5-2021

Study of the Role of the msaABCR operon in Tricarboxylic Acid Cycle Activity and persister cell formation in Staphylococcus aureus

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by

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A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of Honors Requirements

May 2021

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ABSTRACT

Staphylococcus aureus is an important human pathogen that causes wide arrays of infections ranging from minor skin infections to lethal systemic conditions such as infective endocarditis, osteomyelitis, sepsis and pneumonia. These systemic diseases are often difficult to treat due to the presence of persister cells. Persister cells are a phenotypic variant of the bacterial population that exhibit extreme and transient antibiotic tolerance accompanied by a transient halt in growth. Upon cessation of antibiotic treatment, however, persisters resume growth which results in recurrence of infections. This characteristic of persister cells therefore displays high clinical significance. In this study, we show the involvement of the *msaABCR* operon in antibiotic tolerance in *S. aureus* under the clinically relevant bactericidal antibiotic gentamicin. In previous study, the *msaABCR* operon was found to involve in persister cells formation such that the deletion of the *msaABCR* operon showed decreased persister formation in the methicillin resistant *S. aureus* (MRSA) strain USA300 LAC (Sahukhal et al. 2017). The RNA transcriptome of the *msaABCR* deletion mutant showed differential expression of genes that are involved in various metabolic pathways including carbohydrate, amino acid pathways (Sahukhal et al 2017). Considering the importance of metabolism in antibiotic tolerance, first we examined the expression of tricarboxylic acid (TCA) genes to measure the TCA cycle activity. The qRT-PCR results showed that the *msaABCR* deletion mutant indeed has increased expression of TCA genes and higher ATP content (Sahukhal et al., 2017). Furthermore, we evaluated the antibiotic tolerance in TCA cycle mutants in *S. aureus*. The results showed that the transposon mutants of TCA cycle genes exhibit higher antibiotic tolerance as compared to the wild type USA300 LAC. Considering the

importance of energy content in the antibiotic tolerance, we also measured the ATP content and membrane potential. Deletion of TCA genes leads to decreased cellular ATP content as well as reduced membrane potential. We also observed increased pigment production and biofilm production in the TCA mutants. Altogether, these results suggest that the reduced metabolic activity contributes to the antibiotic tolerance as well as virulence in *S. aureus*.

Key words: *Staphylococcus aureus*, antibiotic tolerance, metabolism

DEDICATION

To my mom, Dylan, Deac and Tucker,

Thank you for supporting me endlessly throughout this process. Without my family's love and support, I would have never been able to achieve my goals. I am so incredibly grateful for each of you.

ACKNOWLEDGMENTS

I would first like to thank Dr. Mohamed O. Elasri for taking a chance on a student who had no prior research experience before joining his laboratory. Throughout the process of joining his laboratory, we discussed many aspects of my future and how laboratory experience will help me throughout my life, and for that I am so thankful. I would also like to thank Dr. Gyan Sahukhal for helping me smoothly transition into the laboratory, and for taking time out of his busy schedule to make sure that I felt comfortable and confident in the laboratory. I would also like to thank graduate student Bibek G.C. for providing further insight to any protocol I needed help with while working in the lab. I am also so incredibly thankful for undergraduate student Michael Wilson. Michael helped me tremendously throughout the research process, and I do not think I would have been able to do it without his help. Last but not least, I would like to thank graduate student Shanti Pandey. Shanti has worked tirelessly with me throughout the entire duration of my time in this laboratory. She has guided me every step of the way and has exhibited an immense amount of patience when working with me. Not to mention, she did all of this while also completing her own dissertation. Her unwavering support and patience have made this tiresome process so much easier. She selflessly committed herself to teaching and mentoring an undergraduate student with no prior experience, and I could never express my gratitude for her enough. Thank you to each of these people for always supporting and encouraging me throughout this process.

The work performed was supported by the Mississippi INBRE, Mississippi IDeA Network of Biomedical Research Excellence. Funding was provided by the National Institute of General Medical Sciences grant number P20GM103476.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CA	Community acquired
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CCPE	Catabolite control protein E
CDC	Center for disease control and prevention
CDM	Chemically defined medium
CFU	Colony-forming unit
CNS	Coagulase negative staphylococci
DNA	Deoxyribonucleic acid
HA	Healthcare associated
MHB	Muller hinton broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin-sensitive *S. aureus*
NARSA	Network of antimicrobial resistance
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNA	Ribose nucleic acid

STX	Staphyloxanthin
S. aureus	*Staphylococcus aureus*
TCA	Tricarboxylic acid
TSB	Tryptic soy broth

CHAPTER I – INTRODUCTION

1.1 *Staphylococcus aureus*

S. aureus is a gram-positive coccus, non-motile and non-spore forming bacterial pathogen that belongs to the Firmicutes phylum and Bacilli class. This bacterium is facultative anaerobe measuring 0.7-1.2 μm in diameter. *S. aureus* is the most common pathogen among *Staphylococcus* genus that causes wide arrays of infections in human. In 1882, Ogston examined pus from a surgical abscess and named these bacteria "staphylococci", derived from the Greek words *staphyle,* referring to "the bunch of grape", and *kokkos* denoting "berry", referring to the bacterial structures as observed under microscope (Ogston, 1882). In 1884, Rosenbach noted white and pigmented staphylococci. The species that showed the golden pigmented colonies was then named *S. aureus,* derived from the Latin word aurum meaning "gold" (Licitra, 2013). In 1903, Loeb observed coagulation of blood by *S. aureus* (Loeb, 1903), which is widely known as the coagulase test (clotting of blood plasma) to differentiate *S. aureus* from other coagulase negative staphylococci (CNS) (Loeb, 1903). Likewise, *S. aureus* produces a catalase enzyme, and this property is tested to differentiate the pathogen from streptococci.

S.aureus is an important human pathogen that can cause wide arrays of infection ranging from the minor skin infections to often lethal systematic infections such as osteomyelitis, sepsis, infective endocarditis and pneumonia (Lowy, 1998). *S. aureus* are also commensal bacteria found amongst 30% of the human population (Gorwitz et al., 2008; Kluytmans et al., 1997). However, underlying immunocompromised conditions or loss of skin barrier may result in *S. aureus* opportunistic infections in an individual.

1.1.1 *S. aureus* is a Health Burden Worldwide

S. aureus is a major bacterial pathogen that can cause a remarkably diverse array of infections ranging from acute skin infections to systemic and often life-threatening conditions such as endocarditis, bacteremia, osteomyelitis and pneumonia (Lowy, 1998). *S. aureus* is the prime causative agent of bacteremia and infective endocarditis (Tong et al., 2015). In addition to this, the bacteria are also a major source of osteoarticular infections, pleuropulmonary infections, skin and soft tissue infections, and surgical device related infections (Tong et al., 2015). Despite the clinical relevance, there is an absence of an effective vaccine against *S. aureus.*

The ability of *S. aureus* to cause a variety of infections is mainly due to various virulence factors, antibiotic resistance, and their ability to escape the host immunity (Foster, 2009; Johnson, 2011; Lowy, 1998). Immunocompromised individuals with underlying conditions such as diabetes, vascular diseases, cancer or the patients prescribed with hospital devices such as tips or catheters have a higher risk of getting *S. aureus* infections (https://www.cdc.gov/hai/organisms/staph.html). *S. aureus* was further reported to be the major cause of healthcare associated (HA) infections. In addition, within the past decade, community acquired (CA) *S. aureus* infections have been consistently increasing. In 2004, the methicillin resistant *S. aureus* (MRSA) strain USA300 was reported to be the most common cause (more than 98%) of the emergency cases in soft tissue infections in 11 cities in the US (Moran et al., 2006). In 2017, Centers for Disease Control and Prevention (CDC) reported >119,000 MRSA associated bacteremia cases with ~20,000 deaths in the U. S. alone (Kourtis AP, 2019).

Additionally, MRSA is also reported to be the leading cause of infective endocarditis, accounting for ~50,000 incidences per year. The annual treatment cost for MRSA infections was reported to range between 4 to 30 billion dollars in the US alone (Moran et al., 2006). Due to its ability to cause severe diseases, *S. aureus* infections result in high morbidity and mortality rates, thus imposing a high socio-economic burden worldwide (Kourtis et al., 2019). In addition to North America, CA-MRSA has also been reported in Asia, Europe, South America, and Australia (Chambers & Deleo, 2009).

1.2 Persister Cells

Persister cells are a phenotypic variant of the bacterial population that are extremely tolerant to antibiotics. This tolerance property is transient and is accompanied by a transient halt in growth. Persister cells are characterized as dormant, non- growing cells (Brian P. Conlon, 2014). When a bacterial culture is exposed to a lethal concentration of bactericidal antibiotic, a bulk of the population is killed. However, a small fraction of cells are left intact form a characteristic persister plateau. This antibiotic tolerance property gives rise to a characteristic biphasic killing curve during *in vitro* experiments (Kim Lewis, 2010). Once antibiotic treatment is discontinued, the dormant persister cells are capable of resuming growth and producing a cell population identical to that of the original (Shah et al., 2006). The population produced by the persister cells has also been found to have a similar fraction of antibiotic tolerant vs non tolerant cells to that of the original population (Iris Keren et al., 2004).

Persister cells behave in a manner that allows them to avoid antibiotic killing without genetic modification (Balaban et al., 2004). Antibiotics commonly used on *S. aureus* infections were manufactured to target the mechanisms and processes that are

active during cellular growth (Fauvart et al., 2011). Since persister cells undergo dormancy, antibiotics therefore become ineffective against this specific population (Fauvart et al., 2011).

Because persister cells contain the ability to tolerate antibiotic exposure for extended periods of time and regrow upon the removal of antibiotic treatment, persister cells are thought to be one of the major causes of reoccurring *S. aureus* infections (I. Keren et al., 2004; Wood et al., 2013). The mechanism concerning persister cell formation is not directly understood, but there is evidence that factors such as reduced ATP concentrations contribute to the antibiotic tolerance (Brian P. Conlon et al., 2016).

1.2.1 Mechanisms Underlying Persister Formation is Poorly Understood

Although the persister phenomenon was discovered more than 70 years ago, the mechanisms of persister formation in *S. aureus* are not well understood. Previously, stringent response or dormancy were regarded as the contributing factors for bacterial persistence. However, recent studies have challenged these findings such that the deletion of toxin-antitoxin systems did not decrease persister formation (Dawson et al., 2011; Harms et al., 2017; Svenningsen et al., 2019; Wood et al., 2013); making a persister switch even more of an elusive bacterial phenomenon. Therefore, understanding the mechanisms of persister formation has been extremely challenging, mainly due to their transient nature and small population size.

1.2.2 Reduced Metabolism Increases Persister Cells Formation

Recently, an increasing number of studies have examined the contribution of metabolic genes in the formation of *S. aureus* persister cells. For example, Yee et al. reported the contribution of purine genes in persister cell formation (Yee et al., 2015).

Wang et al. showed that inactivation of succinate dehydrogenase (*sdhA/B*) forms a lower population of persister cells against different stressors, including antibiotics (Wang et al., 2015). Likewise, the mutant of *phoU* showed decreased persisters due to increased metabolic activity (Shang et al., 2020). Recent studies have found that the environmental conditions that have normally been correlated with interrupting transcription, translation or the synthesis of ATP actually increase persister fraction (B. P. Conlon et al., 2016; Shan et al., 2017; Wood et al., 2013). Studies have shown that the inactivation of TCA cycle genes drastically increases persister cells ((Wang et al., 2018; Zalis et al., 2019). A decrease in ATP levels has resulted in a higher persister cell frequency during the exponential growth phase (B. P. Conlon et al., 2016; Zalis et al., 2019), while also noting that persister cell frequency is characterized by lower membrane potentials during the stationary phase or in a polymicrobial culture (Nabb et al., 2019; Wang et al., 2018). The reduced ATP level as the cause for increased persister fraction was also observed in the mutant of carbamoyl phosphate synthetase (*carB*) in the major pathogens *S. aureus, E. coli*, and *P. aeruginosa* (Cameron et al., 2018). These findings highlight the importance of metabolism in persister formation such that the inactivation of TCA enzymes result in a drastic increase in persister frequency.

1.3 Staphylococcal Virulence Factors

Virulence factors are defined as the molecules that increase the potential of a pathogen to cause disease (Heilmann, 2011). Virulence factors have been known to transfer from one strain to another through a process called horizontal gene transfer (Morikawa et al., 2012). *S. aureus* possess a plethora of virulence factors including but not limited to: the production of biofilms, capsules, pigments and proteases. Each of these

virulence factors help *S. aureus* to cause various diseases by aiding in averting the host defense systems (Otto, 2014).

1.3.1 Role of Biofilms in Antibiotic Tolerance

S. aureus biofilms are composed of a matrix of bacteria derived from extracellular polysaccharides, proteins, DNA and host-derived fibrin matrices. These structures provide environmental niches such as low nutrient level and gradient of pH (Kavanaugh & Horswill, 2016). Such unfavorable conditions provide a selection pressure for the generation of antibiotic insensitive persister cells, which are metabolically inactive with the presence of reduced ATP content (Lewis, 2008; Waters et al., 2016). Since the biofilms disperse, these infections can also result in metastatic infections (Lewis, 2001; K. Lewis, 2007).

Considering the importance of biofilms in treatment failures, several approaches have been proposed to effectively kill persisters within *S. aureus* biofilms. For example, Study by Allison et al. in a mouse catheter model showed the eradication of the persisters by potentiating the aminoglycosides drugs with the addition of metabolites such as fructose and mannitol. These metabolites were shown to enhance the gentamicin uptake via generation of proton motive force (PMF) resulting into eradication *of S. aureus* and *E. coli* biofilms (Allison et al., 2011). Likewise, L-arginine via pH mediated mechanism was also shown to facilitate biofilm eradication by gentamicin in catheter-based biofilms formed in mice (Lebeaux et al., 2014). Another approach used an energy independent synthetic acyldepsipeptide antibiotic ADEP4 that could degrade more than 400 proteins. In combination with rifampicin, ADEP4 was shown to eradicate catheter model infection in mice (Conlon et al., 2013). The undeniable link between the recalcitrance of chronic

infections and persister formation is what sparked further research into the topic of persister cells, as well as the urgency to understand the mechanisms behind their formation (Lafleur et al., 2006; Kim Lewis, 2007).

1.4 Role of the *msaABCR* Operon in Persister Cells Formation

The *msaABCR* operon comprises four genes *(msaA, msaB, msaC* and anti-sense *msaR)* were identified and characterized by the Elasri research group (Sahukhal & Elasri, 2014). Among these four genes, *msaB* encoding MsaB (also known as CspA) is the only protein coding gene. However, *msaC, is* a non-protein coding RNA that is essential to regulate the function of the *msaABCR* operon. The anti-sense *msaR* that is complementary to the 5'end of the *msaB* expresses differentially based on the growth phase of *S. aureus* (Sahukhal & Elasri, 2014). Previous studies conducted in Elasri lab have demonstrated the role of the *msaABCR* operon in biofilm development (Sahukhal et al., 2015; Sahukhal & Elasri, 2014), capsule production (Batte et al., 2018), and antibiotic resistance and tolerance in *S. aureus.* The deletion of the *msaABCR* operon was found to form decreased persister cells in both planktonic and biofilm growth conditions as compared to the wild type USA300 LAC in the presence of several clinically relevant antibiotics (Sahukhal et al., 2017).

In this study, we examine the role of *msaABCR* in TCA cycle activity. Furthermore, using the TCA mutants, we also show the importance of metabolism in virulence of *S. aureus.* Altogether, this study suggests that the *msaABCR* operon plays role in the adaptation of *S. arureus* in different environmental stressors as well as via producing several virulence factors that aid the pathogens in invading or evading the host defense systems.

CHAPTER II-MATERIALS AND METHODS

2.1 Growth Conditions and Bacterial Strains

The bacterial strains used for the study were methicillin-resistant *Staphylococcus aureus* (MRSA) USA 300 LAC, isogenic *msaABCR* operon mutant (*msaABCR*) and the complement of the operon strain (complementation). First of all, the cultures of *S. aureus* cells were prepared by inoculating cells from frozen culture. The cells from the frozen culture were placed into 5 ml fresh tryptic soy broth (TSB; Becton, Dickinson and Company, MD, USA) and incubated at 37° C with continuous shaking at a 225 rpm. After 2-3 h of incubation, cells were normalized to OD_{600} nm of 0.05 in 5 ml TSB and incubated further for 16 h to prepare the stationary phase culture. Bacterial strains used in this study are listed in Table 3.2.

The production of the *msaABCR* operon deletion in USA300 LAC achieved through the allelic replacement method as previously described (Batte et al., 2016; Sahukhal & Elasri, 2014). The ORF of the *msaABCR* operon region was then cloned into the pCN34 low-copy-number vector for trans-complementation. For this process, the kanamycin selectable marker was changed to a chloramphenicol resistance marker.

2.2 Generation of Transposon Mutants

The plasmid-cured derivatives of the LAC strain JE2 carrying transposon mutations in the specific coding regions of the desired genes were acquired from the Network of Antimicrobial Resistance in the *S. aureus* (NARSA) collection. The mutations were transferred to USA300 LAC strain or UAMS-1 strain through the generalized transduction process. The bacteriophage φ11 was utilized in this process. Through amplification of the beginning and end of the open reading frame (ORF) for the

designated genes, the transduction of the mutation into the desired strains was confirmed by end-to-end PCR as described previously (Batte et al., 2016; Pandey et al., 2019).

2.3 Persister Assay

The pre-cultures prepared as mentioned above were normalized to OD_{600} nm value of 0.05 in 5 ml of TSB at a flask-to-medium ratio of 10:1. After normalization, the cultures were then incubated at 37° C until stationary growth phase (16 h). The cultures were standardized in 3 ml of TSB. For the measurements of initial colony forming units (CFUs) count, 100 µl of the standardized culture was withdrawn before adding antibiotics, and serial diluted in 900 µl of 1 × phosphate buffer saline (PBS) and plated on the tryptic soy agar (TSA) plates. The rest of the cultures were then exposed to gentamicin (20 µg/ml, 20 × MIC). Then the cultures were incubated at 37° C with continuous shaking. At the designated time after exposure to the antibiotic, 100 µl of each sample was removed and washed with 900 µl of 1× PBS. These samples were then plated and incubated for 24 h to obtain final CFUs count.

Stationary phase cells were exposed to 10 µM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or different concentration of fumarate (15 mM, 30mM and 50 mM) for a thirty minute before exposure to gentamicin. At 24 h post exposure, 100 µl of cells were collected, washed, and plated to obtain a value for the CFUs.

2.4 Measurement of pH and acetate

Cells grown until stationary phase in 5 ml TSB as mentioned above were centrifuged and the supernatant was transferred to new tube. Then pH in the supernatant was measured by Accumet with Fisher brand pH meter. Similarly, cells grown to stationary phase were centrifuged and the supernatant was preserved in -80°C to measure

the acetate accumulation. The acetate was measured in the preserved culture supernatant according to the manufacturer instruction (R-biopharm, Washington, MO).

2.5 RNA Extraction, Reverse Transcription, and qRT-PCR

From the appropriate growth cultures, 500 µl amounts were treated with RNAprotect bacterial agent (Qiagen, Valencia, CA) for 5 minutes at room temperature. Extracting of the total RNA was done through the use of the Qiagen RNeasy kit (Qiagen), and the extracted RNA was analyzed with the NanoDrop spectrophotometer (Thermo-Scientific) in order to observe the quality and concentration of the extracted RNA. The iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) was used for reverse transcription. This process was done by using 1 µg of the total RNA according to the instructions provided by the manufacturer. SYBR Green supermix (Bio-Rad) was used to do qRT-PCR. The relative fold change for gene expression was then calculated through the use of *gyrB* as an endogenous control gene. The primers used in this study are listed in table 3.2.

2.6 Measurement of ATP

The BacTiter-Glo kit (Promega, WI, USA) was used to measure the value for intracellular ATP content. At the designated phase, the cells were adjusted to an OD_{600} nm of 0.025 in 1 ml of TSB. From there, 50 µl aliquots were transferred into the wells of an opaque 96-well plate and mixed with the BacTiter-Glo Reagent. The resultant luminescence was measured at OD_{560} nm after a 5 minute incubation at room temperature.

2.7 Measurement of Membrane Potential

The BacLight Bacterial Membrane Potential kit (Life Technologies, USA) was used to examine membrane potential. The cells when reached stationary phase were washed and normalized to OD_{600nm} of 1.0 in 0.5 ml of filtered PBS. After resuspension, 16.6 µl of fluorescent membrane potential indicator dye 3,3'- diethyloxacarbocyanine iodide ($DIOC_2(3)$) were added and then incubated for 30 minutes at room temperature. The fluorescent signals were recorded for 100,000 cells through the use of a LSRFortessa flow cytometer (BD Biosciences, CA, USA). The negative control cells were incubated in 10 µm CCCP for 30 minutes before adding ($DIOC_2(3)$). The ratio for red fluorescence (PE-A) to green fluorescence (FITC) was calculated and analyzed through the use of FlowJo v10 software.

2.8 Statistical Analysis

The GraphPad Prism software was used to analyze the data. Specifically, the student's t-test (unpaired) or one-way analysis of variance (ANOVA) accompanied by Tukey's multiple comparison test with a *p* value of less than 0.05 being considered as statistically significant.

CHAPTER III-RESULTS

3.1 Measurement of growth kinetics and minimum inhibitory concentration (MIC) values

Before conducting persister assays, we measured the growth kinetics of the mutant of TCA genes citrate synthase (encoded by *gltA*), succinate dehyderogenase (*sucA*) and fumarate hydratase (*fumC*) in the nutrient rich medium TSB. To represent the early, middle and late genes in the TCA cycle, these genes were chosen in this study. The results show that although the exponential growth was similar to the wild type USA300 LAC, the *gltA* and *fumC* mutant showed reduced growth rate as compared to the wild type USA300 LAC measured in the stationary phase (Fig. 3.1). Considering the clinical relevance of antibiotic tolerance, in this study we measured the persister frequency in the presence of aminoglycoside drug gentamicin. To determine the MIC values, standard micro-broth dilution method was followed. Deletion of the *msaABCR* operon as well as the TCA genes *gltA, sucA* and *fumC* did not change the susceptibility of *S. aureus* towards gentamicin (Table 3.1).

Table 3.1 Minimum Inhibitory Concentration (MIC) value of gentamicin

Strains	Minimum Inhibitory Concentration of gentamicin (μg/ml)
USA300 LAC	1
msaABCR	1
Complementation	1
gltA	1
sucA	1
fumC	1

Considering the importance of growth of bacterial cells in persister formation, we also examined the growth kinetics of all the strains in the nutrient rich medium TSB in the absence of antibiotics. The results show that the inactivation of *gltA* and *fumC* reduced the growth rate in USA300 LAC while *sucA* showed similar growth kinetics as comparable to the wild type (Fig. 3.1).

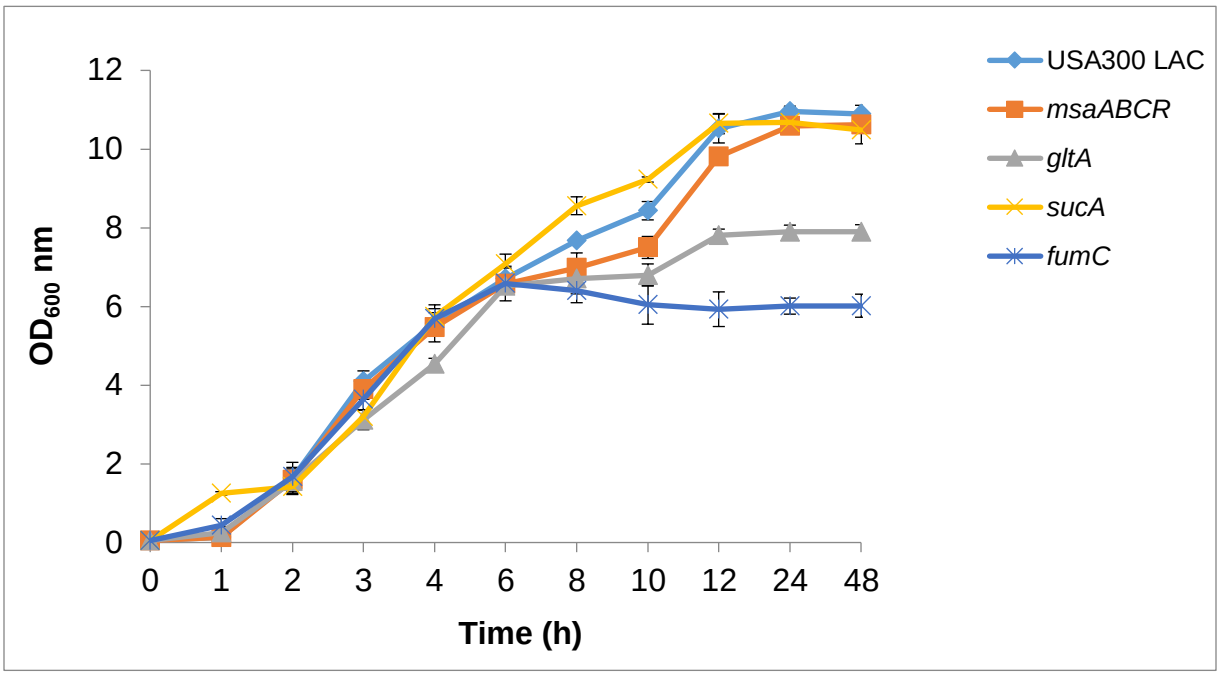

Figure 3.1 Measurement of growth kinetics

Growth rate measurement in nutrient rich TSB medium until the stationary-phase of growth (48 h). The normalized cultures were incubated at 37° C and the absorbance of the culture was taken at OD600 nm each hour. Data represents the OD600 nm readings at the designated time points. Error bars represent standard error (SE) of the mean of the three replicates.

3.2 The *msaABCR* mutant has higher TCA cycle activity and the cellular ATP level

Previous study showed the involvement of the *msaABCR* operon in persister cells formation. Given that the persister cells is associated with the depletion of energy, we

hypothesized that the deletion of the *msaABCR* operon in *S. aureus* cells results in increased metabolic activity resulting in decreased persisters in the *msaABCR* mutant. To test this, we measured the TCA cycle activity by measuring the expression of the TCA genes. Since, the TCA cycle is activated during the late exponential phase to generate ATP when glucose is depleted; we measured the expression of TCA genes citrate synthase (*gltA),* aconitate hydratase *(acnA),* isocitrate dehydrogenase *(icd), 2-oxoglutarate (sucA),* succinyl-CoA synthetase (*sucD),* succinate dehydrogenase *(sdhA/B),* and *f*umarate hydratase *(fumC*) during this growth phase. The qRT-PCR results show increased expression of these genes in the *msaABCR* mutant compared to the wild type USA300 LAC (Figure 3.2A). Furthermore, we also measured the ATP and found that the *msaABCR* mutant contains higher ATP levels relative to the USA300 LAC strain in the late exponential growth (Figure 3.2B).

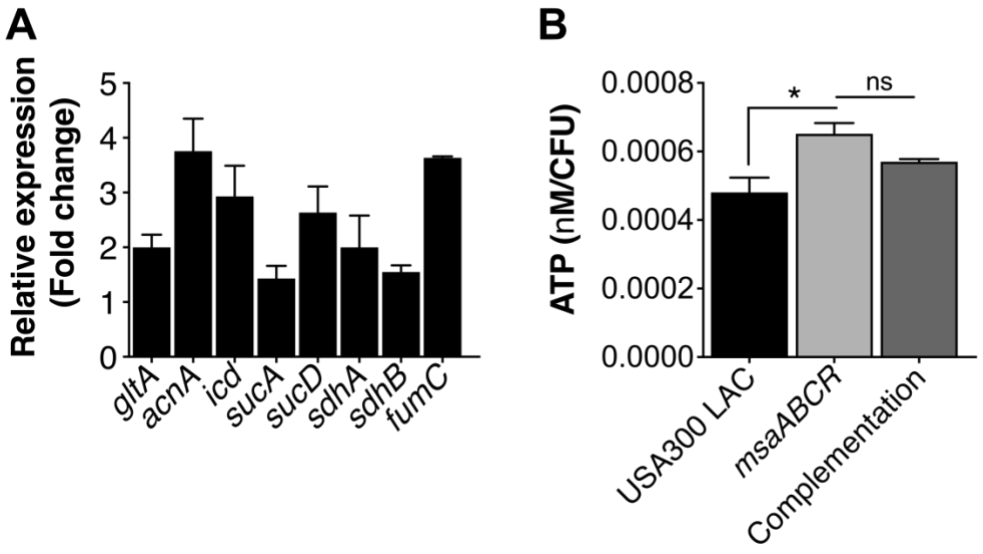

Figure 3.2 Measurement of TCA cycle activity and intracellular ATP level

Fold change expression of TCA genes (*gltA, acnA, icd, sucA, sucD, sdhA/B, and fumC*) in late exponential growth phase for the *msaABCR* mutant compared with the USA300 LAC strain, as measured by qRT-PCR.

(B) The ATP concentrations per CFU of all strains in late exponential phase. The data represent the average of three measurements with two biological replicates. Error bars represent the standard error of the mean (SEM) for three independent experiments. Statistical significance was determined using analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *, $P \leq 0.05$; ns, not significant.

3.3 Inactivation of TCA genes increases persister formation

Since, we observed increased expression of TCA genes in the *msaABCR* mutant, we sought to examine the role of the TCA genes in antibiotic tolerance. For this, we performed the persister assay in mutants of the TCA cycle genes *gltA*, *sucA* and *fumC* in USA300 LAC background in the presence of gentamicin. Consistent with the previous studies, the results showed that the inactivation of TCA genes drastically increases the persister formation in USA300 LAC (Fig. 3.3).

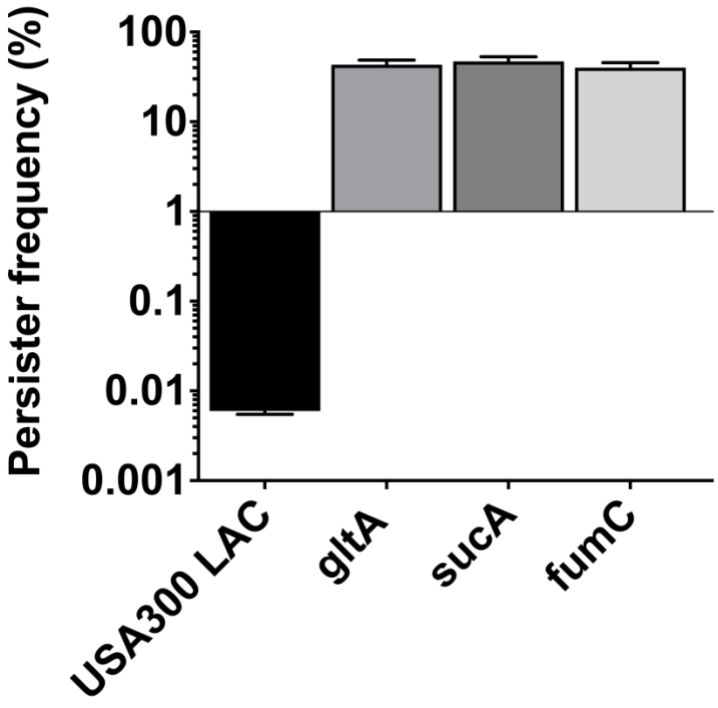

Figure 3.3. Persister assay in TCA mutants in the stationary phase of growth.

Cells grown to stationary phase (16 h) were standardized and were exposed to antibiotic gentamicin (20 × MIC). At 24 h post exposure, cells were harvested, washed in PBS and serially diluted and plated for the persister count. The data represent the fraction of surviving cells as compared to the initial count (before adding antibiotic). Error bars represent the mean of the standard error (SEM) of at least three replicates.

These results show the importance of metabolic genes in the persister formation such that the inactivation of TCA genes lead to increased persister formation.

3.4 Addition of fumarate increases persister formation

Previous studies have shown variation in persister formation in *E. coli* and *P. aeruginosa* by intracellular fumarate concentration (Kim et al., 2016; Meylan et al., 2017). To test whether fumarate increases the persister formation in *S. aureus* cells, we measured the persister frequency in presence of exogenously added fumarate. Our results show that addition of fumarate increased the persister frequency in dose dependent manner both in wild type and *msaABCR* mutant (Fig. 3.4).

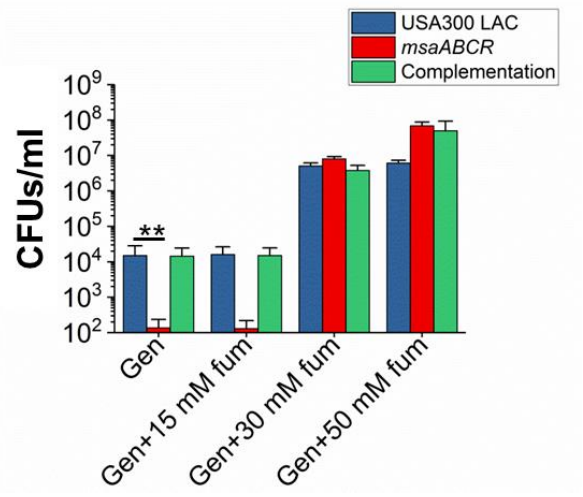

Figure 3.4 Exogenously added fumarate increases the persister frequency

Cells grown to the late exponential phase were divided into two tubes (3 ml each) and exposed to gentamycin and gentamycin with fumarate. The persister frequency was estimated by taking c.f.u counts after 24 h of treatment. Data represent average of three independent experiments. Error bars represent SE. Statistical significance was determined using students t-test to compare the result of *msaABCR* mutant to the wild type. **, $P \leq 0.005$.

3.5 The TCA mutants show decreased pH accompanied by increased acetate content in the stationary growth phase

In *S. aureus*, the TCA cycle is activated when glucose is depleted, and acetate is being catabolized. Deletion of TCA genes therefore obstructs the catabolism of acetate that is accumulated during the exponential phase of growth. As expected, we observed that the culture supernatants of the TCA mutants displayed lower pH as compared to the wild type strain (Fig. 3.5A).

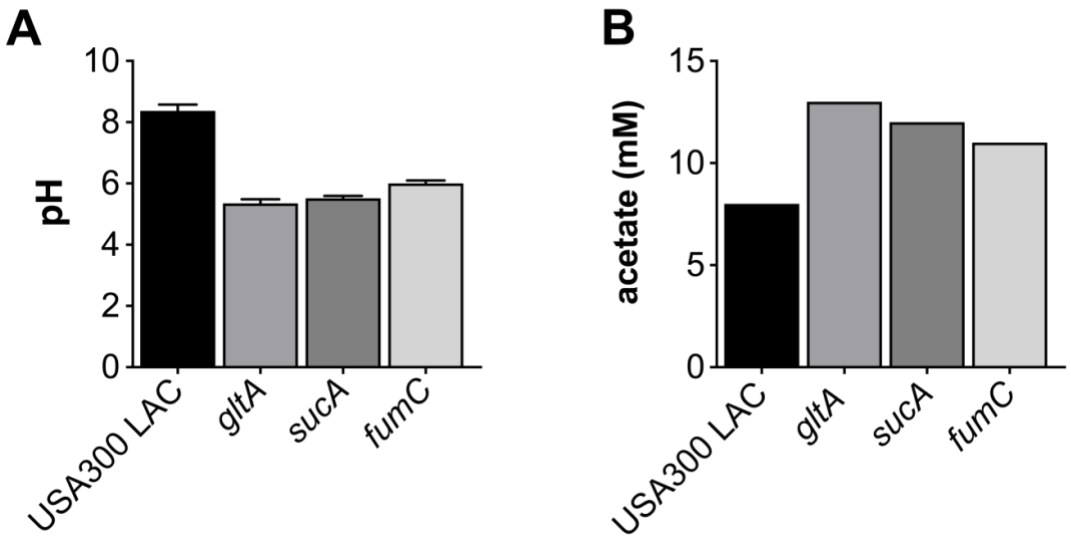

Figure 3.5 Measurement of pH and acetate accumulation in the stationary phase growth

(A) Cells grown in 5 ml TSB to stationary state were centrifuged and supernatant was used to measure the pH. Error bars represent the SEM of pH of the three biological replicates. (B) Acetate was measured in the stationary growth phase of each strain following the manufacturer instruction.

This result led us to examine the acetate accumulation and the results showed that the TCA mutants indeed have increased levels of acetate in the culture supernatant as compared to the wild type USA300 LAC (Fig 3.5 B).

3.6 Decreased pH does not influence persister formation

Since we observed decreased pH in the culture supernatants of the TCA mutants, we further sought to examine whether the increased persister fraction is associated with the acidification of the bacterial culture. To examine this, we used the TSB medium without glucose that would give the comparable pH in the TCA mutants. The absence of glucose as resulted in the similar pH with the wild type strain (Fig. 3.6 A), however, the persister frequency in the TCA mutant *gltA* and *fumC* was still increased and similar to that formed in the TSB medium (Fig. 3.6B).

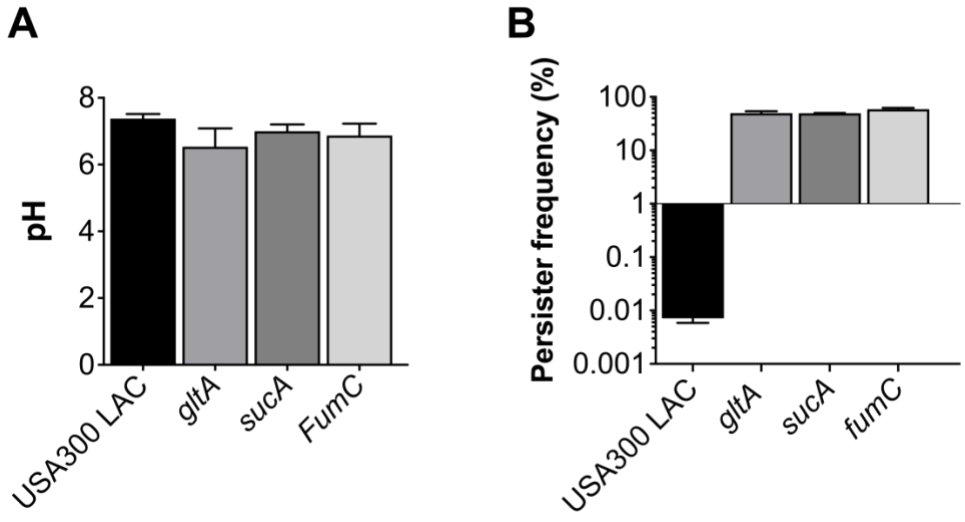

Figure 3.6 Measurement of pH and persister frequency in the glucose free medium

(A) The pH was measured in the supernatant of culture grown for 16 in the glucose free medium. (B) Persister fraction was measured in the stationary state cells grown in glucose free medium. Error bars represent the SEM of three biological replicates.

These results suggested that the decreased pH in the culture media does not contribute to the increased persister fraction in the TCA mutants.

3.7 Reduced ATP level does not confer persister cells formation

In *S. aureus*, increased fraction of persister cells is related to the reduced level of ATP. To examine if the increased persister cells in the TCA mutants is associated with reduced ATP, we measured the ATP content in the TCA mutants. Among the TCA mutants, *gltA* did not differ from the wild type strain while *sucA* and *fumC* mutants showed significantly reduced ATP level (Fig. 3.7). Therefore, these results suggest that ATP alone does not determine the persister frequency in *S. aureus.*

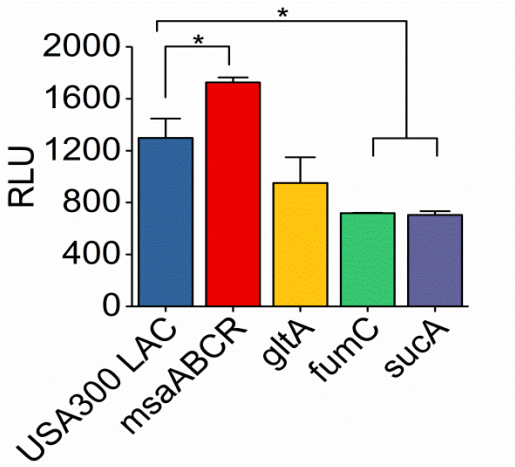

Figure 3.7 Measurement of ATP

ATP level of stationary cultures was tested with BacTiter-Glo™ Microbial Cell Viability Assay kit. Aliquots of 50 µl bacterial suspension in TSB was carefully mixed with equal volume of BacTiter-Glo™ Reagent and incubated at room temperature for 5 minutes before the luminescence was recorded with emission at 560 nm. The data represent the average of three independent RFU value per CFUs of three

biological replicates for each individual sample. Error bars represent SE. Student's t-test was used to compare the results of the wild-type to mutants.

3.8 Membrane potential contributes to the persister formation in TCA mutants

A previous study showed increased persister formation in the TCA mutants is caused due to decreased membrane potential (Wang et al., 2018). To examine this association, we measured the membrane potential in our test strains. We measured the membrane potential via flow cytometry using the fluorescent membrane stain $DiOC_2$ (3) and compared with the USA300 LAC strain. The results showed lower membrane potential in the TCA mutant *fumC* than in the USA300 LAC strain (Fig. 3.8A), suggesting that the reduced membrane potential increases the persister cells formation in the TCA mutant. To further confirm the role of membrane potential in persister formation, we measured the persister fraction in the presence of the proton ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a known inhibitor of PMF generation that dissipates H^+ ion gradients. In the presence of CCCP, the wild type USA300 LAC as well as the *msaABCR* mutant both displayed drastic increase in persister fraction (Fig 3.8B). Altogether, these results suggest that the reduced membrane potential contributes to the increased persister formation in *S. aureus* in the presence of gentamicin.

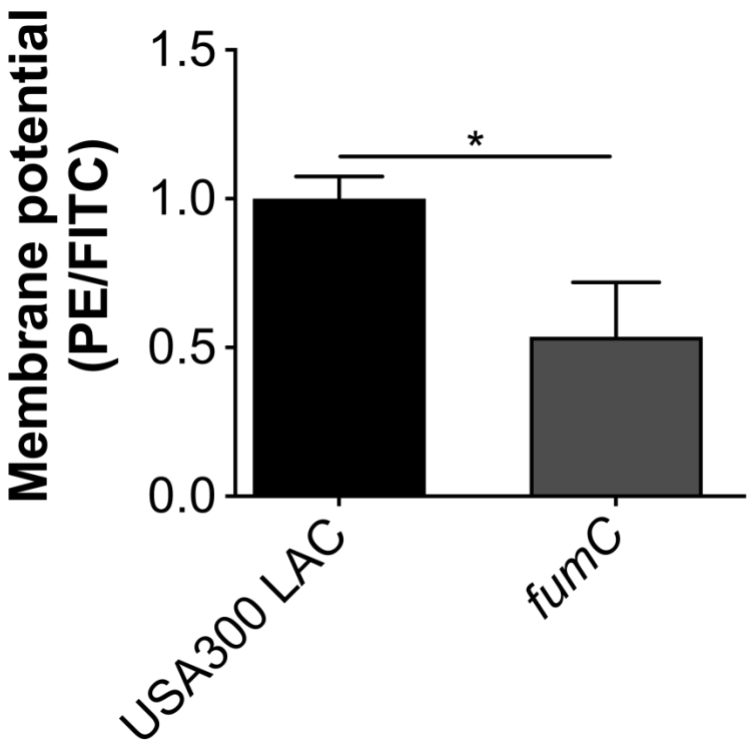

Figure 3.8A Measurement of membrane potential in wild type USA300 LAC and TCA mutant *fumC*.

Stationary-phase cells washed twice with PBS were incubated with 3, 3′-diethyloxacarbocyanine iodide ($DiOC_2(3)$) for 30 min in room temperature. After incubation, the cells were subjected to flow cytometry. The data represents the ratio between channel F3 (red fluorescence) and F1 (green fluorescence), calculated with FlowJo software.

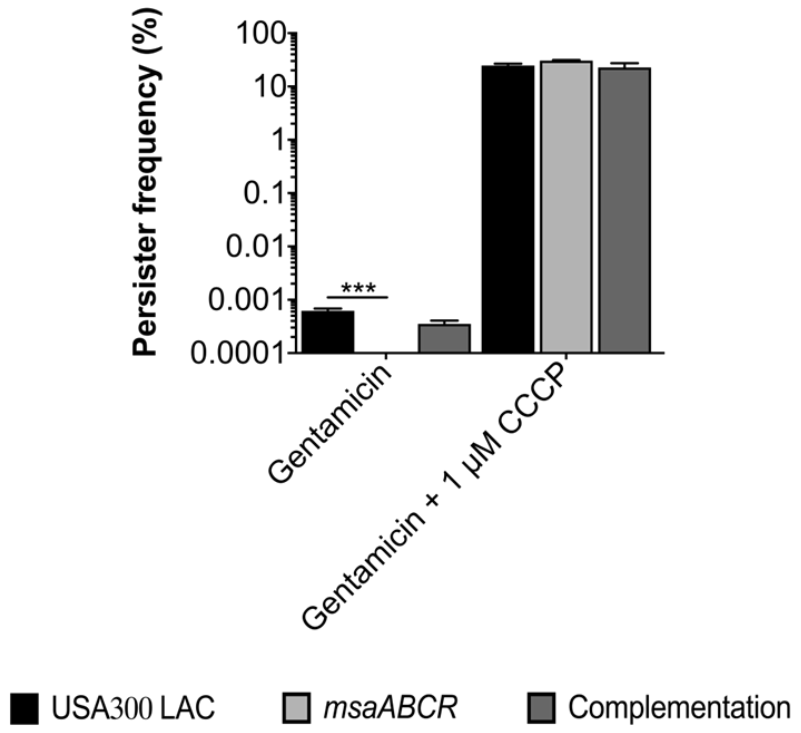

Figure 3.8B Measurement of membrane potential and persister formation in the presence of CCCP

Stationary phase cells pre-incubated for 30 min with different concentrations of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) were exposed to gentamycin (20 µg/ml). At 24 h post exposure, the cells were harvested, washed, and plated for CFU counting. Data represent the percentage of cells survived after antibiotic exposure to the initial population. Data represent the average of three biological replicates. Students t-test was used to compare the results of the *msaABCR* mutant to the wild type. *** p <0.0001.

Table 3.2 List of strains and primers used in this study

Strains	**Description/Genotype**	**Source**
S. aureus USA300 LAC strain	Methicillin resistant	Dr. Lindsey Shaw
msaABCR	*msaABCR* operon deletion mutant in USA300 LAC	(Sahukhal & Elasri, 2014)
Complementation	*msaABCR* complement in *msaABCR* mutant LAC strain	(Sahukhal & Elasri, 2014)
gltA	SAUSA300_1641	This study
sucA	SAUSA300_1306	This study
fumC	SAUSA300_1801	This study
Primers for qRT-PCR	**Sequences (5' ⟶ 3')**	
gltA F	AAATCGTTATGAAAGAGCAATG	
gltA R	GGATTAGGCTTAAGTGGTTCTT	
acnA F	ATAGTGTTGTAACACCTGAATTAT	
acnA R	CATATAGAGGTTGATCAGTTACAT	
icd F	ATTGCTGACATTTTCTTACAAC	
icd R	AGCATCTGAAATATAGTCACCA	
sucA F	CAGATGACGTTGAAGCTACTAT	
sucA R	CGACGATAACCTACTAAATCAA	
sucD F	TTGACTGAAGAAGGTATTGGTC	
sucD R	TTGACTGAAGAAGGTATTGGTC	
sdhA F	TGAAATTATGACAGCAAATGTAAC	
sdhA R	CAATATCTTCATAACGTTTCATCA	
sdhB F	TTCTATGGTTATCAATGGTCGT	
sdhB R	TAACTGGGAAAGTATTCATTGG	
fumC F	TCGAAGTAGTTTATGGTTTTGC	
fumC R	CGTATACAATGGCATCTTTCTT	

CHAPTER IV- DISCUSSION

Persister cells are transiently tolerant to the antibiotics and can resume the growth when the antibiotic treatment is ceased. This property of the persister cells is strongly associated with the recurrence of infections that signify its clinical relevance. In exponential phase, persister cells are produced in smaller fraction while the frequency increases as the culture enters into the stationary phase (B. P. Conlon et al., 2016). Similarly, biofilms provide a favorable niche for the generation of slowly growing, energy depleted antibiotic tolerant persister cells. Given that the *S. aureus* chronic infections are mainly biofilm-associated, understanding the mechanisms underlying the formation of the persister cells is extremely important. In the last two decades, an increasing number of studies have focused on the mechanisms of persister formation. However, understanding the persister phenotype has been extremely challenging because of its small population size and transiency.

In this study, we demonstrate the role of the *msaABCR* in energy generation in *S. aureus*. Likewise, using the mutants of TCA cycle, we show the importance of metabolism in persister cells formation. The slowly growing *S. aureus* cells are more tolerant to antibiotic treatment due to the target inactivation that is caused due to low-energy state (K. Lewis, 2007, 2010). The reduced energy level is indeed associated also with the rare persister population that is generated in the exponential growth phase (Cameron et al., 2018; B. P. Conlon et al., 2016; Shan et al., 2017). These persister cells display low levels of expression of TCA enzymes (Zalis et al., 2019). In our study, we show that the deletion of the *msaABCR* operon in *S. aureus* was defective in persister formation, whereas there was an increased persister fraction among the TCA cycle mutants. These results suggested that the decreased metabolic activity corresponds with increased persister frequency.

The persister fraction is influenced with the reduced growth rate. In our study, although the *gltA* and *fumC* mutants exhibited reduced growth kinetics, the *sucA* mutant showed similar growth kinetics as comparable to the wild type USA300 LAC. These results indicate that the slow growth of the TCA mutants, *gltA* and *fumC*, did not determine the enhanced persister frequency in those mutants.

To correlate the importance of ATP with persister frequency, we measured the ATP level in the tested strains. Although the *sucA* and *fumC* mutants showed reduced ATP content as compared to the USA300 LAC, the *gltA* mutant showed similar level of ATP as with the wild type strain. Likewise, we observed a drastic increase in antibiotic tolerance and reduced intracellular ATP levels with other TCA mutants *sucA* and *fumC*. Our findings from this study indicate that the cellular energy is required for the killing efficiency of bactericidal antibiotics. However, multiple factors aside from ATP appear to cause the antibiotic tolerance. Altogether, these results suggested that the reduced ATP is not the only deterministic factor for persister formation. Previous studies showed that the persister formation in the TCA mutants are caused due to reduced membrane potential against ciprofloxacin (Wang et al., 2018). Consistent with this, we also found that the TCA mutants exhibited reduced membrane potential as compared to the wild type strains. It is known that the proton motive force (PMF) is required for the uptake of gentamicin inside the bacterial cells. The increased uptake correlates with the increased killing of the cells. Therefore, reduced membrane potential in the TCA mutants may have resulted in increased persister fraction in the presence of gentamicin.

Previous studies have demonstrated the association between persister formation and membrane potential. For example, enhanced membrane potential led to decreased persister fraction with enhanced uptake of aminoglycoside antibiotic gentamicin (Allison et al., 2011; Barraud et al., 2013; Shan et al., 2015). Similarly, decreased membrane potential was regarded as the major cause for increased persister frequency in the TCA mutants of *S. aureus* (Wang et al., 2018). The small-colony variants of *S. aureus* display increased resistance towards aminoglycosides due to their low membrane potential (Kriegeskorte et al., 2014). Similarly, disruption of *serA* gene led to increased membrane potential and subsequently decreased persister frequency (Shan et al., 2015). A previous study demonstrated TCA intermediate metabolite fumarate mediated the antibiotic persistence in *E. coli* (Kim et al., 2016). Consistent with these findings, in our study too, we observed increased persister formation in the presence of exogenously added fumarate. However, the mechanism underlying this association is not clearly understood. It is, however, speculated that the accumulated fumarate may cause

the reduction in generation of PMF thereby increasing the persister fraction in the presence of gentamicin. We further need to examine this association in the presence of other antibiotics.

Another study also showed increased antibiotic tolerance caused due to reduced ATP and membrane potential in *S. aureus* cells when grown in a polymicrobial culture (Nabb et al., 2019). Despite these clues, the complete mechanism underlying persister formation based upon the membrane potential is not understood. Given that the persister cells are of tremendous clinical significance, understanding the mechanisms of persister cells is important. *S. aureus* systemic infections that is mainly associated with biofilms, which are difficult to treat. So far, eradication of persisters by aminoglycosides has been achieved in conjunction with the metabolites mannitol and fructose, by an ATP-independent acyldepsipeptide antibiotic (ADEP4), by drugs in combination with reactive oxygen species, and by membrane disruption-mediated clearance in different bacterial systems (Allison et al., 2011; Briers et al., 2014; B. P. Conlon, 2014; Conlon et al., 2013; Cui et al., 2016). However, clinical efficacies of these strategies have yet to be elucidated.

Gentamicin is a clinically relevant antibiotic to treat *S. aureus* infections. Likewise, the evidences show that the drug has been used in many infections without any adverse side effects (Pallagrosi, 1973; Richards et al., 1971). In addition, gentamicin treatment in combination with other drugs has been found to shorten the treatment duration (Tsuji & Rybak, 2005). These findings suggest that gentamicin is a potential antibiotic to treat the *S. aureus* infections. Therefore, we examined the persister cell formation in *S. aureus* strains in the presence of gentamicin stress. Since the deletion of the *msaABCR* operon led to a deficiency in persister formation in the presence of gentamicin, this study indicates that the *msaABCR* operon is a potential target to eradicate the persister cells and overcome treatment failures. Likewise, since the disruption of metabolic genes drastically increases persister frequency, future studies focusing in understanding the complete role of metabolism in the antibiotic tolerance in different bacterial species would be remarkable to overcome the treatment failures.

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