Role of msaA Gene in Regulation of the msaABCR Operon and Biofilm Development in Staphylococcus aureus

Ahmed Alzuway
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ROLE OF MSAA GENE IN REGULATION OF THE MSAABCR OPERON AND
BIOFILM DEVELOPMENT IN STAPHYLOCOCCUS AUREUS

by

Ahmed Alzuway

A Thesis
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Master of Sciences

Approved:

____________________________________
Committee Chair

____________________________________
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Dean of the Graduate School

August 2014
ABSTRACT

ROLE OF MSAA GENE IN REGULATION OF THE MSAABCR OPERON AND BIOFILM DEVELOPMENT IN STAPHYLOCOCCUS AUREUS

by Ahmed Y. Alzuway

August 2014

Staphylococcus aureus is an important human pathogen that causes wide variety of diseases ranging from chronic biofilm associated infection to acute life threatening infection such as bacteremia, pneumonia, osteomyelitis, or endocarditis, despite the progress with antibiotics used in the treatment of bacterial infections. Furthermore, increase use of prosthetic and indwelling devices in modern medical practices has led to increased infections due to S. aureus. Treating S. aureus infections have become difficult owing to its ability to resist most of the antibiotics; this problem is further exacerbated by ability of MRSA strains to form biofilms. Emergence of community-acquired methicillin-resistance staphylococcus aureus (CA-MRSA) strains that cause severe infection among the healthy individual with no predisposing factor further exacerbate the conditions.

CA-MRSA strains produce a vast array of virulence factors that are controlled by a complex network of global virulence regulators, and they make this pathogen more successful compared to other MRSA strains. In previous study, we have shown that the msaABCR is a new four gene operon that globally regulates virulence and biofilm development in S. aureus. Also we have shown that this operon regulates the expression of some well-characterized global regulators like sarA and agrA which play vital roles in S. aureus pathogenesis. In order to study the role of individual gene in this operon, we
deleted the first gene (msaA) of msaABCR operon and found that msaA gene plays an opposing role in the regulation of msaABCR operon in terms of regulation of sarA, extracellular protease production, and biofilm formation. Deletion of msaA led to the over-expression of sarA, decreased protease production, and increased biofilm formation, which is in contrast to msaC and msaABCR deletion mutant which down regulates the expression of sarA, increases the production of protease, and decreases biofilm formation, which suggests a regulatory role for msaA gene in regulation of msaABCR operon

Keywords: Staphylococcus aureus, msaA, msaABCR operon, biofilm, virulence factors
ACKNOWLEDGMENTS

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I would like to express my deepest gratitude to my government who offered me this opportunity to pursue my graduate education in one of the most advanced countries in sciences and education. I would like to thank my little family here: Prof. Hamed Benguzzi and Pro. Micheal Tucci. Last but not least, I would like to thank my parents, my kids, and my brothers for their wishes and prayer which simplified the life abroad and made me succeed.
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INTRODUCTION

Background on *Staphylococcus aureus*

*Staphylococcus aureus* is a gram-positive facultative anaerobic human pathogen that appears under the microscope as grape-like clusters of round, golden-yellow colonies. It is a Latin name that comes from this description (*staphylococcus* means a grape-like clusters and the word *aureus* means golden yellow color). *S. aureus* is a ubiquitous species due to the fact that *S. aureus* is commensal bacterium that mainly colonizes the nasal passages but may also be found regularly on our skin, and colonizes the oral cavity, throat, and gastrointestinal tract. It is estimated that around 30% of healthy people are carriers for this bacterium, and approximately 46% mainly colonize the throat [1, 2].

*S. aureus* possesses many components and products that contribute to its pathogenesis. These components and products are the virulence factors, which include surface associated proteins like fibrinogen, fibronectin, and vitronectin involved in adherence to the host, enzymes like proteases that degrade proteins, lipases which degrade the lipids on the skin and its correlated with abscesses formation, and toxins which damage the host’s cells like heomlysins [3]. All these virulence made *S. aureus* an important human pathogen which has the potential to cause different range of infections and diseases ranging from superficial skin and mucous membrane infections to more serious and life threatening infections through progressing to the circulatory system causing diseases like pneumonia, osteomyelitis, endocarditis, and medically indwelling devices infections [4].
*S. aureus* has become more problematic especially with emergence of Methicillin-resistant *Staphylococcus aureus* (MRSA) [5] mediated by the acquisition of the *mecA* gene, which is carried on mobile genetic element staphylococcal chromosome cassette (scc) and possibly acquired via lateral gene transfer between related staphylococcal specie and confers resistance to all β-lactam agents [6]. The situation worsens by prevalence of Community-associated MRSA (CA-MRSA), which is now most common cause of skin and soft tissue (SSTI) infection in USA [7]. On the other hand, it is able easily to invade healthy people with no predisposing factor [8].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is also recognized as the most frequent cause of biofilm-associated infections because of its characteristic ability to form biofilm, which is a sessile microbial community embedded in a self-produced extracellular polymeric matrix of polysaccharides, teichoic acids, proteins, and eDNA. The bacteria in a biofilm community exhibit an altered phenotype regard to growth, gene expression, and protein production in response to surrounding environmental condition. The biofilm formed in three developmental stages: initial attachment, accumulation (maturation), and detachment or dispersal. At the beginning, the cells create focal point of colonization by adhering to a surface whether it’s biotic or non-biotic surface which mediates through surface expressed colonization factors such as (fibronectin binding protein A&B, elastin binding protein, collagen binding protein, fibrinogen binding protein, and clumping factors). After adhering to the host, the cells start multiplying and aggregate layer by layer to form a mushroom like structure. Finally after the cells has matured, they start disperse to colonize new sites. There are many factors that influence the detachment of the cells from biofilm community like shear force, abundance of
exopolysaccharide, and availability of the enzymes that destroy the matrix such as proteinases and nucleases. The biofilm has the ability to provide the nutrition through sequestering and concentrating the environmental minerals and nutrients; also, it provides protection from the host clearance mechanisms including antimicrobial agents, shear stress, phagocytic elimination, and host immune defenses. Moreover, the biofilms act as a diffusion barrier that reduces penetration of antimicrobial agents [9-11]. However, the biofilm formation is controlled by complex regulatory mechanism, which is influenced by many global regulators, and is still not well understood.

Figure 1. Illustrating the Three Stages of Biofilm Formation (attachment, growth, and dispersal), Source: Center for biofilm Engineering at MSU-Bozeman, 2003.
Regulation of these virulence factors and biofilm formation is a very complex mechanism and requires interaction with several global regulators. Some of these regulators are well identified while others still under investigation. Some of the well-studied global regulators are sarA, agr and sigB, which are major players that are essential for formation and regulation of biofilm formation [12]. We have identified a new regulator msaABCR operon (modulator of sarA), which is required for the full expression of sarA, agr, and sigB and also plays major role in the modulation of several other virulence factors in *S. aureus* [13].

Role of *S. aureus* in Oral Infections

The studies and research investigating the role of *S. aureus* in oral cavity in health and diseases are insufficient, which explains the rarity of clinical and laboratory data regarding *S. aureus* infection of the oral tissues. Some of the oral infections are caused at least in part by *S. aureus*, for example, angular cheilitis, parotitis, and staphylococcal mucositis [14, 15]. However, recent studies suggest that staphylococci can frequently be isolated from the oral cavity of particular groups of patients such as children, people using dental appliances especially the elderly who wear dentures, and some immune compromised groups of patients [16, 17]. Furthermore, periodontist and peri-implantities, which are inflammation of the soft and hard tissues surrounding the teeth and/or dental implant, the *S. aureus* biofilm in oral cavity, acts as a reservoir of oral infections. However, the prevalence of *S. aureus* in the periodontal pocket is 13.4% and 15.8% in the oral cavity. *S. aureus* also shows high affinity to titanium surface, which is widely used in dental filed as in dental implants and screws and plates used in fixation of fractured jaws. A study reported that *S. aureus* is recovered at high levels from infected peri-implant pockets, although this pathogen is not strongly associated with periodontitis,
but it is associated with complicated cases [18]. Osteomyelitis is the infection of the bone, which could affect the jaws as a result of intrusion of \textit{S. aureus} bacteria into the site of oral surgery or the site of extraction. Indeed, \textit{S. aureus} might intrude into the fractured jaws as a result of a road traffic accident, violent assault, or an overlying infection as well and causes severe infection [19, 20].

The Aims of this Study

Understanding and characterizing the mechanism of regulation of \textit{msaABCR} operon by investigating the role of individual gene within the operon and its interaction with its targets will provide us with more information to fully understand the mechanism by which this \textit{msaABCR} operon regulates the virulence factors network in \textit{S. aureus}. Therefore, in this study, we elucidated the role of the first gene in the operon, \textit{msaA} in the regulating of \textit{msaABCR} operon, and biofilm development.
CHAPTER II
CHARACTERIZATION OF MSAA GENE

Abstract

Community-acquired, methicillin-resistant Staphylococcus aureus (CA-MRSA) strains cause serious and severe infections among healthy people. CA-MRSA strains produce a vast array of virulence factors that are controlled by complex network of global virulence regulators. We previously identified the msaABCR operon as a new regulator of virulence and biofilm development. In order to understand the mechanism by which this operon functions we studied the role of individual genes. In this study we studied the role of first gene of an operon, msaA from CA-MRSA strain LAC. We studied different phenotypes (Biofilm formation, pigmentation, protease production, and expression of global regulators, sarA, agr, and sigB) and compared with msaABCR operon deletion mutants. The results showed that deletion of msaA gene results in abolition of agr expression, an increase in biofilm production, and a decrease in protease activity. This phenotype is different from that of the operon deletion mutant suggesting a regulatory role for msaA.

Introduction

The community acquired-MRSA strains are widely prevalent strain. Invading the healthy people out of the hospitals and health care units without risk factors and the evolution of CA-MRSA strains to cause severe infections is more alarming [21]. Moreover, this strain characteristically is able to form biofilm, which is associated with several infections. Bacteria in the biofilm are protected from the host defense and confer resistance to antibiotics; advancement in medical technology, and increased use of prosthetic and indwelling devices, especially in orthopedic, heart devices, and in the
dental field to improve the people’s quality of life has led to the increased number of infections due to S. aureus. And therefore, good understanding of the regulatory mechanism by which this strain controls its virulence factors and biofilm formation and how they interact with their targets will provide us with future therapeutic target to combat this virulent strain and its associated infections.

Materials and Methods

1. Bacterial strain and plasmids.

2. Construction of msaA deletion mutant, and complement.

3. Study the phenotypic characterization of msaA mutant by applying the Following assays:
   a. Biofilm assays (statistic assay, flow cell biofilm, and confocal assays)
   b. Protease assay
   c. Pigmentation assay
   d. Triton X-100 induced Autolysis (autolysis assay)

4. Study the effect of different environmental condition on msaA mutant biofilm.

5. Biofilm detachment assay.

6. RNA isolation and real-time qPCR.

1. Bacterial Strain and Plasmids

S. aureus strains (community-acquired MRSA strain USA300_LAC, restriction-deficient laboratory strain RN4220) and E. coli strain DH5α were used in this study.

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

Table 1

*Plasmids and Strains Used in this Study*

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant Characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJB38</td>
<td>Gram positive shuttle vector</td>
<td>NARSA</td>
</tr>
<tr>
<td>pCN34</td>
<td>pT181-based low copy number E. coli-Staphylococcal shuttle vector</td>
<td>This study</td>
</tr>
<tr>
<td>MOE</td>
<td>pJB38-ΔmsaA vector plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>pMOE592</td>
<td>pJB38-ΔmsaA deletion construct in <em>E. coli</em></td>
<td>This study</td>
</tr>
<tr>
<td>pMOE593</td>
<td>pJB38-ΔmsaA deletion construct in RN4220</td>
<td>This study</td>
</tr>
<tr>
<td>pMOE552</td>
<td>pCN34-msaA construct in <em>E. coli</em></td>
<td>This study</td>
</tr>
<tr>
<td>pMOE553</td>
<td>pCN34-msaA construct in RN4220</td>
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Table 1 (continued).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN4220</td>
<td>Restriction deficient mutant of 8325-4</td>
<td>NARSA</td>
</tr>
<tr>
<td>LAC</td>
<td>CA-MRSA USA300 strains</td>
<td>Dr. Lindsey Shaw</td>
</tr>
<tr>
<td>MOE597</td>
<td>LAC :: Δ msA deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>MOE613</td>
<td>msA gene complement into msA deletion mutant</td>
<td>This study</td>
</tr>
</tbody>
</table>

2. **Construction of msA Deletion Mutant and its Complementation**

The *msaA* genes in *S. aureus* USA300 strain was mutated by deletion using the allelic replacement method, the protocol described by Bae and Schneewind [22]. Where temperature sensitive shuttle vector plasmid pJB38 was used, the flanking regions of *msaA* were amplified using primer pairs *msaA*-upstream F&R and *msaA*-downstream F&R from USA300_LAC strain. The amplified regions were ligated together by T4 ligase at an introduced *BamHI* restriction site, and this ligate product was further amplified by terminal primers (*msaA*-upstr F and *msaA*-dnstr R), and it was inserted into pJB38 temperature sensitive gram positive shuttle vector plasmid after digestion with restriction enzymes KpnI and SalI to produce (pJB38-*msaA* deletion constructs). This construct was first transformed into *Escherichia coli* then transformed to *S.aureus* intermediate strain RN4220 by electroporation, and then subsequently transduced into LAC using Phage
lysate transduction method. Two rounds of temperature shifts were necessary to isolate
the deletion mutant. Deletion of msaA gene in LAC was verified by sequencing and PCR.
The primers used for msaA deletion are shown in Table 2.

The msaA complement was constructed by amplifying wild type msaA gene from
LAC using primers (msaA comp. F and msaA comp.R) that have inserted EcoRI and
BamHI restriction site. The amplified msaA gene was inserted to a low-copy-number
plasmid pCN34, Gram-positive shuttle vector, by restriction digestion using EcorI and
BamHI restriction enzymes to produce (msaA-pCN34 vector plasmid) this construct was
introduced to msaA deletion mutant by transduction and was used as complement for
further study. The primers used for complement construction are shown in Table 2.

Table 2

*Primers Used in this Study*

<table>
<thead>
<tr>
<th>Primer for msaA deletion and complement</th>
</tr>
</thead>
<tbody>
<tr>
<td>msa A upstr F</td>
</tr>
<tr>
<td>ATTGTCTAAAGGGTACCTTTGTTTTACTGATTGTGTC TACCTGT</td>
</tr>
<tr>
<td>msa A upstr R</td>
</tr>
<tr>
<td>CGACTTGAAAGGATCCAAAGTAGTAACATAAGTAAAT TATTAGTT</td>
</tr>
<tr>
<td>msa A dnstr F</td>
</tr>
<tr>
<td>CAGTTACAAAATTGGATCCGATTATGAGGGATGG GGATCC</td>
</tr>
<tr>
<td>msa A dnstr R</td>
</tr>
<tr>
<td>TAGCCCTAGTCACTAAATTTAAAGCATCATATAACA TACCACACTGTCGAC</td>
</tr>
<tr>
<td>msa A compl F</td>
</tr>
<tr>
<td>ACCCGCGGTACCCAAAGCTGCAAACGTTGTAACACTA</td>
</tr>
<tr>
<td>msa A compl R</td>
</tr>
<tr>
<td>AAGCACGTGAATTCATAAAGCGACAATCGTTAA</td>
</tr>
</tbody>
</table>
3. Phenotypic Assays

Static biofilm assays. The microtiter biofilm assay was performed as described by Sambanthamoorthy et al. (2006) [23], with slight modification. In brief, overnight cultures of cells, including wild type, msA mutant, and its complemented strain of USA300 LAC were diluted 1:100 times in TSB supplemented with 3% NaCl and 0.5% glucose and inoculated in the microtiter plates, pre-coated with 20% human plasma. The biofilms were grown for 24 or 48 h with shaking at 150 rpm. The adherent biofilm washed three times and fixed with 100% Ethanol, and dried for 5 mints. They were then stained with 0.5% crystal violet, and dried overnight. After that it eluted in 5% acetic acid, the absorbance was measured at 595 nm using a SpectraMax M5 microplate spectrophotometer system.

Confocal microscopy of msA biofilm. Confocal microscopy of biofilm produced by the msA deletion mutant, the biofilms were grown on microtiter as described above. The 48 h biofilm was stained with live-dead cell stains Syto-9 [1.3 μm] and Toto-3 [2.0 μm] respectively. The stains were prepared in 2 ml of filter-sterilized PBS buffer prior to be used for staining. The Z-stack images of the biofilm were taken under 60X objective with oil immersion. The Syto-9-dye (Green fluorescence) was excited with an argon laser at 488 nm, and the emission band-pass filter used for Syto-9 was 515 ± 15 nm and was used to stain the live cells in the biofilm. The Toto-3-dye (Red fluorescence) was excited using a HeNe 633-nm laser and emissions were detected using a 680 ± 30-nm filter and was used to stain the dead cells and eDNA, then incubated at 30°C in dark for 10 minutes. The images obtained by CLSM were processed using COMSTAT. The total biomass, average and maximum thickness of the biofilm, and amount of dead and eDNA of the biofilm was quantified.
Protease assay. Protease activity assay was performed as described by Sambanthamoorthy et al. (2006) [23]. In brief, 300 μl of the culture supernatant from overnight cultures were mixed with 800 μl of 3 mg azocasein ml⁻¹ in Tris-buffered saline (pH 7.5) and incubated overnight at 37°C in dark. After incubation un-degraded azocasein was precipitated by adding 400 μl of 50% (w/v) trichloroacetic acid, removed by centrifugation, and the amount of acid-soluble azocasein was determined by measuring the absorbance at 340 nm with using water as blank. We took the mean values from a minimum of three independent experiments; each performed in triplicate and was recorded.

Pigmentation assay. A pigmentation assay was performed on cells harvested from overnight cultures, as described by Morikawa et al. [24]. Briefly, 1 ml of the overnightly grown cells were collected and washed twice with water. Then, they were suspended in 1 ml of methanol and heated at 55°C for 3–5 min with casual vortexing. The cells were removed by centrifugation at 15,000-x g for 1 min, and the absorbance of the supernatant was measured at A₄₆₅nm with water used as a blank. Mean values from a minimum of three independent experiments; each performed in triplicate were recorded.

Autolysis assay. Autolysis assay was performed using Triton X-100 as described by Manna et al. (1997) [25]. Overnight bacterial cultures were diluted to an OD 600 of 0.05 in TSB broth contain 1M NaCl. The bacteria were grown until the optical density A₅₈₀ 0.7. The cells were collected by centrifuging at 10,000 rpm for 10 min at 4°C. The cells were washed twice with ice-cold autoclaved water and then were re-suspended in autolysis buffer (50 mM Tris-HCl (pH 7.5) containing 0.1% (v/v) Triton-x-100). The rate of autolysis was measured at A₅₈₀ every 30 minutes intervals for 3 hours.
4. Effect of Different Environmental Condition on Biofilm Formation

Biofilm formation affected by several environmental conditions, such as glucose, osmolarity, ethanol, temperature, and anaerobiosis as reported by (Gotz 2002) [26]. Here we would like to study how the msaA mutant wills response to different concentration of NaCl, glucose, and ethanol.

Effect of sodium chloride. Biofilm assay on 96-well polystyrene plates was performed as described by Heilman et al. (1996) [27]. In brief, overnight cultures normalized to an optical density at 600 nm of 0.05 in TSB containing 0.5% glucose and sodium chloride at concentrations ranging from 0 to 5%. A 200 ul sample of normalized bacterial suspension inoculated into each well of flat-bottom 96-well polystyrene plates over-nightly pre-coated with 20% human plasma and incubated at 37°C for overnight. After incubation, the wells were washed gently with 200 ul of PBS three times, air dried, fixed with 100 ul of absolute Ethanol, and stained with 0.2% crystal violet. The absorbance measured at 595 nm to quantify the remaining biofilm.

Effect of glucose. The same protocol mention above was used with only difference is that the overnight cultured cells normalized in TSB containing 3% NaCl, which supplemented with different glucose concentrations ranging from 0 to 5%.

Effect of ethanol. Follow the same protocol mentioned above; the only difference is that overnight cultured cells normalized in TSB media supplemented with different ethanol concentrations ranges from 0 to 6%, and we further investigated the effect of different ethanol concentration on msaA mutant in TSB media supplemented with 0.5% glucose, and TSB media supplemented with 3% NaCl respectively.
5. **Biofilm Detachment Assay**

We studied the effect of different chemicals and media that affects the biofilm stability. To determine the sensitivity of biofilms to Sodium metaperiodate, DNase I, proteinase K, the biofilm was grown in human plasma coated microtiter plate as described above for 48 hr and treated them as previously described in Rice et al. (2007) [28]. Briefly, the biofilm was grown in biofilm media and incubated at 37°C with shaking at 150 rpm each for every chemical tested. Sodium metaperiodate (10mM), DNase I (10U/ml), and Proteinase K (100ug/ml) were introduced at different point time intervals (0 to 12 hours) in their respectively labeled plates and further incubated till 48 hr. After incubation, the wells were rinsed gently with 200 ul of PBS three times, air dried, fixed with 100 ul of absolute ethanol, and stained with 0.2% crystal violet for 10min. The absorbance was then measured at \(A_{595}\) nm to quantify the remaining biofilm.

6. **RNA Isolation and Real-Time qPCR**

The total RNA was harvested and the Real-time PCR was performed as previously described in Sahukhal and Elasri, 2014 [13]. In brief, overnight cultures of *S. aureus* were diluted to an \(OD_{600}\) of 0.05 in TSB and incubated at 37°C with shaking (200 rpm) until they reached an \(OD_{600}\) of 4.0, and RNA was harvested using Rnasy mini kit. The quality of the total RNA was determined by Nano-drop and a Bioanalyzer (Agilent) and Real time PCR was performed. The constitutively expressed *gyrase A* (*gyrA*) gene was used as an endogenous control gene and was included in all experiments. Analysis of expression of each gene was done based on at least three independent experiments. Two-fold or higher changes in gene expression were considered significant. All the primers used for RT-qPCR are listed in Table 3.
Table 3

*Primers Used in The RT-qPCR*

<table>
<thead>
<tr>
<th>Primers used in RT-qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT msaA F</td>
</tr>
<tr>
<td>TCGATAACTATGTCACAGGCAAATA</td>
</tr>
<tr>
<td>RT msaA R</td>
</tr>
<tr>
<td>TTGTAATCCTCTTCAATCTTTCG</td>
</tr>
<tr>
<td>RT agrA F</td>
</tr>
<tr>
<td>TTTGTCGTCATCGCCATAA</td>
</tr>
<tr>
<td>RT agrA R</td>
</tr>
<tr>
<td>TTAAACGTTTCTCACCAGATGC</td>
</tr>
<tr>
<td>RT sarA F</td>
</tr>
<tr>
<td>TTTGCTTCAGTGATTTCGTTTTATTTACTC</td>
</tr>
<tr>
<td>RT sarA R</td>
</tr>
<tr>
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<td>RT sigB F</td>
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<tr>
<td>RT gyrA F</td>
</tr>
<tr>
<td>GCTCGTTCGGACAAAGAAAGAAA</td>
</tr>
<tr>
<td>RT gyrA R</td>
</tr>
<tr>
<td>TTTGCATCCTACGCACATC</td>
</tr>
</tbody>
</table>

Results

*Deletion of msaA and its Complementation*

One of the early observations in previous study about *msaC* gene was that expression of the upstream genes (SAUSA300_1295 and SAUSA300_1296) were significantly reduced (two-fold) by deletion of *msaC* [29]. Further findings from RACE and Northern analyses of the *msaC* by Sahukhal GS and Elasri MO, (2014) [13] showed
that *msaC* is part of a four-gene operon, *msaABCR* that comprises a gene that encode for a hypothetical protein (SAUSA300_1296), a gene that is similar to the *E. coli* cold-shock gene (*cspA*, encoding SAUSA300_1295), the *msaC* gene (encoding SAUSA300_1294), and anti-sense RNA (*msaR*) (Figure 1). They also showed that all these genes are functionally related and involved in biofilm development, protease production, and regulation of the *sarA*, *agr*, and *sigB* genes. However, the role of individual gene in an operon has not been well elucidated. Moving further, in this study we have characterized the function of first gene of an operon, *msaA*, and its role in the regulation of an operon and in the biofilm development and virulence of *S. aureus*.

We used online bioinformatics tool, I-TASSER to predict the putative function of *msaA*. I-TASSER predicts *msaA* as a conserved hypothetical protein which has strong similarity to the twin-arginine signal-binding protein [30, 31]. In many bacterial systems, twin-arginine transport system is used for translocation of fully folded proteins across the cytoplasmic membrane [32]. However, in *S. aureus*, no such system has been described in detail. Analysis of the predicted *msaA* structure also revealed that it is likely located in the cytoplasm and that it might be involved in the regulation of small GTPase-mediated signal transduction [30, 31]. The contribution of the putative *msaA* protein to the function of the operon remains unclear, and hopefully, by end of this study, we will learn some information in regards.
We used allelic replacement method to make in-frame deletion msaA mutant in USA300_LAC strain. The deletion plasmid (AmsA-pJB38 deletion construct) was constructed and confirmed as described in material and method and shown in (Figure 3-A&B). The allelic replacement protocols were followed as described by Bae T et al. (2006) [22]. We screened several colonies for the deletion construct; the positive with msaA gene deleted is shown in (Figure 3-B).
Figure 3. Gel electrophoresis Picture (A)- Shows Restriction Digestion, to verify presence of deletion construct in RN4220 strain using (kpnI HF and SalI HF) lower band is the insert (~2.6kb) and upper band is the vector plasmid (7kb). (B) Showing Polymerase Chain Reaction (PCR)to verify msA deletion relative to LAC wild type, which was used as control.

Phenotypic Characterization of msA Deletion Mutant

We studied several phenotypes, Biofilm formation, protease production, pigmentation, and rate of cell death to characterize the role of msA gene in S. aureus. Our results show that deletion of msA gene resulted in slight increase in biofilm formation, and decreased of protease activity, and pigmentation; and no significant difference in the rate of cell death compared to wild type (Figure 4). The biofilm formation, protease activity, and autolysis phenotype results are different from that of msA operon deletion mutant, suggesting a regulatory role for msA in this operon.
Figure 4. Biofilm Assay Analysis; shows increased biofilm formation of the *msaA* deletion mutants relative to *msaA* complement and wild type USA300LAC. An error bars representing standardized errors.

Figure 5. Protease Assay Analysis; shows reduction of protease activity of the *msaA* deletion mutants relative to *msaA* complement and wild type USA300LAC. An error bars representing standardized errors.
Figure 6. Pigmentation Assay; shows reduced pigmentation of the *msaA* deletion mutants, relative to *msaA* complement and wild type USA300LAC. An error bars representing standardized errors.

Figure 7. Autolysis Assay Analysis; shows less rate of cell death of the *msaA* deletion mutants relative to *msaA* complement and wild type USA300LAC.
Confocal Analysis of Biofilm Formation

Confocal Microscopic analysis of *msaA* mutant biofilm, show that *msaA* mutant form thick robust biofilm with multiple spots of dead cells compared to relative wild, and *msaA* complement biofilm as shown in Figure 5. Presence of frequent spots of dead cells may be due to the *msaA* gene deletion. Indeed, the extracellular DNA is found as a major component required for initial bacterial attachment to surfaces, as well as for the subsequent early phase of biofilm development. Moreover, evidence is presented that release of extracellular DNA is mainly caused through programmed cell death and activity of the major autolysin Atl [33].

*A*  
*B*  
*C*

*Figure 8.* Confocal Microscopic Analysis of Static Biofilm on Microtiter Plate of (A) wild type LAC, (B) *msaA* mutant, and (C). Complement *msaA*, were grown upto 48 hrs and stained with syto-9 (live cells, green) and toto-3 (dead cells & eDNA, red) under 40X /1.4 Oil DIC objective. Scale bar represents 10 μm.
COMSTST analysis of confocal microscopy of *msaA* deletion mutant biofilm relative to wild type and complement shows a pit increase in live cells biomass in *msaA* mutant (110%) relative to wild type (100%). Furthermore, *msaA* mutant shows more cell death (130%) compared to wild type (100%). This could explain the increase in the biofilm mass as result of release of eDNA and the proteinaseous content of the cells which are main components of biofilm. Moreover analysis by COMSTST shows no differences in biofilm thickness between *msaA* mutant and wild type. COMSTST analysis of confocal microscopy of *msaA* mutant and *LAC* wild type as shown in Figure 9.
Figure 9. COMSTAT analysis of confocal microscopic of msaA deletion mutant biofilm relative to wild type and msaA complement. (A) live cells. (B) dead cells. (C) biofilm thickness.
**Effect of Different Environmental Condition on msaA Mutant Biofilm**

*Effect of different sodium chloride (NaCl) concentration.* There are no significant differences between wild type and msaA mutant biofilm at low concentration of NaCl (0.1\%- 0.5\%). But as the NaCl concentration increase the msaA mutant shows more biofilm formation (Figure 10).

![Graph showing effect of NaCl concentration on biofilm formation](image)

*Figure 10.* Effect of different sodium chloride concentration on msaA mutant biofilm. Concentration in % range from 0-5 Values are shown as the percent activity related to wild type strain USA300 LAC, which represents the average of three independent assays which were done in triplicates. Results are reported as the mean ± S.E. An error bars representing standardized errors.
**Effect of different glucose concentration.** Biofilm formation by both wild type and msaA mutant strains increased as the concentration of glucose increased from 0-3%, and beyond 3% they are not affected by addition of glucose (Figure 11).

*Figure 11.* Effect of different glucose concentration on msaA mutant biofilm. Concentration in % range from 0-5, values are shown as the percent activity related to wild type strain USA300 LAC, which represents the average of three independent assays which were done in triplicates. Results are reported as the mean ± S.E. error bars representing standardized errors.
Effect of Different Ethanol Concentration. The effect of different ethanol concentration on msaA mutant biofilm was investigated in tryptic soy broth (TSB), tryptic soy broth supplemented with 0.5% glucose, and tryptic soy broth supplemented with 3% NaCl.

Different Ethanol Concentration with Tryptic soy Broth (TSB)

Ethanol at low concentration increase biofilm formation in both msaA mutant and wild type strains. But at concentration higher than 3% msaA mutant shows more biofilm related to wild type (Figure 12).

Figure 12. Effect of different ethanol concentration with tryptic soy Broth (TSB) on msaA mutant biofilm. The ethanol concentration in % range from 0-6. Values are shown as the percent activity related to wild type strain USA300 LAC, which represents the average of three independent assays which were done in triplicates. Results are reported as the mean ± S.E. An error bar representing standardized errors.
Different Ethanol Concentration with TSB Supplemented with 0.5% Glucose

A biofilm formed by *msaA* mutant and wild type does not show significant difference at low ethanol concentration, but as the ethanol concentration increases, the *msaA* mutant shows more biofilm compared to wild type (Figure 13).

*Figure 13.* Effect of different ethanol concentration with tryptic soy broth (TSB) supplemented with 0.5% glucose on *msaA* mutant biofilm. The ethanol concentration in % range from 0-6. Values are shown as the percent activity related to wild type strain USA300 LAC, which represents the average of three independent assays which were done in triplicates. Results are reported as the mean ± S.E. An error bar representing standardized errors.
Different Ethanol Concentration with TSB Supplemented with 3% Sodium Chloride

The msaA mutant shows more biofilm formation compared to wild type as the ethanol concentration increases up to 2%, but at concentration higher than 2% the biofilm formation is reduced (Figure 14).

*Figure 14.* Effect of different ethanol concentration with tryptic soy broth (TSB) supplemented with 3% sodium chloride on msaA mutant biofilm. Ethanol concentration in % range from 0-6. Values are shown as the percent activity related to wild type strain USA300 LAC, which represents the average of three independent assays which were done in triplicates. Results are reported as the mean ± S.E. An error bar representing standardized errors.
In the biofilm there is specific microbial surface component recognizing adhesive matrix molecules, and nonspecific molecules are involved in attachment of bacteria to surface. Specific molecule has capacity to bind other proteins while nonspecific molecules bind to polymer surfaces [34-36]. The polysaccharide intercellular adhesion (PIA) also called PNGA, polymeric N-acetyl-glucosamine is responsible for intercellular adhesion in *S. aureus* [37, 38]. Which encoded by *ica* operon [27, 38], then PIA independent biofilm formation reported in 2005 by Toledo-Arana et al. [39]. Also teichoic acid, a cell wall associated protein, reported to play important role in initiation of biofilm formation of *S. aureus* [40, 41] and important of extracellular DNA in biofilm formation reported by Whitchuch et al. (2002) [42] [28]. The PNGA, surface proteins, and extracellular DNA are important components of biofilm; therefore, we examined the effect of proteinase K, DNase I, and sodium metaperiodate on biofilm formed by *msaA* mutant and compared it with wild type.

*Detachment Biofilm Assay*

The result showed both *msaA* mutant and wild type *S. aureus* biofilms were decreased in response to addition of proteinase K or DNase I. And this is correlated with results of previous studies. This proved that proteins and eDNA are important components of *S. aureus* biofilm. [40, 41, 43, 44][28, 42]. As shown in Figure 15, 16. Also, addition of sodium metaperiodate at different time interval show negative effect on biofilm formation for both *msaA* mutant and wild type strains as shown in Figure 17. It is report that sodium metaperiodate affects the PANG (PIA) in biofilm if the biofilm is PIA dependent [45].
Figure 15. Detachment Assay Analysis. The msaA mutant, msaA complement, and wild type performed biofilm show sensitivity to proteinase K. Average of three independent assays, which were done in triplicates; results are reported as the mean ± S.E. An error bar representing standardized errors.

Figure 16. Detachment Assay Analysis. The msaA mutant, msaA complement, and wild type performed biofilm show sensitivity to each of DNase I. Average of three independent assays, which were done in triplicates; results are reported as the mean ± S.E. An error bar representing standardized errors.
Figure 17. Detachment Assay Analysis. The msaA mutant, msaA complement, and wild type performed biofilm show sensitivity to each of sodium metaperiodate. Average of three independent assays, which were done in triplicates; results are reported as the mean ± S.E. An error bar representing standardized errors.
**Relative Expression of Global Regulators, sarA and agr in msaA Deletion Mutant**

After we studied the *msaA* and confirmed that it is a part of *msaABCR* operon, and it regulates fundamental phenotypes in *S. aureus* such as biofilm development and other virulence factors, we found deletion of the *msaA* gene led to severe reduction in expression of *agr* (180 folds) and (2 fold) reduction of *sarA* and *sigB* as shown in Table 4.

Table 4

**Represent Relative Expression of the Global Regulators, sarA, agr and sigB in the msaA Mutant, LAC Wild type, and msaA Complement.**

<table>
<thead>
<tr>
<th>Tested Strains</th>
<th>sarA</th>
<th>agrA</th>
<th>sigB</th>
<th>msaC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC wild type</td>
<td>1*</td>
<td>1*</td>
<td>1*</td>
<td>1*</td>
</tr>
<tr>
<td>msaA mutant</td>
<td>-1.38</td>
<td>-181</td>
<td>-2.07</td>
<td>2.19</td>
</tr>
<tr>
<td>msaA complement</td>
<td>-1.86</td>
<td>-86.4</td>
<td>-1.01</td>
<td>65.4</td>
</tr>
</tbody>
</table>

*Fold Change Transcription Level in the Various Strains Relative to wild-type (USA300 LAC). Values Represent the Mean Ratio for at Least Three Independent Experiments. Note. * Value for wild type strain was considered as 100% or 1 fold for phenotypic assay and gene expression respectively.*
Discussion

The biofilm formation plays a major role in infections and diseases associated with oral cavity, and the bacteria in the biofilm communicates through physical, physiological, and metabolic interaction. Since the oral cavity acts as reservoir for wide variety of microorganism, around 500 species are isolated from oral cavity [57]. That colonizes tooth surface, sub and supra-gingival pocket, tongue, soft hard palate, and buccal mucosa, establishing early biofilm by interaction with suitable substrate. With the new technologies for observing and investigating the bacterial biofilm formation like flow cells system, and imaging technique of the cells in situ with confocal scanning laser microscopy, and the advancement in genetic studies owing to the discovery of gene-transfer systems, all these useful tools provide an access to discover bacterial responses to their environment and open the ways toward better understanding bacterial communication in multispecies biofilm.

The biofilm formation basically follows the same developmental mechanism in various microorganisms; therefore, the mechanism involved in biofilm formation in *S. aureus* is considered a good model to study the biofilm formation at genetic level, which might be used by other microorganisms. A better understanding of this mechanism is necessary to provide novel strategies for treating and preventing oral diseases and other infections associated with biofilm formation.

Previously, in our lab they identified the *msa* gene (modulator of *sarA*) that control expression of *sarA*, which is essential for biofilm development and protease activity in *S. aureus* [23]. Later they found that the *msa* currently referred to as *msaC* is part of a four-gene operon named *msaABCR* that control expression of several virulence regulators and biofilm formation [13]. Indeed, deletion of *msaC* from USA300-LAC
strain resulted in a significant defect in biofilm development, increase in protease activity, reduced pigmentation, and increased cells death [29]. Also, they found that in the msaC deletion mutant, sarA expression was reduced during biofilm growth and decreased expression of upstream genes (msA, masB). Interestingly, the phenotypic characterization of msaC deletion mutant was similar to whole operon deletion mutant. Furthermore, complementation studies in the msaC deletion mutant confirmed that all three genes were required for restoration of the wild type phenotypic features. These findings suggested that the three genes are functionally related and form an operon [13].

Sequence analysis of the genes in the msaABCR operon showed that msaA is conserved hypothetical protein in S. aureus. By using the I-Tasser program to predict protein structure and function, we found that msaA has strong similarity to the twin-arginine signal-binding protein, which involved in twin-arginine translocation system (Tat). The (Tat) system exports the fully folded exoproteins across the bacterial cytoplasmic membrane [46]. For staphylococci, very few information regarding the function of the Tat system exists. However, it is reported that the Tat translocation system can effectively translocate heterologous proteins like protein A or lipase, when it is bind with the signal peptide of iron-dependent peroxidase, since a homologs of TatA and TatC have been determined in S. aureus, and it functions and serves to translocate the iron-dependent peroxidase [32].

The phenotypic characterization of msaA deletion mutant was shown increased in biofilm formation, decreased in protease production, and reduced pigmentation. These results were opposing to msaABCR operon deletion mutant. More interesting the msaA deletion mutant showed abolition of agr expression.
However, a quorum sensing intercellular signaling between bacteria is known to be involved in biofilm development by several bacteria [47]. \textit{S. aureus} have a quorum-sensing system encoded by the accessory gene regulator (\textit{agr}) locus, which responds to and affected by auto-inducing peptide (AIP) molecule. [48] This \textit{agr} quorum-sensing system modulates the expression of virulence factors and involved in biofilm formation in \textit{S. aureus} [49]; however, presence of an active quorum-sensing blocks attachment and development of a biofilm. The \textit{agr} mutants exhibit a high tendency to form potent and robust biofilms, but the cells dispersing from the biofilm have shown displaying of an active \textit{agr} system [12, 50].

\textit{S. aureus} and bacteria, in general, respond to environmental changes by fast and complicated regulation of their gene expression through overlapping global regulatory networks. For instance, it is reported that \textit{agr} global regulator showed response to the changes in nutrient accessibility [51]. Chakrabarti SK, et al. (2000) reported that growth on rich media containing glucose represses the \textit{agr} system[52], and the bacteria response to environmental stress by induction the activity of an alternative sigma factor B which has negative influence on \textit{agr} expression [53].

The \textit{msaA} mutant biofilm formation under different stress condition showed that sodium chloride (NaCl) and glucose induce biofilm formation as their concentrations increased; thus, this effect is concentration dependent [54]. Because NaCl and glucose induce expression of \textit{icaA}-gene and \textit{rbf} gene (regulator biofilm formation), which are important regulators in biofilm formation [55, 56]. At the mean time glucose repress \textit{agr} expression system, which induces the biofilm formation [51].

There are no significant differences between wild type and \textit{msaA} mutant biofilm at low concentration of NaCl (0.1%- 0.5%). But as the NaCl concentration increased the
msaA mutant shows more biofilm formation. Biofilm formation by both wild type and msaA mutant strains increased as the concentration of glucose increased from 0-3%, and beyond 3% they are not affected by addition of glucose. Ethanol at low concentration increase biofilm formation in both msaA mutant and wild type strains. A biofilm formed by msaA mutant and wild type does not show significant difference at low ethanol concentration, but as the ethanol concentration increased the msaA mutant shows more biofilm compared to wild type. The msaA mutant shows more biofilm formation compared to wild type as the ethanol concentration increases up to 2%, but at concentration higher than 2% the biofilm formation is reduced, which could possibly reasoned to denaturation of proteins, which may result from high ethanol concentration. Further research and understanding is required to understand the effect of ethanol and other environmental conditions in the msaA mutant’s biofilm formation.

The result of detachment assays showed that both msaA mutant and wild type S. aureus biofilms were sensitive to proteinase K and DNase I, respectively. The Proteinase K degrades all proteinaseous component of the biofilm and led to either sloughing or detachment of the biofilm. While the DnaseI enzyme acted on the extracellular DNA, which was another important component of the biofilm and aid in biofilm detachment.

Our result showed that both msaA mutant and wild type S. aureus biofilms were affected by the addition of proteinase K or DNase I, and this is correlated with results of previous studies. This proved that proteins and eDNA are important components of S. aureus biofilm [40, 41, 43, 44]. Addition of sodium metaperiodate at different time intervals showed negative effect on biofilm formation in both msaA mutant and wild type strain. Since Sodium metaperiodate would affect the PNAG (PIA) in biofilm if the biofilm is PIA dependent [45]. However, the USA300 strains of S. aureus seems to form
biofilm independent of PNAG pathway, and this might be the reason that Sodium metaperiodate does not have any effect on biofilm formation in \textit{msaA} mutant biofilm compared to the wild type. Overall, the response of \textit{S. aureus} to different stressful environmental conditions by altering expression of various virulence factors and affecting capability to form biofilm formation is protective mechanism to maintain integrity of the cell machinery and ensure its survival.

Conclusion

We characterized \textit{msaA} gene of \textit{msaABCR} operon and studied its role and observed that it plays an opposing role to \textit{msaC} in the regulation of \textit{sarA}, extracellular protease production, and biofilm formation. These findings allow us to define the mechanism of regulation of virulence by the \textit{msaABCR} operon and the role of \textit{msaA} in the regulation of an operon itself.
REFERENCES


