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The msaABCR Operon Mutant *Staphylococcus aureus* is Deficient in Persister Cells

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The University of Southern Mississippi

The *msaABCR* Operon Mutant *Staphylococcus aureus* is Deficient in Persister Cells

by

Aaliyah Cole

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirement for the Degree of
Bachelor of Science
in the Department of Biological Sciences

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Abstract

Persister cells comprise a phenotypic variant that shows extreme antibiotic tolerance resulting in chronic infections. While this phenomenon has posed a great threat in public health, mechanism underlying their formation in *Staphylococcus aureus* remains largely unknown. Increasing evidence of the presence of persister cells in recalcitrant infections underscores the great urgency to unravel the mechanism by which these cells are developed. The Elasri Research group characterized *msaABCR* operon that plays roles in regulation of virulence, biofilm development and antibiotic resistance. It was hypothesized that the operon also plays a role in persister cell formation. In this study, the persister cell fraction in wild type (WT) USA300 LAC, its isogenic *msaABCR* deletion mutant (Δ *msaABCR*) and complemented operon mutant (complement) strains were compared. The present study shows that the Δ *msaABCR* forms fewer persister cells as compared to WT challenged with various antibiotics single as well as in the combinations in exponential as well as in the stationary phase. The complement restored the phenotype as comparable to the WT in most of the drug tested. Likewise, the Δ *msaABCR* also formed less number of persister cells challenged with gentamycin in the presence of metabolite fructose. Taken together, this study suggests that *msaABCR* plays role in the persister formation. Ultimately, the goal is to define the mechanism that causes *msaABCR* to control persistence. This study will bring new insights into the understanding of persister cells formation and consequently overcome failures of staphylococcal infections.

Key Words: Persister cells, *Staphylococcus aureus*, *msaABCR* operon, metabolite, antibiotics

Dedication

Beatrice Cole, Biven Cole, Rosie Thomas, Lynell Brandon, Kristen Dupard

Thank you for your love, prayers, and support.

You have been a true blessing.

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List of Abbreviations

CFU	Colony Forming Unit
PMF	Proton- Motive Force
LAC or WT	Wild-type <i>Staphylococcus aureus</i>
TSA	Tryptic Soy Agar
PBS	Phosphate Buffer Solution
OD	Optical Density
MHB	Mueller Hinton Broth
ATP	Adenosine Triphosphate

Chapter 1: Introduction

In 1944, Bigger discovered small fraction of Staphylococcal cells survived the penicillin treatment that resumed their growth upon removal of the drug, which he termed as persister cells. Bacterial resistance and tolerance to antibiotics lead to treatment failures of many infections. Antibiotic resistance often stems from genetic changes, whereas antibiotic tolerance is a property of a small fraction of phenotypic variants called persister cells. Persister cells undergo dormancy and hence survive antibiotic treatment due to their lack of metabolism, rather than due to genetic changes (Keren, Kaldalu, Spoering, Wang, & Lewis, 2003). Mechanism of persister formation in *S. aureus* is still not understood. Increasing evidences suggest that there is presence of persister cells in recalcitrant bacterial infections (Conon BP., 2014). Persister cells show extreme tolerance to antibiotics (Keren et al., 2004), have little or no cell wall synthesis, and other cellular processes. In tolerant persister cells, antibiotics can bind to the target, but cannot alter it, so no corrupted product is produced, thus cells are not killed. When the antibiotic is removed, the persister cells awake from their dormant state, resume growth and form the regular cells (Lewis K., 2007).

The genetics behind the development of persister cells continues to be researched. A newly characterized operon *msaABCR* has been found to play a role in biofilm formation, virulence, and antibiotic resistance. Hence, it was hypothesized that *msaABCR* operon may also play a role in the development of antibiotic tolerant persister cells. In this study, the number of persister cells formed in the WT USA300 LAC, its isogenic Δ *msaABCR* strain, and the complement in single as well as with the combination of different antibiotics was compared. Also, the number of persister cells challenged with aminoglycoside mainly gentamicin in the

presence of metabolites was compared. This was done to determine the effect of different metabolites that enhance the uptake of aminoglycoside to kill persister cells.

Chapter 2: Literature Review

Mechanism of Persister Formation

Persister cells are a subpopulation of bacteria that are multi-drug tolerant and contribute to chronic and recalcitrant clinical infections such as cystic fibrosis, tuberculosis, pneumonia and invasive infections caused by variety of bacterial pathogens. Persisters are metabolically dormant which makes the cells highly tolerant to traditional antibiotics that are mainly effective against actively growing cells (Kwan, Chowdhury, & Wood, 2015). Persister cells have been discovered to be in high numbers in bacterial biofilms. These cells are believed to be associated with the antibiotic tolerance and reoccurrence infections caused by biofilm producing pathogens. (Fauvart, Groote, & Michiels, 2011). Mechanism of formation of persister cells in *S. aureus* is not well understood. However, a recent study showed that persisters in *S. aureus* are associated with depletion of ATP. In the exponential phase, fewer persister cells are formed and were found to enter the stationary phase early with the depletion of ATP (Conlon BP, 2016). The fraction of persister cells increases as the cells enter the stationary phase. Biofilm harbors more number of persister cells, protecting them from immune invasions and antibiotic effects leading to detrimental antimicrobial challenge and cause the regrowth of the biofilm associated infections (Roberts & Stewart, 2005).

In a recent study, several genes were confirmed to be associated with persister cells formation in *S. aureus*, including *hemB*, *mazF*, and *glpK* (Fu et al., 2009; Singh et al., 2009; Han et al., 2014; Wang, et al., 2015). It was also found that *ureG*, *sdhA*, and *sdhB* were associated with persister cell formation in *S. aureus* under treatment with levofloxacin (Wang, et al., 2015). Also, new evidence has been found that associates a drop in intracellular adenosine triphosphate (ATP) with *S. aureus* persister cells production into the stationary phase. Levels of ATP in the

cell have been found to be predictive of bactericidal antibiotic efficacy and explain bacterial tolerance to antibiotics (Conlon, Rowe, Gandt, Nuxoll, Zalis, & Lewis, 2016).

Isolation of Persister Cells

The first method to isolate persisters was based on the simple sedimentation of surviving cells from a culture that had been lysed with ampicillin antibiotic treatment (Lewis, 2007).

Lewis's study allowed specific genes to be identified that could have contributed to the dormant phenotype: *rmf* stationary state inhibitor of translation; *sulA*, an inhibitor of septation; as well as toxin-antitoxin (TA) loci *relBE*, *dinJ* and *mazEF* (Lewis, 2007). Because persister cells are in their highest frequency during the stationary phase of growth (Lewis, 2007), this phase is an ideal time to isolate and run tests on the cells. The stationary phase is the phase in which the number of dividing cells and the number of dying cells are equal in number in the media. At this point, the amount of nutrients that are being consumed and the toxic wastes of the organisms are accumulating. Because persister cells are slow growing or non-growing cells that form more readily under growth limiting conditions (Fauvart, Groote, & Michiels, 2011), this now nutrient limited environment is ideal for studying the presence of persister cells.

***Staphylococcus aureus*: Organism of Study**

S. aureus, is an opportunistic pathogen that causes many diseases that affect the human population. The diseases that *S. aureus* cause are often chronic and recalcitrant to many common antibiotic treatments (Conlon B. P., 2014). Community-acquired, methicillin-resistant *S. aureus* strains cause localized infections in immune-compromised hosts. In addition, some strains show superior virulence that leads to severe infections even among healthy individuals with no predisposing risk factors (Sahukhal, 2014). *S. aureus* can form biofilms, which have been linked to many diseases. The bacteria biofilms are sheathed in a polysaccharide glycocalyx

which in turn provides the bacteria with protection against the host's immune defenses and antimicrobial drugs (Sambanthamoorthy, Schwartz, & Nagarajan, 2008). Infections that are ascribed to biofilms include but are not limited to childhood middle ear infection, gingivitis, and infections of indwelling devices such as catheters, orthopedic prostheses, and heart valves. It has been found that stationary- state cultures of *S. aureus* produce large populations of persisters (Keren, Kaldalu, Spoering, Wang, & Lewis, 2003). Therefore, studying the virulence factors and the regulators which are believed to contribute to persister cell formation and that are responsible for the development of chronic *S. aureus* infection is essential and can be used as a basis for treatment development.

***msaABCR* Operon**

The *msaC* gene had been identified as a regulator of *sarA*, *agr*, and many other virulence factors (Sahukhal, 2014). The staphylococcal accessory regulator, *sarA*, is a major global regulator that is essential for biofilm formation both *in vitro* and *in vivo* (Sambanthamoorthy, Schwartz, & Nagarajan, 2008). In a study done by Sambanthamoorthy, Schwartz and Nagarajan, the *msa* gene was mutated and the biofilm formation was observed. The study determined that mutation of the *msa* gene in strain of *S. aureus* resulted in a weak biofilm at the accumulation stage resulting in an immature biofilm. This defect was likely mediated by the reduced expression of *sarA* in the *msa* mutant (Sambanthamoorthy, Schwartz, & Nagarajan, 2008). The results suggested that the weak biofilm defect in the *msa* mutant was an intermediate phenotype between the *sarA* mutant and wild-type (Sambanthamoorthy, Schwartz, & Nagarajan, 2008). It was discovered that the *msaABCR* operon was vital in the regulation of virulence and the development of biofilms in *S. aureus*. The two RNAs that were found to regulate the expression of the *msaABCR* operon are *msaC* and *msaR* (Sahukhal, 2014).

Because the *msaABCR* operon has been found to regulate the virulence and biofilm development of *S. aureus*, it is reasonable to study the potential relationship between the formation of persister cells and the *msaABCR* operon. Therefore, in the present study, the goal is to compare the number of persister cells formed by wild-type *S. aureus* and the Δ *msaABCR* challenged with aminoglycoside enabled with metabolites and different combination of antibiotics.

Biphasic Killing Curve

Persister cells are phenotypic variants of the normal cells which show multidrug tolerance to antibiotics (Percival, Hill, Malic, Thomas, & Williams, 2011). When a bacterial population is treated with antibiotics, majority of the cells are killed leaving behind small fraction of cells showing a plateau with no or slow growth for extended period (Fauvart, Groote, & Michiels, 2011). This biphasic killing curve is characteristic of persister cells and depends on the drug used (Fig.1). Upon, removal of the antibiotics, persister cells resume their growth and form the normal population, mechanism by which persister cells cause the relapsing infections (Lewis K, 2010).

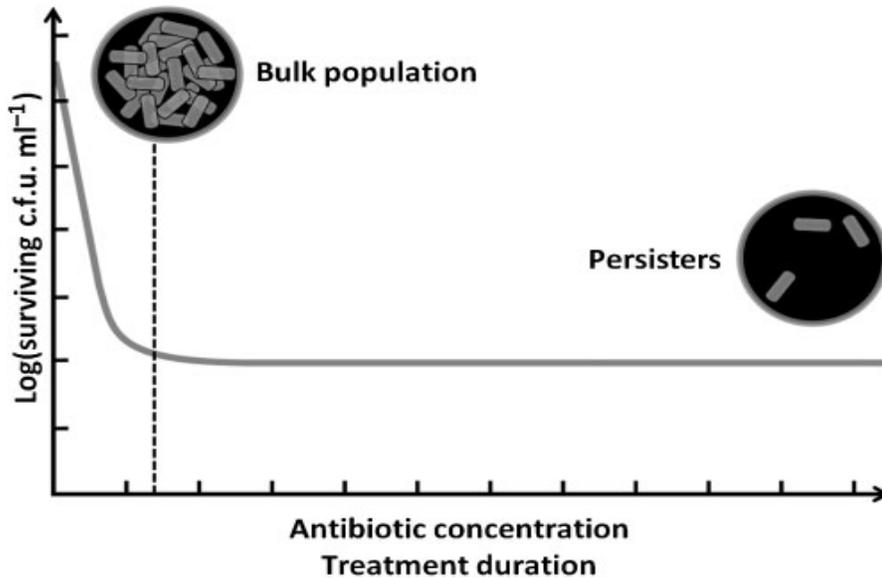


Figure 1. “Biphasic killing pattern in response to antibiotics. Addition of increasing concentrations of antibiotics and/or increasing the treatment duration leads to an initial phase of rapid killing of the bulk population. However, beyond a certain threshold (indicated by a dashed vertical line), a killing plateau is observed as only persister cells remain viable.” (Fauvart, Groote, & Michiels, 2011)

Antibiotic Tolerance

Chronic infections are paradoxically caused by antibiotic sensitive persisters which show transient multidrug tolerance by slowing down their metabolic state and entering dormancy. Antibiotics kill the cells by binding to the active bacterial targets and producing the corrupted product, which are toxic to the cell viability. In antibiotic resistance, the antibiotic is prevented to bind to the target. But in antibiotic tolerance, antibiotics can bind to the target but cannot form the corrupted product; hence the cells are intact in antibiotic tolerance Fig. 2.A (Lewis K., 2010). This idea that persister cells are tolerant rather than resistant has led to the suggestion that persisters are specialized survivor cells (Keren, Kaldalu, Spoering, Wang, & Lewis, 2003).

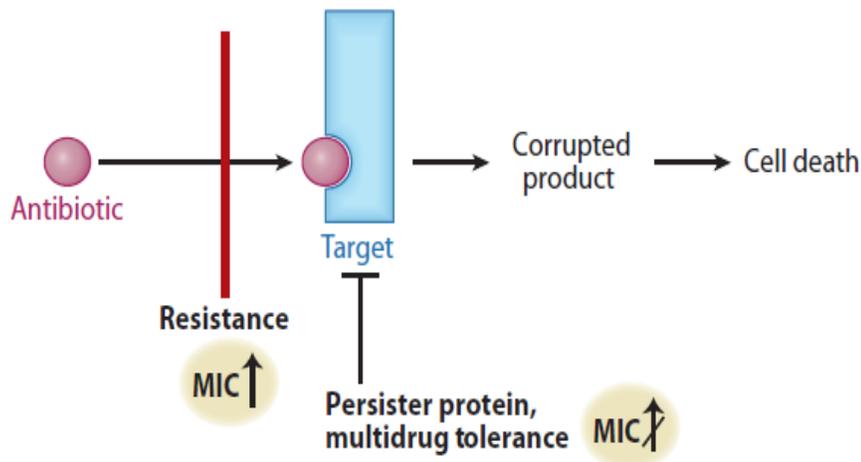


Figure 2. A) Resistance versus Tolerance

Biofilm

Persister cells are found in higher number in staphylococcal biofilm infections. Biofilm acts as a protective layer against the host immune responses and antibiotic treatment which has been hypothesized to be an important determinant in biofilm tolerance to drugs and other host defense factors. The altered physiology of biofilm on the other hand reflects the unique environmental niche and high cell density, which is likely to limit nutrient and oxygen availability, forcing the subset of biofilm cells into a stationary and persister-like state. Cells in this physiology are primed to survive wide-ranging environmental insults including antibiotic challenge (Conlon BP., Rowe SE and Lewis K., 2014). During biofilm infections, cells are killed via antibiotic or host immune defenses but small fraction of cells remain untouched inside the biofilm. On removal of the antibiotic treatment, these persister cells resume their growth and relapse the infection Fig. 2B. (Lewis K., 2010)

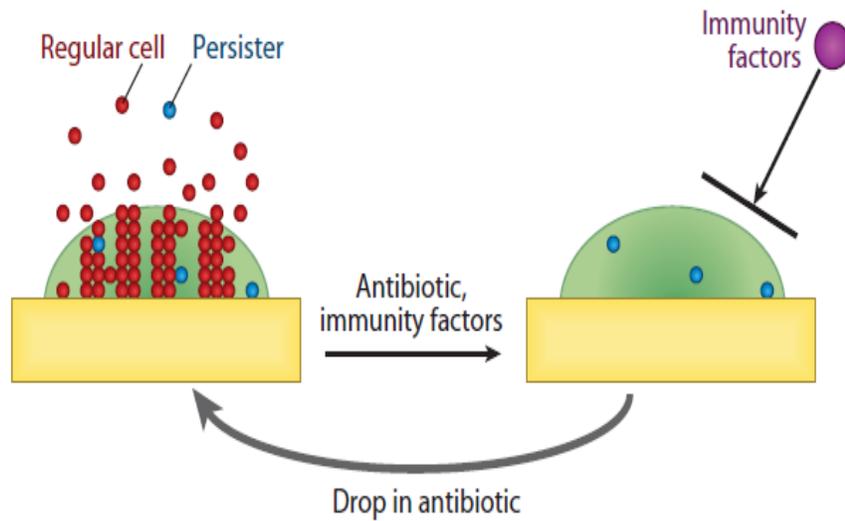


Figure 2.B) Model for relapsing biofilms. (Lewis.K, 2010)

Persister Cells and Infections

In the past, a variety of antibiotics were used to treat *S. aureus* infections, and soon became ineffective treatments due to the extreme adaptability of the bacteria and its ability to develop resistance and/or tolerance. Antibiotics used today include vancomycin, rifampicin, and gentamicin. Recently, teixobactin, a new antibiotic, was discovered to have a significant impact on the treatment of *S. aureus* and other gram-positive and drug-resistant strains of bacteria. It was found to strongly inhibit synthesis of peptidoglycan, and there has been no sign of the development of resistance which suggests that it is not a protein (Ling, 2015). The exploration of antibiotics that not only affect the normal cell population, but that also have the capacity to eradicate persister cells is essential, and provides the basis for the present study.

Metabolism as a Means for Persister Eradication

As with all cells, bacterial cells function and survive by means of metabolism. However, persister cells are traditionally metabolically dormant cells which make them difficult to treat

with most antibiotics. (Kwan, Chowdhury, & Wood, 2015). In a study conducted by Allison, Brynildsen & Collins, metabolic stimuli were shown to enable the killing of persister cells via aminoglycosides which bind to the ribosome and cause the cells to die by mistranslation of RNA. This enabling of killing was reasoned to be due to an increase in the proton-motive force (PMF) which facilitates the uptake of aminoglycosides, a type of antibiotic (Allison, Brynildsen, & Collins, 2011). Mitchell's chemiosmotic hypothesis stated that the proton motive force was the driving force for energy-requiring processes such as solute transport and ATP synthesis (Mitchell, 1966). In a study conducted by Taber, Mueller, and Miller, it was determined that aminoglycoside uptake requires energy; bacterial cells take up aminoglycosides against a concentration gradient. The mechanisms by which this occur remains ambiguous, but after conducting the current study, a relationship between the aminoglycoside uptake and the $\Delta msAABC R$ operon may be determined which could lead to the further exploration of the mechanism on the molecular level.

In the study conducted by Allison, Brynildsen and Collins, it was proposed that certain metabolites such as glucose, fructose, and mannitol were transported into the cytoplasm by phosphotransferase system enzymes (Allison, Brynildsen, & Collins, 2011). After entering glycolysis where NADH is generated, NADH is oxidized by enzymes in the electron transport chain that in turn contributes to PMF. These now increased levels of PMF facilitate the uptake of aminoglycosides which leads to cell death (Allison, Brynildsen, & Collins, 2011). This process can be seen in Figure 3. The results from their study on the metabolite-enabled killing off persister cells that showed the potentiation of each of the sugars tested can be seen in Figure 4.

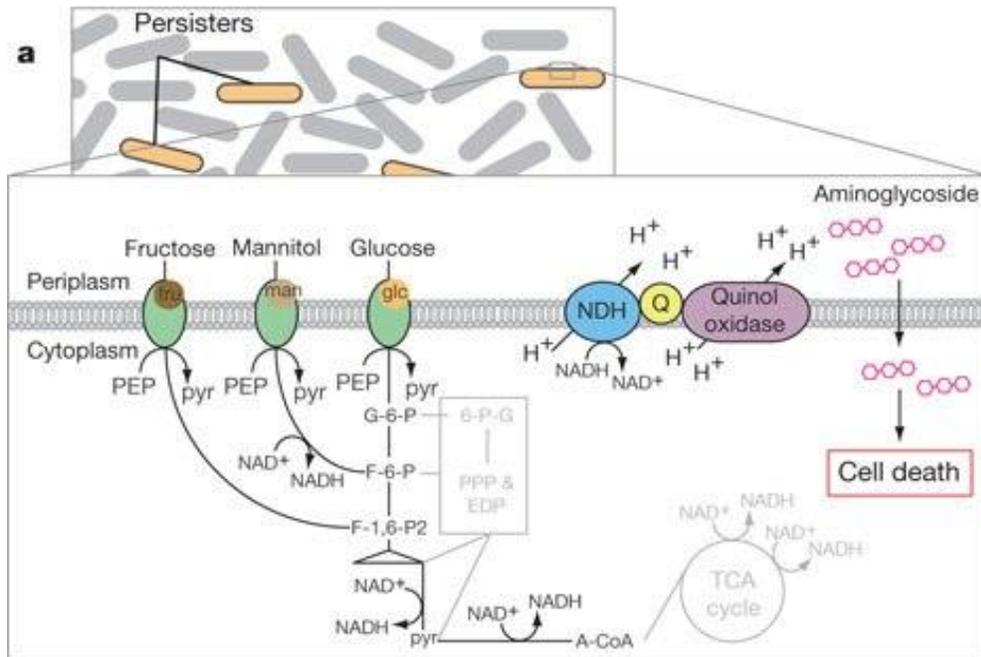


Figure 3. **Mechanism for metabolite eradication of persisters** (a). Metabolite-enabled eradication of persisters occurs via the catabolism of carbon sources (i.e. glucose, fructose, mannitol) which generates NADH. NADH is oxidized by the electron transport chain that contributes to PMF, which facilitates the uptake of aminoglycosides that leads to persister cell death. (Allison, Brynildsen, & Collins, 2011)

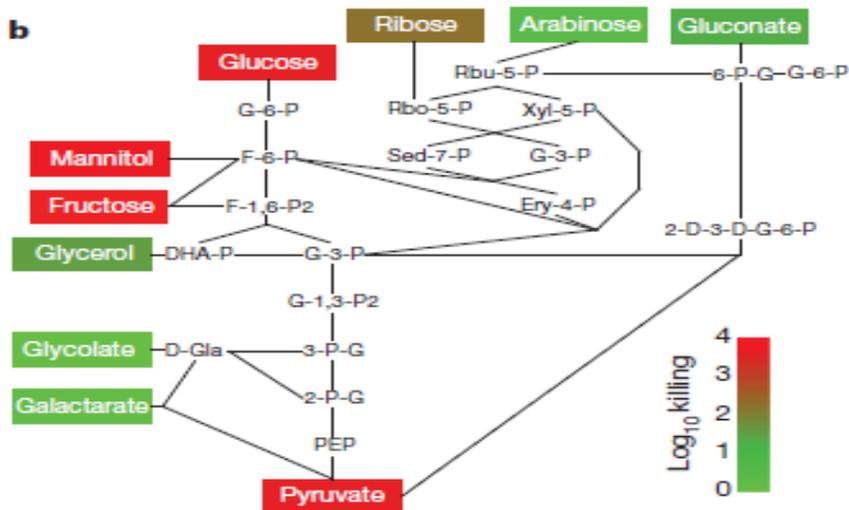


Figure 4. **Metabolite-enabled killing of persister cells.** (Allison, Brynildsen, & Collins, 2011)

Chapter 3: Methods

Bacterial strain and Culture Preparation

Overnight cultures of bacterial strains USA300 LAC, isogenic *msaABCR* mutant strain, and its complement strain were prepared by inoculating small fraction of frozen stock into 5 mL Muller and Hinton Broth (MHB) in 17X100 mm culture tubes for 16 hours with continuous aeration at 225 RPM at 37°C. The overnight cultures were diluted 1:10 and incubated further for 2 hours, and then normalized to OD₆₀₀ 0.02 in warmed 10 mL MHB to use as the starting culture. Exponential phase cells were prepared by incubating starting cultures for ~2 hours until they reached an OD₆₀₀ of 0.5. Stationary phase cells were prepared by inoculating 1µL of starting culture in 10 mL MHB for 16 hours.

Media Preparation

The Mueller Hinton Broth (MHB) and Luria Burtani Broth were prepared as manufacturer's instruction. After thoroughly mixing media in distilled water, the bottles were vented by slightly untwisting the cap, and then autoclaved. The media cooled before it was used to prepare cultures.

Equal Cell Counts

To determine when the growth was reached the desired OD, 100µl of the culture was diluted into 900µl of PBS when using LB. Using the spectrophotometer, the OD of the cells was measured. Upon receiving the OD, the volume of each culture that needed to be added to the fresh media was calculated using the following equation:

$$\frac{\text{Desired concentration} \times \text{Prepared volume of media}}{OD} \times 1000 \mu\text{L}$$

Metabolite Assay

After equal cell counts were achieved, the cultures grew an additional two hours. Then, the initial cell count was taken and plated prior to the addition of the stressors (metabolite or aminoglycoside or both). After the initial cell count was taken, culture was treated with 10 μ g/mL of Gentamycin combined with 0.05% of the desired metabolite (fructose, mannitol, glucose, arabinose, ribose, rhamnose). The cultures grew for two hours. Starting at the two-hour mark and continuing bi-hourly for eight hours, the CFU count was taken via the plating of 100 μ l of each sample onto a TSA plate. The same procedure was followed as with the control group (with sugar only) with the CFU count being taken bi-hourly for eight hours via plating onto TSA plates. The colonies grew overnight and were counted the next day.

Antibiotic Assay

Exponential phase cells were plated for colony forming units (CFU) counts and challenged with the antibiotics daptomycin (10 μ g/ml, 10 \times MIC), vancomycin (25 μ g/ml, 40 \times MIC), linezolid (50 μ g/ml, 10 \times MIC), gentamicin (50 μ g/ml, 10 \times MIC), and rifampicin (2.4 μ g/ml, 40 \times MIC). These concentrations were the minimum concentrations showing similar killing kinetics to that of higher concentrations of the respective antibiotics. At designated time point of each antibiotic, 100 μ L of cells was removed, washed with 1X phosphate buffer saline (PBS) and plated for CFU counts after 24 hrs. Stationary phase cells were plated for initial CFU count and persister cells were enumerated as above at designated time points after adding different concentration of antibiotics in combinations (Table 1). Persister assays were performed as above in exponential as well as stationary phase cells challenged with combination of the antibiotics. The colonies grew overnight and were counted the next day.

Antibiotic Concentrations used (Exponential)			MIC
DAP+RIF	1.24µg/mL	0.312µg/mL	0.0312+0.0078
VAN + RIF	3.12µg/mL	0.074µg/mL	0.156+0.0037
LIN + RIF	3.12µg/mL	0.074µg/mL	0.156+0.0037
DAP + GEN	6.24µg/mL	25µg/mL	0.312+1.25
VAN + GEN	6.24µg/mL	25µg/mL	0.312+1.25
LIN + GEN	6.24µg/mL	50ug/mL	0.312+2.5
Antibiotic Concentrations used (Stationary)			
DAP+RIF	1.24µg/mL	0.312µg/mL	0.0312+0.0078
VAN + RIF	3.12µg/mL	0.074µg/mL	0.156+0.0037
LIN + RIF	3.12µg/mL	0.074µg/mL	0.156+0.0037
DAP + GEN	6.24µg/mL	25µg/mL	0.312+1.25
VAN + GEN	6.24µg/mL	25µg/mL	0.312+1.25
LIN + GEN	6.24µg/mL	50ug/mL	0.312+2.5

Table 1: Concentration of antibiotic used

Dilutions

To complete the dilutions, 100µl aliquots of each experimental culture (those that were treated with the gentamycin and a carbon source) as well as the control cultures (those that have not been treated with gentamycin and a carbon source) were re-suspended in 900µl of PBS and labeled. These initial dilutions were centrifuged at 10,000 RPMs for 5 minutes. Once removed, 700µL of the supernatant was removed from the top and disposed. Then, 700µL of PBS was added back to the culture and mixed well via vortex for approximately 10 seconds to assure an even mix. Then, each tube was serially diluted 10-fold, by extracting 100µL of the culture from the initial tubes and serially diluting it into 900µL tubes of PBS. This was repeated until 5 dilutions were made for each original culture. Upon completing the serial dilutions, the cells were plated onto TSA agar plates using the spreading method. This method of serial dilution was repeated for each strain exposed to a carbon source and the aminoglycoside.

Plating and counting

To plate, 100µl of each dilution from the second dilution to the fifth was separately plated on an individual TSA agar plate. Using a pipette, 100µL of each sample was added to a plate and spread around the plate using a spreader. Once the sample no longer appeared moist on the agar, the top was replaced and the plate was inverted and incubated at 37°C overnight. After the incubation period, the CFUs were counted and compared to the number of CFUs from the control.

Determining Persister Cells

The number of colony forming units (CFUs) per milliliter after exposure to different antibiotics was measured. Persister (surviving fraction) was calculated after incubation by dividing the number of cfu/mL in the culture with the antibiotic by the number of cfu/mL in the culture before adding the antibiotic. The CFUs was counted at designated time points (i.e. 2 hours, 4hours...). The equation used is show below.

$$\frac{\text{No of } \frac{CFU}{mL} \text{ in the culture with the antibiotic}}{\text{No of } \frac{CFU}{mL} \text{ in the culture before antibiotic}}$$

Chapter 4: Results

Metabolite with Aminoglycoside

The treatment of the *ΔmsaABCR* strain with aminoglycosides and metabolites did show a further decrease in cell death in comparison to the WT USA300 LAC. There were seven sugars (fructose, glucose, mannitol, pyruvate, arabinose, rhamnose, and ribose) used in each media and combined with gentamycin. In the LB media, fructose showed good potentiation in gentamycin treatment that most of the persisters were eradicated in the *ΔmsaABCR* and a considerable number of persisters remained in the USA300 LAC. However, in this assay, the complement did not show the resumption of phenotype comparable to the WT strain (Fig. 4). The test with only fructose and gentamycin, no killing was seen in all the three strains tested (Fig. 5 and 6).

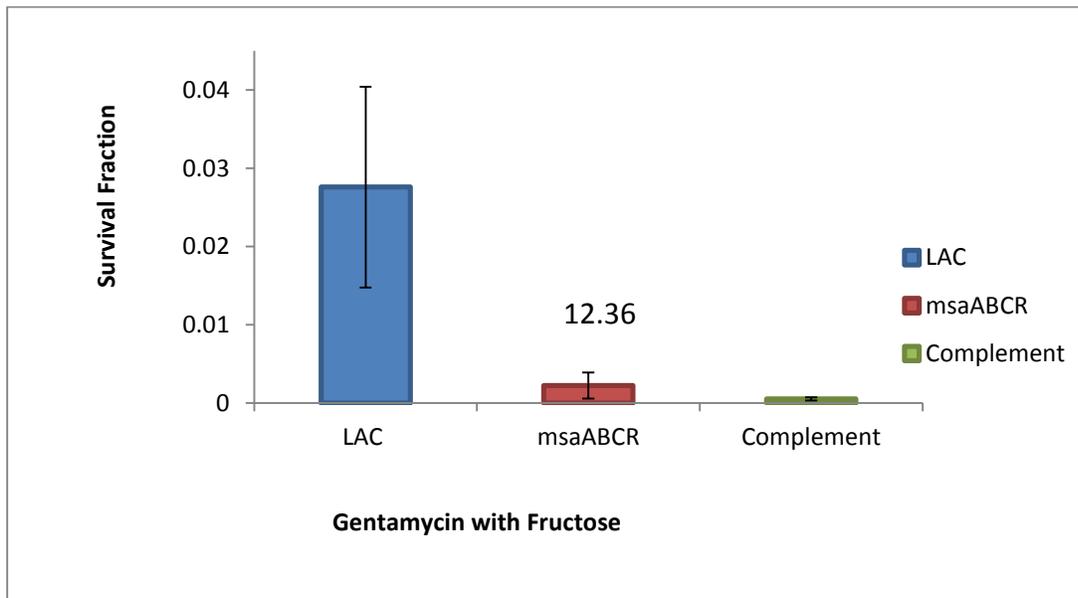


Figure 5. Gentamycin with fructose. There is a significant decline in the number of persisters found in *ΔmsaABCR* (red) strain of *S. aureus* which expresses that on average most of the persisters were eradicated and shows a potential relationship between the uptake of the aminoglycoside and the *msaABCR* operon.

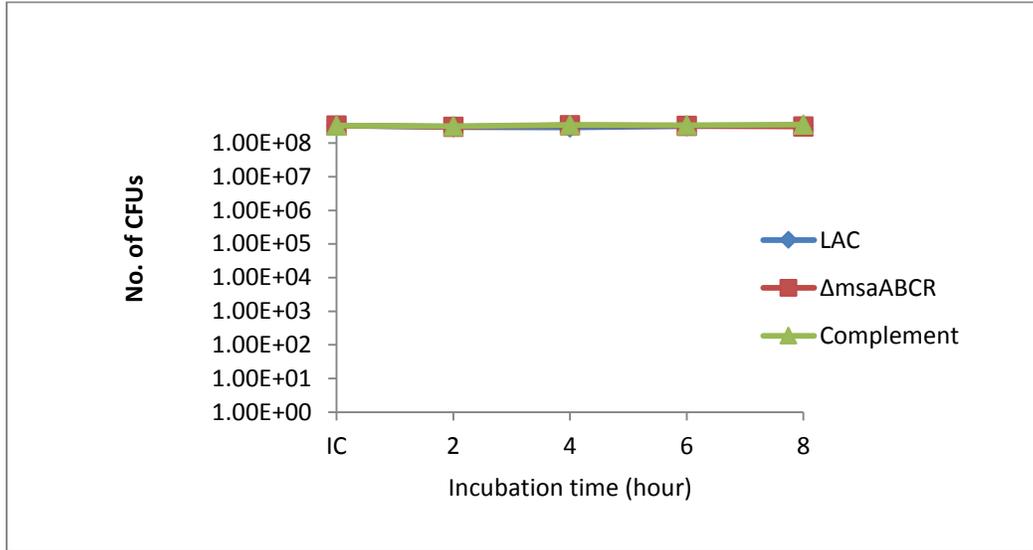


Figure 6. Gentamycin (10 μ l/without fructose). When gentamycin is used alone to treat the *S. aureus* population, there is no killing of persister cells in neither the wild-type LAC (blue), the operon mutant Δ msaABC R (red), nor the complement strain (green).

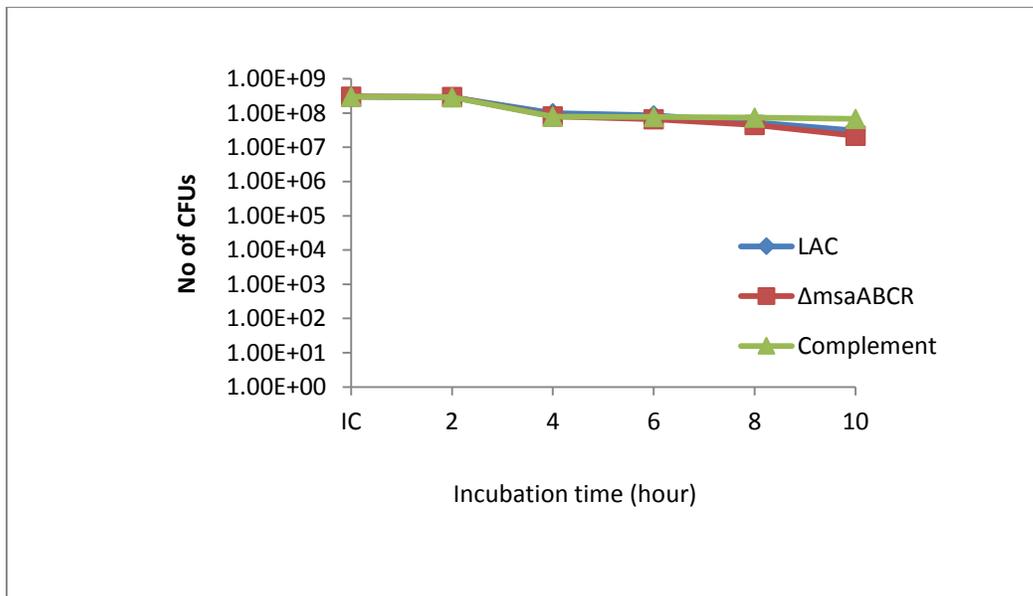


Figure 7. Fructose Only. When fructose is used alone to treat the *S. aureus* population, there is little killing of persister cells in neither the wild-type LAC (blue), the operon mutant Δ msaABC R (red), nor the complement strain (green).

Antibiotic Assay

The treatment of the *AmsaABCR* strain with different combinations of antibiotics showed an overall decrease in persister cells when compared to the USA300 LAC. There were five antibiotics used rifampicin, gentamycin, vancomycin, daptomycin, and linezolid. When vancomycin and rifampicin were combined, *AmsaABCR* generated more than 2 log fold decreased number of persister cells as compared to the WT (Fig.9), and the stationary growth phase formed 4.04-fold less number of persister cells than WT at 48 hours (Fig. 10). When daptomycin and rifampicin were combined, the exponential growth phase showed reduced number of persister cells by 48.40fold compared to wild-type (Fig. 11), and while it decreased by 16.6-fold than wild-type in stationary phase after 48 hours of treatment (Fig. 12). With the combination of linezolid and gentamycin, *AmsaABCR* showed 22.5fold less number of persister cells than wild-type at 24 hours (Fig. 13), and the stationary growth phase showed less by 9.5-fold than wild-type at 48 hours of antibiotic treatment (Fig. 14).

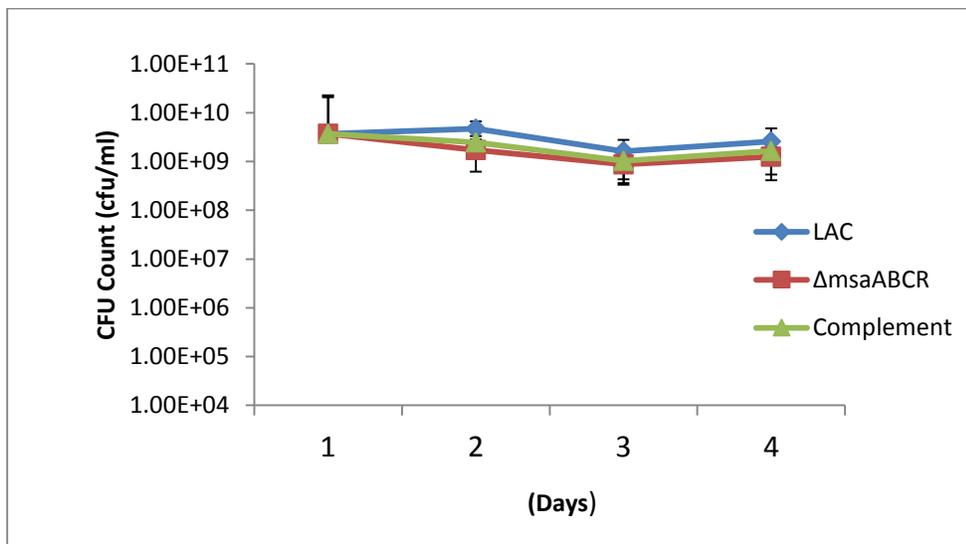


Figure 8. Growth Curve for the stationary growth phase for each of the *S. aureus* strains LAC (blue), *AmsaABCR* mutant (red), and complement (green) in the absence of antibiotics or other stimuli.

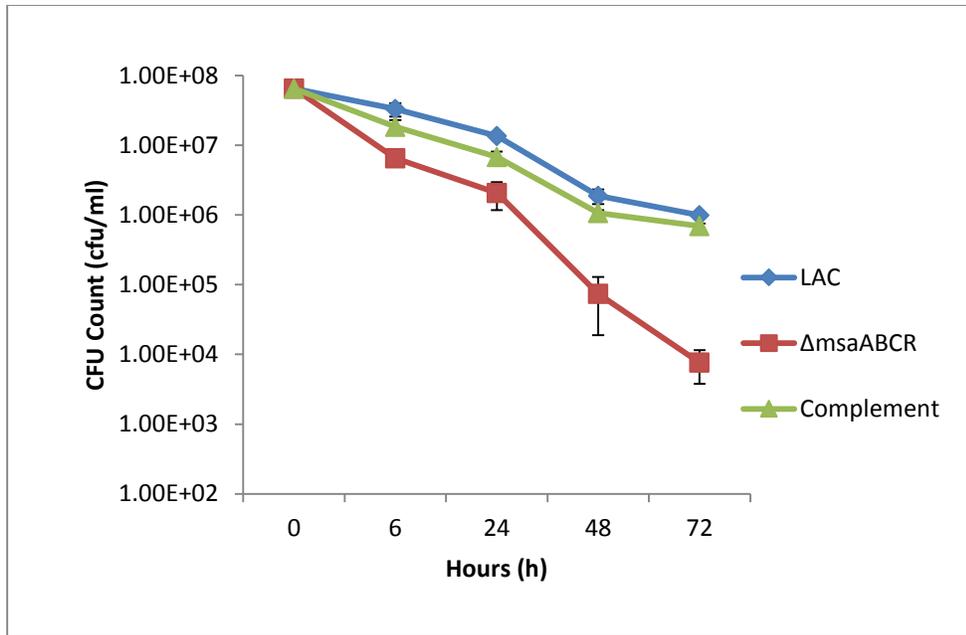


Figure 9. Exponential growth phase results when the three *S. aureus* strains LAC (blue), Δ msaABC R mutant (red), complement (green) are treated with vancomycin at 3.12 μ g/mL concentration and rifampicin at 0.074 μ g/mL concentration. Δ msaABC R expressed change by 2-fold 1/m less than wild-type (LAC) at 72 hours.

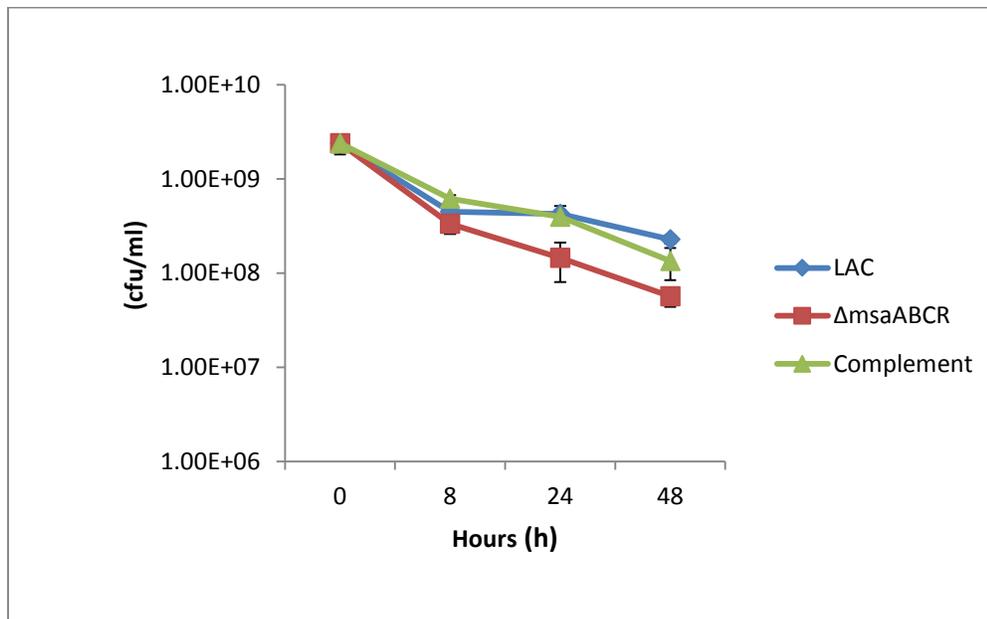


Figure 10. Stationary growth phase results when the three *S. aureus* strains LAC (blue), Δ msaABC R mutant (red), complement (green) are treated with vancomycin at 3.12 μ g/mL concentration and rifampicin at 0.074 μ g/mL concentration. Δ msaABC R expressed change by 4.04-fold 1/m less than wild-type (LAC) at 48 hours.

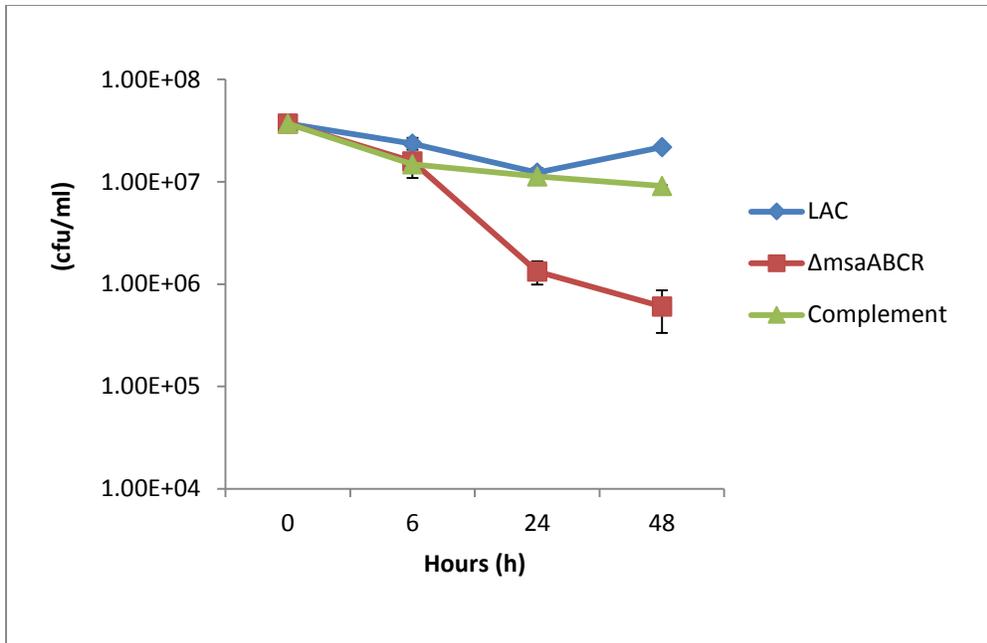


Figure 11. Exponential growth phase results when the three *S. aureus* strains LAC (blue), Δ msaABCR mutant (red), complement (green) are treated with daptomycin at 1.24 μ g/mL concentration and rifampicin at 0.312 μ g/mL concentration. Δ msaABCR expressed change by 48.40-fold 1/m less than wild-type (LAC) at 48 hours.

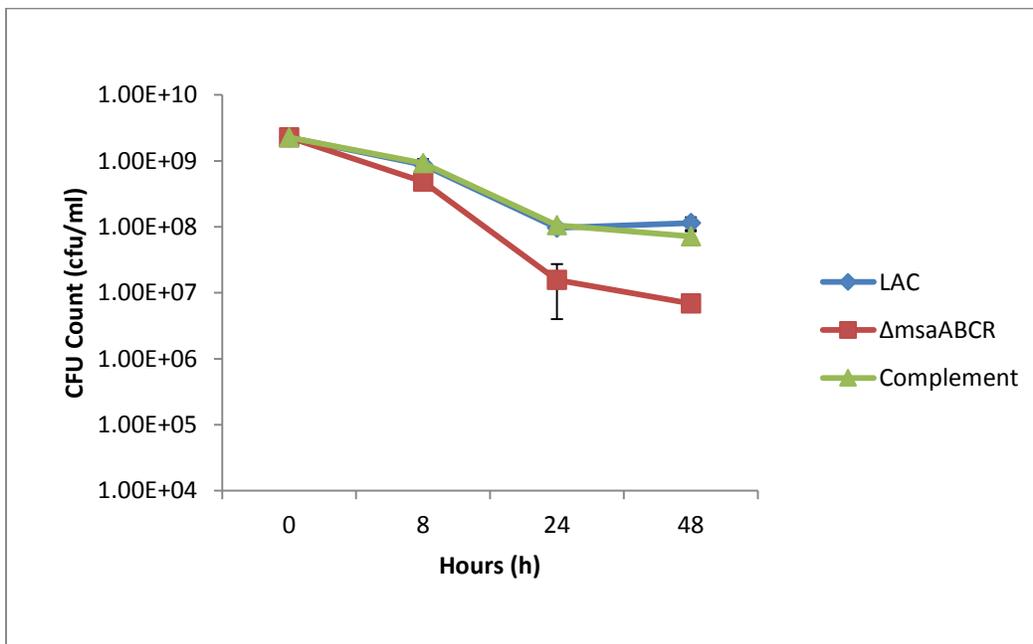


Figure 12. Stationary growth phase results when the three *S. aureus* strains LAC (blue), Δ msaABCR mutant (red), complement (green) are treated with daptomycin at 1.24 μ g/mL concentration and rifampicin at 0.312 μ g/mL concentration. Δ msaABCR expressed change by 16.6-fold 1/m less than wild-type (LAC) at 48 hours.

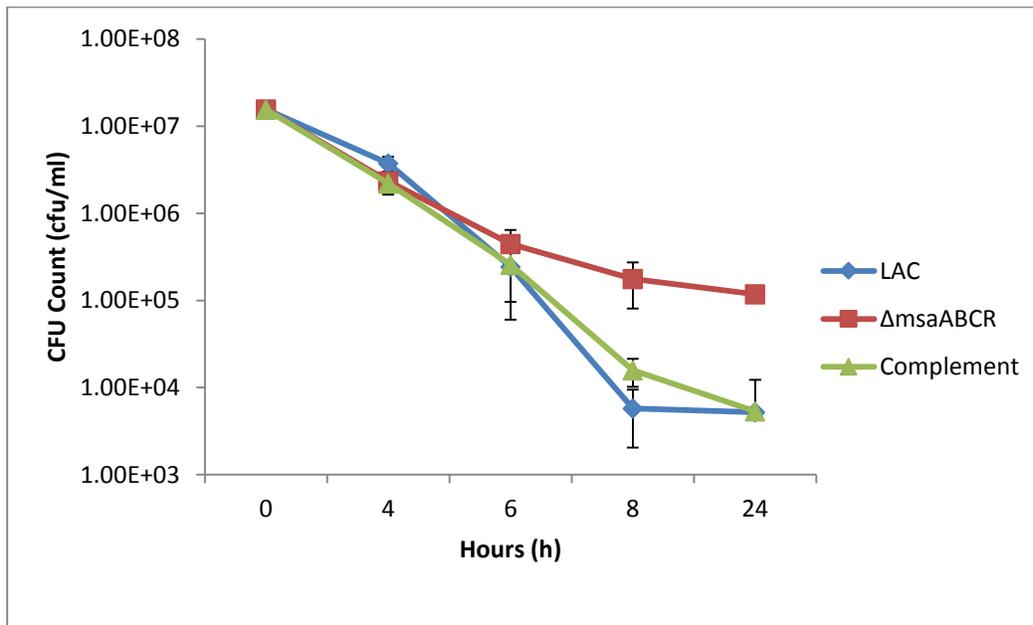


Figure 13. Exponential growth phase results when the three *S. aureus* strains LAC (blue), Δ msaABCR mutant (red), complement (green) are treated with linezolid at 6.24 μ g/mL concentration and gentamycin at 50 μ g/mL concentration. Δ msaABCR expressed change by 22.5-fold 1/m greater than wild-type (LAC) at 24 hours.

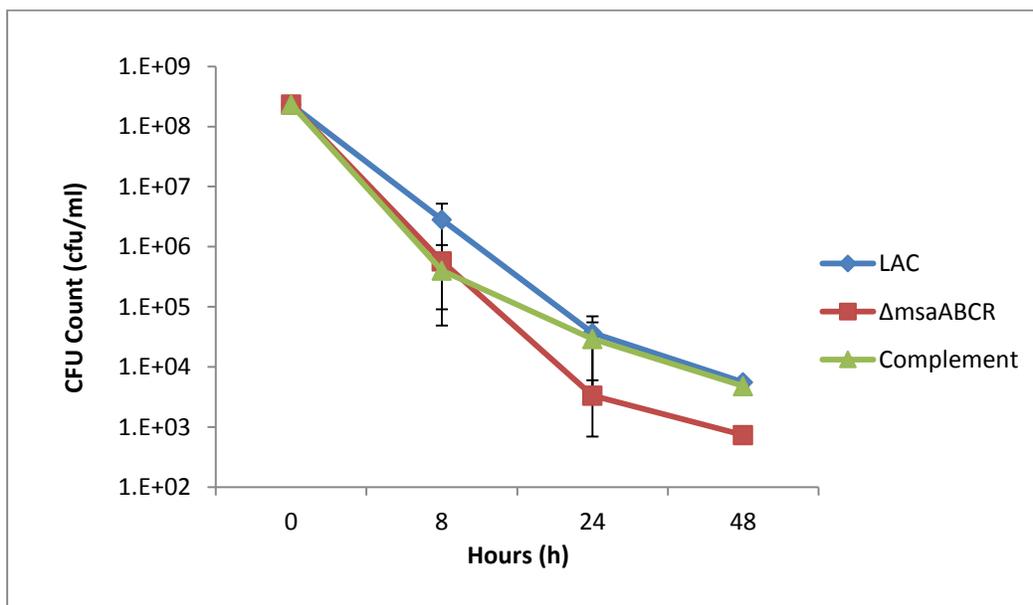


Figure 14. Stationary growth phase results when the three *S. aureus* strains LAC (blue), Δ msaABCR mutant (red), complement (green) are treated with linezolid at 6.24 μ g/mL concentration and gentamycin 50 μ g/mL concentration. Δ msaABCR expressed change by 9.5-fold 1/m less than wild-type (LAC) at 48 hours.

Chapter 5: Discussion and Conclusion

In the study, seven sugars were used to determine the metabolite eradication of persister cells in *S. aureus* via the aminoglycoside, gentamycin. The results from the test conducted without the aminoglycoside shows a constant growth and death rate over time, and then a dramatic decline in the number of cells in the sample. This dramatic change occurred as the cells continued to consume the sugar while growing in the media. Once the cells reached the stationary phase, the amount of sugars consumed and the amount of waste produced increased. This depletion of healthy nutrients and accumulation of toxins lead to an increase in cell death. It is believed that the additional sugar added to the growth media cause the cells to grow and replicate at a much faster rate which lead to the viable cells in the culture reaching the stationary phase sooner.

The result from the test conducted without the metabolite showed a constant growth and death rate over time. In this case, the aminoglycoside could only kill the viable cells leaving the persister cells to maintain the culture. This occurred because of the aminoglycosides weak activity against dormant cells which was mentioned in the study by Allison, Brynildsen, and Collins. Aminoglycoside activity requires energy and therefore needs a metabolite to stimulate the cell to uptake the aminoglycoside, without this stimulant, the aminoglycoside remains ineffective against normally metabolically dormant cells such as persister cells.

In the LB media, there was little potentiation in glucose, pyruvate, arabinose, rhamnose, ribose, and mannitol. Some of the sugars showed no effect on either strain, while some of the sugars seemed only to affect the wild-type strain. However, fructose showed good potentiation in the LB media and showed the killing of the persisters in the *AmsaABCR* operon over the wild-type and the complement. These results are consistent with the results in Allison, Brynildsen, and

Collin's study in that gentamycin and fructose reduce the viability of cells in *S. aureus*. The results obtained from this part of the study illustrate the relationship between the uptake of aminoglycosides into cells and the role of the *msaABCR* in cell death.

It can be concluded that the *msaABCR* coordinated with fructose and gentamycin further increased persister cell death. In the future, the results from this study may be used to determine the mechanism by which the *msaABCR* operon codes for virulence factors in *S. aureus* that lead to recurrent infections. Also, the results from this study can be used to develop medicines that can be used to treat chronic *S. aureus* infections.

In the study, five different antibiotics were used and combined in varying manners. The combinations included vancomycin and rifampicin, daptomycin and rifampicin, linezolid and gentamycin, rifampicin and vancomycin. The results for each combination showed an increase in persister cell death with the Δ *msaABCR* strain having increased persister cell death in majority of the samples. Daptomycin and rifampicin when combined was found to play a significant role on the eradication of persisters in *S. aureus* in the Δ *msaABCR* strain by showing a drastic 48.40-fold 1/m decrease in persister cells from the wild-type. Also, rifampicin and vancomycin showed a significant decrease in persister cells in the mutant *S. aureus*. In the assay with linezolid and gentamycin, the exponential growth phase for the Δ *msaABCR* strain changed by a 22.5-fold 1/m increase in persister cells from the wild-type.

In this study both the exponential growth phase and stationary growth phase were explored because there are two types of persisters. The results of this study showed that the greatest declines in the mutant persister cell populations were expressed in the exponential growth phase for each of the antibiotic combinations except linezolid and gentamycin. This

emphasis on persister cell death shows that the different combinations of antibiotics act more readily on persister cells present while the culture is proliferating.

It can be concluded that the antibiotics used in this study with the exception of the linezolid and gentamycin combinations work more effectively in a nutrient-rich environment. The results of this study can be used in future studies to develop effective antibiotic treatments for eradication of persister cell populations.

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