ABSTRACT

Determining the correlation between accessory gene regulator polymorphisms and *Staphylococcus aureus* sensitivity to 470 nm blue light

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Staphylococcus aureus is a Gram-positive pathogen responsible for minor skin infections, deep tissue infections, and even death. Due to the increasing resistance of *S. aureus* strains to antibiotics, it is important to investigate and develop alternative modes of treatment for bacterial infections. Photodynamic therapy using 470 nm blue light has been identified as a viable alternative to antibiotic treatment, however significant differences in the sensitivity of strains to blue light have been found. This project investigated the role of a genetic component, the accessory gene regulator (*agr*), found to be down-regulated in *S. aureus* isolates exposed to blue light. A statistically significant difference was found between the response of *S. aureus* isolates among *agr* groups, indicating that there exists a genetic basis for the varying responses of isolates to blue light therapy. However, further studies must be conducted to further elucidate the role of *agr* in the response to photodynamic therapy.

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DETERMINING THE CORRELATION BETWEEN ACCESSORY GENE REGULATOR POLYMORPHISMS AND *STAPHYLOCOCCUS AUREUS* SENSITIVITY TO 470 NM BLUE LIGHT

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CHAPTER ONE

Review of Literature

Staphylococcus aureus is a member of the phylum Firmicutes, which are low G+C Gram positive bacteria. *S. aureus* is characterized by golden-colored colonies that grow in a pattern resembling clusters of grapes [1]. The bacteria can typically be found on the skin and asymptomatically colonizing the mucous membranes of the anterior nares. Previous studies have disagreed on the prevalence of *S. aureus* nasal colonization, ranging from 15% to 70% of the population [2]. On the surface of the skin, or in the nasal cavity, *S. aureus* is not harmful to the host, however upon introduction to different locations in the body it has been shown to cause a wide spectrum of diseases, often generally termed "staph" infections. Through the large number of virulence factors and toxins produced by *S. aureus* or by direct invasion of tissues, this bacteria can cause a range of infections from minor skin to deep tissue infections, systemic diseases such as toxic shock syndrome, pneumonia, bacterial sepsis, and even death. Because of the common asymptomatic carriage of *S. aureus* in the nares of humans, spread of the bacteria and subsequent infection can be difficult to contain. While antibiotics have been introduced to treat infections caused by staph, resistance has developed to most that have been introduced. Shortly after the use of penicillin to treat staph infections, resistance developed, with less than 10% of strains being susceptible to this antibiotic today. Resistance to methicillin, nafcillin, oxacillin, and dicloxacillin developed shortly thereafter as well. While antibiotic-resistant staph infections typically were confined to

hospital-acquired infections, the emergence of increasingly aggressive, virulent community-acquired methicillin-resistant *S. aureus* (MRSA) strains has been the cause of increasing concern [1] . Vancomycin, a glycopeptide antibiotic, has remained the only antibiotic to continue to be active against MRSA strains. However, *S. aureus* isolates exhibiting resistance to vancomycin have also been reported [1].

As a result of the increasing resistance to antibiotics and difficulty in treating staph infections, alternative modes of treatment are increasingly important. Photodynamic therapy (PDT) is one such alternative mode of treatment that has shown success in treating multi-drug resistant pathogens [3, 4] . Photodynamic therapy involves the excitement of a photosensitizer by a light source to generate reactive oxygen species (ROS) which then may play a role in inactivating and killing the bacteria. Previously, PDT has been used as a treatment against various cancers, acne, endotracheal tube and catheter biofilms, burn wounds and as a method of nasal decolonization for infection prevention purposes [3, 5-8] . In particular, 470 nm blue light has been shown to have a bactericidal effect on *S. aureus* and to be a viable alternative to killing MRSA as well as other bacteria [9] . While the mechanism of blue light photodynamic therapy remains largely unknown, the generation of reactive oxygen species (ROS) has been shown to play a critical role in the inhibition of *S. aureus* [10] . Upon the excitation of a photosensitizer by light, cytotoxic reactive oxygen species are generated that target various components in the cell, which may include DNA, amino acids and proteins, and enzymes, and result in cell death [11, 12] . DNA damage and the damaging of the cytoplasmic membrane and associated proteins are two mechanisms that have been proposed to explain the inhibition of bacteria [13] . As a result of the important role of ROS

in bacterial inhibition, the identity and interaction of the photosensitizer with the bacterial cells significantly affects the effectiveness of photodynamic inactivation [11, 12] . Previous experiments in our lab have determined that there is a photosensitizer in the growth media used during blue light treatment, which would explain the effective inhibition of *S. aureus* in our experiments without the addition of a photosensitizer [14] .

Various other factors also play a role in the response of bacteria to blue light therapy. In addition, experiments have shown that as bacterial density increases, photodynamic therapy is less effective [9] . Accordingly, increased light penetration and greater surface area of bacteria exposed have been shown to have a greater bactericidal effect [9] . In addition, the wavelength of the applied light used in photodynamic therapy has shown to have a varied effect. While studies have shown success using a range of wavelengths from 300-700 nm, 405 nm blue light in particular has shown the greatest bactericidal efficiency. However we used a longer wavelength of 470 nm to avoid the use of any ultraviolet light [10] .

Even under controlled conditions of concentration, temperature, wavelength, and treatment time, the response of different isolates to blue light therapy shows great variation as well [14] . While previous studies conducted in our lab determined the amount of light necessary to kill 100% of the tested isolates under fixed conditions, at sub-lethal doses of blue light, the sensitivity of different isolates varies [14] . A pressing concern with photodynamic therapy is the potential for *S. aureus* isolates to develop resistance to blue light therapy as they have previously done in response to antibiotic treatment. As a result, this thesis addresses this concern by looking for a genetic component that correlates with the varying sensitivity to blue light.

The *S. aureus* genome is made up of the core genome, which contains all vital genes necessary for survival, and the accessory genome, which contains the diversity within the species. The accessory genome is made up of mobile genetic elements (MGEs) that often are the source of many important virulence factors for the bacterium and are genetically diverse among different strains [15]. MGEs are also capable of inter- and intracellular mobility, allowing *S. aureus* to easily adapt to new environments. The ability for gene transfer has played a large role in the acquisition of antibiotic resistance by strains of *S. aureus*. For example, resistance to methicillin is conferred through the *mecA* gene, found on a large DNA fragment called a staphylococcal cassette chromosome likely acquired from *S. sciuri*. Additionally, vancomycin resistance has also been conferred after the transfer of an MGE containing the gene cluster Tn1546 from vancomycin resistant enterococci to *S. aureus.* Thus, determining the mechanism and genetic basis of variation in blue light inhibition is important to ensure that resistance does not similarly develop to this alternative method of treatment.

In order to determine which genes may be involved in the response to blue light, the transcriptomes of *S. aureus* BUSA 2288, isolated under blue light and no light conditions, were analyzed by undergraduate Bayless Drum [16]. This RNAseq experiment analyzes the messenger RNA to provide a snapshot of the functional activity of the cells. In order to determine the differential expression of genes as a result of treatment with blue-light, computer programs were used to determine the statistical difference in the number of RNA molecules (gene expression) under the two conditions of light and no light. The results of this experiment have been uploaded onto the NCBI GEO database and pair-wise analysis has been performed in GeneSifter (a program provided by Perkins-

Elmer that statistically analyzes the RNAseq data). This analysis identified several membrane-associated proteins such as transporters and those involved in quorum-sensing pathways, which were down-regulated after blue light treatment.

One operon identified by the RNAseq data was the accessory gene regulator (*agr*). The accessory gene regulator is a quorum-sensing system that regulates a large variety of density-dependent virulence and transcription factors [17, 18]. Figure 1 illustrates the *agr* quorum sensing pathway [18]. In this pathway, agrD is transported out of the cell through agrB and converted into an auto inducing peptide (AIP), a signal that can be detected by agrC. When agrC receives the signal from AIP (based on concentration) it activates agrA which increases the expression of both the *agr* operon and the virulence genes controlled by the promoter region P3. In the figure, points of inhibition by Solonamide and Savarin are indicated. These molecules are known to inhibit and kill *S. aureus* through inhibition of *agr* mediated quorum sensing.

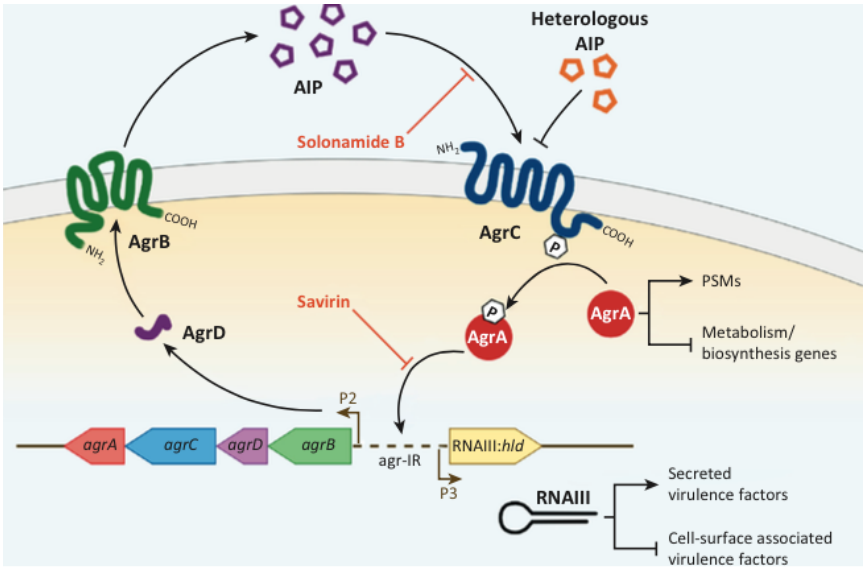


Figure 1: Accessory Gene Regulator quorum-sensing system [18]

In regulating numerous transcription and virulence factors, the *agr* operon controls the expression of various genes affecting cell growth and division, metabolism, and transport, as well as numerous other activities vital to the cell's function. Furthermore, the *agr* operon has been shown to play an important role in the response of *S. aureus* to oxidative stress [19, 20]. Previous studies have shown decreased transcription of the *agr* system following oxidation of the AIP signaling molecule [19]. In addition, Sun et al. have identified an intra-molecular disulfide redox switch within the AgrA response regulator that represses transcription of the *agr* system in response to oxidative stress by inhibiting the DNA binding ability of the AgrA molecule [20]. The hyper-susceptibility to ROS-producing photodynamic therapy of *S. aureus* mutant isolates lacking *agr* function further demonstrates the importance of the *agr* operon in the defense against oxidative stress [4]. In our own lab, previous RNAseq data of undergraduate Bayless Drum also revealed that *agr* components were down-regulated following photodynamic therapy [16].

In addition, *agr* has been categorized into four different groups based on the DNA sequence of the auto-inducing peptide (AIP) produced by agrD, and its receptor, produced by agrC [21]. *Agr* grouping was shown to be related to certain pathotypes such as vancomycin resistance and various toxin productions [22]. Similarly, we proposed that the different polymorphisms of *agr,* which can be differentiated using polymerase chain reaction as shown in Figure 2, could be related to the diversity of blue light response.

CHAPTER TWO

Introduction

Staphylococcus aureus bacteria is the cause of a variety of infections ranging in severity from minor skin infections to severe deep-tissue infections such as bacterial pneumonia, toxic shock syndrome, bacteremia, and even death [1] . The increasing resistance of hospital- and community-acquired infections to antibiotics has led to an increased need for alternative methods of treating "staph" infections. Photodynamic therapy, and in particular blue light therapy, has been shown to be one effective alternative method of treatment against antibiotic-resistant infections [10] . However, the high variation in response of different isolates to sub-lethal levels of blue light raises concerns regarding the possible development of resistance of *S. aureus* isolates to blue light. [14] .

In order to determine a genetic component for the variation in response and further elucidate the mechanism of blue light inhibition, differentially expressed genes were analyzed to identify those with high genetic diversity that may be potential targets of blue light. The quorum-sensing accessory gene regulator system was chosen because of its high degree of diversity as a mutational hotspot, global regulatory role of transcription and virulence factors, toxins, and metabolic pathways across the cell, as well as its role in the cell's response to oxidative stress [4, 17, 23] . In particular, there are four polymorphisms found in the *agrC* gene by which isolates can be categorized into four *agr* groups. The hypothesis of this study is that as a result of blue light-generated

reactive oxygen species acting on the *agr* system, the diversity in blue light responses will be correlated to the different *agr* polymorphisms. It is proposed that blue light-generated ROS decreases the activity of the *agr* system either by inactivating AIP or damaging the membrane protein agrC, the receptor of AIP, disrupting the quorum-sensing pathway of the *agr* system. We suggest that the variations in the agrC protein and the corresponding AIP molecule contribute to the high variation in response of *S. aureus* isolates to blue light treatment because structural variations may result in different molecular targets available to interact with reactive oxygen species generated by blue light illumination.

CHAPTER THREE

Methods

Identifying areas of genetic diversity in Staphylococcus aureus isolates

Since some *S. aureus* isolates are more sensitive to blue light than others, we hypothesized that the mechanism of growth inhibition may have a genetic component. To test this hypothesis, we first identified the genes that were at least 5-fold up or down-regulated after blue light treatment and then we analyzed the amount of genetic diversity for each gene by comparing the amino acid sequences among 11 *S. aureus* strains.

In order to determine the differential expression of genes as a result of treatment with blue light, PerkinElmer's GeneSifter was used to perform a pair-wise analysis on RNAseq data previously acquired from the transcriptomes of BUSA2288 after exposure to blue light and no light [16] . Genes identified to be up or down-regulated by a 5+ fold change after blue light treatment, were further explored using NCBI's Basic Local Alignment Search Tool (BLAST), multiple alignment, and the Scorecons Server [24] . BLASTp was used to compare 31 different protein sequences among 11 different *S. aureus* strains. The multiple alignments generated using BLASTp were exported and analyzed using the Scorecons Server, which scores amino acid variability at each position, with higher scores indicating higher gene diversity [24] . The accessory gene regulator (*agr*) was found to have a high degree of variation, as shown by the high Scorecons value for AgrB in Figure 2.

Locus Tag	Entrez Gene #	Fold Change	Gene Description	Scorecons
SA0204	1122981	Up 28.36	Azoreductase	9.8%
SA2307	1125234	Up 17.05	Glyoxalase	8.6%
SA0203	1122980	Up 15.18	Tandem lipoprotein	8.4%
SA2479	1125409	Up 12.69	Hypothetical protein	5.6%
SAS065	1124734	Down 9.99	Delta hemolysin	22.7%
SA2176	1125104	Down 9.24	Nitrite extrusion protein/transporter	3.7%
SAS089	1125289	Down 8.37	Hypothetical protein	45.1%
SA1630	1124473	Up 7.45	Serine protease	40.9%
SA1842	1124735	Down 7.23	AgrB	**47.1%**
SA2264	1125192	Down 7.11	Hypothetical protein	54.7%
SA0285	1123064	Down 6.29	Membrane hypothetical protein	3.4%
SA0414	1123199	Down 6.16	Membrane protein	23.8%
SA2183	1125111	Down 6.01	Nitrate reductase delta	12.9%
SA2184	1125112	Down 6.01	Nitrate reductase beta	6.5%
SA2182	1125110	Down 6.01	Nitrate reductase gamma	2.5%
SA2185	1125113	Down 6.01	Nitrate reductase alpha	1.6%
SA2189	1125117	Down 5.87	NirR transcriptional regulator	20.8%
SA2187	1125115	Down 5.85	Assimilatory nitrate reductase	4.7%
SA2186	1125114	Down 5.85	Uroporphyrin-III-methyltransferase	11.3%
SA2180	1125108	Down 5.79	Histidine kinase	1.9%
SA2398	1125327	Down 5.68	Membrane hypothetical protein	7.2%
SA0644	1123451	Down 5.59	Membrane hypothetical protein	3.8%
SA0889	1123712	Down 5.59	Membrane hypothetical protein	3.0%
SA2179	1125107	Down 5.59	VraR	73.5%
SA1226	1124065	Down 5.58	Aspartate semi-aldehyde dehydrogenase	2.1%
SA2181	1125109	Down 5.57	NreA	1.6%
SA1637	1124480	Down 5.46	LukD, gamma hemolysin	43.7%
SA2188	1125116	Down 5.36	Nitrite reductase	2.1%
SA0890	1123713	Down 5.25	Oxidoreductase	0.0%
SA0982	1123809	Up 5.05	Sortase B	9.3%

Figure 2: Differentially expressed genes and diversity scores

Agr typing of nasal isolates

In order to test the hypothesis that the *agr* type was correlated to the blue light effect on *S. aureus*, we first determined the *agr* type of 62 nasal isolates. *S. aureus* isolates can be divided into 4 distinct *agr* polymorphisms [25] . For this experiment, archived DNA samples from a total of 62 *S. aureus* nasal isolates collected from healthy undergraduates were screened through polymerase chain reaction (PCR) to determine the *agr* type [26] . PCR was performed according to methods shown in Bibalan et al. in a 25 µL reaction mixture as follows: 12.5 µL AmpliTaq Gold 360 Master Mix™ (Life Technologies, USA), 2.5 µL extracted DNA, 6.25 µL of 10 µM oligonucleotide primer solution (Life Technologies, USA) as shown in Figure 3, and 3.75 µL water [21] . The thermal profile involved an initial denaturation step at 94°C for 6 minutes, followed by 32 cycles of denaturation at 95°C for 45 seconds, primer annealing at 56°C for 60

seconds, and primer elongation at 72°C for 70 seconds, with a final extension step following the cycling at 72°C for 8 minutes.

Agr group	Primers	Product size
	Pan F 5'- ATG CAC ATG GTG CAC ATG C- '3	
agr I	R 5'- GTC ACAAGT ACT ATA AGC TGC GAT – '3	441 bp
agr II	R 5' – TAT TAC TAA TTG AAA AGT GGC CAT AGC – '3	575 bp
agr III	R 5'- GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G – '3	323 bp
agr IV	R 5' – CGA TAA TGC CGT AAT ACC CG – '3	659 bp

Figure 3: PCR primers for *agr* polymorphisms [21]

PCR products were separated through gel electrophoresis in a 1.5% agarose gel stained with ethidium bromide and visualized using a BioRad Gel Doc EZ Imager™. *Agr* types I through IV were determined by the size of DNA fragment products as shown in Figure 2. This screening process was repeated until the *agr* types of all 62 *S. aureus* isolates were determined. A detailed protocol of *agr* typing can be found in Appendix C.

Optical density

In order to determine a possible correlation between *agr* polymorphism and blue light resistance, 29 *S. aureus* isolates were chosen and treated with 470 nm blue light. Ten isolates from *agr* group type I, 10 isolates from *agr* group type II, and 9 isolates from *agr* group type III were tested.

Streak plates were created from cryobead stocks of chosen isolates and grown overnight at 35°C. One colony was picked from each streak plate and placed in 1 mL of Brain-Heart Infusion broth (BHI) in a 24-well plate. Plates were allowed to grow

overnight at 35°C, shaking at 300 RPM. 10 µL of overnight growth was placed in 1 mL of BHI and grown for 2 hours at 35°C, shaking at 300 RPM. The optical density of the 2-hour growth culture plates was taken using a Biotek ELx800™ Absorbance Reader at 600 nm to determine the "pre-treatment OD". Isolates were treated with 30 minutes of 470 nm blue light at 35°C, shaking at 300 RPM in a black 24-well plate (ibidi, USA) according the layout shown in Figure 4. Black well plates prevent the transfer of light from well to well and outside of the plate. A parallel no-light experiment was performed in a Falcon® 24-well clear plate (Corning Inc., USA) simultaneously. Each isolate was tested in triplicate within three different experiments for a total of 9 trials per isolate.

Isolate 1	Isolate 1	Isolate 1	Isolate 2	Isolate 2	Isolate 2
Isolate 3	Isolate 3	Isolate 3	Isolate 4	Isolate 4	Isolate 4
Isolate 5	Isolate 5	Isolate 5	Isolate 6	Isolate 6	Isolate 6
Isolate 7	Isolate 7	Isolate 7	Isolate 8	Isolate 8	Isolate 8

Figure 4: Layout of 24-well plate for blue light treatment

Optical density growth measures were taken 2 hours and 4 hours post-treatment at 600 nm. The "2 hour post-treatment OD" and "4 hour post-treatment OD" were used to calculate the bacterial growth, which was then used to determine percent inhibition for blue light- and no light-treated cultures according to Figure 5. A non-parametric Wilcoxon rank-sum test was performed on the resulting calculations to determine significance of the results. A detailed protocol of the optical density methods can be found in Appendix D.

(NL growth – BL growth)/NL growth ×100

Figure 5: Percent inhibition of blue light treatment

Flow Cytometry

In order to further explore the effect of blue light on *S. aureus* at a finer resolution, a bacterial viability assay was performed using flow cytometry according to a BD Cell Viability Kit™ protocol.

Streak plates were made from cryobead stocks of 12 chosen isolates and grown overnight at 35°C. One colony from each streak plate was chosen and placed in 1 mL BHI in a 24-well plate. Plates were allowed to grow overnight at 35°C, shaking at 300 RPM. Ten µL of overnight growth was placed in 1 mL of BHI and grown for 2 hours at 35°C, shaking at 300 RPM. The 2-hour culture was serially diluted 1:100 resulting in an average concentration of 5×10^5 to 9×10^6 CFU/mL as determined by colony counts. These cells were treated with 30 minutes of 470 nm blue light at 35°C, shaking at 300 RPM. A parallel no-light experiment was performed in a BD 24-well clear plate simultaneously. Cultures were incubated for 4 hours, the prepared for flow cytometry analysis in a 12 x 75 mm tube by adding 50 µL of bacterial culture, 5 µL of thiazole orange dye (TO), and 5 µL of propidium iodide (PI) to 500 µL of BD sheath fluid. Tubes were vortexed, then incubated at room temperature for 5 minutes, and analyzed on BD FACSVerse™ flow cytometer to determine the ratio of live *S. aureus* cells to dead *S. aureus* cells. Settings used were as follows: FSC at 201.4 V, SSC at 365.1 V, FITC at 455.4 V, and PE at 450.2 V. All plots were visualized using logarithmic amplification. A total of 10,000 events were collected. Total *S. aureus* cells, live cells, and dead cells were gated and the populations counted. A non-parametric Wilcoxon rank-sum test was performed to analyze and determine the significance of the results. A detailed protocol of the flow cytometry experiment can be found in Appendix E.

CHAPTER FOUR

Results

Identifying areas of genetic diversity in Staphylococcus aureus isolates

In order to test the hypothesis that blue-light inhibition of *S. aureus* has a genetic component, pair-wise analysis of RNAseq data generated by Bayless Drum and deposited in Gene Expression Omnibus (GEO) accession #GSE62055 was analyzed. Differential gene expression analysis, using PerkinElmer's GeneSifter software, revealed 31 *Staphylococcus aureus* genes with a 5+ or greater fold change in gene expression after exposure to blue light. Notable differentially expressed genes include multiple unspecified membrane and hypothetical proteins, nitrate reductase subunits, and histidine kinase genes.

In order to determine if these genes played a role in the cell's response to blue light, we next determined whether the up- or down-regulated genes were conserved or contained polymorphic regions. We performed multiple alignments of highly up- or down-regulated genes among 11 different *Staphylococcus aureus* isolates and calculated diversity scores using the Scorecons server (Figure 2). The only genes found to have a relatively high degree of diversity, characterized by a Scorecons score greater than 40%, were hypothetical proteins, serine protease, VraR, and LukD γ hemolysin, as shown in Figure 2. VraR, which is a two-component response regulator protein responsible for

vancomycin resistance, and LukD γ hemolysin, a leukocidin toxin, both are genes that play a role in the antibiotic resistance and virulence of *S. aureus* isolates. In addition, AgrB, a subunit involved in the accessory gene regulator was found to have a high degree of diversity, with a Scorecons score of 47.1%. After further investigation, agrA and agrC were also found to have a high degree of diversity (41.9% and 48.9% respectively), although each saw only a 4.09 and 3.97 fold decrease in gene expression. As a result of the numerous genes that *agr* regulates, and the high diversity of each subunit, the accessory gene regulator was chosen for further investigation.

Agr typing of nasal isolates

The *agr* polymorphisms of 62 *Staphylococcus aureus* isolates obtained from the anterior nares of healthy undergraduate students were determined using polymerase chain reaction. *Agr* type I was the most common, characterizing 32 out of 62 isolates (51.6%). The next most common *agr* type was type II with 21 isolates (33.9%), and 9 isolates were *agr* type III (14.5%). None of our randomly chosen isolates were found to be *agr* type IV. Detailed results of *agr* typing can be seen in Figure 6.

Agr type	I		II		III
***S. aureus* isolates**	39	2372	3	1079	240
	349	2414	55	1127	311
	371	2418	109	1130	1026
	405	2455	115	1470	1463
	1152	2458	318	2019	2072
	1215	2472	330	2263	2195
	1240	2503	346	2431	2210
	1278	2669	374	2553	3064
	1307	2671	398	2653	3097
	2040	2672	1046	2819	
	2045	2695			
	2057	2734			
	2069	2860			
	2163	3016			
	2288	3017			
	2344	3180			

Figure 6: Classification of 62 Baylor *S. aureus* nasal isolates by *agr* type

Optical density as a measurement of blue light inhibition

In order to test the hypothesis that *agr* type plays a role in the response to blue-light, we first measured the amount of blue light inhibition for each isolate. Optical density measurements were used to assess the response of *S. aureus* isolates to blue light treatment, as specified in Methods, Chapter Three. The growth of control cultures grown in the dark and of those exposed to a sub-lethal dose of blue light was calculated and compared to assess the overall bactericidal effect of blue light within *agr* groups and among isolates. To normalize the data, analysis was performed using a Wilcoxon non-parametric two-sample test.

With respect to the overall inhibition of *S. aureus*, there was no significant difference between no light and blue light cultures after 2 hours of recovery, but after 4 hours of recovery the culture treated with blue light was inhibited as evidenced in Figure 7 by the mean score of 300.204 compared to no light's 324.796 mean score ($p=0.044$). While blue light inhibition was observed among the total group of isolates tested, when

analyzed by *agr* groups, there was no significant difference between the controls and blue light-treated culture 2 or 4 hours post-treatment.

Recovery time	No Light	Blue Light	P value
2 hours	320.981	314.019	0.417
4 hours	324.796	300.203	0.042

Figure 7: Wilcoxon average scores for difference in growth over time determined by optical density

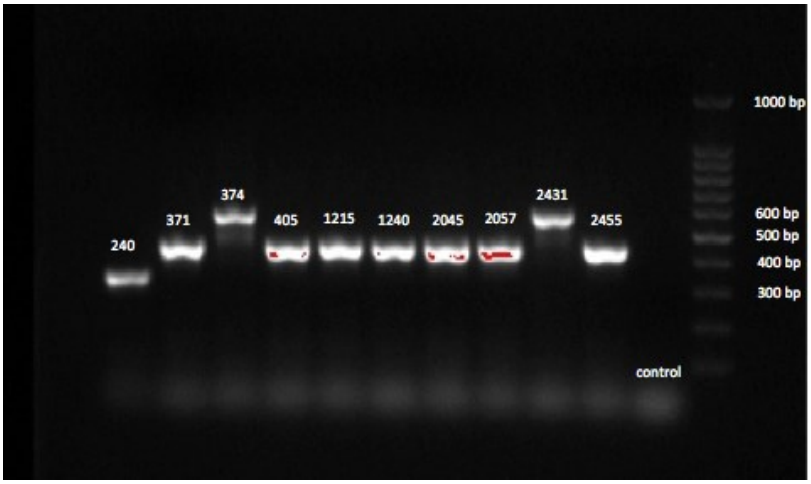


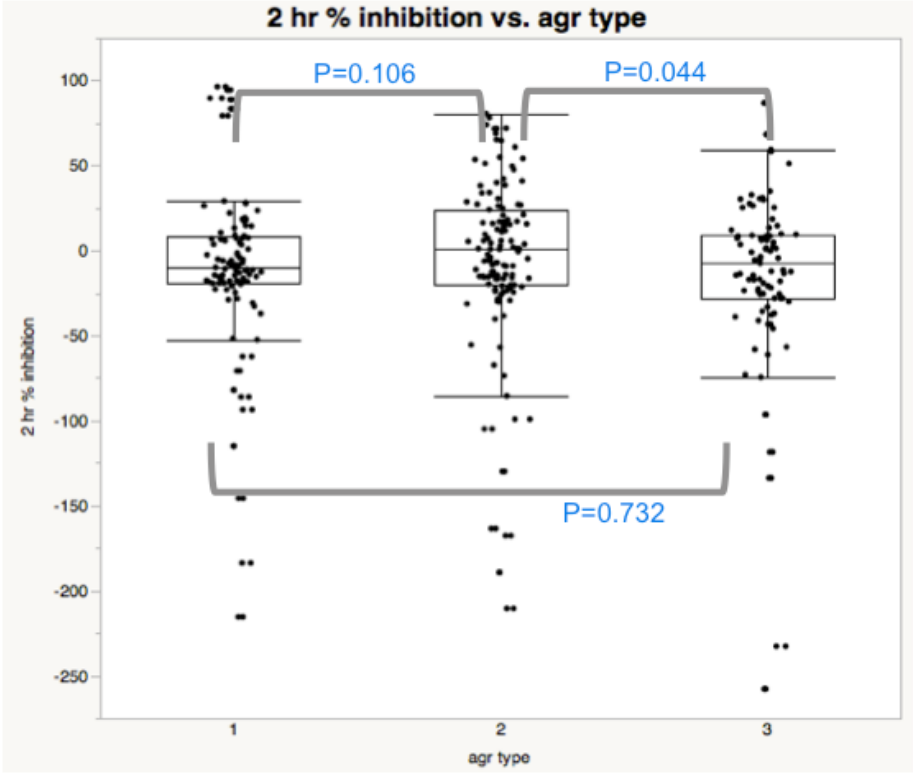
Figure 8: Gel electrophoresis of PCR products to identify *agr* group types

In order to more directly compare the responses within *agr* groups and determine whether a difference in response existed between groups, the percent inhibitions of no light and blue light-treated cultures were calculated and compared. Percent inhibition is calculated as shown in Figure 5, and is used to represent the overall response of isolates to blue light treatment. A positive percent inhibition value reveals that blue light treated cultures grew less than the control group. A negative percent inhibition value reveals that blue light treated cultures grew more after blue light treatment than those in the control group.

Figures 9a and b show the comparison of percent inhibition between all three *agr* groups, and individual comparisons between *agr* groups (*agr* I vs. *agr* II, I vs. III, II vs. III). While there is no distinct trend that can be seen between groups, with the median bars of the boxplots at relatively close values, *agr* groups 2 and 3 showed a significant difference in response after 2 hours of recovery ($p=0.044$), according to a Wilcoxon rank sum comparison. After 4 hours of recovery, no significant difference in optical density was found between any of the *agr* groups.

Figure 9 also shows the large range of variation in response to blue light, with each point representing a percent inhibition value of an isolate. A high degree of variation is observed among isolates as a whole, as well as within *agr* groups. The percent inhibition of *S. aureus* isolates 2 hours post-treatment ranged from -72.6% to 27.2% inhibition after blue light treatment. The percent inhibition 4 hours post-treatment ranged from -17.3% to 33.3% inhibition.

a)



b)

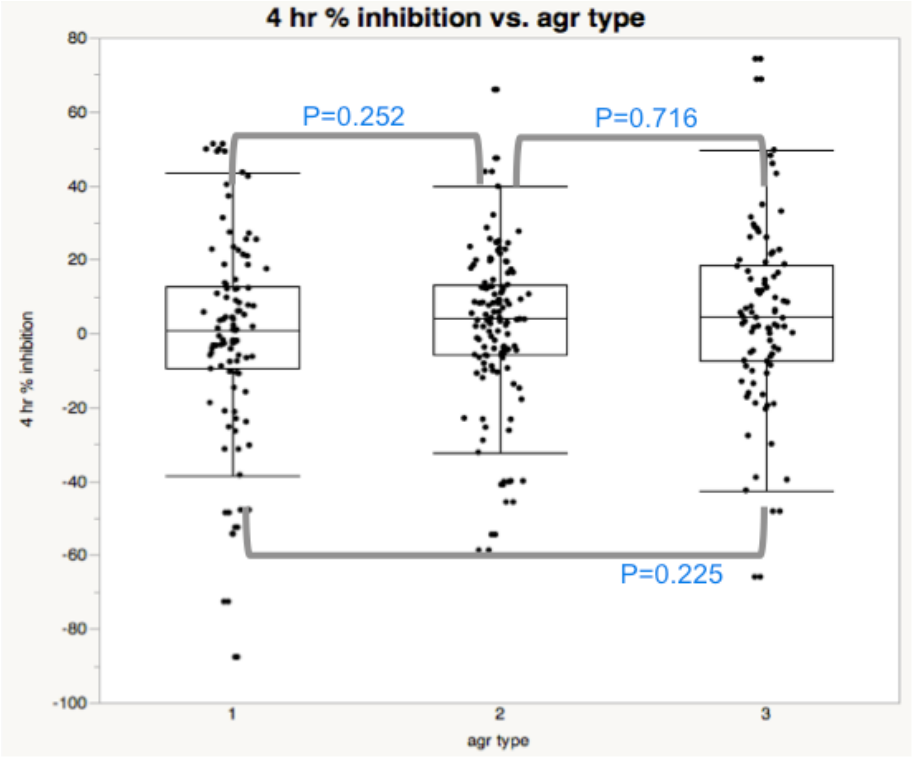


Figure 9: Box and scatterplot visualizations of % inhibition determined by optical density by *agr* group type,
P values for overall comparison: a, P=0.09; b, P=0.34

Live-to-dead flow cytometry cell ratios as a measurement of blue light inhibition

In order to improve the resolution of detecting the effects of blue light at an early time period, blue light treatment was repeated with 12 isolates and the resulting live-to-dead cell ratios were measured through flow cytometry as described in Methods, Chapter Three. Live-to-dead cell ratios provide the improved ability to compare living cells in the control and treated groups.

Significant blue light inhibition (p=0.039) of *S. aureus* was again observed 4 hours post-treatment, as shown by the lower Wilcoxon score of blue light treated cultures compared to control no light cultures, shown in Figure 10. These results were in agreement with the results from the optical density trials, confirming the bactericidal effect of blue light on *S. aureus*.

Agr group	NL mean score	BL mean score	P value	N
Overall	56.041	44.08	0.039	51
1	24.429	18.571	0.125	21
2	18.286	10.714	0.016	14
3	19.389	17.611	0.624	16

Figure 10: Wilcoxon average scores for blue light vs. no light live-to-dead cell ratios 4 hours following blue light treatment

The average Wilcoxon rank sum scores were higher for no light cultures in all three *agr* groups tested, indicating that within each group blue light was also inhibiting cell growth. However, only *agr* group 2 saw a significant blue light inhibition (p=0.016). *Agr* group 3 showed the smallest difference in scores between no light and blue light-treated cultures and also had the largest statistical P-value (p=0.624).

To test the question concerning the role of the *agr* polymorphism in the response of blue light, the live-to-dead ratios of no light control cultures and blue light treated cultures between *agr* groups were analyzed. The percent inhibition was calculated to more easily visualize the amount of blue light inhibition within *agr* groups. These results are visually represented in Figure 11. Blue light had the least inhibitory effect on isolates in *agr* group 3, followed by *agr* group 1, and the most inhibition against isolates in *agr* group 2.

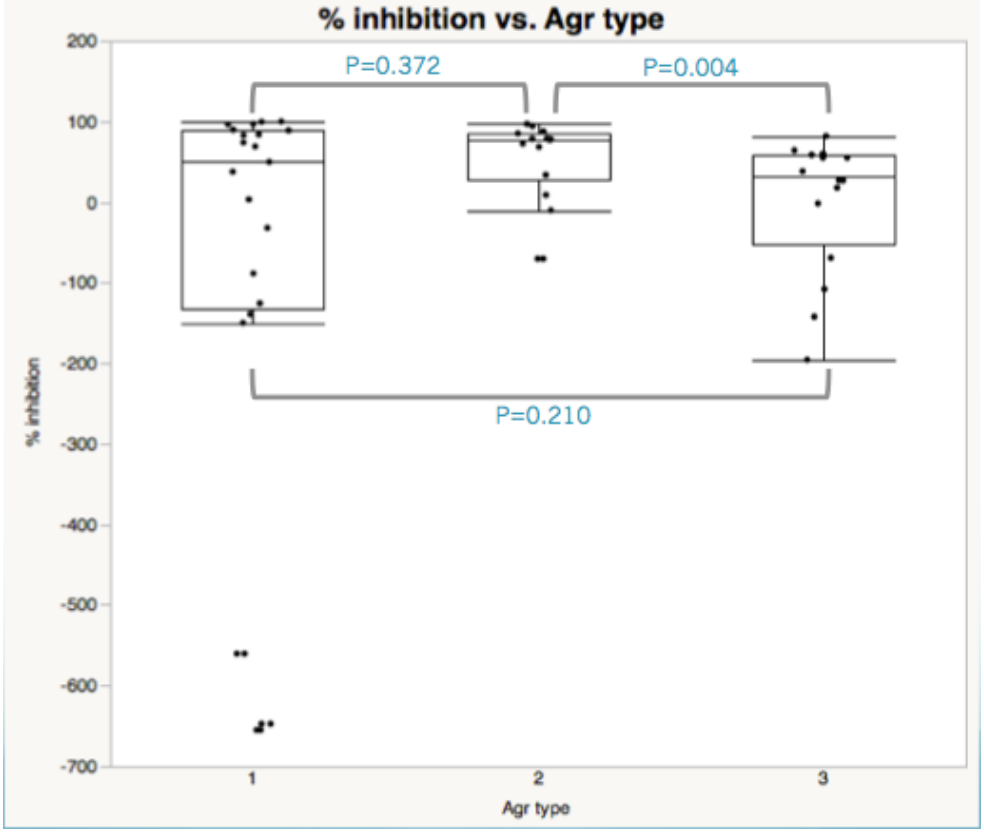


Figure 11: Box and scatterplot visualizations of % inhibition 4 hours post-treatment determined by flow cytometry by *agr* type (Overall: P=0.036)

In order to directly compare the responses of *agr* groups, Wilcoxon rank sum tests were also performed to compare the live-to-dead cell ratios directly between individual *agr* groups (*agr* I vs. *agr* II, I vs. III, II vs. III). While results from the optical density

experiment showed a significant difference only between *agr* groups 2 and 3 and an insignificant difference among all three *agr* groups tested, flow cytometry displayed a significant difference among all *agr* groups (Figure 11, P=0.036) as well as a significant difference between *agr* groups 2 and 3 (P=0.004). These were the same two groups found to differ 2 hours post-treatment in the optical density trials.

CHAPTER FIVE

Conclusion and Discussion

In order to determine the viability of 470 nm blue light therapy as an alternative method of treatment for *Staphylococcus aureus* infections, this project investigated the possible correlation between the accessory gene regulator and *S. aureus* response to blue light exposure. Previous experiments have shown a high degree of variability in the sensitivity of *S. aureus* isolates to blue light treatment [14]. To further elucidate the mechanism of blue light inhibition and prevent the development of resistance to blue light treatment in addition to antibiotic resistance, the accessory gene regulator was investigated in hopes of discovering a possible genetic component for the variation in response. The hypothesis that *agr* polymorphisms play a role in the varying sensitivity of isolates to blue light was supported by our data, which showed a statistical difference in the response of *agr* groups II and III measured with optical density and flow cytometry. Grinholc et al. demonstrated significant differences in response between isolates characterized by *agr* groups I and II, and a significant difference between *agr* III isolates and I, II, and IV isolates [3]. However, in my studies, no significant difference was found among other individual group comparisons (*agr* I vs. II, I vs. III). In comparison to other studies that have investigated the relationship between *agr* and photodynamic therapy, my experiments differ in the addition of a photosensitizer and the measurement of *agr* activity. Rather than adding a porphyrin photosensitizer, this experiment assumed the presence of a sensitizing molecule in the BHI media, which allowed for blue light

inactivation [3] . Additionally, rather than assessing the functional activity of *agr* through a delta-hemolysin assay, or utilizing mutant strains to determine the effect of blue light on *agr*, this experiment assessed the overall response of *S. aureus* to blue light, focusing on cell death rather than specifically *agr* activity.

However, accounting for the different *agr* groups, there was still high degree of unexplained variation within *agr* groups and among individual isolates to blue light treatment, indicating that blue light inhibition has a multifactorial effect not limited to the *agr* polymorphism of isolates. The accessory gene regulator was chosen to be the target of this study because of its wide range of effects across the cell as a result of its regulation of numerous transcription and virulence factors, role in the cell's oxidative stress response, and polymorphisms [17, 20, 21] . Various other global transcriptional regulators such as MgrA, SarZ, and SarA, which also contribute to the oxidative stress sensing and response may also play a role in the response to blue light treatment, and thus also contribute to the variation in response among different isolates [20] .

Ultimately, the differences between responses of *agr* groups 2 and 3 found in this study support the hypothesis that polymorphisms in the accessory gene regulator contribute to the variation in response of isolates to 470 nm blue light treatment. Because the polymorphisms we focused on were found in the *agrC* gene, it is hypothesized that blue light generates reactive oxygen species that attack the AgrC membrane protein and inhibit the function of this protein to varying degrees dependent on the polymorphism. James et al. have demonstrated that the sequestration of the signaling molecule AIP leads to the down-regulation of *agr* in human serum [28] . Sun et al. suggest that oxidation of AIP represses the *agr* regulon by inactivating AIP [20] . In a similar manner, we

hypothesize that blue light could be inhibiting the binding of AIP to a receptor on AgrC and causing *agr* down-regulation. However, while AIP has also been shown to be susceptible to inactivation by strong oxidants such as HClO and ONOO, mild oxidants such as H_2O_2 have not been shown to have the same effect [19]. Instead, blue light-generated ROS could also be oxidizing AgrC, which in a damaged state would fail to activate AgrA, leading to *agr* down-regulation as well. AgrA has been shown to mediate the response to oxidative stress from strong and mild oxidants, however our data on AgrC polymorphisms indicate that this membrane protein is likely also involved in the response to blue light-generated ROS [20].

Certain small molecules have also been developed that target the *agr* system and can be used as potential drugs to treat antibiotic resistant infections. Solonamide B and solonamide analogues have been proven effective as *agr* inhibitors that bind to the AgrC receptor [29]. Similarly, AIP structural analogues have been developed that also inhibit the AIP:AgrC interaction and decrease *agr* activity [30]. These molecules could be used individually, or synergistically with antibiotics and blue light therapy as a more effective method of treating antibiotic-resistant infections.

In addition, while this study focused on the polymorphisms found in *agrC*, *agr* components B and D have also been identified as mutational hotspots [31]. These so-called hyper-mutable genes are also areas of interest that should be further investigated in order to further determine the mechanism of action that the accessory gene regulator employs in the response to blue light response. One such example is the intra-molecular disulfide switch that has been identified in AgrA that mediates the oxidative stress response by

decreasing the binding ability to DNA and causing the down-regulation of the *agr* operon[20] .

In order to expand on this study and further elucidate the effect of the polymorphisms of accessory gene regulator on the mechanism of response to blue light inhibition, in depth investigations into the nature and function of AgrC polymorphisms would be required. In particular, we would be interested in using proteomics to determine the structure of the AgrC protein and the structural differences between *agr* group types. A functional genomics study would also be useful in determining the differences between groups. Finally, a functional assay for delta hemolysin following blue light treatment would be a more precise method of measuring the effect of blue light on the activity of the accessory gene regulator.

Photodynamic therapy has been proven effective as a treatment against acne, bacterial biofilm formation, cancer, burn wounds, and multi-drug resistant pathogens [3-8] . While 470 nm blue light therapy also holds promise as a possible alternative treatment for antibiotic-resistant *S. aureus*, high variation in response of different isolates raises concerns regarding the effectiveness of treating infections *in vivo* and possible development of resistance to blue light as well as to antibiotics. The starting concentration of cells, surface area exposed to light, and duration of exposure has all been shown to influence the effectiveness of photodynamic therapy [9, 14] . Overall blue light inhibition was observed 4 hours post-treatment, but not 2 hours post-treatment. These results support earlier studies, which have shown that the blue light inhibition of *S. aureus* has a delayed response depending on the amount of blue light and starting concentration of the bacterial culture[27] . In addition, no significant blue light inhibition

was observed when analyzing the responses of separate *agr* groups. This could be accounted for by the smaller sample sizes (N) of *agr* groups compared to the whole. Greater blue light inhibition would likely be observed with a longer exposure of bacterial culture to blue light, however a sub-lethal dose of blue light was intentionally used to avoid fully killing the bacterial cells and be able to differentiate the responses of isolates and groups. Our data, in particular, supported previous studies that sub-lethal dosages of blue light can increase the growth of certain *S. aureus* isolates. As a result, for effective clinical use of blue light as a treatment for *S. aureus* infections, treatment exposure lasting 2 hours or greater is recommended [14] . Ultimately, further studies are required to fully elucidate the mechanism of blue light inhibition and develop a standard protocol for the use of blue light in treating *Staphylococcus aureus* infections.

APPENDICES

APPENDIX A

Optical Density Data

Agr I

Isolate	NL 2 hr growth	BL 2 hr growth	2 hr % inhibition	Average % inhibition	NL 4 hr growth	BL 4 hr growth	4 hr % inhibition	Average % inhibition
349	0.162	0.174	-7.407		0.294	0.306	-4.082	
	0.146	0.194	-32.877		0.283	0.308	-8.834	
	0.135	0.166	-22.963		0.264	0.292	-10.606	
	0.161	0.165	-2.484		0.419	0.395	5.728	
	0.153	0.175	-14.379		0.403	0.424	-5.211	
	0.152	0.169	-11.184		0.409	0.4	2.200	
	0.451	0.502	-11.308		0.498	0.386	22.490	
	0.452	0.62	-37.168		0.524	0.545	-4.008	
	0.477	0.365	23.480	-12.921	0.542	0.523	3.506	0.132
1152	0.162	0.246	-51.852		0.431	0.458	-6.265	
	0.125	0.242	-93.600		0.356	0.441	-23.876	
	0.117	0.213	-82.051		0.326	0.428	-31.288	
	0.22	0.236	-7.273		0.857	0.589	31.272	
	0.238	0.236	0.840		0.812	0.592	27.094	
	0.242	0.226	6.612		0.782	0.615	21.355	
	0.436	0.516	-18.349		0.565	0.835	-47.788	
	0.466	0.552	-18.455		0.581	0.863	-48.537	
	0.418	0.478	-14.354	-30.942	0.553	0.634	-14.647	-10.298
2057	0.217	0.245	-12.903		0.602	0.449	25.415	
	0.205	0.241	-17.561		0.62	0.475	23.387	
	0.199	0.209	-5.025		0.549	0.424	22.769	
	0.318	0.346	-8.805		0.705	0.695	1.418	
	0.309	0.335	-8.414		0.694	0.689	0.720	
	0.302	0.318	-5.298		0.702	0.65	7.407	
	0.69	0.63	8.696		0.681	0.726	-6.608	
	0.4	0.513	-28.250		0.48	0.829	-72.708	
	0.345	0.526	-52.464	-14.447	0.532	0.736	-38.346	-4.061
2288	0.501	0.427	14.770		0.653	0.39	40.276	
	0.456	0.434	4.825		0.566	0.461	18.551	
	0.402	0.412	-2.488		0.519	0.629	-21.195	
	0.412	0.297	27.913		0.517	0.679	-31.335	
	0.504	0.393	22.024		0.647	0.622	3.864	
	0.506	0.359	29.051		0.533	0.588	-10.319	
	0.604	0.488	19.205		0.676	0.491	27.367	
	0.547	0.489	10.603		0.644	0.509	20.963	
	0.517	0.478	7.544		0.572	0.472	17.483	
	0.578	0.612	-5.882		0.608	0.534	12.171	
	0.542	0.563	-3.875		0.611	0.56	8.347	
	0.52	0.527	-1.346	10.195	0.555	0.474	14.595	8.397
2344	0.154	0.263	-70.779		0.382	0.483	-26.440	
	0.141	0.229	-62.411		0.356	0.446	-25.281	
	0.108	0.201	-86.111		0.278	0.424	-52.518	
	0.258	0.298	-15.504		0.606	0.618	-1.980	
	0.241	0.284	-17.842		0.578	0.602	-4.152	
	0.233	0.272	-16.738		0.574	0.585	-1.916	
	0.485	0.4	17.526		0.464	0.551	-18.750	
	0.475	0.35	26.316		0.554	0.614	-10.830	
	0.479	0.345	27.975	-21.952	0.457	0.484	-5.908	-16.420

Isolate	NL 2 hr growth	BL 2 hr growth	2 hr % inhibition	Average % inhibition	NL 4 hr growth	BL 4 hr growth	4 hr % inhibition	Average % inhibition
2472	0.274	0.306	-11.679		0.714	0.678	5.042	
	0.268	0.315	-17.537		0.728	0.663	8.929	
	0.289	0.336	-16.263		0.75	0.677	9.733	
	0.316	0.378	-19.620		0.607	0.623	-2.636	
	0.296	0.357	-20.608		0.59	0.601	-1.864	
	0.29	0.332	-14.483		0.593	0.612	-3.204	
	0.468	0.383	18.162		0.526	0.463	11.977	
	0.452	0.368	18.584		0.502	0.472	5.976	
	0.464	0.437	5.819	-6.403	0.511	0.472	7.632	4.621
2734	0.215	0.268	-24.651		0.636	0.608	4.403	
	0.207	0.254	-22.705		0.657	0.586	10.807	
	0.23	0.256	-11.304		0.685	0.601	12.263	
	0.119	0.256	-115.126		0.203	0.381	-87.685	
	0.24	0.009	96.250		0.369	0.353	4.336	
	0.222	0.244	-9.910		0.354	0.288	18.644	
	0.354	0.303	14.407		0.449	0.585	-30.290	
	0.331	0.392	-18.429		0.446	0.688	-54.260	
	0.277	0.293	-5.776	-10.805	0.587	0.516	12.095	-12.187
2860	0.263	0.311	-18.251		0.704	0.691	1.847	
	0.245	0.292	-19.184		0.766	0.665	13.185	
	0.279	0.302	-8.244		0.783	0.676	13.665	
	0.427	0.048	88.759		0.393	0.247	37.150	
	0.464	0.026	94.397		0.466	0.263	43.562	
	0.413	0.043	89.588		0.487	0.28	42.505	
	0.473	0.44	6.977		0.517	0.512	0.967	
	0.473	0.438	7.400		0.519	0.535	-3.083	
	0.461	0.445	3.471	27.212	0.516	0.53	-2.713	16.343
3016	0.234	0.306	-30.769		0.642	0.691	-7.632	
	0.222	0.286	-28.829		0.681	0.655	3.818	
	0.231	0.275	-19.048		0.876	0.653	25.457	
	0.227	0.558	-145.815		0.562	0.604	-7.473	
	0.191	0.542	-183.770		0.597	0.591	1.005	
	0.206	0.65	-215.534		0.605	0.641	-5.950	
	0.472	0.538	-13.983		0.645	0.747	-15.814	
	0.498	0.593	-19.076		0.659	0.811	-23.065	
	0.515	0.496	3.689	-72.570	0.646	0.607	6.037	-2.624
3017	0.243	0.28	-15.226		0.56	0.57	-1.786	
	0.242	0.268	-10.744		0.552	0.57	-3.261	
	0.249	0.28	-12.450		0.569	0.573	-0.703	
	0.637	0.106	83.359		0.66	0.335	49.242	
	0.62	0.104	83.226		0.714	0.358	49.860	
	0.67	0.139	79.254		0.718	0.35	51.253	
	0.493	0.448	9.128		0.587	0.643	-9.540	
	0.492	0.456	7.317		0.591	0.715	-20.981	
	0.481	0.417	13.306	26.352	0.585	0.598	-2.222	12.429

Agr II

Isolate	NL 2 hr growth	BL 2 hr growth	2 hr % inhibition	Average % inhibition	NL 4 hr growth	BL 4 hr growth	4 hr inhibition	Average % inhibition
3	0.232	0.267	-15.086		0.486	0.446	8.230	
	0.217	0.251	-15.668		0.537	0.492	8.380	
	0.214	0.245	-14.486		0.413	0.509	-23.245	
	0.234	0.248	-5.983		0.587	0.472	19.591	
	0.223	0.232	-4.036		0.618	0.473	23.463	
	0.24	0.226	5.833		0.759	0.516	32.016	
	0.132	0.382	-189.394		0.459	0.522	-13.725	
	0.151	0.398	-163.576		0.455	0.536	-17.802	
	0.177	0.474	-167.797	-63.355	0.522	0.502	3.831	4.527
330	0.715	0.156	78.182		0.395	0.419	-6.076	
	0.381	0.265	30.446		0.465	0.332	28.602	
	0.75	0.233	68.933		0.465	0.389	16.344	
	0.545	0.53	2.752		0.588	0.571	2.891	
	0.406	0.57	-40.394		0.588	0.612	-4.082	
	0.41	0.53	-29.268		0.602	0.607	-0.831	
	0.309	0.401	-29.773		0.790	0.476	39.747	
	0.319	0.442	-38.558		0.555	0.485	12.613	
	0.339	0.391	-15.339	2.998	0.552	0.505	8.514	10.858

Isolate	NL 2 hr growth	BL 2 hr growth	2 hr % inhibition	Average % inhibition	NL 4 hr growth	BL 4 hr growth	4 hr % inhibition	Average % inhibition
346	0.191	0.218	-14.136		0.324	0.372	-14.815	
	0.187	0.228	-21.925		0.330	0.389	-17.879	
	0.199	0.232	-16.583		0.358	0.387	-8.101	
	0.231	0.243	-5.195		0.555	0.513	7.568	
	0.22	0.236	-7.273		0.620	0.481	22.419	
	0.213	0.231	-8.451		0.634	0.479	24.448	
	0.42	0.348	17.143		0.498	0.542	-8.835	
	0.429	0.406	5.361		0.564	0.561	0.532	
	0.379	0.413	-8.971	-6.670	0.497	0.546	-9.859	-0.502
374	0.213	0.256	-20.188		0.483	0.443	8.282	
	0.22	0.255	-15.909		0.462	0.446	3.463	
	0.252	0.275	-9.127		0.482	0.479	0.622	
	0.307	0.233	24.104		0.764	0.669	12.435	
	0.29	0.211	27.241		0.755	0.562	25.563	
	0.266	0.264	0.752		0.740	0.779	-5.270	
	0.508	0.256	49.606		0.563	0.450	20.071	
	0.487	0.288	40.862		0.543	0.550	-1.289	
	0.516	0.381	26.163	13.723	0.511	0.457	10.568	8.272
1046	0.222	0.258	-16.216		0.494	0.481	2.632	
	0.208	0.25	-20.192		0.485	0.476	1.856	
	0.204	0.252	-23.529		0.499	0.488	2.204	
	0.26	0.213	18.077		0.606	0.523	13.696	
	0.224	0.199	11.161		0.553	0.530	4.159	
	0.211	0.203	3.791		0.563	0.616	-9.414	
	0.373	0.171	54.155		0.357	0.520	-45.658	
	0.346	0.169	51.156		0.615	0.496	19.350	
	0.339	0.204	39.823	13.136	0.447	0.428	4.251	-0.769
1079	0.118	0.235	-99.153		0.276	0.438	-58.696	
	0.135	0.226	-67.407		0.343	0.433	-26.239	
	0.11	0.204	-85.455		0.298	0.394	-32.215	
	0.188	0.234	-24.468		0.768	0.602	21.615	
	0.183	0.235	-28.415		0.700	0.730	-4.286	
	0.129	0.224	-73.643		0.678	0.719	-6.047	
	0.639	0.468	26.761		0.672	0.633	5.804	
	0.734	0.486	33.787		0.667	0.645	3.298	
	0.688	0.491	28.634	-32.151	0.682	0.657	3.666	-10.344
2431	0.255	0.305	-19.608		0.615	0.643	-4.553	
	0.248	0.308	-24.194		0.631	0.664	-5.230	
	0.243	0.293	-20.576		0.660	0.683	-3.485	
	0.225	0.241	-7.111		0.571	0.538	5.779	
	0.246	0.129	47.561		0.588	0.443	24.660	
	0.26	0.257	1.154		0.593	0.571	3.710	
	0.471	0.391	16.985		0.532	0.513	3.571	
	0.476	0.449	5.672		0.550	0.507	7.818	
	0.489	0.428	12.474	1.373	0.547	0.497	9.141	4.601
2653	0.142	0.176	-23.944		0.433	0.485	-12.009	
	0.108	0.168	-55.556		0.427	0.470	-10.070	
	0.119	0.155	-30.252		0.428	0.453	-5.841	
	0.275	0.229	16.727		0.417	0.332	20.384	
	0.179	0.281	-56.983		0.363	0.291	19.835	
	0.186	0.209	-12.366		0.337	0.327	2.967	
	0.561	0.195	65.241		0.503	0.709	-40.954	
	0.751	0.211	71.904		0.500	0.700	-40.000	
	0.806	0.229	71.588		0.490	0.687	-40.204	
	0.478	0.4	16.318		0.602	0.527	12.458	
	0.473	0.539	-13.953		0.588	0.514	12.585	
	0.483	0.608	-25.880	1.904	0.599	0.512	14.524	-5.527
2819	0.22	0.256	-16.364		0.512	0.531	-3.711	
	0.201	0.264	-31.343		0.606	0.529	12.706	
	0.198	0.24	-21.212		0.555	0.525	5.405	
	0.589	0.208	64.686		0.511	0.269	47.358	
	0.399	0.113	71.679		0.272	0.204	25.000	
	0.43	0.112	73.953		0.303	0.468	-54.455	
	0.512	0.502	1.953		0.639	0.651	-1.878	
	0.566	0.651	-15.018		0.712	0.550	22.753	
	0.526	0.488	7.224	15.062	0.745	0.254	65.906	13.232

Isolate	NL 2 hr growth	BL 2 hr growth	2 hr % inhibition	Average % inhibition	NL 4 hr growth	BL 4 hr growth	4 hr % inhibition	Average % inhibition
115	0.153	0.17	-11.111		0.316	0.328	-3.797	
	0.175	0.176	-0.571		0.307	0.327	-6.515	
	0.16	0.18	-12.500		0.319	0.333	-4.389	
	0.108	0.126	-16.667		0.254	0.249	1.969	
	0.114	0.121	-6.140		0.266	0.244	8.271	
	0.106	0.105	0.943		0.250	0.217	13.200	
	0.153	0.186	-21.569		0.534	0.497	6.929	
	0.185	0.183	1.081		0.600	0.501	16.500	
	0.17	0.178	-4.706		0.610	0.497	18.525	
	0.199	0.197	1.005		0.433	0.434	-0.231	
	0.199	0.18	9.548		0.435	0.450	-3.448	
	0.221	0.184	16.742		0.465	0.427	8.172	
	0.178	0.141	20.787		0.428	0.381	10.981	
	0.176	0.15	14.773		0.461	0.412	10.629	
	0.179	0.151	15.642		0.467	0.425	8.994	
	0.17	0.174	-2.353		0.462	0.444	3.896	
	0.187	0.165	11.765		0.465	0.422	9.247	
	0.195	0.164	15.897		0.477	0.485	-1.677	
	0.266	0.194	27.068		0.343	0.379	-10.496	
	0.339	0.21	38.053		0.363	0.387	-6.612	
	0.361	0.209	42.105		0.385	0.341	11.429	
	0.223	0.513	-130.045		0.475	0.451	5.053	
	0.207	0.643	-210.628		0.455	0.504	-10.769	
	0.245	0.502	-104.898		0.425	0.533	-25.412	
	0.312	0.145	53.526		0.449	0.325	27.617	
	0.297	0.134	54.882		0.452	0.364	19.469	
	0.369	0.072	80.488		0.417	0.321	23.022	
	0.291	0.274	5.842		0.479	0.421	12.109	
	0.253	0.31	-22.530		0.475	0.433	8.842	
	0.251	0.216	13.944		0.540	0.407	24.630	
	0.638	0.393	38.401		0.519	0.638	-22.929	
	0.585	0.386	34.017		0.502	0.531	-5.777	
	0.604	0.236	60.927		0.496	0.279	43.750	
	0.568	0.448	21.127		0.586	0.722	-23.208	
	0.528	0.396	25.000		0.598	0.771	-28.930	
	0.571	0.653	-14.361		0.527	0.434	17.647	
	0.458	0.388	15.284		0.627	0.551	12.121	
	0.47	0.449	4.468		0.635	0.525	17.323	
	0.466	0.506	-8.584	1.453	0.584	0.519	11.130	5.058

Agr III

Isolate	NL 2 hr growth	BL 2 hr growth	2 hr % inhibition	Average % inhibition	NL 4 hr growth	BL 4 hr growth	4 hr % inhibition	Average % inhibition
240	0.138	0.192	-39.130		0.316	0.35	-10.759	
	0.119	0.192	-61.345		0.294	0.35	-19.048	
	0.118	0.185	-56.780		0.29	0.338	-16.552	
	0.194	0.222	-14.433		0.469	0.495	-5.544	
	0.196	0.203	-3.571		0.502	0.481	4.183	
	0.206	0.208	-0.971		0.512	0.49	4.297	
	0.444	0.141	68.243		0.546	0.31	43.223	
	0.481	0.064	86.694		0.578	0.291	49.654	
	0.455	0.19	58.242	4.105	0.593	0.397	33.052	9.167
311	0.387	0.27	30.233		0.457	0.366	19.912	
	0.302	0.441	-46.026		0.399	0.364	8.772	
	0.408	0.409	-0.245		0.398	0.385	3.266	
	0.279	0.379	-35.842		0.504	0.393	22.024	
	0.378	0.446	-17.989		0.594	0.494	16.835	
	0.323	0.564	-74.613		0.494	0.517	-4.656	
	0.209	0.457	-118.660		0.44	0.627	-42.500	
	0.236	0.552	-133.898		0.501	0.651	-29.940	
	0.189	0.629	-232.804	-69.983	0.387	0.642	-65.891	-8.020

Isolate	NL 2 hr growth	BL 2 hr growth	2 hr % inhibition	Average % inhibition	NL 4 hr growth	BL 4 hr growth	4 hr % inhibition	Average % inhibition
1026	0.152	0.263	-73.026		0.299	0.443	-48.161	
	0.163	0.258	-58.282		0.331	0.46	-38.973	
	0.168	0.241	-43.452		0.362	0.436	-20.442	
	0.227	0.258	-13.656		0.644	0.569	11.646	
	0.228	0.238	-4.386		0.581	0.567	2.410	
	0.245	0.224	8.571		0.648	0.561	13.426	
	0.511	0.355	30.528		0.598	0.598	0.000	
	0.571	0.372	34.851		0.626	0.58	7.348	
	0.537	0.371	30.912		0.477	0.567	-18.868	
	0.56	0.553	1.250		0.517	0.457	11.605	
	0.57	0.646	-13.333		0.572	0.484	15.385	
	0.518	0.514	0.772	-8.271	0.556	0.43	22.662	-3.497
1463	0.233	0.293	-25.751		0.554	0.463	16.426	
	0.211	0.291	-37.915		0.556	0.502	9.712	
	0.201	0.268	-33.333		0.601	0.536	10.815	
	0.276	0.31	-12.319		0.817	0.56	31.457	
	0.241	0.276	-14.523		0.791	0.585	26.043	
	0.143	0.281	-96.503		0.73	0.624	14.521	
	0.315	0.296	6.032		0.245	0.127	48.163	
	0.298	0.349	-17.114		0.237	0.061	74.262	
	0.247	0.301	-21.862	-28.143	0.24	0.075	68.750	33.350
2072	0.206	0.268	-30.097		0.492	0.431	12.398	
	0.2	0.256	-28.000		0.536	0.433	19.216	
	0.193	0.242	-25.389		0.58	0.409	29.483	
	0.245	0.273	-11.429		0.561	0.535	4.635	
	0.258	0.272	-5.426		0.569	0.537	5.624	
	0.253	0.266	-5.138		0.555	0.547	1.441	
	0.456	0.32	29.825		0.57	0.537	5.789	
	0.47	0.383	18.511		0.541	0.575	-6.285	
	0.465	0.423	9.032	-5.346	0.536	0.628	-17.164	6.126
2195	0.218	0.279	-27.982		0.425	0.468	-10.118	
	0.226	0.279	-23.451		0.435	0.467	-7.356	
	0.25	0.314	-25.600		0.464	0.527	-13.578	
	0.273	0.325	-19.048		0.602	0.633	-5.150	
	0.22	0.311	-41.364		0.547	0.618	-12.980	
	0.265	0.31	-16.981		0.623	0.615	1.284	
	0.505	0.444	12.079		0.581	0.548	5.680	
	0.513	0.444	13.450		0.577	0.538	6.759	
	0.493	0.445	9.736	-13.240	0.546	0.545	0.183	-3.919
2210	0.28	0.314	-12.143		0.52	0.487	6.346	
	0.25	0.302	-20.800		0.44	0.479	-8.864	
	0.249	0.305	-22.490		0.464	0.484	-4.310	
	0.227	0.265	-16.740		0.791	0.574	27.434	
	0.234	0.247	-5.556		0.817	0.532	34.884	
	0.266	0.262	1.504		0.814	0.579	28.870	
	0.563	0.415	26.288		0.452	0.577	-27.655	
	0.411	0.506	-23.114		0.152	0.544	-257.895	
	0.616	0.25	59.416	-1.515	0.305	0.165	45.902	-17.254
3064	0.225	0.288	-28.000		0.521	0.519	0.384	
	0.217	0.269	-23.963		0.531	0.521	1.883	
	0.239	0.257	-7.531		0.621	0.508	18.196	
	0.452	0.221	51.106		0.561	0.456	18.717	
	0.352	0.263	25.284		0.542	0.481	11.255	
	0.434	0.623	-43.548		0.539	0.528	2.041	
	0.402	0.393	2.239		0.559	0.601	-7.513	
	0.415	0.378	8.916		0.588	0.821	-39.626	
	0.444	0.402	9.459		0.535	0.639	-19.439	
	0.463	0.441	4.752		0.575	0.586	-1.913	
	0.507	0.433	14.596		0.613	0.561	8.483	
	0.505	0.487	3.564	1.406	0.619	0.609	1.616	-0.493
3097	0.222	0.206	7.207		0.59	0.503	14.746	
	0.213	0.2	6.103		0.466	0.506	-8.584	
	0.146	0.2	-36.986		0.447	0.519	-16.107	
	0.489	0.329	32.720		0.594	0.426	28.283	
	0.443	0.321	27.540		0.599	0.47	21.536	
	0.404	0.302	25.248		0.597	0.442	25.963	
	0.54	0.5	7.407		0.573	0.562	1.920	
	0.538	0.492	8.550		0.57	0.591	-3.684	
	0.548	0.504	8.029	9.535	0.574	0.559	2.613	7.409

APPENDIX B

Flow Cytometry Data

Agr I

Isolate	NL Live	NL Dead	BL Live	BL Dead	NL L:D ratio	BL L:D ratio	% Inhibition	Average % Inhibition
3016	9790	1	4850	185	9790.000	26.216	99.732	
3016	9510	53	4770	235	179.434	20.298	88.688	
3016	9936	4	3897	211	2484.000	18.469	99.256	
3016	6606	108	8955	234	61.167	38.269	37.435	
3016	5947	105	5616	377	56.638	14.897	73.699	
3016	5755	107	8433	311	53.785	27.116	49.585	74.73244678
2734	7164	49	6760	7	146.204	965.714	-560.525	
2734	7891	12	6164	30	657.583	205.467	68.754	
2734	8294	2	7877	11	4147.000	716.091	82.732	-136.3460849
2860	9283	36	9637	5	257.861	1927.400	-647.457	
2860	9813	17	9799	9	577.235	1088.778	-88.619	
2860	9577	22	9862	3	435.318	3287.333	-655.156	-463.7441658
2288	647	302	646	228	2.142	2.833	-32.251	
2288	432	353	793	287	1.224	2.763	-125.778	
2288	8498	169	549	282	50.284	1.947	96.128	-20.63378913
3017	9753	39	8665	211	250.077	41.066	83.579	
3017	9775	13	8235	319	751.923	25.815	96.567	
3017	9781	12	9224	109	815.083	84.624	89.618	89.92102533
2344	9315	83	9664	36	112.229	268.444	-139.194	
2344	9643	64	9413	25	150.672	376.520	-149.894	
2344	9554	94	9655	98	101.638	98.520	3.068	-95.34001261

Agr II

Isolate	NL Live	NL Dead	BL Live	BL Dead	NL L:D ratio	BL L:D ratio	% Inhibition	Average % Inhibition
3	9817	34	7909	806	288.735	9.813	96.602	
3	9747	33	6991	404	295.364	17.304	94.141	
3	9658	29	9400	131	333.034	71.756	78.454	
3	8577	154	9489	100	55.695	94.890	-70.375	
3	9591	18	9400	78	532.833	120.513	77.383	55.241
115	8948	461	7769	1260	19.410	6.166	68.233	
115	8087	379	7829	1308	21.338	5.985	71.949	
115	8843	385	7590	1478	22.969	5.135	77.642	
115	8566	261	7274	1761	32.820	4.131	87.414	
115	8713	472	7197	1783	18.460	4.036	78.134	
115	8291	336	7027	1878	24.676	3.742	84.836	78.035
346	8641	35	8986	33	246.886	272.303	-10.295	
346	9015	34	8822	50	265.147	176.440	33.456	
346	8776	44	8759	48	199.455	182.479	8.511	10.557

Agr III

Isolate	NL Live	NL Dead	BL Live	BL Dead	NL L:D ratio	BL L:D ratio	% Inhibition	Average % Inhibition
311	9052	194	8165	434	46.660	18.813	59.680	
311	7295	352	7751	367	20.724	21.120	-1.908	
311	8986	168	8251	427	53.488	19.323	63.874	
311	9467	208	9102	273	45.514	33.341	26.747	
311	9679	130	9163	272	74.454	33.688	54.754	
311	9523	121	9234	282	78.702	32.745	58.394	
311	9467	208	9102	273	45.514	33.341	26.747	
311	9679	130	9163	272	74.454	33.688	54.754	
311	9523	121	9234	282	78.702	32.745	58.394	44.60398582
2195	9699	74	9754	3	131.068	3251.333	-2380.654	
2195	9212	167	8133	11	55.162	739.364	-1240.357	
2195	9321	30	8282	11	310.700	752.909	-142.327	-1254.446171
2210	9681	70	9812	24	138.300	408.833	-195.613	
2210	9815	9	9783	49	1090.556	199.653	81.693	
2210	9468	37	9703	46	255.892	210.935	17.569	-32.11735811
2072	9537	39	9669	19	244.538	508.895	-108.104	
2072	9845	29	9765	17	339.483	574.412	-69.202	
2072	9853	5	9779	8	1970.600	1222.375	37.969	-46.44560457

APPENDIX C

Detailed *agr* Typing Protocol

DNA Extraction

1. Make streak plate from *S. aureus* isolate stock solutions and grow overnight at 35° C.
2. Harvest cells by centrifuging 3 mL of overnight culture for 10 minutes at 10,000 RPM.
3. Re-suspend cell pellet in 200 uL of Lysis Buffer
    a. Lysis Buffer: 1 unit/uL of Achromopeptidase in 10:1 Tris-EDTA buffer
4. Incubate at 37°C for 15 minutes in heat block
5. Immerse tubes in boiling water for 5 minutes
6. Centrifuge at 10,000 g for 1 minute
7. Transfer supernatant into new tube
8. Store in freezer at -20°C

Polymerase Chain Reaction

1. Make 100 μM stock solution of primers.
2. Make "pre-working" solution for each primer:
    a. Put 10 μL of each stock into 40 μL of water. (20 uM solution)
3. Make a 1 μM "working" solution:
    a. Put 15 μL of each "pre-working" solution together into a single clean tube.
4. Perform PCR assay in 25 μL of reaction mixture:
    a. 12.5 μL AmpliTaq Gold 360 Master Mix
    b. 2.5 μL DNA
    c. 6.25 μL of "working" primer solution
    d. 3.75 μL water
5. Thermal profile:
    a. Initial denaturation: 94° C for 6 min
    b. 32 cycles:
        i. Denaturation: 95° C for 45 s
        ii. Primer annealing: 56° C for 1 min
        iii. Primer elongation: 72° for 70 s
    c. Extension: 72° C for 8 min
6. Perform gel electrophoresis in 1.5% agarose gel stained with ethidium bromide.

APPENDIX D

Detailed Optical Density Protocol

1. Make streak plate from *S. aureus* isolate stock solutions and grow overnight at 35° C.
2. Pick a single colony from streak plate and place in well of 24-well plate with 1 mL Brain Heart Infusion broth. Grow overnight at 35°C.
3. Plate 1 - Aliquot 10 µL of each overnight culture in 1 mL BHI in each well of a black 24-well plate as follows:

1	1	1	2	2	2
3	3	3	4	4	4
5	5	5	6	6	6
7	7	7	8	8	8

4. Plate 2 - Aliquot 10 µL of each overnight culture in 1 mL BHI in each well of a 24-well plate in the same manner as above to serve as the no-light control.
5. Incubate and allow both plates to grow for 2 hours at 35°C.
6. Take optical density measurement of both plates at 600 nm for pre-treatment OD measurements.
7. Treat Plate 1 with 30 minutes of blue light, while incubating at 35°C and shaking at 300 RPM. Treat Plate 2 with no light, incubating under the same conditions.
8. Allow growth for 2 hours and take optical density measurements of both plates at 600 nm. Repeat measurements 4 hours post-treatment.

APPENDIX E

Detailed Flow Cytometry Protocol

1. Make streak plate from *S. aureus* isolate stock solutions and grow overnight at 35° C.
2. Pick a single colony from streak plate and place in well of 24-well plate with 1 mL Brain Heart Infusion broth. Grow overnight at 35°C.
3. Aliquot 10 µL of overnight growth into 1 mL of BHI. Allow growth for 2 hours at 35°C.
4. Plate 1 - Aliquot 10 µL of each pre-treatment culture in 1 mL BHI in each well of a black 24-well plate as follows:

1	1	1	2	2	2
3	3	3	4	4	4
5	5	5	6	6	6
7	7	7	8	8	8

5. Plate 2 - Aliquot 10 µL of each pre-treatment culture in 1 mL BHI in each well of a 24-well plate in the same manner as above to serve as the no-light control.
6. Incubate and allow both plates to grow for 2 hours at 35°C.
7. Treat Plate 1 with 30 minutes of blue light, while incubating at 35°C and shaking at 300 RPM. Treat Plate 2 with no light, incubating under the same conditions.
8. Allow growth for 4 hours.
9. Transfer culture from each well into centrifuge tubes.
10. Pellet cells at 5,000 RPM for 1 minute. Remove supernatant.
11. Re-suspend cells in 1 mL of sheath fluid. Vortex for 15 seconds.
12. Pellet cells at 5,000 RPM for 1 minute. Remove supernatant.
13. Re-suspend cells in 1 mL of sheath fluid. Vortex for 15 seconds.
14. Aliquot 50 µL of each cell suspension into 500 mL sheath fluid in a clean round-bottom polystyrene tube.
15. Add 5 µL of propidium iodide dye and 5 µL of thiazole orange dye to each tube. Vortex for 15 seconds and incubate at room temperature for 5 minutes.
16. Perform flow cytometry count with the following settings:
    - FSC: 201.4 V
    - SSC: 365.1 V
    - FITC: 455.4 V
    - PE: 450.2 V

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