ABSTRACT

RNA-SEQ Reveals Changes in the *Staphylococcus aureus*

Transcriptome Following Blue Light Illumination

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Staphylococcus aureus infections are becoming a more serious human health concern as antibiotic resistance among *S. aureus* strains increases. As a result, alternatives to antibiotic treatments are being explored, including photodynamic therapies. It has been shown that blue light exposure will inhibit the growth of both Gram-positive and Gram-negative bacteria, including *S. aureus* growing in culture. However, the mechanism of this inhibition is not well characterized. In an effort to better understand the mechanism of blue light inhibition, a whole transcriptome analysis of *S. aureus* isolate BUSA2288 was performed using RNA-seq to analyze the response to blue light exposure. RNA was extracted from *S. aureus* cultures illuminated with blue light, and control cultures grown in the dark. Transcriptomic comparisons using a cutoff of 5 fold identified a total of 28 down-regulated genes and 6 up-regulated genes in the samples that were exposed to blue light. The differentially regulated genes fall into 8 functional categories with 4 genes of unknown function. These results indicate that blue light does have an effect on the transcriptome of *S. aureus*. Pathways involving reactive oxygen intermediates generated in the broth and excitation and damage to membrane proteins are proposed to explain the killing effect of blue light on *S. aureus*.

Key words: Staphylococcus aureus; phototherapy; reactive oxygen intermediates; RNA-seq; MRSA; antibiotic resistance

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RNA-SEQ REVEALS CHANGES IN THE *STAPHYLOCOCCUS AUREUS* TRANSCRIPTOME FOLLOWING BLUE LIGHT ILLUMINATION

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

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Waco, TX

May 2014

TABLE OF CONTENTS

ACKNOWLEDGMENTS

Thanks to Dr. Adair for her constructive and kind mentorship. Thanks to Dr. Bryan Gibbon, Meredith Rosenthal, Gregg Fox, Carlo Manzana, Kayla Fricke, Jeanne Samake, Suk Namkung, William Jones, Bre Bohart, and Sydney Singleterry for their encouragement and assistance. Thanks to Jerry Milner for construction of the blue light box. Thanks to the Department of Biology and URC for financial support.

I'm blue da ba dee da ba die
- Eiffel 65

CHAPTER ONE

Review of Literature

Staphylococcus aureus in Health Care

S. aureus, a Gram-positive cocci, is normally a commensal bacteria that resides on the human skin or in the nose. However, *S. aureus* can be pathogenic and cause infection of the bloodstream, respiratory tract, heart, soft tissue, and bone (1). Risk factors for infection include hospitalization, open wounds, participation in contact sports, and invasive devices. Antibiotic resistant strains of *S. aureus* are a growing human health concern in and out of the hospital setting. In particular, methicillin resistant *S. aureus* (MRSA) infections are becoming increasingly difficult to treat as strains become multi-drug resistant. One third of people in the United States are colonized with *S. aureus* in their nose asymptomatically while 2% are colonized with MRSA strains in their nose (2). There were an estimated 80,461 cases of invasive MRSA infections in the United States in 2011 (3). These MRSA strains can be categorized as either hospital acquired (HA) if the bacteria were identified within four days after hospitalization (or following a previous hospital stay) or community acquired (CA). CA and HA strains have independent genetic backgrounds and appear to have developed methicillin resistance independent from one another (4). Additionally, there are livestock associated strains of MRSA that have developed methicillin resistance independent of human-associated MRSA (5).

In 1959, methicillin was introduced as a treatment for penicillin resistant *S. aureus* infections. By 1961, some strains in the United Kingdom had acquired methicillin resistance and MRSA has now spread worldwide (1). All methicillin resistant

strains carry the methicillin resistance gene, *mecA*, which encodes a methicillin binding protein and is carried on the staphylococcal cassette chromosome, SCC*mec*. There are eight different SCC*mec* types in *S. aureus* (I-VIII) (6). This mobile genetic element can be acquired from distantly related bacterial species (7). Many MRSA strains are resistant to multiple antibiotics and are typically treated with glycopeptide antibiotics. As a result, in the 1980s vancomycin became the antibiotic of choice for treatment of MRSA infections at many hospitals in the United States. Vancomycin use in hospitals was also increased to treat a growing number of *Clostridium difficile* and coagulase-negative staphylococci infections (8, 9). As a result of this increase in vancomycin use, in 1997 the first strain of vancomycin resistant *S. aureus* (VRSA) was isolated. Several more VRSA strains have been isolated since and appear to have developed from MRSA strains (10, 11).

Photodynamic Therapy

Because of the increase in antibiotic resistance among *S. aureus* strains, alternatives for antibiotic treatments are being explored, including photodynamic therapies. Ultraviolet (UV) light has been shown to destroy bacteria by causing photo-damage to the cell's DNA at a faster rate than the cell can repair (12). However, because UV light is harmful to human tissues its clinical applications are limited. Photodynamic therapy involves pre-treatment with photosensitizers, i.e. molecules that absorb visible light and generate cytotoxic reactive oxygen species after blue light exposure (13). After absorbing light, photosensitizers go to either the singlet or triplet excited state. The triplet state is much more stable and therefore normally interacts with other molecules. A photosensitizer in the triplet-excited state can go through either a type I or type II reaction.

In type I reactions, the excited photosensitizer interacts with a reducing substance in the cell other than oxygen. This reaction produces free radicals and radical ions such as hydroxyl radicals (HO•) and a reduced photosensitizer. These free radicals can interact with oxygen by electron transfer to produce a superoxide ion (O_2^-). The reduced photosensitizer can react with oxygen by proton transfer to produce hydrogen dioxide (14-18).

In type II reactions the excited photosensitizer directly interacts with oxygen in the cell in one of two ways. The excited photosensitizer can transfer its excitement to oxygen to produce singlet oxygen ($^1O_2$) and return the photosensitizer to its original state. The excited photosensitizer can also transfer an electron to oxygen to produce a superoxide ion (O_2^-) and an oxidized photosensitizer. The superoxide ion, hydrogen dioxide, hydroxyl radical, and singlet oxygen are all reactive oxygen species. Reactive oxygen species can directly or indirectly oxidize and damage a variety of molecules inside or outside of the cell including DNA, membranes, and amino acids (14-18).

Photosensitizers involved in microbial phototherapy include several types of pigments including Methylene Blue, Toluidine Blue, Rose Bengal, hematoporphyrin, Photofrin, Malachite Green isothiocyanate, and δ-aminolevulinic acid. Photosensitizers that work in conjunction with blue light exposure specifically include Toluene blue, δ-aminolevulinic acid, and methylene blue. Photodynamic therapy involving photosensitizers that utilize blue light is of particular interest because blue light is in the visible light range, which is not considered harmful to human tissues, and several blue light emitting devices have been approved by the FDA for clinical use. The main wavelengths used in blue light photodynamic therapy are 405nm and 470nm.

Blue Light Therapy

The use of photosensitizing agents in a clinical setting is not always practical and their safety on human tissues is a concern. As a result, the effect of blue light alone on bacterial cultures has been studied. It has been shown that blue light alone will effectively destroy both Gram-positive and Gram-negative bacteria including MRSA, *Staphylococcus epidermidis, S. aureus, Streptococcus pyogenes, Clostridium perfringens, Escherichia coli, Acinetobacter baumannii, Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella pneumonia, Porphyromonas gingivalis*, and others (12, 19-23). The effectiveness of blue light inactivation is dependent upon the type of bacteria, the wavelength of light, and the energy of the light. Maclean has shown that in general, Gram-positive species are more susceptible to blue light inactivation than Gram-negative (12). Table 1 summarizes different studies that have shown blue light of different wavelengths to inhibit growth of bacterial cultures without the use of a photosensitizer. These are all in vitro studies. However, blue light inactivation of *S. aureus* has been demonstrated in vivo on mouse tissues (24).

Organism	Illumination Medium	Dosage (J/cm^2)	Wavelength	Percent Inhibition	Reference Number
MRSA	PBS	7.2	400-420 nm	99%	21
MRSA	PBS	45	405 nm	99.99%	12
MSSA	PBS	36	405 nm	99.99%	12
MRSA	TSA	1 to 60	405 nm	92.1-93.5 %	19
MRSA	TSA	1 to 60	470 nm	90.40%	12
MRSA	NB	120	400-800 nm	83%	23
MSSA	NB	60 to 120	415nm	90%	22
MSSA	NB	60 to 120	455nm	50%	22

Table 1. Blue Light Inactivation of *S. aureus* in vitro. Tryptic soy agar (TSA), phosphate buffered saline (PBS), nutrient broth (NB), Methicillin sensitive *S. aureus* (MSSA).

The mechanism of this inhibition is not well characterized. The eradication of *P. acnes, H. pylori, H. parainfluenzae, P. gingivalis*, and other bacteria is hypothesized to be caused by endogenous porphyrins interacting with the blue light to produce reactive oxygen species(12, 25-29). These reactive oxygen species damage the cell leading to cell death. This hypothesis is supported by five pieces of evidence. First, the bacteria have absorbance peaks that are characteristic of endogenous porphyrins when exposed to blue light(30). Second, endogenous porphyrins accumulate during incubation, and bacteria that have been incubated longer are more susceptible to blue light inactivation (30, 31). Third, porphyrins accumulate during light exposure (32). Fourth, blue light inactivation of bacteria has been shown to be dependent upon the presence of oxygen (16, 25, 29). Fifth, the variance of percent inhibition seen among different strains of bacteria could be attributed to the different types and amounts of porphyrins in the cell wall (33). For example, Gram-positive *S. aureus* produces 2-3 times more coporphyrin than Gram-negative species such as *E. coli* (34), and *S. aureus* is also more sensitive to blue light irradiation than *E. coli.*

Additionally, there has been a recent interest in investigating the role of blue light receptors in plants, fungi, and bacteria. There is particular interest in blue light receptors with LOV and BLUF domains. While the *S. aureus* genome does not contain genes with LOV or BLUF domains, there could be other blue light receptors present in the membrane of these bacteria causing a response to blue light that could lead to growth inhibition.

In order for blue light therapy to be clinically applicable, its safety with human tissues must be assessed. It has been shown that short-term exposure to multiple blue light treatments does not harm human skin (35). However, another study has shown that hydrogen peroxide production increased in cultured human, mouse, and monkey cells after exposure to 445–455 nm light (36). More studies are needed to evaluate the safety of blue light exposure to human tissues.

There are many possible applications for blue light therapy in the clinical setting. Blue light could be used to treat infected surface wounds and burns or to prevent infection. Surgical tools or foreign devices in the human body could emit blue light, reducing the chance of infection. Treatment of endotracheal tubes with blue light may prevent biofilm formation and decrease risk of ventilator-associated pneumonia (37). Blue light is already being used clinically to treat *P. acnes* infection of the skin. Blue light has been suggested as a treatment for gastric conditions caused by *H. pylori* and treatment of periodontal disease involving *P. gingivalis* (25). Additionally, an optical fiber emitting blue light was inserted into the stomach of patients and shown to decrease the presence of *H. Pylori* in the stomach (38).

The S. aureus Genome

Genomics is an area of molecular biology, which is concerned with the structure, function, evolution, and mapping of genomes. Transcriptomics is concerned with the expressed transcriptome in the form of RNAs. Proteomics is concerned with the expression, structure, and interactions of different protein within the cell.

The *S. aureus* genome is around 2.8 mega base pairs in length with around 2,600 protein coding regions (39). The circular chromosome contains a core genome first described by Lindsey and Holden (39). This core genome is conserved across all *S. aureus* strains and may be supplemented by an accessory genome. The accessory genome varies from strain to strain and consists of insertion sequences, mobile genetic islands, bacteriophages, and transposons dispersed throughout the genome. The accessory genome often contains genes that code for virulence factors and antibiotic resistant genes. It is horizontal transfer of these genes that has led to wide spread antibiotic resistance among *S. aureus* strains. For example, SCCmec, a mobile genetic island mentioned earlier, codes for methicillin resistance. Also, the Panton-Valentine leukocidin gene is a virulence factor that codes for a cytotoxin that destroys white blood cells.

Studying the S. aureus Transcriptome

A greater understanding of the genome can be found in functional genomics, which involves studying the transcriptome. The expression profile of bacteria is often determined by microarray in which the RNA sample in question is probed with thousands of gene sequences that are placed on a glass slide called a chip. In this way, one is able to identify which genes are being expressed and to what degree. However, a newer method

of analyzing the transcriptome is becoming popular. RNA-seq, also known as whole transcriptome shotgun sequencing, consists of generating complementary cDNA sequences from an RNA sample, sequencing the cDNA, aligning the sequences to a reference genome, and producing a transcriptome map. The transcriptome map is then analyzed using a software program such as Genesifter®. Raw data aligned to a reference genome is entered into the software program, which uses the sequences as quantitative data points to measure the frequency of expression (40). Pairwise comparisons of gene expression under two different conditions can be conducted to see how a sample is responding to a certain environment. Genesifter® can combine multiple replicates into one pairwise comparison so that statistically significant differences in expression profiles can be identified (41).

The main advantage to RNA-seq over a microarray is that RNA-seq does not require probes to look for expression of a certain set of genes. RNA-seq looks over the entire genome of the organism to produce a global transcriptome map. Also, because of the use of next generation sequencing, errors are minimized, and transcription level quantification is very accurate (42).

RNA-seq data can be used in bacterial studies to form hypotheses that explore gene expression under different conditions or over time as reviewed by Pinto, et al(42). Data acquired through RNA-seq provides the opportunity to ask more detailed questions about how the cell is functioning and responding to its environment at the molecular level. For example, RNA-seq has been used to compare transcription levels for *S. aureus* N315 at four different times during growth(43). This experiment revealed that about 10% of the transcripts were of unknown function, opening up the opportunity for more studies.

RNA-seq can be utilized to form hypotheses about the true function of putative genes and their role in metabolic pathways.

CHAPTER TWO

Introduction

Staphylococcus aureus is normally a commensal organism that resides on the human skin or in the nose of approximately one third of people in the United States(44). However, *S. aureus* can be pathogenic and cause infections of the bloodstream, respiratory tract, heart, soft tissue, and bone (1). Particularly, antibiotic resistant strains of *S. aureus,* such as methicillin resistant *S. aureus* (MRSA), are a growing human health concern in and out of the hospital setting with an estimated 80,461 cases of invasive MRSA infections in the United States in the year 2011 (3).

Because of the increase in antibiotic resistance among *S. aureus* strains, alternatives for antibiotic treatments are being explored, including photodynamic therapies. Ultraviolet (UV) light has been shown to destroy bacteria by causing photo-damage to the cell's DNA at a faster rate than the cell can repair (45). However, because UV light is harmful to human tissues, the clinical applications of UV light therapy are limited. Photodynamic therapy involves the application of light-activated photosensitizers to bacteria. Cytotoxic reactive oxygen species are generated from the photosensitizer after exposure to a certain wavelength of visible light (13). Photodynamic therapy that utilizes 470 nm blue light is of particular interest because blue light in this range is not considered harmful to human tissues, and several blue light emitting devices have been approved by the FDA for clinical use. Since the use of photosensitizing agents in a clinical setting is not always practical or safe, the effect of

blue light without an applied photosensitizer has also been studied (12, 19-23). The inhibitory effect of blue light has been measured in both Gram-positive and Gram-negative bacteria. The degree of blue light inactivation of *Staphylococcus epidermidis, S. aureus, Streptococcus pyogenes, Clostridium perfringens, Escherichia coli, Acinetobacter baumannii, Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella pneumonia, Porphyromonas gingivalis*, and other bacteria varies depending upon the type of bacteria, the wavelength of light, the energy of the light, and the medium used during illumination (12, 19-23). Experiments demonstrating the eradication of *P. acnes, H. pylori, H. parainfluenzae, P. gingivalis*, and other bacteria by blue light hypothesize that an endogenous molecule is serving as the photosensitizer during blue light treatment (12, 25-29). Experiments with *S. aureus* have not revealed an endogenous photosensitizer, although Enwemeka has demonstrated that cells grown on TSA without an added photosensitizer are inhibited by both 405nm and 470nm light (19, 20).

In order to better characterize the mechanism of inhibition, this study explores the response of *S. aureus* to 470nm blue light at the transcriptomic level using RNA-seq. RNA-seq consists of generating complementary cDNA sequences from an RNA sample, sequencing the cDNA, aligning the sequences to a reference genome, and producing a quantitative transcriptome map. RNA-seq data can be used in bacterial studies to form hypotheses exploring gene expression under different conditions or over time as reviewed by Pinto, et al (42). Transcript data acquired through RNA-seq provides the opportunity to investigate how cells respond to environmental factors. Hypotheses can be formed concerning the function of genes involved with inhibited growth. Knowledge of the pathways is desired when considering new or synergistic treatments.

In the present study, we analyzed RNA samples using RNA-seq data generated from *S. aureus* liquid cultures exposed and not exposed to blue light to investigate the cell's response to blue light exposure. Using conservative cut-off parameters, we identified a total of 28 down-regulated genes and 5 up-regulated genes. The differentially regulated genes fall into 8 functional categories with 5 genes encoding conserved proteins of unknown function.

CHAPTER THREE

Methods and Materials

Bacterial Strains

The *S. aureus* isolate used in this study was provided by Tamarah Adair, Baylor University Waco, TX. This MRSA strain that was isolated from the nasal passage of a healthy Baylor University student is referred to as BUSA2288. The consent form, collection procedures, and recording methods were approved by the Baylor University Committee for Protection of Human Subjects in Research, the official University Institutional Review Board for the protection of human subjects or participants in laboratory learning experiences at or connected with Baylor University. The nasal passage sample was collected by swabbing each anterior nare and gently rolling the swab across the surface of a mannitol salt agar (MSA) plate. Fermenting colonies were isolated and purified on tryptic soy agar (TSA) plates. Gram positive, catalase positive, coagulase positive, staphylococcal cultures were identified as *S. aureus* and stored in CRYOCARE beads (Key Scientific Products, Stamford, Texas) for future use. A Kirby Bauer disc diffusion assay was performed on *S. aureus* BUSA2288 and oxacillin resistance was confirmed using Etest (bioMérieux, Inc., Durham, NC) and PCR(46).

Growth conditions

BUSA2288 was grown overnight in 5mL of Brain Heart Infusion (BHI) broth at 37 °C. Broth cultures were routinely inoculated from a single colony on a TSA plate.

The contents of this overnight culture were added to 45mL BHI broth, resulting in a concentration of 1 x 10^8 CFU/ml as measured by colony counts. One mL aliquots of this diluted overnight culture were transferred to each well of two BD Falcon™ non-treated 24-well plates. The control plate was labeled No Light (NL) and the treatment plate was labeled Blue Light (BL).

Light Source

The illumination box was designed and constructed in house and has been shown to effectively inhibit growth or kill *S. aureus* in BHI (47). Twenty-four 1.5 mm Kingbright blue LED lights were attached to a 24-well plate lid. The lights were arranged so that when the modified lid was place on a 24-well plate the lights were 0.5mm above the broth of the individual wells as seen in Figure 1. The LED lights are CIE 127 compliant with a dominant wavelength of 465 nm and a 2θ 1/2 of 16°. The lights were operated at a forward current of 20mA for 2 hours resulting in a total light dosage of 250 J/cm^2 (48). The resistors were placed away from the light box so as to not increase heat inside of the incubator.

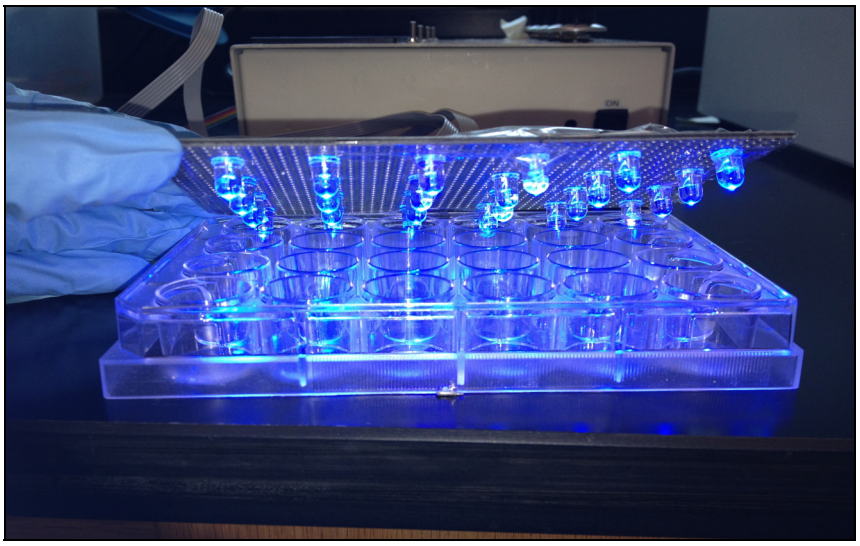

Figure 1: Blue light box.

Light Exposure

BL and NL plates were incubated with shaking at 35 °C for 2 hours with and without blue light exposure respectively. After illumination, the contents of the 24 NL wells were pooled and mixed thoroughly. Colony counts were obtained by diluting the sample and plating in triplicate. Cells were pelleted and resuspended in 800µL Ambion RNA*later* ™ solution and the sample was stored in a refrigerator overnight. This procedure was performed in the same manner for the BL plate.

RNA Extraction and mRNA Enrichment

Total RNA was extracted from the NL and BL samples using a modified phenol chloroform extraction method as follows. The pelleted cells were resuspended in RNAse free water and incubated with an equal volume of 1:1 phenol/chloroform. Phases were separated after centrifugation, and RNA solution was centrifuged at 12,000 x g for 10 minutes. Two times the volume of isopropanol was added to the aqueous layer. The RNA was pelleted, and the pellet was washed with ethanol on ice and resuspended in Elution

Solution (Ambion). Both samples were treated with DNAse Inactivation Reagent (Ambion). Enrichment of the RNA was performed using the MICROBExpress Bacterial mRNA Enrichment Kit (Ambion) which involves using a modified capture hybridization involving magnetic beads to remove 16S and 23S ribosomal RNAs. RNA was analyzed at the University of Oklahoma Science Center's lab for Molecular Biology and Cytometry Research using Agilent Bioanalyzer 2100 to verify the concentration and purity of the mRNA.

RNA-seq

Gene expression for NL and BL samples was analyzed by RNA-seq. Two independent experiments were performed and submitted to the University of Oklahoma Science Center's lab for Molecular Biology and Cytometry Research. Complementary DNA (cDNA) were generated from the RNA samples and then sequenced using the Illumina MiSeq Next Generation Sequencer. BUSA2288 is not a sequenced *S. aureus* strain. In order to determine which reference genomes to align our sequences to we performed a basic local alignment using BLAST and determined the two closest related reference genomes. The cDNA sequences were then aligned to the genomes of MRSA252 (NC_002952) and N315 (NC_002745), in order to create a transcriptome map.

The combined results of both independent experiments were analyzed using the Pairwise Analysis tools in GeneSifter®. GeneSifter® is a software program owned by Geospiza, a PerkinElmer company. The genes were normalized by Mapped Reads using EdgeR statistics including a Benjamini and Hochberg false discovery rate correction. Quality was set at a minimum number of 10 reads to be considered as expressed. The lower threshold for change was 5 fold. The functions of genes with a p-value of 0.05 or

less were determined by analyzing the information recovered from protein BLAST and KEGG pathways.

CHAPTER FOUR

Results

The concentration of starting cultures was 1.22 $x10^8$ CFU/mL for Trial 1 and similarly 1.00 $x10^8$ CFU/mL for Trial 2 as determined by colony counts. Percent inhibition of growth after 2 hours of blue light exposure was 37% for Trial 1 and 50% for Trial 2.

RNA was extracted from the pooled cells, and enriched RNA from the two independent experiments was submitted to University of Oklahoma Science Center's lab for Molecular Biology and Cytometry Research. cDNA was sequenced using the Illumina MiSeq Next Generation Sequencer, and sequences were aligned to the genomes of MRSA252 and N315 in order to create a transcriptome map.

The combined results of both independent experiments were analyzed using the Pairwise Analysis tools in GeneSifter®. In order to identify statistically different expression levels of individual genes we applied the following criteria to the pairwise analysis: (i) Quality must have a minimum number of 10 reads, (ii) the transcript level must change by at least 5-fold, and (iii) the P value for the t test must be 0.05 or less. This resulted in adjusted P values of 0.30 or less. A total of 28 down-regulated genes and 5 up-regulated genes were identified as seen in Table 1. GeneSifter® identified and named 23 of the 33 differentially regulated genes. The remaining 10 genes were placed in categories based on the function of identical matches to annotated *S. aureus* genes. All but 6 of the differentially regulated genes fall into 8 functional categories: amino acid

biosynthesis, cell envelope components, cellular processes, central intermediary metabolism, energy metabolism, protein synthesis, regulatory function, and transport and binding proteins as seen in Table 2. Five genes encode conserved proteins of unknown function. These genes are identified by their locus number rather than their gene name. Two of the genes of unknown function, SA2242 and SAS089, are present in N315 but not MRSA252. Four of the genes of unknown function- SA2264, SAR0291, SAR0292, and SAR2683- are annotated as putative transmembrane genes on NCBI Gene.

Table 2. Differentially regulated genes.

Functional category and gene	n-Fold change	P-value	Locus Tag
1. amino acid biosynthesis			
asd	-5.54	4.41E-03	SAR1406
lysC	-5.3	7.70E-03	SAR1405
2. cell envelope components			
srtB	5.03	9.45E-03	SAR1108
yceI-like	11.74	3.47E-02	SAR2769
3. cellular processes			
sepA	-8.38	3.47E-05	SAR2259
splB	7.45	1.49E-02	SAR1906, SAR1907
4. central intermediary metabolism			
narG	-5.88	5.96E-03	SAR2486
narH	-5.88	5.96E-03	SAR2485
narI	-5.88	5.96E-03	SAR2483
narJ	-5.88	5.96E-03	SAR2484
narK, narT	-9.24	1.25E-03	SAR2475, SAR2476
narT	-9.24	1.44E-03	SAR2476
nasD	-5.46	2.50E-02	SAR2489, SAR2490
nasE	-6.15	1.20E-02	SAR2488
nasF	-6.15	1.20E-02	SAR2487
SAR2490	-7.13	9.08E-03	SAR2490
5. energy metabolism			
acpD	28.06	1.79E-02	SAR0203
SAR2599	18.32	1.14E-02	SAR2599
SAR0742	-5.64	1.13E-02	SAR0742
6. protein synthesis			
SARt023	-10.13	4.01E-02	SARt023
7. regulatory function			
agrB	-8.03	5.29E-04	SAR2123
nreB	-5.56	1.38E-02	SAR2482
nreB, nreC	-5.68	3.52E-02	SAR2480
nreC	-5.88	2.24E-02	SAR2481
RNAIII	-9.88	2.38E-04	SARs022
8. transport and binding proteins			
SAR1005	-5.76	3.68E-03	SAR1005
SAR1010	-5.64	9.06E-03	SAR1010
9. conserved protein, unknown function			
SA2264	-7.11	1.77E-03	SA2264
SAR0291	-6.24	3.48E-04	SAR0291
SAR0292	-6.31	5.52E-03	SAR0292
SAR2683	-5.7	5.96E-03	SAR2683
SAS089	-8.37	1.02E-02	SAS089
10. putative conserved membrane protein			
SAR0455	-5.51	4.12E-02	SAR0455

CHAPTER FIVE

Discussion and Conclusions

The mechanism of inhibition of bacterial growth by blue light is not well characterized. The eradication of *P. acnes, H. pylori, H. parainfluenzae, P. gingivalis*, and other bacteria has been hypothesized to be caused by endogenous porphyrins within the cell interacting with blue light to produce reactive oxygen species(12, 25-29). These reactive oxygen species could then damage the cell leading to cell death. This hypothesis is supported by five pieces of evidence. First, the bacteria have emission peaks that are characteristic of endogenous porphyrins when exposed to blue light(30). Second, endogenous porphyrins accumulate during incubation, and bacteria that have been incubated longer are more susceptible to blue light inactivation (30, 31). Third, porphyrins accumulate during light exposure (32). Fourth, the blue light inactivation of bacteria has been shown to be dependent upon the presence of oxygen (16, 25, 29). Fifth, the variance of percent inhibition seen among different strains of bacteria could be attributed to the different types and amounts of porphyrins in the cell wall (33). For example, Gram-positive *S. aureus* produces 2-3 times more coporphyrin than Gram-negative species such as *E. coli*, and *S. aureus* is also more sensitive to blue light irradiation than *E. coli* (12).

In *S. aureus* experiments, cells grown on TSA are killed by blue light without an added photosensitizer. Also, we have evidence that the inhibitory effect of blue light could be media dependent. For example, bacteria grown in different broths are inhibited

to different degrees (49). Also, treatment of the broth with blue light followed by the addition of the bacteria also inhibits the growth of the bacteria (49). We hypothesize that blue light is generating electrons through a substance in the media that produces reactive oxygen species, including hydroxyl radicals, which damage the cell membrane, cell wall, or cell membrane proteins inhibiting the growth of the bacteria (50). The results of the pairwise analysis were used to investigate the specific targets of this proposed reaction.

Twenty-six of the 33 differentially regulated genes found in this analysis are either transmembrane proteins, regulated by transmembrane proteins, or otherwise related to the outside of the cell. Of these 26 proteins, four are hypothetical proteins with a transmembrane structure. One is a putative membrane protein. We propose the following model to explain the differential regulation of these genes after exposure to blue light beginning with ROS damage to the outer cell. Excitation or other causes of structural change to membrane-bound proteins results in depletion of the signaling pathway regulated by membrane-bound proteins and normal response to oxidative stress. Four examples are given.

First, consider the down-regulated genes *agrB* and RNAIII. These genes are a part of the accessory gene regulator (*agr*) quorum sensing system, which allows the cells to control expression of many virulence genes (51, 52). This system involves two different transcripts, RNAII and RNAIII. RNAIII is a regulatory RNA, known to regulate several virulence factor genes. RNAII encodes for 4 proteins- AgrA, AgrB, AgrC, and AgrD- that form the quorum sensing system. Activated AgrA binds to the promoter for both RNAII and RNAIII to activate transcription of the *agr* locus. The *agrD* gene product is necessary to allow the trans membrane protein AgrB to release autoinducing pherome

(AIP) molecules outside of the cell. AIP is detected by the trans membrane histidine kinase AgrC, which controls the activity of AgrA, the response regulator(53). If the membrane of the cell were disrupted by blue light exposure in a manner that interrupted the function of AgrB and AgrC, a decrease in the transcripts produced by the *agr* quorum sensing system including *agrB* and RNAIII would result, as demonstrated in Figure 2.

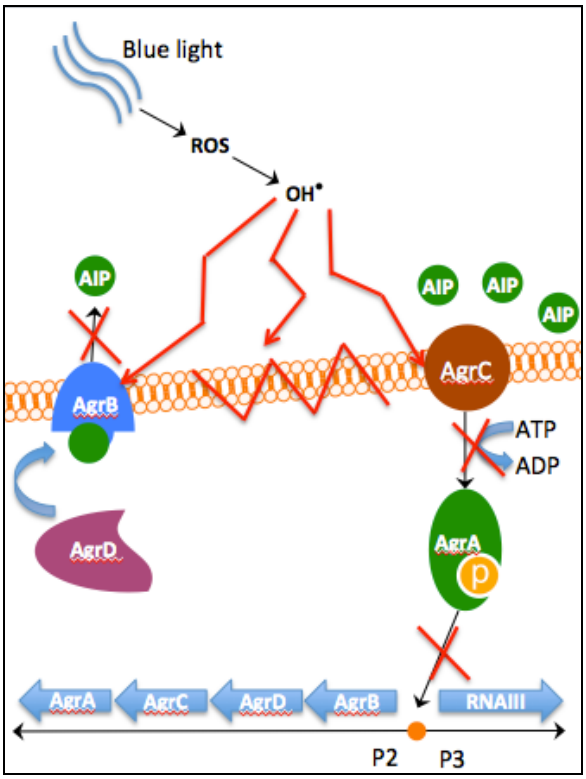

Figure 2. *agr* quorum sensing model.

A second pathway proposed to be involved in cell death involves ferric uptake. The up-regulated gene, *srtB*, is a part of the iron response surface determinants (*isd*) operon, which composes an iron-uptake system. Upstream of the *isd* operon is a ferric uptake repressor (Fur) box. If *S. aureus* is grown in the presence of high iron, Fur binds to the Fur box and inhibits the transcription of the *isd* operon (54). SrtB is required to

anchor other proteins in the operon to the cell wall. These other proteins allow the uptake of iron into the cell (55). Iron cofactors are necessary for the function of the electron transport chain, and the production of hydroxyl radicals quenches iron ions (56, 57)**.** If iron is being quenched by the presence of hydroxyl radicals, the concentration of iron would be low, and the Fur system would not inhibit genes related to iron uptake such as *srtB*. *srtB* could be up-regulated in order to respond to the need for a consistent level of iron within the cell.

Third, SAR2599 is an up-regulated gene that encodes a protein that is structurally similar to an YceI protein that is necessary for isoprenoid synthesis. Isoprenoids are an important functional component of the cell membrane in *S. aureus*. If the cell were overwhelmed by membrane damage, an upregulation of isoprenoid synthesis could be a response to return the damaged membrane to its original composure.

Fourth, four operons connected to nitrogen metabolism are down-regulated (Figure3). *nasD*, *nasE*, and *nasF* are a part of the nitrite reductase operon. Similarly, *narG*, *narH*, *narJ,* and *narI* are a part of the nitrate reductase operon. *narT* and *narK* encode nitrite and nitrate transporter proteins. NreB and NreC compose a two component system that regulates these three operons. The activation of these nitrogen metabolism genes is controlled by the presence of oxygen (58). If oxygen is depleted this is directly detected by NreB which phosphorylates NreC. NreC activates the three nitrogen metabolism operons at the promoter(58). If NreB, which resides in the cell membrane were damaged, the activity of NreC would decrease leading to a down-regulation of the three nitrogen metabolism operons as illustrated in Figure 3.

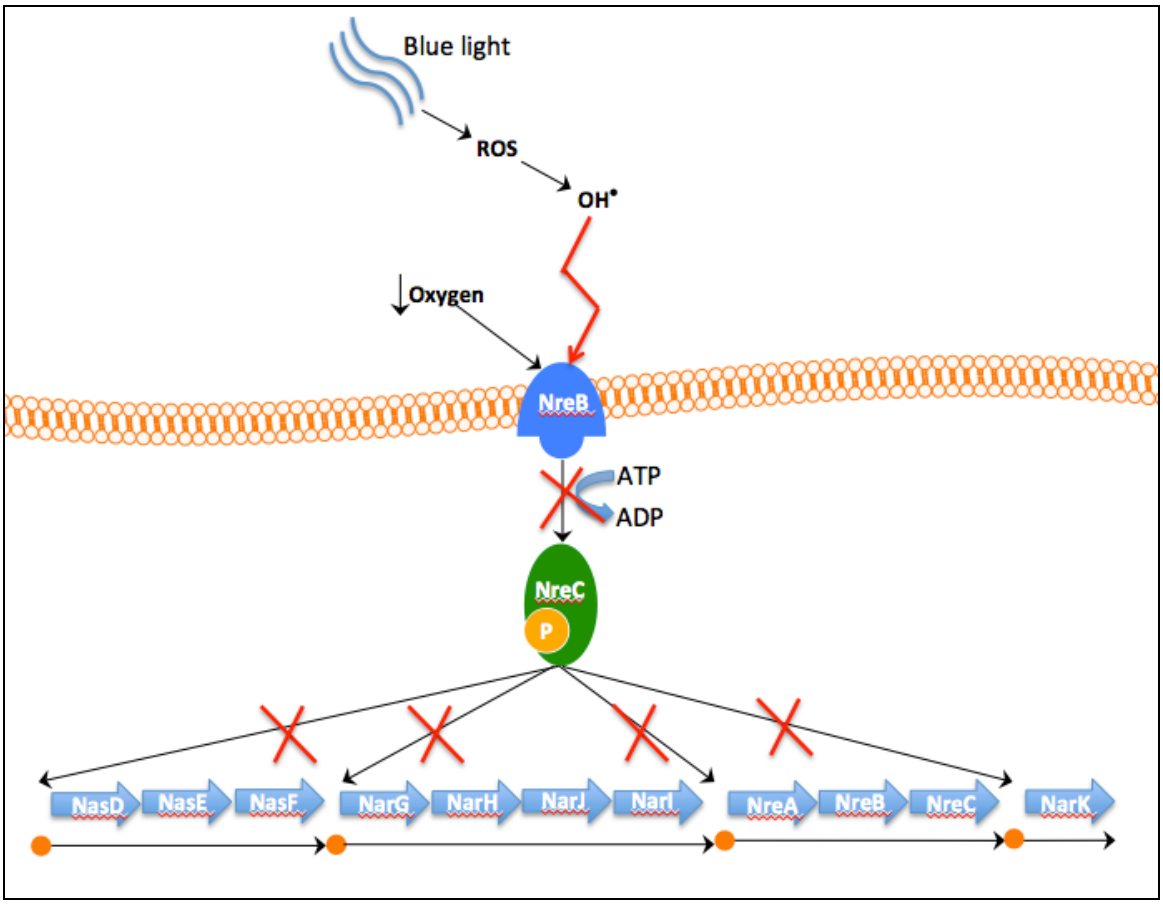

Figure 3. Model involving nitrogen metabolism genes.

Blue light is a potential therapy for antibiotic resistant *S. aureus* infections. The mechanism for the inactivation of bacteria is hypothesized to involve ROS. We have found evidence that supports this hypothesis that involves multiple pathways including essential metabolic pathways and known virulence pathways. Evidence suggests that there is a variation in the inhibitory effect of blue both among different bacteria and among *S. aureus* strains(47). Our results provide information to explain this variation. The variation could be explained by the different membrane and cell wall compositions among different *S. aureus* strains. This, along with the result that multiple pathways are affected by treatment, is promising in regard to the avoidance of resistance. These results

need to be verified using Quantitative RT-PCR, and the genetic response needs to be verified on multiple strains. Blue light still holds the potential for being an alternative treatment for antibiotic resistant *S. aureus* infections.

APPENDICES

APPENDIX A

Protocols

1) S. aureus culture preparation
    a) Prepare the master plate
        i) Inoculate broth with a BUSA2288 cryobead and allow to incubate overnight at 37 °C.
        ii) Streak the overnight broth onto a TSA plate and allow to incubate overnight at 37 °C.
        iii) Select a single colony from this plate and inoculate 5mL BHI. Allow to incubate overnight at 37 °C.
        iv) Streak the liquid culture onto a TSA plate. This is the master plate.
            (1) A fresh master plate was made from the previous master plate once every two weeks.
    b) Experiment culture preparation
        i) Inoculate 5 mL BHI with a streak from the master plate and allow to incubate overnight at 37 °C.
        ii) Add the contents of the overnight culture to 45 mL BHI.
        iii) Transfer 1mL of the diluted overnight culture to each well of two 24-well plates, with one plate labeled No Light (NL) and the other plate labeled Blue Light (BL).
2) Blue light irradiation and plating

a) Blue light treatment

   i) Wrap the NL plate with foil and attach the BL plate to the blue light apparatus.

   ii) Place both plates on the shaker at half speed and incubate the plates at 37 °C for 2 hours.

b) Colony counts

   i) During the 2 hours of treatment, dilute the remaining diluted overnight culture to 10^{-6}, and plate 50 µL of the 10^{-6} culture in triplicate.

   ii) After the 2 hours of treatment, combine the contents of the NL plate into a 50 mL conical tube. Combine the contents of the BL plate into a second 50 mL conical tube.

   iii) Dilute a sample of both the BL and NL liquid culture to 10^{-6}, and plate 50 µL of the 10^{-6} culture in triplicate for both samples.

   iv) Allow all plates to incubate at 37 °C overnight and perform colony counts.

   v) Pellet the contents of the BL and NL conical tubes using a microcentrifuge and resuspend the pellet in 600 µL of RNA later. Store in the refrigerator overnight.

3) RNA extraction

a) Pellet the cells using a centrifuge and remove the supernatant.

b) Add 1 mL RNAse free water and transfer to a new microcentrifuge tube. Pellet and remove supernatant.

c) Add 250 µL of RNase free water and vortex 3 min.

d) Add 250 µL of 1:1 phenol/chloroform.

e) Incubate for 30 min at 70°C vortexing for 1 min every 5 min.

f) Centrifuge at 12,000xg for 10 min.

g) Transfer 200 µL of the top aqueous phase to a new micro centrifuge tube.

h) Add 400 µL isopropanol, vortex 3 min, centrifuge at 12,000xg for 10 min in the freezer, and remove the supernatant.

i) Wash with 200 µL 70% ethanol and centrifuge at 8,000xg for 5 min in the freezer

j) Allow the microcentrifuge tube to dry upside down on a paper towel for 10 min.

k) Add 25 µL elution buffer pre-warmed to 95°C.

l) Store in the freezer.

4) Microbe Express, adapted from Microbe Express Protocol

a) RNA Precipitation

i) Precipitate the RNA by adding the following and mixing well:

(1) 0.1 volume 5 M ammonium acetate or 3 M sodium acetate

(2) 2.5–3 volumes 100% ethanol

ii) Leave the mixture at –20°C overnight, or quick-freeze it in ethanol and dry ice, or in a –70°C freezer for 30 min.

iii) Recover the RNA by centrifugation at ≥12,000 x g for 30 min at 4°C.

iv) Carefully remove and discard the supernatant. Remove the supernatant by gentle aspiration with a fine-tipped pipette.

v) Centrifuge the tube briefly a second time, and aspirate any additional fluid that collects with a fine-tipped pipette.

vi) Add 1 mL ice cold 70% ethanol, and vortex the tube.

vii) Re-pellet the RNA by centrifuging for 10 min at 4°C. Remove the supernatant carefully,

viii) Repeat steps iv-vii.

ix) Dissolve the RNA in ≥15 µL TE.

b) Anneal RNA and Capture Oligonucleotide Mix

i) Pipet 200 µL Binding Buffer into a 1.5 mL tube provided with the kit.

ii) Add total RNA (2–10 µg RNA in a maximum volume of 15 µL) to the Binding Buffer.

iii) Close the tube, and tap or vortex gently to mix.

iv) Add 4 µL of Capture Oligo Mix to the RNA in Binding Buffer.

v) Close the tube and tap or vortex gently to mix, and microfuge briefly to get the mixture to the bottom of the tube.

vi) Heat to 70°C for 10 min Incubating the mixture at 70°C for 10 min denatures secondary structures in RNA, including the 16S and 23S rRNAs. This heat denaturation helps to facilitate maximal hybridization of the rRNAs to the capture oligonucleotides.

vii) Anneal at 37°C for 15 min. The 37°C, 15 min incubation allows the capture oligonucleotides to hybridize to homologous regions of the 16S and 23S rRNAs. The Binding Buffer is optimized to function specifically and efficiently at this temperature. Prepare the Oligo MagBeads as described in the next section during this incubation.

c) Prepare the Oligo MagBeads

i) For each RNA sample, remove 50 µL Oligo MagBeads to a 1.5 mLtube. Oligo MagBeads for up to 10 RNA samples (500 µL) can be processed in a single 1.5 mL tube.

ii) Capture the Oligo MagBeads by placing the tube on a magnetic stand. Leave the tube on the stand until all of the Oligo MagBeads are arranged inside the tube near the magnet. This will take ~3 min with the Ambion Single Place Magnetic Stand.

iii) Carefully remove the supernatant by aspiration, leaving the beads in the tube, and discard the supernatant.

iv) Wash the Oligo MagBeads with an equal volume of Nuclease-free Water.

v) Add Nuclease-free Water to the captured Oligo MagBeads; use a volume of Nuclease-free Water equal to the original volume of the Oligo MagBeads.

vi) Remove the tube from the magnetic stand, and resuspend the beads by brief, gentle vortexing.

vii) Recapture the Oligo MagBeads with a magnetic stand, and carefully aspirate and discard the Nuclease-free Water leaving the beads in the tube.

viii) Equilibrate the Oligo MagBeads with an equal volume of Binding Buffer.

ix) Add Binding Buffer to the captured Oligo MagBeads; use a volume of Binding Buffer equal to the original volume of the Oligo MagBeads.

x) Remove the tube from the magnetic stand, and resuspend the beads by brief, gentle vortexing.

xi) Recapture the Oligo MagBeads with a magnetic stand, and carefully aspirate and discard the Binding Buffer leaving the beads in the tube.

xii) Resuspend the Oligo MagBeads in an equal volume of Binding Buffer, and bring the slurry to 37°C.

xiii) Add fresh Binding Buffer to the captured Oligo MagBeads; use a volume of Binding Buffer equal to the original volume of Oligo MagBeads.

xiv) Remove the tube from the magnetic stand, and resuspend the beads by gently tapping the tube or very gentle vortexing.

xv) Place the Oligo MagBead slurry in a 37°C incubator, and allow the temperature to equilibrate to 37°C before proceeding.

d) Capture the rRNA and Recover the Enriched mRNA

i) Heat the Wash Solution to 37°C.

ii) Gently vortex the tube of washed and equilibrated Oligo MagBeads to resuspend them, and add 50 µL of Oligo MagBeads to the RNA/Capture Oligo Mix.

iii) Very gently vortex or tap the tube to mix and microfuge very briefly to get the mixture to the bottom of the tube.

iv) Incubate 15 min at 37°C. During this step the oligonucleotide sequence on the Oligo Mag-Beads anneals to the Capture Oligonucleotides, and the Capture Oligonucleotides remain hybridized to the 16S and 23S rRNAs. The hybridization "sandwich" of Oligo MagBead: Capture Oligonucleotide: rRNA is formed at this step.

v) Capture the Oligo MagBeads by placing the tube on the Magnetic Stand. Leave the tube on the stand until all of the Oligo MagBeads are arranged inside the tube near the magnet. This will take ~3 min using the Ambion Single Place Magnetic Stand

vi) Aspirate the supernatant which contains the enriched mRNA, being careful not to dislodge the Oligo MagBeads. Transfer it to a Collection Tube on ice.

vii) If a very small amount of Oligo MagBeads are accidentally carried over to the supernatant, they can be removed at the end of the procedure.

viii) Add 100 µL Wash Solution that has been prewarmed to 37°C to the captured Oligo MagBeads.

ix) Remove the tube from the magnetic stand, and resuspend the beads by brief, gentle vortexing in the 37°C Wash Solution

x) This wash step recovers mRNAs that were inadvertently trapped in the rRNA:Capture Oligonucleotide hybrids.

xi) Recapture the Oligo MagBeads, and carefully recover the supernatant. Pool this supernatant with the RNA already in the Collection Tube and proceed immediately to the precipitation step.

e) Precipitate and Resuspend the Enriched mRNA.

i) Add the following to the pooled mRNA from, and briefly vortex to mix. the final concentration will be 100 µg/mL (7 µL)

(1) 1/10th volume 3 M Sodium Acetate (35 µL)

(2) 1/50th volume Glycogen (5 mg/mL).

ii) Add 3 volumes ice cold 100% ethanol (1175 µL), and vortex to mix thoroughly.

iii) Precipitate at –20°C for at least 1 hr

iv) Centrifuge for 30 min at ≥10,000 X g (typically ~13,000 rpm in a microcentrifuge) and carefully decant and discard the supernatant.

v) Do a 70% ethanol wash as follows:

(1) Add 750 µL ice cold 70% ethanol and vortex briefly.

(2) Centrifuge for 5 min at ≥10,000 X g. Discard the supernatant.

vi) Do a second 70% ethanol wash.

vii) Briefly re-spin the tube after discarding the second 70% ethanol wash. Carefully remove any remaining supernatant with a pipettor, being careful not to dislodge the pellet.

viii) Air dry the pellet for 5 min. Do not air dry the pellet for more than 5 min.

ix) Resuspend the RNA pellet in 25µL Tris EDTA.

x) Rehydrate the RNA for 15 min at room temperature. Vortex the sample vigorously if necessary to resuspend the RNA. Collect the sample by brief centrifugation.

xi) If the RNA solution has a brownish color, there is probably a small amount of Oligo Magbeads remaining in the sample. To remove them, put the tube on the magnetic stand for ~3 min and move the enriched mRNA solution to a new RNase-free tube. Enriched mRNA yield from 10 µg of high quality total RNA is typically 1–2.5 µg.

APPENDIX B

Raw data

Sample, Dilution	Colony Count 1	Colony Count 2	Colony Count 3	Average	Standard Deviation
T0, 10-5	50	72	uncountable	61	15.55634919
BL, 10-5	79	68	66	71	7
NL, 10-5	108	118	uncountable	113	7.071067812

Table 3. Trial 1 Colony Counts.

Sample, Dilution	Colony Count 1	Colony Count 2	Colony Count 3	Average	Standard Deviation
T0, 10-6	4	7	4	5	1.732050808
BL, 10-6	2	2	2	2	0
NL, 10-6	1	5	6	4	2.645751311

Table 4. Trial 2 Colony Counts.

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