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The Regulatory Relationship of Transcriptional Regulators MsaB and CodY in Capsule Production in *Staphylococcus aureus*

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by

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Abstract

There are many transcriptional regulators found in pathogenic *Staphylococcus aureus*. Many of these regulators are essential for the organism's ability to switch from commensal form to the virulent pathogenic form. One of these main regulators is CodY. This regulator has been shown to be responsive to nutrient availability during phases of growth. Additionally, we have recently found that MsaB, the only protein coding ORF of the *msaABCR* operon, is a putative co-regulator of capsule along with CodY. To explore these regulator interactions, we produced mutations of *codY* and *msaABCR* individually and a double mutation of *msaABCR/ codY*. We have observed that both MsaB and CodY bind to the *cap* promoter region in very close proximity. CodY binds to this region in early phases of growth in response to nutrients as a repressor of *cap* transcription and MsaB binds to this region in the later phase of growth as an activator of *cap* transcription. Using a chemically defined medium (CDM), we have shown that the *msaABCR* operon and ultimately MsaB is also likely involved in sensing nutrient availability. In this study, we show that the transcriptional regulator, MsaB, interacts with another known DNA binding transcriptional regulator, CodY, in *S. aureus*. These interactions are important in the regulation of sensing environmental nutrients and regulating capsule production in *S. aureus*.

Keywords: *S. aureus*, capsule, MsaB, CodY, ChIP, virulence, infection

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List of Abbreviations

S. aureus	*Staphylococcus aureus*
msa	Modulator of *sarA*
ChIP	Chromatin Immunoprecipitation Assay
CDM	Chemically Defined Media
BCAAs	Branched Chain Amino Acids
DoLR	Drug of Last Resort
OD	Optical Density
LB	Luria-Bertani Broth
TSB	Tryptic Soy Broth

Chapter I: Introduction

The purpose of this study is to gain more insight on the transcriptional regulation of capsule production in *Staphylococcus aureus*. Because of the increasing inefficacy of current microbial treatments, there has been a shift in the field of microbiology to examine molecular pathways involved in the expression of virulence factors employed by the pathogen during a successful infection. This information will lead to the development of more specific treatments that silence expression pathways that make the pathogen more virulent. Gyan Sahukhal and Mohamed Elasri (2014) identified a four-gene operon, *msaABCR*, in *S. aureus*. The study also specifically defined MsaB, the only protein produced by the operon, as a regulator of virulence in *S. aureus*. Another protein, CodY, has been identified as a direct link between virulence and nutrient availability in the different phases of growth (Pohl, et. al 2009). We further examined the role of MsaB and CodY proteins in the regulation and production of capsule in *S. aureus* and studied the different nutrient conditions surrounding the expression of capsule.

Justification

Since the recent discovery of the protein, MsaB, as a regulator, there has been limited published research on the specific actions of MsaB during the expression of capsule. This study is unique because it looks closely at the interaction of CodY and MsaB at the capsule promoter region in *S. aureus*. Research in the field has identified that nutrients influence the production of capsule in *S. aureus*, but there has not been any direct mechanism of this nutrient dependent regulation identified. We gain more insight on the regulation and expression of capsule in *S. aureus* in this study.

Methods

To examine the regulatory relationship between MsaB and ~~CodY, single~~CodY, single mutations of *codY* and *msaABCR* as well as a double *codY/ msaABCR* mutant were analyzed to see how the mutations effected capsule expression compared to the wild type strain. *E. coli* was used as an expression system to express and purify MsaB protein as was done in previous work (Batte, et. al., 2016). This protein was used to perform binding assays and investigate what nutrient conditions hinder or promote capsule production.

The Chromatin Immunoprecipitation assay was used to determine if and in what phase of growth CodY and MsaB bind to the capsule promoter region. ChIP analysis involves using an antibody produced specific to the desired protein to bind to the protein-antigen complex formed in a crosslinked treated total cell lysate (Das et. al, 2004). We harvested cells during the different phases of growth and grown under different conditions to discover when MsaB and CodY are bound to the capsule promoter region.

Outcomes

We hypothesize that both MsaB and CodY bind to the *cap* promoter region in very close proximity in different phases of growth. We hope to determine if CodY binds to this region in early phases of growth as a repressor of *cap* transcription and if MsaB binds to this region in the later phase of growth as an activator of *cap* transcription. Other groups have shown that CodY responds directly to nutrient availability (Pohl et al, 2009). Using chemically defined medium or CDM also determined that MsaB is involved in sensing nutrients in the cell.

As growth continues, and the bacteria start to use up the nutrients in the media, CodY loses its affinity to bind the capsule promoter region and then MsaB binds to promote the capsule expression. In the nutrient depleted condition, MsaB binds to the *cap* promoter region in earlier phases of growth. By observing the activity of the capsule promoter region in the *codY* mutant and *msaABCR* mutant more information was uncovered on the other pathways the organism employs to produce capsule and persist in its' environment.

Significance

Methicillin resistant *S. aureus* is a priority for disease control due to infections being able to quickly escalate to a medical emergency. The rising ineffectiveness of current antibiotics creates a crisis in medicine as microbial mutation and contact continues to increase. As a result, there is a growing section of research focused on the study of the genetic pathways that make *S. aureus* more virulent. This study looks at how the cell's environment plays a role in the regulation of transcriptional factors. This regulation then affects the translation of virulent factors. Capsule is the specific virulence factor looked at in this study. The capsule enables the cell to resist phagocytosis by immune cells (Voyich et. al, 2005). Encapsulated organisms are found in asymptomatic colonization, abscesses and septicemia, which can lead to complex and life threatening infections. Outlining the transcriptional factors involved in the genetic pathway of capsule production in *S. aureus* will lead to the development of a new generation of antimicrobials. These antimicrobials could be developed to directly target certain virulence factors including capsule and lead to hindering the cell's ability to establish

infection. The less virulent cell would be more susceptible to antibiotics and the host's immune cells, which could produce better patient outcomes.

Chapter II: Literature Review

Staphylococcus aureus is one of the major causes of fatal nosocomial infection in hospitals. One of the reasons why this bacterium is so successful at infecting even the healthiest of individuals is because it contains a vast collection of virulence factors (Cassedevall and Pirfoski, 2001). These virulence factors include phenotypic characteristics, such as a polysaccharide capsule, and the bacteria's ability to resist antibiotic treatment. The Centers for Disease Control and Prevention reported the first case of vancomycin resistant *Staphylococcus aureus* in 2002 (Goldrick, 2002). This was alarming because vancomycin is used to treat methicillin resistant *S. aureus* strains as a drug of last resort or (DoLR). The increase in the rate of bacterial success in infection and colonization of the host creates a crisis in healthcare that calls for a more effective treatment and understanding of the transcriptional pathways that lead the production of the virulence factors that *S. aureus* employs during the infection. Not all colonization of *S. aureus* is harmful to the host. In fact, a large percentage of the population is colonized by *S. aureus* and experience no symptoms (Graham et al, 2006). *S. aureus* colonization becomes infectious when the organism switches from commensal to virulent form. An understanding of the environmental conditions and genetic pathways that cause this bacterium to switch from benign to virulent could be used to develop new types of therapies that target these specific pathways and therefore reduce the success of the pathogen.

Genetic Regulation of Virulence in *S. aureus*

The accessory gene regulator, *agr*, and the staphylococcal accessory gene regulator, *sar*, are two important global regulatory pathways for the expression of virulence in *S. aureus* (Dunman, 2001). Agr is responsible for quorum sensing of the cell (Queck et. al, 2008). Quorum sensing involves regulating expression of certain genes in response to cell-population densities toward the later stages of growth, such as the post-exponential phase (Queck et. al, 2008). A study done by Chan and Foster (1998) identifies the *agr* locus is also activated by the protein produced by the *sar* locus, *sarA.* SarA is a regulatory DNA binding protein that can regulate virulence expression through the *agr* locus by binding to the promoter regions, P1 and P2 (Dunman, 2001). SarA is also capable of regulating virulence independent of the *agr* locus (Dunman, 2001). The independent method of activating virulence involves direct interaction between SarA and the regions of noncoding DNA that regulate the transcription of virulence factors (Dunman, 2001). The study identifies SarA as a signal transducer that responds to oxygen and carbon dioxide availability in the cell (Dunman, 2001). Sambanthamoory et. al (2006) discovered a region of DNA that modifies the production and function of SarA and named it *msa*, modulator of *sarA*. This study suggests that it is a membrane protein in which mutation cause a decreased expression of SarA and virulence. These discoveries lead to studies that identified and defined the regulatory mechanisms employed by *msa* or (modulator of *sarA*). Gyan Sahukhal and Mohamed Elasri (2014) examined a *msa* deletion mutant and found that the deletion had an effect of the genes just upstream of *msa*. This study found that the *msa* was a part of a four-gene operon, *msaABCR*, that regulates many of virulence factors in *S. aureus*. The *msaABCR* operon is made up of

msaA, *msaB*, *msaC* (previously known as *msa*), and *msaR*. *msaB* is the only region of the operon that codes for a 66-amino acid protein named MsaB protein. The study identified MsaB as a regulator of several virulence factors, but does not describe how the protein interacts with other factors or what causes the cell to employ that pathway of gene expression. Further research is needed on the relationship between MsaB, SarA, and *agr* in expression of virulence.

Capsule

The main virulence factor of my interest is the production of a capsule, an extracellular polysaccharide covering produced by the cell. The capsule protects the cells during harsh environments by helping it to resist desiccation and nutrient loss. Capsule serotype 5 and 8 are predominately found in clinical environments (O'Riordian and Lee, 2004). The capsule boosts the success of *S. aureus* in the bloodstream by preventing phagocytosis by leukocytes (Karakawa et. al 1998). O'Riordian and Lee (2004) further supported this observation with the finding that the two major opsonins, complement and antibody, were unable to bind to the encapsulated strains and therefore inhibiting leukocytic opsonophagocytosis. Many studies have shown that capsule production is highly sensitive to environmental signals. Herbert et. al (1997) showed that capsule serotype 5 production is dependent of carbon dioxide availability. Lee et al (1993) demonstrated that increased iron levels in growth conditions limited the production of capsule serotype 8. Capsule production is maximally expressed during the post exponential growth phase in *in vitro* models (Pöhlmann-Dietze, 2000). Luong et. al (2002) showed that the *agr* locus plays a role in the expression of capsule by quorum-sensing. Therefore, as the bacteria continue to grow in a set amount of media, the

increasing number of organisms start to deplete the nutrients and the density of the bacteria increases. These factors put the bacteria under stressful conditions in which prompt the production of virulence factors such as the capsule for protection.

The Role of *codY* in *S. aureus*

The idea of the cell sensing nutrients and then adapting its phenotype to best fit its environment lead to studies on the gene, *codY*. A study done by Pohl et. al (2009) demonstrated the sensitivity of CodY protein to nutrients and its regulation of virulence. The study suggests that under conditions of amino acid surplus, CodY represses the virulence genes that would help the bacteria live in limited nutrient environments. *codY* null mutants and wild type strains were grown in chemically defined media (CDM) containing all three-branched chain amino acids (BCAAs), valine, leucine, and isoleucine. Analysis of this data showed that the *codY* mutants grew more slowly than wild type in the CDM. Because *codY* works as a repressor of virulence, it masks many of the virulence factors that would help the bacteria survive in low nutrient environments. Majerczyk et al. (2010) identified that CodY was active by the presence of BCAAs. They also found that CodY controls the expression of virulence genes through sensing nutrients in the cells' environment. They also noted that CodY represses some genes of the *cap* locus directly and through a *agr* locus dependent manner. Pohl et. al (2009) found that isoleucine specifically has a more dominant effect on CodY compared to the other branched chain amino acids (BCAAs). This suggests that genes needed for growth are repressed by CodY during heavy isoleucine concentrations, which indicates that isoleucine is a major ligand regulating CodY repression of virulence. More research is

needed on the interactions on *agr*, SarA, MsaB, and CodY in relation to the environment to completely outline the transcriptional pathway of capsule production in *S. aureus*.

Protein Purification and Expression

Studying the interactions of CodY and MsaB require purified protein samples of each. Bornhorst, et. al (2000) detailed an efficient way of producing a large amount of desired protein using *E. coli* as an expression vector. The construction of the plasmid involves amplifying the region of DNA that codes for the protein from the organism that will be produced. This region is then ligated into a digested plasmid with six amino acid histidine tags. The plasmid is then transformed into *E. coli* and it contains instructions in its own genetic material on how to create the protein from the organism. Harvesting the protein from the *E. coli* involves growing and lysing the cells and then separating the protein from the cell debris by centrifuging the sample. Centrifuging the lysed cells leaves the protein suspended in the media and the large cell fragments spun towards the bottom of the container. Next the lysate is run through a nickel resin column. Histidine binds to the nickel inside the column as the lysate passes through the resin. In this study, buffer containing 10 mM imidazole was used to wash out any undesired proteins that may have bound to the nickel column because they contained one or two histidine tags. After washing multiple times with the low amount of imidazole, eluting the rest of the desired six histidine tagged protein was accomplished by increasing the amount imidazole concentration to 250 mM. This model of protein purification can be used in many different organismal models. Once the protein is purified, it can be used in different binding assays to detect the region of DNA in which the protein associates.

Protein DNA Interaction Studies

Das et. al (2004) described an assay that observes protein and DNA interactions called chromatin immunoprecipitation, (ChIP). ChIP involves extracting the protein and DNA complexes from whole cell fragments using an antibody generated from the protein of interest. ChIP is a useful method for the study due to it allows to test what phase of growth the protein associates or disassociates with the DNA region. This is especially important because the expression of many virulence factors such as capsule have been shown to be growth-phase dependent (Dunman, et.al, 2001).

Chapter III: Methodology

This study uncovers more information about the transcriptional mechanism *S. aureus* utilizes during the different environmental conditions that it encounters, specifically focusing on the genetic pathways involving the MsaB and CodY proteins. CodY has been shown to be a major repressor of virulence factors in response to nutrient availability in the different phases of growth (Pohl et. al, 2009). The protein MsaB has been previously identified as a regulator of virulence factors but little is known about this expression pathway (Batte, et. al, 2016). To assess the relationship between MsaB and CodY in capsule production, we analyzed the capsule expression of a *codY* mutant, *msaABCR* mutant, and a double *msaABCR/codY* mutant compared to wild type to test the effects these mutants made on growth and regulation of capsule production. Large amounts of CodY and MsaB protein were expressed using *E. coli* as an expression vector. The purified protein can then be used to perform binding assay to the capsule promoter region and protein interaction studies.

Growth Curves in Chemically Defined Medium

The mutants were grown with optical density (OD) measured hourly in an extended growth curve for at least 12 hours using chemically define media (CDM). Similar to the study done by Pohl et.al (2009), the bacteria were grown in plain CDM and CDM without branched chain amino acids (BCAAs) valine, isoleucine, leucine. The strains, *msaABCR* mutant, *msaABCR* complement, and wild type were normalized to an OD of 0.05, in CDM and incubated while shaking at 37° Celsius. The OD was measured hourly to observe the growth rate of the *msaABCR* mutant and complement compared to wild type.

Protein Expression and Purification

Purified samples of CodY and MsaB protein are needed to study the interactions and binding sites of each. *E. coli* is an organism commonly used as a protein expression vector because it is highly efficient and cost effective (Hannig, 1998). We created a plasmid constructs with an antibiotic marker that contains the *codY* or *msaB* protein coding region with a six-histidine tag and transform the plasmid into *E. coli* (Charpentier et. al, 2004). The cells were grown in one liter of Luria-Bertani broth (LB) at 37° Celsius for 5 hours. The cell lysate was created by using a sonicator with amplitude of 30% for 20 seconds on and 1 minute off repeated seven times. The lysate was centrifuged at 10,000 G for 20 minutes to remove cell debris. Following the Bornhorst and Falke (2000) detailed protocol of purifying the six-histidine tag *codY* protein using a nickel resin column. The lysate was run through a charged nickel resin column twice. The column was washed with at least 40 ml of wash buffer containing 10 mM imidazole, verifying with a negative Bradford reagent reading of the lysate that all nonspecific proteins were

eluted through. Our protein was eluted with the elution buffer that contains 250 mM imidazole.

Chromatin Immunoprecipitation Assay

Sengupta et. al (2004) detailed the chromatin immunoprecipitation, ChIP, assay is used to determine if and in what phase of growth does MsaB and CodY associate with the *cap* promoter region using a whole cell lysate. The *codY* mutant, *msaABCR* mutant, and wild type were liquid cultured under two conditions (CDM and CDM with no BCAAs) and incubating at 37° Celsius. Cells were then collected during the early-, mid-, late-, and post-exponential phases of growth. The crosslinking reaction was initiated by treating the cells with 1% formaldehyde and 10 nM sodium phosphate. The crosslinking reaction was stopped when 3 M glycine was added to the solution. Spinning and washing the cultures with an equal volume on 0.1 M phosphate buffer removed excess formaldehyde. The cells were pelleted to remove the phosphate buffer and then resuspended in 200 uL of immunoprecipitation (IP) buffer. After the cells were bead beaten the cells were spun to remove cell debris. The lysate was diluted with 1 ml of IP buffer. The diluted antibody was added to 200 uL of clear lysate and incubated at room temperature for 2 hours. The mixture was added to Protein-G-coupled magnetic beads and incubated for one hour. A magnetic tube holder was used to collect the antigen-antibody-bead complex washed with tris buffer saline and ultrapure water. To reverse crosslinks, the beads were resuspended in elution buffer an incubated 65 C overnight. Phenol-chloroform extraction method was used to extract the DNA. Polymerase chain reaction was then used to amplify the DNA with site specific primers.

Chapter IV: Results

Restricted Nutrient Growth Curves

The results of the growth curve in CDM and CDM without BCAAs media are presented in Figure 1 and Figure 2 respectively. All bacteria strains experienced an extended lag phage in both CDM and CDM without BCAAs. There was also a slower growth of all strains of the bacteria in either of the CDM medium compared to a rich medium, tryptic soy broth (TSB). Additionally, all the strains had an extended lag phase initially. However, *msaABCR* operon mutant showed slightly decreased growth rates and patterns as well as a more pronounced extended lag phase compared to wild type and complement in whole CDM media. Interestingly, in the CDM without BCAAs the wild type and complement has a very long lag phase and seem to grow very slowly. However, the *msaABCR* mutant growth is very similar, in a putative "uncontrolled" manner, in CDM without BCAAs compared to whole CDM medium. Taken together, these results suggest that in an unidentified mechanism the *msaB* and ultimately the *msaABCR* operon are sensing and/or responding to changes in nutrients.

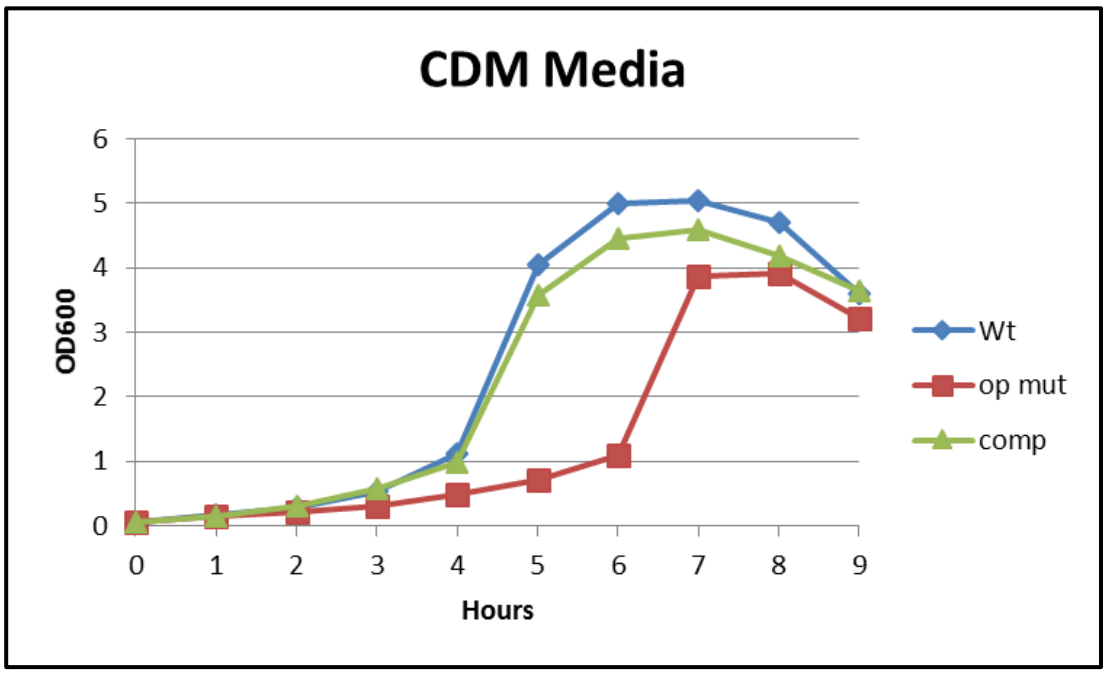

Figure 1 Growth curve in CDM with *S. aureus* strains of wildtype, *msaABCR* mutant, and *msaABCR* compliment for nine hours

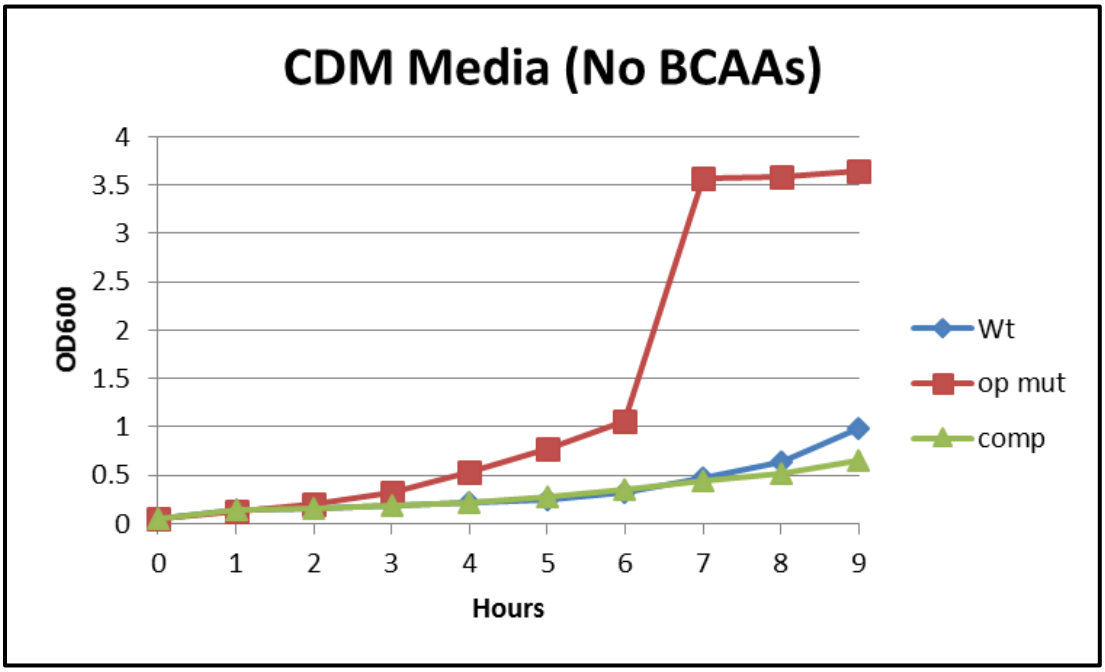

Figure 2 Growth curve in CDM with no BCAAs with *S. aureus* strains of wildtype, *msaABCR* mutant, and *msaABCR* compliment for nine hours

Chromatin Immunoprecipitation Assays

Figure 3 shows results of ChIP analysis of wild type, *msaABCR* operon mutant, and the *codY* mutant. The *codY* mutant had visible bands in all phases of growth. This means that there is binding of MsaB protein to the capsule promoter region in all phases of growth. This differs from wild type in that MsaB only binds to the *cap* promoter region in late- and post-exponential phase of growth. Importantly, these results suggest that in an undetermined mechanism that CodY, when present in wild type, is inhibiting MsaB binding in early phases of growth. The ChIP analysis in CDM media and CDM without BCAAs (nutrient limited conditions) revealed that MsaB binds to the *cap* promoter region in the mid-exponential phases of growth in wild type, independent of CodY presence. Interestingly, in limited nutrient conditions even in conditions when CodY is supposed to be bound (whole CDM) MsaB binds independently of CodY shown in Figure 4. This data suggest that MsaB may be sensing nutrient availability itself altering its binding ability to its target. However, more work needs to be done to establish this putative nutrient sensing mechanism of MsaB.

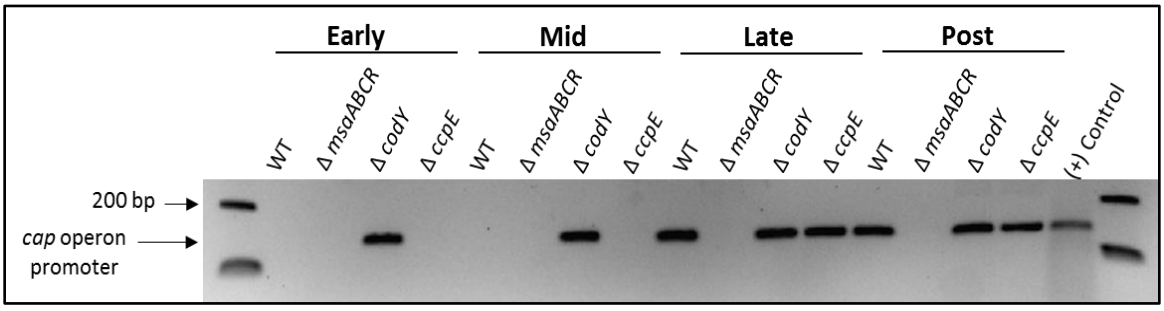

Figure 3 ChIP analysis in TSB of *S. aureus* wildtype, *msaABCR* mutant, *ccpE* mutant, and *codY* mutant in early, mid, late, post phases of growth

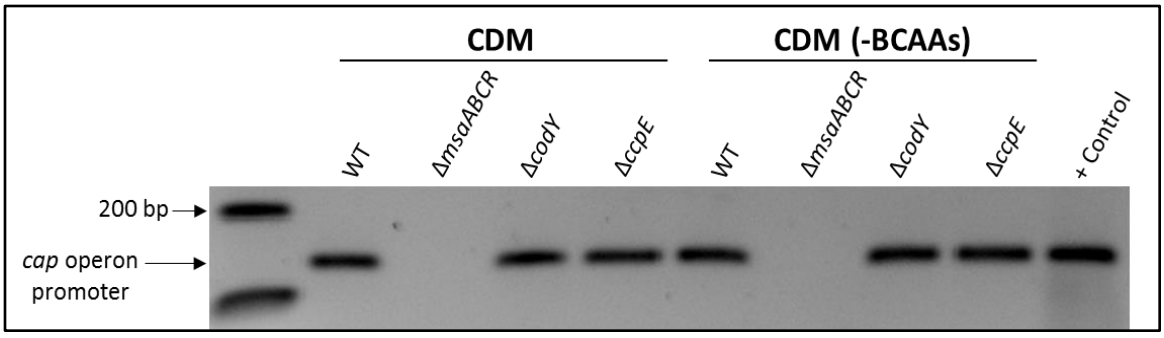

Figure 4 ChIP analysis of CDM and CDM with no BCAAs of *S. aureus* wildtype, *msaABCR* mutant, *ccpE* mutant, and *codY* mutant in mid-exponential phase of growth

Chapter V: Discussion

The *msaABCR* operon plays a role in the growth and regulatory activity or virulence genes in nutrient limited conditions. The deletion of *msaABCR* operon altered growth rate compared to wild type and the *msaABCR* complement, but increased regulatory activity (binding) in wild type in the CDM media suggest that certain mechanisms that the cell normally employs to survive were limited without the genetic information and sensing ability of the *msaABCR* operon. Thus, the *msaABCR* operon mutant is limited in ability to adapt its phenotype to survive in the stressful environment. The similarity of growth in CDM media and CDM media without BCAAs suggest that the *msaABCR* operon mutant is unable to sense and respond to in its environment. However, when MsaB or the *msaABCR* are present in the wild type the organism is able to respond properly to changes in nutrient levels.

Justin Batte (2016) previously showed that the regulation of MsaB and capsule production in *S. aureus* is linked to nutrient availability. MsaB was found to bind to the capsule promoter region only in the late phases of growth (Batte, et al, 2016). In the early phases of growth MsaB does not bind to the capsule promoter region and there is no

capsule production. This coincides with nutrient availability in the cell's environment. In the later phases of growth, there is limited nutrition. This triggers the activation (MsaB binding) resulting in capsule production. The result of the ChIP analysis in CDM media and CDM without BCAAs shows that MsaB is responding to nutrient availability. CDM media is much more minimal in nutrients compared to TSB. The limited environment places enough stress on the cell to produce activation of capsule production, as evidenced by MsaB binding to the capsule promoter region in the mid-exponential phase of growth. Additionally, in rich nutrient conditions in the *codY* mutant, MsaB bound in all phases of growth. Without this global regulator (repressor), the cell does not respond to the nutrients in its environment. This suggests that MsaB binds to its target site when the known repressor of capsule, CodY, loses its affinity to the site and detaches. This detachment of CodY to its region makes the MsaB binding site accessible for binding.

More research is needed to identify other transcriptional factors or nutrient stimuli involved in the translation of capsule production in *S. aureus*. In the future, it is important to uncover more information about specific nutrients and other signals that stimulate this process. We hope to gain more knowledge on how significant the *msaABCR* operon and *codY* is in sensing nutrients as well as what specific nutrients have a dominant effect on the regulation of different virulent factors.

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