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Interregulation between msaABCR operon and ccpE to determine Staphylococcal metabolism and virulence

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by

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ABSTRACT

Staphylococcus aureus is a complex human pathogen that causes problems in both healthcare and community settings. *Staphylococcus* aureus is a gram-positive, sphere shaped bacterium that usually colonizes in the nasal cavity of healthy individuals. *Staphylococcus aureus* infections are a growing health concern due to its ability to produce virulence factors, such as pigmentation, protease production, and capsule formation. Two regulators in *S. aureus* virulence factors are *msaABCR*, which is a newly characterized operon, and *ccpE*. In order to determine the interaction between these two regulators in regulating virulence and metabolism in *S. aureus*, *ccpE* and *ccpE*/*msaABCR* transposon mutants were constructed. The ability for *S. aureus* to survive and adapt is due to its capability to reutilize acetate via TCA pathway, produce pigment and protease, and survive without access to nutrients and oxygen. Various assays such as aconitase activity assay, pigmentation assay, protease assay, and survival assay were performed in these strains and were compared to wild type and *msaABCR* mutant strains. Results from aconitase activity assays showed that *msaABCR* represses aconitase activity most likely via *ccpE*. Whereas, pigmentation assays and protease activity assays showed that *msaABCR* operon activate pigment production and repress protease expression independent of *ccpE*. In addition, we showed the role of *ccpE* in the stationary phase survival of *S. aureus*. This study thus elucidated the complex regulatory network of *ccpE* and *msaABCR* to determine virulence in *S. aureus*.

Keywords: Staphylococcus aureus, msaABCR, ccpE, virulence factors, metabolism

DEDICATION

To my family, especially my father, John, and mother, Christy

Without all of your support and encouragement, this along with the past four years of rigorous coursework would not have been achieved. I am so grateful for all that you all have done for me.

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LIST OF ABBREVIATIONS

Acetyl- CoA	acetyl-coenzyme A
AckA	acetate kinase
agr	accessory gene regulator
AIP	auto-inducing peptide
ATP	adenosine 5'-triphosphate
CidC	pyruvate oxidase
CFU	colony forming unit
F-6P	fructose 6-phosphate
Glc-6P	glucose 6-phosphate
KEGG	Kyoto Encyclopedia of Genes and Genomes
MRSA	methicillin-resistant *Staphylococcus aureus*
msa	modulator of *sarA*
NADH	nicotinamide adenine dinucleotide + hydrogen
OD	optical density
PEP	phosphoenolpyruvate
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
PTA	phosphotransacetylase
sarA	staphyloccal regulator A
S. aureus	*Staphylococcus aureus*
ScpA	staphopain A
ScpB	staphopain B
SCV	small colony variant
TCA	tricarboxylic acid
TSA	tryptic soy agar
TSB	tryptic soy broth
VRSA	vancomycin-resistant *Staphylococcus aureus*
WT	wild-type

INTRODUCTION

Staphylococcus aureus is a human pathogen that leads to numerous infections, both on the superficial level and the systemic level (*1*). *Staphylococcus aureus* infections are a growing concern to human health due to their high morbidity and mortality rates in healthcare and community settings. A major issue when treating *S. aureus* infections is the ability for the pathogen to acquire resistance to antibiotics (*1*). One of the most prevalent strains of *S. aureus* is the methicillin-resistant *S. aureus* (MRSA). MRSA can be spread in a multitude of settings, such as hospitals, other healthcare facilities, and the daily community life. The ability for *S. aureus* to form biofilms is a major reason why *S. aureus* is antibiotic resistant (*2*). Biofilms are vigorous, adherent multicellular communities that allow for *S. aureus* to live on both abiotic and biotic surfaces. These biofilms will allow *S. aureus* to evade a host's immune system and are able to tolerate many unfavorable environmental stressors, such as chemical detergents and disinfectants (*3*).

The reason why *S. aureus* has the ability to infect a variety of tissues and is so hard to fully treat is attributed to the ability of producing diverse virulence factors. *Staphylococcus aureus*'s virulence factors include carotenoid pigment, capsule, biofilms, hemolysins, leukocidins, proteases, enterotoxins, exfoliative toxins, and immune-modulatory factors (*4*). *S. aureus* can produce Staphyloxanthin (STX), which is a golden carotenoid pigment that acts as an important virulence factor due to its antioxidant properties. STX can improve *S. aureus*'s antioxidant properties and its resistance to neutrophils (*5*). The ability for *S. aureus* to form capsules, another virulence factor of the bacterium, is important in the pathogenesis of its infections. The staphylococcal capsule

enhances virulence by hindering phagocytosis, which leads to persistence of the bacterium in the infected host's blood stream (*6*). The virulence of *S. aureus* is determined by proteins in its cell wall and the toxins that are produced during the growth phase of the bacterium. Virulence factors of *S. aureus* allow the bacterium to adapt to a multitude of environmental conditions rapidly and specifically, and therefore allow it to develop infection (*7*).

In previous studies, our lab group has identified the operon *msaABCR*, which was found to be involved in the regulation of *S. aureus* virulence factors, biofilm development, and antibiotic resistance (*1, 8-12*). Previous studies on this operon have shown that the whole *msaABCR* operon is transcribed to *msaABCR* transcript, which encodes two non-coding RNAs, which are *msaA* and *msaC*, and the antisense RNA *msaR*. Only the protein coding gene *msaB* is shown to produce MsaB protein, also known as CspA protein. MsaB protein is shown to function as a transcriptional factor as well as an RNA chaperone. All of these other genes are essential in order for the regulation of *msaB* expression (*1*).

Another key regulator of *S. aureus* virulence factors is the catabolite control protein E (CcpE). CcpE is a transcriptional regulator of the tricarboxylic acid cycle (TCA cycle). CcpE is an important metabolic sensor that permits *S. aureus* to sense and adjust its metabolic state and then coordinate expression of virulence factors. The *ccpE* gene is a main regulator for carotenoid production, tricarboxylic acid cycle activity, and the virulence of *S. aureus* (*13*).

In our previous studies, *msaABCR* operon is shown to repress *ccpE* expression (*10, 14*) . In addition, MsaB protein is shown to bind to the promoter region of the *ccpE* gene (*10, 14*). Based on these evidences, we hypothesized that *msaABCR* and *ccpE* interact to regulate virulence and central metabolism in *S. aureus*. In this study, we aim to determine the interaction between these two regulators in regulating TCA cycle activity and virulence in *S. aureus*.

LITERATURE REVIEW

2.1 Background of *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium that is mutualistic by colonizing in the nasal cavity and skin of healthy individuals. *Staphylococcus aureus c*an cause a wide range of diseases and infections that can be systemic and fatal. Infection can occur following implanted device contamination and is most common in individuals that are immunocompromised (*7*). *Staphylococcus aureus* causes various suppurative diseases, food poisoning, pneumonia, and toxic shock syndrome. It also can cause serious issues via nosocomial infection in hospitals and other healthcare settings, especially the methicillin-resistant strain of *S. aureus* (MRSA) (*4*).

MRSA is a strain of *S. aureus* that is resistant to several antibiotics. MRSA most often causes skin infections, but in more serious cases, can cause pneumonia and blood stream infections. If MRSA is left untreated, the infection can become very severe and lead to sepsis. Beginning in the 1990s, the prevalence of MRSA strains has significantly increased and now accounts for one third of all *S. aureus* infections worldwide (30). Another strain of *S. aureus* that causes issues in treating the pathogen is vancomycin-resistant *Staphylococcus aureus* (VRSA). Vancomycin is a glycopeptide antibiotic that is widely used by health care workers to treat MRSA infections and the amount of VRSA strains that have infected people has increased (*7, 11*). *S. aureus*'s way of being able to be resistant to antibiotics, adapt to many different environments, and cause numerous and diverse amount of diseases make it a very successful, dangerous, and important human pathogen.

2.2 Genetic virulence regulation of *S. aureus*

The ability of *S. aureus* to infect a wide variety of tissues and cause numerous diseases is due to its expression of virulence factors that allow the bacterium to adapt and survive in different types of conditions. *S. aureus*'s virulence factors are categorized as surface-associated proteins, secreted proteases, toxins, and immune modulators. Expression of these virulence factors in *S. aureus* is precisely coordinated by a wide variety of genetic regulators. Understanding how virulence is regulated is extremely important for finding ways to effectively prevent and treat *S. aureus* infections.

In order for *S. aureus* to successfully adapt, it utilizes many genetic regulators that synchronize the release of certain virulence factors according to the state of the external environment. Some of these genetic regulators include stress response sigma factor B (*sigB*), staphylococcal accessory regulator A (*sarA*), and the accessory gene regulator (*agr*) (*15*). There are signals that are known to release a regulatory response which includes changes in the pH, carbon dioxide levels, cell density, and the concentration of auto inducing peptides (AIPs) (*16*). The two most consistent global regulators to show fundamental roles in the virulence regulation of *S. aureus* are *agr* and *sarA*.

The *agr* locus was first described by Peng et. al (1988) (*17*) and is found to be widespread in staphylococci. The *agr* operon in *S. aureus* directly regulates virulence genes using a two-component system that employs quorum sensing to recognize and respond to the shifts in cell density. A functional *agr* operon allows *S. aureus* to evade

host defenses, spread within the host, and degrade host cells and tissues (*16*). The *sarA* operon was first identified by Cheung et. al (*18*) and was found to affect the expression of exoproteins and surface proteins. It has been shown that the major regulatory DNA binding protein SarA, is an *agr*-independent regulator of transcription of nearly 100 genes, many of which are associated with virulence (*12, 19, 20*).

One of the important virulence factors in *S. aureus* is the Staphyloxanthin (STX). The carotenoid produced by *S. aureus,* STX, has an important role in the fitness, oxidative response and host immune response of the bacterium. The first step in the STX-biosynthetic pathway is catalyzed by the dehyrdosqualene synthase, CrtM. This results in the formation of dehydrosqualene (4,4'-diapophytoene by head to head condensation of two molecules of farnesyl-diphosphate). The dehydrosqualene desaturase CrtN dehydrogenates dehyrdrosqualene and forms intermediate 4,4'-diaponeurosporene, which is then oxidized, glycosylated, and finally esterified to produce the carotenoid STX (*5*).

S. aureus also produces an array of secreted proteases that serve as virulence factors for the bacterium. These proteases also serve as an aid in acquiring nutrients from the infected host. Proteases that are encoded by *S. aureus* include four major extracellular proteases: staphylococcal serine protease (V8 protease; SspA), cysteine protease (SspB), metalloprotease (aureolysin; Aur), and staphopain (Scp). Operons that encode these proteases are positively regulated by Agr and negatively regulated by SarA. The proteases have also been shown to cleave specific host proteins (*21*).

In addition to these global regulators, there have been several recent identified genetic regulators that have been shown to be involved in the virulence regulation of *S. aureus*

but call for further studies to be done on them (*14*). One of these regulators is the *msaABCR* operon, which is an operon of main focus in this study and in our lab.

sigB	Sigma factor B
sarA	Staphylococcal accessory regulator A
Agr	Accessory gene regulator
STX	Staphyloxanthin
V8 protease; SspA	Staphylococcal serine proteases
SspB	Cysteine protease
Aureolysin; Aur	Metalloprotease
Scp	Staphopain

Table 1. List of some genetic regulators and virulence factors in *Staphylococcus aureus*.

2.3 Metabolism of *S. aureus*

All bacteria require carbon and energy in order to replicate. Pathogenic bacteria, such as *S. aureus*, derive their carbon and energy from a host organism. This is done by the synthesis of a wide assortment of virulence determinants that are capable of killing host cells and then catabolizing their macromolecules. All macromolecules in this bacterium can be synthesized from 13 biosynthetic intermediates that are derived from the glycolytic, pentose phosphate, and tricarboxylic acid (TCA) cycle pathways (*22*). Carbohydrates are catabolized primarily through the glycolytic and pentose phosphate pathways, but the TCA cycle activity is largely repressed when nutrients are abundant in the culture medium (*23*). Glycolysis produces two molecules of pyruvate for every molecule of glucose consumed and during this, two molecules of NAD+ are reduced to NADH. In aerobically grown staphylococci, pyruvate is oxidized to acetyl coenzyme A (acetyl-CoA) and CO_2 by the pyruvate dehydrogenase complex (*24*). Acetyl-CoA can be further oxidized by the TCA cycle when grown in the presence of certain citric acid cycle

intermediates, but the amount of acetyl-CoA that enters the TCA cycle is low during nutrient-rich growth. In the exponential growth phase, acetyl-CoA is used to generate the small phosphodonor acetyl-phosphate, which is ultimately excreted as acetate. Acetyl-CoA is hydrolyzed to acetate by the phosphotransacetylase-acetate kinase (Pta-AckA) pathway, which generates one molecule of ATP through substrate level phosphorylation (*25*). The exit from the exponential phase of growth happens when the concentration of an essential nutrient, such as glucose, decreases to the level when it can no longer sustain rapid growth or by the addition of growth-inhibitory molecules, like lactic acid and acetic acid (*22*). Entry into post-exponential growth happens when there are favorable conditions and it corresponds with the catabolism of a non-preferred carbon source (*26*). This requires TCA cycle activity and occurs simultaneously with a large increase in TCA cycle enzymatic activity. An ADP-forming acetyl-CoA synthetase (ACD) catalyzes the conversion of acetyl-CoA to acetate as part of the acetate overflow mechanism. Functioning ACD involved in acetate formation and of AMP-forming acetyl-CoA synthesases (ACSs) activate acetate excretion (*27*). Naturally occurring mutations in *S. aureus* can alter the ability of the bacterium to catabolize acetate as a TCA function and has been found to be involved in the virulence, survival, and persistence of *S. aureus* (*28*). Due to the fact that the TCA cycle provides biosynthetic intermediates, ATP, and a reducing potential, this transition to the TCA cycle-driven metabolism drastically changes the staphylococcal metabolome. This increases the biosynthetic precursors that are available to use in a set of amino acid and nucleic acid biosynthetic pathways. This also leads to an increase in oxidative phosphorylation activity (*29*).

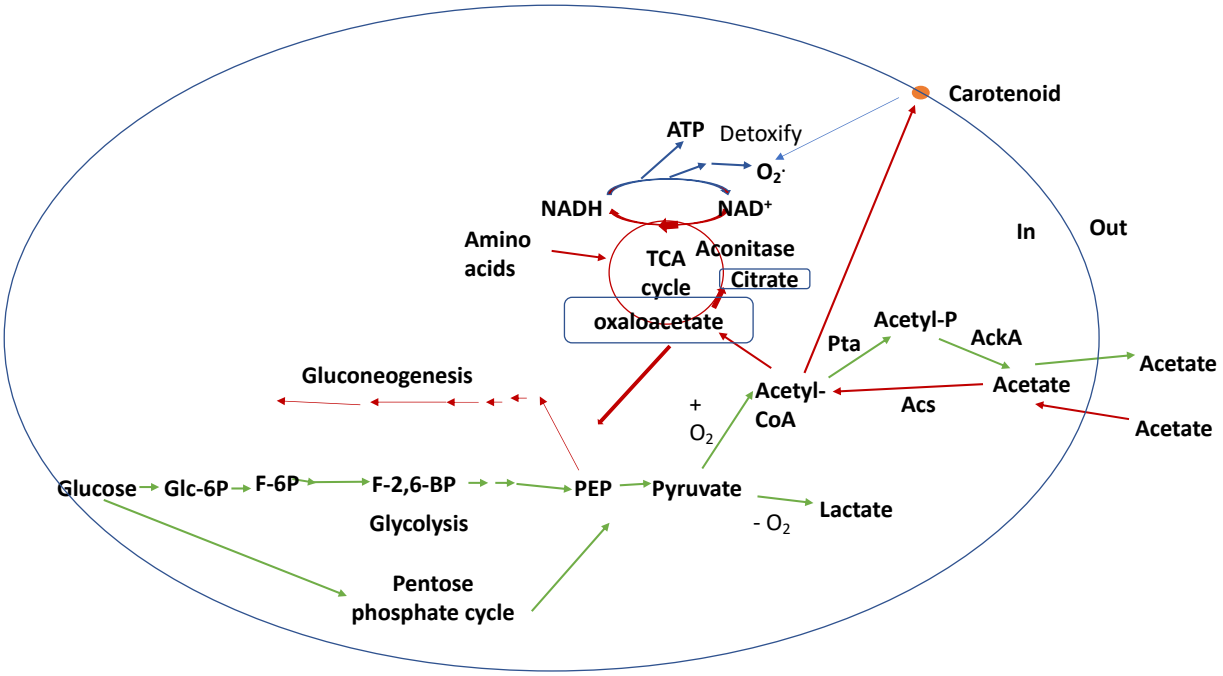

Figure 1. Schematic representation of glucose catabolism by S. aureus when growing in TSB. Green arrows, reactions or pathways used primarily during the exponential phase of growth; red arrows, reactions or pathways used in the post exponential growth phase

2.4 *msaABCR* operon

As previously mentioned, our lab group has identified in past studies an operon called *msaABCR*. This four-gene operon has been found to be involved in the regulation of phenotypic virulence traits, such as the development of biofilms, capsule production, pigment production and proteases expression, resistance against antibiotics, and antibiotic persistence in *S. aureus* (*1*, *8-12*). The operon codes for functional noncoding RNAs, which are *msaA*, *msaC*, and *msaR*. These three noncoding RNAs help regulate *msaB*, which is the only transcript of the whole operon that encodes for a protein MsaB. Characterization of the *msaABCR* operon shows that the MsaB protein is a recognized

transcription factor that binds target DNA in response to the nutrient availability (*10*). The *msaABCR* operon also regulates the expression of regulator proteins that are encoded by *sarA* and *agr*. The deletion of the *msaABCR* operon will alter the expression of these regulators (*1*). The *msaABCR* operon is a negative regulator of proteases. It has been found that the deletion of the *msaABCR* operon in *S. aureus* increased the protease production that lead to an increased processing of the major autolysin, Atl. An uncontrolled processed major autolysin causes an increased autolysis of cells and is responsible for defective biofilm formation (*9*). All previous studies have shown that the *msaABCR* operon is linked with many important phenotypes such as pigmentation, capsule production, and biofilm development (*1, 8-12*). The deletion of the *msaABCR* operon also reduces cell wall thickness, which leads to a decreased resistance to vancomycin in the vancomycin- intermediate *S. aureus*. The *msaABCR* operon was found to control the balance between cell wall synthesis and cell wall hydrolysis, which are both required in order to maintain a vigorous cell wall and to obtain resistance to cell-wall targeting antibiotics like vancomycin and the β- lactams (*8*).

2.5 CcpE

The transcriptional regulator catabolite control protein E (CcpE) is a critical citrate-sensing regulator that modulates metabolic state, virulence factor expression, and bacterial virulence in *S. aureus* (*13*). CcpE controls the transcription of the tricarboxylic acid (TCA) cycle genes. CcpE also is a regulator for virulence determinant biosynthesis and pathogenesis. It has been found that CcpE is a negative regulator of virulence in *S.*

aureus (15). CcpE directly affects the transcription of the aconitase-encoding gene *citB*, which increases the TCA cycle activity and decreases pigment production in *S. aureus* (*30*). Because TCA cycle activity and pigment production affect virulence determinant synthesis and infectivity of *S. aureus*, it was found that CcpE modulates the expression of virulence factors and pathogenicity in the pathogen (*15*). Even though CcpE is a positive regulator for TCA cycle activity, its regulon is primarily virulence-associated genes. Because it binds to and activates CcpE, citrate appears to be a key catabolite for the coordination of the *S. aureus* metabolic state with the bacterial virulence of *S. aureus*. Changes in the TCA cycle activity provide a survival advantage for *S. aureus* during infection (*13*). Deletion of *ccpE* affects the transcription of virulence factors such as *capA*, which is the first gene in the capsule biosynthetic operon, *hla*, which is an encoding α −toxin, and *psma* (*15*). It is clear in multiple studies that CcpE controls many important virulence-associated traits and the expression of a multitude of virulence-associated genes in *S. aureus*.

2.6 Hypothesis and aim of study

This project aided in the understanding of the interrelation between the *msaABCR* operon and CcpE in regulating staphylococcal virulence regulation. Previous studies have shown that *msaABCR* regulates virulence expression, antibiotic resistance, biofilm formation, oxidative stress response, and persistent infection (*1, 8-12*). The *msaABCR* operon also directly regulates Catabolite Control Protein (CcpE) expression. CcpE affects tricarboxylic acid (TCA) cycle activity by sensing the first intermediate of the TCA

cycle, which is citrate, regulating virulence and pathogenesis of *S. aureus*. CcpE is a major positive regulator for the TCA cycle, but its regulon is made up of mostly genes that are involved in the pathogenesis of *S. aureus*. It is found that CcpE is a vital metabolic sensor that will cause *S. aureus* to be able to sense and subsequently modify its metabolic state, thus regulating expression of virulence factors (*13*). We hypothesized that the *msaABCR* operon and the *ccpE* gene have interrelated mechanisms to regulate staphylococcal virulence. Our aim was to understand the complex and interrelated regulatory mechanisms between the *msaABCR* operon and CcpE to regulate protease production, pigmentation, survival (fitness), and aconitase activity. Ultimately, this project will add more insight towards our final goal to develop an alternative therapeutic target to treat *S. aureus* related infections by targeting global regulators like the *msaABCR* operon. The anticipated contribution that this research project will make to the field of study is to increase the knowledge about the global regulatory network of *S. aureus* virulence.

MATERIALS AND METHODS

3.1 Bacterial strains and Growth conditions

The strain used was the clinically significant community-obtained MRSA strain USA300 LAC. The allelic replacement method was utilized in order to generate *msaABCR*-deletion mutants USA300 LAC. The *msaABCR* and *ccpE* double mutant and *ccpE* mutant were made by the transduction of bursa aureulis transposon *ccpE* mutants that were acquired from the Nebraska Transposon Mutant Library to USA300 LAC *msaABCR* mutant using Φ11 *Staphylococcus aureus* strains were grown in tryptic soy agar (TSA) or tryptic soy broth (TSB).

Strains or plasmids	**Relevant characteristics**	**Reference/ source**
USA300 LAC	CA-MRSA USA300 strain	Dr. Shaw
USA300 LAC *msaABCR*	USA300 LAC *msaABCR*-deletion mutant	Sahukhal & Elasri(*1*)
USA300 LAC *msaABCR* complementation	(pCN34-*msaABCR* operon) complementation into USA300 LAC *msaABCR*-deletion mutant	Sahukhal & Elasri(*1*)
USA300 LAC *ccpE:Tn*	LAC *ccpE* mutant	This study
USA300 LAC *msaABCR*/*ccpE:Tn*	LAC *msaABCR/ccpE* mutant	This study

Table 2. Strains used in this study. This table lists each bacterial strain used in the experiments for this study and the source for each strain.

3.2 Construction of transposon mutant

The plasmid-cured derivative of the LAC strain JE2 that encompassed the transposon mutation in the specific coding regions of the desired genes was obtained from the Network of Antimicrobial Resistance in the *S. aureus* (NARSA) collection. The mutated strain was then transferred to USA300 LAC strain through the generalized transduction process. The bacteriophage $\varphi 11$ was used in this process. Successful transduction was verified using PCR and electrophoresis. For PCR amplification, primers at the beginning and end of the open reading frame (ORF) of *ccpE* gene were designed. DNA from mutants was used as template DNA for PCR reaction. Primers required for confirmation are listed in table 3.

Primer	Sequence (5′ to 3′)	Reference
Primers for Tn mutant verification		
ccpE Mut F	CACCAACAACAAAAGAAAAACTAAG	This study
ccpE Mut R	ACAAACCTTAAATTAGTCTAAAA	This study

Table 3. List of primers used in this study.

3.3 Media Preparation

By following the instructions made by the manufacturers, $Bacto^{TM}$Tryptic Soy Broth (TSB) and $Bacto^{TM}$ Tryptic Soy Agar (TSA) were prepared by combining the correct volume of distilled water and the correct amount of the powered media. The

bottles containing the media were sterilized by the process of autoclaving before use. The plates used in the experiments were made by using the TSA media.

3.4 Bacterial growth normalization of liquid cultures

A spectrophotometer was used to measure the optical densities (OD) of the starting cultures at 600 nm in order to make certain that all the cultures had equal amounts of bacterial cell growth. For preparation of the starting culture, the overnight cultures were diluted ten times and then grown for another two hours. Then, the two hour grown cultures were each used as an original culture to normalize to OD_{600} in the required volume for each assay. The volume of the original overnight culture resuspended in the correct volume for each assay was found by using the calculation of first (1) multiplying the desired bacterial cell density (OD_{600} of 0.05) and the volume of the freshly prepared media needed for resuspension, then (2) dividing by the OD_{600} measurement of the original culture, and lastly, (3) multiplying by 1,000 uL. All of these calculations can be simplified into the following equation:

Volume (uL) of original culture to resuspend in freshly prepared media=

$$\frac{[Desired\ bacterial\ cell\ density\ (OD)\ x\ Volume\ of\ freshly\ prepared\ media\ (mL)]}{OD\ measurement}$$

x1000uL

3.5 Aconitase activity assay

Aconitase activity was measured as described previously with some modifications (*15*). Cell pellets were harvested from 2mL culture by centrifugation at 10,000 x g for 10 min, washed with 50 mM Tris HCl buffer, re-suspended in 1000 µl of lysis buffer (90 mM Tris, pH 8.0, and 100 µM fluorocitrate). Bacteria were mechanically disrupted in a Fast Prep instrument at a speed of 6.5 for 45 s for 3 times. The cellular lysates were clarified using a 10-minute high-speed centrifugation at 10,000 x g. 10 µL of lysate was added to 190 µl assay buffer [100 mM Tris-HCl (pH 8.0), 50 mM trisodium citrate]. The amount of aconitate produced was quantified by measuring the Abs240. A molar absorption coefficient of 3.6 mM−1 cm−1 was used and 1 U aconitase was defined as the enzyme activity that catalyzes the formation of 1 µm aconitate per min. Enzymatic activity was standardized with respect to the total protein concentration and subsequently to that of the WT. Values are shown as the percent activity relative to the wild type strain USA300 LAC, which was set as 100%. The mean values from a minimum of three independent experiments, each performed in triplicate, were recorded.

3.6 Pigmentation assay

A pigmentation assay was performed on the cells harvested from the overnight cultures. An optical density (OD) reading of OD_{600} was recorded for all of the strains. 990 uL of PBS and 10 uL of the cells were mixed in a cuvette in order to take the OD reading in the spectrometer. 1000 uL of PBS was used as the blank. 1 ml of the cells were harvested and washed twice with PBS. They were then suspended in 1 ml of methanol and heated at 55 $°C$ for ten minutes with occasional vortexing in between. The cells were

removed by centrifugation at 15,000 x g for one minute. The supernatant was transferred to the cuvette, and the absorbance of the supernatant was measured at 465 nm with methanol used as the blank. Values are shown as the percent activity relative to the wild type strain USA300 LAC, which was set as 100%. The mean values from a minimum of three independent experiments, each performed in triplicate, were recorded.

3.7 Survival assay

The strains were grown in 5mL TSB plus 14 mM glucose in a 50 mL flask overnight at 37°C with shaking at 220 rpm. The 72-hour cultures were serial diluted to 10^{-7}, with 10^{-5},10^{-6}, and 10^{-7} being plated on a TSA plate. The plates were placed in the incubator at 37°C for 24 hours. The CFU was counted after the 24-hour of incubation in order to calculate live bacteria from culture. The mean values from a minimum of three independent experiments, each performed in triplicate, were recorded.

3.8 Protease assay

The strains were grown in 5 ml TSB in a 50 mL flask overnight at 37°C with shaking at 220 rpm. The cultures were standardized to the lowest optical density (OD) at 600 nm. 1.5 ml of standardized broth was then spun down at 10,000 x g, and then, the supernatant was collected. The supernatant was filter sterilized using a 0.45 um syringe filter. Two microcentrifuge tubes (1.5 ml) were set up for each sample and include a TSB control. 300 uL of the supernatant and 800 uL of Azocasein solution (3 mg/ml in Tris-Buffered saline pH 7.5) were mixed. The tubes were incubated at 37°C overnight in the

dark by wrapping the whole rack of tubes in foil. The un-degraded Azocasein was precipitated by adding 400 uL of 50% trichloacetic acid (TCA). Then, the tubes were centrifuged for ten minutes at 15,000 x g in order for pellets to precipitate. The OD of the supernatant at 340 nm was measured, using water as a blank. Values are shown as the percent activity relative to the wild type strain USA300 LAC, which was set as 100%. The mean values from a minimum of three independent experiments, each performed in triplicate, were recorded.

3.9 Statistical Analysis

In this study, all of the statistical analysis to test for significance were performed by using one-way ANOVA, followed by a post-hoc Turkey test with OriginPro software (Originlab, Northampton, MA). A significance level of 0.05 was set as the cutoff value to determine the significant difference between the test groups.

RESULTS

4.1 Construction of transposon mutant

We constructed the *ccpE* transposon mutant and *ccpE*/*msaABCR* double mutant and it was verified using PCR and gel electrophoresis. For PCR amplification, primers at the beginning and end of the open reading frame (ORF) for the *ccpE* gene was designed. We observed ~ 867 bp band for USA300 LAC wild type and *msaABCR* mutant strain, which was actual size of *ccpE* ORF. The *ccpE* transposon mutant and *ccpE*/*msaABCR* double mutant showed a higher molecular weight than wild type and *msaABCR* mutant respectively in the gel (Figure 2). This result confirmed construction of *ccpE* transposon mutant and *ccpE*/*msaABCR* double mutant in USA300 LAC.

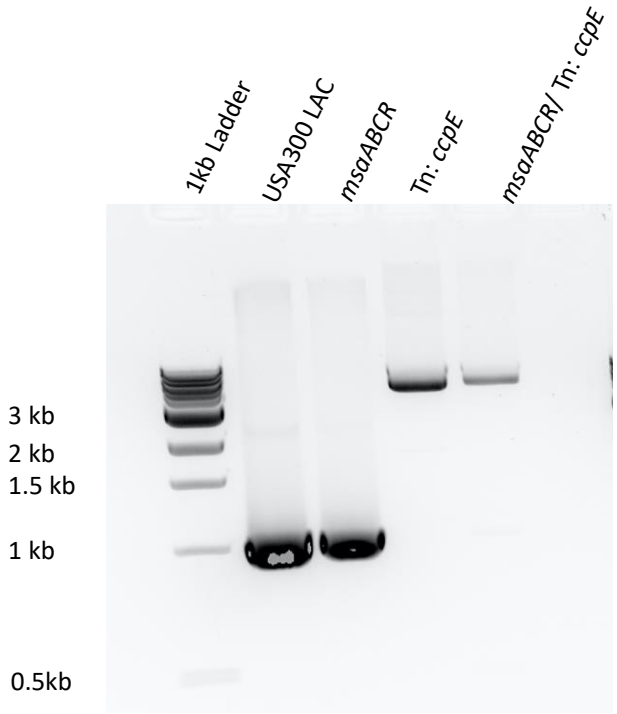

Figure 2. PCR verification of constructed transposon mutants. PCR with end to end primer flanking ORF of ccpE gene was used to verify the presence of presence of transposon in mutant strain. PCR reaction was separated in agarose gel electrophoresis.

4.2 *msaABCR* regulates aconitase activity via *ccpE.*

Our recent studies showed that *msaABCR* operon directly regulates *ccpE* expression. CcpE is shown to directly regulate the transcription of the aconitase-encoding gene *citB*, which increases the TCA cycle activity (*30*). We performed aconitase activity assay in *msaABCR* mutant and *msaABCR*/*ccpE* mutant in order to elucidate the roles of the *msaABCR* operon and *ccpE* in TCA cycle activity. As shown in Figure 3, *msaABCR* mutant showed increased aconitase activity compared to wild type levels. The aconitase activity was significantly decreased in the *ccpE* mutant compared to the WT. However, deletion of *ccpE* in *msaABCR* mutant caused significant decrease in aconitase activity comparable to *ccpE* mutant aconitase activity. These results suggest regulation of aconitase activity by *msaABCR* operon is via *ccpE* expression.

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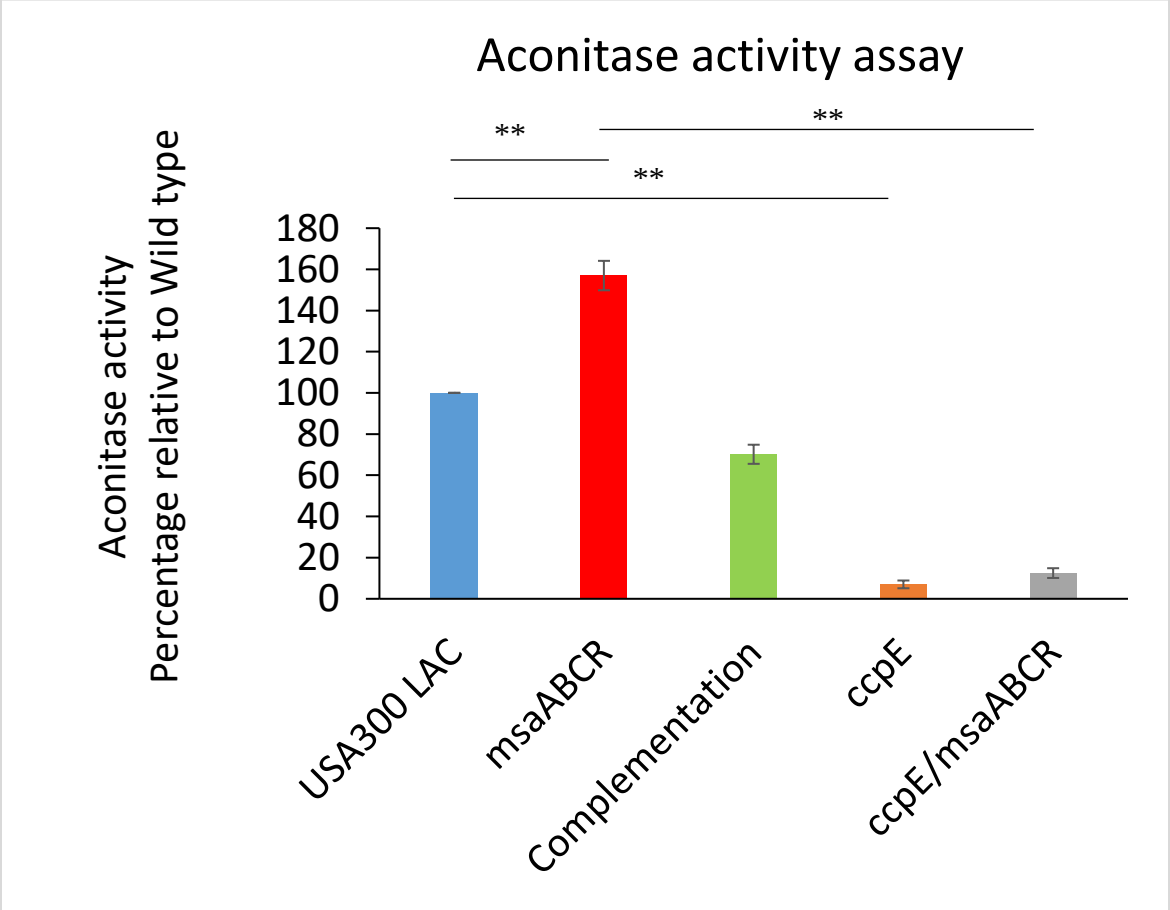

*Figure 3. Aconitase activity of the USA300 LAC, msaABCR mutant, complementation, msaABCR mutant and msaABCR/ccpE mutant strains grown aerobically in TSB supplemented with 0.25% glucose for 12 hr. Enzymatic activity was standardized with respect to the total protein concentration and subsequently to that of the WT. Values are shown as the percent activity relative to the wild type strain USA300 LAC, which was set as 100%. The mean values from a minimum of three independent experiments, each performed in triplicate, were recorded. All of the values were analyzed using one-way ANOVA followed by a post-hoc Turkey test. Error bars indicate standard errors of the means. A P-value of <0.05 was considered statistically significant (** p-value <0.05)*

4.3 *msaABCR* and *ccpE* regulates pigment production.

Most *S. aureus* strains produce the carotenoid pigment called staphyloxanthin, which is responsible for the yellowish-orange appearance of the bacterium (*15*). *ccpE* and

the *msaABCR* operon have been shown to directly regulate pigment production in *S. aureus* (*13-15, 30*). We performed the pigmentation assay in order to quantify the pigment produced by *S. aureus*. We measured the carotenoid (pigment) concentration in three different types of growth conditions- growth in TSA plates with and without glucose for 16 hr, and growth in TSB for 16 hr in *ccpE* transposon mutant and *ccpE/msaABCR* double mutant.

4.3.1 Carotenoid pigmentation when grown in TSB for 16 hours

As shown in previous studies, the *msaABCR* mutant produced a reduced amount of pigmentation and the *ccpE* mutant produced a higher level of carotenoid pigmentation compared to wild type strain when grown for 16 hrs in TSB (*13, 14, 30*). Also, the *msaABCR*/*ccpE* mutant produced pigments in levels comparable to *msaABCR* mutant, which was significantly lower than wild type and *ccpE* mutant (Figure 4). When *ccpE* is deleted in the double mutant, the pigment production is significantly reduced, which suggests that *msaABCR* regulates pigment production independent of the *ccpE* gene. This is significant for our research.

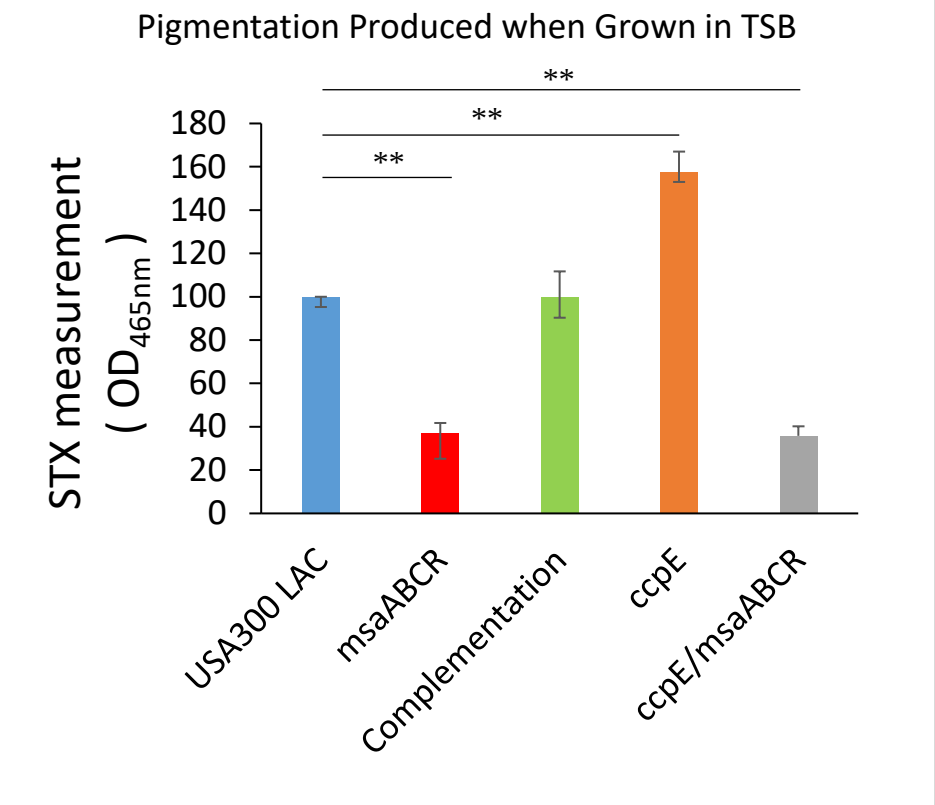

*Figure 4. Pigmentation assay of carotenoid concentration when grown in TSB for 16 hours. The results of the pigmentation assay which compares the optical density of the pigmentation for the wild type, the complementation, the msaABCR deletion mutant, the ccpE deletion mutant, and the ccpE/msaABCR deletion mutant using the absorbance readings at 465 nm. Values are shown as the percent activity relative to the wild type strain USA300 LAC, which was set as 100%. The mean values from a minimum of three independent experiments, each performed in triplicate, were recorded. All of the values were analyzed using one-way ANOVA followed by a post-hoc Turkey test. Error bars indicate standard errors of the means. A P-value of <0.05 was considered statistically significant (**p-value <0.05)*

4.3.2 Carotenoid concentration when grown in TSA plates with and without glucose for 16 hours

The glucose concentration was altered to see how pigment production was affected when grown in different mediums. As shown in Figures 5 and 6, *msaABCR*

produces significantly less pigment than the WT (USA300 LAC), while *ccpE* produces more pigment when grown in TSA plate with glucose and without glucose for 16 hours. However, interestingly, the deletion of *ccpE* in the *msaABCR* mutant reverses the pigmentation production to higher level than WT under both the conditions. The *ccpE/msaABCR* mutant still produces less pigment than *ccpE* mutant, suggesting that *msaABCR* regulates pigment production independent of *ccpE* gene.

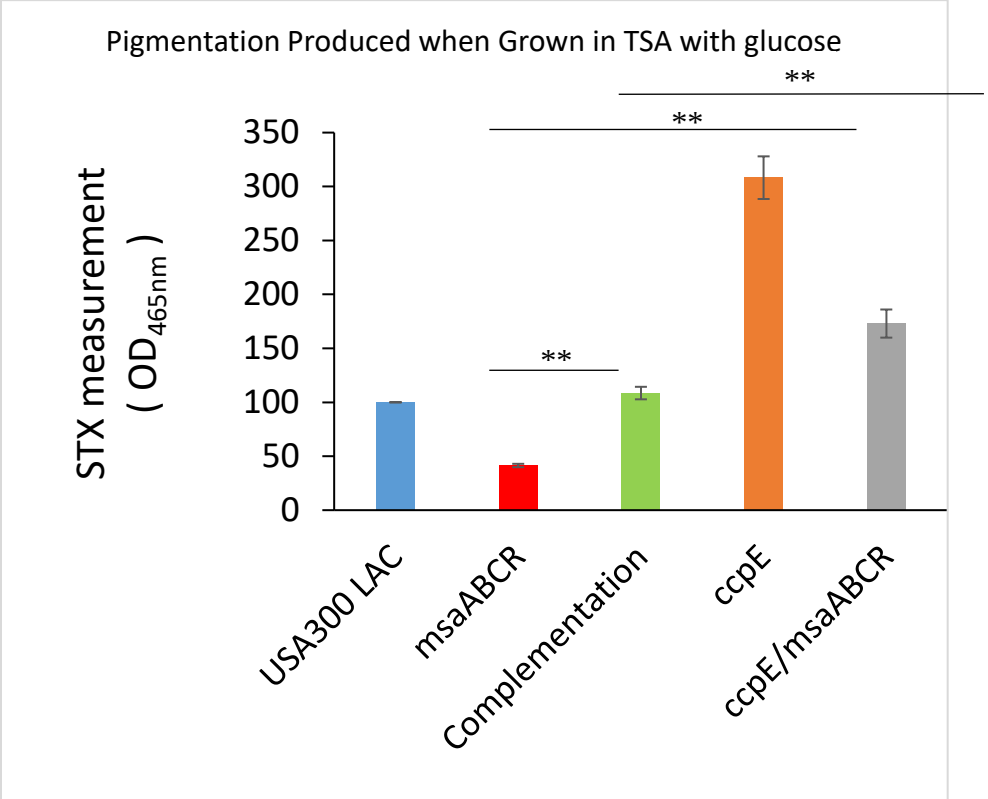

Figure 5. Pigmentation assay of carotenoid concentration when grown in TSA plates for 24 hours. The results of the pigmentation assay which compares optical density of the pigmentation for the wild type, complementation, msaABCR deletion mutant, ccpE deletion mutant, and the ccpE/msaABCR deletion mutant using the absorbance readings at 465 nm. Values are shown as the percent activity relative to the wild type strain USA300 LAC, which was set as 100%. The mean values from a minimum of three independent experiments, each performed in triplicate, were recorded. All of the values

*were analyzed using one-way ANOVA followed by a post-hoc Turkey test. Error bars indicate standard errors of the means. A P-value of <0.05 was considered statistically significant (**p-value <0.05)*

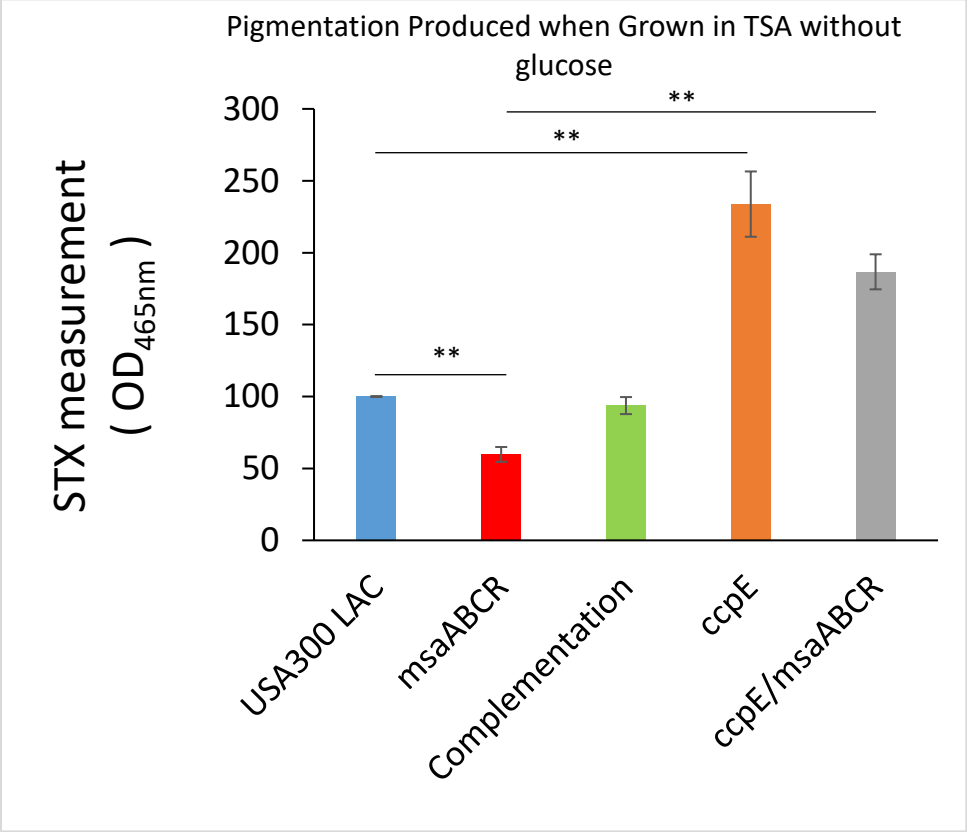

*Figure 6. Pigmentation assay of carotenoid concentration when grown in TSB without Glucose agar plates. The results of the pigmentation assay which compares the optical density of the pigmentation for the wild type, the complementation, the msaABCR deletion mutant, the ccpE deletion mutant, and the ccpE/msaABCR deletion mutant using the absorbance readings at 465 nm. Values are shown as the percent activity relative to the wild type strain USA300 LAC, which was set as 100%. The mean values from a minimum of three independent experiments, each performed in triplicate, were recorded. All of the values were analyzed using one-way ANOVA followed by a post-hoc Turkey test. Error bars indicate standard errors of the means. A P-value of <0.05 was considered statistically significant (**p-value <0.05)*

4.4 *msaABCR* regulates proteases activity independent of *ccpE*

The *msaABCR* operon was shown to repress protease activity in *S. aureus*. We therefore performed the protease assay in order to see how the deletion of *ccpE* in the *msaABCR* mutant affects protease production. As shown in Figure 7, *msaABCR* mutant had significantly higher exoprotease activity compared to the WT, while *ccpE* had a slightly reduced activity compared to the wild type. In addition, deletion of *ccpE* in *msaABCR* mutant, did not affect protease activity, showing that *msaABCR* operon regulates protease activity independent of *ccpE*.

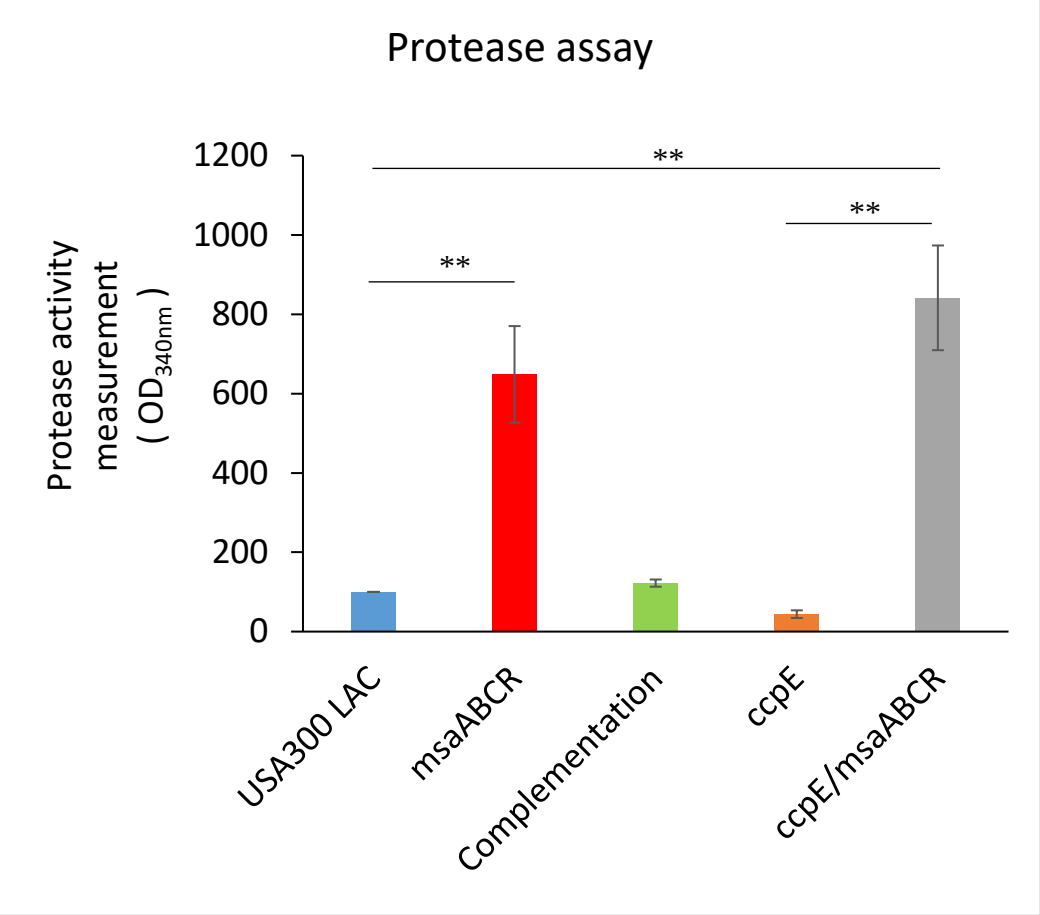

*Figure 7. Protease assay. The results of the protease assay which compares the optical density of the protease production for the wild type, the complementation, the msaABCR deletion mutant, the ccpE deletion mutant, and the ccpE/msaABCR deletion mutant using the absorbance readings at 340 nm. Values are shown as the percent activity relative to the wild type strain USA300 LAC, which was set as 100%. The mean values from a minimum of three independent experiments, each performed in triplicate, were recorded. All of the values were analyzed using one-way ANOVA followed by a post-hoc Turkey test. Error bars indicate standard errors of the means. A P-value of <0.05 was considered statistically significant (**p-value <0.05)*

4.5 Survival assay

Survival assay was performed in order to see the survival of the strains after growth without nutrients or oxygen for a 24-hour period in TSB with 14 mM of Glucose. As shown in Figure 8, *msaABCR* had a similar rate of survival compared to the WT, while *ccpE* had a decreased rate of survival. Interestingly, when *ccpE* is deleted from *msaABCR*, the rate of survival was comparable to wild type level and *msaABCR* mutant level.

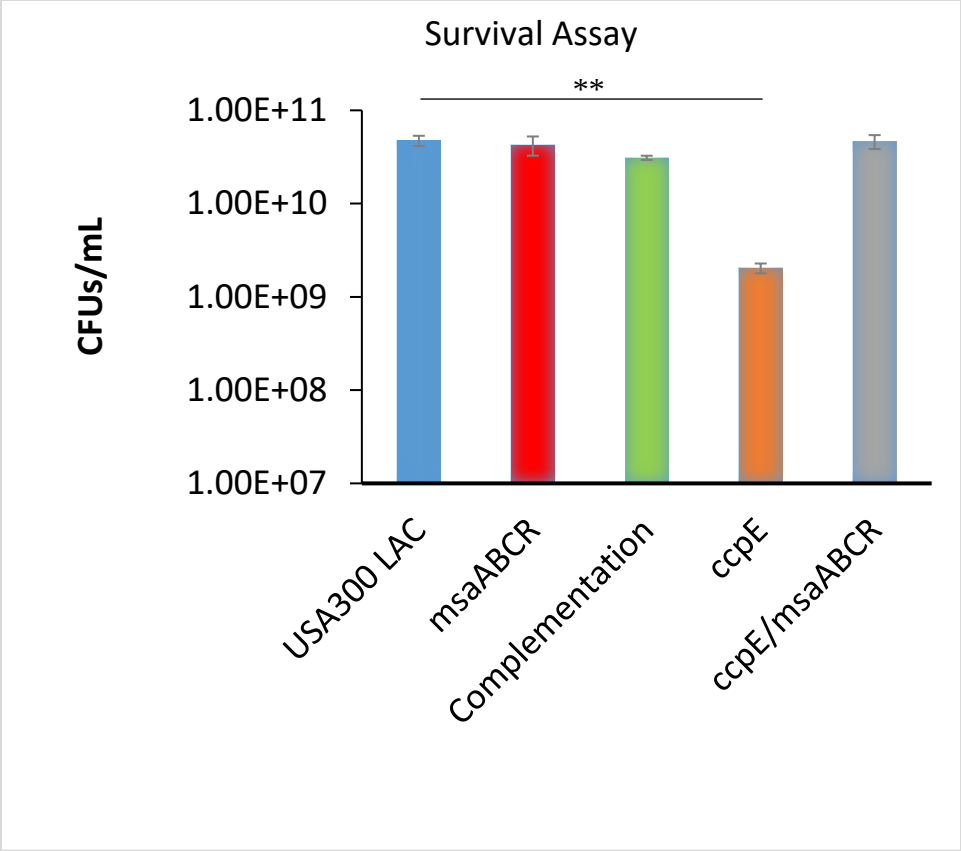

*Figure 8. Survival assay. The results of the survival assay which compares the CFU of the wild type, the complementation, the msaABCR deletion mutant, the ccpE deletion mutant, and the ccpE/msaABCR deletion mutant. All of the values were analyzed using one-way ANOVA followed by a post-hoc Turkey test. Error bars indicate standard errors of the means. A P-value of <0.05 was considered statistically significant (**p-value <0.05)*

DISCUSSION

Staphylococcus aureus is a versatile and dangerous human pathogen that causes antibiotic-resistant and lingering infections. Its success as a pathogenic bacterium is largely attributed to its wide array of virulence factors. The *msaABCR* operon, characterized by Dr. Elasri's lab, has been shown to have an important role in the regulation of *S. aureus*'s virulence factors expression, such as biofilm formation, pigmentation, protease production, and capsule synthesis, antibiotic resistance and persister formation (*1, 8-12, 14*). Another key regulator *of S. aureus*'s virulence factors is the catabolite control protein E (CcpE). CcpE is a transcriptional regulator of the tricarboxylic acid cycle (TCA cycle). CcpE is an important metabolic sensor that allows *S. aureus* to sense and adapt its metabolic state, and then coordinate its expression of virulence factors (*13*). Recent study from Dr. Elasri showed that the msaABCR operon directly regulates ccpE expression. (*14*). In this study, I aimed to understand the interrelation between the *msaABCR* operon and CcpE in regulating *S. aureus* virulence.

In a previous study, the inactivation of *ccpE* revealed that CcpE is a positive transcriptional effector of an enzyme of the TCA cycle called aconitase (*15*). CcpE is also shown to act as an important metabolic sensor that allows *S. aureus* to sense and adapt its metabolic state, and then coordinate its expression of virulence factors (*13*). In this study, we performed an aconitase activity assay in *msaABCR* mutant and *msaABCR/ccpE* deletion mutant in order to see the role of *msaABCR* operon in regulation TCA cycle activity. In our study, it was found that the *msaABCR* deletion mutant had increased aconitase activity compared to the WT, while the *ccpE* deletion mutant showed a significantly lower level of aconitase activity. This study supported previous studies on

the role CcpE on regulation of aconitase activity (*14*). However, deletion of *ccpE* in *msaABCR* mutant, decreased aconitase activity much lower than that of the WT. In fact, aconitase activity of *msaABCR/ccpE* double mutant was comparable to *ccpE* only mutant. These studies showed that *msaABCR* negatively affects the aconitase activity. Also, these data suggest that *msaABCR* operon regulates aconitase activity via *ccpE*.

Staphyloxanthin is a carotenoid pigment that is produced by *S. aureus* that acts an important virulence factor for the bacterium because it is shown to have antioxidant properties and the ability to resist neutrophils (*5*). In order to analyze the role of *msaABCR* operon and *ccpE* gene in pigment production, we analyzed pigment production in *msaABCR* mutant, *ccpE* mutant and *ccpE/msaABCR* mutant. We performed the assay in three different growth conditions- growth in TSA plates, growth in TSB medium, and growth in TSA plate without glucose in order to see how the pigment production is affected when grown in different types of medium. In all three conditions, we observed that the *msaABCR* mutant and *ccpE/msaABCR* mutant produced significantly less pigment compared to wild type and the *ccpE* mutant, respectively, suggesting that *msaABCR* regulates pigment production independent of *ccpE* gene (Fig.4, Fig.5, Fig.6) Moreover, deletion of *ccpE* in *msaABCR* mutant reversed pigment formation to higher levels than wild type level when grow in TSA plate with and without glucose (Fig. 5, Fig. 6). However, deletion of *ccpE* in *msaABCR* mutant did not affect pigmentation when grown in TSB medium (Fig. 4). Acetyl-CoA is a common precursor for staphyloxanthin synthesis, substrate for Pta-Ack pathway to generate acetate, and substrate for TCA pathway for carbon catabolism (*31*). It's assumed that Acetyl-CoA is fed to produce acetate at a higher rate when grown in liquid growth medium than in solid medium as

bacteria can diffuse acetate produced by cell in liquid medium easily than in solid medium. The increased pigment production by *msaABCR/ccpE* compared to *msaABCR* mutant in solid medium is best described by the fact that Acetyl-CoA is force fed to pigment synthesis pathway in *msaABCR/ccpE* when grown in TSA medium with and without glucose.

Extracellular proteases are another virulence factor of *S. aureus*. Protease assay was performed to measure the protease activity from extracellular proteases produced by each strain and to see if *ccpE* has an effect on protease production. Our results showed that (Figure 6) *ccpE* does not have major roles in protease production as shown in previous studies (*15, 21*). The deletion of *ccpE* in *msaABCR* also did not affect protease activity comparing to *msaABCR* mutant.

S. aureus are shown to survive at constant level from 24 hour up to 7 days when grown in TSB medium. The aconitase activity has been shown to contribute to stationary phase survival in *S. aureus* (*13, 15*). In order to see how *ccpE* affects stationary phase survival, we measured CFUs from cultures grown for 72 hours. Figure 8 showed that the *ccpE* mutant's viability was decreased significantly compared to wild type level. Decreased survival of *ccpE* mutant may be due to lower TCA cycle activity in *ccpE* mutant leading to lower reutilization of acetate and other nutrients in stationary growth phase. It is also shown in Figure 8 that the deletion of *msaABCR* rescues the survival in *ccpE* mutant *S. aureus*. The reason for this intriguing result needs to be further analyzed in future studies.

In conclusion, this study shows that *ccpE* and *msaABCR* may be interrelated in the regulation of virulence and central metabolism of *Staphylococcus aureus*. These

results help understand the relationship that *ccpE* and *msaABCR* have in regulating virulence. This project and other similar research projects may lead to a development of alternative therapeutic targets to treat *S. aureus* infections by targeting the global regulators such as the *msaABCR* operon and *ccpE*. In future studies, we plan to see how *ccpE* and *msaABCR* are related in oxidative stress response, biofilm formation, and antibiotic resistance. Continued research is important and extremely needed for understanding how *S. aureus* produces chronic infection and for developing future treatments in developing the infections. It is also needed to learn how to manage this virulent, antibiotic-resistant bacterium in community and healthcare settings

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