5-2021

Phenotypic Characterizations of msaABCR operon deletion in Staphylococcus Epidermidis RP62A

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Phenotypic Characterizations of *msaABCR* operon deletion in *Staphylococcus Epidermidis* RP62A

by

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

May 2021

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ABSTRACT

Staphylococcus epidermidis is a human pathogen that is increasingly known for its role in hospital infections associated with implantable medical devices. Antibiotic resistance has become a concerning issue for these infections as this bacteria have various virulence traits that help to evade immune response and antibiotic treatment. Currently, the most effective way to treat *S. epidermidis* infection is removal of the implant and long-term antibiotic treatment. *S. epidermidis* causes infection by expressing several protein factors that induce biofilm formation, the bacteria's primary virulence mechanism. The purpose of this study was to perform the phenotypic characterizations of the *msaABCR* operon in *S. epidermidis,* and we hypothesized that *msaABCR* may play a role in biofilm formation, protease production, urease production, and deoxyribonuclease production. To test this hypothesis, we constructed a mutant strain by deleting the *msaABCR* operon from the wild-type strain RP62A. The mutant was later used in several phenotypic assays to observe its activity in biofilm formation, protease production, PIA quantification, and urease production. The *msaABCR* mutant of RP62A showed increased biofilm relative to RP62A after 24hr incubation. However, the *msaABCR* mutant showed reduced biofilm compared to RP62A after 48- and 72 hr incubation. Increased protease and urease production was also observed in the *msaABCR* mutant relative to RP62A. This study provides insights on the role of the *msaABCR* operon in *S. epidermidis* virulence mechanisms as it relates to biofilm formation.

Keywords: *Staphylococcus epidermidis*; *msaABCR*; RP62A; biofilm formation; protease production; PIA quantification

ACKNOWLEDGMENTS

First, I would like to thank Dr. Mohamed O. Elasri for allowing me to join his lab team and conduct undergraduate research. His support has enabled me to grow in knowledge and confidence. Also, I would like to thank Dr. Gyan Sahukhal for his guidance and confidence in me throughout my research process. His mentorship played a significant role in completing my thesis, and I could not have finished this process without him. Lastly, I would like to thank Shanti Pandey and GC Bibek for their technical support and guidance as I conducted experiments.

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Aap	Accumulation-associated protein
AMP	Antimicrobial Peptides
CoNS	Coagulase-Negative Staphylococci
CVC	Central Venous Catheters
ECM	Extracellular Matrix
eDNA	Extracellular DNA
Embp	Extracellular matrix binding protein
Fbe	Fibrinogen binding protein
FMRI	Foreign Material Related Infections
MSCRAMMS	Microbial Surface Components Recognizing Adhesive Matrix Molecules
PCR	Polymerase Chain Reaction
PIA	Polysaccharide Intracellular Adhesin
PBS	Phosphate Buffered Saline
PSM	Phenol-soluble Modulin
SCOPE	Surveillance and Control of Pathogens of Epidemiological Importance
Sdr	Serine-aspartate repeat
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

CHAPTER I: INTRODUCTION

Staphylococci are Gram-positive microorganisms that can cause nosocomial infections in human. Staphylococci are also associated with chronic infection related to medical implants inserted in human body (1). *Staphylococcus epidermidis* was classified as a coagulase-negative staphylococci (CoNS), a heterogeneous group lacking coagulase, a blood-clotting enzyme (2). As a generally harmless and beneficial organism of the human skin flora, *S. epidermidis* majorly has been found to colonize moist areas of the body, including the armpit, conjunctiva, and groin (2). As a commensal bacterium for the skin and for maintaining homeostatic functions (3), it has additionally become known for its relation to nosocomial infections.

Today, *S. epidermidis* has shown to be an invasive, opportunistic pathogen in foreign material related infections (FMRI) (4) and in procedures that use implanted medical devices (3). The materials used in these procedures can be natural or synthetic and are referred to as biomaterials. Using biomaterials as implants has shown to replace or repair compromised tissue. However, this material has become vulnerable to several problems, bacterial infections being the most prominent (5, 6). *S. epidermidis* infections have occurred in procedures involving central venous catheters (CVC), artificial heart valves, prosthetic joints, prosthetic valves, and cerebrospinal fluid (7, 8). Patients with predisposing factors, such as congenital immune defects or concomitant medical conditions, have higher risks of developing a disease from a *S. epidermidis* infection (3, 9). A study revealed *S. epidermidis* to be the cause of a majority of hospital-acquired bloodstream infections related to catheters in the United States (3, 10). Additionally, the Surveillance and Control of Pathogens of Epidemiological Importance (SCOPE) revealed

the leading cause of hospital-acquired bloodstream infections to be from CoNS (3, 10). Out of the CoNS isolates, eighty percent were identified to be *S. epidermidis* (3). Annually, approximately 80,000 central venous catheter infections occur in the United States, caused by *S. epidermidis (1, 11)*. Therefore, understanding the mechanisms by which biofilm formation in *S. epidermidis* strains occurs and its association with the bacteria's virulence is important for developing new methods to prevent future occurrences of infection.

S. epidermidis's increasing emergence as a pathogen has been connected with its ability to form biofilm on medical devices (2). *Staphylococcus aureus*, which is another Gram-positive bacteria, posses several virulence factors to cause infection besides forming robust biofilms in humans. However, compared to *S. aureus*, *S. epidermidis* does not produce a similar level of virulence factors, but instead, its abilities to form biofilms on the surface of medical devices and on native tissues has shown to be the virulence mechanism causing infections (1). The three stages of biofilm development include primary attachment, proliferation of matured biofilm, and detachment (1). Research has shown that multi-functional protein factors, Aap (accumulation-associated protein) and Embp (extracellular matrix binding protein), are prominent contributors to the pathogen's biofilm formation abilities of adhering on the surface and intracellularly (3). Treatment of infections caused by the bacterium have shown to be difficult because of its extreme resistance toward antibiotics. Factors derived from the bacteria, such as PIA (polysaccharide intracellular adhesin), Aap, and Embp, have been responsible for protecting *S. epidermidis* from host cell-mediated effector cells (1). The abilities of these factors have contributed to the bacteria's ability to evade the host immune system and

survive antibiotic treatments. Despite the current methods of treatment for implant infections through high doses of antimicrobials and antibiotics, surgical removal of the implant along with long-term antimicrobial therapy have shown to be more effective (6). Previous studies from our lab showed that the *msaABCR* operon plays role in several different staphylococcal phenotypes like biofilm formation, virulence regulation, persister cells formation, cell wall biosynthesis, and antibiotic resistance in *Staphylococcus aureus* strains. Therefore, we hypothesized that the *msaABCR* operon might also be involved in *S. epidermidis* phenotypes like biofilm formation and virulence.

CHAPTER II: LITERATURE REVIEW

2.1 Stages of Biofilm Development

The mechanism of biofilm formation in *S. epidermidis* requires functional factors that can promote binding to the surface, promote cell-cell aggregation, and decompose matrix components (3,12). In the first stage of biofilm development, bacteria colonize the surface through adhesion. Despite the absence of specific receptor-ligand interactions, studies have shown the bacteria's expression of cell surface proteins with extracellular matrix (ECM) binding activities mediates interactions with host ECM components. These proteins are thought to play a pivotal role in initiating infection because of the vast amounts of ECM material that covers foreign materials as they enter the body (3,7,13). In the second stage of biofilm formation, the accumulation process, the process of forming channels takes place to transport nutrients to the deeper layers of the biofilm matrix (14; 1). The development and proliferation of the multilayer biofilm matrix is highly dependent on the expression of intercellular adhesin molecules. In the final stage of biofilm formation in *S. epidermidis* the biofilm clusters detach and spread to other areas of the body.

2.2 Structural and Multifunctional Factors in *S. epidermidis* Biofilm Formation

Three serine-aspartate repeat (Sdr) proteins, SdrF, SdrG and SdrH have been identified because of their role during primary attachment (3, 15). As microbial surface components recognizing adhesive matrix molecules (MSCRAMMS), Sdr proteins adhere to the implant surface through reversible non-covalent interactions (1, 13, 14). Irreversibly, the bacterial cells adhere to surfaces through dipole-hydrophobic interactions and ionic and hydrogen bonding. Additional surface molecules,

lipopolysaccharides and exopolysaccharides, also play a role in bacterial adhesion (6). It has been shown that the SdrG protein (called fibrinogen binding protein (Fbe)) on the surface of bacterial cells is necessary for attachment to the surface of the fibrinogen-coated implant. SdrG has revealed a "dock, lock, and latch" mechanism for bacterial-ECM interactions (3, 16, 17). The SdrF proteins have shown to be necessary for attachment of bacteria to implant surfaces coated with collagen I (3, 18).

S. epidermidis autolysin AtlE is a protein whose role in cell wall turnover and binding to unmodified polystyrene is pivotal to bacteria attachment (3, 19). The recruitment and activation of AtlE has appeared to prompt changes in the hydrophobicity of the bacterial surface (3, 20). Specifically, in S. *epidermidis*, AtlE controls much of the autolysis that occurs (3, 21). AtlE has also revealed a role in extracellular DNA (eDNA) mediated S. *epidermidis* biofilm formation (3, 22). Released during cell lysis, studies have revealed eDNA to be a structural element in the formation of biofilm. Observations of *S. epidermidis* 1457 and *S. epidermidis* RP62A have shown that bacterial attachment to glass surfaces is evaded in the addition of DNase I. Aside from its role in attachment, eDNA has been known to have a role in stabilizing biofilm formation, as a result of its adhesion functions (3, 22, 23).

One of the most important adhesins studied in *S. epidermidis* biofilm is PIA (3, 7). First identified in *S. epidermidis* 1457 and RP62A, PIA, a product of the *icaADBC* operon, consists of a major polysaccharide I and a minor polysaccharide II (1, 3, 24). As the "hemagglutinin" of *S. epidermidis,* the expression of all *icaADBC* genes is required for PIA to function (3, 25). As an adhesin, PIA embeds adjacent S. *epidermidis* cells in an

interlaced structure of extracellular matrix (1, 26, 27). In PIA-independent biofilms, additional proteins, such as Embp and Aap, function as intracellular adhesins (1, 28).

Identified in 90% of strains of *S. epidermidis* isolates, Embp has become known as a significant factor in primary attachment and biofilm accumulation (3, 29). The structural component of Embp consists of FIVAR-GA repeats, which has contributed to the Fibrinogen-binding activity of the molecule (3, 30). A study revealed over-expression of Embp to enhance adherence on surfaces that were Fn-coated. Aside from boosting adherence, Embp-Fn interactions also showed to be important for accumulation on plastic surfaces. Aside from its role in bacterial attachment, Embp has also shown to function in bacterial accumulation by acting as an intercellular adhesin. These functions were identified in a study that revealed the overexpression of Embp1, a shortened isoform (3, 31). The upregulation of Embp has also become known to correlate with resistance against phagocytosis (3, 28). Recently, studies have identified Embp as a likely candidate for preventive approaches against implant infections because of the inhibition anti-Embp antibodies demonstrated in *S. epidermidis* biofilm formation (3, 32).

Another molecule, Aap, has become known for its function in primary attachment, biofilm accumulation, and immune escape. Aap is a protein with an A- and B-domain, linked to the cell wall via its C-terminal anchor region. Localized to the surface of the bacteria, only minimum aggregates are found in the biofilm matrix (3, 27, 28, 33). Studies have revealed the formation of extended fibers in Aap that cluster and protrude away from the cell wall (3, 26). One study revealed varying recombinant proteins found within the B-domain of Aap, along with two regions (G5 domain and E-region). The interconnectedness of the two domains have shown to have thermodynamic

stabilities, which has led to the explanation of Aap's formation of projecting fibers in harsh conditions (3, 34). Additional studies have revealed the B domain's role in bacterial accumulation, revealing Aap's function as an intercellular adhesin (3, 27, 25). Another mechanism involving the modification of surface epitopes by B repeat variations has been hypothesized to contribute to immune escape (3, 29). Aside from the B domain, the A domain of Aap has been discovered to play a role in bacterial attachment to artificial surfaces or epithelial cells (3, 36, 37). In a study investigating bacterial attachment in *S. epidermidis* isolates, a processed Aap isoform, lacking the A domain, revealed to have no effect on bacterial adherence. However, in a mature, unprocessed Aap containing the A domain, adherence was almost completely inhibited, following the deletion of Aap (3, 36). Therefore, it was supported that mature, unprocessed Aap required the A domain for Aap-mediated adherence (3, 38). Studies have shown improved adherence in Aap expressing the A domain; therefore, it has been concluded that Aap's function in *S. epidermidis* biofilm formation is characterized by the protein's structurally distinctive domains (3, 27, 38).

Phenol-soluble modulins (PSMs), pro-inflammatory peptides, have revealed a pivotal role in biofilm dispersal (1, 39, 40). Producing six PSM peptides, δ-toxin and ß-PSM have shown to be the most prevalent in *S. epidermidis* (1, 41, 42). Studies have identified ß-PSM as the peptide responsible for promoting detachment and dispersal of biofilm in infections (3, 40). It is believed that PSM contributes to biofilm dispersal by disrupting the non-covalent interactions between molecules in the biofilm matrix (1, 39).

2.3 Molecular Interactions and Regulation in *S. epidermidis* Biofilm Formation

The *S. epidermidis* biofilm types (PIA-, Aap-, or Embp-) differ by morphological properties. The production of Aap in PIA-dependent biofilms forms a dense layer of bacteria that evenly covers the surface. However, in biofilms that rely on Embp, cells are not clustered, but produce a small amount of extracellular matrix containing Embp. Washing procedures have shown PIA-dependent biofilms to be more stable compared to Aap- or Embp-dependent biofilms (3, 28). Due to these properties, S. *epidermidis* is able to use adhesins to adapt to varying, changing environments. For example, in circumstances with stress exposure and factors of innate immunity, such as CVC infections, *S. epidermidis* strains are more likely to express *icaADBC* and form PIA-dependent biofilms (3, 7, 43).

Research has revealed the production of PIA, Aap, or Embp to be active during biofilm accumulation to promote cell aggregation. However, studies have shown a majority of *S. epidermidis* strains to carry all three genes, with none being sufficient on their own for mature biofilm formation (31, 44, 45). Other studies have shown that the intercellular adhesins play important roles during biofilm formation. Data has revealed stronger biofilm formation in strains with both *aap* and *icaADBC* as compared to strains with only *aap* or *icaADBC* (3, 46). Further bioinformatics have been analyzed to reveal the possible interaction between *aap*, *embp*, and PIA. The interaction between these adhesins has shown possible because of the Aap B domain binding activity and the Embp FIVAR regions sugar binding. As a result of the adhesins' binding activity, interactions involving PIA have shown to be possible but not yet fully supported. Functional substitution among the three genes has also become evident in several studies. In a

treatment involving protease, the expression of *icaADBC* and PIA revealed to protect a Embp-producing *S. epidermidis* strain from proteolytic inhibition (3, 31). The biological properties of each adhesin molecule may provide an explanation as to how *S. epidermidis* can survive immune responses and changing host environments during colonization and infection. Through analysis of *S. epidermidis* strains, this idea was supported as the bacteria can differentially use specific adhesins to adapt to specific environments, such as those in the presence of host effector cells or osmotic stress (3, 28).

2.4 Immune Evasion in *S. epidermidis* and Medical Relevance

The innate immune response against staphylococcal infections involves the recruitment of effector cells by the complement system. The purpose of the effector cells is to label the bacteria to be identified and killed. The three pathways of complement activation (classical, alternative, and lectin) play a role in the immune response to *S. epidermidis* infection, producing an attack complex to kill the cells. While the alternative pathway plays a small role, it is believed that the classical and lectin pathways play a larger role in the rapid killing of the bacterial cells (1, 47). Biofilm formation in *S. epidermidis* has shown to protect the bacteria from uptake by effector cells (1, 48).

PIA has shown to play an important role in *S. epidermidis* immune evasion. Studies have revealed PIA-producing *S. epidermidis* strains to be more virulent than isogenic PIA mutant strains (3, 49). As a prominent factor in biofilm accumulation, studies have also revealed a mechanism in which PIA forms a positively charged "capsule" around the bacteria. This mechanism has been known to protect *S. epidermidis* from immune recognition (1, 50). Additional mechanisms of PIA include the prevention of neutrophil attacks when bacterial clusters are broken down and protection against host

antimicrobial peptides (AMPs) (1, 24). Other adhesins, Aap and Embp, have also shown to protect *S. epidermidis* strains from phagocytosis (28, 31). In a catheter infection model, *icaADBC* and *aap* were inactivated and results were compared. While the inactivation of the *ica* operon had no effect on bacterial colonization, *S. epidermidis* was almost unable to infect the model when *aap* was inactivated (3, 38).

Studies have suggested that PSMs are the only gene products in *S. epidermidis* that possess cytolytic functions (1, 51). PSMδ, specifically, has revealed highly cytolytic activity towards human neutrophils, contributing to the bacteria's pathogenesis. Several mechanisms of PSM have resulted in a low inflammatory profile, potentially helping *S. epidermidis* evade the immune system and colonize medical device surfaces (1, 52). A recent study has observed PSM-mec, which is expressed in methicillin-resistant bacterial isolates, as a potential cause of bloodstream infections related to *S. epidermidis* implant infections and antibiotic resistance within *S. epidermidis* (1, 53).

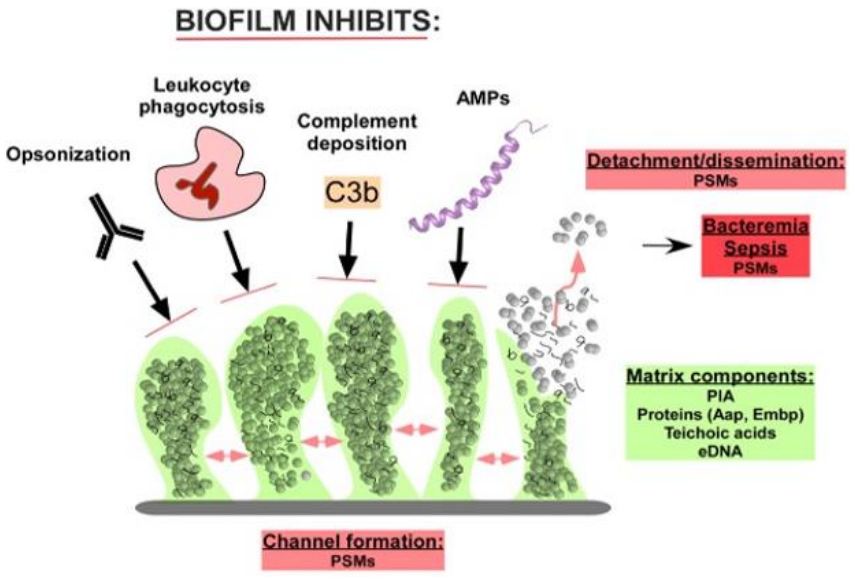

Figure 1: Representation of the biofilm matrix. (Composition and function of *Staphylococcus epidermidis* biofilms in immune evasion: Le et al. 2018. Immune Evasion Mechanisms of *Staphylococcus epidermidis* Biofilm Infection. Front Microbial (1)).

Bacteremia has become a known result of bacterial dispersal from *S. epidermidis* biofilm on implant devices. The presence of CoNS in the bloodstream can result in further infection, leading to acute sepsis. With *S. epidermidis* being the most prevalent in these conditions, the immunocompromised and neonates have shown to be the most susceptible (2, 52). With *S. epidermidis* infections becoming more frequent, the emergence of antibiotic-resistant strains has become a large concern. The current, most effective method of treatment involves surgical removal along with long-term antibiotic therapy (2, 54). Because the immune response is not enough to treat the infection, a majority of *S. epidermidis* infections become chronic. The mechanism of biofilm formation and the function/regulation of *S. epidermidis* factors have provided more knowledge on the bacteria's virulence. With virulence linked to biofilm formation, additional studies on colonization and host immune responses could potentially discover more effective methods for treating and preventing implant infections related to *S. epidermidis* (3).

2.5 *msaABCR* operon

Previously our laboratory characterized the *msaABCR* is a four-gene operon, containing genes (*msaA*, *msaB*, *msaC*, and anti-sense *msaR*) (58). As a non-protein coding RNA, *msaC*, has shown to be a regulator of the operon. Additionally, the anti-sense *msaR* was also shown to be expressed differentially on growth-dependent fashion, thus indicating its role in the regulation and/or expression of the *msaABCR* operon. The anti-sense *msaR* has also been found to be complementary to the 5' end of the *msaB* gene. The only apparent protein coding gene found in the operon is *msaB* which encodes MsaB. Our previous studies have shown that, in *S. aureus*, the MsaB protein in the

msaABCR operon is the dual transcriptional regulator, functioning as both an activator and repressor (55-57). Our findings revealed that the operon positively regulates biofilm development and represses protease production, indicating its role in *S. aureus* virulence. Previous studies showed that deletion of the *msaABCR* operon resulted in increased processing of Atl and increased protease activity. These increases caused autolysis to occur which resulted in defective biofilm in *S. aureus* (58).

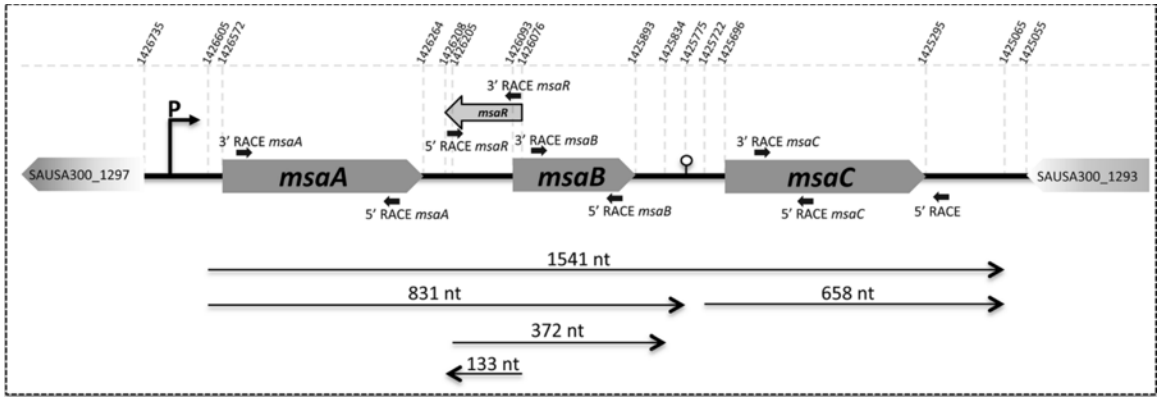

Figure 2: Representation of the msaABCR operon. Identification of the RNAs was done by rapid amplification of cDNA ends (RACE) and Northern blot analysis, which is represented by the long thin arrows with nucleotides length and direction. Short thick arrows represent the direction and position of gene-specific primers used in various reactions (57).

CHAPTER III: METHODS

3.1 Bacterial Strains and Media Used.

Staphylococcus epidermidis strain RP62A and its *msaABCR* mutant were used in this research. *S. epidermidis* strains were grown in tryptic soy broth (TSB) medium. The bacterial strains used in this study is shown in Table 1.

Table 1. Bacterial Strains and Plasmid used in this study.

Strains	**Description/Genotype**	**Source**
*Staphylococcus epidermidis (*RP62A*)*	Methicillin resistant	NARSA
msaABCR mutant	*msaABCR* operon deletion mutant in RP62A	(57)
Complementation	*msaABCR*+pCN34.*msaABCR* in *msaABCR* mutant RP62A	This Study
pCN34 plasmid	Low copy number shuttle vector	NARSA

3.2 Deletion of msaABCR operon from S. epidermidis RP62A

A mutagenesis protocol was used to construct a nonpolar, in-frame deletion of the *msaABCR* operon in the *S. epidermidis* RP62A as previously described (57). The flanking regions of the *msaABCR* operon were amplified by polymerase chain reaction (PCR) and ligated together at a *Bam*HI restriction site. The primers used to amplify the *msaABCR* operon flanking regions are listed in Table 2. Using the Gateway BP Clonase Enzyme Mix (Invitrogen Inc.), the PCR product was inserted into a temperature-sensitive plasmid $pKOR_1$. The $pKOR_1$-*msa* operon deletion construct was introduced into *S epidermidis*

RP62A. The culture was grown in TSB with 10µg / ml of chloramphenicol (CAM) at 30°C. Cells were plated on tryptic soy argar (TSA) containing CAM at 43°C, a non-permissive temperature for $pKOR_1$ replication. Colonies were picked and allowed to grow in TSB at 30°C and plated on TSA containing 100 ng/ml of anhydrotetracycline, which induces antisense secY RNA expression promotes loss of plasmid. Two rounds of temperature shifts were necessary to isolate the deletion mutant. The deletion of *msaABCR* in RP62A was verified by PCR, and functional assays were performed.

3.3 Construction of Complemented Strain

Competent *S. epidermidis* cells were prepared to increase the permeability of the *S. epidermidis* cell membranes for transformation. 250 ml of B media (1% peptone, 0.5% yeast extract, 0.1% glucose, 0.5% NaCl, and 0.1% K_2HPO_4) were prepared prior to preparation of the cells. The pH of B media was adjusted to 7.4. The overnight culture of *S. epidermidis*, grown in 25 ml of B media, was diluted to OD_{600} of 0.1 into 25 ml of pre-warmed B media and shaken at 250 rpm. The cells were grown to an OD_{600} of 2.0 and diluted into 50 ml of pre-warmed B media to an OD_{600} of 0.1. Once cells reached mid-exponential phase (OD_{600} = 0.5-0.65), they were pelleted by centrifugation at 4ºC for 10 min. The cells were washed with 1, 1/2, 1/20, and 1/50, volumes of cold 10% glycerol, pelleting between washes at 4ºC. The final cell pellet was resuspended in 700 µl of cold 10% glycerol. The sample was aliquoted to 60 µl and stored at -80ºC. To transform *S. epidermidis* cells with plasmid DNA, electroporation was performed. The prepared cells were thawed at room temperature for 5 min. The recombinant plasmid (pCN34 containing the functional copy of *msaABCR* operon) was added to the RP62A competent

cells and incubated at room temperature for 30 min. At room temperature, Electroporation was performed at 2kV, 25 uF capacitance, and 100 ohms resistance. The cells were resuspended in 950 µl of rich medium, with antibiotics added if necessary. The cells were shaken at 250 rpm for 4 hr at 37ºC and plated on selective media (TSB containing 50 ug/ml of kanamycin). The *msaABCR* operon gene in the complemented strain was under the control of its native promoter. The primers used to make the complementation plasmid pCN34.*msaABCR* are listed in Table 2.

Table 2. List of Primers used in this study.

Primer	Sequence (5′ to 3′)	Reference
Primers for *msaABCR* operon deletion		
Upstr F	ATTGGGATGGTACCTGGCCGTTCAATTGTTGGA	This study
Upstr R	ATGCCCAGGATCCGAATTATGACTATCTCA	This study
Dnstr F	AATACTGGATCCTTTGTTCGTATGTAGTAAATTC	This study
Dnstr R	GCATTCTTGGTCGACGTGTATACGAGTCTACTAAGTTA	This study
Primers for complement construct		
FP	AGCGCGGATCCTATCAATATGATTTCGCTTA	This study
RP	ATGAGGAATTCGTTGCGTGTAAAATCTAAATCCTACA	This study

3.4 Biofilm Assay

To study the effect of varying growth conditions on the formation of biofilm in the wild type RP62A, *msaABCR* mutant, and complemented strain, a modified biofilm assay was performed as previously described (57, 59). Briefly, overnight cultures of *S. epidermidis* cells were grown. Nine wells were inoculated with 1.5 ml of culture diluted to an OD_{600} of 0.05 in TSB supplemented with 0.25% glucose and 3% NaCl. Three wells,

pre-coated with 25% human plasma, were inoculated with 1.5 ml of culture diluted to an OD_{600} of 0.05 in TSB supplemented with 0.25% glucose and 3% NaCl. Nine wells were inoculated with 1.5 ml of culture diluted to an OD_{600} of 0.05 in TSB. Three wells, pre-coated with 25% human plasma, were inoculated with 1.5 ml of culture diluted to an OD_{600} of 0.05 in TSB. Plates were incubated with shaking at 150 rpm for 24, 48, or 72 hours. The biofilm was washed with 1x PBS, fixed with 100% ethanol, stained with crystal violet, washed three times with 1x PBS, and eluted with 5% acetic acid. The eluted stain was transferred to a microtiter plate, and the biofilm was quantitated at 595 nm. Three separate experiments were performed in triplicate, and the mean values were recorded.

3.5 Congo Red Binding Assay

To detect extracellular polysaccharide in *S. epidermidis,* slight modifications of a Congo red binding assay were performed. Briefly, overnight cultures were diluted to an OD_{600} and normalized to the smallest OD reading in TSB. Plates were inoculated with 10 µl of cells and incubated at 37ºC for 24 hours. The colonies were observed for color-producing pigmentation. Overnight cultures were diluted to an OD_{600} of 0.05 in TSB. 100 µl of cells were inoculated in 5 mL Congo Red broth and incubated at 37ºC for 24 hours. Cells were removed by centrifugation, and the absorbance of the supernatant was measured at 498 nm.

3.6 Protease Assay

Assays were performed to determine the protease activity of *S. epidermidis* cells. Protease activity was measured from the supernatants of overnight cultures as previously described (57, 59). Briefly, overnight cultures were diluted to an OD_{560} of 0.05 in TSB. Centrifugation for 5 min at 15,000 rpm was used to harvest and separate the cells. The supernatant was collected, and filter sterilized using a 0.45 um syringe filter. 300 µl of supernatant was mixed with 800 µl of 3 mg azocasein ml^{-1} in Tris-buffered saline (pH 7.5). TSB was the blank control. The samples were incubated overnight in the dark at 37ºC. 400 µl of 50% trichloracetic acid was added to precipitate undegraded azocasein and centrifugation for 10 min at 15,000 rpm was used. The absorbance, OD_{340}, was measured. Two separate experiments were performed in triplicate, and the mean values were recorded.

3.7 Urease Assay

Assays were performed to determine urease activity of *S. epidermidis* cells. Stuart's Urea Broth was made and filter sterilized. Cells were inoculated in 5mL TSB and incubated at 37ºC for 2 hours. The cultures were diluted to an OD_{600} of in Urea Broth and incubated at 37ºC for 24 hours. Following incubation, 1 mL of culture was centrifuged at 12,000 rpm for 5 min. In a microtiter plate, 200 µl of supernatant was added to the wells and the absorbance at 560 nm was measured. Two separate experiments were performed in triplicate, and the mean values were recorded.

3.8 DNase Test

To determine the production of deoxyribonuclease in *S. epidermidis* cells, a DNase test was performed. DNase Test Agar with toluidine blue was made. Overnight cultures were diluted to an OD_{600} and normalized to the smallest OD reading in TSB. The agar plates were inoculated with cells and incubated at 37ºC for 24 hours. The zone of clearance as a result of nuclease activity was assessed.

3.9 Statistical Analysis

In this study, all the statistical analyses to test for significance were performed by using one-way ANOVA followed by a post-hoc Tukey 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.

CHAPTER IV: RESULTS

4.1 Biofilm Formation

4.1.1 Biofilm Formation by the msaABCR mutant of RP62A

We performed microtiter-based biofilm formation assay in TSB and Biofilm media. The Biofilm plates were harvested after 24-, 48-, and 72 hr for the absolute quantification of biofilm after the fixing and staining process. After 24 hr of incubation, we observed that the *msaABCR* mutant formed 2.35- and 2.64- fold increased biofilm in TSB and Biofilm medium respectively relative to wild type RP62A (Fig 3).

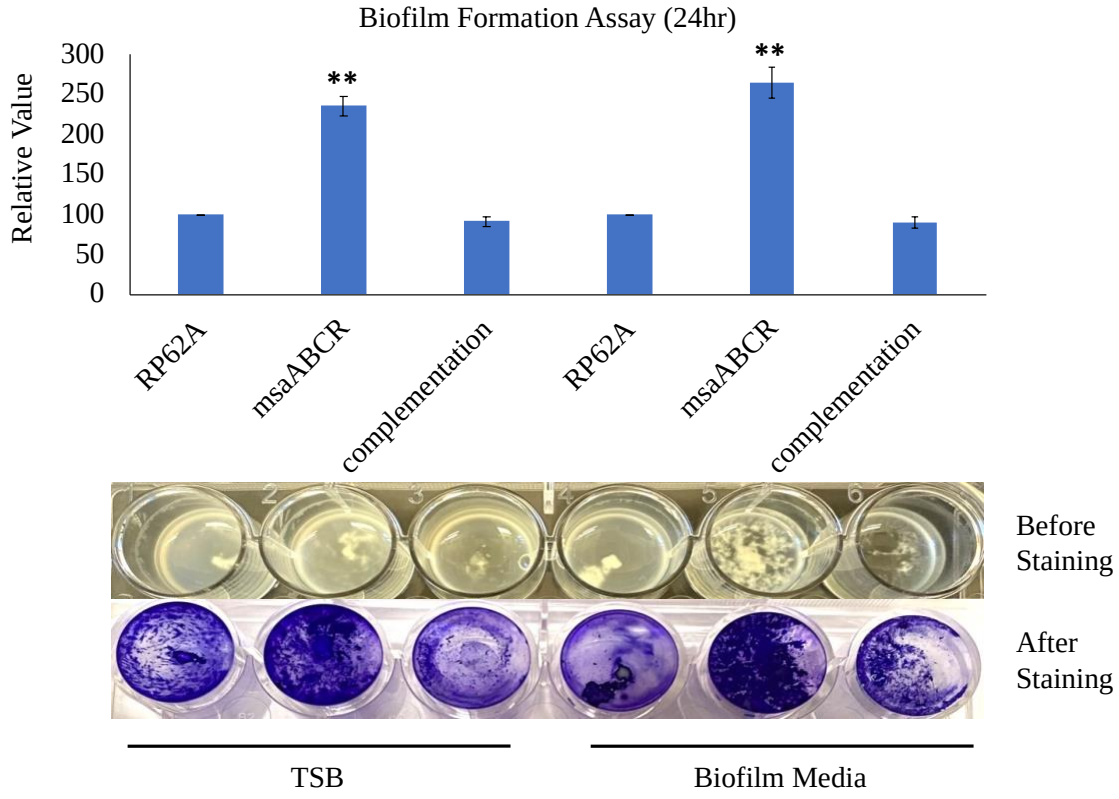

Figure 3: Biofilm Formation Assay (24hr). Graph comparing the biofilm growth of *S. epidermidis* after 24hr incubation with growth conditions of TSB and Biofilm media (TSB supplemented with 0.25% dextrose and 3.0% NaCl). Values show the percent activity relative to wild-type RP62A, set as 100%. All the values were analyzed using one-way ANOVA followed by a post-hoc Tukey test. Error bars indicate standard errors of the means. A *P*-value of < 0.05 was considered statistically significant (**p-value < 0.05).

Interestingly, after 48 hr of incubation, the *msaABCR* mutant showed reduced biofilm formation in TSB media, but showed increased biofilm formation in biofilm media (Fig 4). Likewise, after 72 hr, the *msaABCR* mutant showed reduced biofilm formation (> 2-fold) in both TSB and biofilm media (Fig 5). These results suggest that the *msaABCR* mutants' biofilm formation is dependent in growth and the media conditions. Although, the *msaABCR* mutant of RP62A showed increased biofilm formation during the initial stages, it showed defective biofilm formation at the later stages (after 48 hr).

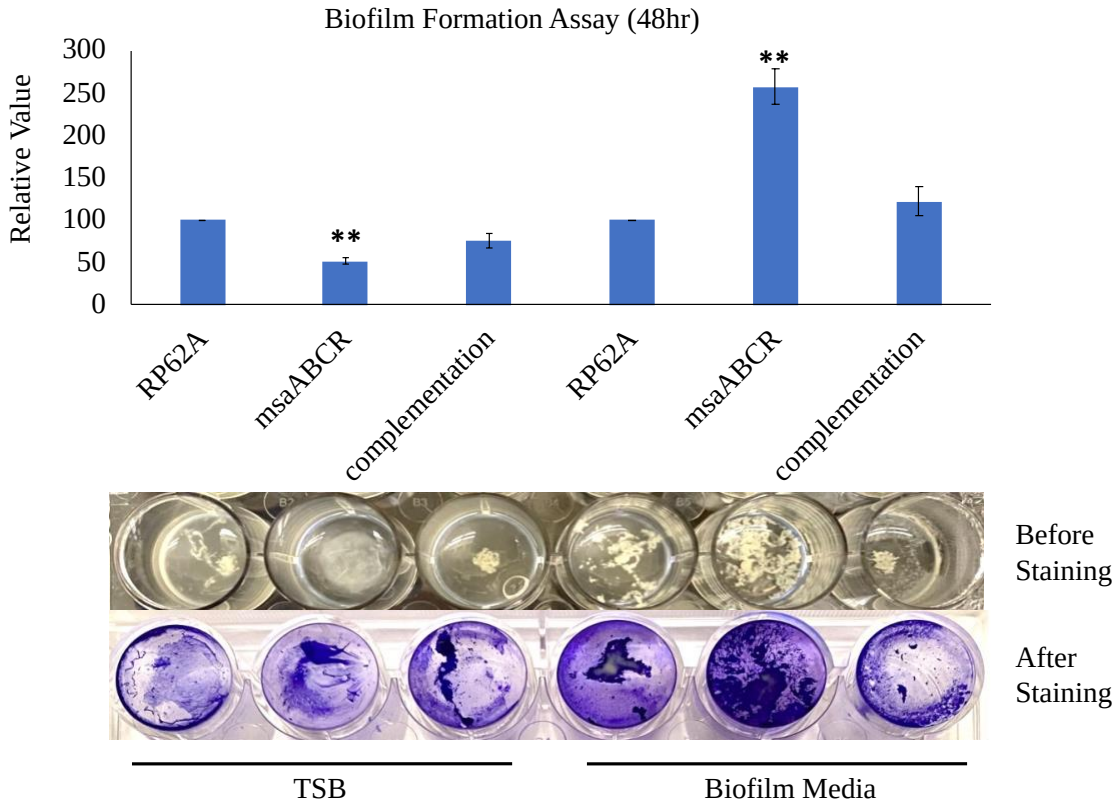

Figure 4: Biofilm Formation Assay (48hr). Graph comparing the biofilm growth of *S. epidermidis* after 48hr incubation with growth conditions of TSB and TSB supplemented with 0.25% dextrose and 3.0% NaCl. Values show the percent activity relative to RP62A, set as 100%. All the values were analyzed using one-way ANOVA followed by a post-hoc Tukey test. Error bars indicate standard errors of the means. A *P*-value of < 0.05 was considered statistically significant (***p*-value < 0.05).

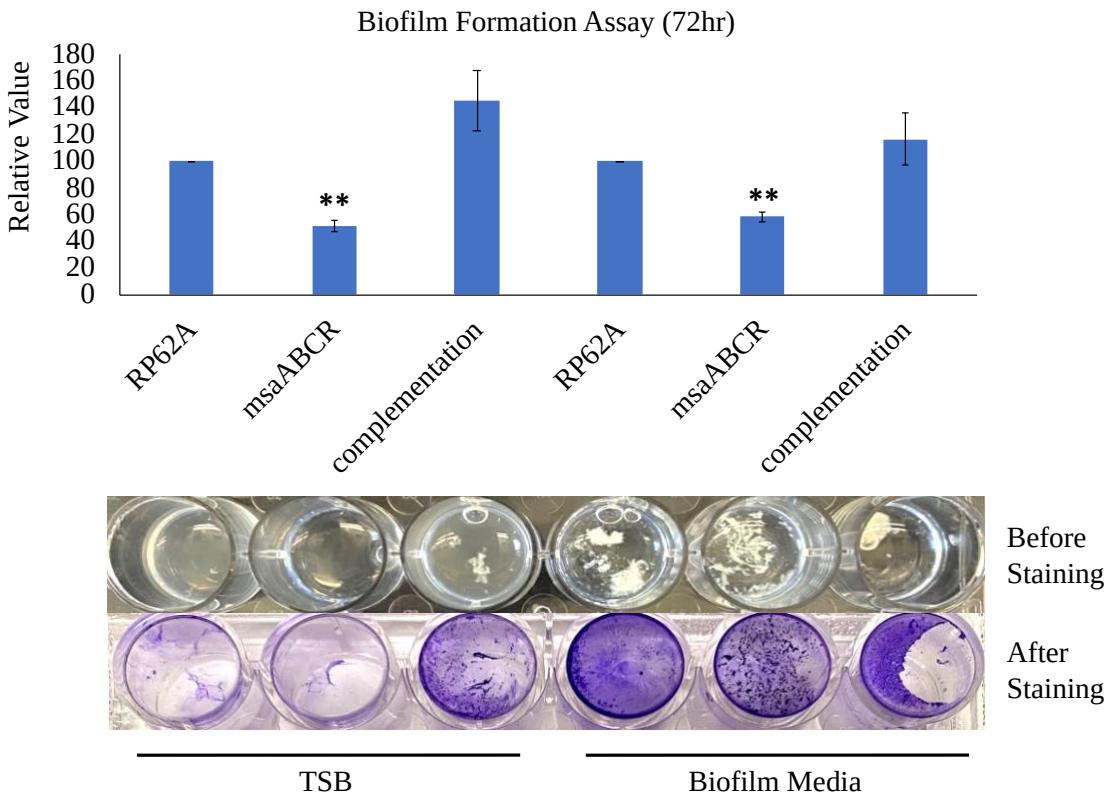

Figure 5: Biofilm Formation Assay (72hr). Graph comparing the biofilm growth of *S. epidermidis* after 72hr incubation with growth conditions of TSB and TSB supplemented with 0.25% dextrose and 3.0% NaCl. Values show the percent activity relative to RP62A, set as 100%. All the values were analyzed using one-way ANOVA followed by a post-hoc Tukey test. Error bars indicate standard errors of the means. A *P*-value of < 0.05 was considered statistically significant (***p*-value < 0.05).

We also tested the biofilm formation in the microtiter plate after coating the wells with human plasma. Coating with human plasma provides several proteins that are necessary to initiate staphylococcal binding during biofilm formation inside the host. When we coat the microtiter plates with human plasma, the *msaABCR* mutant formed 2.72-fold increased biofilm in TSB and 3.94-fold increased biofilm in biofilm media relative to RP62A respectively (Fig 6). However, interestingly, after 48 and 72 hr of incubation the *msaABCR* mutant formed significantly reduced biofilm relative to RP62A in TSB (Fig 7

and 8). Contrarily, in biofilm media, 2.56- and 2.04 - fold increased biofilm formation was observed in the *msaABCR* mutant relative to RP62A after 48 and 72 hr (Fig 7 and 8).

4.1.2 Biofilm Formation in Presence of Human Plasma

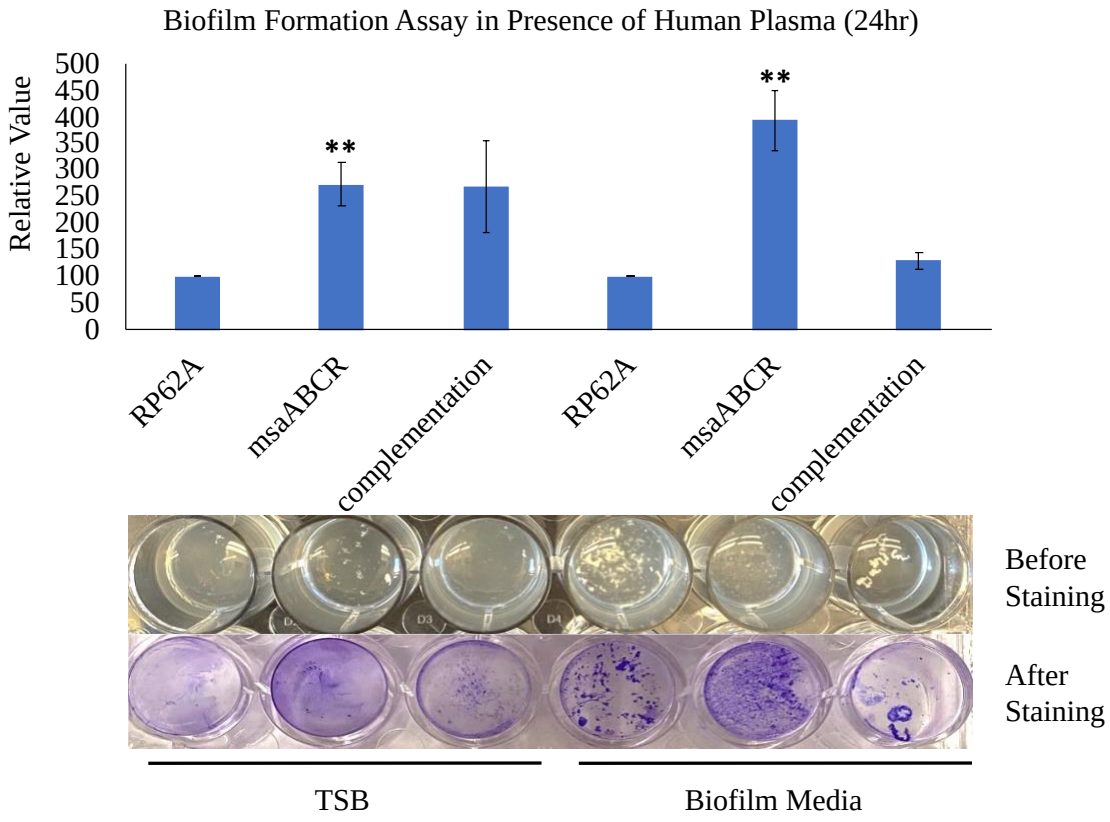

Figure 6: Biofilm Formation Assay in Presence of Human Plasma (24hr). Graph comparing the biofilm growth of *S. epidermidis* after 24hr incubation in the presence of human plasma with growth conditions of TSB and TSB supplemented with 0.25% dextrose and 3.0% NaCl. Values show the percent activity relative to RP62A, set as 100%. All the values were analyzed using one-way ANOVA followed by a post-hoc Tukey test. Error bars indicate standard errors of the means. A *P*-value of < 0.05 was considered statistically significant (**p-value < 0.05).

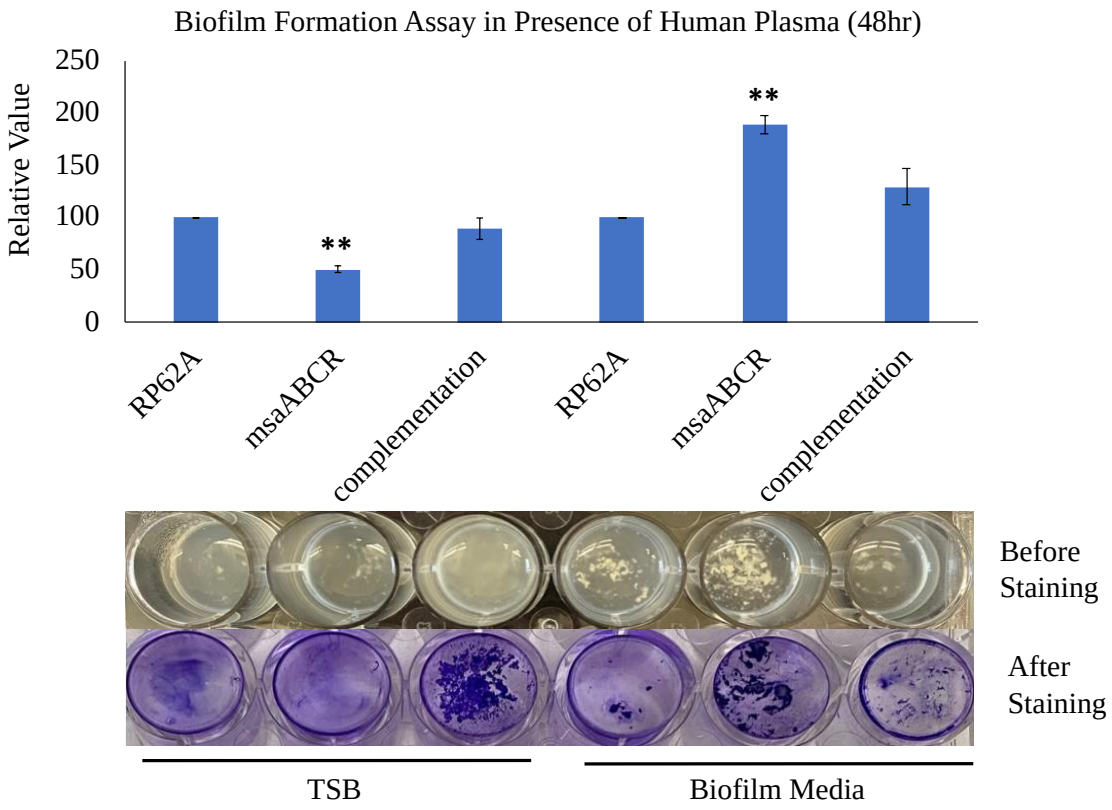

Figure 7: Biofilm Formation Assay in Presence of Human Plasma (48hr). Graph comparing the biofilm growth of *S. epidermidis* after 48hr incubation in the presence of human plasma with growth conditions of TSB and TSB supplemented with 0.25% dextrose and 3.0% NaCl. Values show the percent activity relative to RP62A, set as 100%. All the values were analyzed using one-way ANOVA followed by a post-hoc Tukey test. Error bars indicate standard errors of the means. A *P*-value of < 0.05 was considered statistically significant (**p-value < 0.05).

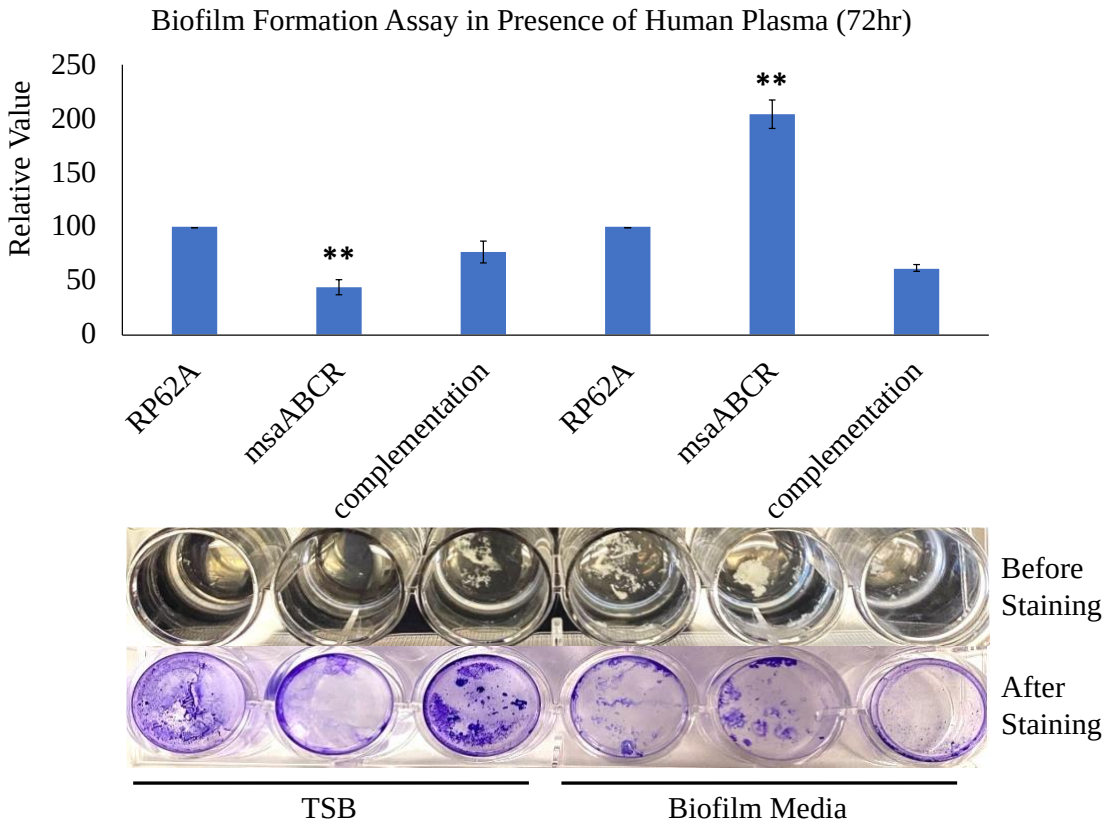

Figure 8: Biofilm Formation Assay in Presence of Human Plasma (72hr). Graph comparing the biofilm growth of *S. epidermidis* after 72hr incubation in the presence of human plasma with growth conditions of TSB and TSB supplemented with 0.25% dextrose and 3.0% NaCl. Values show the percent activity relative to RP62A, set as 100%. All the values were analyzed using one-way ANOVA followed by a post-hoc Tukey test. Error bars indicate standard errors of the means. A *P*-value of < 0.05 was considered statistically significant (***p*-value < 0.05).

These results suggest that, in presence of human plasma, the *msaABCR* mutant showed increased biofilm formation during the initial stages but are defective in later stages of biofilm formation in TSB media. However, in biofilm media the *msaABCR* mutant showed increased biofilm formation in all stages of incubation period.

4.2 Virulence Assays

RP62A of staphylococci produced extracellular proteins, extracellular DNA (eDNA), and polysaccharides in its biofilm matrix during the biofilm developmental processes. In *S. aureus* strains the *msaABCR* operon regulates proteases production and nucleases activity to regulate biofilm formation. So, in this study, we tested if *msaABCR* operon in RP62A regulates proteases production, nuclease production, or PIA production to regulate biofilm formation.

4.2.1 Protease Production

A protease production assay was performed in TSB. After overnight incubation with azocasein and precipitation with 50% Trichloracetic acid, the OD of the supernatant was measured at an absorbance of 340 nm to determine protease activity. We observed that the *msaABCR* mutant did not show any increase in protease activity relative to RP62A (Fig 9). Interestingly, we observed the complementation strains showed significantly reduced protease production relative to RP62A strains. However, we do not understand the reason why the complementation strains showed reduced protease production.

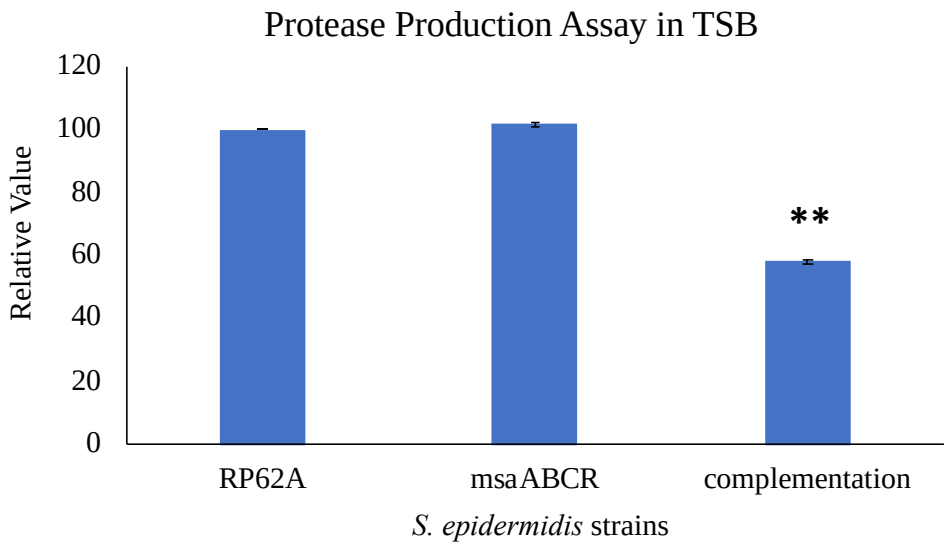

Figure 9: Protease Production Assay in TSB. Graph comparing the protease production of strain RP62A, *msa* mutant strain, and complementation. Values show the percent activity relative to RP62A, set as 100%. All the values were analyzed using one-way ANOVA followed by a post-hoc Tukey test. Error bars indicate standard errors of the means. A *P*-value of < 0.05 was considered statistically significant (**p-value < 0.05).

4.2.2 PIA Production

We spot inoculated Congo Red Agar plates with overnight *S. epidermidis* cultures that were diluted to an OD_{600} and normalized to the smallest OD reading in TSB. As seen in Figure 10, we observed that RP62A strains produced slightly darker zone relative to *msaABCR* mutant. To further confirm this, we performed congo-red binding assay, which showed that the RP62A strain binds more congo-red relative to the *msaABCR* mutant (Figure 11). This result suggests that the *msaABCR* produced less PIA compared to wild type RP62A. In this assay too, the complementation is not showing the RP62A behavior and the reason behind this is not well understood.

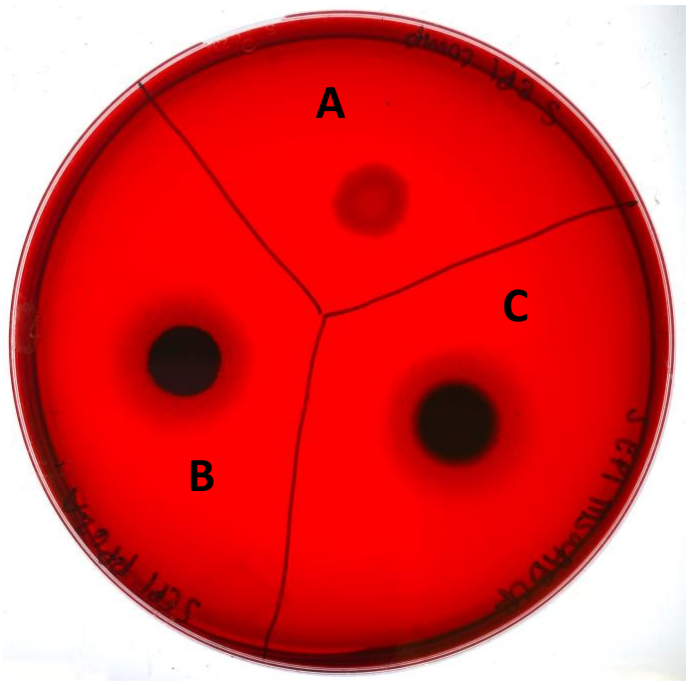

Figure 10: PIA Production in Cong-Red Agar. Image showing the colony colors of strain complementation (A), RP62A (B), and isogenic *msaABCR* mutant (C) in Congo Red Agar.

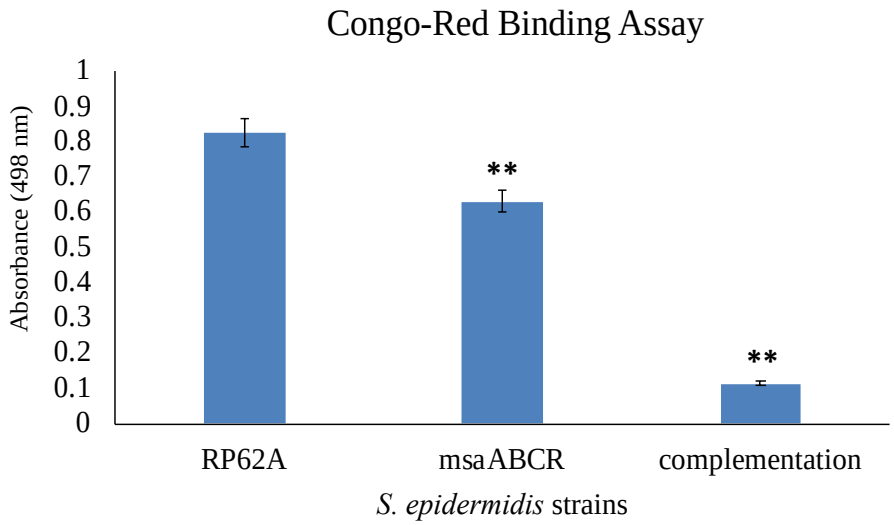

Figure 11: Congo-Red Binding Assay. Graph representing the Congo-Red binding assay to show PIA production. Values show the average amount of Congo-Red absorbed by the cells. All the values were analyzed using one-way ANOVA followed by a post-hoc Tukey test. Error bars indicate standard errors of the means. A *P*-value of < 0.05 was considered statistically significant (**p-value < 0.05).

Congo-red dye was allowed to bind the cells grown overnight. The supernatant was collected, and absorbance reading was taken at 498 nm. The bar graph shows that the *msaABCR* mutant binds less congo-red thus indicating that *msaABCR* mutant produces less PIA. In this assay, we did not observe any complementation in the complemented strains. At this point we are unable to explain the complementation phenotype.

4.2.3 Urease Production

We performed a urease production assay in Urea Broth. After overnight incubation and centrifugation, the supernatant was measured at an absorbance of 560 nm to determine urease activity. We observed that the *msaABCR* mutant showed 4.83-fold increased urease production relative to RP62A (Fig 12).

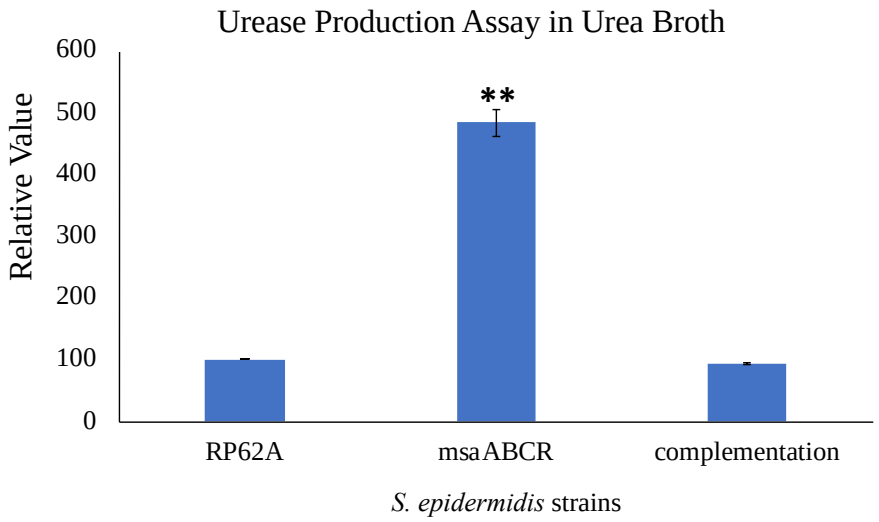

Figure 12: Urease Production Assay in Urea Broth. Graph comparing the urease production of strain RP62A, *msa* mutant strain, and complementation. Values show the percent activity relative to RP62A, set as 100%. All the values were analyzed using one-way ANOVA followed by a post-hoc Tukey test. Error bars indicate standard errors of the means. A *P*-value of < 0.05 was considered statistically significant (**p-value < 0.05).

4.2.4 Nuclease Production

We spot inoculated Dnase Test Agar plates containing toluidine blue with overnight *S. epidermidis* cultures that were diluted to an OD_{600} and normalized to the smallest OD reading in TSB. As seen in Figure 13, we observed that all the test strains (RP62A, *msaABCR* mutant, and complementation) did not produce any zone of clearance, which indicates that none of the strain produced deoxyribonuclease to the detection limit of this test.

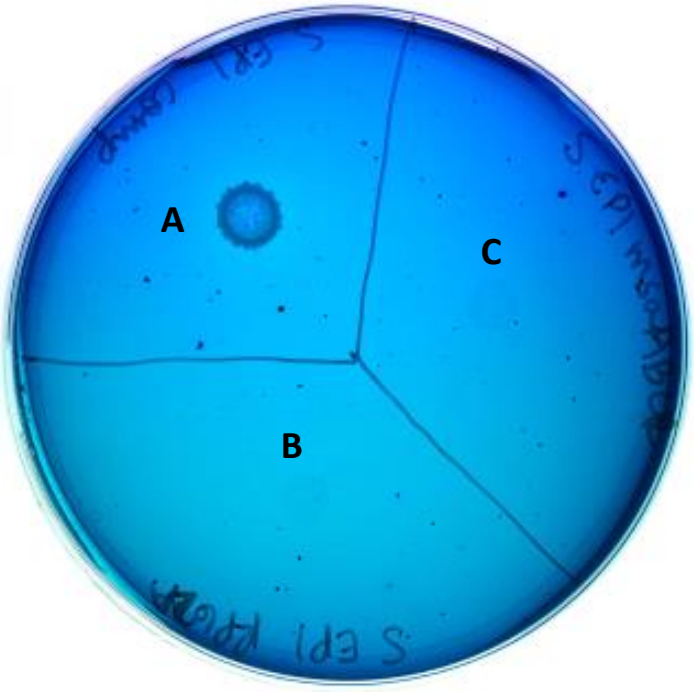

Figure 13:Nuclease Production in DNase Test Agar. Image showing the colonies of strain complementation (A), RP62A (B), and isogenic *msaABCR* mutant (C) in DTA with toluidine blue.

CHAPTER V: DISCUSSION

Previously, our studies showed that the *msaABCR* operon positively regulates biofilm formation in *Staphylococcus aureus* (57, 58). We showed that *msaABCR* operon positively regulates extracellular proteases, which in turn modulates murein hydrolases activity that has resulted in increased cell death to regulate biofilm formation in *S. aureus*. In this study, we examined if the *msaABCR* operon regulates biofilm formation in *Staphylococcus epidermidis* RP62A strains. Interestingly, we observed that mutation of *msaABCR* operon has induced biofilm formation relative to RP62A except during the later stages of biofilm after 72 hr. We also tested the effect of growth condition (TSB vs. biofilm media) and coating the microtiter plate with human plasma on the biofilm formation. In absence of plasma coating, the *msaABCR* mutant showed increased biofilm formation in TSB media condition after 24 hr but showed reduced biofilm formation after 48 hr of incubation. In biofilm media, the *msaABCR* mutant showed increased biofilm formation till 48 hr but reduced biofilm formation after 72 hr of incubation. When the microtiter plates were coated with human plasma, we observed similar effect in TSB growth conditions, however in biofilm media growth condition, *msaABCR* mutant showed increased biofilm formation during all incubation periods. These results were in contrast to our prior observations where *msaABCR* mutants always showed defective biofilm formation in all growth conditions in *S. aureus*. These differences might be because the strain we used in this study is different (*S. aureus* vs. *S. epidermidis*). Another reason may be the mode of biofilm development in RP62A is different from the *S. aureus* strains. *S. aureus* formed protein and eDNA mediated biofilm formation, whereas RP62A forms PIA dependent biofilm formation.

We also examined the effect of *msaABCR* operon mutation on protease production, nuclease production, and PIA production. In contrast to the effect of *msaABCR* operon mutation in *S. aureus* strains, we observed no effect on protease production and nuclease production. However, our result showed that the *msaABCR* produce less PIA relative to the wild type RP62A. PIA is one of the important components of RP62A biofilm matrix. Since the *msaABCR* mutant produced less PIA, this might be the explanation for the defective biofilm formation in the later stages of biofilm development by the mutant. In *S. epidermidis*, the *icaADBC* operon produces PIA, which acts as a structural factor in biofilm formation. Our findings suggest that the *msaABCR* operon could regulate the *icaADBC* operon to produce PIA. Indeed, further experiments are necessary to fully explain the other defects and/or explain the role of *msaABCR* operon in regulating PIA production during the biofilm development.

Previous studies have concluded that urease neutralizes acids, thereby protecting bacteria in acidic environments (61). In our study, the *msaABCR* mutant showed significantly higher urease production relative to RP62A (Fig 10). The findings in urease production could indicate that the *msaABCR* operon represses urease production in *S. epidermidis*. With biofilm formation as its primary virulence mechanism, the *msaABCR* operon could play a role in resistance towards *S. epidermidis* biofilm formation due to the lack of urease activity.

In conclusion, this study shows that the *msaABCR* operon in *S. epidermidis* RP62A plays different roles in regulating biofilm formation and virulence (proteases, nucleases) than we observed in *S. aureus* strains.

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