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The University of Southern Mississippi

IDENTIFICATION AND CHARACTERIZATION OF *MSAB* GENE INVOLVED IN BIOFILM FORMATION AND VIRULENCE IN *STAPHYLOCOCCUS AUREUS*

by

Amelsaad Elbarasi

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 Science

Approved:

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Committee Chair

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Dean of the Graduate School

August 2014

ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF *MSAB* GENE INVOLVED IN BIOFILM FORMATION AND VIRULENCE IN *STAPHYLOCOCCUS AUREUS*

by Amelsaad Elbarasi

August 2014

Staphylococcus aureus is an important human pathogen that causes a wide variety of life-threatening infections ranging from minor skin and oral infections to severe infections, such as bacteremia, pneumonia, osteomyelitis, or endocarditis due to the presence and secretion of a large number of virulence factors that are controlled by global virulence regulators in complex networks. Furthermore, *S. aureus* infections have become a threat to public health because of their high potential to form biofilm, and their ability to resist a wide range of antibiotics has exacerbated further. Therefore, understanding the regulatory networks and developing a drug targeting these networks has the potential to stand as therapeutic targets for future treatment of antibiotic resistant infections.

In a previous study *msaC* was identified as the modulator of *sarA*, a new global virulence regulator that controls the expression of *sarA* and biofilm development. Furthermore, it has also been shown that *msaC* is a part of four-gene operon, *msaABCR* operon, which includes four-genes: SAUSA300_1296 (*msaA*), SAUSA300_1295 (*msaB*), SAUSA300_1294 (*msaC*), and antisense RNA, *msaR.* The mechanism of regulation of *msaABCR* operon and the function of individual genes were not clearly known yet. This study defines the role of *msaB*, the second gene of the *msaABCR* operon, which will help shed some light on the regulation of *msaABCR*. We deleted *msaB* gene from

USA300_LAC, and studied the major *msaB* phenotypes: pigmentation, protease production, biofilm formation, and rate of cell death. Deletion of *msaB* resulted in the similar *msaC* and/or *msaABCR* deletion mutant, thus showing the importance of this gene in this operon. The mutant showed decreased pigmentation, increased extracellular protease production, decreased biofilm formation, and increased rate of cell death. Deletion of the *msaB* gene also resulted in the decreased expression of some key regulators, like *sarA* and *agr* that play major roles in the regulation of virulence and biofilm formation in *S. aureus*, similar to *msaC* and *msaABCR* operon deletion mutant.

Thus, this study identifies the role of *msaB,* in the *msaABCR* operon, that will help us define the mechanism of regulation of virulence and biofilm formation by the *msaABCR* operon and provides a step to investigate the stimulatory signals that the *msaABCR* operon responds to during pathogenesis.

DEDICATION

I dedicate this master thesis work to my parents, Boushnaf and Magboula; my dear husband, Ahmed Alzuway; my uncle, Hamed Benghuzzi; my aunts, Shelly and Oriada; and finally to my handsome kids, Yahya, Yarra, and Yazen.

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Furthermore, I would like to acknowledge with much appreciation the crucial role of Gyan S. Sahukhal for his mentorship, guidance, and support of me throughout this project. I would also like to thank him for making the RT-qPCR for expression of genes. However, it would not have been possible without the kind support and help of many individuals like my committee members, Dr. Glen Shearer and Dr. Shahid Karim, and my colleagues, all of whom I thank for their knowledge, technical support, and for creating a good environment in developing this project. Last but not least, many thanks go to all my family members for their prayers, support, and encouragement throughout my graduate study.

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CHAPTER I

INTRODUCTION

Staphylococcus aureus

Staphylococcus was first identified in 1880 in Aberdeen, United Kingdom, by the surgeon Sir Alexander Ogston in pus from a surgical abscess in a knee joint, and this name was later appended to staphylococcus aureus (*S. aureus*) by Rosenbach, who was credited by the official system of nomenclature at that time. *S. aureus* is an anaerobic facultative gram-positive bacterium; it is a member of the Firmicutes, and it's also known as golden staph due to its ability to produce a carotenoid pigmentation as is shown in Figure 1. *S. aureus* is positive for catalase and nitrate reduction, and this feature differentiates it from other micrococcaceae like streptococci, and enterococci. It is estimated that 20% of the human population is a long-term carrier of *S. aureus* [1] which can be found as part of the normal skin flora, anterior nares, throat, axilla, perirectal area, and groin area [1, 2].

Figure 1. Staphylococcus aureus has been grown on TSA (Tryptic Soy Agar) media. The strain is producing a yellow pigmentation (Golden *Staph*).

Although *S. aureus* is not always pathogenic, it may cause a wide variety of life-threatening infections when it enters in the human body; its ability to acquire resistance to antibiotics very rapidly and its ability to form biofilm to cause biofilm-associated infection is very alarming. *S. aureus* is an important human pathogen associated with one of the most common cause of healthcare and community-associated infections [3]. Even though, it has been over 100 years since the discovery of *S. aureus*, the mode of action is still controversial and it has to be fully elucidated. Since human are considered a natural reservoir of *S. aureus*, it is estimated that around 30 to 50 percent of the human population is persistently colonized with both methicillin-sensitive and methicillin-resistant isolates [4, 5].

The frequencies of both community-acquired and hospital-acquired staphylococcal infections have increased steadily over the past 20 years, with little change in overall mortality [6], and during the period from 1990 through 1992, *S. aureus* was the most common causes of nosocomial infection according to data from the National Nosocomial Infections Surveillance system of the Centers for Disease Control and Prevention (CDC), and they spread rapidly to populate many hospitals and diverse geographic regions with unique processes and yet undefined pathogenicity [7, 8].

Impact of *S. aureus* on Oral Cavity

The oral cavity acts as a reservoir for many pathogens that may cause local or systemic infections [9]; for instance, saliva and the periodontal pocket serve as an ecological niche appropriate for hosting microorganisms that could act as opportunistic pathogens. There are over 700 different bacterial species in the oral microflora [10], and those species have the ability to colonize the whole mouth such as oral mucosa, hard

palate, tongue, teeth, carious lesions, and periodontal pocket. The distribution of microflora in the oral cavity is not random, most species prefer certain sites over others due to the particular local environment those sites provide [11]. Cuesta Al et al. 2010 [12] showed that the oral cavity and sub-gingival pocket are ecological niches for microorganisms that may act as opportunistic pathogens, such as *S. aureus* and especially methicillin-resistant *S. aureus* (MRSA) is one of them. Early detection of MRSA is a matter of concern to Public Health that can help prevent further infections.

Passariello et al. 2012 [13] also found that the oral cavity is an important site of *S. aureus* colonization and showed that conditions modifying the oral environment, such as the presence of periodontitis and fixed prosthetic restorations, promote *S. aureus* and may favor the spread of more pathogenic strains. Information regarding the risk of methicillin-resistant *Staphylococcus aureus* (MRSA) infection transmission in dental healthcare settings was incomplete only a few years ago; therefore, MRSA infection control guidelines were necessarily based on data extrapolated from other fields. Recent publications have made it possible to review such risk. The relationship among these pathogens, periodontal bacteria, and periodontal clinical status is poorly understood.

S. aureus colonizes and causes infection of the oral cavity and this is an important aspect in its pathogenesis because it has been isolated from a wide variety of oral infective conditions, as angular parotitis and cheilitis [14]. Recently, staphylococcal infection has been shown in a clinical condition, which classified as staphylococcal mucositis, emerging as a clinical problem in many debilitated old patients and those suffering from oral Crohn's disease [15]. Also, higher caries rates of Coagulase negative staphylococci and *S. aureus*, or both, appear in patients and have the ability to develop

joint infections, which raises the possibility of the oral cavity serving as a potential source for spread of bacteremia to compromised joint spaces [16].

Furthermore, *Staphylococci* isolated from sub-gingival samples of patients with advanced periodontitis were tested for leukocidal activity. Intact organisms, bacterial sonicator, or bacterial culture supernatants were incubated with human neutrophils that had been pre-labeled with 51chromium. The most common of *S. aureus* periodontal isolates provoked dose-dependent extracellular release of the radiolabel. By contrast, other strains of *staphylococci* had marginal or no demonstrable leukocidal activity. Leukocidal activity was heat-sensitive and was neutralized by horse anti-leukocidin antibodies. The results indicate that *S. aureus* colonizing human periodontal lesions elaborate a leukocidin that may conceivably destroy neutrophils that immigrate into the gingival region. This could adversely affect the overall status of this antimicrobial defense system in the gingival area and contribute to the pathogenicity of *S. aureus* as well as other potential periodontopathic organisms [17].

Biofilms in the Oral Cavity

Accumulation of bacterial biofilm (plaques) on tooth surfaces results in some of the most prevalent bacterial-induced diseases of man—caries and inflammatory periodontal diseases [18]. The former is due to the accumulation of the biofilm supragingivally and involves dissolution of enamel and dentine. The latter stem from a build-up of plaque at and below the gingival margin and the irreversible phase of the disease, chronic periodontitis, that is characterized by detachment of the gingiva from the root surface of the tooth, resulting in a periodontal pocket and, ultimately, resorption of the alevolar bone occurs and leads to tooth loss. There are several other studies conducted

to solve the puzzle of staphylococci pathogenesis in the oral cavity but it still remains unclear and requires more research in this area.

The *S. aureus* Virulence and Biofilm Development

The ability of *S. aureus* to infect a variety of tissues in the human body is due to its expression of a wide variety of virulence factors such as surface-associated proteins, secreted proteases, toxins, and immune modulators [19]. Expression of these virulence factors in is carefully coordinated by a variety of regulators that include trans-acting global regulators, alternative sigma factors, and small non-coding RNAs [20]. The community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strains often cause localized infections by producing various enzymes, such as protease, lipase, and hyaluronidase, that destroy the tissues in immune-compromised hosts. These strains show enhanced virulence leading to severe infections among healthy individuals without any predisposing risk factors; however, their role in the pathogenesis of disease is still not well understood.

One of the major factors responsible for the establishment of chronic infection by *S. aureus* is its ability to form biofilm virtually on any host surfaces [21]. *S. aureus* can form biofilms on host surfaces like heart valves, bone [22], cartilage, and medical implants like catheters and orthopedic devices [23, 24].

A biofilm is an aggregation of any group of microorganisms, in which the cells stick to each other and upon a solid surface, typically with a fluid of microbes bathing [25]. Biofilms can be found everywhere on living or non-living surfaces and can be prevalent in natural, industrial, and hospital settings. The main components of mature biofilm are approximately 5-25% bacterial cells and 75-95% glycocalyx matrix [26].

These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS). Biofilm EPS, which is also referred to as slime (although not everything described as slime is a biofilm), is a polymeric conglomeration generally composed of extracellular DNA, polysaccharide and proteins. These microbial communities form micro-colonies that interact with each other using very sophisticated communication methods (i.e., quorum-sensing) [27]. The development of unique microbiological tools to detect and assess the various biofilms around us is a tremendously important focus of research in many laboratories.

The *S. aureus* biofilm forms in three distinct stages as shown in Figure 2:

1. The free-floating cells attach to the surfaces by means of adhesions or cell wall components, which also help other microorganisms to attach to the surface because they cannot do it on their own.
2. Accumulation of the multilayered cells via subdivision, and then they start production of their self-polysaccharide matrix, which help maturation of the biofilm.
3. Detachment of biofilm cells and dispersal to new distant sites for colonization or to infect other areas, and this is the most important stage in the biofilm life cycle.

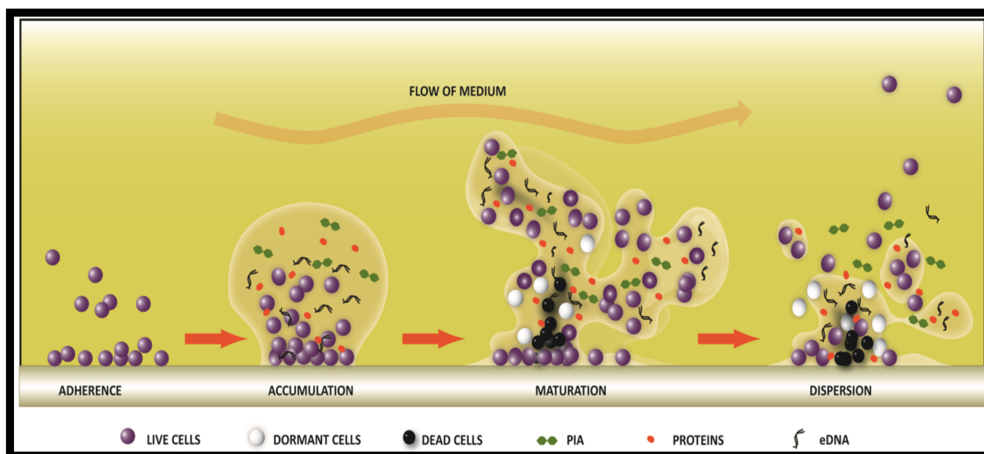

Figure 2. Schematic represents stages of biofilm formation.

This fully matured stage is characterized by an overall shift in gene expression patterns total different from the planktonic stages and offering increased resistance to antimicrobials. Once they are fully developed biofilm, via production of some biofilm destabilizing proteins like phenol soluble modulins (psms), disassembly of biofilm occurs resulting in free-floating planktonic cells (dispersion) that are able to re-initiate the biofilm development process in another site [28]. Several factors like extracellular matrix, reduced metabolic state of bacteria within the biofilm, and efficient spread of antibiotic resistance mechanisms via gene transfer within the biofilm community contribute to the biofilm recalcitrance [29].

Global Regulators in *S. aureus*

The *S. aureus* genome has 124 putative transcriptional regulators [30], and several global regulators have been identified, which include the *agrA* operon, the *sarA* gene family, the *saePQRS* operon, and the genes *arlRS*, *lytSR*, *srrAb*, *hssRS*, *vraSR*, and *graSR* [31, 32]. Several other regulators have also been identified, though they are not as well characterized (e.g., *htrA*, *ccpA, msrR,* and *svrR*) [33].

The first and most important globular regulator gene in *S. aureus* genome is *Staphylococcal* accessory regulator A (*SarA*); its family of homologs is transcriptional regulators that control the expression of several genes [34], including virulence factors, and biofilm formation. The *sarA* locus comprises three promoters (P1, P2, and P3) that drive expression of three transcripts, *sarB*, *sarC*, and *sarA*, respectively; however, the mechanism of regulation of *sarA* is not fully understood [35]. Indeed, the environmental signals that modulate the expression of *SarA* or its activity are not known either. Inactivation of *sarA* leads to a reduction in biofilm formation [36], and it is epistatic to *agr* (accessory gene regulator) during biofilm formation [37, 38]. *SarA* mutants are deficient in biofilm formation and show reduced expression of the *icaADBC* operon, increased protease and nuclease activity, and degradation of surface-associated proteins like *FnbA*, *FnbB*, and *spa* [36, 39, 40].

The next globular regulator, accessory gene regulator *(agr*) is also a global regulator that encodes a quorum-sensing system in *S. aureus*, and the main effector molecule of *agr* is RNAIII, which is expressed in temporal fashion, reaching a maximum in the transition from the post-exponential to the stationary growth phase. Repression of *agr* is also important for biofilm formation [41].

The *agr* system regulates a large number of virulence factors, especially exoprotein genes like *hla, hlb, hld, hlCB, sspABC, splABDF, aur,* and many others, whereas it decreases the expression of several cell wall-associated protein genes like *fnbA, fnbB, spa,* and *coa* [42].

The other important global regulator is the alternate sigma factor B (*sigB*), which responds to environmental stress during stationary growth phases [43, 44]. The *sigB* gene

regulates its targets via other regulatory genes or by acting directly on the promoters of virulence genes. Although *sigB* positively regulates expression of *sarC* and negatively regulates *agr*, its effect on *sarA* is not yet known [45, 46]. It seems that *sigB* acts in opposition to RNAIII, as most of the exoenzymes and toxins were negatively influenced by *sigB*, while the expression of several adhesions was increased [47].

We recently identified a new global regulator named *msaABCR* operon, modulator *sarA* that plays a role in autolysis, biofilm formation, and virulence in the clinical isolate of community acquired *S. aureus* strain USA300_LAC [48]. In addition, it also regulates the expression of some other well studied global regulators including *sarA*, *agr*, and *sigB*, which plays a role in pathogenesis. So understanding virulence regulation during growth under different environmental conditions (e.g., biofilm development) is imperative for the effective prevention and treatment of *S. aureus* infections.

In this study, we deleted the second gene of *msaABCR* operon, *msaB* gene, by an allelic gene replacement method and studied the phenotypes of *msaB* gene and compared with the *msaC* and *msaABCR* deletion mutant in order to elucidate the role of this gene in the operon.

Although it was not clear if *msaB* gene regulated virulence genes directly via its effect on global regulators (*SarA* and *agr* genes) or if it has a different pathway, we did not discover it yet in this experiment. Since *sarA* has been shown to be essential for biofilm development in several strains [36], it is not clear if the *msaB* defect is due to the reduction in *sarA* or other factors.

Sequence analysis of the *msaB* gene showed it is conserved among *S. aureus* strains and encodes a protein that is a cold shock domain, which is a member of a family of small cold-shock proteins (*CSPs*) that are induced during bacterial cell response to stress like low temperatures. Bioinformatics analysis also showed that this protein preferentially binds single-strand RNA and DNA and appears to play an important role in cell physiology under both normal and cold-shock conditions. Although, the function of *CSPs* in cold-shock adaption has not yet been elucidated in detail, a number of experimental evidence suggests that *CSPs* bind messenger RNA (mRNA) and regulates ribosomal translation, the rate of m-RNA degradation, and the termination of transcription [49]. Interestingly, the cold shock protein family has shown structural similarity in different bacterial species (for instance, *E.coli*, *Mycobacterium tuberculosis*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Yersinia pestis*, and *Haemophilus influenzae*) and in other important species such as human, *Caldosporium herbarum*, and *C.elegans* [50].

The Goals of This Project

Our previous study showed that *msaABCR* is an operon that contains three non-coding RNAs, *msaA, msaC,* and *msaR*, and it interacts with other global regulators like *sarA, agr,* and *sigB*; however, the mechanism of regulation and role of each gene in the operon has been studied. In this study we elucidate the role of *msaB* in the *msaABCR* operon to regulate the virulence and biofilm formation in *S. aureus*.

CHAPTER II

CHARACTERIZATION OF *MSAB* GENE

Abstract

Staphylococcus aureus is an important human pathogen that causes a wide variety of infections because *S. aureus* produces a vast array of virulence factors that are controlled by global virulence regulators. Previously identified *msaABCR* is a new global virulence regulator that controls the expression of *sarA, agr*, and *sigB* and virulence and biofilm development in *S. aureus* [48].. To define the role of individual genes in the operon, we deleted *msaB* gene from USA300_LAC strain and compared its phenotype to single *msaC* mutant and *msaABCDR* operon deletion mutant.

Our preliminary data showed that the studied phenotypes of *msaB* deletion mutants are similar to both *msaC* and *msaABCDR* operon deletion mutants, and showed decreased pigmentation, increased extracellular protease activity, increased cell death, and reduced biofilm formation. The whole *msaABCR* operon gene is required for the full complementation of *msaB* gene, which also supports our previous findings that all genes in an operon in cis are required for the function of this operon. Defining the mechanism of action of the *msaABCDR* operon locus, its role in virulence and biofilm development will build more information for understanding *S. aureus* network virulence in order to combat their outbreak in the near future.

Introduction

The *S. aureus* causes life-threatening infections because it produces large numbers of virulence factors and posses complex regulatory networks that control these virulence factors [51]. *S. aureus* infections have become a threat to public health due to

its ability to resist a wide range of antibiotics. Therefore, understanding the function of complex regulatory networks that regulate the virulence factors and pathogenicity of *S. aureus* is important to develop potential future therapeutic targets against these pathogens, which are very inflexible against antibiotic treatment [52]. Some of the important global regulators of virulence in *S. aureus* are *msaABCR*, *sarA*, *sigB,* and *agr*. We have previously shown that *msaABCR* operon positively regulates the expression of some global regulators like *sarA, agr,* and *sigB,* which they are very well studied global regulators that globally regulate virulence and biofilm development in *S. aureus*. In this study, we studied the role the second gene of *msaABCR* operon, *msaB* gene, in order to elucidate the role of this gene in the operon.

Materials and Methods

The Bacterial Strains and Plasmids

All strains and plasmids used in this study are listed in Table 1 below. The *S. aureus* strains (community-acquired MRSA strain USA300_LAC, restriction-deficient laboratory strain RN4220), *E. coli* strain DH5α, pJB38 gram positive shuttle vector, and pCN34 low copy plasmid vector were used in this study. The *S. aureus* strains were grown in tryptic soy broth (TSB) medium. Antibiotics (chloramphenicol (10 μg/ml), erythromycin (10 μg/ml), and kanamycin (50 μg/ml) were used in TSB or TSA where needed. Similarly*, E. coli* strains were grown in LB broth with ampicillin (100 μg/ml) added where needed.

Table 1

Bacteria Strains and Plasmids Used in This Study

Plasmids		
Plasmid	Relevant Characteristics	Source
pJB38	Gram positive shuttle vector	NARSA
pCN34	pT181-based low copy number E. coli-Staphylococcal shuttle vector	NARSA
pMOE 526	pJB38-Δ*msaB* vector plasmid in *Ecoli*	This study
pMOE 528	pJB38-Δ*msaB* vector plasmid in *RN4220*	This study
pMOE 586	pCN34-*msaB* gene in *Ecoli* .::*msaB*gene complement	This study
pMOE 595	pCN34-*msaB* gene in *RN4220*.::*msaB*gene complement	This study

Table 1 (continued).

Strains	Relevant characteristics	Reference or source
RN4220	Restriction deficient mutant of 8325-4	NARSA Dr. Lindsey Shaw
LAC	CA-MRSA USA300 strains	This study
MOE 531	LAC:: Δ *msaA* deletion mutant	This study
MOE 596	*MsaB* gene complement into *msaA* deletion mutant	This study

Construction of msaB Deletion Mutant and its Complementation

The upstream and downstream flanking regions of *msaB* gene were amplified by using high fidelity PCR reaction using primers *MsaB*_upstr_F/R and *MsaB*_dnstr_F/R respectively (All primers used in this study are listed in Table 2 below). The upstream and down-stream PCR products were digested with bamHI and ligated to amplify the ligated PCR product using the terminal primers *MsaB*_upstr_F and *MsaB*_dnstr_R. The amplified ligated PCR product and pJB38 plasmid vectors were again digested with knpI and salI, purified, and then ligated to construct the deletion plasmid construct pJB38_*msaB*. The plasmid pJB38 is an allelic replacement vector, which is a temperature

sensitive shuttle vector was used to successfully delete other *S. aureus* genes; one of the special features of pJB38 is that it offers an inducible counter selection with anhyrotetracycline (aTc) that selects for chromosomal excision and loss of plasmid without the use of an antibiotic. The pJB38_*msaB* deletion plasmid construct was transduced in wild type *S. aureus* strain USA300_LAC, and deletion procedures were followed as described in Sahukhal and Elasri, 2014 [48].

The *msaB* complement was constructed by amplifying the wild type *msaB* gene from wild type LAC strains with complmsaB F/R primers, that contain restriction site BamHI and EcoRI, respectively. The amplified *msaB* gene and gram-positive low copy number shuttle vector, pCN34, was digested with BamHI and EcoRI, and ligated with the amplified *msaB* gene. The pCN34 vector containing *msaB* gene (pCN34-*msaB*) was then inserted into RN4220 by electro-transformation. The transformants containing pCN34-*msaB* were confirmed by restriction digestion and sequencing. Finally, pCN34-*msaB* gene was transduced into an *msaB* deletion construct and was used as the complement strain for the study.

Table 2

The Primers Used in This Study

Primer for *msaB* deletion and *msaB* complement	
MsaB upstr F	GGGGACAAGTTTGTACAA AAAAGCAGGCTGCTTTAAATCAGCGATTAATGTTCG TTTG
*Msa*B upstr R	AACGTTGTTAAAGGATCCTTCTTAGATTTGAATCAT

Table 2 (continued).

MsaB dnstr F	CCTTGTTTCAGGATCCGAAACCTCCAAGACTAAAATTCAT
MsaB dnstr R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTTTGGATTTATCAATTCAATATGGCTTAGC
MsaB compl F	AGGATCTGCAGATATTTTATAGTTTCATTG
MsaB compl R	ACCTCGTCGTTGGGATCCATA

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 [48]. 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 RNeasy 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 has been used as an endogenous control gene and has been included in all experiments.

Analysis of expression of each gene has been done based on at least three individual experiments, and two-fold or higher changes in gene expression have been considered significant. All the primers used for RT-qPCR are listed in Table 3 below.

Table 3

Primers Used in RT-qPCR

Primers used in RT-qPCR analysis	
RT *msaB* F	TTTATCGAAGTTGAAGGAGAAAATG
RT *msaB* R	ACTCAACAGCTTGACCTTCTTCTAA
RT *sarA* F	TTTGCTTCAGTGATTCGTTTATTTACTC
RT *sarA* R	GTAATGAGCATGATGAAAGAACTGTATT
RT *agrA* F	TTTGTCGTCAATCGCCATAA
RT *agrA* R	TTTAACGTTTCTCACCGATGC
RT *gyrA* F	GCTCGTTCGTGACAAGAAAA
RT *gyrA* R	TTTGCATCCTTACGCACATC

Phenotypic Assays

1. *Triton X-100 induced autolysis.* Autolysis assay was performed using Triton X-100 as described by Manna et al. 1997 [53]. 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 A_{580} 0.7. The cells were collected by centrifuging at 10,000 rpm for 10 min at 4˚C, and then the cells were washed twice with ice-cold autoclaved water and then were 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 A_{580} every 30 min intervals for 3 hrs.

2. *Pigmentation assay*. A pigmentation assay was performed on cells harvested from overnight cultures, as described by Morikawa et al. 2001 [54]. Briefly, 1 ml of the over-nightly grown cells were harvested and washed twice with water. They were then suspended in 1 ml of methanol and heated at 55°C for 3–5 min with occasional vortexing. The cells were removed by centrifugation at 15,000-x g for 1 min, and the absorbance of the supernatant was measured at 465 nm with water as a blank. Mean values from a minimum of three independent experiments, each performed in triplicate, were recorded.

3. *Protease assay*. Protease activity assay was performed as described by Sambanthamoorthy et al. 2006 [55]. 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 at 340 nm using water as blank. We took the mean values from a minimum of three independent experiments, each performed in triplicate, and recorded.

4. *Microtiter plate Biofilm assay*. The microtiter biofilm assay was performed as described in Sambanthamoorthy et al. 2008 [56] but with slight modification. Briefly, overnight cultures of cells, including wild type, mutant, and the complemented strain of USA300 LAC were diluted 1:100 times in TSB supplemented with 3% NaCl and 0.25% glucose and inoculated in microtiter plates pre-coated with 20% human plasma. Cultures were incubated for 24 or 48 hrs with shaking at 150 rpm. The adherent biofilm was quantitated at 615 nm after washing and staining with crystal violet and elution with 5% acetic acid. Absorbance readings were made at 615 nm using a SpectraMax M5

microplate spectrophotometer system. Experiments were performed in triplicate and three independent experiments were performed for each of this assay.

Confocal Microscopy of Biofilm

For confocal microscopy assay of biofilm produced by the *msaB* deletion mutant, the biofilms were grown on microtiter as described above. The 48 hrs 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 using to stain the dead cells and eDNA, then incubated at 30°C in dark for 10 minutes. The images obtained by CLSM were analyzed using the COMSTAT program where the total biomass of the biofilms, thickness of the biofilm, and amount of live and dead cells within the biofilm were quantified.

Effect of Different Environmental Conditions on Static Biofilm Formation by msaB Mutant

To study the effect of different environmental conditions that affect biofilm, the static microtiter biofilm was grown as described above, but in the presence of different concentrations of NaCl, glucose, and ethanol tested. To test the different concentrations of NaCl, the TSB with 0.5% glucose concentration supplemented with sodium chloride at concentrations ranging from 0 to 6%, were used.

To test the different concentrations of glucose, the TSB with 3% NaCl supplemented with glucose at concentrations ranging from 0 to 5% was used. Similarly, to test the effect of different concentrations of ethanol, TSB with 3% NaCl, supplemented with ethanol at concentrations 0.5% to 6%, and TSB with 0.5% Glucose, supplemented with ethanol at concentrations 0.5% to 6%, were used. The biofilms were grown in presence of different concentrations of NaCl, glucose, and ethanol, as mentioned earlier in separate microtiter plate, incubated for 48 hrs, washed three times and quantified as described above.

Biofilm Detachment Assays

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 hrs and treated them as previously described in Rice et al. 2007 [57]. 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 hrs) in their respectively labeled plates and further incubated till 48 hrs. 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 10 min. The absorbance was then measured at 595 nm to quantify the remaining biofilm.

Results

Construction of msaB Deletion Mutation in CA-MRSA strain USA300_LAC

We amplified the upstream and downstream flanking region of *msaB* gene and inserted them into a deletion vector pJB38, to construct the deletion plasmid, pJB38-*msaB*. Once we transduced the deletion plasmid into wild type LAC strain, we performed the deletion steps as described in the Material and Methods section. We screened and confirmed the *msaB* deletion from LAC by using PCR (Figure 3) and sequencing of the PCR product. We then introduced *msaB* gene (pCN34-msaB) into *msaB* deletion mutant and used it as the complement construct for further study and we verified the *msaB* mutant complement construct by both sequencing of the PCR product and by using restriction digestion of two restriction enzymes, which are Bam HI and EcoRI, as show in Figure 4.

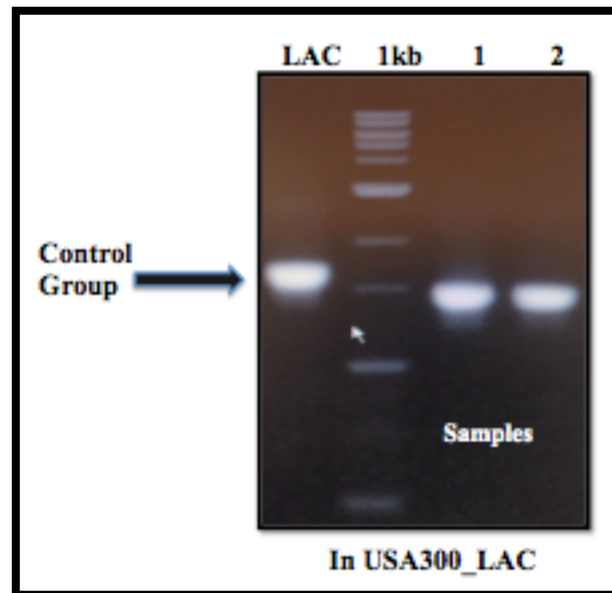

Figure 3. Gel electrophoresis shows poly chain reaction (PCR) using terminal primers to verify *msaB* deletion mutant construct in USA300_LAC wild type (the construct size around1.5kb).

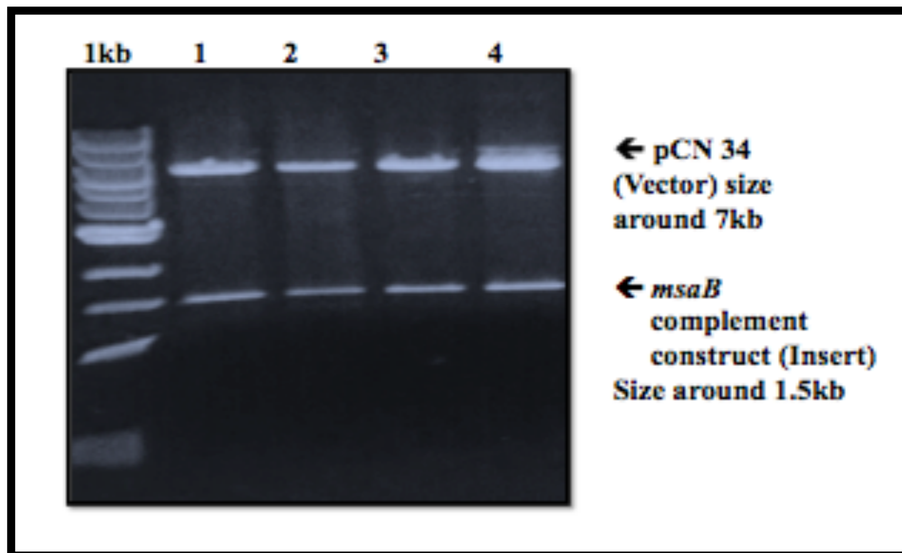

Figure 4. Gel electrophoresis shows restriction digestion to verify the presence of *msaB* complement construct in pCN34-*msaB* complement construct by using two restriction enzymes, Bam HI and EcorI. Upper bands refer to pCN34 plasmid (Vector), which size around 7 KB, and lower bands refer to *msaB* complement construct (Insert), which size around 1.5 KB.

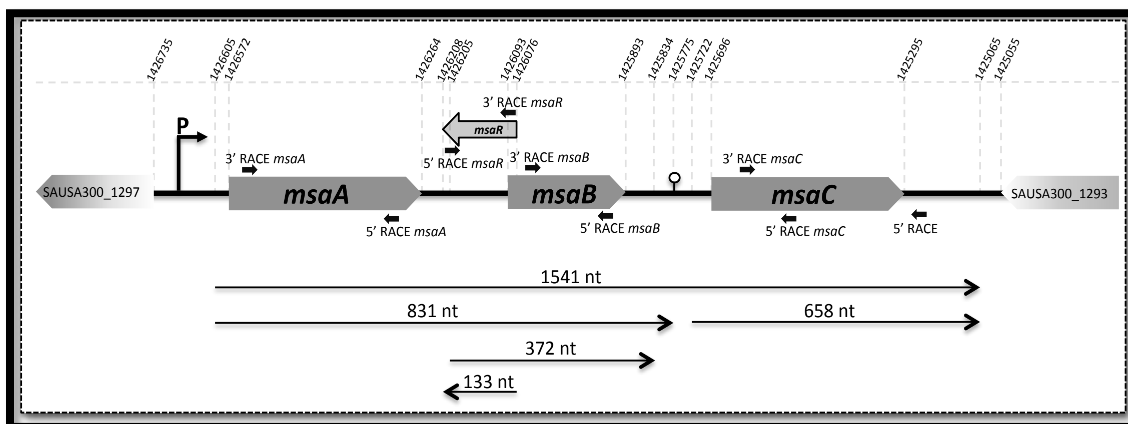

Figure 5. Shows the *msaABCR* operon map, which include of the *msaA*, *msaB, msaC*, and msaR genes (short thick grey arrows). Also it shows different alternative forms of the RNA transcripts (long thin black arrows) formed by the *msaABCR* operon [48].

Relative Expression of Global Regulators sarA and agr

We measured the expression of global regulators *sarA* and *agr* by RT-qPCR in the *msaB* deletion mutant. We observed decrease in expression of *sarA* and *agr* (Table 4), similar to *msaC* and *msaABCR* operon deletion mutant [48].

Table 4

Relative Expression of the Global Regulator, SarA, and Agr in the MsaB mutant and its Complement Relative to Wild Type (USA300_LAC)

Genes	*MsaB* deletion mutation	*MsaB* complement
MsaB	-16.767	-2.23
SarA	-2.14	137.18
Agr	-2.29	-2.24

Note: The fold change transcription level in various strains relative to wild-type USA300_ LAC, and the values represent the mean ratio of three independent experiments.

Phenotypic Characterization of msaB Deletion Mutant

We studied several phenotypes in the *msaB* deletion mutant and compared with its wild type and complement. The *msaB* deletion mutant showed a significant defect in biofilm formation, pigmentation, and showed increased protease production, as well as increased rate of cell death (Figures 6, 7, 8, & 9). The phenotypes we observed were similar to the *msaC* deletion and *msaABCR* operon deletion mutant. This supports the previous result that *msaB* is an important part of *msaABCR* operon. Reintroduction of

msaB cloned in the low copy plasmid pCN34 into the deletion mutants led to an increased biofilm formation, increased pigmentation, decreased protease, and decreased cell autolysis, confirming that all the phenotypic characteristics we saw were due to deletion of the *msaB* gene.

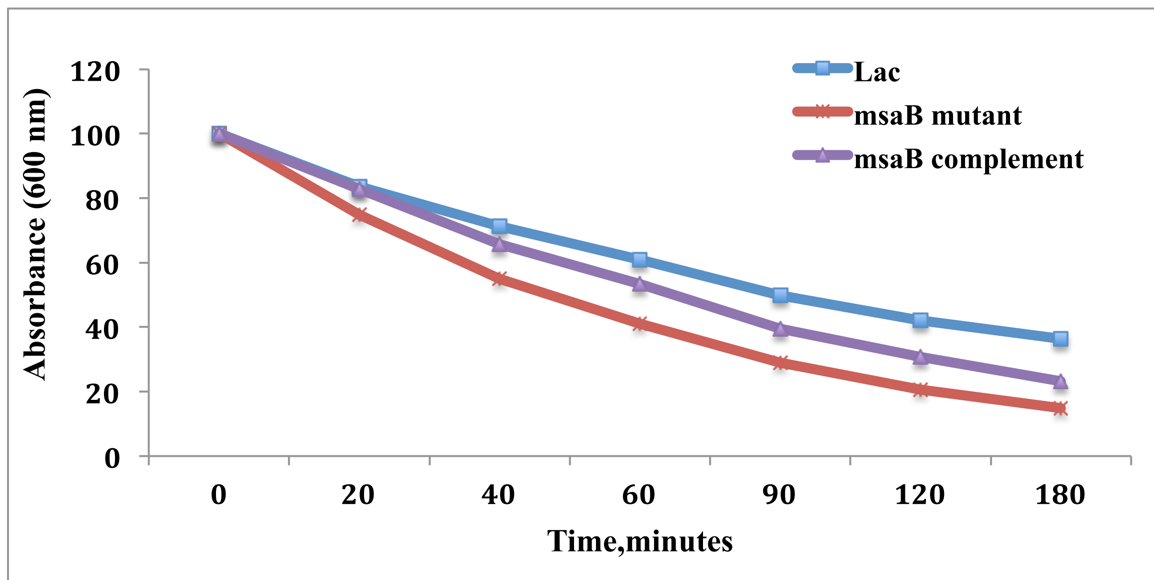

Figure 6. Phenotypic characterization of *msaB* deletion mutant and *msaB* mutant complement. Comparison of the *msaB* deletion mutants relative to complement mutant and wild type USA300_LAC. Phenotypic assay was done on Triton X-100 induced lytic assay. Values represent the average of three independent assays, each of which was done in triplicates. Results are reported as the mean ± S.E, and the standardized errors are represented as error bars.

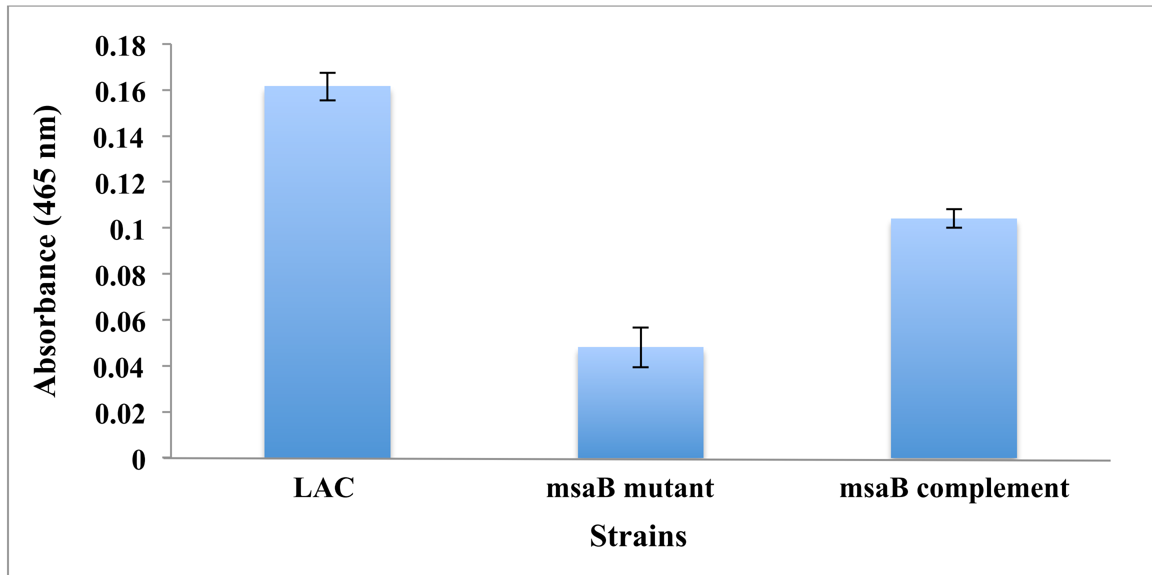

Figure 7. Phenotypic characterization of *msaB* deletion mutant and *msaB* mutant complement. Comparison of the *msaB* deletion mutants relative to complement mutant and wild type USA300_LAC. Phenotypic assay was done on pigmentation, and values represent the average of three independent assays, which were done in triplicates. Results are reported as the mean ± S.E, and the standardized errors are represented as error bars.

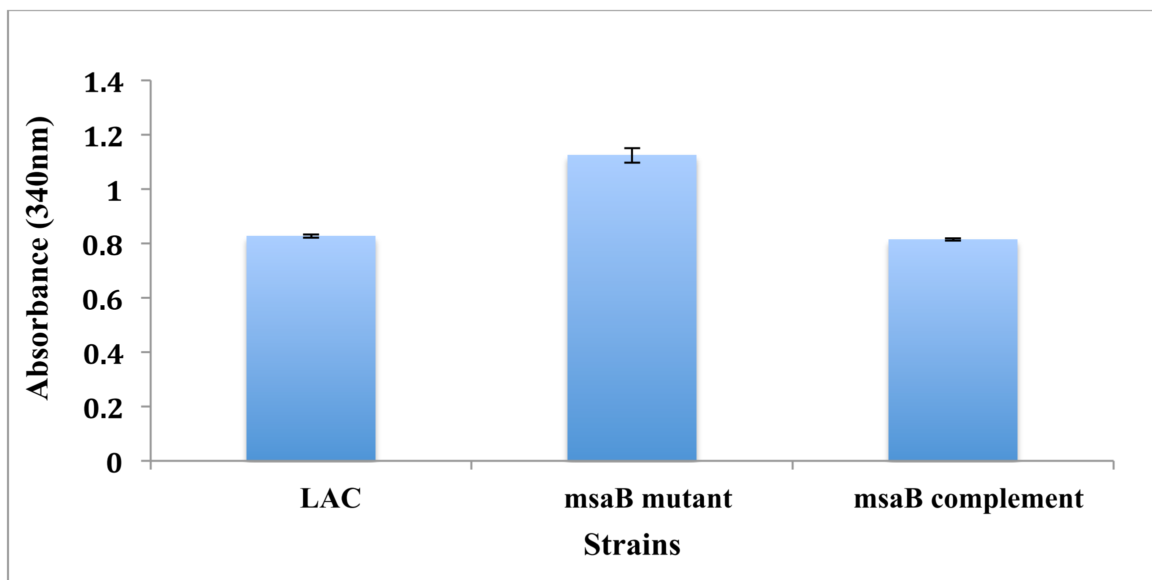

Figure 8. Phenotypic characterization of *msaB* deletion mutant and *msaB* mutant complement. Comparison of the *msaB* deletion mutants relative to complement mutant and wild type USA300_LAC. Phenotypic assay was done on extracellular protease activity, and values represent the average of three independent assays, which were done in triplicates. Results are reported as the mean ± S.E, and the standardized errors are represented as error bars.

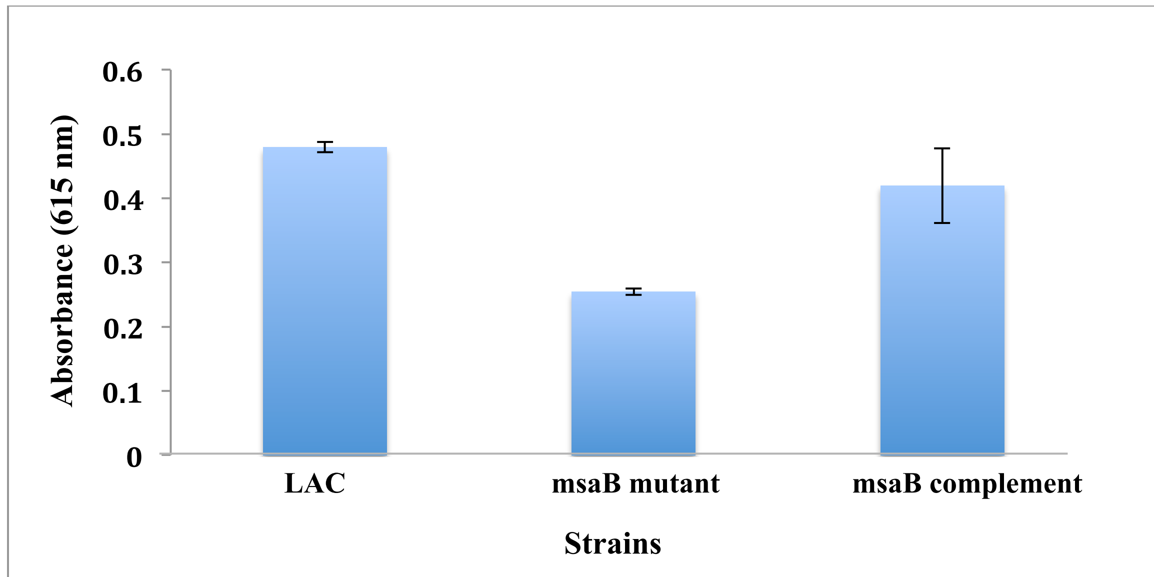

Figure 9. Phenotypic characterization of *msaB* deletion mutant and *msaB* mutant complement. Comparison of the *msaB* deletion mutants relative to complement mutant and wild type USA300_LAC. Phenotypic assay was done on biofilm formation, and values represent the average of three independent assays, which were done in triplicates. Results are reported as the mean ± S.E, and the standardized errors are represented as error bars.

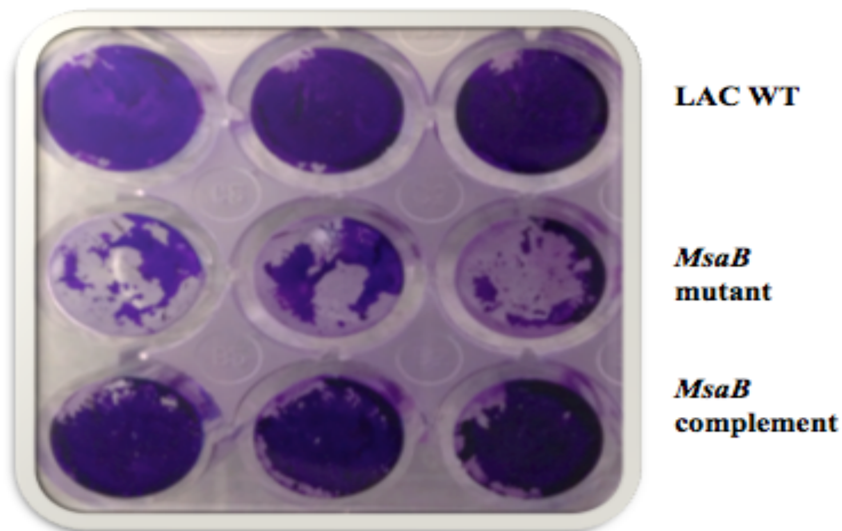

Figure 10. Biofilm formation in *msaB* mutant in microtiter plates. The wild type strain LAC (First row), the *msaB* mutant strain (Second row), and the complement *msaB* mutant strain (Third row) were grown in TSB supplement with 3% NaCl and 0.5% glucose, cultures were incubated for 48 hrs in the wells of microtiter plates with pre-coating with plasma proteins. The violet staining is an indication of biofilm formation in the wells.

Confocal Microscopy of Biofilm

We further analyzed the biofilm formation by the *msaB* deletion mutant by confocal microscopy after staining with live/dead staining with syto-9 and toto-3 and compared with WT LAC and its complement. We observed that *msaB* mutant biofilm is unable to form mature peculiar tower-like structure (white arrow head) and more aggregation of cells death compared to the LAC and complement (Figure 11). This suggests that increased cell death might be responsible for the biofilm defect in the *msaB* mutant, while the importance of other factors still needs to be tested.

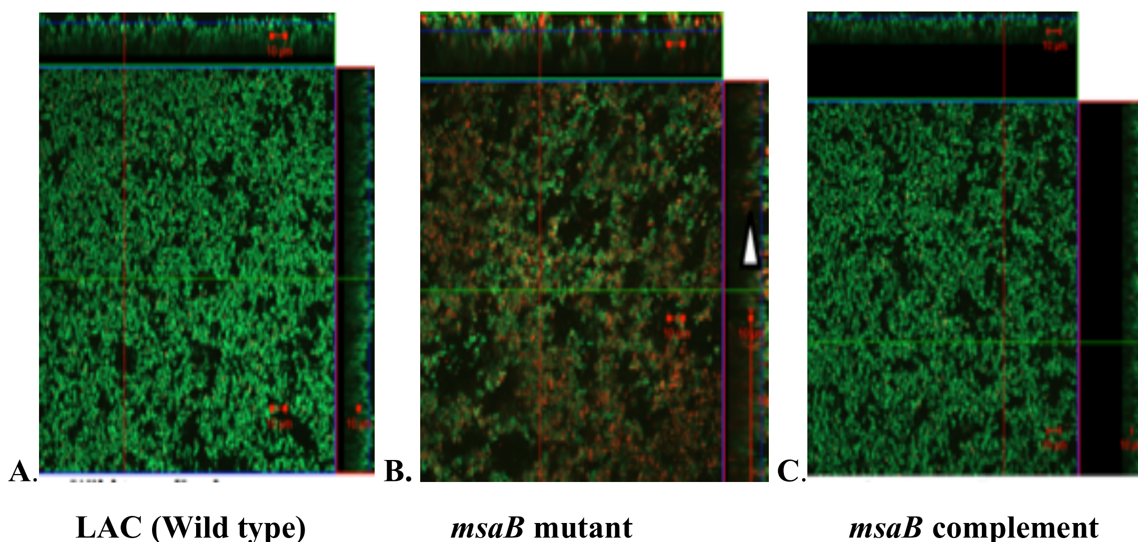

Figure 11. Confocal microscopy (Z-stack images) of static biofilm on microtiter plate. (A) LAC wild type, (B) *msaB* mutant, and (C) Complement *msaB* mutant were grown up to 48 hours and stained with Syto-8dye stain (staining live cells with green fluorescence stain) and Toto-3dye stain (staining dead cells and eDNA with red fluorescence stain). They stained under 40X /1.4 Oil DIC objectives, and these images are representative of three independent experiments with similar results with scale bar represents 10 µm.

COMSTAT Analysis of Confocal Microscopy of MsaB Deletion Mutant Biofilm

We performed COMSTAT analysis for the biofilm formation by the *msaB* deletion mutant and compared with the wild type and its complement. The analysis showed the presence of significantly less live cell biomass by the mutant, 40% compared to 100% by the wild type. The complement produced the cell biomass intermediate between the wild type and complement. Moreover, the mutant showed the presence of increased cell death (169%) compared to wild type (100%). This analysis suggests that increased number of cell death might be responsible for the defective biofilm formation by the *msaB* mutant. Further analysis by the COMSTAT also revealed that the mutant has significantly reduced biofilm thickness (12 μm) compared to wild type (24 um). All the results are shown in Figure 12.

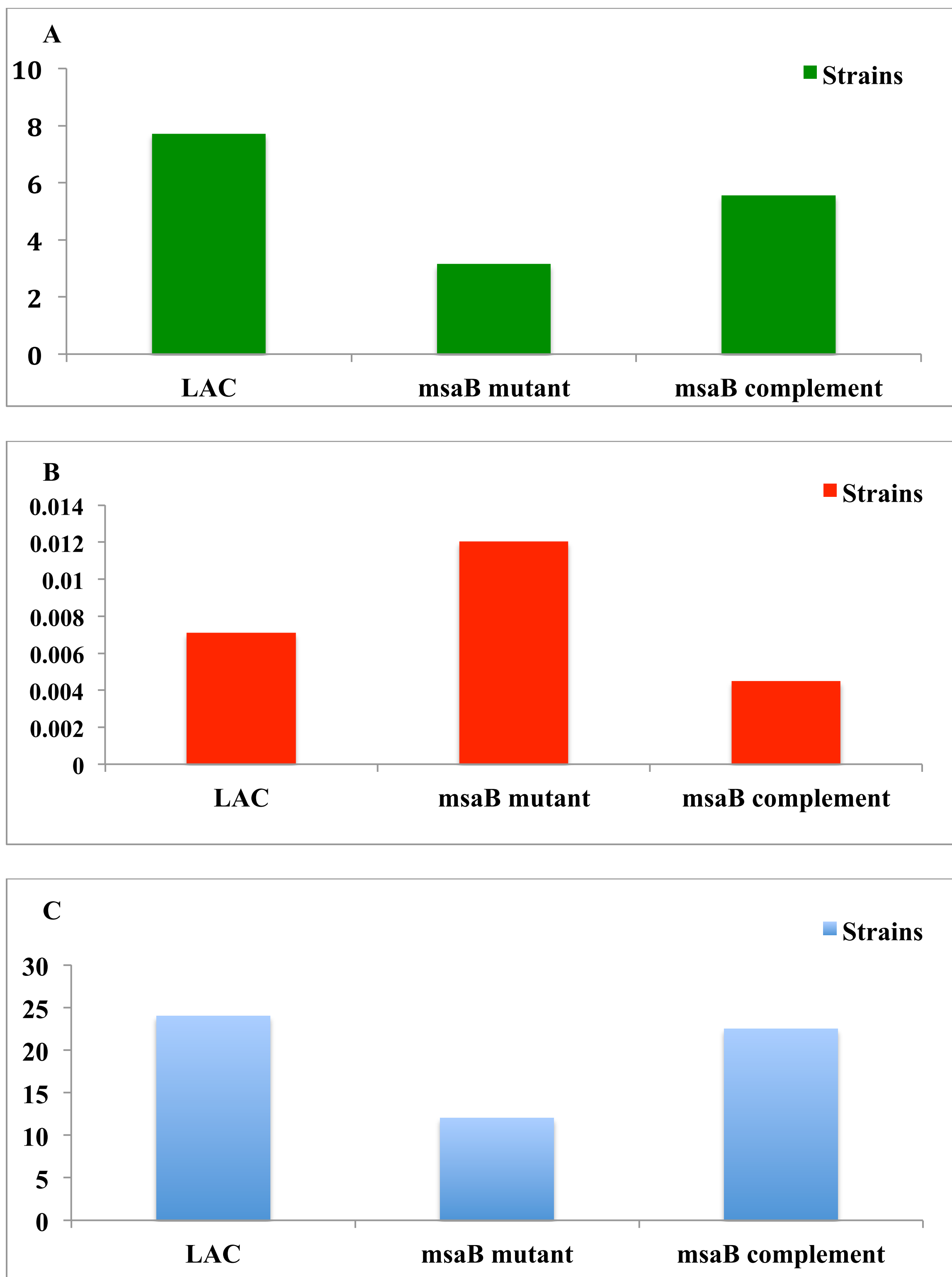

Figure 12. COMSTAT analysis of (A) Live cells, which appear as green columns (B), Dead cells, which appear as red columns, and (C) Biofilm thickness (appear as blue columns) of confocal microscopy of msaB deletion mutant biofilm.

Effect of Different Environmental Conditions on msaB Biofilm

We examined different environmental conditions that effect biofilm formation on *msaB* mutant and compared them with *msaB* complement and the wild type USA300_LAC. We first examined the effects of osmotic stress (NaCl), which was found to induce biofilm formation in biofilm positive strains when grown under stress-induced conditions [58]. So in order to test whether *msaB* gene was involved in transduction of the osmolarity signal for biofilm formation, we assayed biofilm formation by wild type (LAC), *msaB* and complement *msaB* in the presence of different NaCl concentrations. We found no significant difference in the biofilm formation by the *msaB* mutant in any of the concentration of NaCl tested compared to its wild type and complement (Figure 13). Furthermore, we also tested the effect of ethanol in combination with either NaCl or Glucose in TSB. As shown in Figure 10, the biofilm produced by the *msaB* deletion mutant is not significantly different when grown in TSB/ethanol (Figure 14 A) and TSB/ethanol/NaCl (Figure 14 C); however, interestingly *msaB* deletion mutant produced significantly increased biofilm in TSB/ethanol/glucose (Figure 14 B). We still need further investigation of why *msaB* mutant produces more biofilm in TSB/ethanol/glucose.

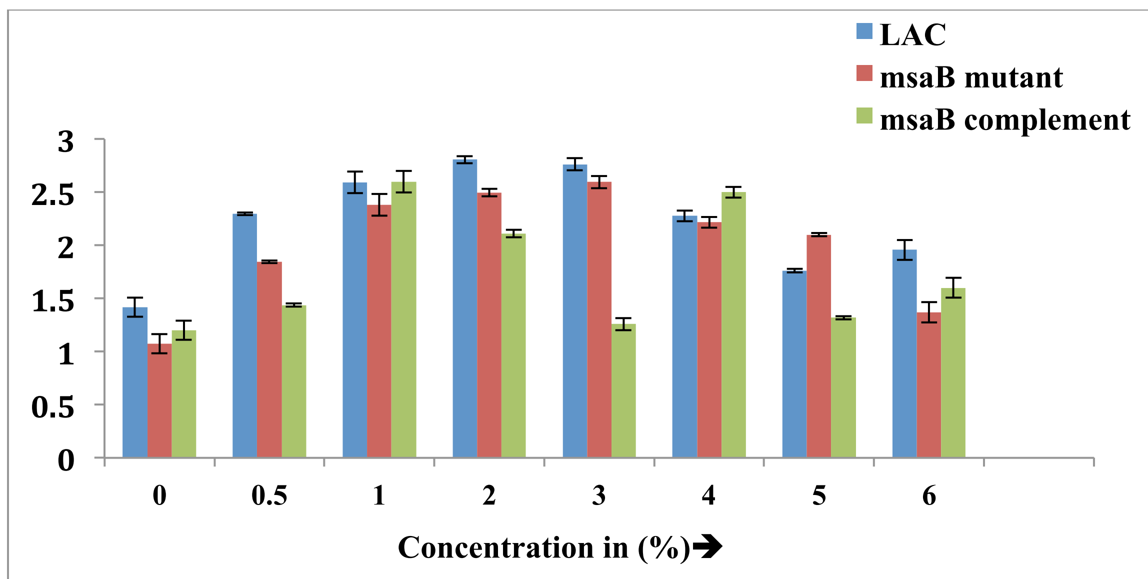

Figure 13. Effect of different sodium chloride concentration, 0%, 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, on biofilm development in *msaB mutant* strains compared with wild type (*LAC*) and its *msaB* complement; the standardized errors are represented as error bars.

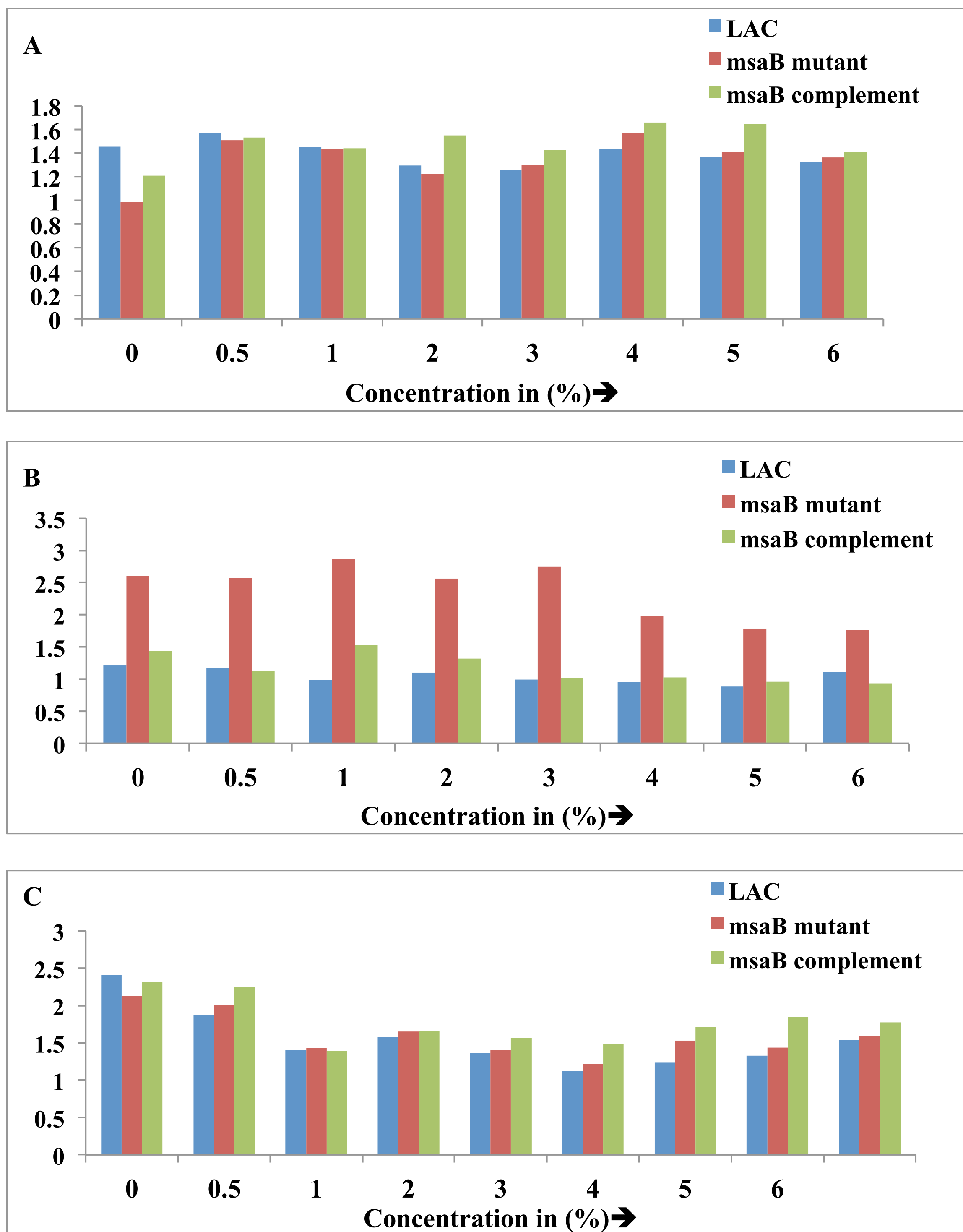

Figure 14. Effect of ethanol on biofilm formation, (A) shows different ethanol concentration, (B) shows different ethanol concentration with constant of 0.5% glucose concentration, and (C) shows different ethanol concentration with constant of 3% sodium chloride concentration, in *msaB* mutant strain, *msaB* complement in comparison to the USA300_LAC wild type.

Glucose has been shown to induce the multicellular aggregation step of biofilm formation, increases PIA production [59], and thus induces biofilm formation. We studied the effect of different glucose concentrations (0 to 4%) in the biofilm formation by *msaB* mutant. We observed defective biofilm formation by the *msaB* deletion mutant at low concentration of glucose. But as the concentration of glucose increased from 1% onwards, *msaB* restored the biofilm formation back to wild type level. We still need further explanation of and research on why *msaB* biofilm defect is glucose dependent, whereas the wild type and complement does not respond much to the change in glucose concentration (Figure 15).

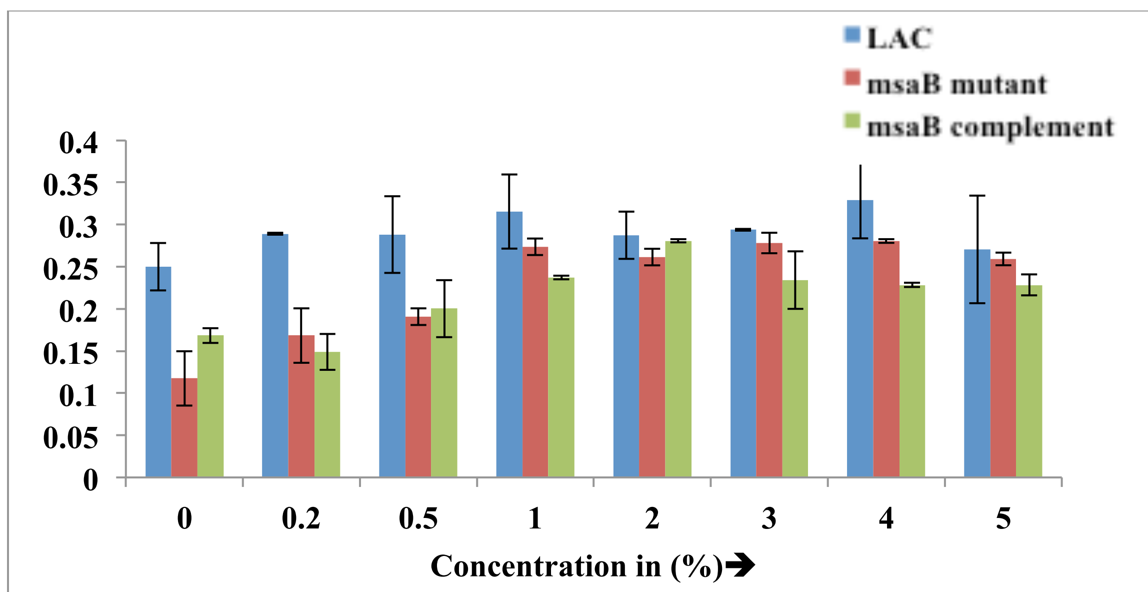

Figure 15. Effect of different glucose concentration, 0%, 0.2%, 0.5%, 1%, 2%, 3%, 4%, & 5%, on biofilm development in *msaB* mutant strain (Red column), *msaB* complement (Green column), and LAC wild type (Blue column); the standardized errors are represented as error bars.

Biofilm Detachment Assay Results

Sodium-metaperiodate (NaMP) affects the production of Poly-N-acetyl glucosamine (PNAG) also known as Polysaccharide intracellular adhesion (PIA). If

biofilm is disrupted by the addition of NaMP, then it is intracellular adhesion mediated PIA-depended biofilm and if the strain is resistant to the added NaMP, then the biofilm formation is PNAG independent [60]. We found that the *msaB* mutant and its wild type and complement strains were resistant to NaMP and does not affect the biofilm formation in the presence of NaMP (Figure 16).

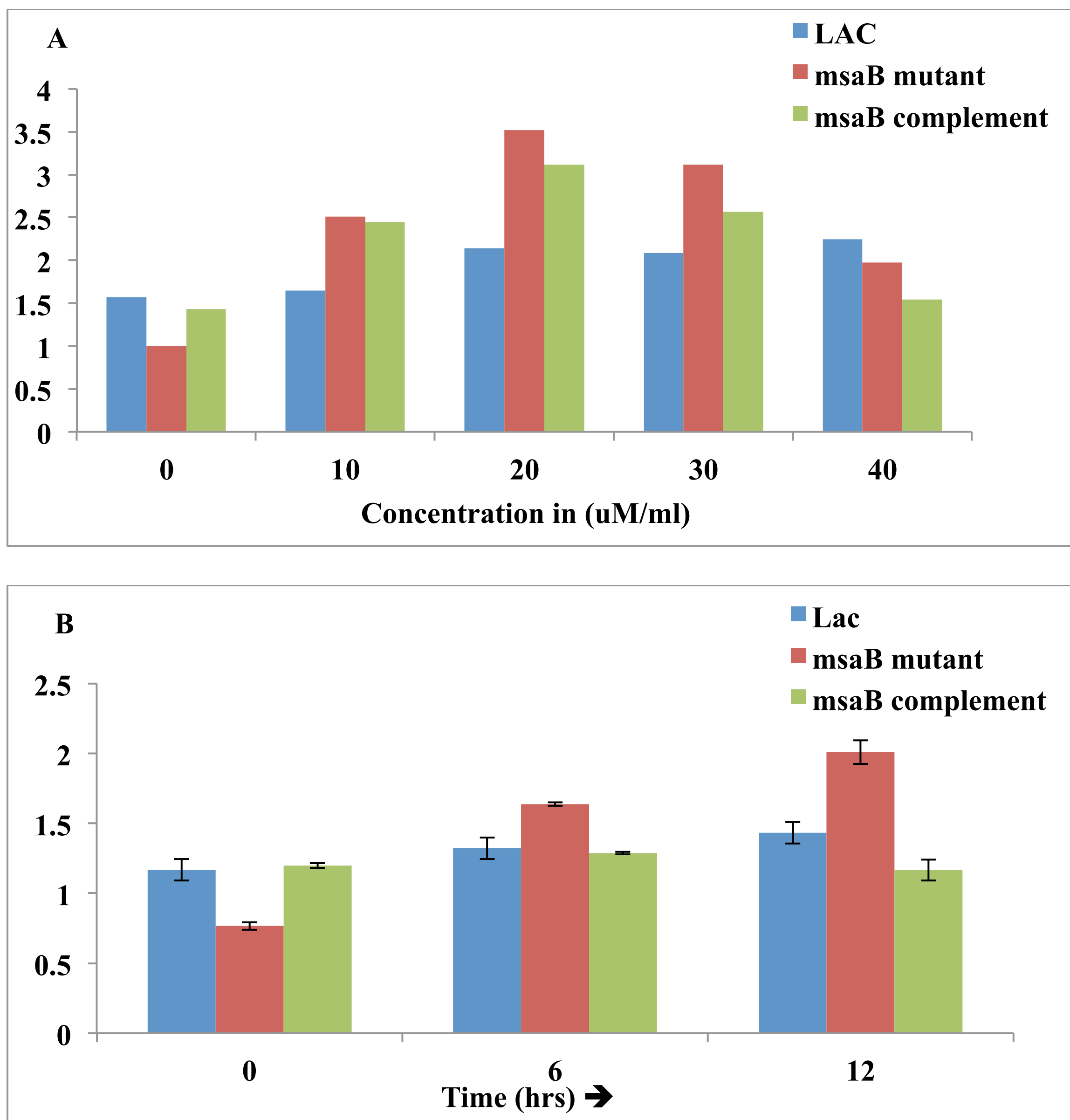

Figure 16. Effect of sodium metaperiodate on biofilm formation in *msaB* mutant, *msaB* mutant complement, and wild type strains at different NaMP concentrations with 24 hrs incubation (A), and at only one concentration of 20uM/ml, with different times intervals; at 0 hour, 6 hrs, and 12 hrs (B); the standardized errors are represented as error bars.

Biofilms were exposed to DNase I (2U per well) at 6 hrs, and at 48 hrs of biofilm development, the wells were washed and stained with crystal violet and the amount of biofilm remaining was quantified at 595 nm. The addition of DNase I reduced the biofilms in both wild and *msaB* mutant, thus indicating the importance of eDNA in the

biofilm formation (Figure 17). Since there is no significant difference between the wild type and the *msaB* deletion mutant, results of this study suggest that the eDNA is an important component to the composition of biofilm; however, it is not responsible for *msaB* mutant's biofilm defect.

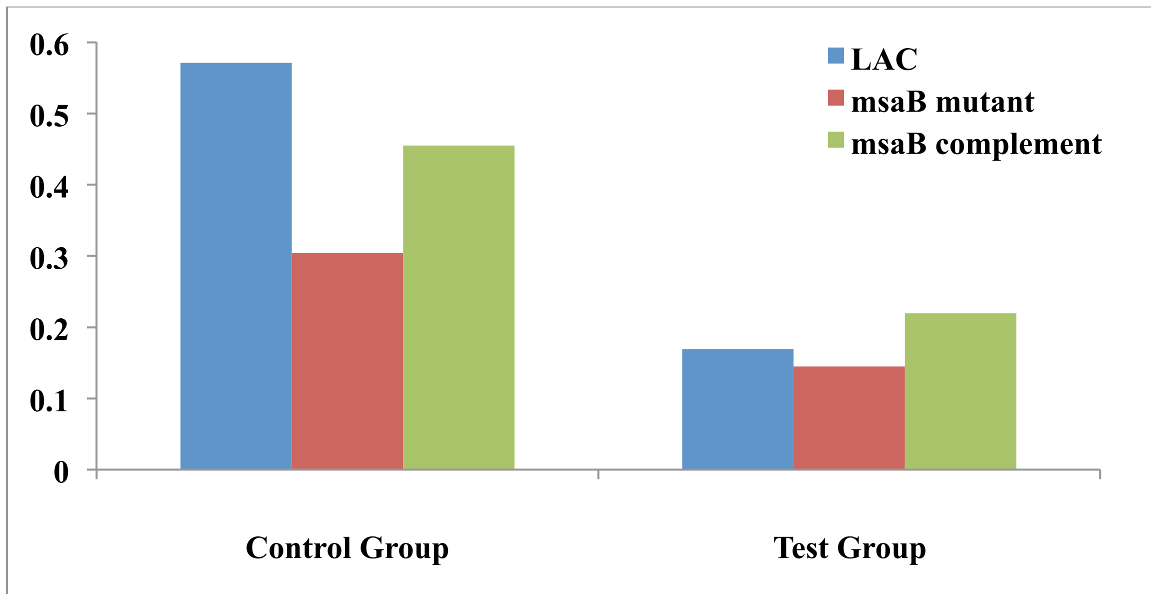

Figure 17. Effect of DNaseI on biofilm formation in *msaB* mutant strain (Red bar), compared to the LAC wild type (Blue bar) and *msaB* complement (Green bar) strains. It shows decreased both wild and mutant strains after added DNase I (Test Group) compared to control group (no DNase I was added).

Addition of Proteinase K at any time point disrupted the biofilms in both wild and *msaB* mutant strains, so we can concluded that both the wild type (LAC) and *msaB* mutant strains were sensitive to DNaseI and proteinase K, and the protein and DNase I are important in biofilm structure (Figure 18).

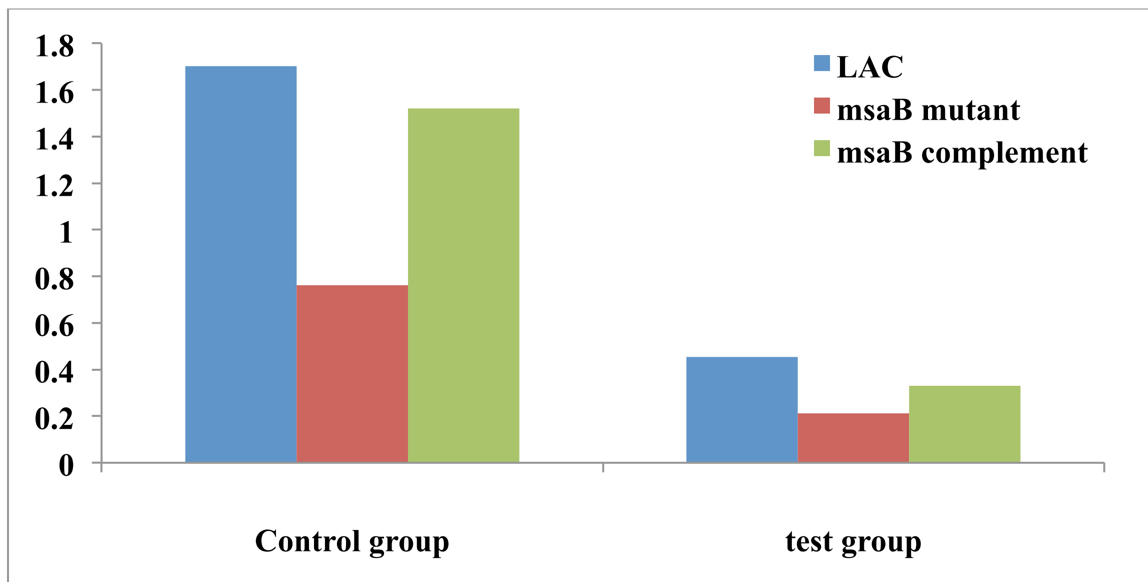

Figure 18. Effect of Proteinase K on biofilm formation in *msaB* mutant strain (Red bar), compared to the LAC wild type (Blue bar) and *msaB* complement (Green bar) strains. It shows decreased both wild and mutant strains after added proteinase k enzyme (Test Group) compared to control group (no proteinase K was added).

Discussion

For hundreds of years, scientists and dentists have been looking for efficient methods to control oral diseases, since the oral ecosystem view has become more widely accepted and oral bacterial interactions have been more elucidated [61]. Most infections of the oral cavity, including the major dental diseases, caries and periodontitis, are opportunistic in nature [62]. They are caused or maintained by microorganisms of the resident or transient flora normally present in low numbers and not pathogenic, but in certain circumstances develop infections.

Mucosal infections have some degree of specificity (e.g., *Candida spp*., *Staphylococcus aureus*, and enterics), and a microbiological test can be interpreted accurately for clinical diagnosis and choice of treatment. Sub-epithelial or deep infection include a number of species from the resident flora, mainly anaerobes whose role in the infections is difficult to interpret; however, microbiological tests for the presence of certain bacterial species could be used for treatment control, risk-evaluation, and even for patient motivation in the prevention of these diseases. Microbiological diagnosis can be used in general practice for several purposes and in various situations that can be of great value for the dental patient [63].

The occurrence of *staphylococci* in the oral cavity was examined using saliva and supra-gingival plaque specimens from 56 systemically and periodentally healthy adults aged 22-43 years old. Nine *staphylococcus* species and 334 isolates were identified, and *S. aureus* was the most frequent species (46.4%), followed by *staphylococcus epidermidis* (41.1%) and other species [64].

The ability of *Staphylococcus spp* and *Candida spp* to form a biofilm and live within certain niches allows them to develop mechanisms that increase persistence, such as the evasion of host defenses and antibiotic efficacy [12]. These microorganisms can easily be or become resistant to antibiotics and lead to super infection. While the diversity of organisms present in the oral cavity is well accepted, there remains considerable controversy as to whether *Staphylococcus* spp. play a role in the ecology of the normal oral flora [65].

Surprisingly, little detailed work has been done on the qualitative and quantitative aspects of colonization or infection either by coagulase-negative *staphylococci* (CNS) or

S. aureus [65]. The *S. aureus* is especially interesting in light of the present difficulty in eradicating carriers of methicillin-resistant *S. aureus* (MRSA) from the oropharynx of the affected individuals. Information on the risk of methicillin-resistant *Staphylococcus aureus* (MRSA) infection transmission in dental healthcare settings was incomplete only a few years ago; therefore, MRSA infection control guidelines were necessarily based on data extrapolated from other fields. Recently, publication of specific studies has made it possible to review such risk [66].

In this study we studied the role of the *msaB* gene, which is a part of *msaABCR* operon that regulates biofilm development and other virulence factors in *S. aureus*. One of the interesting findings is the important role of *msaB* in biofilm formation in the *S. aureus* stains. Identification of the *msaB* will add insight into the complex network of virulence regulation in *S. aureus*.

Despite the identification of numerous regulatory elements in *S. aureus*, it is still unclear how this organism achieves the coordinated expression of virulence factors in the host. It has been suggested that the unique regulation pattern of toxins and the biofilm formation in these strains are primarily responsible for their increased virulence and epidemic spread. Biofilm infections are extremely challenging to treat and the presence of biofilms causes numerous problems in the field of medicine, interfering with clinical therapeutics.

The environmental factors, such as glucose, osmolarity, ethanol, temperature, and anaerobiosis, have been reported to affect biofilm formation [67]. Glucose has been shown to induce the multicellular aggregation step of biofilm formation [68]. Osmotic stress was found to induce biofilm formation [58]. Biofilm positive strains increased

when grown under stress-induced conditions (NaCl, EthOH, sub-inhibitory levels of tetracycline). However, induction of biofilm by NaCl is dependent on the concentration of NaCl used [69]. Ethanol was shown to induce biofilm formation through icaR, which represses the icaADBC genes [70]. IcaR was recently shown to bind to the promoter region upstream of the icaA gene [71]. In contrast the effects of glucose and NaCl are not mediated through icaR [70, 72]. Depending on the strain, ethanol at various concentrations either positively or negatively regulates biofilm formation.

One of the key phenotypes of the *msaB* mutant is the decreased formation of the biofilm formation. Indeed, there are several independent regulators and environmental stimuli which contribute to establish sessile communities of *S. aureus* [73], although the complete picture is still unclear and under investigation. COMSTAT analysis of confocal microscopy of the biofilm formation by the *msaB* deletion mutant showed the presence of significantly less live cell biomass by the mutant, 40% compared to 100% by the wild type. The complement produced the cell biomass intermediate between the wild type and complement. Moreover, the mutant showed the presence of increased cell death (169%) compared to wild type (100%). This analysis suggests that increased number of cell death might be responsible for the defective biofilm formation by the *msaB* mutant. Further analysis by the COMSTAT also revealed that the mutant has significantly reduced biofilm thickness (12 μm) compared to wild type (24 um).

In this study we have shown that *msaB* acts as a regulator of several genes that are involved in protease production, virulence, and biofilm development. The *msaB* gene encodes a 66-amino acid polypeptide that showed homology with cold-shock proteins of *E*. *coli* (*CspA*, 60%) and Bacillus subtilis (*CspB*, 76%). Based on sequence homology*, S.*

aureus produces three proteins (*CspA*, *CspB*, and *CspC*) that may be associated with cold-shock stress [74, 75], however, Anderson et al., 2006 [76] showed that only *CspB* responds to cold shock in *S. aureus.* This was confirmed by proteomic studies that showed increased expression of *CspB* under cold shock, while *CspA* was not differentially expressed.

Studies have also shown that the *CspA* transcript is more abundant than *CspB* and *CspC* under normal growth conditions at 37°C, while the *CspB* predominates at 15°C [76]. In addition, collective studies also have shown that the *CspA* is an important gene for the cationic antimicrobial peptide of human lysosomal cathepsin G, and regulates pigmentation in *S. aureus* through a *sigB*-dependent mechanism [74, 77]. This indicates that *CspA* has biological functions other than the cold-shock response. In *E. coli*, the *CspA* mRNA is a thermo sensor that modulates translation of the cold-shock protein (*CspA*). The *CspA* mRNA in *E. coli* undergoes post-transcriptional modification in response to environmental variations, such as a temperature shift from 37°C to 10°C [78, 79]. This RNA-dependent regulation of gene expression allows *E. coli* to rapidly adapt and respond to its environment.

Further studies have shown that the *CspA* gene in *E. coli* produces a single-stranded nucleic acid-binding protein and an RNA chaperone. This protein is one of the most abundant proteins during early growth phase, and its expression is even higher during cold shock, accounting for 2% of the total proteins in the cell [80, 81]. Since *msaB* (*CspA*) is not directly involved in cold shock in *S. aureus*, it is not clear if it has maintained the same mechanism of regulation or functions as its homolog in *E. coli*. With an increase in the number of *S. aureus* infections, clinicians may encounter additional

complications such as antibiotics-resistance; therefore, understanding the virulence mechanisms of this microorganism is warranted to establish adequate diagnosis and provide proper treatment modalities.

Furthermore, we examined different environmental conditions that effect biofilm formation on *msaB* mutant and compared them with *msaB* complement and the wild type USA300_LAC. We first examined the effects of osmotic stress (NaCl), which was found to induce biofilm formation in biofilm positive strains when grown under stress-induced conditions [58]. We found that in the presence of different NaCl concentrations there is no significant difference in the biofilm formation by the *msaB* mutant in any of the concentrations of NaCl tested compared to its wild type and complement (Figure 13). We also tested the effect of ethanol in combination with either NaCl or glucose in TSB. As shown in Figure 14, the biofilm produced by the *msaB* deletion mutant is not significantly different when grown in TSB/ethanol (Figure 14 A) and TSB/ethanol/NaCl (Figure 14 C); however, interestingly *msaB* deletion mutant produced significantly increased biofilm in TSB/ethanol/glucose (Figure 14 B). Further investigation is needed to understand why *msaB* mutant produces more biofilm in TSB/ethanol/glucose.

Glucose has been shown to induce the multicellular aggregation step of biofilm formation, which increases PIA production [59], and thus induces biofilm formation. We found the defective biofilm formation by the *msaB* deletion mutant at low concentration of glucose, whereas, as the concentration of glucose increases above 1%, the mutant phenotype is restored in terms of biofilm formation back to wild type level. We still need further explanation of and research on why *msaB* biofilm defect is glucose dependent,

whereas the wild type and complement does not respond much to the change in glucose concentration (Figure 15).

Conclusion

In conclusion, we defined the role of *msaB* gene, a part of a four-gene operon, *msaABCR,* that regulates virulence and biofilm development in *S. aureus*. We studied the role of *msaB* gene in regulating important global regulators of virulence genes like *sarA* and *agr*. We also studied its role in pathogenesis in terms of protease activity, autolysis, pigmentation, and biofilm formation. The *msaB* gene showed decreased pigmentation, increased protease activity, increased cell death, and reduced biofilm formation.

These findings suggest that *msaB* is also an important gene in the *msaABCR* operon that regulates other global regulators and virulence in *S. aureus*. Further research is needed to address the mechanism of regulation of this operon and its interaction with its target genes.

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