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Bacterial Growth in the Presence of Metal Ions and the Quorum Sensing Molecule, AIP

(Auto Inducing Peptide)

A Major Qualifying Project Report:

Submitted to the faculty of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements of the

Degree of Bachelor of Science

By

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Jared Watson

Approved:

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Professor Christopher R. Lambert, Advisor

Abstract

The bacteriocidal effect of metal ions on microbes has been well known for many years. The growth kinetics of bacterial cultures are also known to be strongly influenced by the presence of quorum sensing compounds. In this study we look at the effect of metal ions in combination with culture medium in which bacteria have been grown, the conditioned medium will contain quorum sensing compounds. The organism we study is *Staphylococcus aureus* (*S. aureus*). *S. aureus* produces a quorum sensing molecule called autoinducing peptide (AIP). As a first step to looking at this mechanism we have looked at the toxicity of copper and silver ions in the presence of conditioned media.

Acknowledgements

I would like to thank Professor Christopher Lambert for all his time and effort in helping to complete this project. Thank you to Achilles Gatsonis for assistance in laboratory work and research on the subject. Thank you to John Cvitkovic for creating the model of AIP binding with Cu^{2+}. I would like to thank the WPI Chemistry Department and Professor Nikolaos A. Gatsonis for financial support.

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Introduction

The importance of *Staphylococcus aureus*

One of the major threats to the health and safety of hospital patients is healthcare-associated infections (HAIs). Each day nearly 1 in 25 patients in hospitals in the U.S. have an HAI[1]. HAIs can be contracted by bacteria introduced to the body in several ways including intravenous catheters and surgery[2]. Patients in hospitals often have compromised immune systems due to illness, surgery or age. These conditions cause them to be particularly susceptible to developing an infection and making recovery a longer process.

One of the leading causes of both hospital and community-associated infections is *Staphylococcus aureus (S. aureus)*. *S. aureus* is commonly found on humans, often located externally on the skin or in the nasal passages. Most people will be unaware they carry the bacteria as it usually causes no significant effects. However, when *S. aureus* is able to invade a wound or surgical site it can cause infections including sepsis, endocarditis (infection in the heart), osteomyelitis (infection of bone), bacterium (infection in the blood) and pneumonia (inflammation of the lungs), the leading type of infection in the United States[3][4]. The toxins produced by *S. aureus* include hemolysins and leukotoxins which can lead to toxic shock syndrome, a potentially deadly condition[5]. In the U.S. *S. aureus* infections developed in hospitals cause more deaths per year than HIV/AIDS[6].

Antibiotic resistance

Antibiotic resistance development in infectious bacteria is a major health concern around the world[7]. Some species of bacteria that previously have been easily treatable due to the discovery of effective antibiotics such as penicillin, methicillin and tetracycline have become more difficult to treat, leading to longer infections with a higher risk of disability or death[8]. Bacteria have several common mechanisms to resist antibiotics including deactivation of the

antibiotic through reaction, removing the substance quickly enough that it does not kill the cell or alterations in the cell membrane that disallow attachment with the antibiotic.

Treatment of *S. aureus* infections in the first half of the twentieth century was done primary through the use of benzylpenicillin (Penicillin G) which is a β-lactam antibiotic. In the 1950s some strains of *S. aureus* already showed resistance to benzylpenicillin and other β-lactam antibiotics. The resistant bacteria produced the β-lactamase enzyme which would deactivate the antibiotic. Researchers worked to find derivatives of penicillin that would not be affected by β-lactamase and in 1959 they produced methicillin. Methicillin is very similar to Penicillin G but the phenol group is disubstituted with methoxy groups. The methoxy groups reduced the affinity for β-lactamase by increasing the steric hindrance around the amide bond. The effectiveness of methicillin in the treatment of *S. aureus* was however, short lived. When the drug was used clinically strains of Staph aureus with a resistance to methicillin were isolated almost immediately[4]. Referred to as methicillin-resistant *S. aureus* (MRSA) it has become one of the most concerning antibiotic resistant bacteria in the United States.

The mechanism of methicillin resistance is not β-lactamase, instead another penicillin binding protein is expressed referred to as PBP2a. The variety of antibiotics that have been used to treat MRSA since then has resulted in strains with multi-resistance and the search for new antistaphylococcal agents is an urgent issue[4].

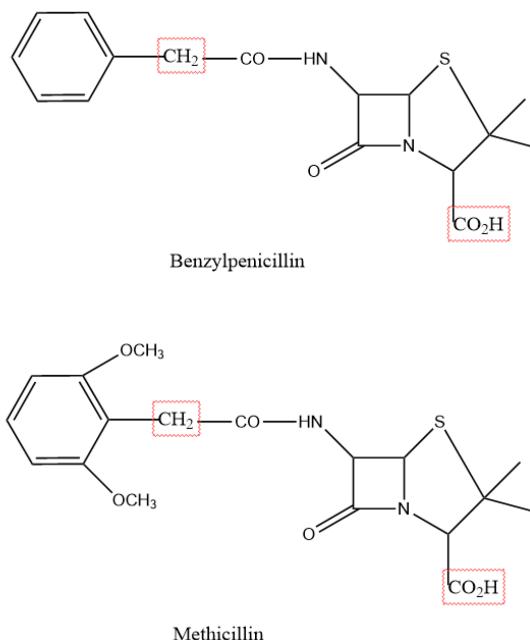

Figure 1: Structure of Benzylpenicillin and Methicillin

Antimicrobial properties of metal ions

One of the possible answers to antibiotic resistance is the use of metal ions. Metals such as copper and silver have been shown to have antimicrobial properties. Copper was even recognized as the first metallic antimicrobial agent by the American Environmental Protection Agency[9]. Copper is currently used for this property in a variety of industries such as water treatment. Silver is another commonly used metal and has been used in wound care, bone prosthesis, cardiac devices and surgical implements. The antimicrobial effects are due to the bioactive silver ion (Ag^+). Silver nitrate at concentrations of 1% or greater is a caustic irritant but at lower concentrations (typically 0.5%) it is an effective antibacterial and has been found to be especially useful for inhibiting the growth of *P. aeruginosa* for patients suffering from burn wounds[10].

The exact mechanism by which silver kills bacteria is still under study, it has been shown that the silver ions react with thiol groups in the proteins of the bacteria which results in deactivation of the bacteria. Micromolar levels of dissolved silver ions resulted in the uncoupling

of respiratory electron transport from oxidative phosphorylation which caused an impairment in DNA replication and interfered with membrane permeability.

A recent development in antibacterial applications of silver is silver nanoparticles, these small particles between 10 nm and 100 nm in diameter have been used in apparel, paints, appliances, cosmetics and wound care for their antibacterial properties. Nanoparticles are capable of more readily penetrating the cell membrane of bacteria, the larger sizes (20-80 nm diameter) are then ionized inside the cell and exhibit similar antibacterial mechanisms to regularly sized silver ions. Silver nanoparticles are of particular interest because they show an increase in efficacy when combined with certain natural or synthetic compounds such as a 32 fold efficacy increase against MRSA when also treated with phenazine-1-carboxamide[11].

Metal ions have the ability to inhibit bacteria that have developed resistance to other antibiotics such as penicillin or methicillin. Studies have already shown bacteria that have developed some resistance to silver, the first silver-ion resistant bacterium was isolated in the 1960's from a patient whose burn wound was treated with silver nitrate. Silver resistant strains have also been found in non-clinical environments in the presence of silver such as silver mines. Many researchers believed that the varied effects of silver nanoparticles on the physiology and reproduction of bacteria would mean that bacteria would have difficulty developing a resistance. However, a study by Grave *et al.* showed that after 200 generations exposed to 10 nm silver nanoparticles the bacteria began to exhibit greater fitness in the presence of various sizes of nanoparticles and silver ions than control strains. Studies have also been performed with copper nanoparticles (1-10 nm) that showed that copper also exhibits the membrane altering effects of silver. Both silver nanoparticles and copper nanoparticles showed greater effectiveness in E. Coli than in *S. aureus*. One study attributes this to differences in the concentration of peptidoglycan in the cell membrane, a substance not found in mammalian cells which explains why silver nanoparticles are toxic to bacteria and not humans[11]

Quorum Sensing

Many bacteria use quorum sensing systems to alter their growth kinetics. *S. aureus* uses two systems of quorum sensing to regulate is virulence factors. In the first system an RNAIII-activating protein (RAP) is produced during bacterial growth. As RAP reaches a certain concentration it causes the phosphorylation of the protein TRAP. This phosphorylation is responsible for the activation of the accessory gene regulator system (agr) which is the second quorum sensing system for staph aureus. Two promoters (P2 and P3) in the agr locus produce RNAII and RNAIII. RNAIII transcribes the genes of many virulence factors, such as toxins, cell surface proteins and enzymes. RNAII is responsible for encoding four genes, agrA agrB, agrC and agrD which work together to synthesize RNAIII. The products of agrB and agrD combine to produce a small protein called the autoinducing peptide. agrC produces a transmembrane AIP receptor, AgrC. AIP bonds to AgrC and causes it to phosphorylate and activate AgrA. The phosphorylated AgrA then activates P2 and P3, increasing the production of RNAIII[12].

RNAIII produces delta-toxin and controls other genes which produce Protein A, Coagulases and Rot (Repressor of toxins) which is used to regulate the virulence gene expression[13][14]. Protein A is an immunoglobulin-binding protein that exists both on the surface of the bacteria and is secreted into the environment. It binds to the Fc sites of antibodies and Fab regions of B-cell receptors. The binding prevents opsonophagocytosis (phagocytosis initiated by opsonin) which results in the death of B-cells. By decreasing the number of B-cells producing antibodies the bacteria can more easily infect the host[15]. Delta-toxin is only one of the group of peptides secreted by *S. aureus* that shows significant, non-specific cytolytic activity[5]. Coagulases are polypeptides that activate prothrombin which converts fibrinogen to fibrin which results in the clotting of plasma and blood. *Staph.* bacteria binding to fibrin or fibrinogen is what causes the formation of abscesses and increases the persistence of the bacteria in the host[16]. The exact regulatory effects of Rot are not completely understood but it

has been demonstrated to negatively regulate lipase, hemolysin and protease proteins which are associated with tissue invasion. Rot also positively regulates the expression of genes that encode cell surface adesins[17].

Different strains of *S. aureus* bacteria are activated by a specific AIP type and inhibited by the others, there are four types of AIP, AIP I-IV. Each type of AIP has roughly the same cyclic shape but several changes in amino acid composition and molar mass. A previous study has used ultra high performance liquid chromatography and electrospray ionization mass spectrometry with an LTQ orbitrap mass spectrometer to quantitatively analyze the concentration of autoinducing peptide present in medium used for the growth of *S. aureus*. Hiyas *et al.* observed the presence of AIP in cultures of *S. aureus* in tryptic soy broth (TSB) maintained at 37°C after four hours from seeding and a maximum concentration of 13 +/- 2 μM after 16 hours. The concentration of AIP then leveled off until the last recorded time at 21 hours[18].

Hypothesis

This project was designed to test if AIP would increase the antibacterial properties of copper or silver ions. Our theory was that the cyclic structure of AIP may chelate metal ions such as Cu^{2+} or Ag^{+} and then carry the ion to the cell, increasing the rate of ion and cell interaction. This potential binding between AIP and Cu^{2+} is shown in figure 2. The modeling indicated that the binding between the peptide and ion would be quite weak which we will investigate with future research.

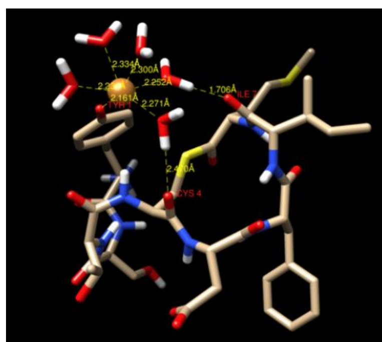

Figure 2: Model of AIP binding Cu^{2+}

Methods

1.1 Receival and initial growth

All tests were performed with *Staphylococcus aureus* subsp. Aureus Rosenbach. Obtained from ATCC, strain designation NCTC8532 [IAM 12544, R. Hugh 2605]. Lot #63532852.

A dried pellet of *Staphylococcus aureus* (*S. aureus, S. aureus*) bacteria was rehydrated on November 1, 2017. The pellet was dissolved in 2 mL of Brain Heart Infusion Broth (BHI) and then transferred to an additional 5 mL of BHI. The bacteria were incubated overnight at 37°C. A new culture was seeded in 10 mL of BHI each day for three days. At this time the culture was seeded in Tryptic Soy Broth (TSB) instead and all future cultures were done with TSB

1.2 Freezing bacteria

To prepare a frozen stock of *S. aureus* 1 mL of S. Aur. was incubated in 75 mL of Tryptic Soy Broth (TSB) overnight at 37°C. 50 mL of DMSO was filtered by vacuum filtration and then sterilized through heating. 7.6 mL of vacuum filtered and sterilized DMSO was added to the 76 mL of *S. Aureus* and TSB. 1.5mL centrifuge tubes were sterilized in the autoclave and about 1 mL of the bacteria/DMSO solution was added to 50 tubes. The tubes were labelled and stored in the -80°C freezer.

1.3 *S. Aureus* Growth Curve

1 mL *S. aureus* in TSB was seeded in 75 mL of TSB and the culture maintained at room temperature with gentle shaking. Immediately, 3 mL of culture was placed into a quartz cuvette (3 mL was the amount required to fill the cuvette) and the absorbance was measured in a lambda 35 spectrometer. The scan range was set from 200-700 nm, an interval of 1 nm and

scan speed of 480. After being scanned the 3 mL sample was disposed of in bleach and the cuvette was cleaned with 70% ethanol and water, then allowed to dry. A new sample was analyzed every hour, after the 5th sample measurements were done 30-40 minutes apart due to the sharp rise in absorbance.

1.4 Purification of AIP

S. aureus was incubated in TSB overnight at room temperature. The culture was centrifuged at 7000 rpm and 4°C for 20 minutes. The supernatant was removed and placed into sterile tubes. The pellet was killed in bleach and removed from the tube. The centrifuge tubes were cleaned using 10% bleach, 70% isopropyl alcohol and rinsed with filtered water. The supernatant was returned to the centrifuge tubes and the process was repeated before a third centrifugation. The retrieved bacteria-free medium was then frozen. The medium was lyophilized for 24 hours and stored in the freezer. The solid was dissolved into the minimal amount of pH 4, 30% acetonitrile. Centrifugation was attempted at 8000G and 12000G for twenty minutes but no pellet was observed. The sample was filtered through a 0.2 micron filter and stored in the freezer overnight. The sample was left to evaporate under a nitrogen stream for 5 hours on each of the next two days (10 hours total). The sample became a thick syrupy liquid but did not dry completely. 0.75mL of filtered water was added to completely dissolve the sample and the liquid became less viscous. The sample was then filtered through another 0.2 micron filter and stored in the freezer until use.

1.5 Growth Curve with crude AIP

Growth curves were performed at 37°C in 1 cm quartz cuvettes. 2.5mL of TSB was added to each cuvette, to the control was added additional dry TSB power to account for the concentrated addition TSB added in the used medium (AIP from Batch 1 - 207 mg. AIP from batch 2 - 73 mg. AIP from batch 3 - 177 mg). 100μL of used medium was added to the

appropriate cuvette and 100μL of filtered water was added to the control. 0.5mL of *S. aureus* was added to each cuvette from frozen stock. The cuvettes were then incubated at room temperature and the absorbance was measured from 800-400 nm every thirty minutes. The absorbances at 670 nm were plotted between each measurement and the experiment was stopped when growth stopped (about four hours).

1.6 Growth Curves with added TSB and multiple concentrations of copper sulfate

Growth curves were performed as in step 1.5 but five cultures were done. Each culture contained an additional 73 mg of TSB powder. One culture was the control containing no copper sulfate. The other four cultures contained 1 mM, 0.3 mM, 0.1 mM and .03 mM copper sulfate.

1.7 Growth Curve with added TSB, AIP and copper sulfate

Growth curves were performed as in step 1.5 except the culture containing 100μL of crude AIP also contained 1mM copper sulfate. The crude AIP used was from batch 2 therefore the control had 73 mg of additional dry TSB powder.

1.8 Growth curve with added TSB, AIP and silver nitrate

Growth curves were performed as in step 1.5 except the culture containing 100μL of crude AIP also contained 1 mM silver nitrate. The crude AIP used was from batch 2 therefore the control had 73 mg of additional dry TSB powder.

1.9 Growth Curves with various concentrations of crude AIP

Growth curves were performed as in step 1.5 except there were five cultures. The experimental cultures contained 100 μL, 50 μL, 10 μL and 1 μL of crude medium. The medium used was from batch 3 therefore 177 mg of dry TSB powder was added to the control. The

culture containing 100 µL was accidentally spilled after 1 hour and more measurements could be taken for that sample.

1.10 Growth curve with equimolar copper sulfate and crude AIP

Growth curves were performed as in step 1.5 except there were 4 cultures. One culture contained 10 µM copper sulfate, another contained 10 µM crude AIP and one contained both 10 µM copper sulfate and 10 µM crude AIP. The crude AIP was from batch 3 therefore 177 mg of dry TSB powder was added to the control.

1.11 Growth curves used various concentrations of copper sulfate with no added TSB

Growth curves were performed as in step 1.5. Five cultures were prepared, one control containing no added TSB and four others cultures containing 100 µM, 10 µM, 5 µM and 1 µM with no added TSB.

1.12 Growth curves with equimolar copper sulfate and crude AIP, no added TSB

Growth curves were performed as in step 1.11 but an additional culture was included which contained no additional dry TSB powder, copper sulfate or crude AIP.

Results and Discussion

Growth curves of S. Aureus in TSB at room temperature and 37°C.

The absorbance at 670 nm was recorded and used to construct a growth curve. A lag time of 200 minutes was observed before the early exponential phase. The culture reached mid exponential phase after 260 minutes and the late exponential phase ended after 590 minutes. It was determined that performing growth curves at 37°C would provide a more reasonable experiment length. A growth curve was performed as in procedure 1.3 but was maintained at 37°C in an incubator for the experiment. The culture was only removed when filling a cuvette for measurement. Under these conditions the culture entered the early exponential phase after only 75 minutes and reached the late exponential phase after 300 minutes, about half the time that a room temperature culture would need.

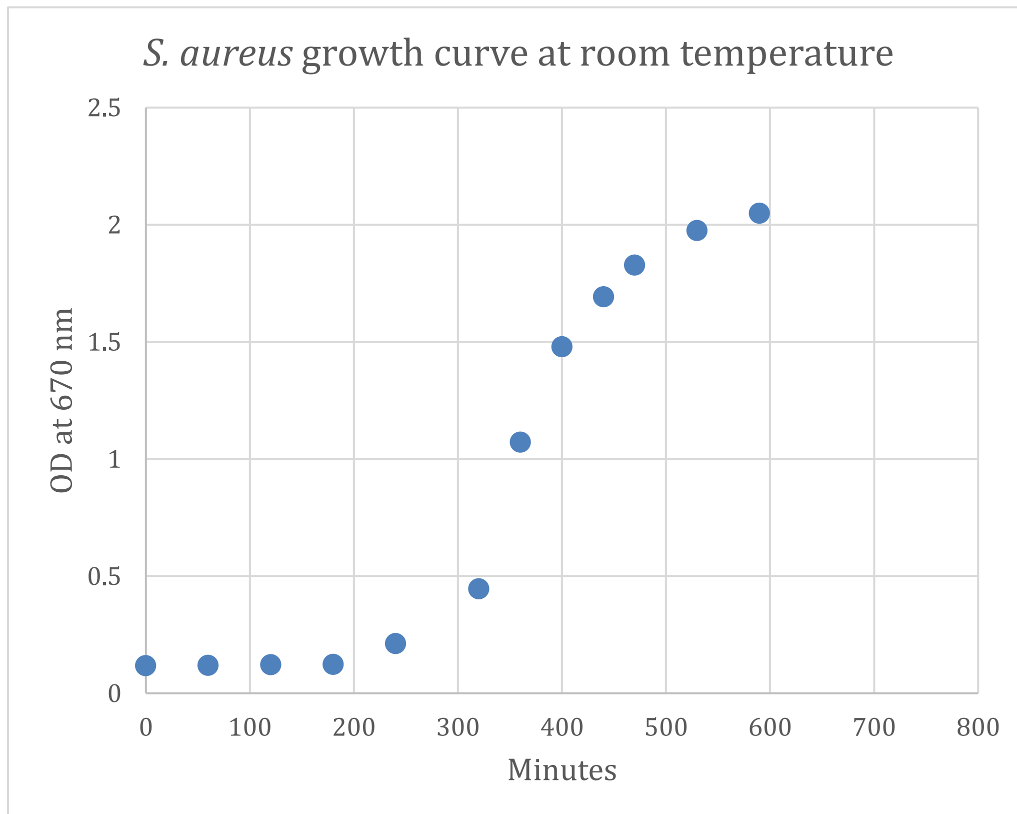

Figure 3: ***S. aureus*** **growth curve at room temp.**

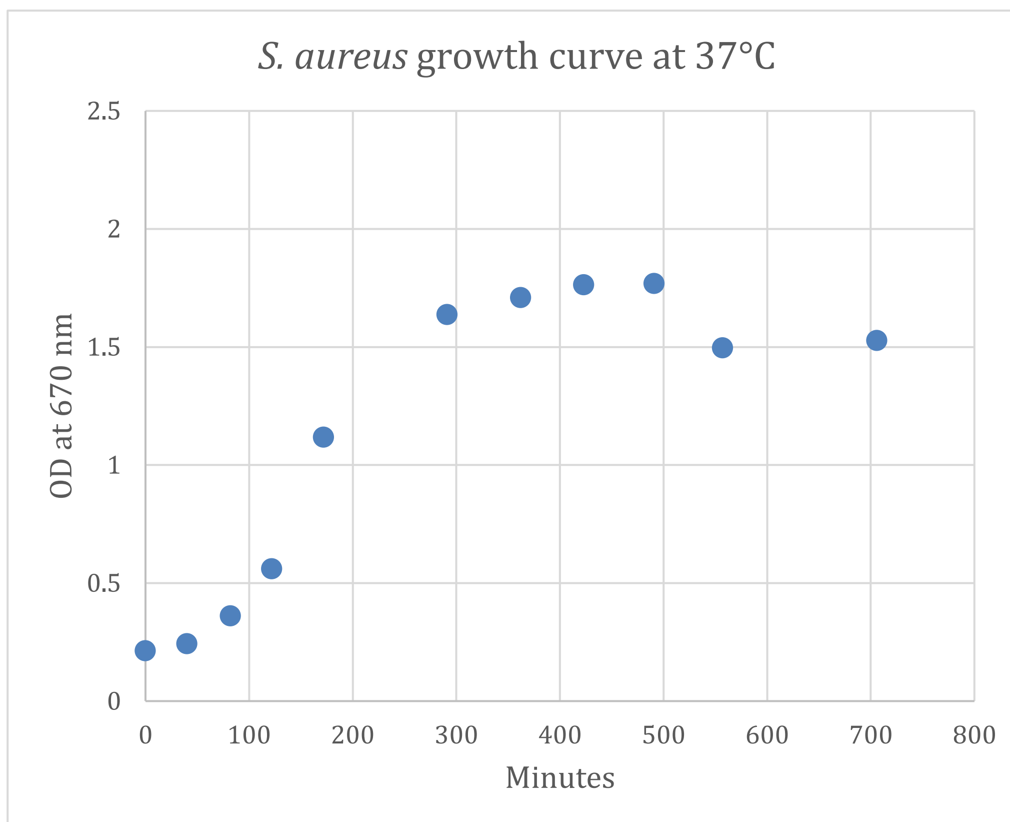

Figure 4: ***S. aureus*** **growth curve at 37°C**

Growth of S. aureus with used medium (crude AIP)

After the method for purification of AIP from used medium chosen (from Balaban *et al.*[19]), cultures were grown using the medium that was expected to contain AIP. A change in the growth kinetics, either as increased total growth or faster growth would have indicated activation of quorum sensing due to the presence of AIP in the crude medium. Cultures containing AIP grew to only 63-70% of the optical density of the controls. Growth did end about 50 minutes but due to the decrease in overall growth, the growth speed was slower than the control cultures. Another important feature was that the control cultures for this experiment grew significantly less than growth curves that were performed using a 75 mL culture. We believe this is due to the much smaller culture size (3 mL) and the lack of a rotator resulting in reduced gas exchange

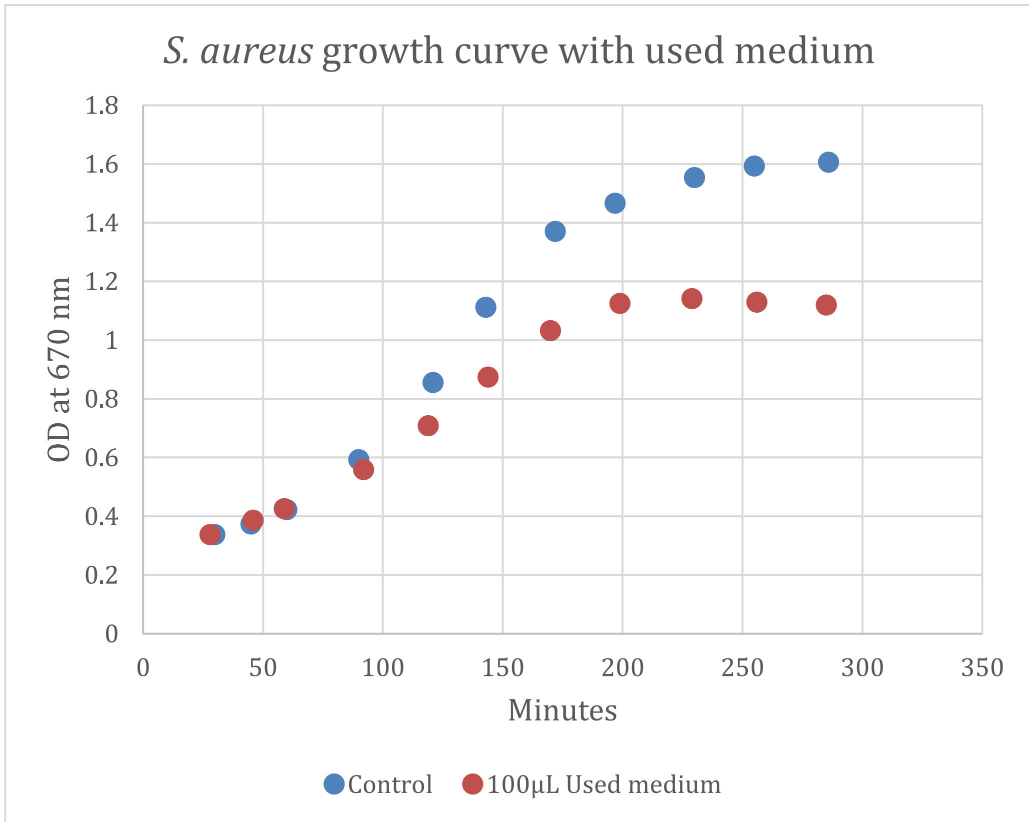

Figure 5: ***S. aureus* growth curve with used medium**

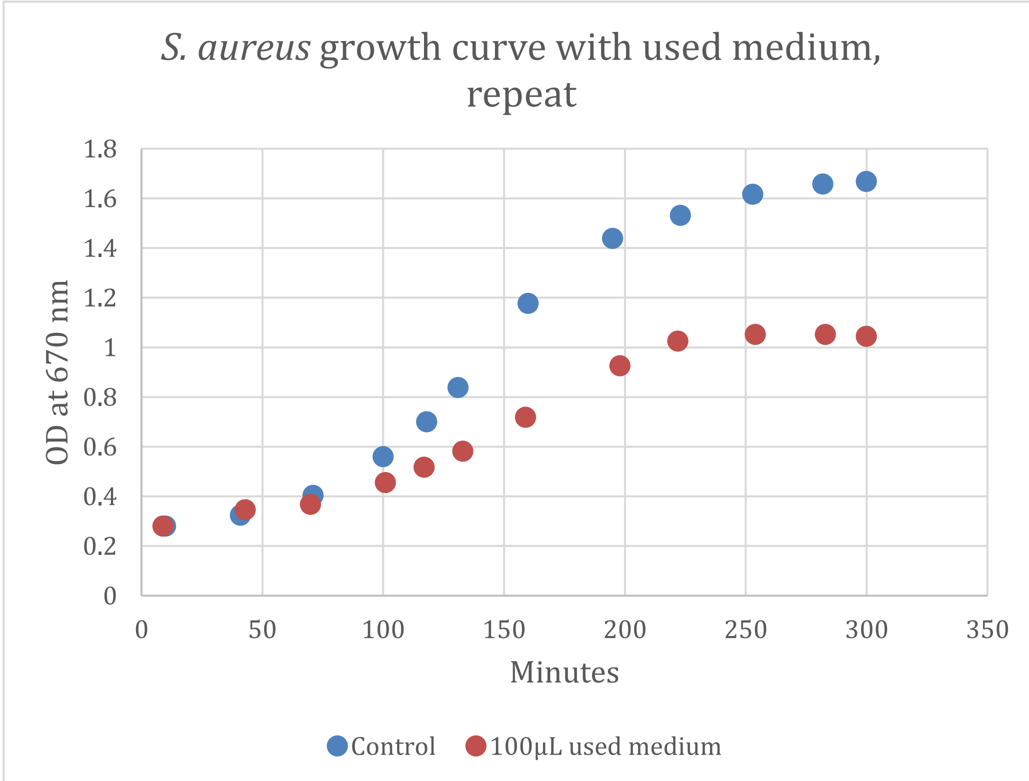

Figure 6: ***S. aureus* growth curve with used medium, repeat**

S. aureus growth in presence metal ion and crude AIP

Copper Sulfate Pentahydrate

Method 1.7 was used observe changes in growth kinetics when a culture contained both copper sulfate and AIP. The control curve, containing only high concentration tryptic soy broth (TSB powder was added to account for concentrated medium added to the test culture) grew reached peak absorption (1.13) after 344 minutes. The test culture containing used medium (Crude AIP) and 1mM copper sulfate reached peak absorption (0.75) after 345 minutes. The test culture reached 66% of the absorbance of the control, both cultures underwent the early, mid and late exponential phases at the same time. The difference in growth but consistency in timing resulted in a similarly shaped growth curve but the test culture had a much lower slope.

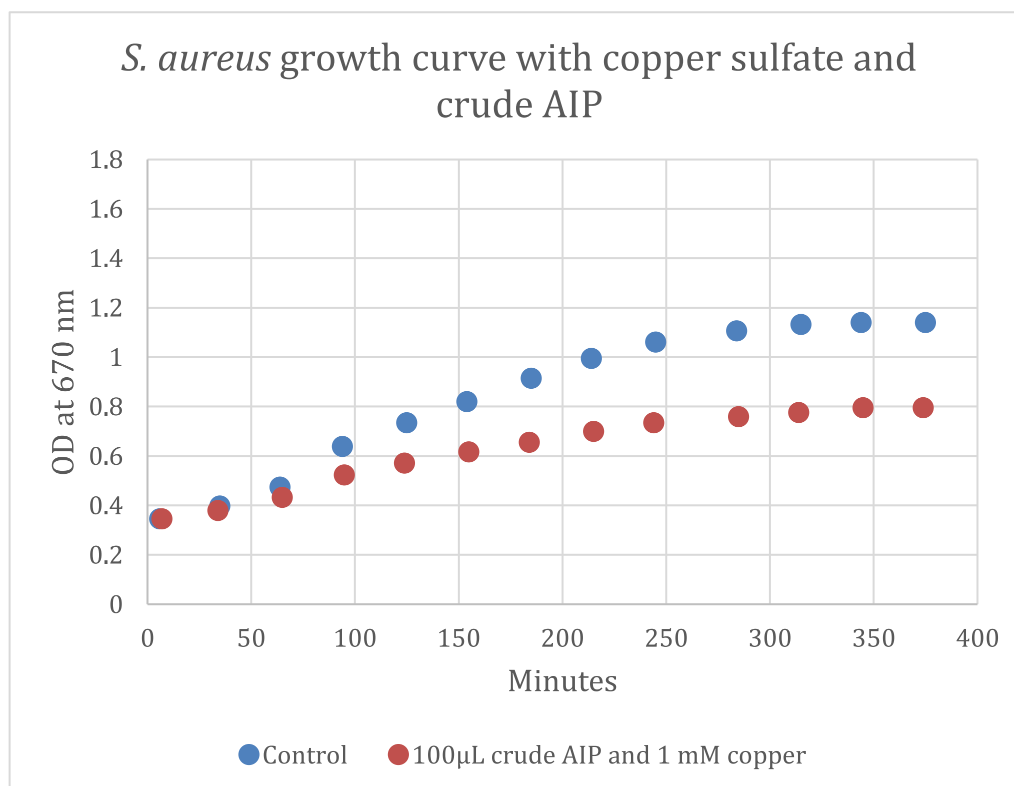

Figure 7: ***S. aureus*** **growth curve with copper sulfate and crude AIP**

Silver Nitrate

Method 1.7 was again used to perform two growth curves, copper sulfate was replaced with silver nitrate. The control culture reached peak absorbance (1.51) after 280 minutes. The test culture reached peak absorbance (0.97) after 221 minutes and then decreased over the next 90 minutes (to 0.91). The silver nitrate culture reached 64% of the absorbance of the control culture but completed growth 1 hour faster (80% of the time required for the control). In comparison to the experiment performed using copper sulfate, silver nitrate did not just reduce the overall growth. The change in time of peak absorption and subsequent decrease in absorbance showed the silver nitrate had a stronger antibacterial effect than copper sulfate.

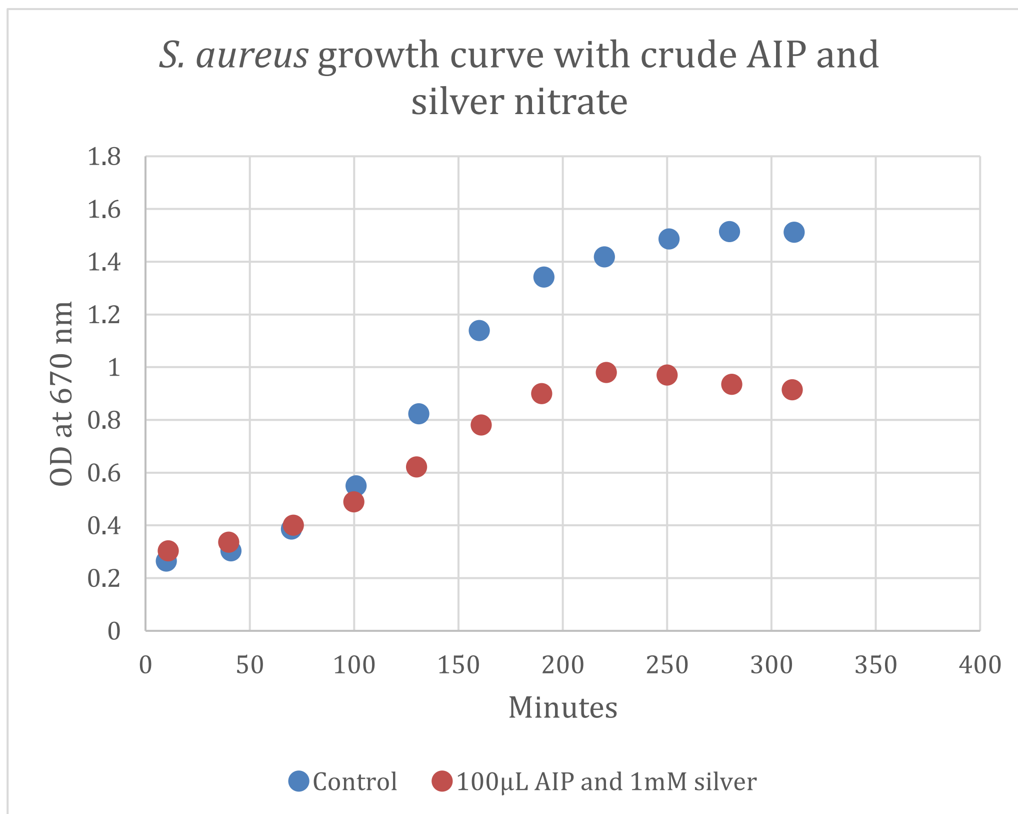

Figure 8: ***S. aureus*** **growth curve with crude AIP and silver nitrate**

Finding the minimal effective concentration of copper sulfate pentahydrate

Multiple concentrations of copper sulfate were tested to identify which concentration would have a minor but significant impact that could be increased by any synergistic effect with AIP. For the first experiment each culture contained additional dry TSB powder. The two lowest concentration cultures, 30µM and 100µM were determined to have had too small of an impact for use in further tests. The culture containing 1000µM did not exit the exponential phase by the time the experiment stopped but it did show much slower growth than any of the other cultures. Due to the low yields of the purification method of AIP it was best to use the minimal possible concentration of metal ions. It was decided that 300µM copper sulfate showed a significant change in growth kinetics while being an attainable concentration of AIP.

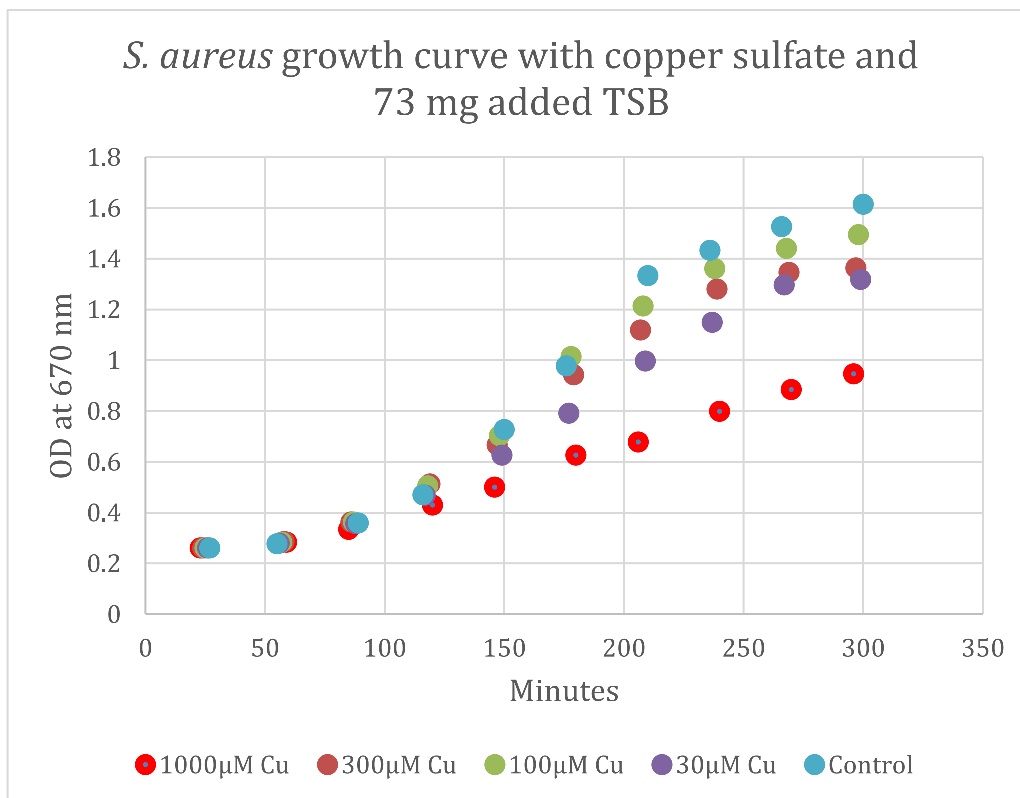

Figure 9: ***S. aureus*** **growth curve with copper sulfate and 73 mg added TSB**

A second experiment was then performed using concentrations of copper sulfate closer to the concentrations of AIP produced in medium during growth before concentration with the lyophilizer (10µM). None of these lower concentrations showed an appreciable difference in growth from the control. This experiment was performed without additional TSB, and at this time it was decided that adding additional TSB to the control culture to account for nutrients left in the concentrated used medium was causing a large amount of growth in controls that resulted in a poor comparison to the test cultures.

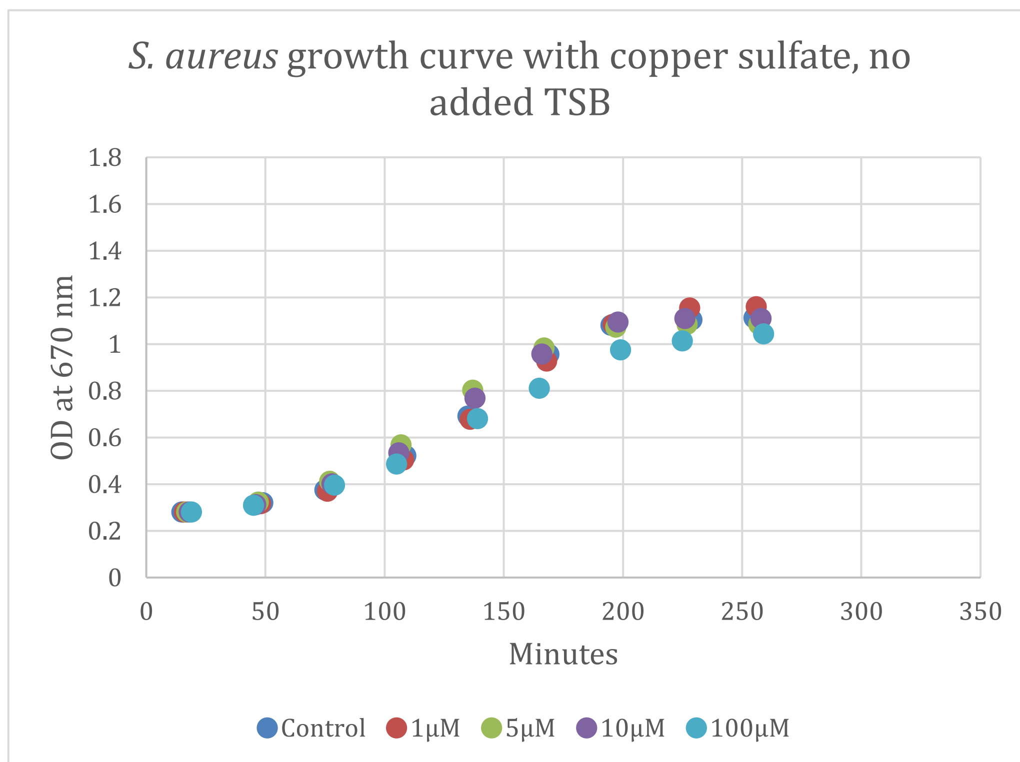

Figure 10: *S. aureus* growth curve with copper sulfate, no added TSB

Growth curves containing various concentrations of crude AIP

Method 1.9 was used to observe if different concentrations of crude AIP would cause a difference in growth that would be indicative of activation of the quorum sensing system. 50µL of crude AIP resulted in a concentration of 10µM in the 3 mL culture, this is the expected concentration for used medium before concentration through lyophilization. The different

cultures did not show any consistent evidence that the concentration of crude AIP changed the growth kinetics of the culture.

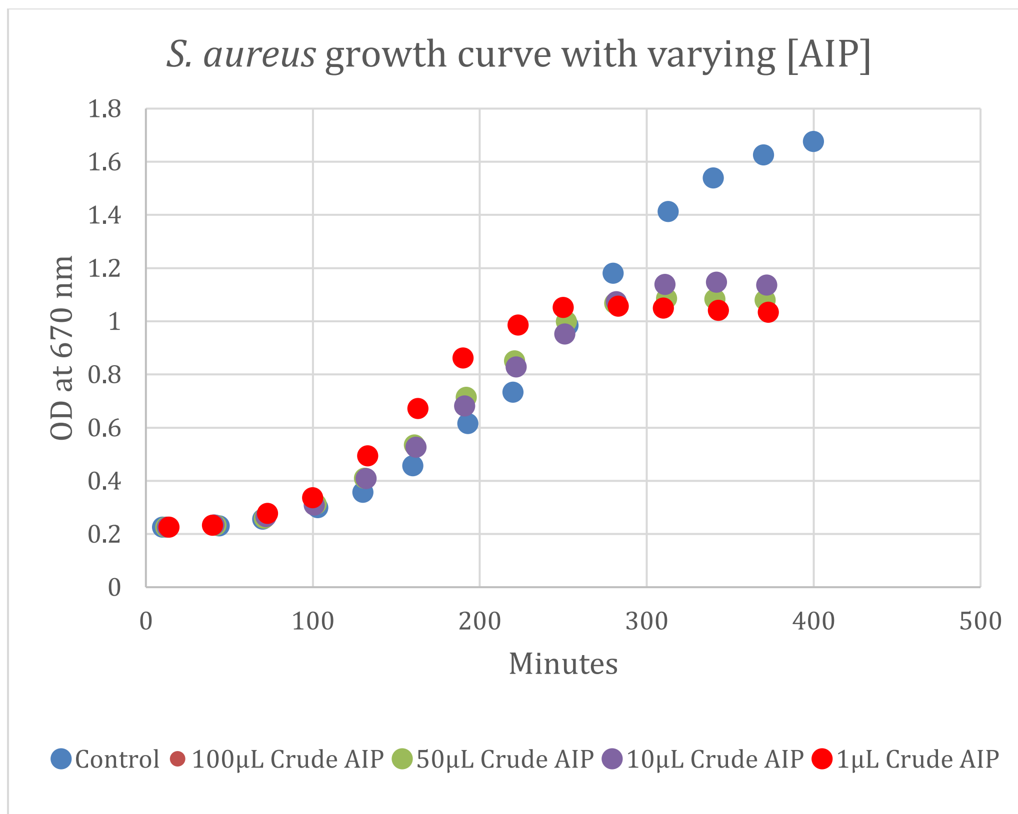

Figure 11: ***S. aureus*** **growth curve with varying [AIP]**

Growth curves with equimolar concentration of copper sulfate and crude AIP

Method 1.12 was used to test if using equimolar concentrations of crude AIP and copper sulfate would cause an increase in the antibacterial effect. The culture containing both AIP and copper sulfate grew slightly slower (30 minutes) than the cultures containing just copper sulfate or crude AIP which were nearly identical.

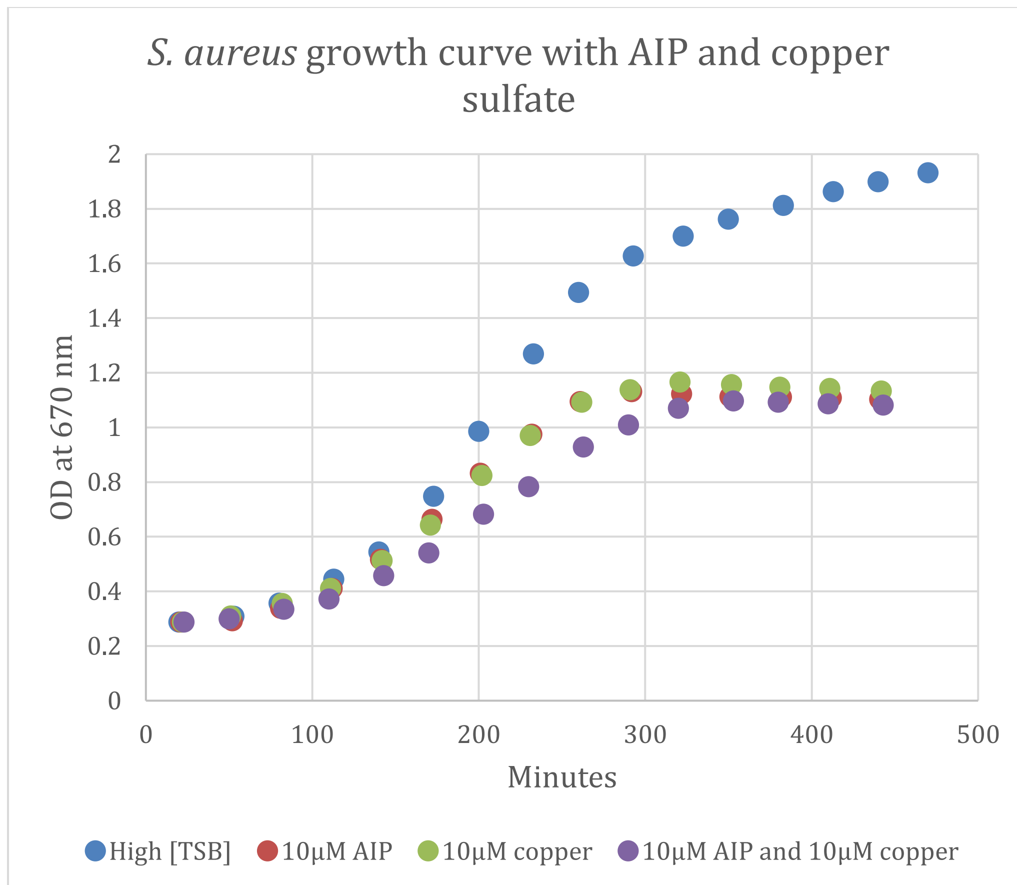

Figure 12: *S. aureus* growth curve with AIP and copper sulfate

The experiment was performed again including another control but this one also did not include additional dry TSB powder. This follow up experiment showed that the cultures containing crude AIP or metal ions showed no difference to a culture containing only the standard amount of TSB. The culture containing both crude AIP and metal ion also had more overall growth and reached peak absorbance faster than the cultures containing only crude AIP or copper sulfate. This change in relationship showed that the combination of crude AIP and copper sulfate was not producing an observable increase in antibacterial activity.

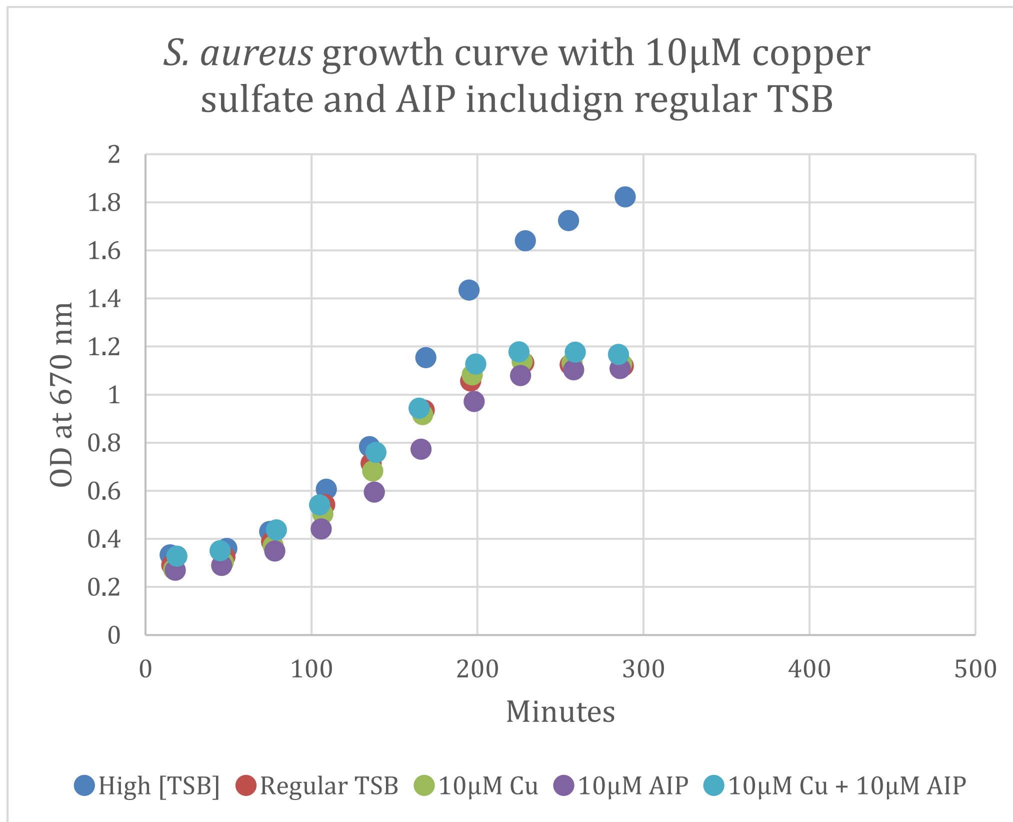

Figure 13: *S. aureus* growth curve with 10µM copper sulfate and AIP including regular TSB

Finding the minimal effective concentration of silver

Using method 1.11 Multiple concentrations of copper silver nitrate were tested to identify which concentration would have a minor but significant impact that could be increased by any synergistic effect with AIP. The lowest concentration culture, 30µM was determined to have had too small of an impact for use in further tests. The cultures containing 300µM and 1000µM showed virtually no growth, this left no room to observe an increase in efficacy due to AIP. It was decided that 100µM copper sulfate showed a significant change in growth kinetics while being an attainable concentration of AIP.

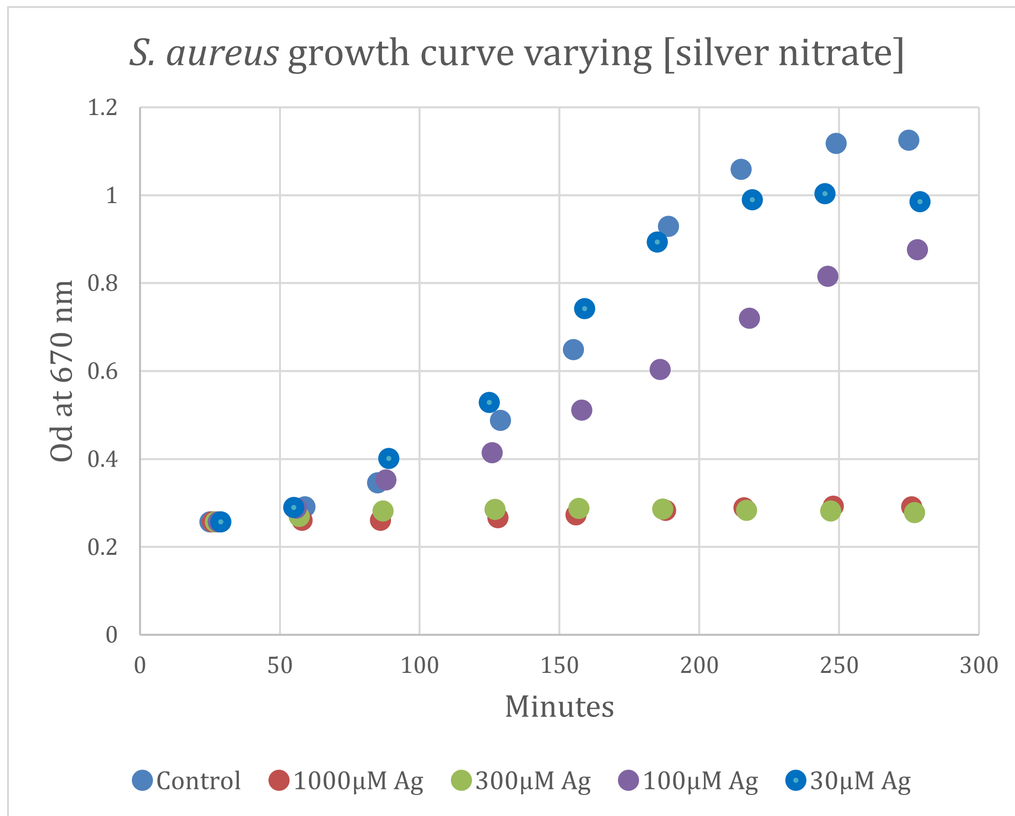

Figure 14: *S. aureus* growth curve varying [silver nitrate]

Advantages of low volume cuvette cultures

The primary method for determining growth curves for this project was by using only a small volume of medium (TSB) and 0.5mL of bacteria culture from frozen stock in a 3 mL quartz cuvette maintained at 37°C. Using the cuvette as the culture container the risk of contamination from liquid transfer was reduced. Cultures also completed growth on average after just four hours. Cultures that contained high concentration of TSB grew for a longer period of time and to higher absorbances but the use of increased concentration cultures was determined to be detrimental compared to regularly mixed TSB (30g/L). In similar studies performed by Chudobova *et al.* their cultures of *S. aureus* contained in microwell plates completed growth after nearly 20 hours. The method used in this project allowed for full growth curves to be performed in just 5 hours, including preparation time.

Another advantage of the method used here was the consistency of results. Starting absorbances for each culture showed some variation (from 0.00 to 0.12 OD) but each culture is seeded by the same frozen stock. Control cultures that did not include a high concentration of TSB reliably grew to an absorbance of 1.11 ± 0.05. The consistency allowed for easy data comparison. When testing a new frozen stock of bacteria two cultures were perform, both in regular TSB but one was from stock frozen on November 7, 2017 and one from stock frozen on May 24, 2018. The lag time for the more recently frozen bacteria was missed during data collection but through time shifting the slope we were able to compare the slope of each curve and conclude that the growth was consistent between cultures. Both of these cultures were comparable to the control cultures run in other experiments and grew to the expected absorbance range (1.11 ± 0.05).

One other method for analysis of growth kinetics was attempted during this project, using a Live/Dead Assay kit using propidium iodide and SYTO 9 dyes to stain the live (green) and dead (red) bacteria. The fluorescence of the peak wavelength of each dye was then measured. The fluorescence of samples that were expected to be the same (repeat wells with identical contents) had fluorescence values that varied as much as 16% (85 RFU to 99 RFU). It was also difficult to maintain consistent levels of fluorescence between cultures. A culture (incubated for 24 hours) diluted to an absorbance of 0.15 provided fluorescence values of 545 RFU but an identically prepared culture the next day was then off scale (>1000 RFU). The inconsistency in data meant that this method would not be usable for valid experiments.

Conclusions and Future Work

With no observable indication of quorum sensing activation when used medium was added to growth cultures I was unable to prove the presence or concentration of AIP in the used medium after purification. All tests performed to observe any synergistic affect between metal ions and autoinducing peptide were inconclusive due to the lack of veritably of AIP but no significant change was observed. In future work we will use synthesized AIP so that we can be certain of the concentration used. We were unable to determine which type of AIP was produced by the strain of *S. aureus* we used in this project. AIPI (75% purity) was ordered (from Selleckchem) as it is the most common, and the activation of growth due to quorum sensing is not required, the AIP can still bind the metal ion and introduce it to the cell which would theoretically increase antibacterial activity. Using the synthesized AIP we will perform methods 1.7, 1.8 and 1.9. Repeating these experiments will allow us to observe the effects of AIP individually and in combination with copper sulfate or silver nitrate while under more controlled conditions. We will also perform growth curves using 300μM silver nitrate and 300μM synthesized AIP to compare the effectiveness of the copper and silver ions.

Appendix A

Growing Staph aureus in Dulbeccos phosphate buffered saline (DPBS)

35 mL of *S. aureus* that was overnight at room temperature was centrifuged at 7,000 RPM (3,840g) for 20 minutes at 4°C in a JA20 rotor. The bacteria formed a white pellet at the bottom of the centrifuge tube and the supernatant appeared clear. The supernatant was removed and disposed of in bleach. The pellet was resuspended in 10 mL of DPBS that contained 5g/L glucose. The sample was centrifuged again under the same conditions and resuspended a second time in another 10 mL of DPBS and glucose solution. 1mL of the bacteria was then seeded in 10 mL of DPBS and glucose solution which was allowed to incubate overnight at room temperature.

After overnight incubation the culture showed no signs of growth and dead bacteria were visibly collecting in the bottom of the culture tube.

Live/Dead assay of Staph aureus in the presence of copper ions

40 mL of *S. aureus* was incubated overnight at 37°C and centrifuged at 3,840g and 4°C for 20 minutes. The pellet was resuspended in 10 of 8 g/L sodium chloride and 1 g/L glucose solution and the OD was determined to be 2.16. The solution was diluted to an OD of .30 using the salt solution. 2 mL of the diluted bacteria and 4 mL of 50X diluted dye (50X diluted, 6 µL of each component in 50 mL of filtered water) were added to two test tubes and allowed to incubate for 15 minutes. 2 mL of salt solution was added to one of the tubes (this is the control tube) and 200 µL of the control solution was transferred to a microwell plate. The fluorescence of the sample was scanned from 490-700 nm with an emission wavelength of 470 nm, slit widths of 3.0 nm and a scan speed of 500. 2 mL of 3.0 mM Cu was added to the second tube and a 200 µL sample was scanned immediately. New samples of both tubes were then scanned every five minutes until the change in intensity slowed, at this point a sample was

taken every 30 minutes until there was no change between samples. The time it took for the ratio of live/dead bacteria to become stable was two hours.

This experiment was carried out again with copper sulfate replaced by silver nitrate. A small amount of white precipitate formed on the addition of the metal solution, this was expected to be silver chloride.

Fluorescence measurements were very time consuming and difficult to perform. The fluorometer had a very narrow range before moving off scale which meant using the same settings for multiple experiments was often impossible. Analysis of the data was made difficult and it was determined that measuring the absorbance of growth curves would be the best method of analysis.

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