The Effects of the Unnatural Amino Acid TTO- 53 on the Biofilm of *Staphylococcus aureus*

By

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Abstract

There are over 30 different species of staphylococci and many cause infection that can lead to hospitalization or death. The member most associated with chronic infection is *Staphylococcus aureus. Staphylococcus aureus* is a Gram-positive bacterium and usually harmless. It can be found on the skin of 15-40% of people in America. *S. aureus* spreads through contact with contaminated surfaces and skin to skin contact. Additionally, *S. aureus* has evolved a number of strategies to become more resistant to antibiotics. One strategy important to facilitating antibiotic resistance and resisting unfavorable environmental changes is biofilm. Biofilm is an extracellular matrix made up of extracellular DNA, proteins, and polysaccharides and acts as extra protection for the bacteria cells. Biofilm development happens in three phases, attachment, maturation, and dispersal phase. During attachment, cells aggregate due to poly-N-acetylglucosamine introducing a positive charge to the negatively charged bacteria. Cells will mature and aggregate through cell-to-cell communication with the use of autoinducer, using a regulatory system known as quorum sensing. Quorum sensing is regulated by a large number of genes involved in both assembly and disassembly. We have previously demonstrated that an unnatural amino acid, TTO-53 is able to disrupt biofilm in a several other organisms. The goal of this research was to determine if TTO- 53 demonstrates similar effects on the biofilm of *Staphylococcus aureus* and how this is occurring. Our data indicated that there are disruptive

effects occurring in biofilm when TTO-53 is introduced to the environment. This disruption is not due to cell death. There is evidence that TTO- 53 is impacting regulation of several biofilm genes. But it is still unknown how TTO-53 is causing disruption.

The Effects of the Unnatural Amino Acid TTO- 53 on the Biofilm of *Staphylococcus aureus*

A Thesis

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Chapter 1: Introduction

There are over 30 different species of staphylococci and many cause infection that can lead to hospitalization or death.[3] Within the staphylococcus genus, one of those most associated with chronic infection is *staphylococcus aureus*. In the United States of America this bacterium is the leading cause in health care associated infections.[32] *Staphylococcus aureus* is a Gram-positive non motile and cocci bacterium, that was first discovered sometime in the 1880's.[18] *Staphylococcus aureus* is usually harmless and can be found on the skin of 15–40 % of people in United States.[30] *S. aureus* spreads through contact with contaminated surfaces and skin to skin contact. If infection occurs, it can hospitalize or kill the patient due to complications of various infections. Blood stream infections, toxic shock syndrome, mastitis, meningitis, pneumonia, and osteomyelitis are among the more common infections in hospital patients. People with burns, deep wounds and compromised immune systems have an increased susceptibility to a higher severity of infection.[18] Along with the complications and infections caused by this bacterium, its resistance to antibiotics has become increasingly concerning. *S. aureus* has evolved and become more resistant to antibiotics, aiding in resistance to antibiotics and unfavorable environmental changes is biofilm.[28]

Biofilm Phases

Biofilm is extra protection for the bacteria against antibiotics, unfavorable environmental changes, and host defenses, making the bacteria less susceptible to treatments. Biofilm is an extracellular matrix, made of extracellular DNA (eDNA), proteins, and polysaccharides that can form on biotic and abiotic surfaces.[23] Biofilm has three phases, attachment, maturation, and dispersal phase as shown in Figure 1. In the first phase, attachment, the *S. aureus* cells adhere to the surface through different mechanisms such as, eDNA, proteins and adhesin molecules.[22]

After attachment the cells start to aggregate and mature. During maturation, the cells aggregate, meaning the cells are forming a cluster and intercellular adhesion is occurring due to poly-N-acetylglucosamine (PIA).[20] This molecule is important because it introduces a positive charge to the negatively charged bacteria cells, which keeps the cells and surface glued together.[27] PIA makes up most of the outer matrix creating the 3-diminsional towers shapes of the biofilm.[20] To ensure cells within the biofilm are receiving nutrients, channels are created within the biofilm to allow nutrients to reach the cells deep in the biofilm.[22] Lastly, disassembly of the biofilm will occur and mature cells disperse landing on another surface and restart this cycle.[20]

All bacteria produce biofilm but diverge in characteristics of biofilm assembly and disassembly. For instance, when *pseudomonas aeruginosa* is grown in cultures within a conical or 96-well plates, the biofilm will stick to the sides creating a ring due to it being a motile bacterium whereas, *S. aureus* being nonmotile, biofilm will assemble at the bottom. These bacteria also use different proteins and molecules during the stages of biofilm formation. When it comes to the systems of biofilm assembly and disassembly *P. aeruginosa* utilizes four systems, Iqs, Rhl, Las, and Pqs. *S. aureus* mainly relay on one system, agr system.[33]

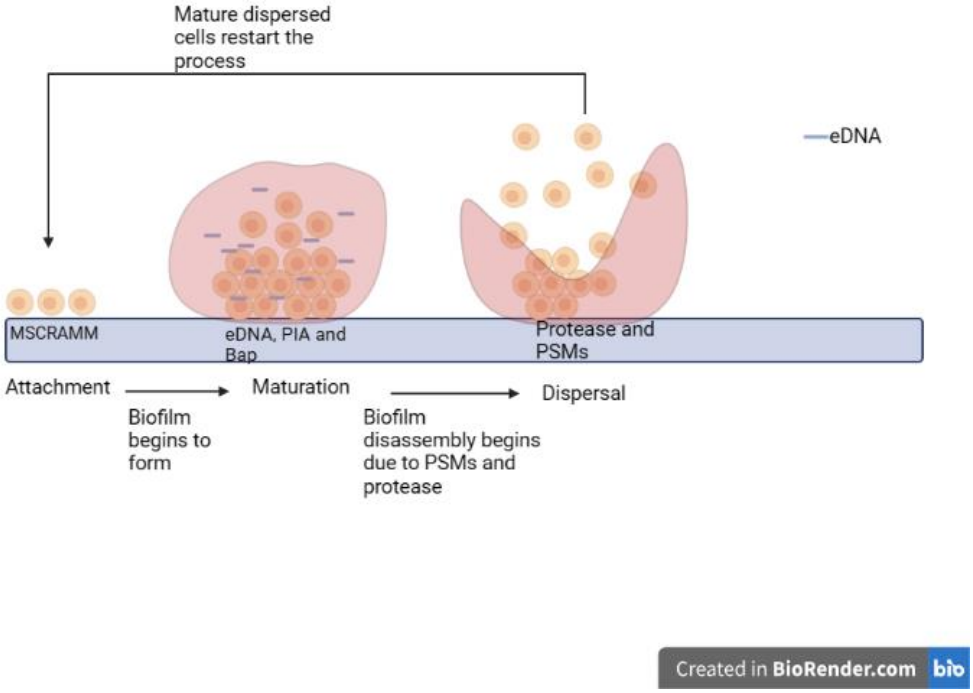

Figure 1. *The phases of biofilm, proteins and protease at certain phases. The long blue rectangle represents the surface, the bacterial cells are represented by the light orange circles. The transparent red structure surrounding the cells is the biofilm.*

The Accessory Gene Regulatory System

Cells use quorum sensing to control the phases of biofilm, motility [22] and communication among cells.[28] Quorum sensing is a method of cells communicating to each other with the use of autoinducer peptides (AIP). An autoinducer peptide is a five-residue cyclic thiolactone ring, and functions as a chemical signal.[34] Changes in concentration of autoinducer peptides indicate a change in concentration of bacterial cells within the environment. When the threshold for autoinducer concentration is reached this results in activation of genes to make the bacteria adapt to changes occurring.[22] The accessory gene regulatory (Agr) system regulates cell to cell communication and interaction between the bacteria and host, factors of virulence, and different toxins. This system also helps *S. aureus* to adapt to environmental changes and is responsible for quorum sensing. The genes in the Agr system work in pairs, AgrA-AgrC and AgrB-AgrD as

shown in Figure 2. The regulation and transportation of AIP is controlled by AgrB and D genes. The AgrD is a ribosomal peptide and precursor of AIP that is responsible for encoding the autoinducing peptide signal. The integral membrane bound peptidase, AgrB can process and transport AIP into the environment after cleaving the AgrD from the mature AIP. The role of the signal peptidase, SpsB is to release the mature AIP into the extracellular environment. The pair AgrA and AgrC become activated when in the extracellular environment, the concentration of AIPs passes a threshold which in turn triggers intercellular communication, known as quorum sensing. In the extracellular environment a certain level of AIPs needs to accumulate in order for AgrC, a histidine sensor kinase, to sense its signal.[14] When the signal is sensed AIP binds to the extracellular domain of the AgrC.[9] This binding will cause a conformation change of the cytoplasmic helix resulting in autophosphorylation.[31] After phosphorylation of AgrC, the phosphate is received by AgrA, a response regulator. The phosphorylated AgrA will be able to bind with the P2 and P3 promoters that mediate the expression of RNAII and RNAIII, which are adjacent transcriptional regions.[14]

The difference between RNAII and RNAIII is the four genes of the *agrBDAC* operon is encoded by the RNAII. The *agrBDAC* operon encodes the components for quorum sensing, including the detection, signal, and processing. The RNAIII transcript regulates the expression of Agr- dependent target genes and acts as the main effector molecule. RNAIII regulates the expression of virulence factors such as hemolysin δ, enterotoxins, exotoxins, proteases, and other factors that contribute to the virulence of *S. aureus*. RNAIII also inhibits cell surface proteins from being expressed. AgrA after being phosphorylated can also bind with α and β phenol-soluble modulin promoters to drive PSMs expression. The inhibition of cell surface proteins due RNAIII and the PSMs being expressed will aid in the dispersal phase of biofilm. [14]

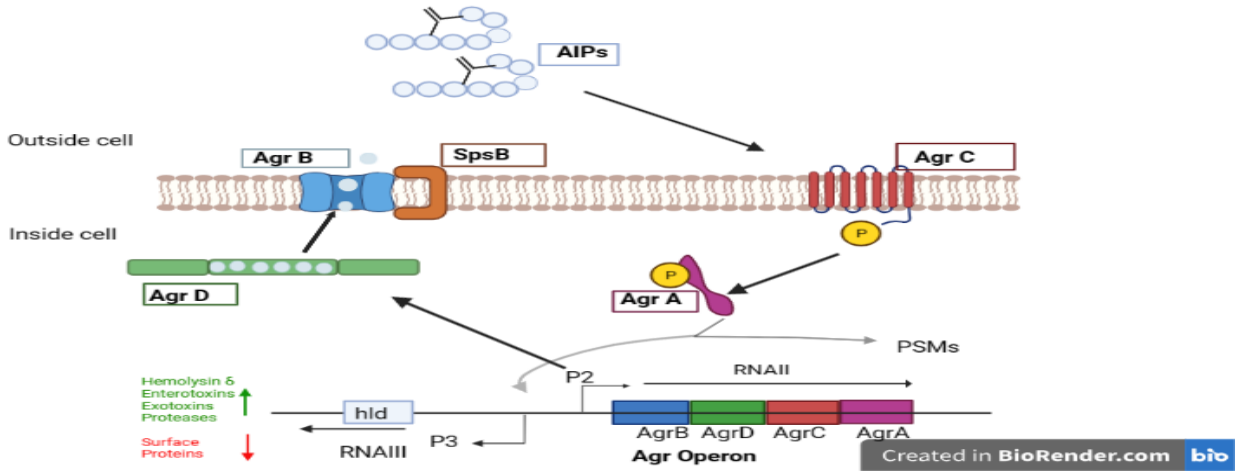

***Figure 2**. The pathway for accessory gene regulatory system. Agr B (blue) cleaves Agr D (green) from the AIPs (light blue circles). SpsB (orange) helps Agr B move the mature AIPs outside the cell. Once the AIPs concentration passes a threshold, Agr C (red) will sense their signal and bind together. Agr C will then be auto phosphorylated. Agr A (purple) will receive a phosphate from Agr C allowing it to bind to P2, P3, and PSMs. This system will act a continuous feedback loop.*

Phenol-Soluble Modulins (PSM) and Proteases

The Agr system also regulates the dispersal phase of *S. aureus* biofilm using Agr A, B, C, and D when induction of phenol-soluble modulins (PSMs) and proteases occurs. Phenol-soluble modulins are peptides derived from Staphylococci that are amphipathic, short and have surfactant properties.[13] α PSMs are about 20-25 amino acids while β PSMs are bigger in length of about 40-45 amino acids both forming α-helix structure. [5] The PSMs aid in biofilm disassembly and play a key role in the pathogenesis of *S. aureus,* causing lysis of different types of human cells.[16]

The cleavage of peptide bonds is catalyzed by a class of enzymatic molecules known as protease, that can be extracellular and intracellular. Proteases are found in all living organisms and aid in anything from regulatory activity to general protein degradation. Due to evidence of

extracellular proteins and enzymes having a role in biofilm structure, its predicted that the extracellular proteases are involved in the regulation of detachment and dispersal of biofilm. The extracellular proteases of *S. aureus* are metalloprotease (Aur), two cysteine proteases (Staphopains ScpA and SspB) and seven serine proteases (SspA and Sp1A-F).[23] A single mon-cistronic operon encodes aureolysin. SspA and B are co-transcribed together, encoded by a polycistronic operon as well as ScpA protease. Aureolysin goes from Pro- aureolysin (inactive) to an active form due to auto proteolytic activation. Then through a mediated cleavage by Aureolysin, SspA is activated, which then will allow the activation of SspB. Similar to aureolysin, ScpA protease activates through self-auto proteolytic cleavage. [23] In studies of bacterial cultures, various proteases were shown to aid in dispersal of established biofilms and reduced biofilm formation. In research done by Mootz *et al*. staphopains protease were found to impact biofilm integrity indicating these proteases aid in regulation of biofilm formation. In the same study SspB and ScpA were overexpressed and purified to have therapeutic properties against different strains of *S. aureus*. The results demonstrated that ScpA had inhibitory properties and was able to disperse biofilm that was already established. Also, both these proteases, SspB and ScpA, played a role in elimination of biofilm formation.[15]

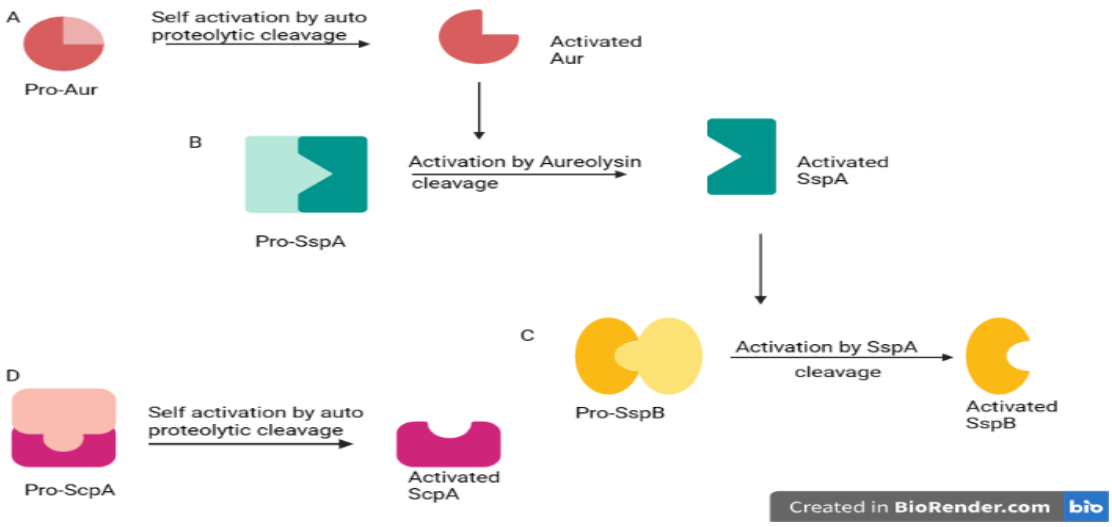

Figure 3A. *Proteolytic activation of S. aureus proteases. The red, blue, yellow and purple represent the active and inactive states of aureolysin, SspAB and ScpA (respectively).*

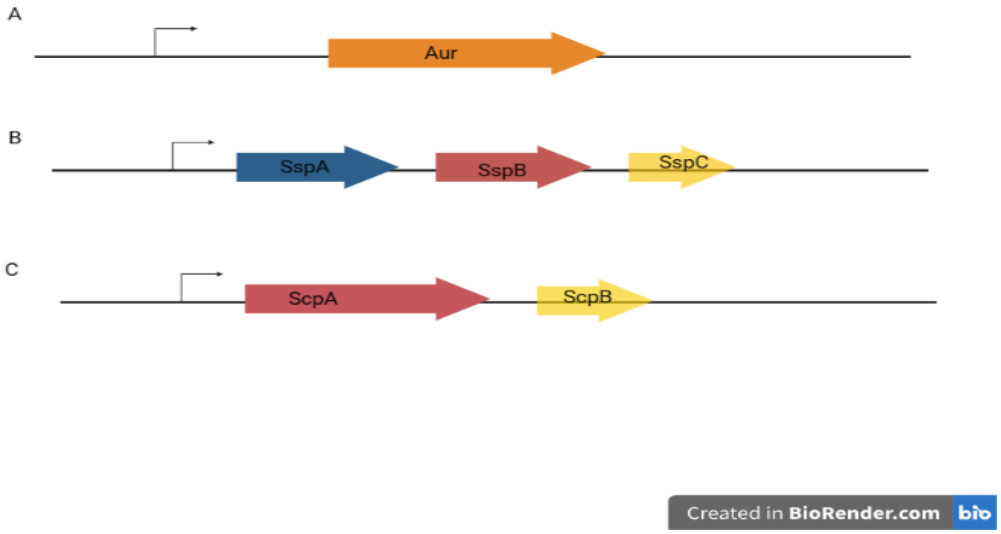

Figure 3B. *A is represents the single mon-cistronic operon for aureolysin (orange). B represents the poly-cistronic operon for SspA (blue), and SspB (red). C represents the mon-cistronic operon for ScpA (red).*

Biofilm Proteins

Playing a vital role in biofilm are various proteins. Microbial surface components recognizing adhesive matrix molecules proteins are used by *S. aureus* to bind with the receptors of host cells reversibly and irreversibly. There are 3 regions to these proteins to allow this binding. The first region is a binding domain, the second region is the domain that spans over the

cell wall and the third region is a non-covalent binding area for the MSCRAMM protein and host cell on the bacterial surface. These MSCRAMM proteins include collagen adhesion protein, clumping factor proteins, fibronectin binding proteins and *S. aureus* surface proteins, which include, SasG, SasX, ClfB, FnBP, Bap and Sdr. The *sasG* gene when over expressed contributes to increased biofilm formation by inhibiting the ClfA and ClfB clumping proteins. The SasG protein may also aid in colonization due to promoting epithelial cells adhesion. SasG protein helps with cell-cell adhesion, due to Zn+2 dependent cleaved SasG B domains that have homophilic protein interactions that the SasG will use to covalently bind to the cell wall. The SasX protein decreases phagocytosis of neutrophils due to the increased cell aggregation which results in an increase of biofilm formation.[10]

Within the biofilm of *S. aureus* there are significant alterations in gene expression. This variation is impacted by several different factors. There are 12 genes that encode for the formation of *S. aureus* biofilm listed as the following, collagen binding protein (*cna* gene), clumping factor protein (*clfA and B* genes), elastin binding protein (*ebps* gene), fibrinogen-binding proteins (*fib* gene), fibronectin-binding proteins (*fnbA* and *B* genes), intercellular adhesion protein (*icaA, B, C* and *D* genes) and laminin binding protein (*eno* gene). The *cna* genes encode their corresponding collagen binding protein and are responsible for promoting surface adherence. Recognition of the surface fibrinogen binding protein is encoded and facilitated by the *fib* gene. The *clfAB* genes promotes biofilm development by aiding in the *S. aureus* colonization within the host and binds with soluble fibrinogen resulting in an immune evasion causing virulence. These genes also bind with surface fibrinogen of the host through the cell wall anchored proteins that are encoded by the *clfA* and *B*. The *fnbAB* genes assist in the biofilm formation. The initiation of biofilm formation and the process of cell-to-cell adherence is

encoded by *icaABCD* genes. Both the *ebps* and *eno* genes have similar functions as the other genes mentioned above, aiding in formation of biofilm and host colonization.[13] These proteins play a vital role in biofilm and represent potential targets for the effects of TTO-53.

Protein	Function
Biofilm-associated protein (Bap)	*Biofilm development*
ClfAB	Cell attachment
Cna	Cell attachment
Ebps	Aids Biofilm formation and host cell colonization
Eno	Aids Biofilm formation and host colonization
fib	Recognition of the surface fibrinogen binding protein
FnbpAB	Host cell colonization and aids in biofilm formation
IcaABCD	Cell attachment/ cell to cell attachment
Microbial surface components recognizing adhesive matrix molecules proteins	Used to bind with the receptors of host cells reversibly and irreversibly
SasG	Inihibit ClfAB resulting in increased biofilm formation. Aids in colonization and cell to cell adhesion
SasX	Increases biofilm formation

Table 1 *is a list of proteins associated with biofilm and their functions.*

Antimicrobial Peptides

Antimicrobial peptides have been used as therapeutic agents and disruptors of biofilm. Antimicrobial peptides can be used against fungi, bacteria, parasites and viruses. AMPs were discovered in 1939 by Rene Dubos, when he extracted an antimicrobial agent from the soil strain *Bacillus.* There have been more than 5,000 AMPs discovered since then. AMPs can be found in a variety of places such as, organs, tissues, prokaryotes, eukaryotes, epithelial cells, phagocytes, lymph and lymphocytes. There are four different types of structures AMPs are grouped into, loop, β-sheet, extended, and α-helix.[2] To classify a peptide as antimicrobial, a minimal

bactericidal concentration (MBC) needs to be performed. This test is to determine the lowest concentration of agent to kill 99.9% of bacteria present in sample. To determine if the antimicrobial peptide has anti biofilm properties, a minimum biofilm inhibitory concentration (MBIC) would be done. The MBIC is the lowest concentration of antimicrobial agent needed to inhibit the formation of biofilm. If an AMP is anti-biofilm the minimum inhibition concentration needs to be higher than the MBIC.[24] AMPs are made up of about 10 -100 amino acids. Due to the positive charges on the AMP, it will easily bind to the bacteria membrane due to the membrane negative charges.[2] These peptides can easily be degraded with bacterial enzymes, such as proteolytic enzymes. Therefore, unnatural amnio acids are added to the AMPs to prevent degradation.[11]

The unnatural amino acid TTO-53 is a synthetically synthesized analog containing three dipeptide units. The three dipeptide units contain both Tetrahydroisoquinolinecarboxylic acid (Tic) and Octahydroindolecarboxyl acid (Oic) as a pair. Other advantages of unnatural amino acids other than preventing degradation are providing the AMP with other physicochemical properties that natural 20 RNA encode amino acids cannot. Also, AMP and the unnatural amino acid form an α-helix providing metabolic stability and flexibility of conformation through its distinctive schemes of hydrogen bonding. Conformation flexibility enables selective interaction with different membranes of various chemical compositions. There are three spacers that define structural components. Spacers play an important role because they contribute to the conformation flexibility allowing the peptide to adapt to membranes of diverse chemical composition. Chemical composition of a membrane is that structure and molecules that make up the organism's cell membrane. If a peptide can adapt to an organism's changing environment of its membrane it will be a major advantage because it supports, the AMP having antimicrobial

activity against a variety of pathogens. Spacer 1 is the difference between the Tic-Oic dipeptide units and defines the structure. Spacer 2 determines the molecule surface charge density and the distance between polypeptide back bone and the nitrogen side chain. The distance between the C-terminal Lys residues and the last Tic residue is defined by spacer 3. This unnatural amino acid was used in previous studies of and was used in thesis research involving *S. aureus*. [11]

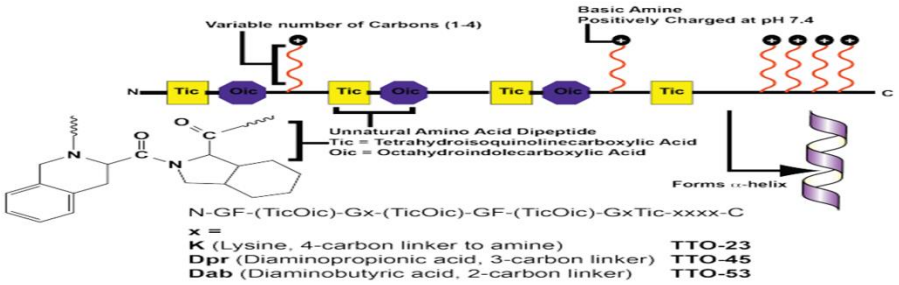

***Figure 4A**. The structure of TTO-53 with AMP. The three dipeptide units are represented by the yellow square and dark blue octagon. The red wavy lines with positive charge represents the AMP.*

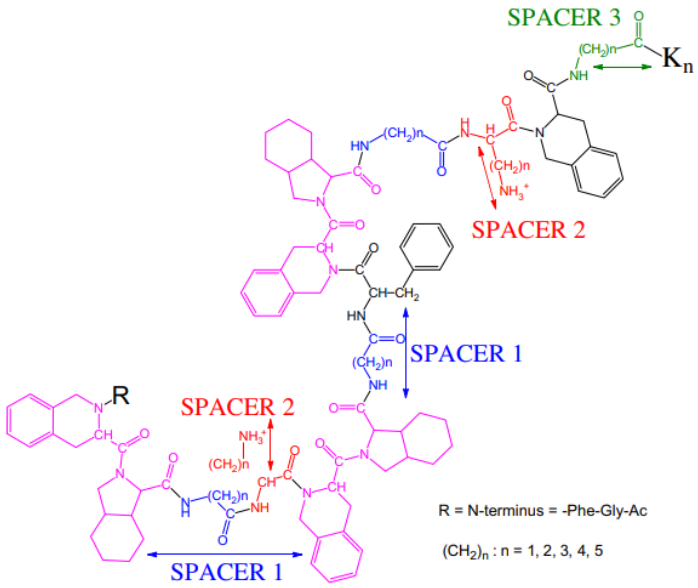

***Figure 4B**. The spacers for a basic TTO peptide structure. Pink is depicting the Tic-Oic dipeptide units. SPACER 1, 2, and 3 are in blue, red and green (respectively).*

The previous research of Sergey Vinogradov *et al.* demonstrated that certain unnatural amino acids were effective at disrupting the biofilm in *Pseudomonas aeruginosa*. Results from

the established biofilm and inhibition biofilm assay indicated the three analogs TTO- 23, 45 and 53 were effective but TTO-53 was the most effective at inhibiting and disrupting established biofilm growth. Another way the effects of the unnatural amino acids were measured was through an electrochemical sensor utilizing two methods, Cyclic Voltammetry (CV) and Square Wave Voltammetry (SWV). This measured the ability of unnatural amino acids to disrupt the biofilm at different concentrations over various time intervals and resulted in the TTO-53 being the most effective.[35]

Chapter 2: Experimental

Hypothesis/Aims:

There are many components involved with biofilm formation and dispersal such as proteins, PSMs, and proteases. The *agr* systems regulate quorum sensing and play an important role in dispersal phase due to producing the PSMs and proteases. Biofilm, proteins and toxins produced by *S. aureus* aid in the bacteria's resistance to treatment.[14] Disrupting biofilm will be vital to making *S. aureus* more susceptible to antibiotics and other therapeutic agents resulting in better treatment. Therefore, determining if unnatural amino acid, TTO-53 had the same disruptive and inhibiting effects on *S. aureus* biofilm as seen in previous research above and how, is important to combating this pathogen. It's not known how TTO-53 interacts with the bacteria but some other antimicrobial peptides have been shown to interact with organisms, protein involved in biological processes, cell division, or with the biofilm itself.[7]

I hypothesized that TTO-53 would disrupt biofilm and did so by triggering a biofilm disassembly pathway. If disruption and inhibiting effects observed in previous research occurred in the biofilm of *S. aureus* this would of been an indication that pathways for disassembly are occurring due to altered gene expression of biofilm signaling pathways. After optimizing growth of biofilm production for *S. aureus*, the first part of aim one was to determine if TTO-53 changes levels of biofilm. The second part of aim one was to determine if TTO-53 impacts bacterial viability. The second aim was to identify potential mis-regulation of biofilm assembly/disassembly genes.

Optimization of Biofilm Production

Optimizing biofilm production for our specific *S. aureus* experiments is important because the optimal conditions for biofilm production vary greatly in the literature and seem to be strain and even lab-specific. One variable which is fairly consistent between papers is the addition of glucose to standard medium to induce biofilm, and another is the use of highly enriched media. Glucose is an important carbohydrate for *S. aureus* and is a common additive in papers using base media such as Luria broth [4,17], so was chosen for further study, and Brain Heart Infusion broth was chosen as the enriched medium based on previous studies where it was used successfully to induce biofilm production.[1]

Six *S. aureus* cultures were prepared containing, Luria Bertani (LB), Brain Heart Infusion (BHI), LB+ *S. aureus* + glucose, LB + *S. aureus*, BHI + *S. aureus* + glucose and BHI + *S. aureus*. These cultures were used to inoculate a 96-well plate. Following a 24 hour and 72-hour incubation period, wells were stained with 0.1% crystal violet for 30 minutes then rinsed twice with PBS. Biofilm- bound dye was then solubilized with 30% acetic acid and visualized using a plate reader at 450 nm.

Brain Heart Infusion + *S. aureus* and LB + Glucose Environments Produce the Highest Biofilm Production.

Figure 5 shows the basic lay out for the plates, and the different color rectangles correlate to various mediums. In Figure 6 and 7, is data from a 24-hour incubation plate and 72-hour hour incubation plate. As seen in Figure 6 the 24-incubation plate, the LB + *S. aureus* + glucose wells had the highest absorbance meaning these wells had the highest biofilm production. In Figure 7, the 72-hour incubation plate, the BHI + *S. aureus* wells had the highest absorbance indicating

this section had the highest biofilm production. This is vital because the 24-hour incubation biofilm will be better to use with the electrochemical sensor because it's a quicker process but for the biofilm assays the 72-hour incubation along with the BHI + *S. aureus* media was used.

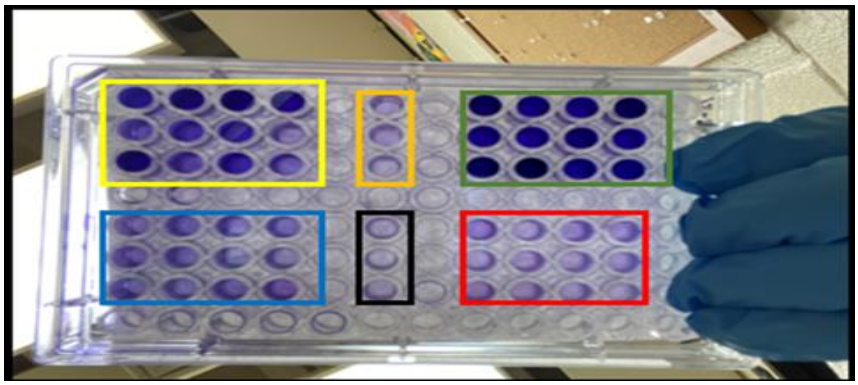

Figure 5*. Stained 96-well plate inoculated with different substances. Dark yellow: Luria Bertani (LB) Black: Brain Heart Infusion (BHI) Red: LB, + S. aureus and glucose Green: LB and S. aureus. Blue: BHI + S. aureus and glucose. Light yellow: BHI and S. aureus.*

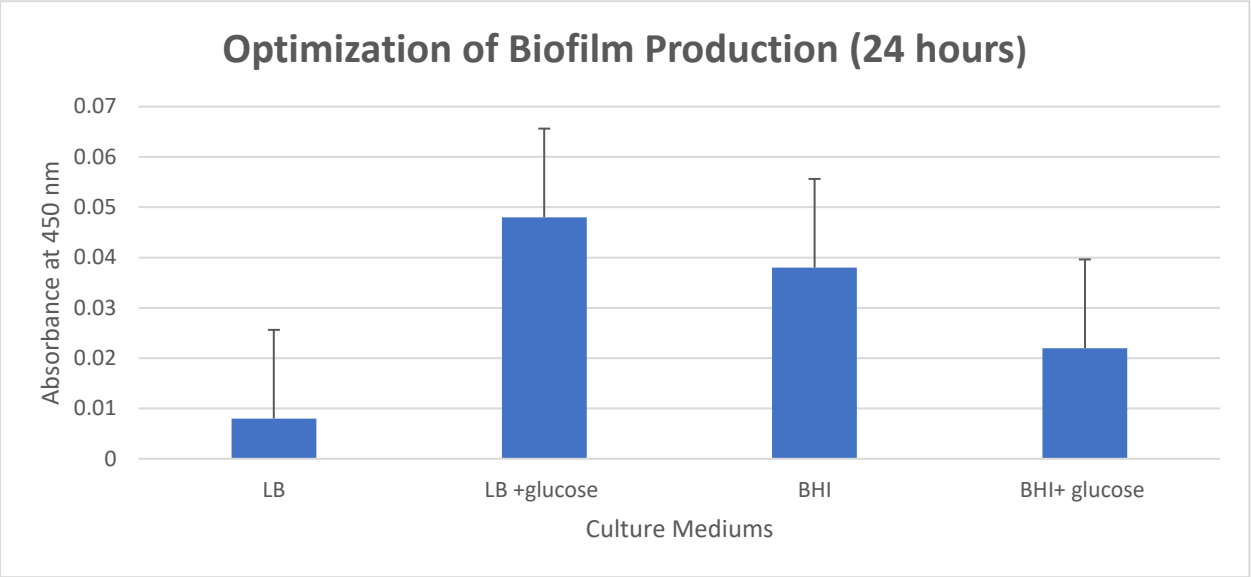

Figure 6. *24-hour incubation plate measuring biofilm levels in different culture medium. The X axis represents the culture mediums. The absorbances measured at 450 nm of each culture are represented on the Y axis. Error bars are determined based on standard deviation of the absorbances.*

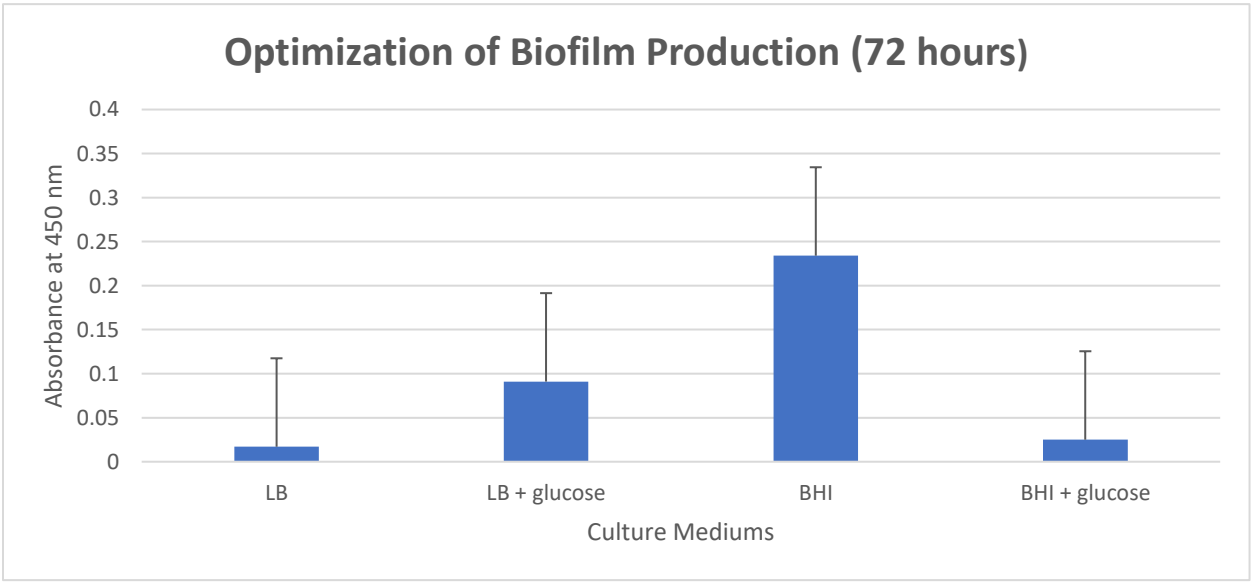

Figure 7. *72-hour incubation plate measuring biofilm levels in different culture medium. The X axis represents the culture mediums. The absorbances measured at 450 nm of each culture are represented on the Y axis. Error bars are determined based on standard deviation of the absorbances.*

Established Biofilm Assays

After establishing optimal conditions for the bacteria, two biological assays were used to measure the effect of the unnatural amino acid, TTO-53, on *Staphylococcus aureus* biofilm for the first part of aim one. The first assay was an established biofilm assay, this measured the ability of TTO-53 disturbance on the *S. aureus* biofilm after its already grown. The following procedures are the same performed by Sergey Vinogradov but some of the procedures have been changed to accommodate *S. aureus.*[35]

The methods for established biofilm assay are as follows. Utilizing a 96-well plate, the BHI and *S. aureus* mixture was added to the wells and left to incubate for 72 hours at a temperature 37 ℃ allowing biofilm to be established but no peptide was added in this stage. Then to the experimental and the controls of the experiment wells, various concentrations of 1mM peptide indicated in Figure 8, were added and then was left to incubate another 72 hours.[35]

After incubation the plate was washed with PBS several times and stained with 0.1 % crystal violet dye. The crystal violet is left to incubate to allow the dye to be absorbed into the biofilm. 30 % acetic acid was added to the wells, then the absorbance of the wells was be measured by a multiskan FC spectrophotometer at a wavelength of 620 nm.[35]

TTO-53 Disrupts Established Biofilm.

When the absorbance is higher this indicates a higher biofilm production but the lower the absorbance, the lower the biofilm production. Figure 8 shows the averaged results of multiple establsihed biofilm assays. 0.3 mM was the most effective disruptive concentration in these assays due to having the lowest absorbance when compared to the control absorbance. Thus, the results support that TTO-53 disrupts established biofilm at a concentration of 0.3 mM under the

conditions tested. Lastly, the concentrations with asterisks above are statistically significant due to standard deviations of error bars outside the range of the control standard deviation. This means that there is variability between the concentrations and aren't reliable results.

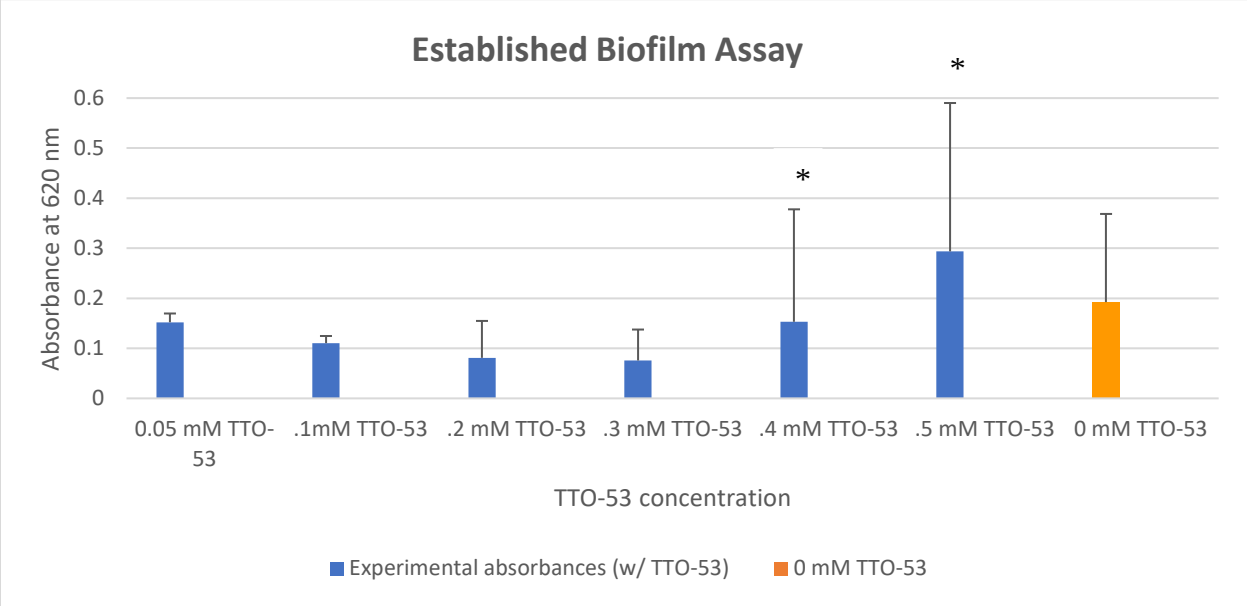

Figure 8. *Established Biofilm Assays. The orange column indicates no peptide. The blue columns are the averages of experimental absorbances of three established biofilm assays. Error bars are based on the standard deviation of all absorbances of all 6 concentrations and control from the assays. The asterisk represents statistically significant data.*

Inhibition Biofilm Assays

The second biological assay used to measure the effect of the unnatural amino acid, TTO-53, on *Staphylococcus aureus* biofilm is the inhibition biofilm assay, which measures the ability of TTO-53 to inhibit biofilm growth. As stated above the following procedures are adapted from Sergey Vinogradov but have been changed to accommodate *S. aureus*. [35]

The methods for the inhibition biofilm assay are as follows. Utilizing a 96-well plate, the BHI and *S. aureus* mixture was added to the wells along with various concentrations of 1mM

peptide indicated in *Figure 9*. The peptide was added to the experimental and the controls of the experiment wells and then was left to incubate for 72 hours.[35]

After incubation the plate was washed with PBS several times and stained with 0.1 % crystal violet dye. 30 % acetic acid was added to the wells, then the plate will be read on a multiskan FC spectrophotometer at a wavelength of 620 nm.[35]

TTO-53 Inhibits Biofilm Growth.

Figure 9 shows the combined results of multiple biofilm inhibition assays. In conparsion of the control versus experimaental absorbances, 0.3 mM had the lowest absorbance, indicating that 0.3 mM was the most effective disruptive concentration in these assays. Therefore, results support that TTO-53 interferes with the initial formation of biofilm. The concentrations with asterisks above are statistically significant due error bars outside the Standard deviation range from control. This indicates that there is variability between the concentration and aren't reliable results.

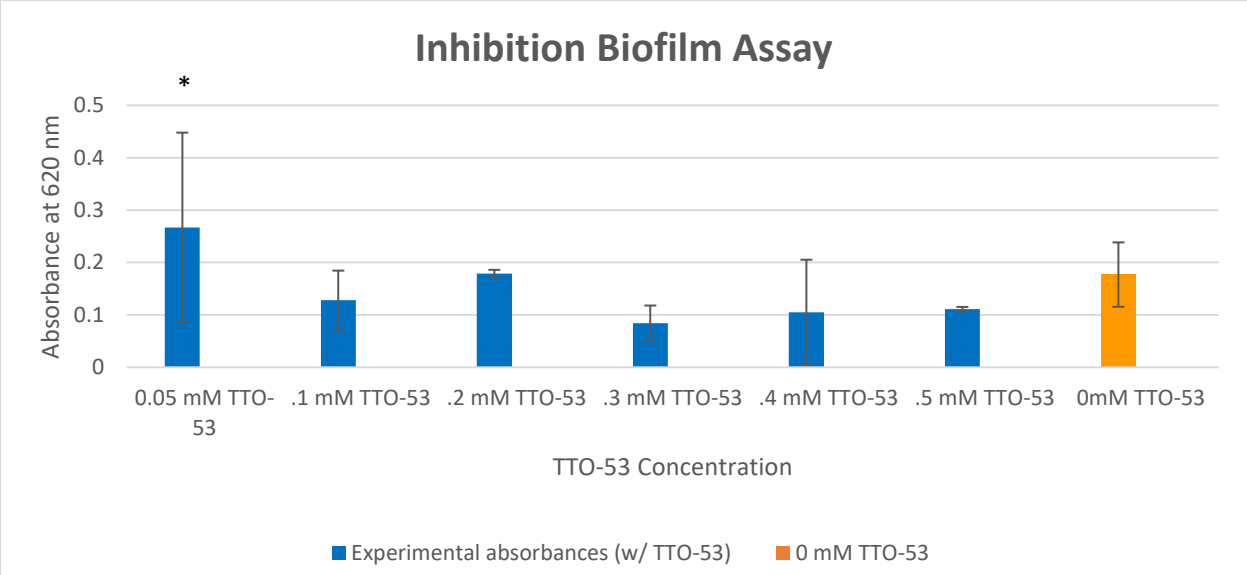

Figure 9. *Inhibition Biofilm Assay. The orange column indicates no peptide. The blue columns are the averages of experimental absorbances of two inhibition assays. Error bars are adjusted based on the standard deviation of all absorbances of all 6 concentrations and control from the assays. The asterisk represents statistically significant data.*

Cell Viability Assay

The data from the established and inhibition biofilm assay supported that TTO-53 is disrupting established biofilm and inhibiting biofilm growth. It's still not known how it's causing disruption. One possibility is that TTO-53 effects on biofilm is due to bactericidal effects of the compound. Therefore, the second part of aim one was to determine if the peptide is altering biofilm levels due to cell killing by examining cell viability through direct viable cell counts. To maintain consistency, cell viability counts were performed on the same cultures used for inhibition biofilm assays in Figure 9.

Cells from the wells used for inhibition biofilm measurements were first 10-fold serially diluted in PBS. Then three 25 µL aliquots of each dilution was plated on a Luria Bertani plate. Colonies numbers were averaged and multiplied by the total dilution to determine the starting concentration in colony forming units per ml (CFU/ml).

TTO-53 Dependent Biofilm Disruption Is Not Due To Cell Death.

Results of the viability assay are shown in Figure 10. Based on the average CFU/ml for each TTO-53 concentration, there is no significant difference in cell viability in any of the concentrations tested despite observed differences in biofilm concentration observesd for these same cultures in Figure 9. This data suggest that biofilm disruption is not due to losses in cell viability.

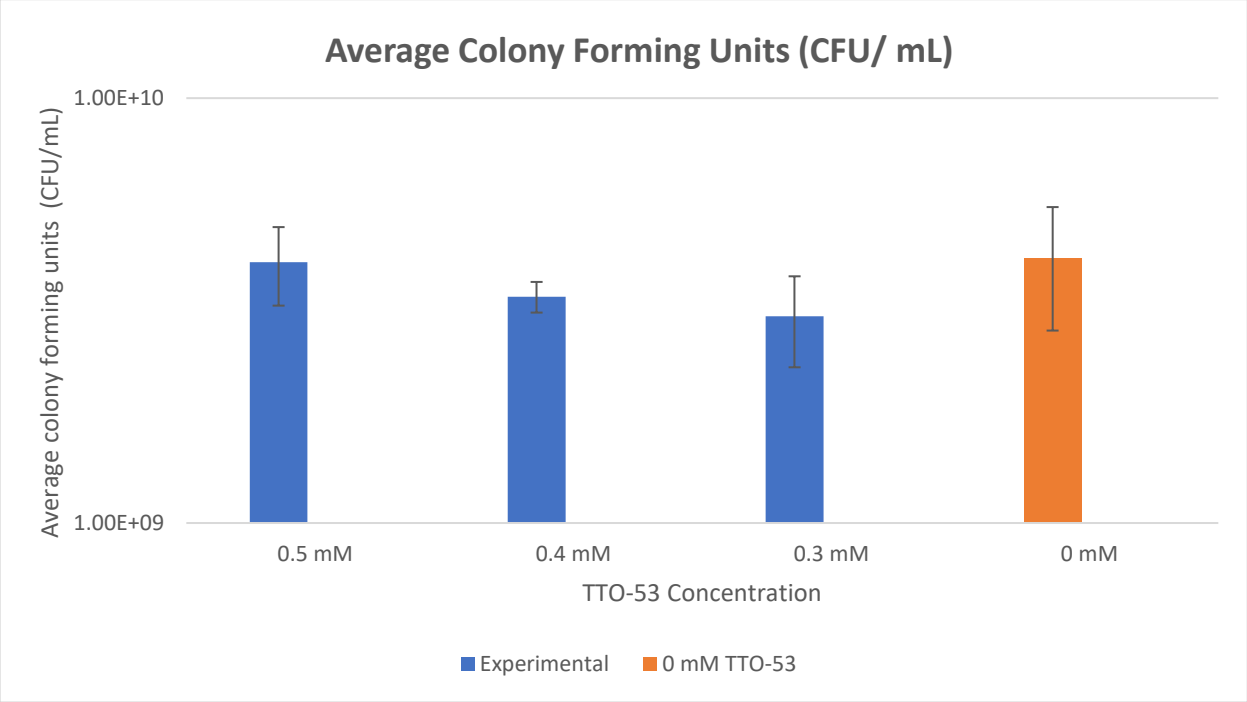

Figure 10. *Average colony forming units (CFU/mL). The blue columns are the experimental and the orange columns is the control.*

Real Time PCR

The data supports that biofilm disrupting isn't occurring through cell death. The next aim was to utilize Real Time PCR to determine if biofilm assembly and disassembly genes were being affected when TTO-53 is introduced at various concentrations. Genes, *fnbAB*, *cna*, *clfAB* of cell surface proteins[13] and *sspB and sspA*, protease genes [15, 23], were examined due to their

expression being down and up regulated (respectively) by RNAIII, aiding in the disassembly of biofilm.[25] The advantage of Real Time PCR is that it allows the gene expression to be quantified in real time.[12] It was predicted that the *sspAB* would be upregulated while the cell surface genes would be downregulated.

cDNA was amplified from samples at 25 minutes post-peptide exposure using SuperScript "lV" Master Mix kit. Total cDNA was determined using a nano-spectrophotometer, and concentrations were adjusted to 1ng/ul. Samples were set up using primers for *sspAB, clfA, clfB, fnbA, fnbB, cna* and *rpod* and *gyrA* were included as constitutive controls. The cycle parameters were 95 ℃ for 190 seconds, 45.0 ℃ for 30 seconds then at end of the cycle the temperature heated up to 72 ℃ for 15 seconds with Invitrogen SuperScript "lV" master mix kit being used for the RT analysis.

TTO-53 is causing Mis-regulation of Disassembly /Assembly Biofilm Genes.

It was predicted that *sspAB* would be upregulated while *fnbA, fnbB, clfA, clfB* and *cna* were to be downregulated in the presence of TTO-53. The results in Figure 11 are the RT fold difference of the targeted genes and were normalized against *rpod* and *gyra* as constitutive controls to allow slight difference in cDNA levels. When the RT fold is negative this indicates a down regulation of genes and a positive RT fold difference shows a up regulation of genes. Figure 11 shows *clfB* was the only gene upregulated and the rest were downregulated, due to the negative and positive RT fold differences. The RT- PCR results do indicate there is mis-regulation occurring but not in the predicted way.

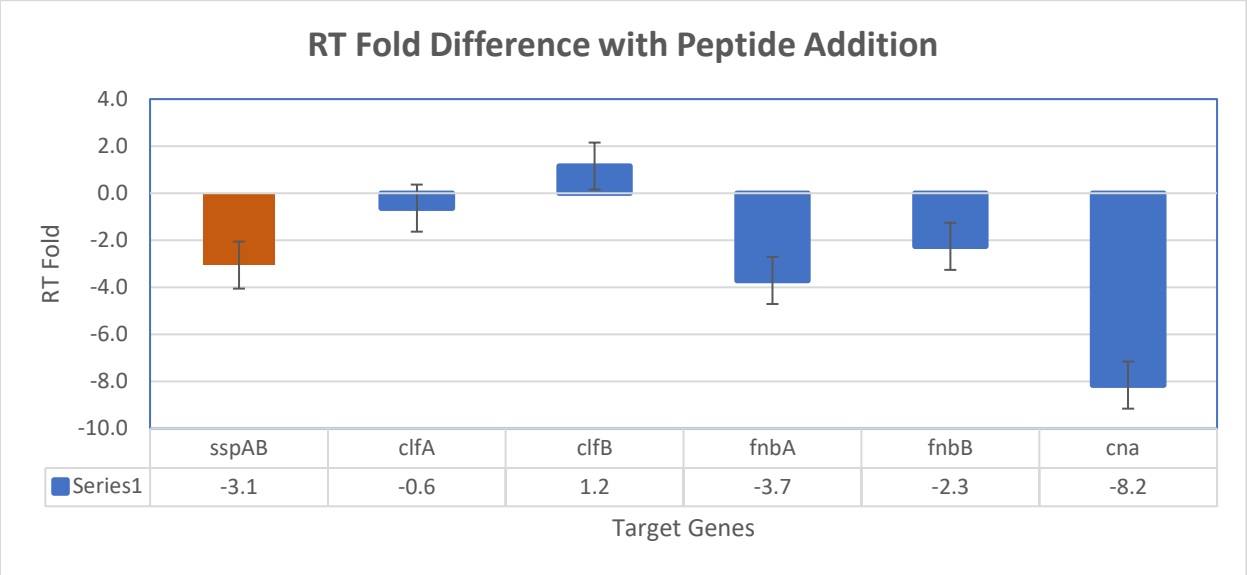

Figure 11. *This is a graph showing the RT Fold Difference of sspAB,(red) clfA, clfB, fnbA, fnabB, and can (blue) in the presence of peptide. The bar in red was predicted to be upregulated and the blue bars were predicted to be downregulated.*

Discussion

Both *S. aureus* and *P. aeruginosa* are biofilm producing bacteria, but they differ in structural molecules and pathways used for biofilm. *P. aeruginosa* utilizes polysaccharides, Psl, alginate, and Pel while *S. aureus* utilizes PIA.[10a] The same disruptive pattern seen with *P. aeruginosa* were replicated in *S. aureus*. This shows that TTO-53 is able to adapt and disrupt different bacterial membranes but how, is still unanswered. But this is an advantage to research because this supports that TTO-53 is effective against multiple organisms as a potential future therapeutic agent. Data from Figures 8 and 9 indicate that TTO-53 is disrupting established biofilm and inhibiting biofilm growth. Due to the statistically significant data (0.5 and 0.05 mM) in established and inhibition assay this could mean that the peptide is dose dependent because of the 0.05 mM and 0.5 mM having higher absorbance levels than the control indicating the more biofilm was being produced. The reason for this phenomenon isn't certain but TTO-53 may be

interacting with the biofilm differently depending on the dosage. The colony forming unit's results support that cell death is not the cause of biofilm disruption indicating the peptide is disrupting biofilm through other means. Real Time PCR was done to determine if TTO-53 is disrupting quorum sensing regulation pathways or biofilm formation and dispersal genes. It was predicted that *sspA* and *B* would be up regulated due to both playing a role in the disassembly of biofilm and downregulation of *clfAB, fnbAB,* and *cna* due these genes helping with biofilm assembly and being down regulated by RNA III of the agr system to disperse biofilm. Conversely results in Figure 11 show all the genes were down regulated except *clfB*. Based on the RT results, TTO-53 is impacting the regulation of these target genes. It would be beneficial to do more rounds of RT PCR to see if the results in Figure 11 can be replicated.

To get a better understanding of *S. aureus* biofilm, flow cell plates would be useful. This method will allow examining the structure and behavior of biofilm in a dynamic environment in the presence of various concentrations of TTO-53 versus no TTO-53 present.[19] Another method that would give insight on how TTO-53 may be disrupting *S. aureus* biofilm is electrochemistry. The electrochemical sensor is another type of assay that was utilized in Sergey's research. The advantage of electrochemistry is that it's more sensitive and faster. Therefore, may be a viable option for further characterizing the optimal concentration, but the technique will require significant optimization.[36]

Chapter 3: Future Directions

This project supports the fact that TTO-53 inhibits biofilm growth and disrupts established biofilm. It's still not known how TTO-53 is causing biofilm disruption. The cell viability assay ruled out cell death as a cause. Disruption may be due to mis-regulation of biofilm pathways, since RT PCR results indicated TTO-53 is impacting regulation of assembly and disassembly genes. For future directions of this project more RT PCR runs should be done to get a better understanding of the impact TTO-53 is having on the regulation of targeted genes mentioned above and use various concentrations of peptide since only .5 mM was used for RT PCR. Depending on those results, more genes should be chosen as targets for RT PCR to gain more knowledge on how impacting regulation of genes could affect the biofilm structure and the bacteria itself. Performing the same biological assays and RT PCR with other unnatural amino acids such as, TTO-23 and 45 may also show the same disruptive biofilm patterns or be even more effective. This could allow us to see if the previous results can be replicated. It would be interesting to see if the concentrations that were statistically significant remain significant even with different peptides.

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Appendix

The following procedures are the same performed by Sergey Vinogradov but some of the procedures have been changed to accommodate *S. aureus*.[35]

Established Biofilm Assay Protocol

The methods for established biofilm assay are as follows. Utilizing a 96-well plate, the 200 uL BHI and S. aureus medium was added to the wells and left to incubate for 72 hours at a temperature 37 ℃ allowing biofilm to be established but no peptide was added in this stage. Then to the experimental and the controls of the experiment wells, various concentrations of 1mM peptide indicated in Figure 8 and 9, were added and then was left to incubate another 72 hours. [35]

After incubation the solution within the wells was discarded and then the plate was washed with PBS several times. After rinsing the wells were stained with 200 µL of 0.1 % crystal violet dye. This was then left to incubate for 30 minutes. The plates were then rinsed and left to dry for 24 hours. 200 µL 30 % acetic acid was added to the wells left to incubate for 30 minutes. Afterward the absorbance of the wells were be measured by a multiskan FC spectrophotometer at a wavelength of 620 nm.[35]

Inhibition Biofilm Assay Protocol

The methods for established biofilm assay are as follows. Utilizing a 96-well plate, the 200 µL BHI and S. aureus medium was added to the wells along with various concentrations of

1mM peptide indicated in Figure 8 and 9 added to the experimental and the controls of the experiment wells. Then the plate was left to incubate for 72 hours at a temperature 37 ℃. [35]

After incubation the solution within the wells was discarded and then the plate was washed with PBS several times. After rinsing the wells were stained with 200 µL of 0.1 % crystal violet dye. This was then left to incubate for 30 minutes. The plates were then rinsed and left to dry for 24 hours. 200 µL 30 % acetic acid was added to the wells left to incubate for 30 minutes. Afterward the absorbance of the wells were be measured by a multiskan FC spectrophotometer at a wavelength of 620 nm.[35]

Cell Viability Assay Protocol

Utilizing a 96-well plate, 200 µL of the BHI and *S. aureus* culture was added to the wells along with *.3 mM, .4 mM, and .5 mM*. these concentrations were the most effective of the inhibition assays. The peptide was added to the experimental and the controls of the experiment wells and then was left to incubate for 24 hours.

After 24-hour incubation cells from the wells used for inhibition biofilm measurements were first 10-fold serially diluted in PBS. Then three 25 µL aliquots of each dilution was plated on a Luria Bertani plate. Colonies numbers were averaged and multiplied by the total dilution to determine the starting concentration in colony forming units per ml (CFU/ml).

Real Time PCR Protocol

First purification of RNA was done using RNeasy Mini Kit 50 protocol. The purified RNA was then reverse transcribed resulting in cDNA, that was amplified from samples at 25 minutes post-peptide exposure using SuperScript “IV VILO” Master Mix kit. In PCR tubes Total

cDNA was determined using a nano-spectrophotometer, and concentrations were adjusted to 1ng/ul. Samples were set up using primers for *sspAB, clfA, clfB, fnbA, fnbB, cna* and *rpod* and *gyrA* were included as constitutive controls. The cycle parameters were 95 ℃ for 190 seconds, 45.0 ℃ for 30 seconds then at end of the cycle the temperature heated up to 72 ℃ for 15 seconds with Invitrogen SuperScript “IV VILO” kit and cyber green being used for the RT analysis.