneumoniae*, *E. coli*), and lung infections in Cystic Fibrosis patients (*Pseudomonas aeruginosa*)[5]. Best known as “plaque” in the oral cavity, bacterial communities also attach to teeth enamel and dental implants, causing dental caries or periodontal diseases such as gingivitis[6]. In addition to formation in the human body, biofilms are found in natural settings, such as streams. Microbes in the overlying water assemble on rocks, forming a slimy substance[7]. Biofilms may even be found in man-made environments, including hospital devices and pipelines. For example, with many surgical implants, infections stemming from biofilm formation cannot solely be treated by antimicrobial therapy. Only complete removal of the implant allows the infection to be treated[8].

Due to the ineffectiveness of traditional methods in reducing the highly resistant biofilms, the use of phage therapy has become an important research area. Phage therapy is the use of bacteriophage, bacteria specific viruses, for the treatment of bacterial infections. Bacteriophages kill bacteria by inducing bacterial lysis. In this process, phages attach to the cell surface and inject their genetic material into the cell. Phage DNA is synthesized within the cell, eventually leading to the production of phage head, tail, and lysis proteins[9]. These components assemble into hundreds of phages and burst, or lyse, the cell. These new phages then infect and kill surrounding bacterial cells. Phage therapy for human infections first found application in the 1920s and 1930s. However, with the discovery of sulfa and penicillin drugs in the 1940s, phage therapy was set aside. While antibiotic drugs have been quite successful in treating infections in the intervening decades, the rapid spread of antibiotic-resistant bacteria in the past two decades has caused a resurgence of interest in phage therapy. Some researchers have already conducted studies using phage therapy to reduce biofilm count on medical catheters. They found that pre-treating catheters reduced biofilm count by nearly a three-log reduction (99.9% decrease), while treating the catheters immediately after bacterial inoculation resulted in just over a two-log reduction (99%)[10].

This study focuses on biofilms that form in aviation jet fuel tanks, which result in a number of problems. Biofilms can cause clogging of thin fuel lines, changes in the chemical makeup of jet fuel, and even corrosion of fuel system infrastructures. We propose to treat these biofilms with bacteriophage, using PAO1 as our model organism. PAO1 is a strain of *P. aeruginosa* that can grow using jet fuel as its sole carbon source. Ripp and Miller determined that the phage UT1, isolated from natural freshwater, was

effective at reducing *P. aeruginosa* biofilms[11]. Their data suggests that, under starvation conditions, UT1 adopts a pseudolysogenic cycle. The process of pseudolysogeny is one of survival, as the development of a bacteriophage is stalled when conditions are not optimal[12]. Another study also found phage PEV2, isolated from a sewage treatment plant, to be effective against *P. aeruginosa*[13]. PEV2 is an obligatorily lytic phage that can infect PAO1 both aerobically and anaerobically. Finally, the phage SN-T was also isolated from a sewage plant, but with *Sphaerotilus natans* as its host[14]. It was more closely examined due to its ability to form plaques on multiple bacterial hosts. The ability to infect multiple bacterial hosts makes it particularly interesting for phage therapy. Our goal is determine which of these three phage types, alone or in combination, is most effective in preventing or reducing the biofilms in an experimental system.

Materials and Methods

Fuel Isolate Screening

A library of bacteria isolated from aviation fuels (Table 1) was screened by the spot lysis test for susceptibility to phages UT1, SN-T, and PEV2. Each bacterium was grown on a Luria Bertani broth (LB) agar plate. After distinct colonies formed, a single colony was taken from the plate using a sterile inoculating loop and deposited in a sterile test tube with 4 mL of LB broth. The tube was capped loosely, placed on a gyratory shaker at 180 RPM, and allowed to grow overnight in a 37°C incubator. The following day, if the overnight culture was cloudy, 100 μL of the overnight culture was added to 4 mL of melted LB top agar and mixed by vortexing. The mixture was immediately poured over an LB agar plate pre-warmed to 37°C. After the top agar solidified, 10 μL of each bacteriophage UT1 ($1.15x10^{11}$ plaque forming units (PFU)/mL), SN-T ($3.19x10^{9}$ PFU/mL), and PEV2 ($7.2x10^{9}$ PFU/mL) were spotted onto the bacterial lawn. Two plates were made for each fuel isolate sample: one incubated at room temperature (RT) and one at 37°C. The following day, the plates were observed for phage-induced lysis.

Efficiency of Plating

To distinguish between lysis from without (when the bacterial cell membrane is overwhelmed by phages) and true infection, each fuel isolate that exhibited lysis in the screening phase (Table 1) was tested for the efficiency of plating (EOP) by that phage. When there was more than a single isolate of a genus, we tested one for EOP (e.g., only one *Bacillus sp.* isolate of the five in Table 1 was tested for EOP). As described above, an overnight culture of each bacterium was used to make a lawn and allowed to solidify in duplicate. In a 96-well plate, stocks of UT1, SN-T, and PEV2 were serially diluted to 10^{2}

PFU/mL. The last six dilutions (10^2-10^7 PFU/mL) of each were spotted onto the bacterial host lawn in 10 μL aliquots. The original, undiluted stock cultures of phage were also spotted. Several of the bacteria that appear in Table 2 were not used, as some were the same genus.

Biofilm Assay

Only *P. aeruginosa* PAO1 was used for the 16-hour biofilm assay. Two variants of this procedure were performed: 1) phage was added at the same time as the bacterial inoculum (inhibition), and 2) biofilms were allowed to form before the addition of phage (remediation).

Inhibition

To each well of a sterile 96-well plate, 200 μL of mixture containing PAO1 at $1x10^5$ colony forming units/mL or PAO1 at $1x10^5$ CFU/mL with phage at a multiplicity of infection (MOI) of 100 was added. For wells containing multiple phages, the total MOI was 100, being composed of equal parts of each phage in the mixture. Plates were incubated at 37°C for exactly 16 hours without shaking.

Remediation

To each well of a sterile 96-well plate, 200 μL of mixture containing PAO1 at $1x10^5$ CFU/mL was added and then incubated at 37°C for exactly 16 hours without shaking. At 16 hours, the supernatant was removed from each well and 200 μL of MSG or MSG with phage at $1x10^7$ PFU/mL was added to the appropriate well. For wells containing multiple phages, the total PFU/mL was $1x10^7$, being composed of equal parts of each phage in the mixture. The plate was then incubated for 24 hours at 37°C without shaking.

Crystal Violet Stain

To determine the biomass formed in each well, 50 μL of crystal violet stain was added to each well and incubated at room temperature for thirty minutes. The plate was carefully washed with MilliQ (purified) water and allowed to dry overnight. The next day, 200 μL of 97.5% ethanol was added to each well and incubated at room temperature for ten minutes to extract the crystal violet from the biofilms. 150 μL of this crystal violet extract was transferred to a new 96-well plate, and absorbance was measured using a plate reader at 590 nm.

Viable Cell Counts

To determine the number of viable cells attached to the well surface, the supernatant from the wells was removed, 200 μL of fresh MSG was added to each well, and the biofilm was mechanically resuspended using a pipet tip. The resuspended biofilms were serially diluted and plated on LB agar according to the drop plate method[15]. Plates were incubated overnight at 37°C and results were reported as colony forming units (CFU) per centimeter^2.

Results

Of the 59 bacteria isolated from fuel, 30 showed some degree of susceptibility to phage (Table 1). There were a few instances in which there was a different degree of clearing for the same bacterium at different temperatures. *Burkholderia cepacia* (#70) did not have any clearing from SN-T at 37°C but had substantial haziness at room temperature (RT); a *Microbacterium* (#81) had a similar result when exposed to UT1. Both a *Staphylococcus sp.* (#78) and an unknown bacterial isolate (#94) showed substantial haziness at 37°C and clearing with a hazy background at RT for phage SN-T and PEV2. The other cases that showed differing results between temperatures (#71, 72, 76, 77, 109) had greater degrees of clearing at 37°C than at RT. SN-T and PEV2 displayed a broader range of activity, affecting 29 and 22 isolates, respectively, while UT1 only affected 12.

In order to ensure that these results were due to true infection from the phage and not lysis from without, the efficiency of plating (EOP) test was performed. Of bacterial isolates that showed susceptibility in the spot lysis assay, a representative from each genus was subjected to EOP (Table 2). If no lysis occurred in any of the dilutions, we concluded that the results of the screening were due to lysis from without and the bacterium was not used in the 16-hour biofilm assay. Unfortunately, none of the fuel isolates showed susceptibility to phage at lower concentrations (Table 2). As a result, biofilm inhibition and remediation trials with phages UT1, SN-T, and PEV2 were done solely with PAO1.

Crystal violet staining was used as an estimate of biofilm biomass where greater absorbance from crystal violet was equated with greater biomass. The results for

inhibition tests in Figure 2 showed that SN-T (fold change of -0.2812 ±0.02389), PEV2 (-0.3951 ±0.06932), UT1/PEV2 (-0.5104 ±0.03419), SN-T/PEV2 (-0.4280 ±0.03490), and UT1/SN-T/PEV2 (-0.7395 ±0.02276) treatments significantly reduced biomass of PAO1 biofilms formed in 96-well plates. UT1 and UT1/SN-T, with statistically significant changes, had an increase in biomass. These UT1 and UT1/SN-T results parallel those from the remediation trials (Figure 3) in which treatment with UT1/SN-T, UT1/PEV2, SN-T/PEV2, and UT1/SN-T/PEV2 resulted in statistically significant increases in biomass. Individual treatments of UT1, SN-T, and PEV2 were statistically similar to the PAO1 control. In the remediation trial, no decrease in biomass was observed.

As crystal violet estimates biofilm biomass, which is composed not only of cells but also the EPS, viable cell counts were also performed to account for possible differences in EPS resulting from phage treatment. Indeed it was found that viable cell counts yielded different results from crystal violet. The inhibition results (Figure 4) showed that UT1 (log reduction -1.107 ±0.1695), PEV2 (-2.664 ±0.001968), UT1/SN-T (-0.08951 ±0.2073), UT1/PEV2 (-2.526 ±0.1883), SN-T/PEV2 (-2.580 ±0.01864), and UT1/SN-T/PEV2 (-2.628 ±0.02232) treatments significantly reduced the number of living cells. For example, PEV2 displayed almost a three-log reduction compared to the PAO1 control, which is about a 99.9% reduction. In other words, the number of viable cells in wells with PEV2 treatments is about 1000 times smaller than those in the PAO1 control wells. However, when phages were applied to an already established PAO1 biofilm in the remediation assay (Figure 5), there were no statistically significant differences between any treatment and the control, i.e. no reduction in the number of live cells in the PAO1 biofilms.

Discussion

The advantage of phage therapy is its self-replicating nature of infection. The hope was to use a small amount of phage to reduce a large amount of biofilm. Additionally, finding a broad-host range phage would be extremely advantageous, since most biofilms are mixed-species. Past studies suggest that these bacteriophages play a key role in phage ecology and gene transfer in nature[12]. If a broad-host range phage cannot be utilized, each bacterium in the biofilm would require its own phage and efficiency would be reduced.

With the initial fuel isolate screening, not all bacteria were susceptible to the phage tested, and those that were showed different degrees of clearing. This was not unexpected as bacteriophages can be very specific for a bacterial host, and even those that exhibit broader host ranges do not infect all bacterial genera. From the efficiency of plating tests, it was determined that initial susceptibility was not due to true infection. If a true infection occurred, the bacterial lawns would be affected at lower concentrations of the phage and individual plaques would have been observed. One possible reason cell death occurred only by a high concentration of phage is lysis from without. During this process, the sheer number of phage attached to the cell membrane causes leaky junctions, eventually destroying it without the formation of new phage[16].

Although previous studies have shown that UT1, SN-T, and PEV2 exhibit a broad host-range, they could not utilize any of the bacterial isolates in this study as hosts. These bacteria may lack the receptor(s) necessary for the phage to gain entry or may have evaded infection through other means such restriction modification systems (destroying phage genetic material before replication), or programmed cell death. Under the

conditions tested, phage cocktails appeared to be most effective. Additionally, phages were more effective at inhibiting biofilm formation than at remediating biofilms.

Some of the results from the crystal violet absorbance readings were unexpected. Though some decrease in biomass was observed, UT1 and UT1/SN-T treatments in inhibition testing and UT1/SN-T, UT1/PEV2, SN-T/PEV2, and UT1/SN-T/PEV2 treatments in remediation testing led to an increase in absorbance. This increase may have been a result of cell debris, phage biomass, or DNA, carbohydrates, and polysaccharides from the EPS. However, the increased biomass was not indicative of the number of living cells still attached to the well wall. In the inhibition trials, wells treated with UT1 nearly doubled in biomass compared to the PAO1 uninfected control, yet, when the viable cell number was determined, there was a one-log reduction from the control, a statistically significant decrease. On the other hand, there was a significant reduction in biomass with SN-T treatment, but no statistically significant change in the number of live cells.

While under current conditions phage treatment was unable to reduce biomass of viable cell number of established PAO1 biofilms, several modifications to the testing methods might be considered. First, allowing phage to incubate longer with the formed biofilms, where the EPS is well established, might determine if the phages need additional time to diffuse through the EPS or to replicate and lyse the cells. Another approach might be to use the phages in combination with chemical compounds that break down the protective EPS, allowing phages greater access to bacterial cells. This process could also be faster than the prior, as the phages could diffuse into the bacterial cells much more quickly. Finally, it would be beneficial to test the best-performing phage

cocktails in a fuel model system. It is possible that the susceptibility of bacteria to phages is entirely different in the presence of fuel or confined space rather than the wells of a plastic plate. On the other hand, the fuel could actually be detrimental to phage structure and destroy them before they even have a chance to attach to the bacterial cell membrane.

Our study has demonstrated 1) combinations of phages inhibit biofilm formation to a greater extent than phage used singly and 2) under the conditions tested, phage therapy was more effective in preventing biofilm formation than it was in remediating established biofilms. While the phages tested did not exhibit the broad-host range activity expected, novel phages could be isolated that pose this activity. Overall, phage therapy appears to be a promising method of reducing bacterial biofilms in aviation fuel systems.

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Tables and Figures

Table 1. Bacterial isolates from aviation fuel tanks tested for susceptibility to phages UT1, SN-T, and PEV2. Numbers represent differing degrees of susceptibility to the phage. 0= no clearing, 1= formation of individual plaques, 2= substantial haziness throughout the cleared zone, 3= clearing throughout with a faintly hazy background, and 4= complete clearing. Those with two numbers had different results at 37°C/room temperature.

Organism	**Plate ID #**	**UT1**	**SN-T**	**PEV-2**
?	7	0	0	0
Clostridium sporosphaeroides	9	0	0	0
Indeterminate	11	0	0	0
?	12	0	0	0
?	14	0	0	0
?	15	0	0	0
Methylobacterium	18	0	0	0
Clostridium	19	0	0	0
Curtobacterium	21	0	0	0
?	22	0	0	0
?	23	0	0	0
?	24	0	0	0
?	25	0	0	0
Staphylococcaceae	27	0	0	0
Curtobacterium	30	0	0	0
Mycobacterium	31	0	0	0
?	32	0	0	0
?	34	0	0	0
?	35	0	0	0
Burkholderia	36	NT	NT	NT
Propioribacterium acnes	38	NT	NT	NT
Burkholderia	40	2	0	0
Rahnella aquatillis	41	0	0	0
?	42	0	0	0
?	43	0	0	0
Staphylococcaceae	45	0	3	3
Mycobacterium	47	0	2	2
Mold	49	0	0	0

Burkholderia	50	0	2	0
Rhodococcus equi	51	0	0	0
?	52	0	0	0
Staphylococcaceae	54	0	0	0
Arthrobacter nitroguajacolicus	56	0	2	0
Rothia amarae	58	2	2	0
Staphylococcus epidermis	59	3	3	0
Arthrobacter nitroguajacolicus	63	0	2	0
Microbacterium	64	0	2	0
Staphylococcaceae	66	2	2	2
Staphylococcaceae	67	3	3	3
Undetermined	68	2	2	2
Burkholderia cepacia	70	0	"0/2"	0
Indeterminate	71	2	"4/2"	"4/2"
Microbacterium	72	"0/2"	"4/2"	"4/2"
Bacillus	76	2	"4/2"	"4/2"
Bacillus	77	2	"4/2"	"4/2"
Staphylococcus	78	3	"2/3"	"2/3"
Bacillus	79	0	2	2
Curtobacterium	80	0	4	4
Microbacterium	81	2	3	3
Staphylococcus	82	0	3	3
Fungus	83	0	2	2
Unable to clone	94	0	"2/3"	"2/3"
Bacillus	97	0	2	2
Bacillus	100	0	0	0
Bacillus	101	0	2	2
Microbacterium	102	0	2	2
Microbacterium	103	0	2	2
Staphylococcus	107	0	3	3
Micrococcus	109	0	"4/3"	"4/3"

Table 2. Efficiency of plating results for bacterial fuel isolates that showed some degree of susceptibility to phages UT1, SN-T, and PEV2.

Organism	Plate ID #	Individual plaques (Y/N)
Burkholderia	50	N
Arthrobacter nitroguajacolicus	56	N
Rothia amarae	58	N
Staphylococcus epidermis	59	N
Bacillus	76	N
Bacillus	77	N
Staphylococcus	78	N
Microbacterium	81	N
Bacillus	101	N
Staphylococcus	107	N

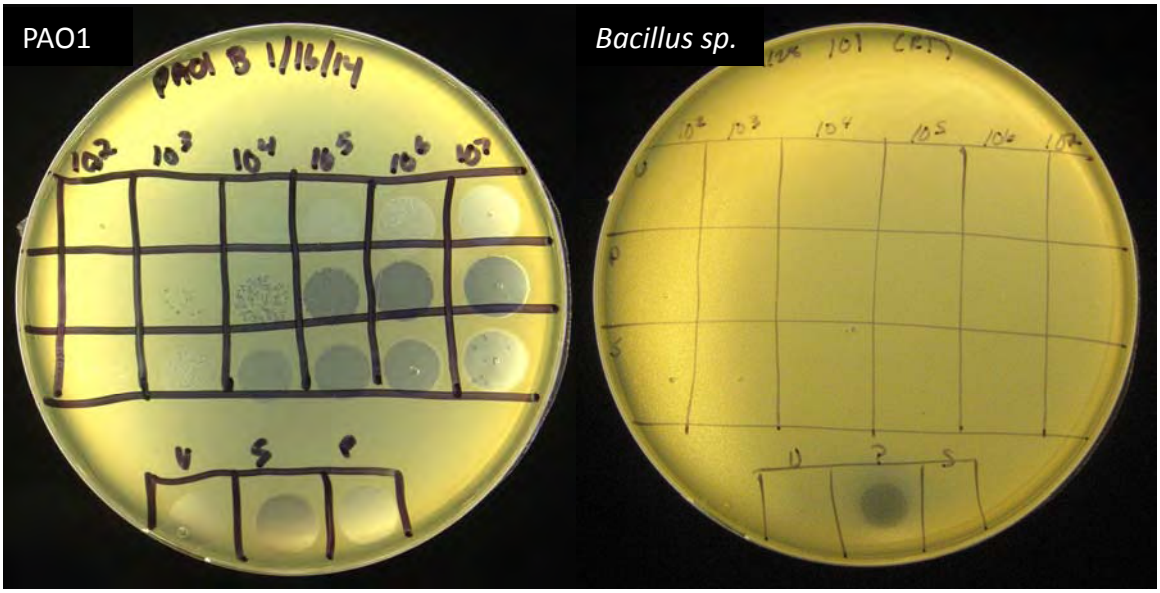

Figure 1. Efficiency of plating results for #101, *Bacillus sp.* compared to the PAO1 control. Absence of individual plaques indicates that clearing in the spot lysis assay was not due to true infection. Similar results were found for all fuel isolates tested.

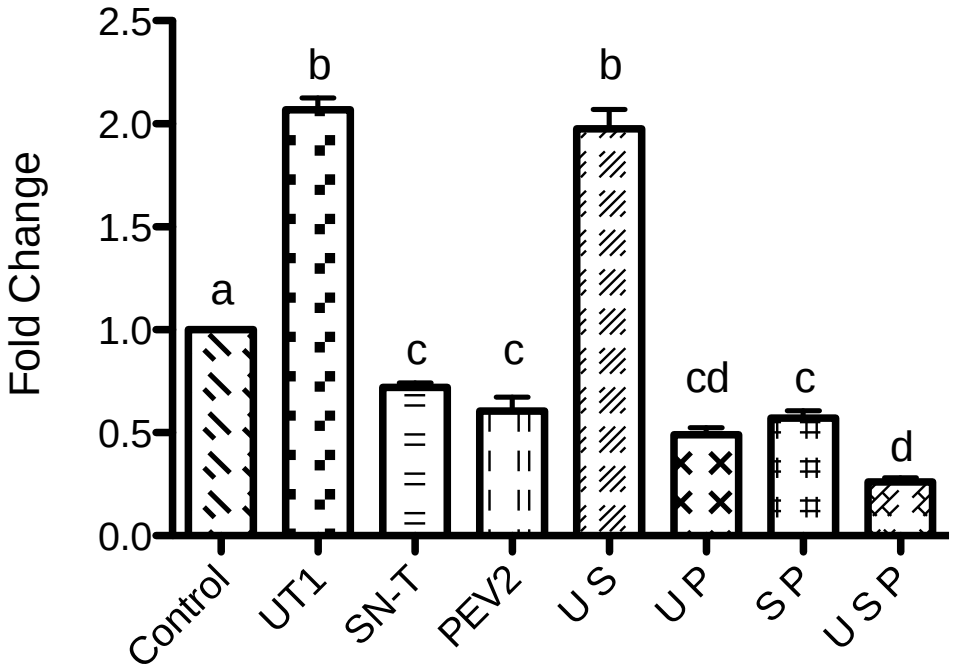

Figure 2. Fold change in crystal violet absorbance for inhibition biofilm assays compared to the PAO1 control. Treatment results with the same letter are not statistically significantly different. Those with different letters have statistically significant differences in biomass.

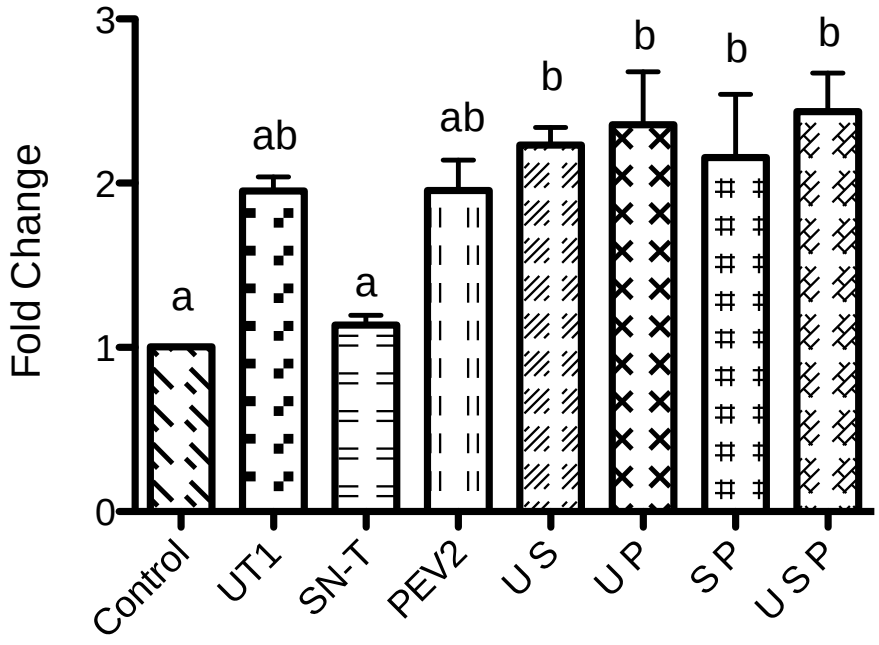

Figure 3. Fold change in crystal violet absorbance for remediation biofilm assays represented as fold change compared to the PAO1 control. Treatment results with the same letter are not statistically significantly different. Those with different letters have statistically significant differences in biomass.

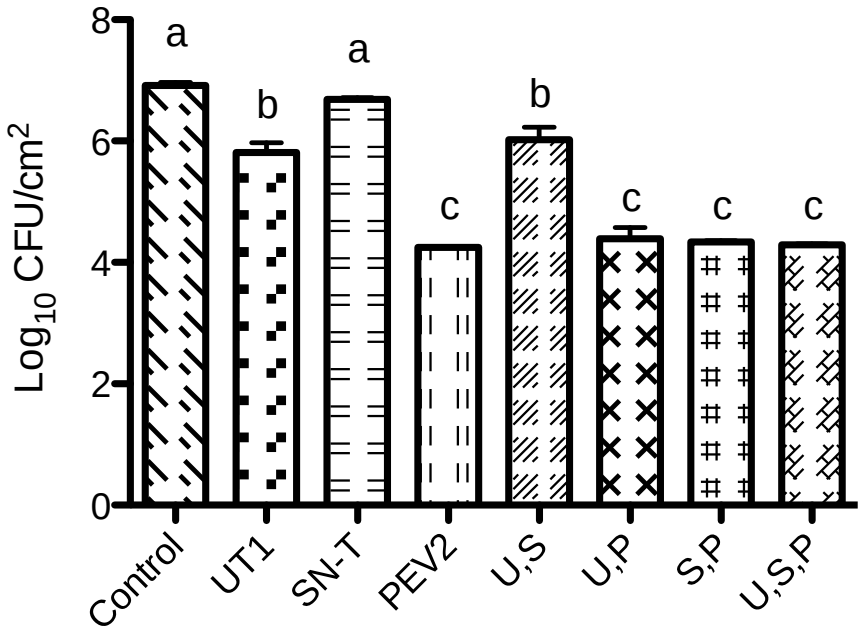

Figure 4. Colony-forming units (CFU) per cm^2 of PAO1 biofilms from inhibition assays. The number of live cells that remain attached to the well surface is represented on a log_{10} scale. Treatment results with the same letter are not statistically significantly different. Those with different letters have statistically significant differences in CFU/cm^2.

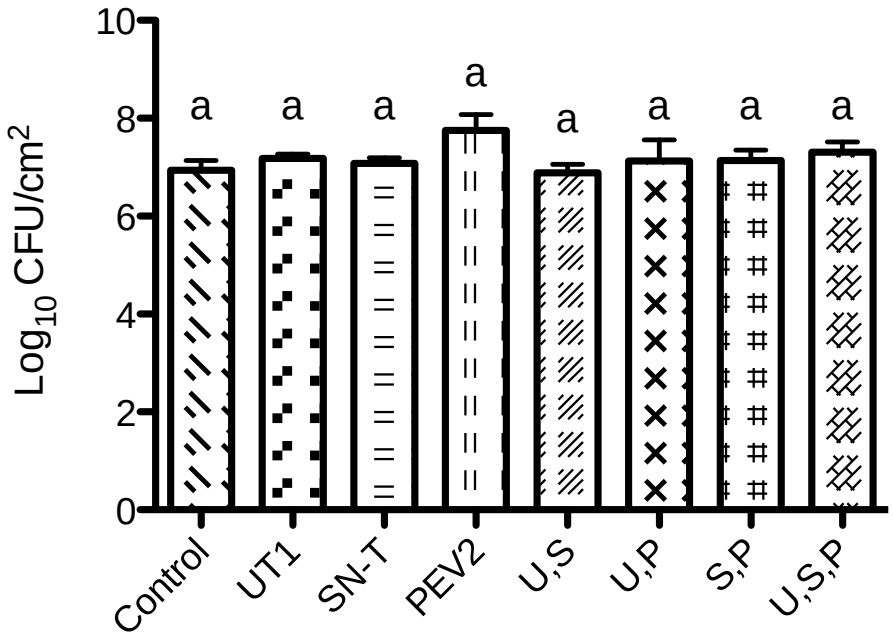

Figure 5. Colony-forming units (CFU) per cm^2 of PAO1 biofilms from remediation assays. The number of live cells that remain attached to the well surface is represented on a log_{10} scale. Treatment results with the same letter are not statistically significantly different. Those with different letters have statistically significant differences in CFU/cm^2.