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The effect of truncated complement constructs on biofilm formation, protease production, and pigmentation

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by

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Abstract

The *msaABCR* operon regulates virulence factors in *Staphylococcus aureus*, a Gram-positive commensal organism that colonizes healthy individuals but can also be a human pathogen. These virulence factors include biofilm development, pigmentation, and extracellular protease production. The operon consists of the *msaB* gene, which produces a coding transcript, the *msaA* gene and the *msaC* gene, which produce noncoding RNAs, and *msaR*, which produces an antisense RNA. The latter three transcript regions of the operon are referred to as untranslated regions (UTRs) and are essential for the function of the operon, but only *msaB* encodes a protein. The mechanism of regulation by which these individual genes from the operon contribute to MsaB production and regulate virulence factors is still unknown. The purpose of this study is to find the region of the *msaABCR* transcript that is required to regulate virulence factors. Truncated complement constructs were made with altered versions of the 5' *msaA* region or the 3' *msaC* region to determine which sections of the transcript are necessary to produce virulence phenotypes. Various assays were conducted using these constructs to determine the effect of the alterations on biofilm formation, protease production, and pigmentation. This was done by comparing the effects of the constructs to the wild type strain, *msaABCR* mutant strain, and *msaABCR* complemented strain. These results could contribute to the discovery of the regulatory mechanisms of the *msaABCR* operon, which is a critical component in preventing and treating *S. aureus* infections. The results showed that the 5'UTR (*msaA*) and 3' UTR (*msaC*) region of the operon transcript are required for its full function in regulating virulence and biofilm development in *S. aureu*s. However, we still do not know the exact mechanism by which UTR regions contribute to the regulation and functioning of *msaABCR* operon and/or MsaB production (Sahukhal & Elasri, 2014).

Keywords: *Staphylococcus aureus*, msaABCR, virulence factors, phenotypic assay, biofilm

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List of Abbreviations

Agr	accessory gene regulator
Atl	major autolysin
Biofilm media	TSB supplemented with 3% sodium chloride and 0.5% glucose
bp	base pairs
eDNA	extracellular DNA
EtBr	ethidium bromide
FP	forward primer
kb	kilobase pair
LB	Luria–Bertani media
MRSA	methicillin resistant *Staphylococcus aureus*
Na citrate	sodium citrate
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIA	poly-N-acetylglucosamine
RP	reverse primer
rpm	rotations per minute
TBE	tris-borate-EDTA
TC	truncated complement construct
TCA	trichloroacetic acid
TSA	tryptic soy agar
TSB	tryptic soy broth

sarA	staphylococcal accessory regulator A
sigB	sigma factor B
UTR	untranslated region
UV	ultraviolet light

Chapter 1: Introduction and Literature Review

Staphylococcus aureus is a Gram-positive commensal organism that primarily colonizes the nose of healthy individuals but can also be a human pathogen. The process by which the bacterium adapts from a commensal lifestyle to a pathogenic one is determined by several regulatory loci of *S. aureus*. The fine-tuning of these regulatory loci is necessary for the organism to alter basic metabolic processes and to activate multiple virulence factors, which are vital in successful colonization and infection (Batte et al., 2018). *S. aureus* causes a wide range of acute and chronic infections that include superficial skin and soft tissue infections such as furuncles, impetigo, and abscesses, as well as deep wound infections. They also cause more severe, invasive biofilm associated infections such as osteomyelitis, endocarditis, necrotizing pneumonia, and bacteremia. In addition, *S. aureus* is becoming increasingly resistant to multiple antibiotics, making it a growing threat to public health. There is a pressing need to understand the complex regulatory networks used by *S. aureus* to cause disease (Nagarajan & Elasri, 2008). Several global regulators modulate virulence of *S. aureus* strains, yet we do not fully understand the regulation of virulence factors that contribute to the prevalence of these strains.

One of the most important aspects of staphylococcal infections is biofilm development within the host, which allows the bacterium to develop a resistance to the host immune response and to antimicrobial agents. Biofilm development is extremely complex and involves several regulators that function to sustain cell survival and proliferation within the host. *S. aureus* produces a multilayered biofilm characterized by high levels of organization and an extracellular polymeric matrix which is comprised of

poly-*N*-acetylglucosamine (PIA), extracellular DNA (eDNA), and several heterogeneous proteins (Sahukhal et al., 2015). Expression of virulence factors in *S. aureus* is maintained by a variety of regulators that include trans-acting global regulators, alternative sigma factors, and small non-coding RNAs.

Developing an understanding of virulence regulation is crucial in providing effective prevention and treatment of *S. aureus* infections. An increasing number of loci that contribute to regulatory control have been identified, several of which play a significant role in regulating virulence factors, such as stress response sigma factor B (*sigB*), staphylococcal accessory regulator A (*sarA*), and the accessory gene regulator (*agr*) (Sahukhal et al., 2015; Sambanthamoorthy et al., 2006). Transcriptional regulators such as the one encoded by *sarA* are major contributors in the regulation of biofilm development. The SarA protein regulates over 100 genes in *S. aureus,* and of these, several are associated with virulence (Nagarajan & Elasri, 2008). Gram-positive bacteria like *S. aureus* respond to stress by expressing alternative sigma factors, such as the sigma B factor, which is encoded by *sigB.* The presence of antibiotics, such as methicillin or vancomycin, has been shown to activate the expression of sigma B in *S. aureus,* indicating that this sigma factor contributes to antibiotic resistance (Samanta & Elasri, 2014). The *S. aureus* bacterium can control the expression of genes that encode virulence factors during the infection process as a response to environmental changes within the host.

The bacteria that form biofilms are surrounded by a polysaccharide glycocalyx, which provides them with protection against host defenses and antimicrobial drugs. Staphylococcal biofilms form in two distinct stages. The first stage includes primary

adhesion to surfaces by means of adhesins or cell wall components. The second stage is characterized by the buildup of multilayered clusters of cells, formed by producing a polysaccharide. Cells are also able to detach from the biofilm and migrate to distant sites within the host for colonization or infection (Sambanthamoorthy et al., 2008).

Our lab group previously identified a new operon, *msaABCR*, which is involved in the regulation of phenotypic virulence traits, including biofilm development and antibiotic resistance in *S. aureus* (Sahukhal et al., 2015; Sahukhal & Elasri, 2014; Samanta & Elasri, 2014; Sambanthamoorthy et al., 2008; Sambanthamoorthy et al., 2006). Former experiments related to this operon have indicated that the complete *msaABCR* operon is transcribed and the transcript is post-transcriptionally processed to produce the msaB transcript, which produces the MsaB protein. In addition to the MsaB transcription factor, the *msaABCR* operon encodes two noncoding RNAs, msaA and msaC, and the antisense RNA msaR, which are essential for the function of the operon (Sahukhal & Elasri, 2014). Additionally, researchers from the Elasri lab group have found that the MsaB effector protein is the only protein encoded within the operon's transcript (Batte et al., 2016).

The *msaABCR* operon is essential for regulating the fundamental phenotype of *S. aureus*. Several studies have shown that the *msaABCR* operon plays a role in regulation of biofilm development, antibiotic resistance, and many other virulence factors (Sahukhal et al., 2015; Sahukhal & Elasri, 2014; Samanta & Elasri, 2014; Sambanthamoorthy et al., 2008; Sambanthamoorthy et al., 2006). The operon was identified as an additional positive regulator of biofilm formation (Sahukhal et al., 2015). Characterization of the *msaABCR* operon has shown that the MsaB protein is a recognized transcription factor

that binds target DNA in response to nutrient availability (Sahukhal et al., 2017). This operon also regulates the expression of the regulator proteins encoded by *sarA*, *agr*, and *sigB*. The deletion of the *msaABCR* operon alters the expression of these global regulators (Sahukhal & Elasri, 2014).

The *msaABCR* operon regulates biofilm development by controlling the rate of autolysis, and the operon acts as a negative regulator of proteases (Sahukhal et al., 2015). Previous studies have shown that the rate of autolysis is regulated by *msaABCR* through the presence of proteases. When the operon has been deleted, excessive amounts of extracellular proteases are produced, increasing the processing of the major autolysin, Atl (Sahukhal et al., 2015). This increased lysis rate causes uncontrolled cell death and causes a defect in biofilm formation in mutant strains that lack the operon. Therefore, the *msaABCR* operon plays a key role in maintaining the balance between autolysis and growth within the staphylococcal biofilm (Sahukhal et al., 2015).

The mechanism of regulation for this operon and the role of individual genes in MsaB production is not fully understood (Batte et al., 2016). Although much has been discovered regarding phenotypes with which the operon is associated, there is not a clear picture of the contributions made by each gene yet. This study attempts to find the minimum region of the *msaABCR* transcript that is necessary for the regulation of MsaB production by making a series of truncated complement constructs from both the 5’ and 3’ UTR regions of the *msaABCR* operon transcript as described in the methods. These truncated complement constructs will allow for examination of the role of the 5’ and 3’ end of the operon in regulating virulence factors and observation of the subsequent impact of these mutations on the phenotype, specifically biofilm development, protease

production, and pigmentation. The results of these assays could aid in finding an important clue regarding the regulation of the *msaABCR* operon. *Staphylococcus aureus* strains (community-acquired MRSA strain USA300_LAC, restriction-deficient laboratory strain RN4220) and *E. coli* strain DH5α were used to make these constructs. In the United States, USA300_LAC has been the dominant strain causing methicillin resistant *S. aureus* (MRSA) infections in community settings (Samanta et al., 2015). For these reasons, discovering the mechanism of *msaABCR* operon regulation is critical to discovering how to best prevent and treat staphylococcal infections.

Chapter 2: Methods

Bacterial strains, culture conditions and plasmids

The plasmids and bacterial strains used for this study are shown in Table 1. *S. aureus* strains were grown in tryptic soy broth (TSB) medium. Antibiotics (chloramphenicol (10 μg/ml), erythromycin (10 μg/ml), and kanamycin (50 μg/ml)) were used in TSB or tryptic soy agar (TSA) where needed. Similarly, *E. coli* strains were grown in LB broth with ampicillin (100 μg/ml) added where needed.

Plasmids		
Plasmid	**Relevant characteristics**	**Source**
pCN34	pT181-based low copy number *E. coli-Staphylococcus* shuttle vector	NARSA
pMOE 403	pCN34-*msaABCR* operon::*msaABCR* operon complement	Sahukhal & Elasri, 2014
pMOE 792	pCN34-*msaAB$_{his}$CR* operon::*msaABCR* operon with 6XHis-tagged MsaB	Dr. Sahukhal
pMOE TC-2	pCN34-*msaAB$_{his}$CR* operon::247 bp deletion from 5' end of *msaABCR* operon	This Study
pMOE TC-3	pCN34-*msaAB$_{his}$CR* operon::181 bp deletion from 5' end of *msaABCR* operon	This Study
pMOE TC-4	pCN34-*msaAB$_{his}$CR* operon::115 bp deletion from 5' end of *msaABCR* operon	This Study
pMOE TC-5	pCN34-*msaAB$_{his}$CR* operon::6 bp deletion from 5' end of *msaABCR* operon	This Study

pMOE TC-6	pCN34-*msaAB$_{his}$CR* operon::75 bp deletion from 3' end of *msaABCR* operon	This Study
pMOE TC-7	pCN34-*msaAB$_{his}$CR* operon::218 bp deletion from 5' end of *msaABCR* operon	This Study
Strains		
Strain	**Relevant characteristics**	**Reference or source**
RN4220 (*S. aureus*)	Restriction deficient mutant of 8325-4	NARSA
LAC (*S. aureus*)	CA-MRSA USA300 strains	Dr. Lindsey Shaw
MOE 401 (*S. aureus*)	LAC :: Δ *msaABCR* operon deletion mutant	Sahukhal & Elasri, 2014
DH5α (*E. coli*)	Chemically induced competent cells	NEB
TC-2 (*S. aureus*)	pCN34-TC-2 in *msaABCR* deletion mutant	This Study
TC-3 (*S. aureus*)	pCN34-TC-3 in *msaABCR* deletion mutant	This Study
TC-4 (*S. aureus*)	pCN34-TC-4 in *msaABCR* deletion mutant	This Study
TC-5 (*S. aureus*)	pCN34-TC-5 in *msaABCR* deletion mutant	This Study
TC-6 (*S. aureus*)	pCN34-TC-6 in *msaABCR* deletion mutant	This Study
TC-7 (*S. aureus*)	pCN34-TC-7 in *msaABCR* deletion mutant	This Study

Table 1. Strains and plasmids used in this study. This table lists each plasmid and bacterial strain used in the experiments for this study and the source for each strain.

Cloning, purification, and ligation of plasmids

The first step in making a truncated complement construct was to extract the desired plasmid pCN34 from *E. coli* cells. This was done by harvesting and purifying the plasmid using the Zymo Wizard purification kit. This kit included resuspension buffer, lysis solution, neutralization solution, and wash buffer, as well as spin columns and

collection tubes. In order to harvest the plasmid, cells containing the plasmid were gently added to a 1.5 ml microcentrifuge tube. This was done near a flame in order to minimize contamination. Following this, 250 µl of resuspension buffer was added to the cells, and the tube was vortexed until no clumps of cells remained. Once this step was completed, 250 µl of lysis solution was added to the tube. The tube was then inverted until the solution became clear and viscous. Next, 350 µl of neutralization solution was added to the tube and the tube was inverted until the solution became cloudy. This step was performed fairly quickly in order to prevent overlysis of the cells.

The tubes were placed in a centrifuge for 5 minutes at 10000 x *g*, and the supernatant that formed in the tube during the centrifuge step was transferred into a spin column that was placed in a collection tube. The collection tube was centrifuged for 1 minute at 10000 x *g* and the residue was discarded. The spin column was washed with 500 µl of wash buffer and centrifuged under the same conditions. This residue was thrown out and the wash step was repeated. After washing, the column was spun in the centrifuge for the dry spin at the same conditions. Then, the spin column was transferred into a new microcentrifuge tube and eluted with 50 µl of water. This was allowed to incubate for 1-2 minutes and then centrifuged for 2 minutes at 10000 x *g*. The concentration of the plasmid was recorded by taking the measurement using a Nanodrop spectrophotometry system using water for the blank. The plasmid tubes were stored at 4°C.

The truncated complemented constructs were amplified by using the primers listed in Table 2 and using pMOE 792 as a template DNA. This was done using polymerase chain reaction (PCR). PCR uses a combination of specific primers that are

added to the template DNA in order to amplify genes in a specific way. A PCR reaction mixture is made of Q5 master mix (NEB), a forward/5' end primer (FP), a reverse/ 3' end primer (RP), plasmid DNA (pMOE 792), and water added to a 0.5 ml microcentrifuge tube. The amount of each component added varied. For these PCR reactions, the amount of master mix was equal to half of the total reaction volume. For example, a 50 µl reaction contained 25 µl of the Q5 master mix. For these PCR reactions, the concentration of both the FP and RP stock solution was diluted to 10 µM, and the concentration of template DNA was 50 ng/µl. The amount of water added was equal to the remaining desired volume. In this example, the amount of water added was 19 µl because the amount added for the primers and DNA was 6 µl and the master mix was 25 µl. If the total desired volume was 50 µl, the remaining volume for water was 19 µl. The water was added first followed by the master mix, the primers, and then the DNA. The tubes were vortexed and spun down in a centrifuge.

The PCR reaction mixtures were made using the primers listed in Table 2. The reactions created recombinant plasmid constructs named TC-2 through TC-7, where TC stands for truncated complement. Once the PCR reaction tubes were assembled, they were placed in the thermocycler where the PCR reaction takes place. The thermocycler settings were selected for the correct reaction volume and temperatures for each step including the annealing temperature, which is primer specific. These settings were 95°C for 30 s for the initial denaturation step; thirty cycles of 98°C for 10 s for denaturation, the specific primer temperature for 10 s for annealing of the nucleotides to the plasmid, and 72°C for 30 s for the extension; and 68°C for 5 minutes for increased extension. The samples were kept in the thermocycler at an infinite hold of 16°C to stabilize the reaction.

Truncated Complement Construct	Primers	Primer sequence
TC-2	CspA F	ATTGCGAAGATTGTGAAGAGGATTTACAAATATT
	DND R	AAGCACGTGAAGTTATAAAGCGACAATCGTTAAGA
TC-3	Pro 1295 IV F	AGTTCGATAACTATGTCACAGGCAAATATAATATT
	DND R	AAGCACGTGAAGTTATAAAGCGACAATCGTTAAGA
TC-4	Pro 1295 III F	ATCAATATCAAGATTTTGATGACATGTTTAAGCAC
	DND R	AAGCACGTGAAGTTATAAAGCGACAATCGTTAAGA
TC-5	FP-HP proless	ATAGAGTTTTTGAAGTATGGAAGGGGTCTTTA
	DND R	AAGCACGTGAAGTTATAAAGCGACAATCGTTAAGA
TC-6	Pro 1296 F	ACTTGTAAATGTATATGTCTCATTTTTACCACCTCA
	msa op TC-2 R	ATGCTTGGACATATACTCTAAAAAAGGAAGAACAG
TC-7	Pro 1296 F	ACTTGTAAATGTATATGTCTCATTTTTACCACCTCA
	msa op TC- 3 R	AGCATCATGAAATTAGATTTTATGCTTTACTTGCT

Table 2. Truncated complement constructs and primers used. Each forward primer contained a BamHI restriction site and the reverse primers contained an EcoRI restriction site. Each primer pair mentioned above was used to amplify the respective truncated complement constructs.

Gel electrophoresis was done following PCR to confirm the presence of the amplified genes. The gel for the electrophoresis is a 1% agarose gel where 1 g of agarose was dissolved in 100 ml of 10X Tris-borate-EDTA (TBE) buffer in a beaker or flask. For this buffer, the Tris base concentration was 1 M. The boric acid was 1 M, and the EDTA was 0.02 M. The pH of the buffer was 8.0, and the working concentration was 0.5X. The solution was microwaved to ensure the agarose was properly dissolved. Once dissolved, approximately 1.5 µl of ethidium bromide (EtBr) was mixed into the solution by swirling the beaker or flask. A gel casting tray and comb were then assembled and the mixture

was poured into the casting tray evenly. The gel was poured in a manner that ensured that there were no bubbles as to prevent holes from forming in the gel.

The gel box was assembled for electrophoresis by filling it with TBE buffer, and once the gel had solidified, the comb was gently removed and the tray was placed into the gel box so that it was submerged in the buffer. The gel was placed so that the top of the gel was placed on the same side as the negative electrode. The PCR reaction samples were removed from the thermocycler and a fraction of the product was added to a new 0.5 ml microcentrifuge tube. Before loading the samples, they were mixed with 5 μl of loading dye before they were vortexed and spun down in a centrifuge. An equivalent volume to the sample of a 1 kilobase pair (kb) ladder was loaded into the first well of the agarose gel in the gel box. The samples were added to the remaining wells in the gel and the lid was placed on top of the gel box. The cords were plugged into the power supply, the voltage was set to 100 V, and the electrophoresis was started. It was verified that the chamber was working by looking for bubbles near the negative electrode. The DNA fragments in the sample moved from the negative electrode to the positive and were separated by weight. The electrophoresis was monitered for approximately 30 minutes before the gel was examined using ultraviolet (UV) light. The EtBr allowed the fragments that were dyed to appear orange in the gel when viewed under UV light

The PCR product was purified using the Zymo Wizard kit in a similar manner to plasmid purification. A volume of membrane binding solution equal to the volume of the PCR product being purified was added to the sample. This mixture was transferred into a spin column that was placed in a collection tube and incubated for 1 minute. The collection tube was centrifuged for 1 minute at 10000 x *g* and the residue was thrown out.

The spin column was then washed with 500 µl of wash buffer and centrifuged at the same conditions. This residue was thrown out and the wash step was repeated. After washing, the column and collection tube were spun in the centrifuge for the dry spin under the same conditions. Then, the spin column was transferred into a new microcentrifuge tube and eluted with 50 µl of water. This was allowed to incubate for 1-2 minutes and centrifuged for 2 minutes at 10000 x *g*. The concentration of the purified DNA product from the reaction was recorded by taking the measurement using Nanodrop as previously mentioned. The tubes containing the purified PCR products were stored at 4°C.

After purifying the PCR products, the constructs must be digested using restriction enzymes so that sticky ends are created for ligating the PCR product to the recombinant plasmid in the next step. Restriction digestion reactions were composed of Cutsmart buffer, two restriction enzymes, DNA, and water added to 0.5 ml microcentrifuge tubes. For these restriction digestion reactions, restriction enzymes BamHI and EcoRI were used. Restriction digestion reactions contained 10% of the total reaction volume as the amount of Cutsmart buffer. For example, a 20 µl reaction contained 2 µl of Cutsmart. Restriction digestion reaction components were added in a similar manner to PCR reaction components. 10 µl of water was added to each tube. Then, 2 µl of cutsmart buffer and 1.5 µl of each restriction enzyme were added. Lastly, 5 µl of the PCR products, which served as the DNA in these reactions, was added to each tube. The tubes were vortexed and spun down in a centrifuge.

Once the reaction tubes were assembled, the restriction digestion was performed, and a gel electrophoresis was done in the same manner as for PCR reactions to confirm the plasmid was digested correctly. The restriction digestion gel showed two bands as in

Figure 2. The restriction digestion bands were cut out of the gel and placed in a 1.5 ml microcentrifuge tube in order to purify them using the Zymo Wizard kit. To dissolve the gel, 10 µl of membrane binding solution was added per 10 mg (milligrams) of gel slice. The tube was vortexed and incubated in the hot water bath at 50°C until the gel was completely dissolved.

The mixture was transferred into a spin column and a collection tube and incubated for 1 minute. The collection tube was centrifuged for 1 minute at 10000x*g*, and the residue was thrown out. The spin column was washed with 500 µl of wash buffer and centrifuged under the same conditions. Thhis residue was thrown out and the wash step was repeated. After washing, the column and collection tube were spun in the centrifuge for the dry spin at the same conditions. Then, the spin column was transferred into a new microcentrifuge tube and eluted with 50 µl of water. The samples were incubated for 1-2 minutes and then centrifuged for 2 minutes at 10000x*g*. The concentration was recorded using Nanodrop, and stored at -20°C.

After digesting the plasmids, the DNA fragments were cloned into a gram-positive low copy number shuttle vector (pCN34) through a ligation process. The ligation step inserts the digested PCR products into pCN34 using a ligase enzyme, a ligase buffer, and water combined in a 0.5 ml microcentrifuge tube. The amounts of pCN34 and amplicon that were added depend on the concentrations recorded from Nanodrop. The water was added first followed by the ligase buffer. The plasmid and amplicon were added next, and the ligase enzyme was added last. The tubes were placed in the thermocycler, and the setting for ligation was selected which were 4°C overnight. The

reaction volume and temperature were checked, and once the reaction was completed, the ligation products were stored at -20°C.

Moving the plasmid into *E. coli*

The next phase after ligating the truncated complement plasmid to pCN34 was to move this recombinant plasmid into DH5α *E. coli* cells through transformation. To do this, 5 µl of the ligation product was added to 50 µl of *E. coli* cells in a 1.5 ml microcentrifuge tube and incubated on ice for 30 minutes. After this incubation period, the tubes were placed into a water bath at 42°C for 30 s in order to heat shock the cells. Then 450 µl of SOC media wass added to each tube, and they were placed in the incubator at 37°C for 45 minutes. The cells were then plated on Luria–Bertani (LB) plates supplemented with ampicillin (100µg/ml) with 50 µl spread onto the first plate, 100 µl spread on the second plate, and the rest being spread on a third plate. These plates were placed in the incubator and allowed to grow overnight.

After growing the plates overnight, colonies were selected and a colony PCR was performed. Colony PCR is a PCR reaction that uses the selected colony in place of DNA. For these PCR reactions, the amount of master mix was equal to half of the total reaction volume as in standard PCR reactions. Because these PCR reactions use a colony in place of DNA, both the FP and RP were 1.25 µl each, and the amount of master mix was 12.5 µl. The amount of water added was equal to the remaining desired volume. If the total desired volume was 25 µl, the remaining volume for water was 10 µl. The water was added first followed by the master mix, the primers, and then the colony was added

to the tube by lightly touching the previously selected colony with a pipette tip and placing it in the tube. This was done near an open flame to prevent contamination.

The tubes were then vortexed and spun in a centrifuge. Colony PCR reaction mixtures were made using the same primers as the initial PCR reactions. These primers are listed in Table 2. Once the PCR reaction tubes were assembled, they were placed in the thermocycler and the settings for PCR (95°C for 30 s for the initial denaturation step; thirty cycles of 98°C for 10 s for denaturation, the specific primer temperature for 10 s for annealing of the nucleotides to the plasmid, and 72°C for 30 s for the extension; 68°C for 5 minutes for increased extension; and infinite hold of 16°C to stabilize the reaction) were selected. After the colony PCR reaction was finished, gel electrophoresis was done to verify the amplification of the correct genes. The PCR gel should look like the PCR gel from the initial PCR gel, the results of which are shown in Figure 1.

After verifying the colony PCR, selected positive colonies were subcultured onto LB ampicillin plates and grown in the incubator overnight. The plasmid was then extracted from these subcultured plates using the same Zymo Wizard kit and protocol. The concentration of the plasmid was recorded using Nanodrop and the tubes were stored at 4°C. The next step after harvesting and purifying the plasmid s to perform a restriction digestion on the plasmid. For these restriction digestion reactions, the same restriction enzymes were used as before, BamHI and EcoRI. Once the reaction tubes were assembled, the restriction digestion procedure was performed. The reaction volume and temperatures for each step were verified, and a gel electrophoresis was performed once the thermocycler reaction finished. The restriction digestion gel should show two bands like the first restriction digestion reaction which is shown in Figure 2.

Moving the plasmid into RN4220 *S. aureus*

After moving the plasmid to *E. coli*, the next step is to move it into a restriction deficient mutant, RN4220, through electroporation. This was done by adding 2 µl of the plasmid extracted from *E. coli* to 50 µl of competent RN4220 cells and letting it incubate for 30 minutes. Tthe plasmid and cell mixture were transferred to electroporation cuvettes on ice. The micropulser was set to bacteria and the cuvette were placed inside. The pulse button was pressed and the voltage was checked to verify that it worked. 500 µl of B2 media was added immediately and the cuvettes were put on ice until all of them had been pulsed. The contents of the electroporation cuvette were transferred to a 1.5 ml microcentrifuge tube and incubated for 1 hour shaking. The cells were then plated on LB ampicillin plates with 50 µl spread onto the first plate, 100 µl spread on the second plate, and the rest being spread on a third plate. These plates were placed in the incubator and allowed to grow overnight.

After growing the plates, the plasmid was harvested from RN4220. This was done using the Zymo Wizard kit. The steps for extracting the plasmid from staph cells differ in that lysostaphin must be added to RN4220 due to the difference in cell wall between Gram negative and Gram positive cells. Lysostaphin breaks down the peptidoglycan in Gram positive cell walls and allows for the extraction of the plasmid. In this experiment, 10 µl of lysostaphin was added after adding resuspension buffer and the samples were allowed to incubate for 30 minutes. Following this incubation, the lysis step was performed and all other steps were performed as previously described. After purifying the extracted plasmid, the concentration was recorded using Nanodrop, and this plasmid was used to perform an restriction digestion the same way as previously described where the

amount of plasmid added depends on the concentration. After making the reaction tubes, the restriction digestion was performed as well as a gel electrophoresis where the results looked like the gel in Figure 2.

Moving the plasmid into USA300_LAC *S. aureus*

After harvesting the plasmid from RN4220, the next step is to insert the plasmid into the USA300_LAC *msaABCR* deletion mutant through phage farming and transduction. RN4220 plasmid cells were added to a 1.5 ml microcentrifuge tube and suspendeds in 1 ml of tryptic soy broth (TSB). 200 µl of the bacterial solution was added to four test tubes with 200 µl of one of the four phage Φ11 dilution stock solutions. This phage is a transducing phage used to move plasmids from RN4220 to USA300. 40 µl of 500 micromolar (µM) $CaCl_2$ and 5 ml of top agar were added to each tube, and each tube was poured over a tryptic soy agar (TSA) plate. The plates were incubated lid up overnight at room temperature.

After incubating the plates overnight, one of the four plates was chosen for each strain to use for the transduction step. 4 ml of TSB was added to the selected plates and sthe top agar layer was scraped into a 15 ml tube. The tube was vortexed until its contents were completely suspended and uniform and were then spun in the centrifuge for 10 minutes at 10000x*g*. The supernatant was filter sterilized into new 15 ml tubes using 0.45 µm filters, and the new tubes were placed on ice. Cells from a plate of the recipient strain USA300_LAC *msaABCR* deletion mutant were added to a 1.5 ml microcentrifuge tube with 1 ml of TSB. The cells were suspended and 200 µl of the bacterial solution, 100 µl of phage lysate, 400 µl of TSB, and 20 µl $CaCl_2$ were added to new 1.5 ml tubes. The

tubes were incubated shaking for 20 minutes. After the incubation, 400 µl of 0.02 molar (M) sodium citrate (Na citrate) was added to each tube and they were placec on ice for 2 to 3 minutes. The tubes were spun in the centrifuge for 5 minutes at 10000x*g* and the supernatant was discarded. The pellet was resuspend in 400 µl Na citrate and plated on TSA plates supplemented with Na citrate and kanamycin (50 µg/ml). 50 µl, 100 µl, and the rest of the sample were spread on three plates in the same manner as previous steps.

The plates were incubated overnight, and selected colonies were subcultured in the same manner that the *E. coli* cells were subcultured. The plates were thenallowed to incubate overnight. The plasmid was harvested from the USA300_LAC bacteria in the exact same way as for RN4220, and the concentration of the plasmid was recorded. The tubes were stored at 4°C. After harvesting the plasmid, a restriction digestion was performed using this plasmid and the same restriction enzymes that were used throughout this experiment in order to verify the digestion product through gel electrophoresis.

Phenotypic Assays

To conduct a biofilm assay, 200 µl of human plasma was added to the required number of wells needed to measure each construct in triplicate using a 96 well microtiter plate and was incubated overnight at 4°C. Cultures of each strain were made in biofilm media, which is TSB supplemented with 3% sodium chloride and 0.5% glucose, and allowed to incubate overnight. After incubating the wells overnight, the plasma was removed from the wells by gentle aspiration. The cells were normalized to 0.1 optical density (OD) at 600 nm in 5 ml of biofilm media, and the 96 well plate was inoculated with 200 µl of the diluted bacterial cultures. The plate was placed in the incubator

overnight. Following this incubation, the bacterial cultures were aspirated from the wells and each well was washed gently three times with 200 µl of phosphate-buffered saline (PBS).

After washing, the biofilm was fixed in the wells with 200 µl of 100% ethanol and the ethanol was immediately aspirate off. The plate was allowed to dry for 10 minutes with the lid off inside of a sterile hood. Once the plate had dried, the biofilm was stained by adding 200 µl of crystal violet to each well for 2 minutes, and then the crystal violet was aspirated from the wells. The wells were washed three times with 200 µl of PBS in the same manner as the first wash step. The plate was allowed to dry overnight with the lid off inside of the hood. To take the measurement, 50 µl of acetic acid was added to each well, and the plate was placed in the microplate reader. The system was set to read the absorbance at 595 nanometers (nm).

To conduct protease and pigmentation assays, the strains were grown in 10 ml of TSB overnight. The cells were normalized to 0.1 OD at 600 nm in 10 ml of TSB, and 1 ml of the standardized culture was placed in a 1.5 microcentrifuge tube. The tubes were centrifuged and the supernatant was filter sterilized using a 0.45 µm filter. The pellet was used for the pigmentation assay, and the filtered supernatant was used for the protease assay. To conduct the pigmentation assay, the pellet was dissolved in 1 ml of 100% methanol by vortexing the solution. This solution was placed in the water bath for 10 minutes at 58°C. The pellet was vortexed periodically to help it dissolve. The tube was centrifuged for 5 minutes at 10000x*g*. The OD of the supernatant was taken at 465 nm using water for the blank.

To conduct the protease assay, three 1.5 ml tubes were set up per sample including the controls. An azocasein solution (3 mg/ml in PBS) was prepared and filter sterilized using 0.45 µg filters. 300 µl of supernatant and 800 µl of azocasein was added to each tube. The tubes were incubated overnight in the dark. To ensure that the reaction was completely in the dark, the tray of tubes was completely wrapped in aluminum foil. After incubating, the un-degraded azocasein was precipitated out by adding 400 µl of freshly prepared 50 % trichloroacetic acid (TCA). After adding the TCA, the tubes were centrifuged for 10 minutes at 15000x*g* to pellet the precipitate. The OD of the supernatant was measured at 340 nm in the spectrophotometer using water as the blank.

Chapter 3: Results

The truncated complement constructs were made, inserted in the *msaABCR* deletion mutant, and used in this study to study the role of 5' and 3' UTR region of *msaABCR* operon in the virulence and biofilm development in *S. aureus*. We studied different *msaABCR* operon related phenotypes such as pigmentation, extracellular protease production, and biofilm formation. The truncated complement constructs were amplified by using a combination of primers (Sahukhal & Elasri, 2014) shown in Table 2. A representative photograph of a PCR amplification is shown in Figure 1. Once the truncated complements were inserted in the pCN34 plasmids, they were used to transform *E. coli*. The plasmid constructs were verified by using PCR and restriction digestion before we moved the plasmids into the *S. aureus* RN4220 strains. A representative photograph of a restriction digestion is shown in Figure 2. The TC-2, TC-3, TC-4, and TC-5 constructs were 5' constructs, meaning that they lacked base pairs (bp) from that end of the transcript, and were 1355 bp, 1421 bp, 1487 bp, and 1604 bp ling, respectively (Figure 3). The TC-6 and TC-7 constructs were 3' constructs and were missing base pairs from the 3' end of the transcript. The 3' constructs were 1513 bp and 1656 bp long, respectively (Figure 3).

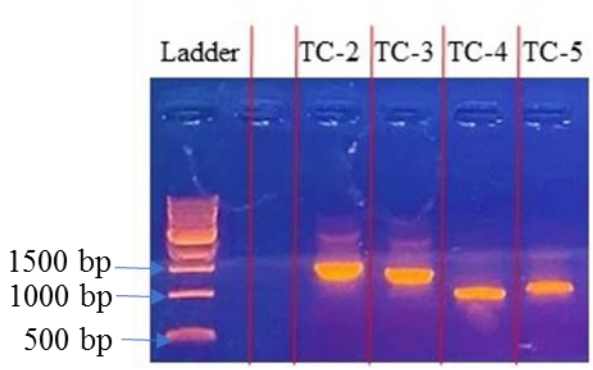

Figure 1. Agarose gel electrophoresis of PCR products. The figure showing the representatives of PCR fragments amplified to make the truncated complement constructs.

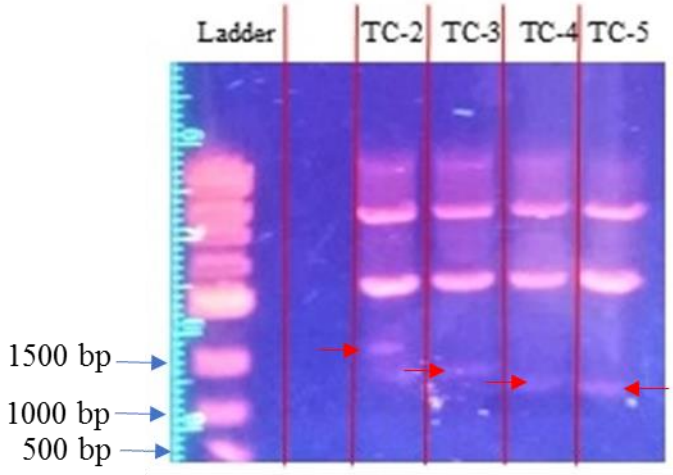

Figure 2. Restriction digestion gel electrophoresis. Restriction digestion were performed to confirm the truncated complement constructs. The representative picture is shown above. The red arrow denotes the fragments that were inserted in the plasmid pCN34.

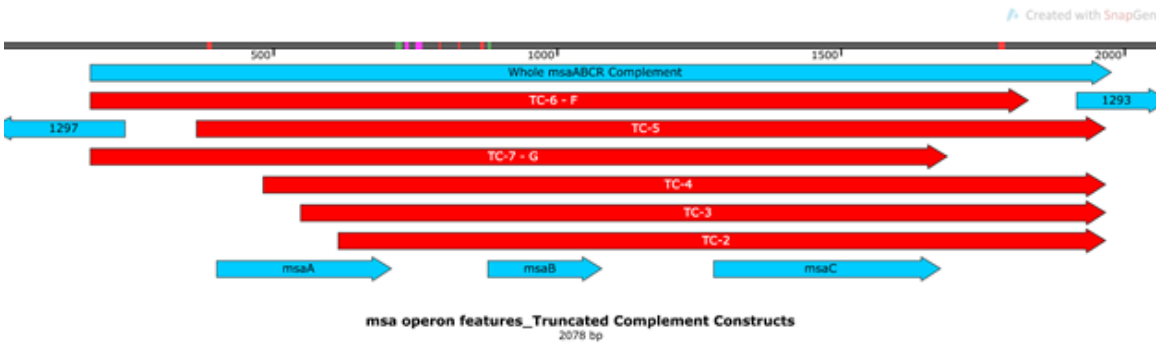

Figure 3. Truncated complement constructs; TC-2 through TC-7 were constructed using pMOE 792 as templated DNA. TC-1 to TC-5 constructs included a series of 5' UTR end truncation from the 5' end of *msaABCR* operon while TC-6 and TC-7 constructs included a series of truncation from the 3' end of the transcripts. All the truncated complement constructs were inserted into pCN34 plasmid. The constructs were transduced to *msaABCR* deletion mutants and used as truncated complements to study the major *msaABCR* phenotypes.

The full *msaABCR* operon transcript with its complete intergenic region complemented to the wild type level in terms of pigmentation, protease production, biofilm development, and MsaB production. However, in this study we constructed several complement constructs that contained truncated regions either in 5' UTR region or in 3' UTR region of *msaABCR* operon as listed in Table 1 and studied the level of complementation in the *msaABCR* deletion mutant.

We performed pigmentation assay, in which we quantified pigmentation produced by *S. aureus*. Our result showed that *msaABCR* operon mutant do not produce any pigmentation, whereas wild type strain produce pigments. The *msaABCR* operon complement produce huge amount of pigments that exceeds wild type level. All the truncated compelement constructs also produce pigments to wild type level, except TC-6 that failed to produce pigment. This result showed that the 3' UTR region of *msaABCR* operon might play role in pigmentation (Figure 4).

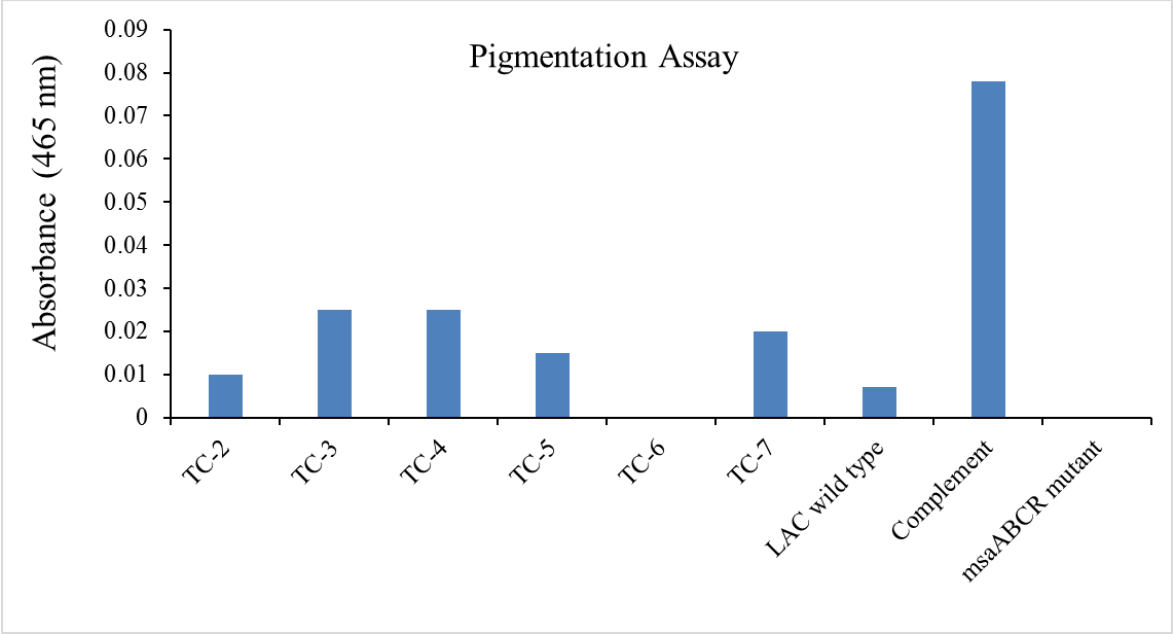

Figure 4. Pigmentation assay. The results of the pigmentation assay which compares the optical density of the pigmentation for each construct, the wild type, complement, *msaABCR* deletion mutant, and a control using the absorbance readings at 465 nm.

The results of the protease assay showed that all truncated complement including wild type and *msaABCR* deletion mutant showed similar level of protease production. (Figure 5). The results from the protease assay was not very informative at this time. However, Sahukhal and Elasri, 2014 showed that the *msaABCR* deletion mutant produced increased level of protease production. In fact, in this study we found similar proteases phenotypes in all the strains (Figure 5).

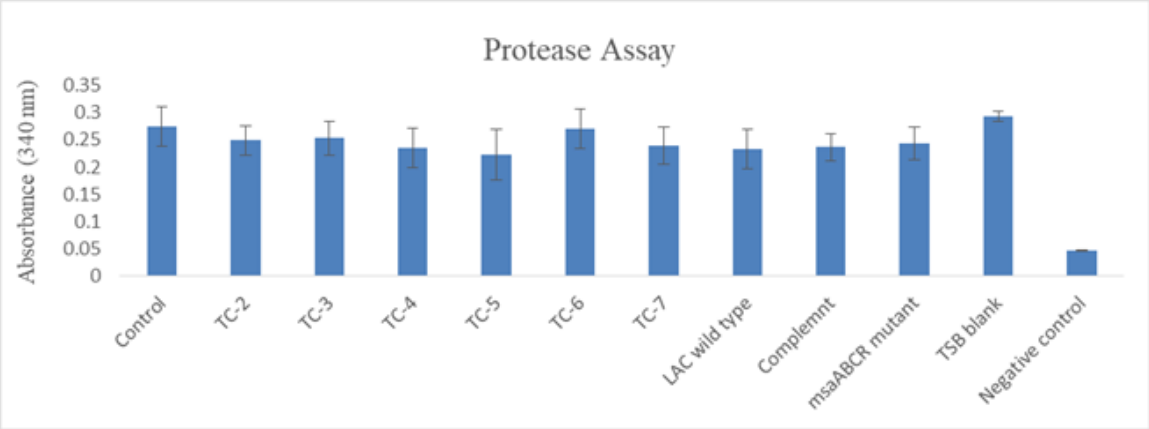

Figure 5. Protease assay; The results of the protease assay which compares the optical density of the protease production for each construct, the wild type, complement, *msaABCR* deletion mutant, a positive control, a negative control, and a blank using the absorbance readings at 340 nminutes. The standard deviation is also shown in the error bars.

We also performed the static microtiter biofilm formation assay as described in methods. The results showed that the *msaABCR* deletion mutant is defective in biofilm formation. The *msaABCR* operon complement fully restored the defective biofilm formation in the *msaABCR* mutant. Likewise, the TC-2, TC-3, TC-5, and TC-7 complements also fully restored the biofilm phenotype in the *msaABCR* mutant. However, the TC-4 and TC-6 complements were unable to restore the biofilm phenotype of the *msaABCR* mutant (Figure 6 and Figure 7. Overall, the results showed that both the 5’ and 3’ UTR regions of the *msaABCR* operon are important for the regulation of virulence factors. However, does these UTR region plays role in the MsaB production is not known, so further study should be conducted.

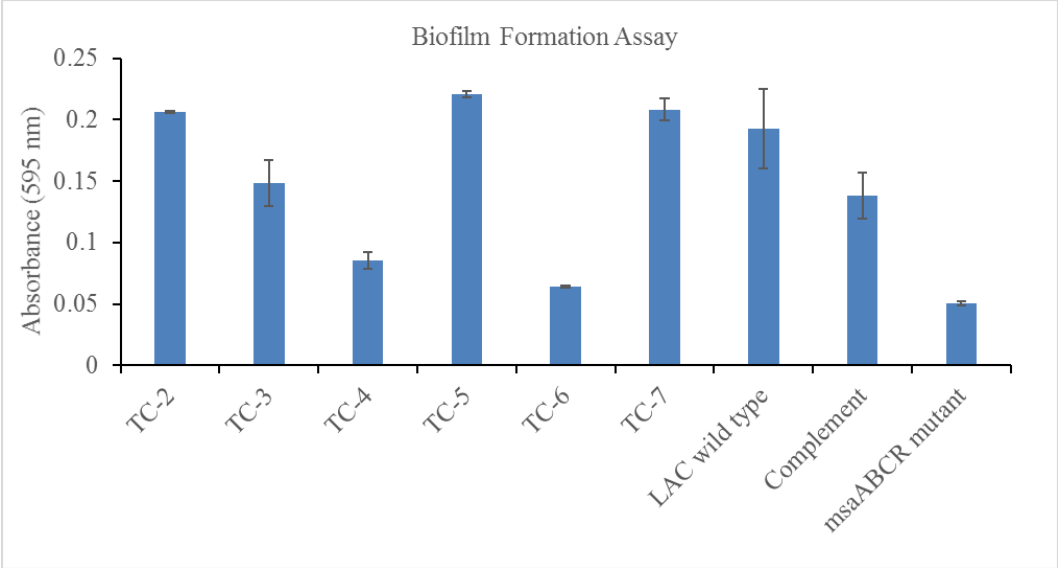

Figure 6. Biofilm assay; The results of the biofilm formation assay which compares the absorbance of the biofilm formation at 595 nm for each construct, the wild type, complement, *msaABCR* deletion mutant, and a control. The standard deviation is also shown in the error bars.

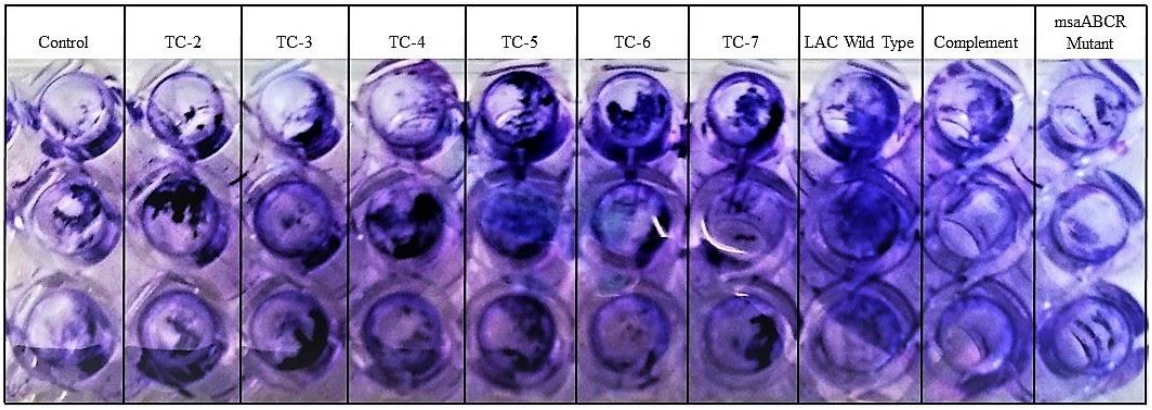

Figure 7. Biofilm production; The results of the biofilm formation assay which shows the biofilms formed in the microtiter plate for each construct, the wild type, complement, *msaABCR* deletion mutant, and a control. The absorbance of these biofilms was used to create the graph in Figure 6.

Chapter 4: Discussion

A newly characterized *msaABCR* operon plays important role in regulating several important staphylococcal virulence like biofilm formation, pigmentation, capsule production, and protease production. The *msaABCR* operon is a four-gene operon that contain *msaA, msaB, msaC,* and *msaR*. The only protein coding gene is *msaB* and produce a protein called MsaB, which contains cold shock domain and is a transcriptional factor, that binds DNA to regulate its target gene. The non-coding genes *msaA* is located in the 5' UTR region and *msaC* is located in the 3' UTR region of *msaABCR* operon. Although we know that the full intact *msaABCR* operon transcript with its complete intergenic region is required to fully complement the *msaABCR* deletion mutant, we still do not fully understand the contribution of 5' UTR (*msaA*) and 3' UTR (*msaC*) in the function of *msaABCR* operon. Therefore, in this study we constructed a series of several truncated complement constructs from 5' UTR end and 3' UTR end of *msaABCR* operon and used as a complement in the *msaABCR* mutant.

This study helps us to understand the role of the 5' and 3' UTR region of the *msaABCR* operon in regulating the phenotypes like formation of biofilms, production of extracellular proteases, and pigmentation. The results of the study showed that the full *msaABCR* operon transcript with its complete intergenic region complemented the *msaABCR* mutant back to wild type level in terms of pigmentation, protease production, and biofilm development as observed in previous studies.

The results fron this study indeed indicated that the 5' and 3' UTR region of *msaABCR* operon does contribute to the *msaABCR* operon related phenotypes. For instances, TC-6 is unable to produce pigments, and TC-4 and TC-6 are unable to

complement biofilm formation. The results of this study should be further analyzed, and the study should be repeated to confirm these findings.

Further analysis of these results should confirm that both the 5’ and 3’ UTR region of the operon are crucial in regulating virulence in *S. aureus*. Analysis of the alterations to the genome were inconclusive in that the study must be replicated to confirm the findings and verify that the results of these experiments are accurate. Repetition of this experiment will be more insightful as to its implications in the biology of *S. aureus* and the regulation of its virulence factors in relation to the *msaABCR* operon. Furthermore, the effect of 5’ and 3’ UTR truncation in stabilization of msaABCR transcript, msaABCR transcript processing and MsaB production should be studied in detail to better understand the precise role of these UTR regions in msaABCR related phenotypes in *S. aureus*.

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