EFFECTS OF *N*-ACYL-L- HOMOSERINE LACTONE LACTONASE ON DISRUPTION OF PREFORMED BIOFILMS, BACTERIAL RECRUITMENT, AND PREVENTION OF BIOFILMS

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ABSTRACT

EFFECT OF *N*-ACYL-L-HOMOSERINE LACTONE LACTONASE ON DISRUPTION OF PREFORMED BIOFILMS, BACTERIAL RECRUITMENT, AND PREVENTION OF BIOFILMS

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Biofouling is the accumulation of biofilms on inanimate surfaces in aqueous environments. Biofouling can produce economic as well as medicinal consequences such as decreased flow efficiency of chemicals through pipes, increased drag on ships and interference with proper function of medical implants resulting in diseases. Biofilms

found in nature are typically composed of mixed species containing bacteria such as *Pseudomonas aeruginosa*. *P. aeruginosa* is normally found in soil and marine environments but is also an opportunistic pathogen of humans and lower eukaryotes. *P. aeruginosa* and many other organisms utilize a quorum-sensing pathway for cellular communication including the secretion of acylated homoserine lactones (AHLs). Lactonases are enzymes naturally expressed by certain bacteria that have been found to disrupt (quench) the AHL quorum-sensing pathway, but the effects of lactonase on biofilm stability and bacterial recruitment has not been well studied. In this study, wild type *P. aeruginosa* was co-cultured with DH5α *Escherichia coli* containing a cloned lactonase gene, *aiiA,* from *Bacillus thuringiensis* under the control of isopropyl-β-D-thiogalactopyranoside (IPTG) inducible promoter to determine the effects of the AHL lactonase on biofilm stability and the effects on bacterial recruitment. Plate counts revealed that biofilm formation of PA01 and DH5α *E. coli* pMAL-t-*aiiA* co-culture was thicker than when PA01 was grown in a monoculture. A moderately significant lower difference was observed when a 48-hour monoculture of PA01 was exposed to DH5α *E. coli* pMAL-t-*aiiA* expressing lactonase when compared to exposure of PA01 to DH5α *E. coli* pMAL-t-*aiiA* not expressing lactonase. Bacterial recruitment was not affected by the

presence of DH5α *E. coli* pMAL-t-*aiiA* expressing lactonase. The results indicate that the lactonase has a small impact on biofilm stability but not bacterial recruitment.

I. INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, bacillus shaped opportunistic pathogen of humans and lower eukaryotes (46), and is normally found in soil, freshwater and other marine environments (20). *P. aeruginosa* can be associated with urinary tract infections (UTIs), respiratory tract infections, soft tissue infections, bacteremia, bone and joint infections, sepsis, pulmonary infections, and a variety of systemic infections (50, 52). *P. aeruginosa* is becoming more prevalent as an opportunistic pathogen, and is the most common Gram-negative found in nosocomial infections (53). *P. aeruginosa* affects immunocompromised patients such as burn patients, cancer patients, and patients with cystic fibrosis (CF). A chronic infection of *P. aeruginosa* can lead to lung failure and even death in CF patients (28). Even though *P. aeruginosa* is an opportunistic pathogen causing various and sometimes fatal infections, it can be associated with biofouling in non-medical settings.

Biofouling is the accumulation of microbial biofilms on inanimate objects such as ship hulls in marine environments. Biofouling can greatly decrease flow efficiency of

chemicals through pipes, increase drag on boats or ships (4, 36), interfere with medical implants such as indwelling catheters (48). Accumulation of biofilms on ship hulls or underwater piping can have significant economic consequences, including increased drag and corrosion. The related decrease in fuel efficiency (36) from a major product supplier can lead to an increase in the overall price of their products. In the medical field, long-term urinary catheters are sometimes used on patients who cannot urinate normally due to paralysis or other bladder malfunctions (44). Unfortunately, catheters used for indwelling catheterization are changed every 8-12 weeks allowing for a substantial amount of biofouling that can lead to sepsis or other associated complications (48). According to Saint (44), urinary tract infections (UTI) caused by long-term catheterization accounts for up to 40%–approximately 1 million UTIs annually–of all nosocomial infections, which can lead to severe complications. UTIs are generally associated with infected urine flowing through the catheter, which can be associated with biofilm formation such as that from *P. aeruginosa* on the luminal surface of the catheter.

Biofilms are fixed or immobile microbial communities associated with surfaces that together secrete a gelatinous extracellular polymeric matrix, which completely envelop the community (23, 57, 59). Biofilms are used primarily for protection against antibiotics (23, 24, 59) and from host immune defenses, which explains why biofilms are

associated with several types of chronic infections. It has been well established that biofilms can be up to 1000 times more resistant to host defenses and antibiotics than their planktonic counterparts (5, 23). A study conducted by Nickel et al. (38) showed that planktonic *P. aeruginosa* have a minimal inhibitory concentration to tobramycin of <1.0 μg/ml compared to a minimal inhibitory concentration of 1000 μg/ml when living in biofilms. Biofilms in nature are found as mixed species rather than monocultures. There is evidence to suggest that biofilms in nature grow as mixed species to form commensal relationships to increase chances of survival (1). Biofilm formation begins with the attachment of planktonic primary colonizers such as *P. aeruginosa* to nutrient rich surfaces, which then begin to secrete an extracellular polymatrix. It is thought that once primary colonizers attach to a surface and begin to secrete an extracellular polymatrix, secondary bacteria species are recruited to the surface (6, 32). Recruitment of secondary species to form commensal relationships can be advantageous to bacteria such as *P. aeruginosa* by allowing the sharing of metabolic activity, migration, protection, and removal of dead biomass (1, 6, 9). McLean et al. (32) conducted an experiment on aquatic biofilms, and found that acylated homoserine lactones (AHLs) are secreted within these mixed species biofilms suggesting interspecies communication to form commensal relationships. Interspecies communication via secretion of small diffusible molecules

such as AHLs is referred to as quorum-sensing (18). Fuqua et al. (17) suggest that quorum-sensing plays a key role in the species dynamics of microbial biofilms. It is thought that bacteria incapable of producing their own AHLs can still respond to AHLs secreted by other bacteria such as *P. aeruginosa* (54).

Quorum-sensing is utilized by many Gram-positive and Gram-negative bacteria as a way to monitor cell density and control global gene expression (13, 16, 21). Gram-positives such as *Staphylococcus aureus* secrete peptide-based signals that interact with a sensor of the histidine kinase pathway to accomplish quorum-sensing (9). Gram-negative bacteria utilize a few different cellular pathways to accomplish quorum-sensing including secretion of quinolones, secretion of butyrolactones, and secretion of AHLs. AHLs are small diffusible molecules produced by *las* and *rhl*, which are homologues of the *lux* genes responsible for population density-dependent bioluminescence originally found in *Vibrio fishceri* (37). The genes that were identified in *V. fishceri* were *luxI* and *luxR*; *luxI* is an AHL synthase and *luxR* is an AHL response regulator (14). A Gram-negative bacterium known to utilize the AHL quorum-sensing pathway is *P. aeruginosa*.

P. aeruginosa utilizes two AHL quorum-sensing pathways–*las* and *rhl*–to detect cell or population density, secrete density dependent virulence factors, form mature biofilms, and communicate between cells (18, 29, 45, 47, 60). According to Huang et al.

(26), *las* and *rhl* operate in a type of hierarchy where *las* regulates *rhl*. Together, they control an estimated 6 to 11% of *P. aeruginosa* genome (55, 58). Like many signaling pathways, the AHL quorum-sensing signal pathway is a cascade of events beginning with activation of *lasR* and *lasI* (55). According to Nouwens et al. (39), the quorum-sensing genes associated with *las* and *rhl* are both composed of two genes; one of which (*lasR* and *rhlR*) encodes a transcriptional regulatory protein LasR and RhlR. The other gene (*lasI* and *rhlI*) encodes an autoinducer synthase LasI and RhlI. The metabolic pathway of *las* and *rhl* controls the production of AHLs and virulence factor production of *P. aeruginosa*.

The AHL quorum-sensing pathway begins with the production of the AHL autoinducer *N*-(3-oxododecanoyl)-$_L$-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butanoyl-$_L$-homoserine lactone (C_4-HSL) by *lasI* or *rhlI* respectively at basal levels at low population density (7, 17, 42, 47). Once the autoinducer is synthesized, it is moved down its concentration gradient and diffuses into the environment (17, 30, 59). It can then move in and out of cell membranes through diffusion or active transport (10, 41). As the population continues to grow and becomes denser, the AHL autoinducer begins to accumulate. After a certain population threshold concentration has been reached, the autoinducer binds with its cognate transcriptional regulator LasR or RhlR produced by

lasR and *rhlR* respectively (13, 35). The new complex binds upstream on the operon, which then turns on target genes and synthesize virulence factors such as exoproteases, hemolysins, pyocyanin, pyoverdin, cyanide, and others (47). The virulence factors, pyoverdin and pyocyanin act as siderophores and are secreted by *P. aeruginosa* to induce apoptosis of neutrophils. Inducing apoptosis of neutrophils allows *P. aeruginosa* to obtain nutrient requirements and reduce proinflammatory response aiding in its survival (2, 34, 50). According to De Kievit et al. (8), *P. aeruginosa* emits density dependent virulence factors to ensure the bacteria can achieve a high enough population count to sustain life within the host.

Prevention of biofilms would have great economic and medical advantages. Prevention of biofilms would decrease biofouling and decrease the incidence of UTIs due to biofouling of indwelling catheterization or other medical implants. Some bacteria are naturally capable of producing signals referred to as quorum-sensing inhibitors (QSI) that are capable of disrupting (quenching) the AHL quorum-sensing pathway (11, 22), which could prevent biofilm formation. AHL lactonases and AHL acylases are examples of enzymes that can be expressed by certain bacteria as quorum-sensing inhibitors (21). The quenching of the AHL quorum-sensing pathway by *N*-acyl-L-homoserine lactone lactonase has been shown to interfere with secretion of virulence factors (47). Dong et al.

(11) isolated a lactonase gene *aiiA* from *Bacillus thuringiensis*, which has been found to inactivate AHL signals by hydrolyzing the lactone ring (30). According to Dong et al. (12), when *Erwinia caratova*–a known AHL producing bacteria associated with cabbage rot is grown in the presence of *B. thuringiensis*, the amount of AHLs secreted by *E. caratova* were significantly lowered thereby attenuating the secretion of virulence factors (12, 30). Due to the hydrolysis of the lactone rings, cabbage rot caused by *E. caratova* was inhibited or severely retarded. In another experiment, Zhu et al. (61) was able to control infection by *E. caratova* on cactus stems and potatoes by using *aiiA* isolated from *B. thuringiensis*. Zhang and Dong (60) imply that expression of AHL degrading enzymes such as AHL lactonases would significantly boost the bacteria's competitive edge to ensure survival.

The biological activity of biofilms stability when exposed to *N*-acyl-L-homoserine lactone lactonase has not been well studied. This study was undertaken to determine the effects of the AHL degrading enzyme *N*-acyl-L-homoserine lactone lactonase on prevention of wild type *P. aeruginosa* (PA01) biofilm formation, disruption of PA01 preformed biofilms, and effect on bacterial recruitment to a surface. It was hypothesized that when PA01 is co-cultured with DH5α *E. coli* pMAL-t-*aiiA*, AHL secretion by PA01 would be quenched or attenuated due to hydrolysis of the lactone ring of the AHL, which

would prevent PA01 biofilm formation, disrupt preformed PA01 biofilms, and effect bacterial recruitment.

II. MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains used in this study are listed in Table 1. The *aiiA* from *B. thuringiensis* was previously cloned by Thomas et al. (51) into DH5α *E. coli* inducing it with 0.3mM isopropyl-β-D-thiogalactopyranoside (IPTG). All strains with the exception of DH5α *E. coli* pMAL-t-*aiiA* were maintained on LB agar. *C. violaceum* 31532 and 12472 were grown at 30°C, and PA01 and DH5α *E. coli* pMAL-t-*aiiA* were grown at 37°C. DH5α *E. coli* pMAL-t-*aiiA* was grown on LB agar supplemented with 50μg/ml ampicillin. After growth on agar, all strains were wrapped with parafilm and stored at 20°C until needed and stored for one week before re-growing.

Table 1: Strains and growth conditions of bacteria used in this study

Strain	Description	Purpose	Source or Reference
Chromobacterium violaceum 31532	Non-pigmented, C6HSL production	AHL lactonase activity	McClean et al. (31)
Chromobacterium violaceum 12472	Type strain	QSI indicator, AHL lactonase activity	McLean
DH5α *E coli* pMAL-t-*aiiA*	AHL degrading Lactonase	Hydrolyze lactone ring	Thomas (51)
Pseudomonas aeruginosa PA01	Wild Type	Secretion of AHLs	McLean

***N*-acyl-L-homoserine lactone lactonase activity assay.** To ensure that AHL lactonase was being induced *in vitro* by 0.3mM IPTG, an enzyme assay was conducted by using indicator strains of *C. violaceum* 31532 and *C. violaceum* 12472. DH5α *E. coli* pMAL-t-*aiiA* was grown at 37°C via shaking at 100rpm, while *C. violaceum* 31532 and 12472 were grown at 30°C and no shaking. 25μL of 50mg/ml ampicillin and 2.5ml of 0.3mM IPTG was added to the overnight culture of DH5α *E. coli* pMAL-t-*aiiA*. After 24-hour incubation without shaking, 3ml of DH5α *E. coli* pMAL-t-*aiiA* was transferred to 5 15ml conical vials (3ml in each conical vial). Then the *C. violaceum* 31532 was centrifuged in 1.5ml centrifuge tubes for 10 minutes at 13,200 x G. The 1.5ml supernatant was decanted into a 3ml culture of DH5α *E. coli* pMAL-t-*aiiA* and incubated for its designated period of time at 37°C without shaking. The incubation periods were 0.5hr, 1hr, 1.5hr, 2hr, and 4hr. After the designated incubation time had elapsed, 2-1.5ml centrifuge tubes were filled with the treated supernatant and DH5α *E. coli* pMAL-t-*aiiA* co-culture and centrifuged for 10 minutes at 13,200 x G. The supernatant from the 2-1.5ml centrifuge tubes was then decanted into 3ml of *C. violaceum* 12472. The culture was then minimally vortexed to mix and then incubated at 30°C for 24 hours. After 24 hours of incubation, the samples were removed from the incubator and minimally vortexed to suspend cells. Then, OD_{600} spectrophotometer readings were taken using 1ml

from each culture; spectrophotometer was blanked with 1ml LB. The absorbance readings from each of the samples were plotted and graphed using SigmaPlot v8.0.

***N*-acyl-L-homoserine lactone lactonase activity overlay assay**. As described by McLean et al. (33), an overlay assay was conducted to ensure AHL lactonase *aiiA* was being induced by 0.3mM IPTG. Briefly, 0.5µl of *C. violaceum* 12472 overnight was transferred to 5ml of 1% soft agar overlay and vortexed. The entire contents of the soft agar tube was poured over a plate of LB agar plate containing 0.3mM IPTG freshly streaked with PA01 and DH5α *E. coli* pMAL-t-*aiiA* in a cross design as in Figure 1. The plate was incubated at 30°C for 24-hours. The overlay procedure was repeated with the exception of the media used. Instead of streaking the bacteria onto an LB plate containing 0.3mM IPTG, 150µL of 0.3mM IPTG was pipetted directly onto the DH5α *E. coli* pMAL-t-*aiiA* prior to pouring contents of soft agar overlay onto the plate.

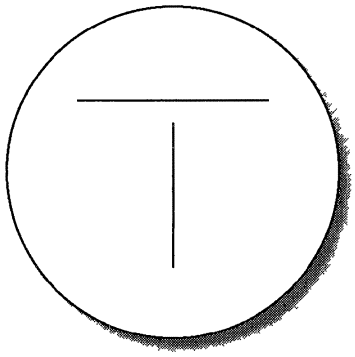

Figure 1: Soft agar overlay bacteria design to test for AHL lactonase activity by inhibition of AHLs. Top horizontal line represents PA01, and the bottom vertical line represents DH5α *E. coli* pMAL-t-*aiiA*.

Disruption of preformed PA01 biofilms assay. To test the ability of AHL lactonase to disrupt preformed PA01 biofilms, 8mm silicone disks were placed into fresh LB cultures of PA01 and incubated for 48 hours via shaking at 100rpm at 37°C. The silicone disks were washed at room temperature in diH_2O to remove any non-adherent bacteria and transferred to 24-hour cultures of DH5α *E. coli* pMAL-t-*aiiA*; both cultures contained 50μg/ml amp but one culture contained 0.3mM IPTG. Both cultures were incubated at 37°C for 48 hours via shaking at 100rpm. After 48-hour incubation, the silicone disks were washed, sonicated for 10 minutes, and vortexed for 30 seconds. 10-fold serial dilutions were conducted from the sonicated sample. The dilution plates were incubated for 24 hours at 37°C and quantified to determine CFU/ml. SigmaPlot and SigmaStat were used to plot and run ANOVA and t-Test statistical analysis of the average log CFU.

96-well plate assay. To test for prevention of PA01 biofilm formation, the protocol from O'Toole (40) was used. Briefly, three separate trials were conducted each with 9 samples of 3 different cultures. A FisherBrand 96-well microtiter plate was inoculated with 100µl of a 1:100 dilution of a PA01 monoculture, a DH5α *E. coli* pMAL-t-*aiiA* monoculture, and a co-culture of PA01 DH5α *E. coli* pMAL-t-*aiiA* and incubated 24-hours at 37°C via shaking at 100rpm; nine wells for each culture were used. Cultures with DH5α *E. coli* pMAL-t-*aiiA* contained 0.3mM IPTG and 50µg/ml amp.

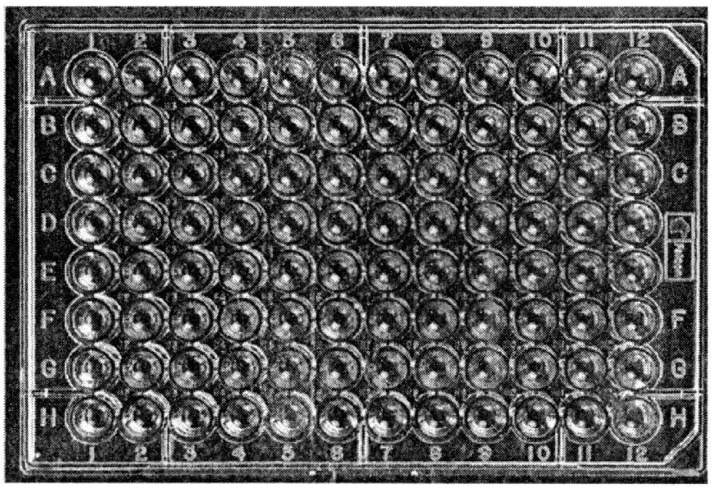

Figure 2: Example of a 96-Well microtiter plate used in testing for 96-well plate assay for PA01 biofilm prevention. Row A 1-9 was assigned for PA01; row C 1-9 was used for DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50µg/ml amp; row E 1-9 was used for PA01 DH5α *E. coli* pMAL-t-*aiiA* co-culture with 0.3mM IPTG and 50µg/ml amp.

The microtiter plate was inverted into a waste container to remove biofilms and then submerged into clean water. This was repeated three times to remove non-adherent bacteria. Then, 125µL of freshly prepared 1% w/v crystal violet was added to each of the

wells and incubated at room temperature for 10 minutes. Following incubation, the plates were washed and inverted onto a stack of paper towels to remove residual water and crystal violet. 200μL of 95% EtOH were added after a 30 minute dry time to each of the wells and mixed several times. The plates were covered and incubated at room temperature for 15 minutes. 125μL of the mixture was then transferred to new previously unused wells and read at OD_{600} using a SpectraMax microtiter plate reader. The OD_{600} readings were plotted and graphed using SigmaPlot.

Bacterial recruitment assay. To determine if PA01 AHL degradation via AHL lactonase had any effect on recruitment of various bacteria, a modified protocol of McLean et al. (32) was used. Spent culture supernatants of overnight cultures of the following bacteria were prepared by centrifugation for 10 minutes at 10,000 x G and filter sterilized using Fisherbrand 0.22μm syringe filter to filter out any remnant bacteria left in the supernatant: PA01, PA01 with 0.3mM IPTG and 50μg/ml amp, PA01 with 0.3mM IPTG but without amp, PA01 without IPTG and with 50μg/ml amp, co-culture PA01/DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50μg/ml amp, co-culture PA01/DH5α *E. coli* pMAL-t-*aiiA* without IPTG and with 50μg/ml amp, and co-culture with 250μL each at OD_{600} .120nm of PA01 and DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM

IPTG and 50μg/ml amp; uninoculated LB was also used as a control. 1000 molecular weight cutoff (MCWO) dialysis tube was inoculated with 1ml of one of the spent supernatant cultures and clamped on both ends with a Spectra clamps; one clamp was weighted. Once the dialysis tube was inoculated, it was placed into a beaker of diH_2O of sterile water for transport. Field studies were conducted at Spring Lake in San Marcos Texas. Duplicate dialysis tubes were placed at random 25cm apart submerged in the water and attached to the wire fencing on the wooden deck. The inoculated tubes were allowed to incubate for 5 hours and then retrieved for analysis.

The contents of the dialysis tubes were discarded and then gently dipped into sterile diH_2O to remove non-adherent bacteria. Approximately 3cm section of the dialysis tube was placed into a scintillation vial containing 10ml of diH_2O and sonicated for 10 minutes. Following sonication, 10-fold serial dilutions were performed and then plated onto R2A, and incubated at 30°C for 48 hours. Then colony morphology was observed, and plates were quantified to determine CFU/ml.

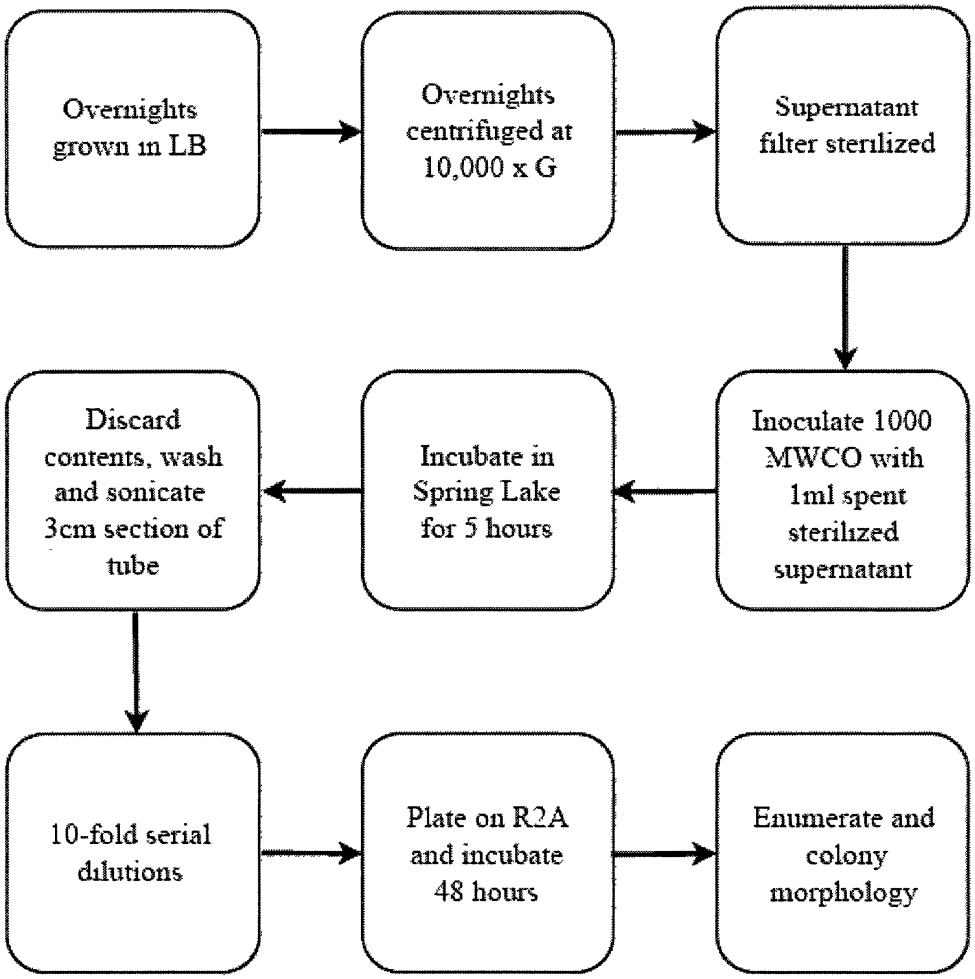

Figure 3: Flowchart of bacterial recruitment assay.

III. RESULTS

N-acyl-L-homoserine lactone lactonase activity assay

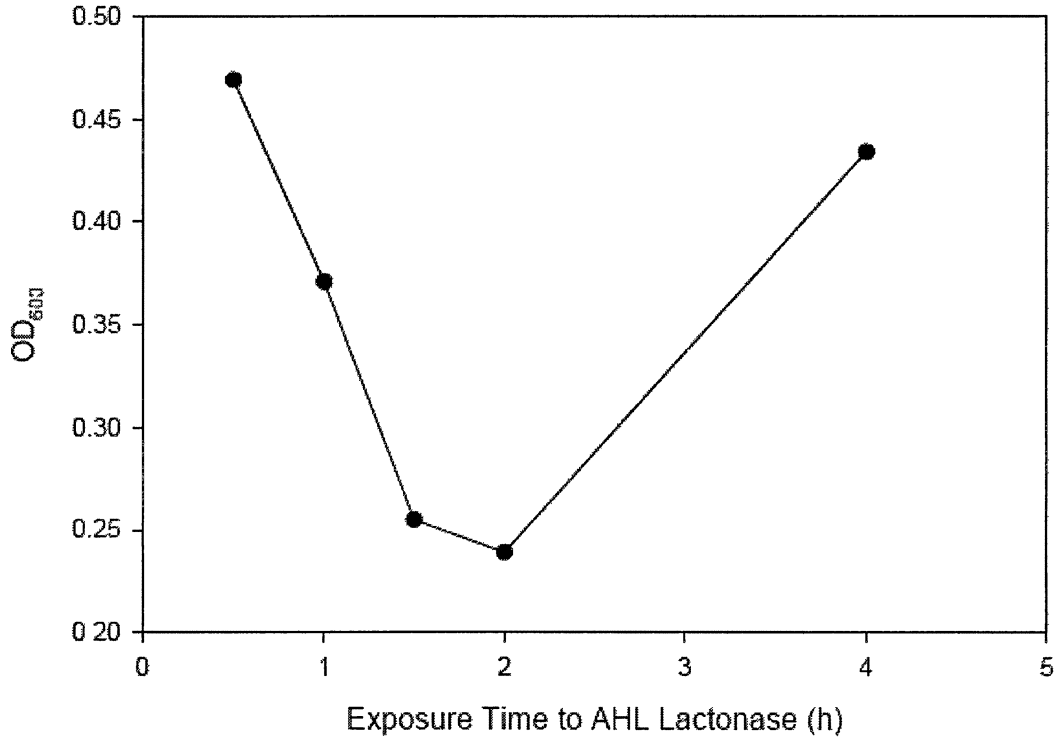

Figure 4: AHL lactonase *aiiA* expression assay using *C. violaceum* 31532, *C. violaceum* 12472, and DH5α *E. coli* pMAL-t-*aiiA* using OD_{600} versus incubation times in hours.

OD_{600} readings in Figure 3 show the AHL lactonase activity using *C. violaceum* 31532, *C. violaceum* 12472, and DH5α *E. coli* pMAL-t-*aiiA*. A decrease in absorbance

indicate an increase in AHL lactonase activity, which was seen between the incubation times of 0.5 hours to 1.5 hours. Between 1.5 hours and 2 hours, a small decrease in activity was observed, and a substantial decrease in activity was observed between the 2-hour and 4-hour incubation time period

_N_-acyl-L-homoserine lactone lactonase overlay assay

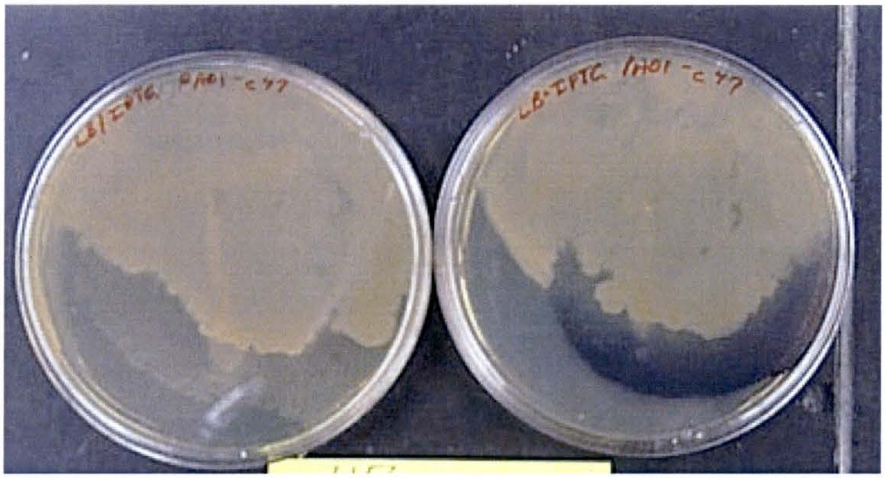

Figure 5: 0.3mM IPTG LB agar plates with cross design of PA01 and DH5*α E. coli* pMAL-t-*aiiA* overlaid with 5ml soft agar overlay containing 0.5µl *C. violaceum* 12472.

0.3mM IPTG LB was made to test the AHL lactonase activity. Duplicate plates of PA01 and DH5α *E. coli* pMAL-t-*aiiA* were streaked in a cross design as in Figure 1. A white halo in the purple pigment secreted by *C. violaceum* 12472 should have been seen around PA01, but instead, the entire contents of the overlay were white without the presence of a halo around PA01. The presence of a white halo in the *C. violaceum* 12472 purple violacein would indicate the presence of AHLs.

Figure 6: PA01 and DH5α *E. coli* pMAL-t-*aiiA* grown in cross design overlaid with 5ml soft agar overlay containing 0.5µl *C. violaceum* 12472. 150ul of 0.3mM IPTG was spread over DH5α *E. coli* pMAL-t-*aiiA*.

PA01 and DH5α *E. coli* pMAL-t-*aiiA* were streaked onto LB/Amp 50µg/ml in cross design as in Figure 1. 150µl of 0.3mM IPTG was streaked onto the plate in an attempt to induce expression of *aiiA*. No white halo in the purple violacein was observed around PA01, but instead, a solid white overlay was observed. However, on plate 2, pyoverdin production was noticed as well as the absence of a white halo in the violacein overlay. Also, PA01 over grew DH5α *E. coli* pMAL-t-*aiiA* on plate 2.

Disruption of preformed PA01 biofilms

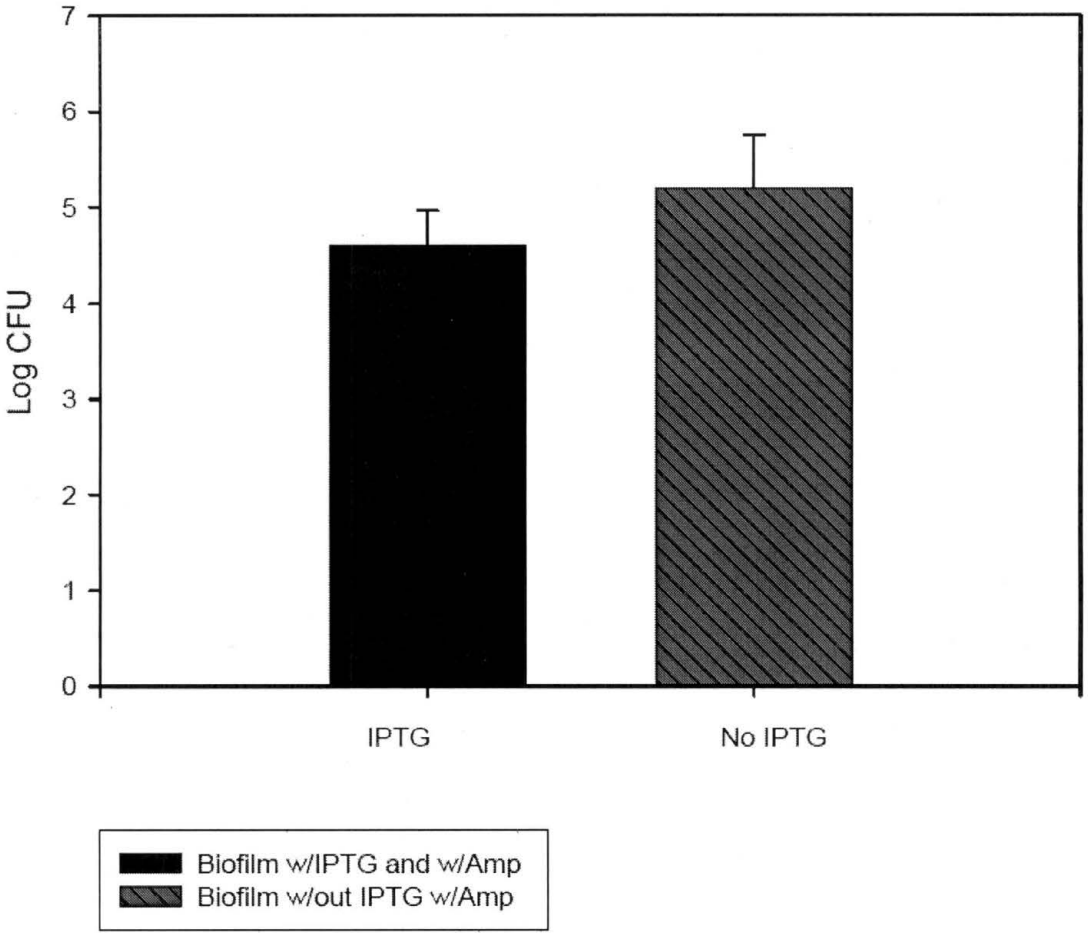

Figure 7: Disruption of preformed PA01 Biofilms grown with and without 0.3mM IPTG. The first bar represents the average from 3 trials of CFU/ml of PA01 preformed biofilm when placed into a 24-hour culture of DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50μg/ml amp. The second bar represents the average from 3 trials of CFU/ml of PA01 preformed when placed into a 24-hour culture of DH5α *E. coli* pMAL-t-*aiiA* with 50μg/ml amp but without IPTG.

To test for disruption of preformed PA01 biofilms, 8mm silicone disks were placed in a PA01 LB broth monoculture for 48 hours and then transferred to a 24-hour culture of DH5α *E. coli* pMAL-t-*aiiA* with or without 0.3mM IPTG; both DH5α *E. coli*

pMAL-t-*aiiA* cultures contained 50μg/ml amp. The PA01 preformed biofilm disks that were placed into a 24-hour culture of DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG had an average of 4.5 Log CFU count. The PA01 preformed biofilm disks that were placed into a 24-hour culture of DH5α *E. coli* pMAL-t-*aiiA* without IPTG had an average of 5.1 Log CFU count.

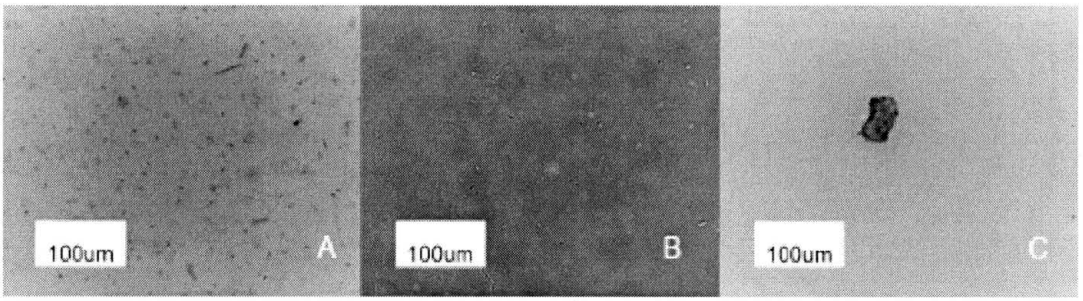

Figure 8: Epifluorescence microscopy of a co-culture of PA01 and DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50μg/ml amp. (A) culture after 48 hours incubation of PA01 silicone disk in DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50μg/ml; (B) wash of silicone disks after 48 hours incubation of PA01 silicone disk in DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50μg/ml; (C) biofilm after 48 hours incubation of PA01 silicone disk in DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50μg/ml.

All of the microscopy pictures in Figures 8 and 9 were taken from silicone disks that were grown for 48 hours in a monoculture of PA01 and then transferred to monoculture of DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50μg/ml. After 48 hours of incubation in DH5α *E. coli* pMAL-t-*aiiA*, the disks were washed and sonicated. Figure 8A and 9A shows PA01 disks incubated 48 hours in DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50μg/ml amp. Figure 8B and 9B shows the disk wash in 5ml

diH_2O, and Figure 8C and 9C shows the bacteria remaining on the disk after sonication in 5ml diH_2O. As anticipated, a higher concentration of bacteria was seen on the disks prior to sonication. Figures 8A and 9A have the highest concentration of bacteria compared to Figures 8C and 9C. Although the concentration of bacteria noticeably decreases from Figures 8A and 9A to 8C and 9C, the concentration does not differ significantly between PA01 biofilms treated with AHL lactonase (Figure 8) versus PA01 biofilms not treated with AHL lactonase (Figure 9).

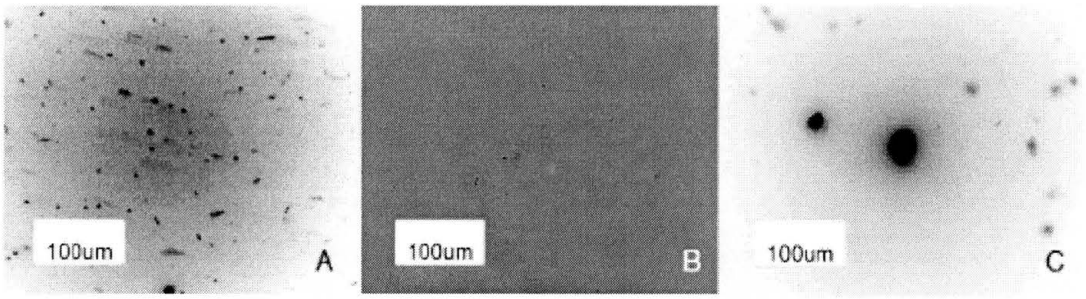

Figure 9: Epifluorescence microscopy of a co-culture of PA01 and DH5α *E. coli* pMAL-t-*aiiA* with 50μg/ml amp but without IPTG. A through C correspond with D through F; (A, D) culture after 48 hours incubation of PA01 silicone disk in DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50μg/ml; (B, E) wash of silicone disks after 48 hours incubation of PA01 silicone disk in DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50μg/ml; (C, F) biofilm after 48 hours incubation of PA01 silicone disk in DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50μg/ml.

96-well plate assay

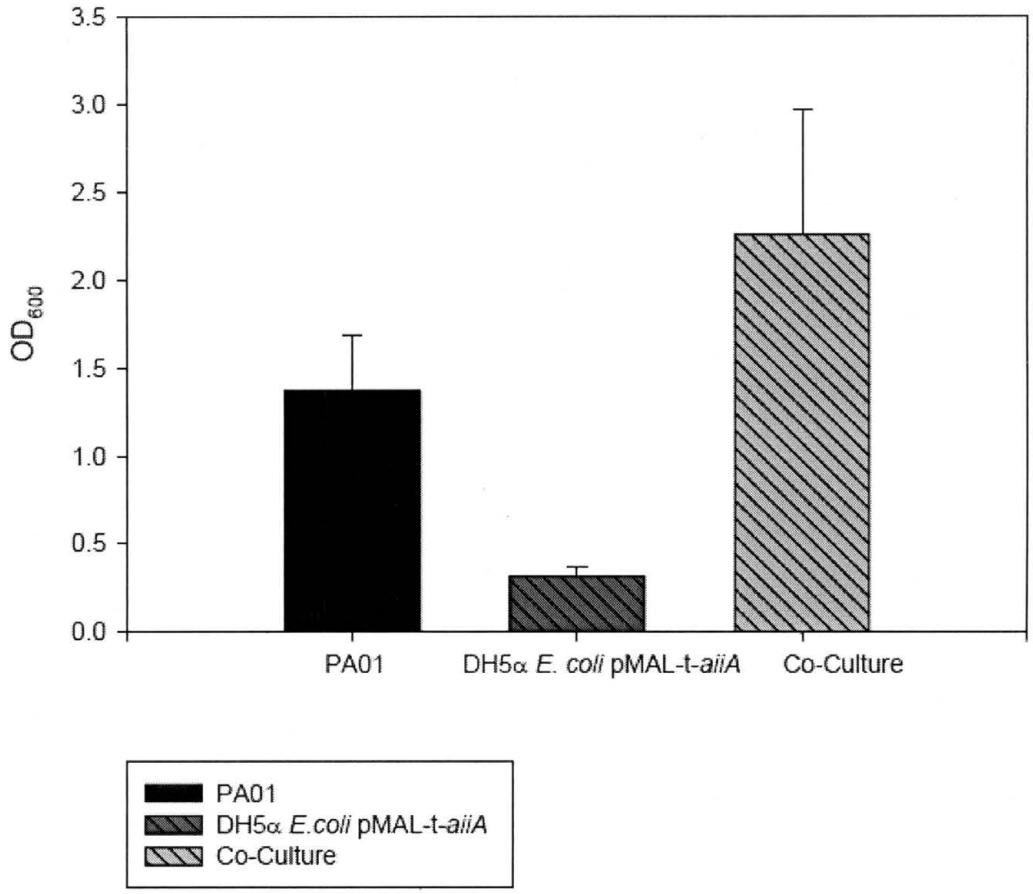

Figure 10: PA01 biofilm prevention using AHL lactonase *aiiA*. The first bar represents PA01 monoculture; the second bar represents DH5α *E. coli* pMAL-t-*aiiA* monoculture with 0.3mM IPTG and 50μg/ml amp; the third bar represents a co-culture of PA01 and DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50μg/ml amp.

The chart represents an average OD_{600} reading of 3 trials with 9 samples for each culture. AHL lactonase *aiiA* was induced with 0.3mM IPTG in an attempt to prevent or attenuate PA01 biofilm formation. PA01 when grown as a monoculture grew to an average OD_{600} reading of 1.3, and DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and

50μg/ml amp grown as a monoculture grew to an average OD_{600} reading of .38. When PA01 was co-cultured with DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG and 50μg/ml amp, the average OD_{600} reading was 2.28, which is significantly more dense than when grown in a monoculture.

Bacterial recruitment assay

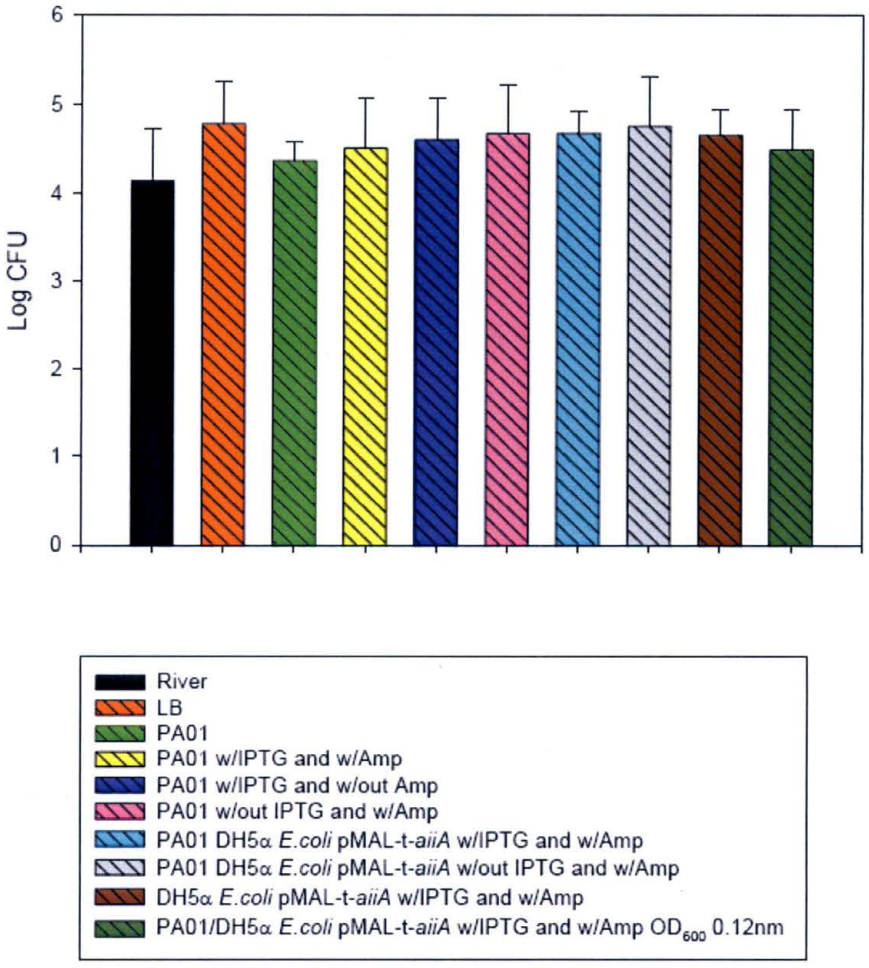

Figure 11: Effect of AHL Lactonase *aiiA* on bacterial recruitment. Each of the samples is a log average of CFU of enumerated bacteria after 10-fold serial dilutions.

AHL lactonase *aiiA* was induced with 0.3mM IPTG in overnight cultures of DH5α *E. coli* pMAL-t-*aiiA*, PA01 co-cultured with DH5α *E. coli* pMAL-t-*aiiA* without IPTG, PA01 co-cultured with DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG, and PA01 mixed with DH5α *E. coli* pMAL-t-*aiiA* with 0.3mM IPTG at an OD_{600} at 0.12 for both cultures. As implied in Figure 11 and confirmed by ANOVA, there was no significant difference in average log CFU of bacterial recruitment between any of the samples. Each of the samples had an average log CFU around 4.5.

IV. DISCUSSION

Biofilms are the accumulation of sessile bacterial communities, which can interfere with the normal function of devices such as indwelling catheters (4, 36). It has been suggested that quorum-sensing via the secretion of small diffusible molecules including AHLs by Gram-negative bacteria plays a vital role in biofilm formation (7, 32). Quorum-sensing via the secretion and accumulation of AHLs is used by many bacteria including *P. aeruginosa* to form mature biofilms and secrete population dependent virulence factors (6, 13, 35). According to Costerton and Lewandowski (6), water channels are formed within mature biofilms for nutrient transport and cellular communication via AHLs. Mutant *P. aeruginosa* lacking the ability to secrete *N*-(3-oxo-C_{12}-HSL) were unable to form mature biofilms and lacked proper water channels (6, 7). Reimmann et al. (43) found that destruction of the *N*-(3-oxo-C_{12}-HSL) reduced expression of virulence factors and swarming motility in PA01.

Recent research have focused heavily on the ability of *N*-acyl-L-homoserine lactone lactonase to quench the AHL quorum-sensing pathway by degrading the AHL

signal (11, 12, 25, 27, 51, 61). The most studied AHL lactonase to date is the AiiA isolated from *B. thuringiensis* by Dong et al. (10, 11). In an experiment conducted by Dong et al., the AiiA AHL lactonase was shown to quench AHL quorum-sensing pathway of *E. caratova* thereby reducing the level of cabbage rot (11). Thomas et al. (51) was able to transform the *aiiA* from *B. thuringiensis* into DH5α *E. coli* yielding DH5α *E. coli* pMAL-t-*aiiA*, which was used in this experiment to test for PA01 biofilm stability and test for effects on biofilm bacterial recruitment. Certainly quorum-sensing inhibitors as well as others show promise in the disruption of biofilm formation (19), but at this time, an effective method of preventing and removing biofilms has yet to be discovered.

_N_-acyl-L-homoserine lactone lactonase activity

In order to determine if the lactonase gene *aiiA* from *B. thuringiensis* was being induced *in vitro*, an enzyme assay was performed using *C. violaceum* 31532, *C. violaceum* 12472, and DH5α *E. coli* pMAL-t-*aiiA*. After conducting the AHL lactonase assay using the two indicator strains of *C. violaceum*, it was evident that the gene *aiiA* was being induced *in vitro*, and the AHL lactonase was being expressed. An increase in AHL lactonase activity was noticed between the incubation periods of 0.5 hour to 1.5

hours, but then decreased at the 2-hour incubation period up to the 4-hour incubation period. It is possible that after 1.5 hours of incubation, the AHL lactonase became exhausted, which is why a decrease in activity was observed.

An overlay assay was also used to test for AHL lactonase activity, but the observed results were different than expected. According to previous experiments conducted by McLean, Pierson, and Fuqua (33), a white halo should have been observed around PA01 indicating an inhibition of *C. violaceum* 12472 violacein pigment due to secreted AHLs from PA01. AHLs inhibit pigment production due to a competitive inhibition of the *C. violaceum* cognate C_6HSL by $3OC_{12}$HSL and C_4HSL. The signals competitively bind C_6HSL secreted by *C. violaceum* 12472 (33). However, a white halo–quorum-sensing inhibition–wasn't observed for the overlay assay. Instead of observing a white halo, the entire contents of the *C. violaceum* 12472 overlay were white without any visual indication of violacein production. Since DH5α *E. coli* is not known to secrete AHLs of its own (49, 54), this indicates that PA01 is secreting AHLs that are competitively binding with C_6HSL secreted by *C. violaceum* 12472. Since the PA01 outgrew DH5α *E. coli* pMAL-t-*aiiA* in the overlay assay, there was no way to tell if the lactonase was being induced by 0.3mM IPTG *in vitro*. When 150μL of 0.3mM IPTG was spread over DH5α *E. coli* pMAL-t-*aiiA*, pyoverdin expression from PA01 was

noticeably evident. Pyoverdin is a virulence factor secreted by PA01 as a siderophore that acts as an iron chelator during iron starvation (34). Since *P. aeruginosa* can utilize many different carbon sources (52), it is possible that IPTG was serving as a carbon source for PA01, and therefore PA01 had to express pyoverdin to maintain iron requirements (34). Alternatively, IPTG or co-culture conditions may independently induce pyoverdin synthesis. Pyoverdin is synthesized by PA01 to induce apoptosis of neutrophils (15) to satisfy nutrient, such as iron, requirements and reduce proinflammatory response. Sio et al. (47), found a gene–PA2385–within the PA01 genome that encodes for an acylase thought to be on the pyoverdin synthesis operon. It is possible that pyoverdin is synthesized by PA01 due to a direct competition of the acylase found in the PA2385 gene with the induced *aiiA* by 0.3mM IPTG, and is produced to ensure survival. Similar pyoverdin synthesis was noticed in a co-culture experiment conducted by Weber and McLean 2007 (56).

Disruption of preformed PA01 biofilms

To test if the AHL lactonase had any effect on prevention of PA01 biofilm formation, a 96-well plate assay was conducted. The statistical analysis revealed that there was no significant difference between the PA01 cultures treated with the AHL

lactonase and the PA01 cultures not treated with AHL lactonase. However, before transforming the CFU/ml data into an average log CFU, there was a 10-fold difference in every trial between the cultures containing the induced AHL lactonase versus cultures that did not contain induced AHL lactonase. The epifluorescence microscopy of the bacteria from the silicone disks revealed that there wasn't a significant difference in concentration between the treated PA01 silicone disks versus the untreated PA01 silicone disks. Although there was no statistical significance, this 10-fold difference in CFU/ml would indicate that the AHL lactonase does have some effect on removal of preformed PA01 biofilms.

96-well plate assay

To test for prevention of PA01 biofilm formation, DH5α *E. coli* pMAL-t-*aiiA* was used to cleave the lactone ring of *N*-(3-oxo-C_{12}-HSL) autoinducer. Statistical analysis revealed the AHL lactonase did not significantly reduce the quantity of biofilm formation when compared to the PA01 monoculture biofilm. In contrast to what was hypothesized, the OD_{600} readings indicated a significant increase in biofilm formation when PA01 was co-cultured with DH5α *E. coli* pMAL-t-*aiiA* induced with 0.3mM IPTG. This again could be due to PA01 utilizing IPTG as a carbon source. This phenomenon

could also be due to PA01 increasing its growth rate to out compete DH5α *E. coli* pMAL-t-*aiiA*. An et al. (3) found that the growth rate of *P. aeruginosa* increased when co-cultured with *Agrobacterium tumefaciens* to the point of what they referred to as blanketing *A. tumefaciens*, which is directly related to secretion of AHLs.

Bacterial recruitment assay

McLean et al. (32) suggested that AHLs have an effect on biofilm bacterial recruitment. According to McLean, bacteria are attracted to the surface of established biofilms in marine environments via the secretion of AHLs. The AHL lactonase AiiA was used to quench the autoinducer signal secreted by PA01 to determine if that would have any effect on bacterial recruitment. In theory, if the AHL lactonase did quench the quorum-sensing autoinducer *N*-(3-oxo-C_{12}-HSL), then the quantity (CFU/ml) of bacteria would be less in cultures treated with the AHL lactonase versus cultures not treated with the AHL lactonase. Three separate trials were conducted with each trial having 20 samples of various controls and treatments, and there was no statistical significance in average log CFU between the controls and treated cultures. Species identification was not done for this experiment; bacteria were only enumerated using 10-fold serial dilutions. However, colony morphology between each of the 20 samples did not appear

to vary. Again, it may be possible that the AHL lactonase AiiA is having an effect, but not to the point of causing significant differences.

V. CONCLUSIONS

In conclusion, the *N*-acyl-L-homoserine lactone lactonase does seem to have a small but insignificant effect on biofilm stability. However, in the case of bacterial recruitment, the data indicate that there was no significant difference between the average log CFUs of the controls versus the average log CFUs of the monocultures and the co-cultures of PA01 and DH5α *E. coli* pMAL-t-*aiiA*.

Since the *aiiA* isolated from *B. thuringiensis* cloned into DH5α *E. coli* is cytoplasmic, future experiments should focus on exporting the lactonase out of the cytoplasm. In theory, if the AHL lactonase could be exported from the cytoplasm, then the chances of it interacting with AHLs should increase. It is in my opinion that the interactions of the AHL lactonase and the AHLs are being restricted since the AHL lactonase is not secreted or exported from the cytoplasm. Increased interactions between AHLs and the AHL lactonase should disrupt the quorum-sensing pathway of PA01 and other Gram-negative bacteria.

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