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Role of *MSA* in Immune Evasion, Persistence, and Protease Regulation in the Human Pathogenic Strains of *Staphylococcus aureus*

Maria Deepa Basco
University of Southern Mississippi

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The University of Southern Mississippi

ROLE OF *MSA* IN IMMUNE EVASION, PERSISTENCE AND PROTEASE

REGULATION IN THE HUMAN PATHOGENIC STRAINS OF

STAPHYLOCOCCUS AUREUS

by

Maria Deepa Shanthini Basco

Abstract of a Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

August 2013

ABSTRACT

ROLE OF *MSA* IN IMMUNE EVASION, PERSISTENCE AND PROTEASE REGULATION IN THE HUMAN PATHOGENIC STRAINS OF *STAPHYLOCOCCUS AUREUS*

by Maria Deepa Shanthini Basco

August 2013

Opportunistic pathogens like *Staphylococcus aureus* on entering the host can stay colonized at the foci of infection or evade the immune system to disseminate to other sites. In this study we investigated the regulatory influence of the modulator of *sarA* (*msa*) on immune evasion and host persistence, employing the hospital-acquired strain *S. aureus* UAMS-1 and community-acquired strain *S. aureus* USA300 LAC. In the murine sepsis model, mutation of the *msa* gene in LAC showed no change in dissemination of infection; however, in UAMS-1 a decrease in microbial load was observed in the lungs. Differential regulation by the *msa* gene was also observed in the blood survival and neutrophil assays. Several evasion factors were found to be regulated by *msa*, namely the *scn*, *clfA*, *spa*, *aur*, and *sak* genes. Interestingly, the combination of factors and the regulation of these factors differed in the two strains.

S. aureus form biofilms on post-surgical wounds, prosthetic devices, and various host tissues that are resilient to immunological clearance and antimicrobial treatments. Biofilm detachment is a stage of biofilm development that aids in metastasis of infection. Proteases are one of the factors that trigger biofilm detachment. In our study, we observed *msa* to regulate proteases of *S. aureus* strain LAC when they are not in the form of a biofilm community; however, when they form biofilms the regulatory effect on

proteases by the *msa* gene is absent. Thus, we show the environment-dependent behavior of the *msa* gene.

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Approved:

Mohamed O. Elasri
Director

Gordon Cannon

Glen Shearer

Timothy McLean

Vijay Rangachari

Susan A. Siltanen
Dean of the Graduate School

August 2013

DEDICATION

I dedicate this dissertation to my parents, J. Louisa and Jonas Basco, and my sisters, Merlyn Jyothi and Angelina Satya Priya, for the continuous prayers, encouragement, and support.

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TABLE OF CONTENTS

ABSTRACT	ii
DEDICATION	vi
ACKNOWLEDGMENTS	vi
LIST OF TABLES	xii
LIST OF ILLUSTRATIONS	xiii
CHAPTER	
I. INTRODUCTION	1
<i>Staphylococcus aureus</i>	
Virulence and Immune Evasion Factors	
Global Regulation	
Pathogenesis	
Methicillin Resistant <i>Staphylococcus aureus</i> (MRSA)	
Hospital-Acquired <i>Staphylococcus aureus</i>	
Community-Acquired <i>Staphylococcus aureus</i>	
II. BACKGROUND	23
The <i>msa</i> Gene	
Strain <i>S. aureus</i> UAMS-1	
Strain <i>S. aureus</i> USA300 – 0114 LAC	
Comparison of <i>S. aureus</i> community acquired isolate USA300 LAC vs hospital acquired isolate UAMS-1	
III. DELETION OF THE <i>MSA</i> GENE	27
Introduction	
Materials and Methods	
Results	
Discussion	
IV. <i>MSA</i> REGULATES IMMUNE EVASION, HOST PERSISTENCE AND DISSEMINATION IN THE CLINICAL ISOLATES OF <i>STAPHYLOCOCCUS AUREUS</i>	39

	Abstract	
	Introduction	
	Materials and Methods	
	Results	
	Discussion	
V	REGULATION OF PROTEASES BY <i>MSA</i> IN BIOFILM AND NON-BIOFILM CONDITIONS	74
	Abstract	
	Introduction	
	Methods and Materials	
	Results	
	Discussion	
	APPENDIX	84
	REFERENCES	85

LIST OF TABLES

Table

1.	Examples of MSCRAMMS	5
2.	Cytotoxins produced by <i>S. aureus</i>	7
3.	Immune evading factors produced by <i>S. aureus</i>	8
4.	Regulation of virulence genes by <i>agr</i>	13
5.	Some of the sites of infections caused by <i>S. aureus</i>	18
6.	Genes previously known to be regulated by <i>msa</i>	23
7.	Comparison of <i>S. aureus</i> community acquired isolate USA300 LAC vs hospital acquired isolate UAMS-1	25
8.	Primers used for isolating the flanking regions of the <i>msa</i> gene	30
9.	Primers used for RT-qPCR	46
10.	Statistical Significance by Mann Whitney test of UAMS-1 strains in the sepsis model	49
11.	Statistical Significance by Mann Whitney test of UAMS-1 strains in the sepsis model	51
12.	Relative fold change in the expression of the genes required for phagocytic evasion on deleting the <i>msa</i> gene	60
13.	Primers for RT- qPCR	77
14.	Relative Expression of proteases	80
15.	Relative Expression of proteases in 50 hr mature biofilm	81
16.	Relative Expression of proteases in detached cells from 50 hr mature biofilm	81

LIST OF ILLUSTRATIONS

Figure

1.	Quadrant streak of <i>Staphylococcus aureus</i> colonies showing the typical golden yellow colonies	2
2.	Gram staining of <i>Staphylococcus aureus</i> showing grape-like clusters of staphylococcal cells	2
3.	Some of the infections caused by <i>S. aureus</i> causing (A) a cutaneous abscess1 of <i>S. aureus</i> , (B) an osteomyelitis infection caused by <i>S. aureus</i> , and (C) an infection of a of subclavian catheter	4
4.	MSCRAMMs ((Microbial Surface Components Recognizing Adhesive Matrix Molecules) of <i>S. aureus</i>	6
5.	Schematic representation of the process of coagulation by <i>S. aureus</i>	8
6.	The <i>agr</i> quorum system of <i>S. aureus</i>	11
7.	The <i>sarA</i> system and the influence of SarA on other genes	15
8.	Pathogenesis model of <i>S. aureus</i>	17
9.	Schematic representation of the temperature sensitive pKOR1 vector employed to create deletion mutants of <i>S. aureus</i>	28
10.	Schematic representation of the insert preparation containing the Upstream and Downstream regions of <i>msa</i> gene to be inserted into the pKOR1 vector.....	28
11.	Schematic representation of the expected <i>msa</i> mutation	31
12.	PCR with <i>msa</i> primers to verify the deletion of the <i>msa</i> gene in the <i>S. aureus</i> strains of LAC, UAMS-1, RN6390 and COL	33
13.	PCR with primers specific for the ACME region (unique for USA300 LAC strains) to confirm the genetic background of <i>S. aureus</i> LAC strains	33
14.	Verification by PCR of <i>S. aureus</i> UAMS-1 (unique for UAMS-1 strain) by the presence of the <i>cna</i> gene of <i>S. aureus</i> UAMS-1 strains	34
15.	Tetracycline test with <i>S. aureus</i> COL strains confirming the strains of <i>S. aureus</i> COL strains	34

16.	Growth Curve of <i>S. aureus</i> UAMS-1 strains showing that the deletion of the <i>msa</i> gene of <i>S. aureus</i> UAMS-1 does not influence the viability of <i>S. aureus</i> UAMS-1	35
17.	Growth Curve of <i>S. aureus</i> LAC strain showing that the deletion of the <i>msa</i> gene of <i>S. aureus</i> UAMS-1 does not influence the viability of <i>S. aureus</i> UAMS-1	35
18.	Biofilm Assay of <i>S. aureus</i> COL strains showing the weak biofilm formation in the <i>msa</i> mutants	36
19.	Protease Activity of <i>S. aureus</i> COL strains showing increased protease activity in the <i>msa</i> mutants compared to the wild type strains	37
20.	Hemolytic Assay of <i>S. aureus</i> strains of COL showing reduced hemolytic activity on deleting the <i>msa</i> gene	37
21.	Bacterial load of wild type and mutant strains of <i>S. aureus</i> LAC strains in the various organs of the murine sepsis model	48
22.	Bacterial load of wild type and mutant strains of <i>S. aureus</i> UAMS-1 strains in the various organs of the murine sepsis model	50
23.	Increased percentage survival of the <i>msa</i> mutants of <i>S. aureus</i> LAC strains in whole human gender-pooled blood compared to the wild type and complement strains	51
24.	Decreased percentage survival of the <i>msa</i> mutants of <i>S. aureus</i> UAMS-1 strains in whole human gender-pooled blood compared to the wild type and complement strains	51
25.	Growth Curve of UAMS-1 strains showing no effect on the growth of UAMS-1 strains on deleting the <i>msa</i> gene	52
26.	Growth Curve of LAC strains showing no effect on the growth of LAC strains on deleting the <i>msa</i> gene	52
27.	Viability Assay of the strains <i>S. aureus</i> LAC and UAMS-1 showing no change change in viability on deleting the <i>msa</i> gene	53
28.	Percentage of PMN migrating in response to the supernatant of the strains of LAC and UAMS-1	54

29a.	Scanning Electron Microscopy of spherical neutrophils in the absence of <i>S. aureus</i> (control)	55
29b.	Scanning Electron Microscopy of neutrophils showing no change in shape in the presence of <i>S. aureus</i> LAC strains	55
29c.	Scanning Electron Microscopy of neutrophils comparing the extent of branching of neutrophils in the presence of UAMS-1 strains	56
30.	TEM image showing the intracellular presence of <i>S. aureus</i> (A) LAC strains and (B) UAMS-1 in human neutrophils	57
31.	Phagocytosis Survival Assay of <i>S. aureus</i> LAC strains showing no difference in intracellular survival on deleting the <i>msa</i> gene	57
32.	Phagocytosis Survival Assay of <i>S. aureus</i> UAMS-1 strains showing a decrease in intracellular survival on deleting the <i>msa</i> gene	57
33.	Detection of <i>sea</i> , <i>sak</i> , <i>chp</i> and <i>scn</i> genes in <i>S. aureus</i> LAC wild type (1), LAC Δ <i>msa</i> (2), LAC complement (3), UAMS-1 (4), UAMS-1 Δ <i>msa</i> (5) and UAMS-1 Comp (6)	58
34.	Quantification of ClfA levels based on the ability to bind to fibrinogen revealed reduced ClfA levels in the mutants of <i>S. aureus</i> LAC	60
35.	Quantification of ClfA levels based on the ability to bind to fibrinogen revealed reduced ClfA levels in the mutants of <i>S. aureus</i> UAMS-1	61
36.	Western Blot with anti-Spa antibodies showing no difference in Spa levels between the wild type and mutant strains of LAC and UAMS-1	61
37.	Hemolytic activity showing no difference between the mutant and wild type strains of <i>S. aureus</i> LAC	62
38.	Protease activity of <i>S. aureus</i> LAC strains using azocasein as a substrate showed increased protease activity on deleting the <i>msa</i> gene in <i>S. aureus</i> LAC strains	63
39.	Protease activity of <i>S. aureus</i> UAMS-1 strains using azocasein as a substrate showed increased protease activity on deleting the <i>msa</i> gene in <i>S. aureus</i> UAMS-1 strains	64
40.	Mutation of <i>msa</i> in LAC did not influence susceptibility of <i>S. aureus</i> LAC strains to HNP-1	65

41.	Mutation of <i>msa</i> in UAMS-1 did not influence susceptibility of <i>S. aureus</i> UAMS-1 strains to HNP-1	65
42.	Schematic representation of the four levels of phagocytic evasion by <i>S. aureus</i>	71
43.	Stages of biofilm development of <i>S. aureus</i>	75
44.	Flow cell set up employed for <i>in vitro</i> growth of biofilms of <i>S. aureus</i>	78
45.	Biofilm Detachment Assay showing no difference in biofilm detachment on deleting the <i>msa</i> gene from <i>S. aureus</i> LAC strains	79
46.	Protease activity of non-biofilm cells of <i>S. aureus</i> LAC strains using azocasein	80
47.	Protease Activity of the biofilm effluents of LAC strains of <i>S. aureus</i>	81

CHAPTER I

INTRODUCTION

*Staphylococcus aureus**Taxonomic Classification*

Domain: Bacteria

Kingdom: Eubacteria

Phylum: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Staphylococcaceae

Genus: *Staphylococcus*

Species: *aureus*

Nomenclature

Staphylococcus, discovered back in the 1880s by Alexander Ogston while studying the cause of pus-formation (Ogston, 1880), is currently one of the most successful gram positive pathogens. Ogston investigated stained smears obtained from an abscess under a microscope and observed chains and groups of spherical organisms. He conducted experiments with the pus obtained from abscesses on guinea pigs and mice, and confirmed the pus-forming abscesses were caused by the spherical organisms (Ogston, 1880). In 1881, Ogston published a report describing *Staphylococcus* (then referred to as *Micrococcus*) to cause septicemia as well (Ogston, 1881). In 1882, Ogston renamed the *Micrococci* as *Staphylococci*, deriving its prefix from the greek word *staphyle* which meant bunch of grapes. Owing to a golden pigmentation which was

unique to a group of *Staphylococci*, Anton J. Rosenbach in 1884 named them *Staphylococcus aureus* (*aurum* in Latin meant golden) (Rosenbach, 1884) (Figure 1).

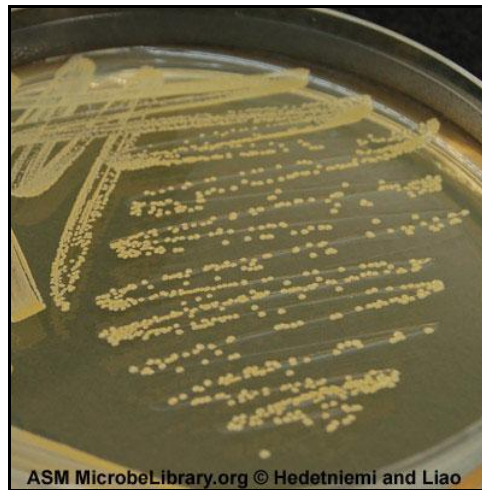


Figure 1. Quadrant streak of *Staphylococcus aureus* colonies showing the typical golden yellow colonies (Hedetniemi & Liao, 2010).

Staphylococci

Staphylococci grow to an average diameter of 0.6–1.2 μm and are non-spore forming and non-motile. They are facultative anaerobes capable of growing in an aerobic and anaerobic environment. *Staphylococci* are highly adaptable microbes, capable of growing in a pH range of 5 to 9. They are also resistant to desiccation and high salt concentrations (Graham & Wilkinson, 1992).

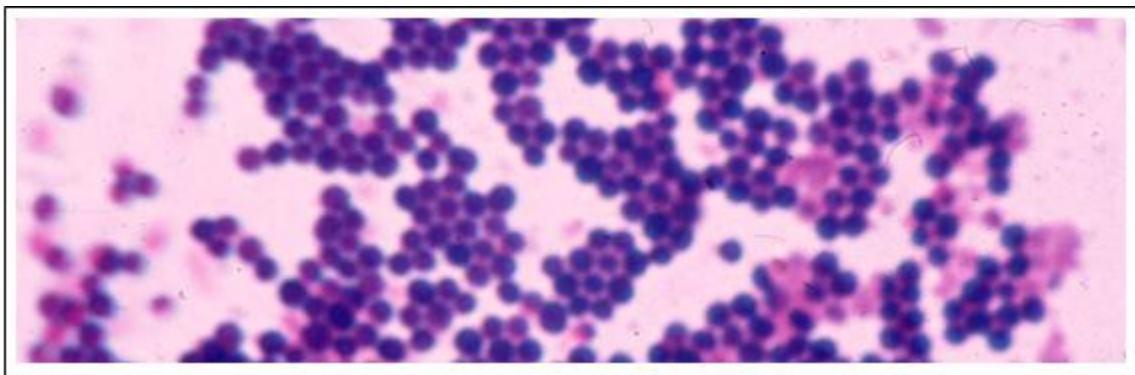


Figure 2. Gram staining of *Staphylococcus aureus* showing grape-like clusters of staphylococcal cells (Wistreich, 2011).

The genus of *Staphylococcus* contains mostly pathogens. *S. epidermidis*, *S. saprophyticus*, *S. lugdunensis*, *S. haemolyticus*, *S. warneri*, *S. schleiferi*, and *S. intermedius* are some of pathogenic members of this genus. Based on the ability to produce coagulase, bacteria belonging to the genus *Staphylococcus* are differentiated into coagulase-positive and coagulase-negative staphylococci. *S. aureus* is one of the major pathogens that belong to the coagulase-positive staphylococci. Coagulase-negative staphylococcus has several pathologically relevant members like *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*, and *S. sciuri*.

S. aureus is commonly found in mammals as part of their normal flora. It is found usually in the nasal passages, axillae, skin, mouth, and along the gastrointestinal and vaginal tract (Smith, Noble, Bensch, Ahlin, Jacobson, & Latham, 1982; Williams, 1946, 1963). According to a study conducted by Peacock and colleagues in 2001, 20% of the population was permanent and 60% were transient nasal carriers of *S. aureus* (Peacock, de Silva, & Lowy, 2001). Thus, human beings are good reservoirs of *S. aureus*. Only 30% of the nasal colonization is permanent, leaving the majority to short-termed colonization (von Eiff, Machka, Stammer, & Peters, 2001). Different strains of *S. aureus* have varying preferential sites of colonization depending on the presence of colonization and adhesion factors. For instance, community acquired Methicillin Resistant *S. aureus* (CA-MRSA) preferentially colonize the skin and throat (Mertz et al., 2007). Colonization of *S. aureus* is very important to study, as it is important in the determination of the transmission of infections and possibly in the treatment regimens.

S. aureus causes superficial skin infections like impetigo, cellulitis, folliculitis, and severe scalded skin syndrome. When *S. aureus* invades the blood stream, it causes bacteremia and infections of the organs in the path of the blood stream like endocarditis,

osteomyelitis, and septic arthritis. Staphylococcal pneumonia, otitis media, and meningitis are some of the other clinical manifestations of *S. aureus* infections. Owing to the variety of enterotoxins produced, *S. aureus* is also a major contaminant in the food industry. *S. aureus* causes staphylococcal food poisoning with characteristic symptoms of stomach pain and diarrhea.



Figure 3. Some of the infections caused by *S. aureus*: (A) a cutaneous abscess¹ of *S. aureus* (Morgan, 2010), (B) an osteomyelitis infection caused by *S. aureus* (Antimicrobe, 2010) and (C) an infection of a of subclavian catheter (Griffin & Hamilton, 2010).

Virulence and Immune Evasion Factors

S. aureus codes for an arsenal of virulence factors that are surface contained or released into the environment. Described below are some of these factors that help in adhesion, colonization, immune evasion, and infection dissemination.

Protein A is a cell wall associated protein that has multiple virulence-associated functions. This surface exposed protein competitively binds to the Fc region of immunoglobulins like IgG, IgA, and IgE (Peterson, Verhoef, Sabath, & Quie, 1977; Lofdahl, Guss, Uhlen, Philipson, & Lindberg, 1983), thus preventing the staphylococcal cells from being marked for phagocytosis (Gemmell, Tree, Patel, O'Reilly, & Foster, 1991). Protein A can behave as a superantigen if it interacts with the IgM molecules on the B-cells (DeDent, McAdow, & Schneewind, 2007; Dossett, Kronvall, Williams, &

Quie 1969; Romagnani et al., 1981) leading to an increased immune response and more damage to the host. Menzies and Kourteva in 1998 also revealed the apoptotic effect of protein A on monocytes and macrophages (Menzies & Kourteva, 1998). Thus, protein A was proposed to be a very important virulence factor of *S. aureus*.

MSCRAMMS (Microbial Surface Components Recognizing Adhesive Matrix Molecules) are surface exposed proteins that are covalently attached to the peptidoglycan layer. These proteins bind to host surfaces to aid in establishing an infection (Foster & McDevitt, 1994).

Table 1

Examples of MSCRAMMS

MSCRAMMS	Host protein	Reference
Fibronectin-binding protein (<i>fnbpA</i> and <i>fnbpB</i>)	Fibronectin	(Flock et al., 1987; Signas et al 1989; Jonsson, Signas, Muller, & Lindberg 1991; Schwarz-Linek et al., 2003)
Clumping factor (<i>clfA</i> and <i>clfB</i>)	Fibrinogen	(McDevitt, Francois, Vaudaux, & Foster, 1994; Bayer, Sullam, Ramos, Li, Cheung, & Yeaman, 1995)
Collagen adhesion (<i>cna</i>)	Collagen	(Patti et al., 1992; Gillaspay, Lee, Sau, Cheung, & Smeltzer 1998)

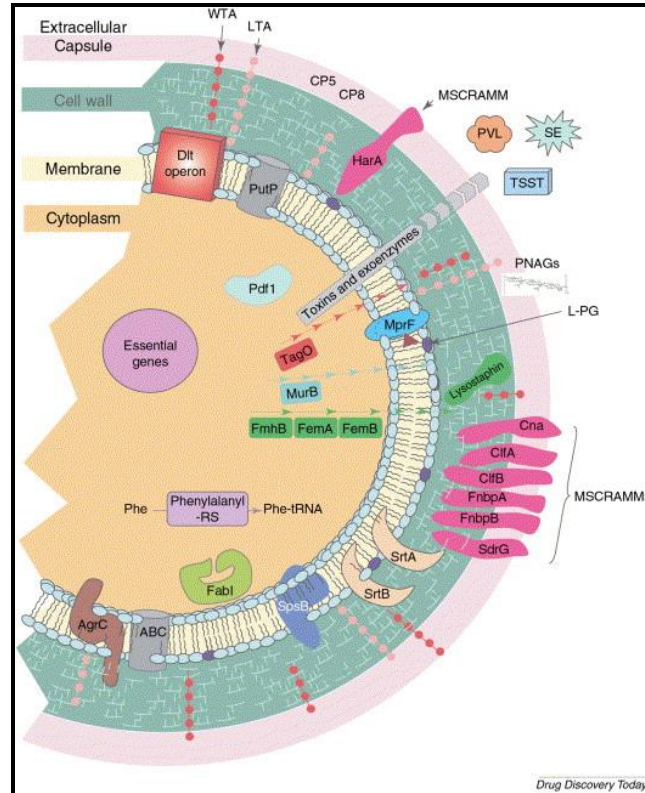


Figure 4. MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules) of *S. aureus* (Garcia-Lara, Masalha & Foster, 2005).

S. aureus synthesizes a capsule (Lee, Xu, Albus, & Livolsi, 1994, Lee, Liang, Hook, & Brown., 2004b) around itself that protects the bacteria and prevents it from complement activation and opsonophagocytosis by preventing the neutrophil receptor from gaining access to the staphylococcal proteins (Thakker, Park, Carey, & Lee 1998; Nilsson, Lee, Bremell, Ryden, & Tarkowski, 1997). Of the 11 capsule serotypes that have been identified, serotypes 5 and 8 have been found associated with human infections. Serotypes 5 and 8 are produced in small quantities and have proven to be difficult to study. Overexpression studies have shown that large quantities of capsule 8 showed longer persistence of the strains in the blood, liver, and spleen of mice in a sepsis model. The enhanced capsule production also showed increased resistance to

opsonophagocytosis (Luong, Sau, Gomez, Lee et al., 2004a, 2004b). *S. aureus* also produces several cytotoxins that have the ability to lyse leukocytes, monocytes and RBCs (Wardenburg, Bae, Otto, DeLeo, & Schneewind, 2007) (Table 2).

Table 2

Cytotoxins produced by S. aureus

Cytotoxins	Role in virulence	References
α – toxin	Lyse red blood cells, platelets, endothelial and epithelial cells	(Gray & Kehoe, 1984; Patel, Nowlan, Weavers, & Foster, 1987; Bhakdi et al., 1988, 1989; Bhakdi & Tranum-Jensen 1991; Bayer, Ramos, Menzies, Yeaman, Shen, & Cheung, 1997)
β – toxin	Lysis of erythrocytes	(Projan, Kornblum, Kreiswirth, Moghazeh, Eisner, & Novick 1989)
γ – toxin and Leukocidin	Lysis of leukocytes	(Jayasinghe & Bayley, 2005)

Several enzymatic virulence factors are secreted by *S. aureus* like proteases, lipases, and coagulases. *Staphylococcus* expresses various proteases. Unlike most of the extracellular secreted enzymes of *S. aureus*, coagulase is a surface associated enzyme (Kaida, Miyata, Yoshizawa, Igarashi, & Iwanaga, 1989) that coagulates serum by the reaction illustrated below (Lowy, 2000).

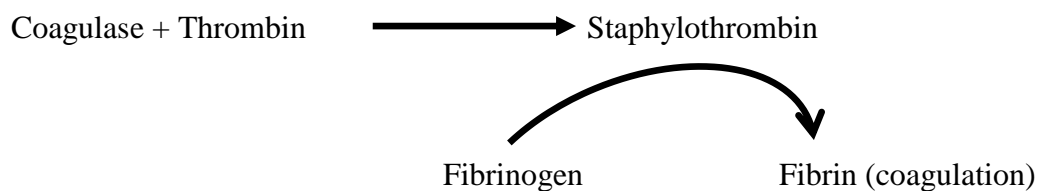


Figure 5. Schematic representation of the process of coagulation by *S. aureus*.

For a pathogen like *S. aureus* to establish an infection in the host, it is essential for the pathogen to withstand the immunological attack by the host. Thus, *S. aureus* has several immune evading mechanisms while encountering the host innate and adaptive immune system. In this study, the focus is on the evasion of phagocytosis, a common immune response that clears the host of pathogens and foreign bodies. Listed in Table 3 are some of the evading mechanisms and factors employed by *S. aureus* at the various stages of phagocytosis.

Table 3

Immune evading factors produced by S. aureus

Virulence Factors	Mechanism of Evasion	Reference
Preventing Chemotaxis		
Chemotaxis Inhibitory Protein (CHIPs)	Binds to neutrophil receptors for formyl peptides and C5a. Thus decreasing chemotaxis.	(de Haas et al., 2004; Haas et al., 2004)
MHC Class II analogous protein/extracellular adherence protein (Eap)	Competes with lymphocyte-function-associated antigen (LFA-1) for binding intercellular adhesion molecule-1 (ICAM-1). Prevents the binding of neutrophils with endothelial cells. Thus inhibiting chemotaxis and extravasation to the site of infection.	(Chavakis, T. et al., 2002)

Table 3 (continued).

Virulence Factors	Mechanism of Evasion	Reference
Preventing Opsonization		
Protein A (Spa)	Competitive binding to Fc region of immunoglobulin like Ig G, IgA and IgE. It also reduces the platelet aggregation	(Uhlen, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L., Lindberg, 1984; Lofdahl, Guss, Uhlen, Philipson & Lindberg, 1983; Gemmell, Tree, Patel, O'Reilly, & Foster, 1991)
Clumping Factor (ClfA)	Clump together by binding to each other and the fibrinogen to coat the cells and causing (1) Inhibition of opsonin deposition (2) Steric hindrance for opsonophagocytosis.	(Palmqvist, Patti, Tarkowski & Josefsson, 2004)
Capsular polysaccharide (Cap)	Prevent opsonin deposition, or access to neutrophil receptors.	(Thakker, Park, Carey, & Lee 1998; Nilsson et al 1997)
Staphylokinase (Sak)	Plasminogen activator protein Dissolve fibrin clots Degrades IgG and C3 (after opsonins deposition has been initiated) Binds to defensin (an antimicrobial peptide)	(Rooijackers, van Wamel, Ruyken, van Kessel, & van Strijp, 2005a)
Staphylococcus Complement Inhibitor (SCIN)	Prevents the formation of C3b formation. Thus, interrupting the complement activation pathways.	(Rooijackers et al., 2005b)

Table 3 (continued).

Virulence Factors	Mechanism of Evasion	Reference
Escape a Phagosome		
α – Hemolysin, γ – Hemolysin, Panton-Valentine Leukocidin, Leukocidin E/D	Assemble into a heptamer and form a pore in the membranes of the host cells. Escape from endosomes and phagocytes	(Montoya & Gouaux, 2003) (Menestrina et al., 2003)
Intracellular Survival		
Aureolysin (Aur)	Cleaves the antimicrobial peptide human defensin peptide cathelicidin LL-37.	(Sieprawska-Lupa, M. et al., 2004)

Global Regulation

The synthesis and action of virulence factors are coordinated and controlled by regulators. These regulators could be transcriptional regulators like Sigma factor B (Bischoff et al., 2004; Ziebandt et al., 2001), two-component systems like Staphylococcal accessory element (Sae) (Novick & Jiang, 2003), or RNA molecules like RNA III of the *agr* system (Janzon & Arvidson, 1990; Novick, Ross, Projan, Kornblum, Kreiswirth, & Moghazeh, 1993). Some the well-studied global regulators are the accessory gene regulator (Agr), staphylococcal accessory element (Sae), and the staphylococcal accessory regulator (SarA).

The Agr System

The Agr system (accessory gene regulator) was first studied in an *agr* mutant with a Tn551 transposon inserted into the *agr* locus (Mallonee, Glatz & Pattee, 1982; Recsei,

Kreiswirth, O'Reilly, Schlievert, Gruss, & Novick, 1986). Cloning and characterization of the *agr* gene showed the *agr* locus to code for a two-component system (Peng et al., 1988). The *agr* locus consists of two transcripts: RNA II and RNA III that are under the promoters P2 and P3 respectively. The genes that code for RNA II are the *agr B*, *agr D*, *agr C*, and *agr A*, and the proteins coded by them regulate the RNA III production. The RNA III also codes for the *hld*, delta hemolysin. The production of RNA III is regulated by the *agrBDCA* genes. The *agr* system plays an important role in the synthesis of secreted proteins like toxic shock syndrome toxin (TSST-1), hemolysins, serine protease, and surface-associated proteins like protein A, coagulase, and fibronectin binding protein (Lindberg et al., 1990).

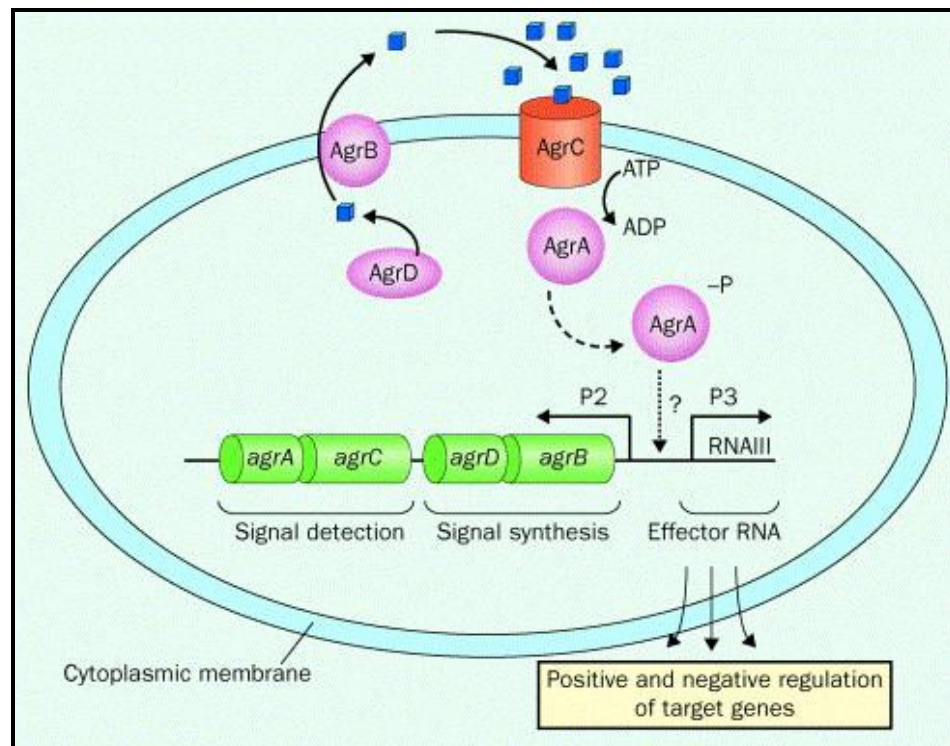


Figure 6. The *agr* quorum system of *S. aureus*. (Camara, Williams, & Hardman, 2002)

A mutant of the *hld* gene did not produce an *agr* mutant, thus was not the effector of the *agr* system (Novick et al., 1993). Although when the role of RNA III in an *agr* system was studied by introducing a plasmid carrying the gene for RNA III into an *agr* strain it produced an *agr*⁺ phenotype. The *agr* complementation was also performed with a RNA II, which failed to produce an *agr*⁺ phenotype. Thus, RNA III was concluded to be the regulator of *agr* system. RNA III regulation of transcription and translation of target genes was independent of each other (Novick et al., 1993). The RNA III was observed to reach a maximum expression during the post-exponential phase (Vandenesch, Kornblum, & Novick, 1991).

The Agr A and Agr C are the response regulators and the histidine kinase components of the two-component system. The Agr C is a 46 kDa transmembrane protein that consists of a sensor that recognizes AIP and a cytoplasmic transmitter that contains a histidine residue (Lina et al., 1998). This activation of Agr C is dose-dependent, and the maximal activation occurs at an optimum concentration of AIP. Activation of Agr C leads to autoinduction of *agrBDCA* and the synthesis of RNA III (Ji, Beavis, & Novick 1997). The RNA III acts as the effector of the *agr* system, which increases transcription of secreted proteins and decreases transcription of surface proteins. The Agr D consists of an N-terminal region, followed by the AIP that gets cleaved off on processing and a C-terminal region. Agr B has the endopeptidase activity by which it modifies the Agr D and secretes the AIP produced out of the cell (Zhang, Gray, Novick, & Ji 2002; Zhang & Ji, 2004; Zhang, Lin & Ji, 2004).

The *agr* system is classified into *agr* types I–IV, based on varying regions (hypervariable regions) (Dufour et al., 2002) in *agrBDCA* operon and they respectively

recognize the AIP types I – IV (Ji, Beavis, & Novick, 1997, 1997; Jarraud et al., 2000; McDowell et al., 2001; Otto, Echner, Voelter, & Gotz, 2001).

The regulatory influence of *agr* on other genes is imparted by the binding of AgrA, binding of the RNA III molecule to the virulence genes under regulation or indirectly through the interaction of RNA III and Rot (repressor of toxins) (Geisinger, Adhikari, Jin, Ross, & Novick, 2006). Dunman et al., (2001) showed that *agr* increased expression of proteases, lipases, ureases, toxins (encoded by *hla*, *hld*, and *hly*), and enterotoxins (SEB, SEC and SED) via the binding of RNA III component of the *agr* system (Dunman et al., 2001). Other factors regulated by *agr* are listed in Table 4.

Table 4

Regulation of virulence genes by agr

Genes Positively Regulated by <i>agr</i>	References
α - hemolysin (<i>hla</i>)	(Morfeldt, Taylor, von Gabain & Arvidson, 1995; Novick et al., 1993; Xiong et al., 2006)
Aureolysin (<i>aur</i>)	(Oscarsson, Tegmark-Wisell & Arvidson 2006)
Serine Protease (<i>ssp</i>)	(Oscarsson, Tegmark-Wisell & Arvidson 2006)
Staphylokinase (<i>sak</i>)	(Shaw, Golonka, Potempa & Foster, 2004)
Polysaccharide capsule (<i>cap</i>)	(Luong, Sau, Gomez, Lee et al., 2004a, 2004b; Dassy, Hogan, Foster & Fournier, 1993)

Table 4 (continued).

Genes Negatively Regulated by <i>agr</i>	References
Protein A (<i>spa</i>)	(Novick et al., 1993, Benito, Kolb, Romby, Lina, Etienne & Vandenesch, 2000)
Coagulase (<i>coa</i>)	(Chevalier et al., 2010)
Fibronectin binding protein (<i>fnb</i>)	(Saravia-Otten, Muller & Arvidson 1997)

The SarA System

The SarA (staphylococcal accessory regulator) is a 124kDa dimeric DNA binding protein. The *sarA* locus that codes for SarA has three transcripts *sar A*, *sar B*, and *sar C* that are produced under three promoters P1, P2, and P3 respectively (Bayer et al., 1996; Manna, Bayer, & Cheung 1998). The P1 and P2 promoters require the sigma A, while the P3 promoter requires the sigma B factor (Deora, Tseng, & Misra, 1997; Manna, Bayer, & Cheung, 1998). This DNA binding protein is expressed in the mid- and late-exponential phase of growth (Rechtin et al., 1999; Bayer et al., 1996).

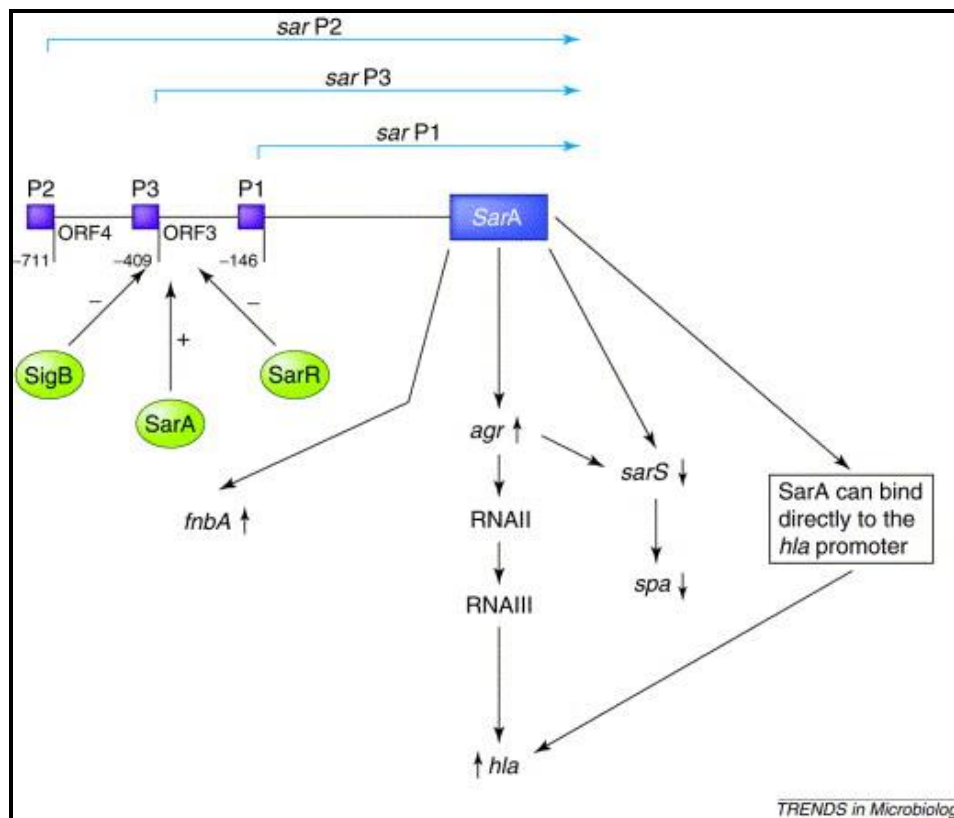


Figure 7. The *sarA* system and the influence of SarA on other genes. (Cheung & Zhang, 2001)

In *in vitro* studies, Sar A has been observed to bind to several promoter regions of genes like *hla*, *fnA*, and *fnB* and upregulate them (Cheung, Koomey, Butler, Projan, & Fischetti, 1992; Cheung & Ying, 1994, Wolz et al., 2000). The *sar A* however, down-regulates expression of genes like the serine protease (SspA) and cysteine protease (ScpA) (Lindsay & Foster, 1999). The *ica* gene locus encodes the PIA (Polysaccharide Intercellular Adhesin) biosynthesis. This gene locus consists of the *ica A*, *ica D*, *ica B*, and the *ica C* genes along with the negative regulator *ica R*. The *ica A* promoter and the *ica R* are regulated by the *sar A* (Tormo et al., 2005b).

Pathogenesis

S. aureus are opportunistic pathogens that cause a variety of infections once they gain access to the host through hair follicles, open skin of cuts, incisions, injections, wounds, surgical wounds, or in-dwelling catheters. The infections caused by *S. aureus* range from localized superficial infections like abscesses, impetigo, and carbuncles to invasive infections like septic arthritis, osteomyelitis, and endocarditis (Figure 7) (Lowy, 1998).

S. aureus is one of the major causative agents of the gram-positive mediated sepsis. It is not the infecting pathogen by itself but the excessive host immunological response to this invader that causes the increased damage to the host (Angus et al., 2001). In an epidemiology study conducted on the incidence of sepsis from 1979 to 2000, *S. aureus* was reported to be the causative agent responsible for 52% of the cases (Martin, Mannino, Eaton, & Moss, 2003). Angus et al. (2001) reported a sepsis-associated mortality percentage of about 30, which increased to 40% with senior citizens in spite of being under hospital care and antibiotic treatment. Of this percentage, 40 % was incurred by gram positive infections (Angus et al., 2001; Alberti et al., 2002).

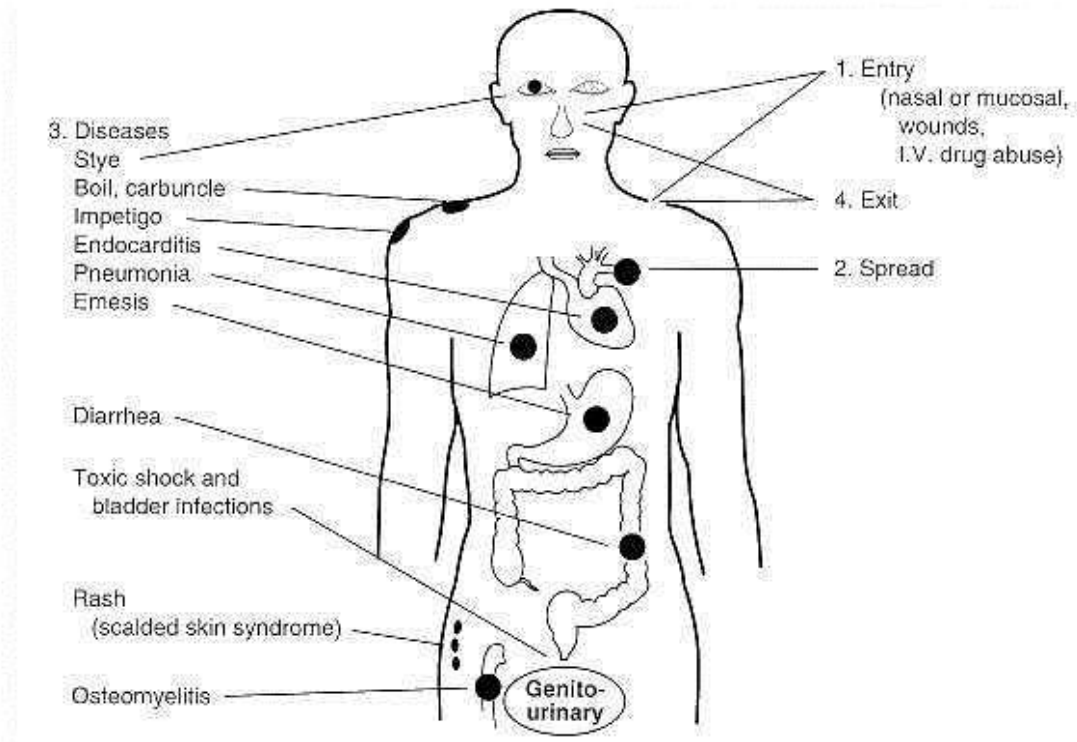


Figure 8. Pathogenesis model of *S. aureus* (Clark & Company, 1998)

S. aureus is subject to attack by both the innate and acquired immune system. On entering the host, *S. aureus* can establish an infection either at the site of entry, or if it evades the immune system it could even cause severe systemic infections (Table 5). Infection leads to inflammation that signals phagocytes to the site. These phagocytes secrete antimicrobial substances to kill the bacteria as well as secrete signals to attract more phagocytes (chemotaxis) to the site of infection to clear the pathogens. The outcome of the immunological attack decides the fate of the staphylococcal invasion. This determines the extent of infection dissemination and severity. The other factors that ease the establishment of a staphylococcal infection are age and compromised immune systems.

Table 5

Some of the sites of infections caused by S. aureus

Sites for Infections	<i>S. aureus</i> infected
Skin and Soft Tissue Infections (SSTIs)	76% of total purulent SSTIs in 11 emergency departments ^a
In-dwelling Device Infections	7.3% of all <i>S. aureus</i> infections ^b
Post-surgical Infections	10.1% of all <i>S. aureus</i> infections ^b
Urinary Tract Infections	3.6% of all <i>S. aureus</i> infections ^b

Note. ^a – In 2004 (Moran et al., 2006); ^b – In 1999 – 2000 (Kuehnert, et al., 2005)

Methicillin Resistant *Staphylococcus aureus* (MRSA)

Methicillin Resistant *Staphylococcus aureus* are *S. aureus* that have the ability to build resistance to most antibiotics like the penicillins–flucloxacillin, oxacillin, and methicillin. Methicillin was the antibiotic used to treat penicillin resistant *S. aureus* infections in the past until the 1960s when the first case of MRSA was found in the United Kingdom (Jevons, 1961). In a study conducted with data from 1,268 ICU sections of 337 hospitals from 1992 to 2003 alone, the MRSA positive infections increased from 35.9% to 64.4% among the *S. aureus* isolates (Klevens et al., 2006). Based on the statistical information by the Centers for Disease Control and Prevention of the *S. aureus* and MRSA Surveillance Summary 2007 in the US, there have been 292,000 cases of hospitalizations that are positive for *S. aureus* infections annually (Kuehnert et al., 2005).

MRSA infections cause a large degree of stress on the healthcare sector owing to its high mortality rate, lack of treatment success, and cost. Several types of genotypic typing have been conducted ever since the realization of the endemic spread of MRSA

infections. Some of the methods used to classify the various *S. aureus* isolates are classifications by antibiotic susceptibility, any phenotypic characteristic, sequence of *spa* gene that encodes for the Protein A, Pulsed-field gel electrophoresis (PFGE, DNA-based finger printing method), and Multilocus Enzyme Electrophoresis (MLEE). These methods were soon taken over by Multilocus sequence typing (MLST). Genotypic typing comparisons of the first MRSA isolate in the UK in 1961 and the MSSA that were prevalent in Denmark in the 1950s revealed no difference (Crisostomo, Westh, Tomasz, Chung, Oliveira, & Lencastre, 2001) except for the mobile genetic element Staphylococcal Cassette Chromosome – SCCmec (Katayama, Ito, & Hiramatsu, 2000). The SCCmec element carries the *mecA* gene that codes for the PBP 2' or PBP2a (Penicillin binding protein 2'), which confers antibiotic resistance to β -lactam antibiotics (Hartman & Tomasz, 1984; Reynolds & Brown, 1985; Utsui & Yokota, 1985). Crisostomo and group thus suggested that MRSA strains might have descended from MSSA isolates (Crisostomo et al., 2001). Unlike HA-MRSA SCCmec types of I–III, CA-MRSA strains carry the SCCmec type IV (Okuma et al., 2002, Vandenesch et al., 2003).

While most infections were found associated with individuals who have had a prior exposure to the healthcare setting or have been in close contact with other individuals (Saravolatz, Markowitz, Arking, Pohlod, & Fisher, 1982a; Saravolatz, Pohlod, & Arking, 1982b; Thompson, Cabezudo, & Wenzel, 1982; Boyce, 1998; Graffunder & Venezia, 2002; Lodise, McKinnon, & Rybak, 2003), Community Acquired-MRSA infections were found in individuals who have had no prior encounters to the clinical setting or any pre-disposing factors (Crum et al., 2006; Naimi et al., 2003). In an epidemiological study conducted from July 2004 to December 2005 in the US, it was reported that of the invasive infections recorded, 58.4% of them were community

onset HA-associated, 26.6% hospital onset HA-acquired, and 13.7% were CA-MRSA infections (Klebens, Morrison, & Nadle, 2007).

Hospital-Acquired *Staphylococcus aureus*

Based on the epidemiologic classification of invasive MRSA infections, health care associated MRSA infections were classified into two types: (a) community onset and (b) hospital onset. Community onset health care-associated MRSA infections were defined as “cases with at least 1 of the following health care risk factors: (1) presence of an invasive device at time of admission; (2) history of MRSA infection or colonization; (3) history of surgery, hospitalization, dialysis, or residence in a long-term care facility in previous 12 mo preceding culture date” (Klebens et al., 2007, p. 1765). Hospital onset health care-associated MRSA infections were defined as “Cases with positive culture result from a normally sterile site obtained >48 h after hospital admission. These cases might also have ≥ 1 of the community-onset risk factors” (Klebens et al., 2007, p. 1765). In a study conducted between the years 1992–2003, HA-MRSA causing infections reached a high percentage of 64.4% of the infections of intensive care units in the US (Klebens et al., 2006). Based on the antibiotic resistance gene, the type of recombinase gene and the number of them, the SCCmec are classified into five classes (Ito et al., 2002; Okuma et al., 2002). SCCmec found in HA-MRSA isolates are SCCmec I-III (Ito et al., 2001; Enright et al., 2002). The PFGE types USA100, USA200, and USA500 are common among HA-MRSA infections.

Community-Acquired *Staphylococcus aureus*

Based on the epidemiologic classification of invasive MRSA infections, cases of community-acquired infections were defined as “cases with no documented community-onset health care risk factor” (Klebens et al., 2007, p. 1765). Community-acquired

MRSA strains were first reported in 1981 in Detroit among intravenous drug abusers (Saravolatz et al., 1982a). Mortality-associated CA-MRSA infections were soon reported a few years later in 1997 in Minnesota and North Dakota (CDC, 1999). In 2006, Moran, et al. published evidence of CA-MRSA causing SSTIs in emergency departments. CA-MRSA reported had a diverse prevalence from children under child care (Adcock, Pastor, Medley, Patterson, & Murphy, 1998), to sports members (CDC report 2003; Begier et al., 2004), to correctional facilities (CDC, 2003). Infections among the community without prior exposure to a health setting was not the only alarming feature of CA-MRSA infections, these SSTI causing strains also caused severe, invasive infections that have been morbidity-associated ones (Naimi et al., 2003; Zetola, Francis, Nuermberger, & Bishai, 2005; Kaplan et al., 2005; Fridkin et al., 2005).

Unlike most strains of *S. aureus*, CA-MRSA strains reside relatively longer on the human skin than in the nasal passages (Mertz et al., 2007; Shurland et al., 2007). This site of colonization makes pathogens like CA-MRSA very accessible for transmission, which could be one of the contributing factors to the contagiousity of CA-MRSA infections (Nguyen, Bancroft, Mascola, Guevara, & Yasuda, 2007).

Pulse-field gel electrophoresis (a RFLP based technique) is one the most common and established modes of local-level genotypic typing followed in the United States. The Center for Disease Control and Prevention identified two notorious clones of CA-MRSA strains prevalent in the US by PFGE: USA300 and USA400 (McDougal et al., 2003). The USA300 PFGE type (Tenover et al., 2006) was among the most pathogenically dominant CA-MRSA strains (Li et al., 2009). The other PFGE types associated with the CA-MRSA strains were USA400, USA1000, and USA1100 (McDougal, Wenming, Patel & Tenover, 2004).

Strains of USA300 are MRSA (Diep et al., 2006). Presence of the genes of Panton-Valentine leukocidin (PVL), enterotoxin Q, enterotoxin K, and arginine catabolic mobile element (ACME) are unique to the USA300 strains (Diep et al., 2006). The ACME is a 30.9 kb acquired genetic element that carries the *arc* gene cluster. The *arc* gene cluster codes for the enzymes required for conversion of L-arginine to metabolically important products like ATP, carbon-dioxide, and ammonia. Nitric oxide (NO) is an important player in the immune system of the host; thus, unavailability of L-arginine, the precursor of NO, is believed to aid in surviving the hostile immunological attacks on bacteria (Diep et al., 2006; Degnan et al., 1998, 2000).

CHAPTER II
BACKGROUND

The *msa* Gene

While screening using a Tn551 library, a novel gene was identified to be involved in the regulation of *sar A* – the *msa* (Modulator of *sar A*) gene. Mutation of this *msa* gene has been seen to affect the expression of *sar A* and several virulence-related genes in the *S aureus* strains COL, RN6390, and UAMS-1 (Sambanthamoorthy, Smeltzer, & Elasri, 2006) (Table 2.1). The mutant of *msa* was also observed to be unable to form mature biofilms, a hallmark feature of *S. aureus*. (Sambanthamoorthy, Smeltzer, & Elasri, 2006; Sambanthamoorthy, Schwartz, Nagarajan, & Elasri, 2008).

Table 6

Genes previously known to be regulated by msa

Extracellular factors	Surface factors
α -hemolysin(<i>hla</i>)	Clumping factor A (<i>clfA</i>)
Aureolysin (<i>aur</i>)	Protein A (<i>spa</i>)
V8 proteases(<i>ssp</i>)	Collagen adhesin (<i>cna</i>)
Lipase (<i>lip</i>)	Fibronectin (<i>fnb</i>)

The sequence of the *msa* sequence was analyzed by computational analysis and predicted to be a 15.67kDa transmembrane protein with three transmembrane regions. The positions of the potential phosphorylation sites further suggested that the Msa protein could be a membrane protein functioning as a signal transducer (Nagarajan & Elasri,

2007). Additional research on the *msa* mutant by microarray analysis showed that the *msa* gene regulated several genes (unpublished data) which, strongly suggested that the *msa* gene could be a global regulator like *sarA* or the *agr* system. At the amino acid level, the Msa sequence shows homology and conservation across several strains like RF122, MRSA252, MSSA476, MW2, COL, Mu50, N315, and NCTC 8325 (Nagarajan & Elasri, 2007).

Strain *S. aureus* UAMS-1

The *S. aureus* strain UAMS-1 was isolated from an osteomyelitis patient from the McClellan Veterans Hospital in Little Rock, Arkansas (Gillaspy et al., 1995). Animal model studies have shown UAMS-1 strains to be very proficient in osteomyelitis and septic arthritis (Blevins et al., 2003; Smeltzer et al., 1997). UAMS-1 strains of *S. aureus* are methicillin susceptible, host acquired strains (Gillaspy et al., 1995). The α -hemolysin gene, *hla* of UAMS-1 carries a nonsense mutation thus UAMS-1 is unable to synthesize the toxin (Cassat et al., 2006) but this has not influenced the pathogenicity of UAMS-1 strains. UAMS-1 strain was used for this study as a representative of a hospital acquired strain.

Strain *S. aureus* USA300 – 0114 LAC

Most clinically relevant strains belonging to the PFGE type USA300 are of the subtype USA300-0114 (McDougal et al., 2003; Tenover et al., 2006). The USA300 strain FPR3757 was isolated from an abscess on the wrist of a HIV-positive male at the San Francisco General Hospital (Pan et al., 2005). This strain was used in this study as a representative of a community acquired clinical isolate.

Comparison of *S. aureus* Community-Acquired Isolate USA300 LAC vs Hospital-
Acquired Isolate UAMS-1

The regulatory role of the *msa* gene was studied in the genetic backgrounds of LAC and UAMS-1. Here, a comparison was made between these two strains to understand the behavior of the *msa* gene in these two genetic backgrounds. Nagarajan et al. showed conservation of the *msa* gene across very strains of *S. aureus*, yet the regulatory behavior of the *msa* gene has showed variations across these strains (Nagarajan & Elasri, 2007; Sambanthamoorthy, Smeltzer, & Elasri, 2006). Thus, to better understand the regulation by the *msa* gene in the strains of *S. aureus*, previous findings on the genetic content of LAC and UAMS-1 were gathered and compared in Table 7.

Table 7

Comparison of S. aureus community acquired isolate USA300 LAC vs hospital acquired isolate UAMS-1

	USA300 LAC	References	UAMS-1	References
First isolated	Abscess isolate, USA, 2000	(Diep et al., 2006)	Bone isolate from a Osteomyelitis patient, Little Rock, AR, 1995	(Gillaspy et al., 1995)
PFGE	USA300 (subtype 0114)	(Kennedy et al., 2005, Francis et al., 2005, Miller et al., 2005)	USA200	

Table 7 (continued).

	USA300 LAC	References	UAMS-1	References
MLST	ST8	(Enright et al., 2000, Miller et al., 2005)	ST30	(Cassat et al., 2005)
Spa type	YHGFMBQBLO, <i>spa1</i>	(Shopsin et al., 1999, Miller et al., 2005)	WGKAKAQ, <i>spa33</i>	(Cassat et al., 2005)
SCCmec	SCCmec Iva	(Ma XX et al., 2002, Robinson et al., 2005, Miller et al., 2005)	None	(Gillaspy et al., 1995)
Methicillin susceptibility	MRSA	(Diep et al., 2006)	MSSA	(Gillaspy et al., 1995)
Agr subgroub	Type I	(McDougal et al., 2003)	III	(Cassat et al., 2005)
ACME	Present (Type I)	(Diep et al., 2006)	Absent	(Zhu et al., 2007; Fey & Olson, 2010)

CHAPTER III

DELETION OF THE *MSA* GENE

Introduction

Previous to this study, the role of *msa* was studied using an insertional mutant which, provided good evidence that *msa* is required in regulating virulence and biofilm formation (Sambanthamoorthy, Smeltzer, & Elasri, 2006; Sambanthamoorthy, Schwartz, Nagarajan, & Elasri, 2008). But, insertional mutants have several disadvantages. There is always a possibility of a truncated product being formed due to the upstream part of the open reading frame in question being intact. Additionally, if the gene is part of an operon, the insertional mutation can hamper the transcription of the neighboring genes. Taking all these potential polar effects of insertional mutants into consideration, the *msa* gene was mutated by deletion.

A temperature sensitive shuttle vector pKOR1 was used to delete the *msa* gene from *S. aureus* strains COL, LAC, RN6390, and UAMS-1. At the non-permissive temperature of 43°C, pKOR1 integrates into the chromosome and at 30°C, which is the permissive temperature the plasmid eviction is favored. This eviction facilitates the loss of the *msa* gene. One of the special features of pKOR1 is that it offers an inducible counter selection with anhydrotetracycline (ATc) that selects for chromosomal excision and loss of plasmid without the use of an antibiotic.

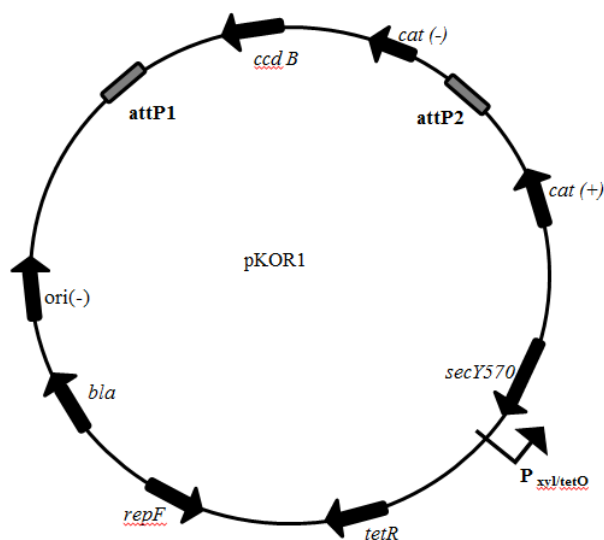


Figure 9. Schematic representation of the temperature sensitive pKOR1 vector employed to create deletion mutants of *S. aureus*. Labels indicated: *attP* sites = attachment sites P1 and P2, *cat* sites = chloramphenicol resistance sites for gram positive [*cat* (+)] and gram negative [*cat* (-)] hosts, *bla* = ampicillin resistance gene, *tetR* = tetracycline resistance gene, *P_{xyl/tetO}* = promoter producing the antisense product for the protein required for protein translocation.

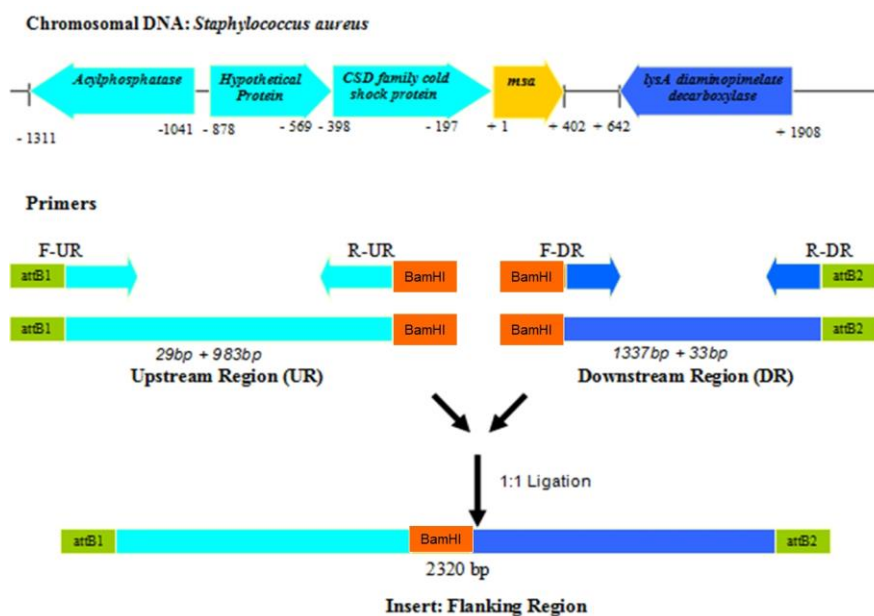


Figure 10. Schematic representation of the insert preparation containing the Upstream and Downstream regions of *msa* gene to be inserted into the pKOR1 vector.

Materials and Methods

Bacteria and Growth Conditions

The bacterial strains of *Staphylococcus aureus* were grown in Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) at the required temperatures and in the presence of appropriate antibiotics. The plasmid pKOR1 was used to delete the *msa* gene from the chromosomal DNA. The pKOR1 transformations were carried out in *Escherichia coli* and *S. aureus* RN4220. The Phage ϕ 11 was also employed to transfer pKOR1 from *S. aureus* RN4220 to the strains of *S. aureus* COL, USA300 LAC (Los Angeles county clone), RN6390, UAMS-1.

Allelic replacement with pKOR1

The gene *msa* in *S. aureus* strains were mutated by deletion using the mutagenesis protocol described by Bae and Schneewind (Bae & Schneewind, 2006). Briefly, the flanking regions of the *msa* gene were amplified from *Staphylococcus aureus* with primers listed in Table 8 by PCR. The DNA fragments isolated were digested with the restriction enzyme BamHI and ligated using T4 DNA ligase. This ligated fragment was amplified using the F-UR and R-DR primers and was inserted into pKOR1 using the Gateway BP Clonase Enzyme Mix (Invitrogen's Corp.). The plasmid thus formed pKOR1- Δ *msa* was introduced into TOP 10 cells (Invitrogen's Corp.) by chemical transformation and then into *S. aureus* RN4220 by electroporation. The plasmids isolated were verified by sequencing and transferred into the respective strains of *S. aureus* by ϕ 11-mediated phage transduction. The plasmids isolated were again verified by PCR and sequencing.

S. aureus strain containing the pKOR1- Δ *msa* was cultured overnight in TSB at 30°C in the presence of chloramphenicol (7.5 μ g/ml). A plate of TSA containing

chloramphenicol (7.5µg/ml) was streaked with the overnight culture and incubated at 43°C overnight. The colonies thus formed were picked and inoculated in plain TSB and allowed to grow overnight at 30°C. The following day a dilution of 10⁻⁴ were prepared from the overnight cultures and plated on TSA containing 100ng/ml of anhydrotetracycline. This temperature shift from 30°C to 43°C was repeated once more.

The colonies thus formed were picked and streaked on both plain TSA and TSA with chloramphenicol. The colonies that grew only on TSA plates were selected and analyzed for mutation of the *msa* gene. The co-integration protocol followed is as described by Bae and Schneewind (2006).

Table 8

Primers used for isolating the flanking regions of the msa gene

Regions	Primer	Sequence
Upstream Region (UR)	F-UR	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTGCGTCA TCCCCTTG
	R-UR	ATTAGGATCCAACAACGTTTGCAGCTTGTG
Downstream Region (DR)	F-DR	TGTTGGATCCTGGTATGTATATGATGCT
	R-DR	GGGGACCACTTTGTACAAGAAAGCTGGGTTACCAT CGCGCATTTA

Note. Region in italics are the *att sites* and the region underlined the Restriction site of BamHI.

Verification of the Deletion Mutant

The mutants of *msa* were verified by the following methods:

- (1) PCR: In order to verify the mutation of the *msa* gene from the chromosome of *S. aureus*, chromosomal DNA was isolated from the potential mutants and tested by

PCR using the (A) F-UR and the R-DR primers (Figure 11) and (B) the *msa* open reading frame primers.

(2) Sequencing: The regions amplified by PCR using the F-UR and R-DR primers were cloned into pCR2.1 (TOPO vector, Invitrogen Inc.). These vectors were sent for sequencing with F-UR, R-DR primers, and the F-UR-SEQ primer.

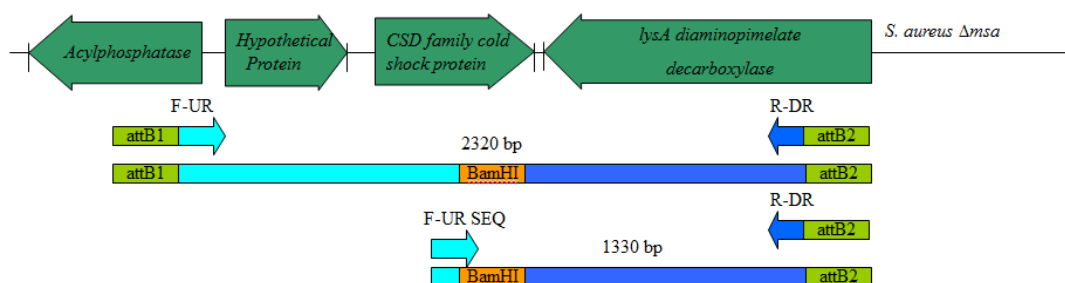


Figure 11. Schematic representation of the expected *msa* mutation.

Complementation

The open reading frame of *msa* along with the upstream region containing the *msa* promoter was inserted into pLi50 and introduced into the *msa* mutant strains to serve as the complement. Since, pLi50 was a high copy number vector, complementation was also attempted with a low copy vector pCN34 (requested from NARSA).

Phenotypic Characterization

Biofilm assay. A 20% solution of human plasma was prepared and 200 μ l of this solution was added to the wells of a microtiter plate and incubated at 4°C to coat the wells. The next day the plasma was carefully aspirated and the wells were washed thrice with 1X PBS. Overnight cultures of the testing strains were normalized (with TSB containing 3% NaCl and 0.5% glucose) and added to the respective wells. The plates were incubated at 37°C. The contents of the wells were aspirated and washed with 1X

PBS. Following washing, 100% of ethanol was used for fixing (10 min) and crystal violet for staining (2 min). The stain was washed with PBS and the plate was allowed to dry overnight inverted.

Protease assay. The strains were grown overnight in TSB at 37°C. The following day 300µl of the culture supernatant was filtered and incubated overnight at 37°C with 800 µl of Azocasein (3mg/ml) in Tris buffered saline (pH 7.5). The undegraded azocasein was precipitated by 50% trichloroacetic acid and the protease activity was assayed by measuring the amount of acid-soluble azocasein spectrophotometrically at 340 nm (Smeltzer et al., 1993).

Hemolytic assay. The strains were grown in TSB at 37°C with shaking at 120 rpm. The following day, the cultures were normalized to the lowest OD at 560nm. Supernatants were collected and filtered. Ten microliters of supernatant was mixed with 1ml of 2% rabbit blood in 10mM Tris HCl (pH 7.5) – 0.9% NaCl. This was incubated at 37°C for 15 minutes. The lysed cells were separated from the supernatant and the supernatant was used to measure optical density (OD) at 405nm. TSB and 1% SDS were used as negative and positive controls respectively (Blevins et al., 2002).

Results

Mutants of *S. aureus* strains LAC, UAMS-1, RN6390 and COL were verified as described. Figure 12 shows the absence of the *msa* gene in the mutant strains of *S. aureus*. Individual strains of LAC, UAMS-1 and COL were also verified by the ACME region (Figure 13), *cna* gene (Figure 14), and tetracycline resistance (Figure 3.7) respectively.

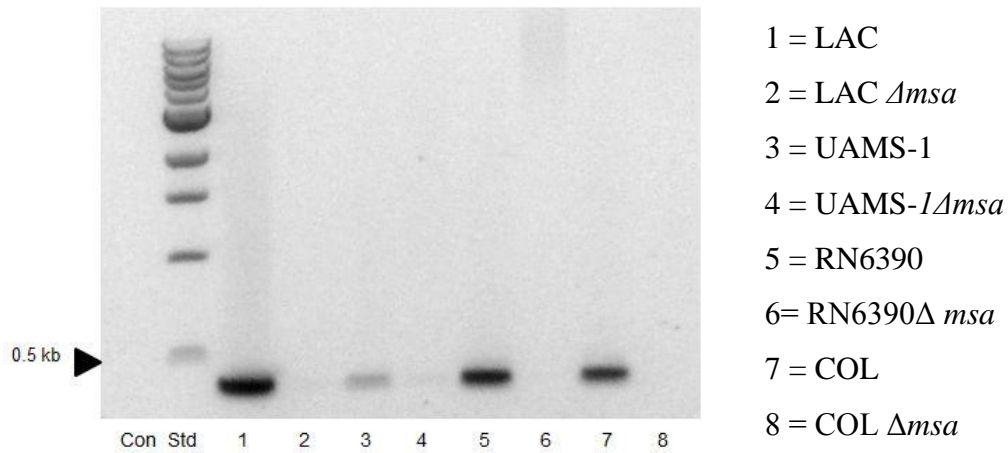


Figure 12. PCR with *msa* primers to verify the deletion of the *msa* gene in the *S. aureus* strains of LAC, UAMS-1, RN6390, and COL.

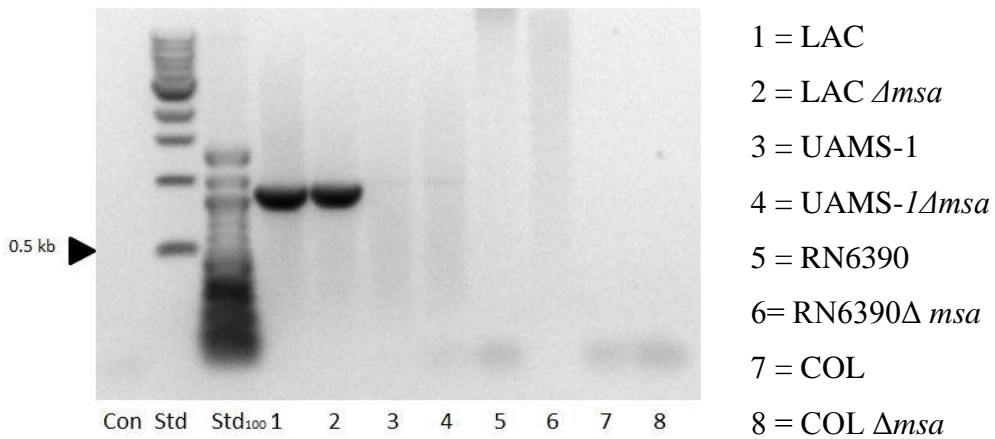


Figure 13. PCR with primers specific for the ACME region (unique for USA300 LAC strains) to confirm the genetic background of *S. aureus* LAC strains.

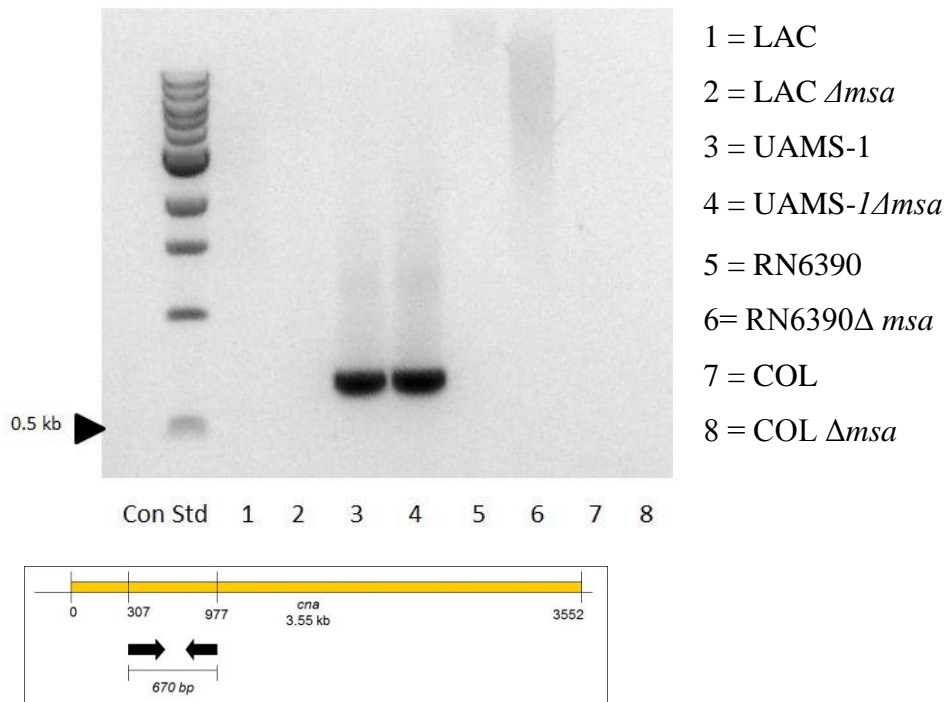


Figure 14. Verification by PCR of *S. aureus* UAMS-1 (unique for UAMS-1 strain) by the presence of the *cna* gene of *S. aureus* UAMS-1 strains.

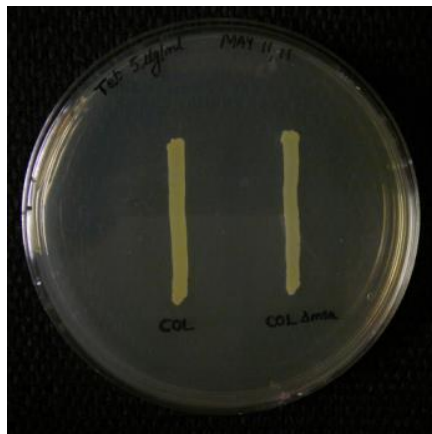


Figure 15. Tetracycline test with *S. aureus* COL strains confirming the strains of *S. aureus* COL strains.

Growth Curve

Growth curves of the strains of *S. aureus* LAC and UAMS-1 strains showed that deletion of the *msa* gene did not affect the growth of the strains (Figures 16 and 17).

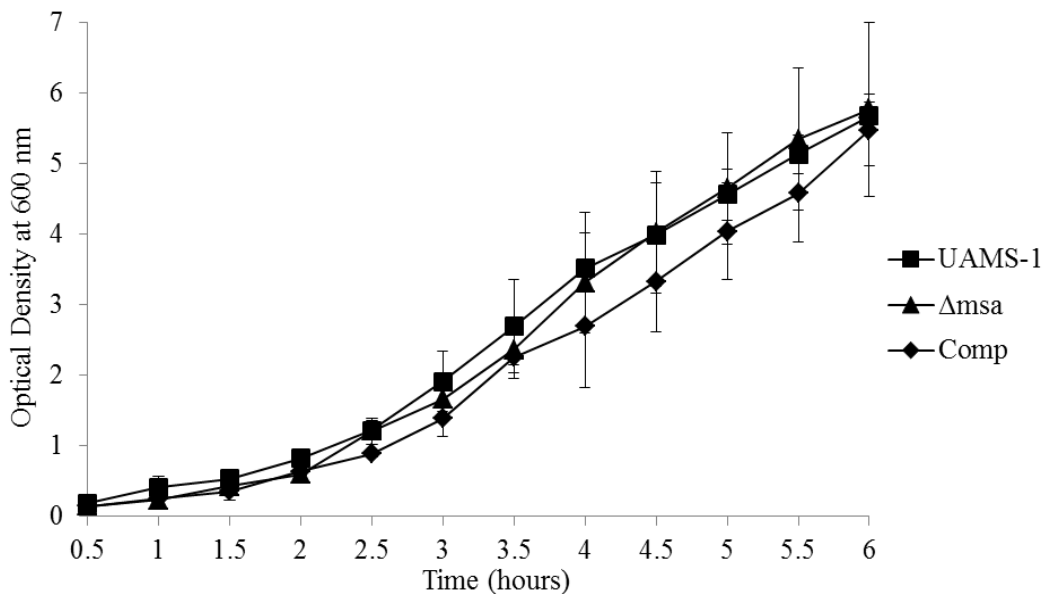


Figure 16. Growth Curve of *S. aureus* UAMS-1 strains showing that the deletion of the *msa* gene of *S. aureus* UAMS-1 does not influence the viability of *S. aureus* UAMS-1.

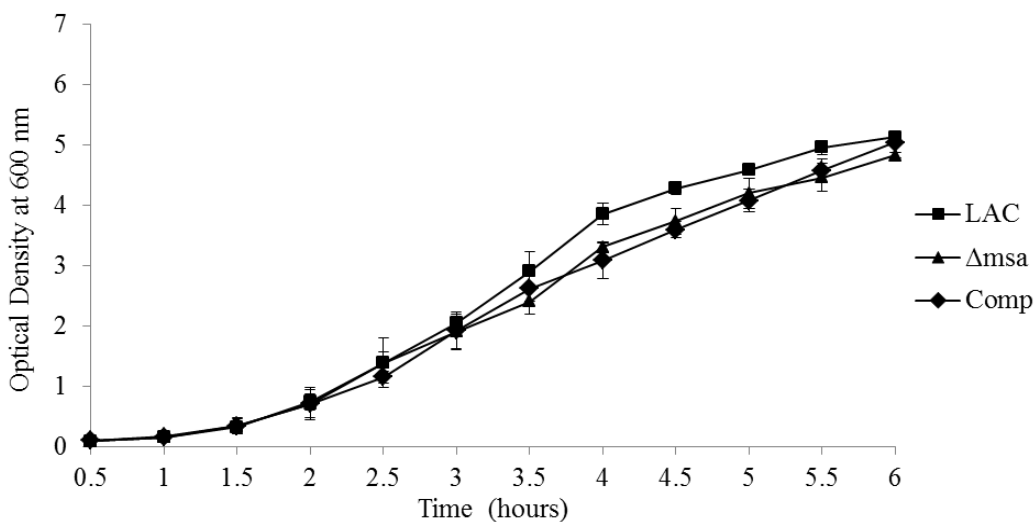


Figure 17. Growth Curve of *S. aureus* LAC strains showing that the deletion of the *msa* gene of *S. aureus* LAC does not influence the viability of *S. aureus* LAC.

Phenotypic Characterization

Biofilm Assay. Wild type strains of *S. aureus* COL have the ability to produce biofilms and Sambanthamoorthy et al. (2006, 2008) showed that insertional mutation of *msa* reduced the biofilm formation. These previous findings matched the observations of the biofilm assay with the deletion mutants of *S. aureus* COL (Figure 18).

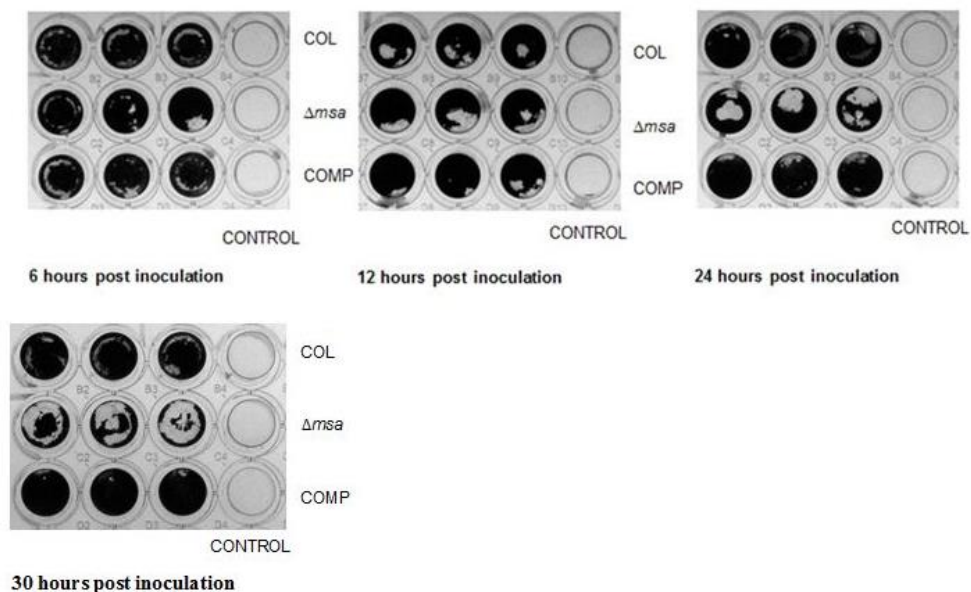


Figure 18. Biofilm Assay of *S. aureus* COL strains showing the weak biofilm formation in the *msa* mutants. Control wells contained uninoculated media to represent negative controls.

Protease assay. Protease activity of *S. aureus* COL was also assessed and showed that deletion of the *msa* gene increased the protease activity of the strains (Figure 19). This phenotype was also observed in the insertional mutants of *S. aureus* COL (Sambanthamoorthy, Smeltzer, & Elasri, 2006).

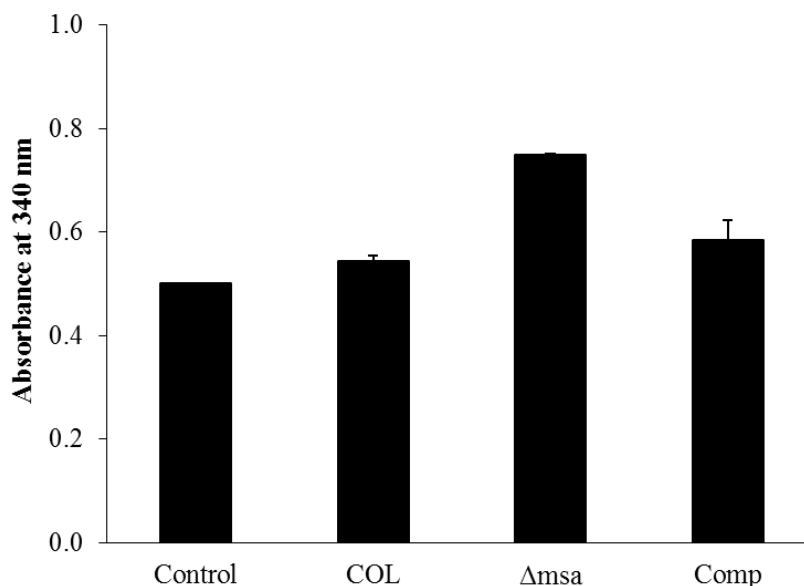


Figure 19. Protease Activity of *S. aureus* COL strains showing increased protease activity in the *msa* mutants compared to the wild type strains. Un-inoculated media was used as a negative control.

Hemolytic assay. Deletion of the *msa* gene reduced hemolytic activity of the *S. aureus* COL strain. The reduced hemolytic activity of the deletion mutants of *S. aureus* COL (Figure 20) were similar to that observed by Sambanthamoorthy et al. (2006).

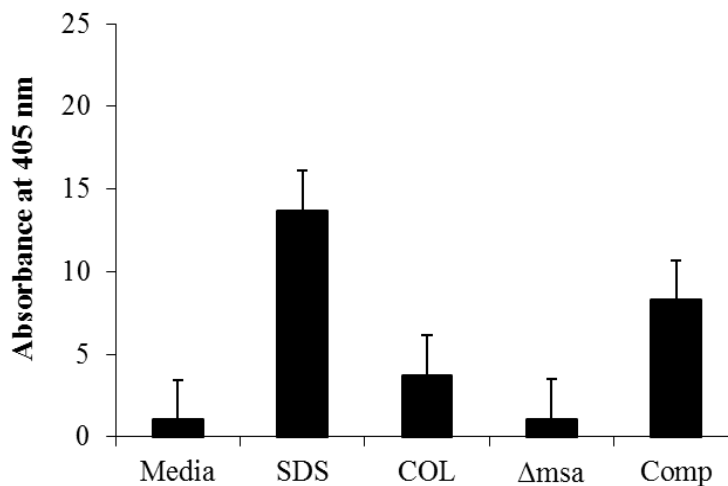


Figure 20. Hemolytic Assay of *S. aureus* strains of COL showing reduced hemolytic activity on deleting the *msa* gene. Un-inoculated media and 2% SDS solution were used as a negative and positive control, respectively.

Discussion

The *msa* gene was successfully deleted from the *S. aureus* strains of COL, USA300 LAC, UAMS-1, and RN6390. Phenotypic characterizations observed with the *msa* mutant of COL were similar to that of the insertional mutants (Sambanthamoorthy, Smeltzer, & Elasri, 2006; Sambanthamoorthy, Schwartz, Nagarajan, & Elasri, 2008).

Further studies on virulence and immune evasion were conducted with mutants in the USA300 LAC and UAMS-1 strains. Hence, to ensure the mutation of *msa* did not affect the viability of the strains, growth of the wild type was monitored along with the mutant. As seen in Figures 16 and 17, mutation of *msa* did not affect the growth and viability of the strains. Thus, the deletion mutants of the *msa* gene were employed to explore the features of global regulation in *S. aureus*.

CHAPTER IV

MSA REGULATES IMMUNE EVASION, HOST PERSISTENCE AND
DISSEMINATION IN THE CLINICAL ISOLATES OF *STAPHYLOCOCCUS AUREUS*

Abstract

Staphylococcus aureus is an opportunistic pathogen residing usually on the skin and nasal passages of humans. On gaining entry into the host, *S. aureus* has the ability to colonize and cause localized infections or spread and infect organs on the path of dissemination. The *msa* gene of *S. aureus* has been previously observed to regulate several virulence genes. Here in this study, we examined the influence of the *msa* gene on the regulation of immune evasion, host persistence, and dissemination employing the hospital-acquired strain *S. aureus* UAMS-1 and community-acquired strain *S. aureus* USA300 LAC. The murine sepsis model used to study host persistence and dissemination, showed that deleting the *msa* gene of UAMS-1 decreased the microbial surviving load in the lung, whereas the mutation in LAC showed no change in dissemination and colonization. The *msa* mutant in UAMS-1 also showed a decrease in survival in blood and in phagocytic-intracellular survival while in LAC the mutation showed no deterrence. On investigating the immune evasion factors regulated by the *msa* gene, we found significant fold changes in the expression of the *scn*, *clfA*, *spa*, *aur* and *sak* genes that play specific roles of evasion at the various stages of phagocytic uptake, namely chemotaxis, opsonization, and phagocytosis. Comparing the regulation of the *msa* gene on the individual factors in the two strains we observed the differential regulatory behaviour by the *msa* gene. While in LAC, the *msa* mutation decreased only the *clfA* levels in UAMS-1, the *msa* mutation decreased *scn*, *clfA*, *spa*, *aur*, and *sak* genes. In addition, the *msa* gene increased *aur* levels in LAC, but in UAMS-1 it decreased the *aur*

levels. Thus, we have not only observed the regulatory effect of *msa* on staphylococcal immune evasion but also the strain-dependent variation of this regulation. In addition, this study shows more evidence of the undeterred virulence of the community acquired USA300 LAC strains.

Introduction

Staphylococcus aureus, we know causes a broad spectrum of infections that range from superficial skin infections like folliculitis, and carbuncles, to more severe endocarditis, and osteomyelitis. Additionally, *S. aureus* causes sepsis—a clinical syndrome caused by entry of a pathogen into the blood stream whose severe clinical manifestations are caused more by the host response to the pathogen than the pathogen itself. Sepsis is characterized by the extensive inflammation along the path of the blood stream which includes the liver, kidney, lungs, and the cardiovascular system on the whole. Components of the *S. aureus* cells like peptidoglycan or the sheer presence of the organism can trigger the release of cytokines, chemokines, and several inflammatory mediators from the epithelial, endothelial, and other stimulated cells of the immune system. Thus, in order to study the role of the *msa* gene in host persistence and metastasis of infection we employed a murine sepsis model.

One of the most common causes of septicemia caused by post-surgical infections, tissue trauma, and indwelling device-related infections is due to MRSA. Kreisel and colleagues, while studying cases of bacteremia caused by USA300 and non-USA300 MRSA, pointed out that 25% of the cases were caused by USA300 MRSA, the skin and soft tissue infection causing strains (Kreisel et al., 2011).

CA-MRSA strains have been observed to be more resilient than the Hospital acquired MRSA (HA-MRSA) strains (Voyich et al., 2005). Voyich et al. pointed out that

the increased virulence seen with USA300 strains was due to its ability to resist PMN killing and cause host cell lysis possibly by the secreted and surface associated virulence factors (Voyich et al., 2005; Diep et al., 2006; Foster, 2005). The hallmark of sepsis is the severe host-associated damage caused by the immune system. This severity and longevity is largely contributed by the persistence of the *S. aureus* CA-MRSA strains in the host. This recalcitrance of CA-MRSA strains is still not completely understood. Studying the behavior of *msa* in the sepsis model, phagocytic evasion, and infection dissemination we hoped would help understand staphylococcal sepsis and virulence and shed light on the recalcitrant pathogenicity of USA300 strains of *S. aureus*.

The *msa* gene has been seen to regulate several virulence factors and, thus, proposed to play a vital role in virulence (Sambanthamoorthy, Smeltzer, & Elasri, 2006). These virulence factors have also been observed to play a role in immune evasion. Persistence of the pathogen in sepsis is largely contributed by the fact that the pathogen can co-exist with the very immune factors that are trying to destroy it; in other words, sepsis is possible by immune evasion. Thus, we investigated the role of *msa* in immune evasion especially in the phagocytic evasion of the neutrophils, an important member of the innate immune system. Here, we observed the role of *msa* in immunological clearance of a CA-isolate (USA300 LAC strain) in comparison with a HA-clinical isolate (UAMS-1). Employing these two strains, we studied if and how *msa* is one of the regulators required for the evasion of the innate immune system and pathogen persistence in a host.

Methods and Materials

Strains and Growth Conditions

The isolates *S. aureus* UAMS-1 and that of USA300 lineage LAC were used for this study. The *msa* gene was mutated by allelic replacement using pKOR-1 as described in Chapter III. Complementation was achieved using pCN34. All cultures were grown in TSB, Todd Hewitt Broth (THB), pooled human blood (Bioreclamation LLC), or horse blood (Thermo Fisher Scientific Inc.) according to the experimental need at 37°C. The complements of the *msa* mutants were selected by kanamycin (50 µg/ml).

Sepsis Model

Ten six-week old CD1 Swiss, outbred and immunocompetent mice (Charles River Laboratories) were inoculated with 10^8 of wild type and mutant bacteria belonging to the strains of USA300 LAC and UAMS-1, while control mice were inoculated with PBS. These bacteria were administered in the tail intravenously in a 100 µl bolus (in DPBS). Mice were monitored every 3rd hour for 24 hours followed by every 8th hour for 72 hours.

Blood Survival Assay

To assess the ability of *S. aureus* to survive in blood, the strains of *S. aureus* USA300 LAC and UAMS-1 were inoculated in gender pooled whole human blood and incubated at 37°C for 3 hours. Samples were collected at the 0 and 3rd hour, serially diluted and plated. The colony forming units were counted after 18 hours of incubation at 37°C.

Growth Curve and Viability Assays

The bacterial strains were grown in TSB with a starting OD₆₀₀ = 0.05. Optical density was measured every 30 min and plotted against time. For viability counts, cells

were collected at the exponential phase of growth, serially diluted and plated on TSA. The colony forming units were counted after 18 hours of incubation at 37°C.

Neutrophil Isolation

Neutrophils were isolated from blood obtained from healthy human volunteers. An informed consent approved by the Institutional Review Board at The University of Southern Mississippi was obtained from the volunteers. Briefly, Ficoll-Plaque PLUS (GE Healthcare Biosciences) was mixed with freshly obtained whole blood at a ratio of 1:2. This mixture was centrifuged at 400 x g for 30 – 40 min at 20°C. The layers containing plasma, lymphocyte, and Ficoll were removed. The pellet was re-suspended to its original volume. Equal volume of dextran/saline solution was added and mixed gently. This suspension was allowed to stand at 25-45 degree angle for an hour at room temperature followed by a 90 degree angle for 10 min. The suspension separated into two distinct layers: the upper clear PMN layer and the lower layer. The PMN layer was carefully transferred to a clean tube and spun down for 1500 rpm for 10 min. The resulting pellet was re-suspended in 10 ml of 0.2% NaCl and the RBCs were lysed by gently pipetting up and down for 1-2 min. Following this, 1.6% NaCl was added and spun down at 1500 rpm for 10 min. The resulting PMNs were re-suspended in the desired volume of RPMI plus 10% Fetal Bovine Serum.

In vitro Phagocytosis Assay

Overnight cultures of *S. aureus* were normalized and grown for 100 min before collecting them by centrifugation. These cells were washed using 1X PBS and opsonized by incubating them with 10% orthologous human serum. Post opsonization, cells were collected by centrifugation and re-suspended in RPMI 1640 medium. Neutrophils and bacteria were mixed at a MOI of 1:100 and incubated at 37°C and 5% CO₂. Samples

were collected every 15 min for serial dilution and plating on Todd-Hewitt Agar. Gentamicin (final concentration of 400 µg/ml) was added to kill the extracellular bacteria. Neutrophils were collected by centrifugation, washed with PBS, and lysed in 37°C warm 0.02% Triton X containing PBS. This enables enumeration of the surviving intracellular bacteria only. Colonies were counted after 15 hours of incubation at 37°C and represented in a log plot as CFU/ml against time (minute).

Neutrophil Activation Assay

Neutrophils activation was assessed according to a modified version of the protocol described by Gouwy and group (Gouwy, Struyf, Catusse, Proost, & Van Damme, 2004). Briefly, post opsonization, cells were prepared similar to the phagocytosis assay, mixed with bacteria and incubated for 10 minutes at 37°C and 5% CO₂. Samples were arrested by fixing them with 2% glutaraldehyde. Neutrophil activation was assessed by the change of shape using a scanning electron microscope.

Scanning Electron Microscopy

After fixation with 2% glutaraldehyde, samples were washed with PBS and serially dehydrated by consecutive incubations in 25%, 50%, 75%, 90%, and 100% ethanol and HDMS. Samples were mounted onto specimen mounts and sputter coated with platinum (5 nm). Images were acquired on a FEI Quanta 200 Scanning Electron Microscopy (FEI Company, OR.)

Detection of the Immune Evasion Cluster

The immune evasion cluster (IEC) of USA300 and UAMS-1 was detected by polymerase chain reaction (PCR) using the primers described by van Wamel and group (van Wamel Rooijackers, Ruyken, Van Kessel, & Van Strijp, 2006).

Chemotaxis Assay

The Chemotaxis Assay will be conducted according the protocol described by Durr et al., 2006. Migarated PMNs were stained with BCECF AM (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein- acetoxymethyl) and enumerated by flow cytometry.

Clumping Factor Quantification Assay

ClfA binds to both soluble and insoluble fibrinogen and this binding was assayed using fibrinogen as described by (Que et al., 2000). Briefly, two-fold serial dilutions of 1 mg/ml of fibrinogen (100 μ l) were tested with 20 μ l of cells washed in ice cold 0.9M NaCl and resuspended in 1/10 of the initial volume in the same NaCl solution. Both strains were mixed with the fibrinogen dilutions and incubated at 4°C for 24 hours. The wells were washed with 1X PBS thrice, fixed with 100% ethanol and stained with crystal violet. The plate was allowed to dry after washing off the excess dye. The wells were washed with 1X PBS. Hundred percent of alcohol was added to each well and allowed to stand for 10 min for the dye to release itself from the cells. The absorbance of the released dye was measured at 595 nm.

Protease Assay

Filter sterilized culture supernatants were collected from post exponentially growing wild type and mutant strains of USA300 LAC and UAMS-1. Azocasein (3 mg/ml) prepared in Tris Buffered saline (pH 7.5) was added to the supernatant and incubated in the dark at 37°C. After overnight incubation, trichloroacetic acid (50%) was added to precipitate the un-degraded azocasein. Centrifugation separated the precipitate. Protease activity was assayed by measuring the absorbance (340 nm) of the supernatant. In order to quantify the metalloproteases, EGTA (1 mM) was employed.

Hemolytic Assay

Hemolytic assay was conducted according to the protocol described in Chapter III.

RNA Isolation and Real Time qPCR

RNA was isolated according to the protocol described by Sambanthamoorthy, et al. (Sambanthamoorthy, Smeltzer, & Elasri, 2006). The primers used are described in Table 9. Gyrase (*gyr*) was used as an endogenous control.

Table 9

Primers used for RT-qPCR

Gene	Primer Sequence (5' to 3')	Reference
<i>clfa FP</i>	CCT GAT GAG CCT GGT GAA AT	(Sambanthamoorthy, Smeltzer, & Elasri, 2006)
<i>clfa RP</i>	ATC GCT GCC AGA ATC TGA AC	
<i>chp FP</i>	ACCGTTTCCTACAAATGA	(Burian, Wolz & Goerke, 2010)
<i>chp RP</i>	TTCAGCAAGTGGTGTATTC	
<i>scn FP</i>	TTGCCAACATCGAATGAA	(Burian, Wolz & Goerke, 2010)
<i>scn RP</i>	CATTGCTTTTTGACCTGAA	
<i>map FP</i>	AATAATAATGAAGCGTCTGC	(Burian, Wolz & Goerke, 2010)
<i>map RP</i>	CCTACTTTCAAATCGANAAC	
<i>sak FP</i>	TGTAGTCCCAGGTTTAATAGG	(Burian, Wolz & Goerke, 2010)
<i>sak RP</i>	CGCGAGTTATTTTGAACC	
<i>spa FP</i>	AGA TGA CCC AAG CCA AAG TG	(Sambanthamoorthy, Smeltzer, & Elasri, 2006)
<i>spa RP</i>	GCT TTC GGT GCT TGA GAT TC	

Table 9 (continued).

Gene	Primer Sequence (5' to 3')	Reference
<i>hla FP</i>	GAA AGG TAC CAT TGC TGG	(Sambanthamoorthy, Smeltzer, & Elasri, 2006)
<i>hla RP</i>	TCA AAG GCC AGG CTA AAC CAC TT	
<i>aur FP</i>	GCA CTT TAT CAC CAG CAG CA	(Sambanthamoorthy, Smeltzer, & Elasri, 2006)
<i>aur RP</i>	TTG ACC GCA TCA CTC TTT TG	
<i>gyr FP</i>	CAT TGC CAG ATG TTC GTG AC	(Sambanthamoorthy, Smeltzer, & Elasri, 2006)
<i>gyr RP</i>	CCG GTG TCA TAC CTT GTT CA	

Western Blot

Cells were collected at the appropriate growth phase and washed with PBS before lysing them with lysostaphin and glass beads. Lysates were loaded on SDS-PAGE gels and immunoblots were tested for protein A, using anti-Spa antibodies (Sigma-Aldrich).

Defensin Susceptibility Assay

Susceptibility to defensins was assessed as described by Jin et al. (2004). Briefly, cultures were grown in THB and normalized to an OD of 0.05. To these cultures Human Neutrophil Protein-1(HNP-1) and HNP – 2 (5 µg/ml) was added separately. Viability was tested by collecting samples after 6 hours of incubation at 37°C. Samples collected were serially diluted and plated on horse blood agar. Colonies were enumerated after 18 hours of incubation at 37°C.

Results

msa is Required for Persistence and Dissemination of *S. aureus*

Persistence and dissemination of the wild type and *msa* mutants of *S. aureus* strains were studied using a murine sepsis model. No difference was seen between the wild type and the *msa* mutants of LAC strains (Figure 21). On the other hand there was a decrease in bacterial load in the lungs of the mouse infected with the *msa* mutant of UAMS-1 compared to the wild type (Figure 22).

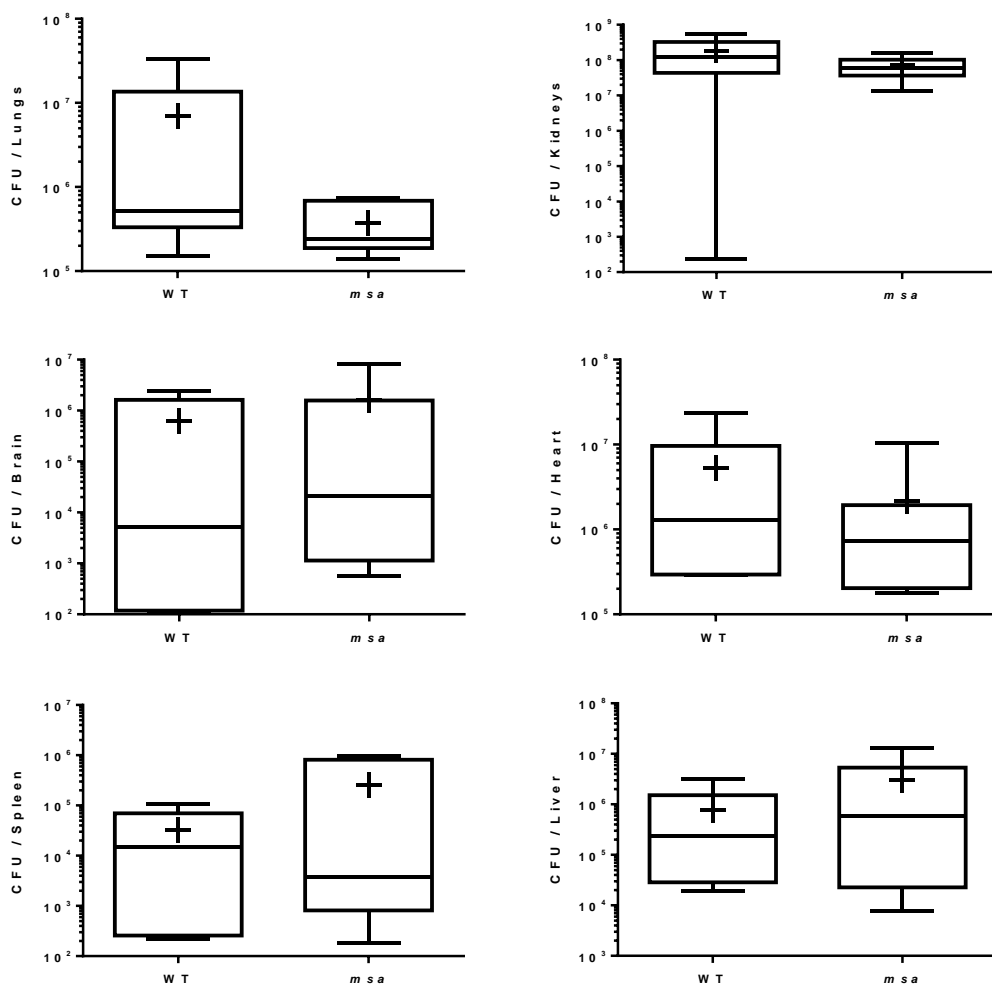


Figure 21. Bacterial load of wild type and mutant strains of *S. aureus* LAC strains in the various organs of the murine sepsis model. In these box and whisker graphs, the solid line is the median and the + is the mean. The boxes represent the interquartile range (IQR). No statistical significance seen (Mann Whitney test): $P < 0.05$.

Table 10

Statistical Significance by Mann Whitney test of LAC strains in the sepsis model

Organ	Significance by Mann Whitney test
Lungs	0.362
Kidneys	0.362
Brain	0.356
Heart	0.524
Spleen	0.798
Liver	0.700

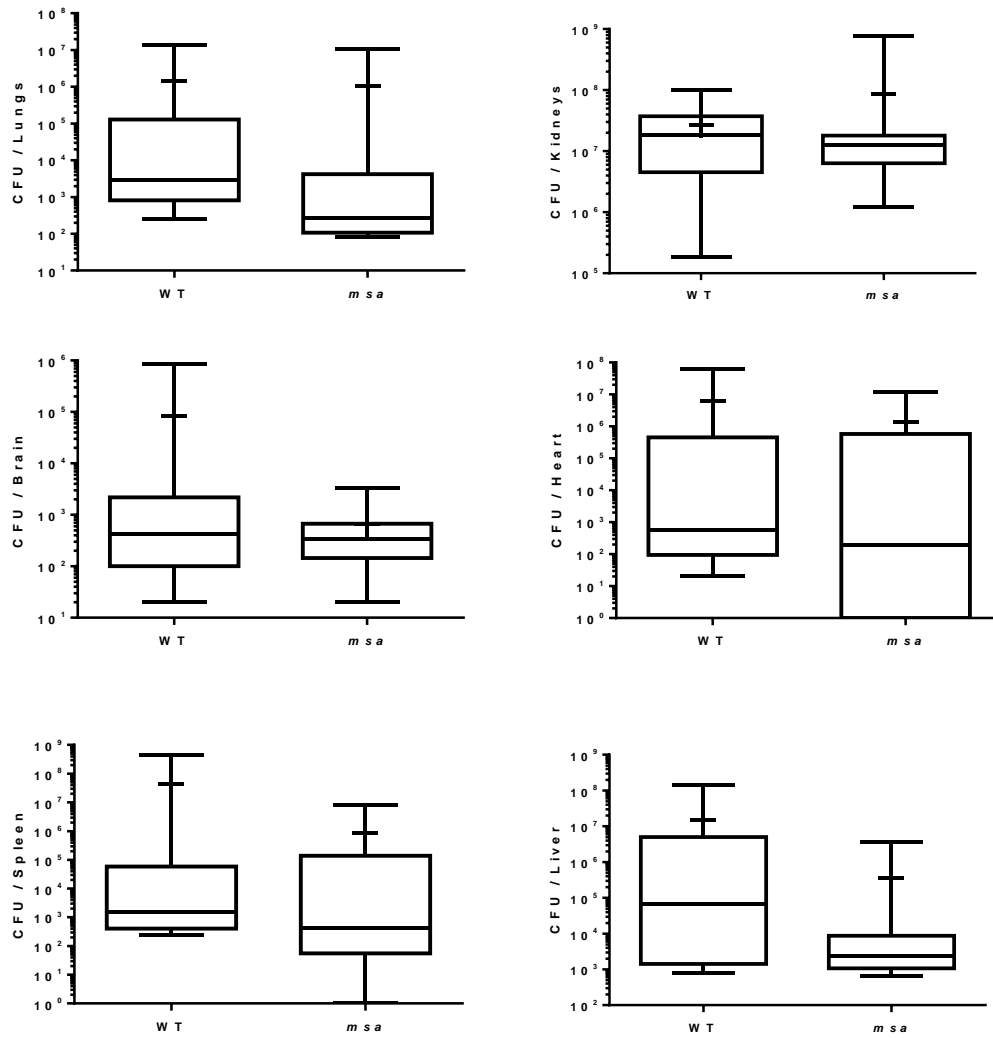


Figure 22. Bacterial load of wild type and mutant strains of *S. aureus* UAMS-1 strains in the various organs of the murine sepsis model. In these box and whisker graphs, the solid line is the median and the + is the mean. The boxes represent the interquartile range (IQR). Statistical significance (Mann Whitney test) seen only in the case of Lungs: $P < 0.05$.

Table 11

Statistical Significance by Mann Whitney test of UAMS-1 strains in the sepsis model

Organ	Significance by Mann Whitney test
Lungs	0.024
Kidneys	0.670
Brain	0.645
Heart	0.362
Spleen	0.210
Liver	0.188

Deletion of msa Influences Survival in Blood

Mutant and wild type strains of USA300 LAC and UAMS-1 were tested for their ability to survive in gender pooled whole human blood. Mutation in USA300 LAC increased its ability to survive (Figure 23) while in UAMS-1 it decreased the survival in blood (Fig. 24). When comparing the wild types of the two strains of USA300 LAC and UAMS-1, clearly the osteomyelitis strain UAMS-1 survived better and multiplied more than the skin and the SSTI causing USA300 LAC.

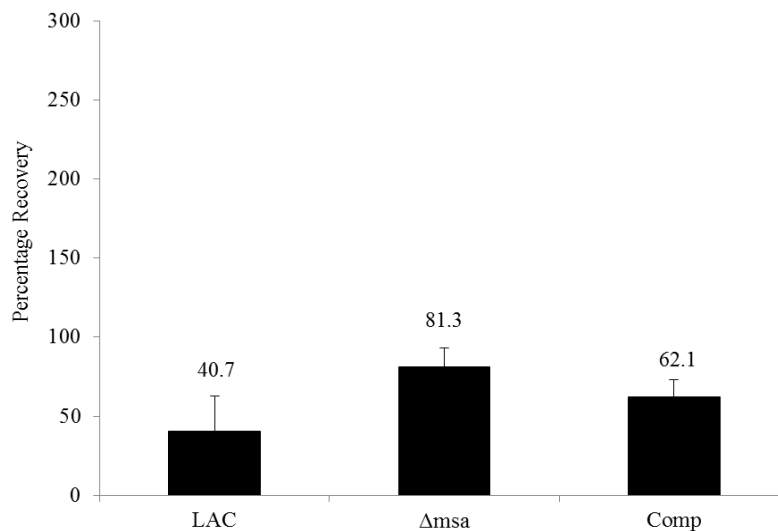


Figure 23. Increased percentage survival of the *msa* mutants of *S. aureus* LAC strains in whole human gender-pooled blood compared to the wild type and complement strains. Deletion of the *msa* gene revealed increased percentage survival in the *msa* mutants of *S. aureus* LAC strains.

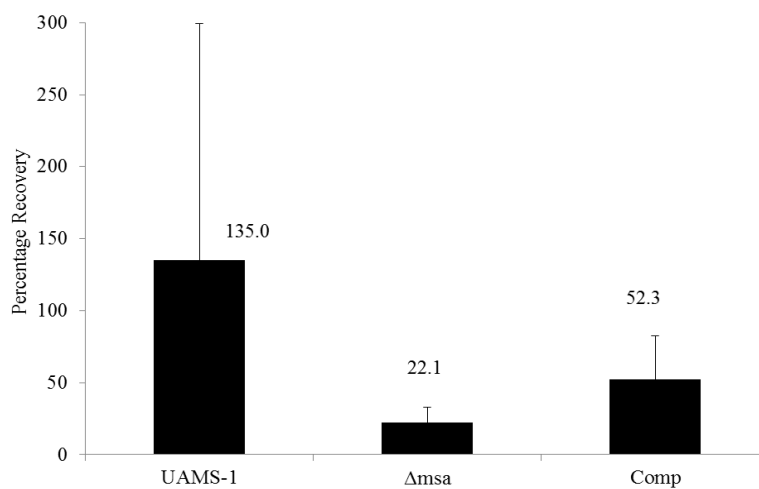


Figure 24. Decreased percentage survival of the *msa* mutants of *S. aureus* UAMS-1 strains in whole human gender-pooled blood compared to the wild type and complement strains. Deletion of the *msa* gene revealed decreased percentage survival in the *msa* mutants of *S. aureus* UAMS-1 strains.

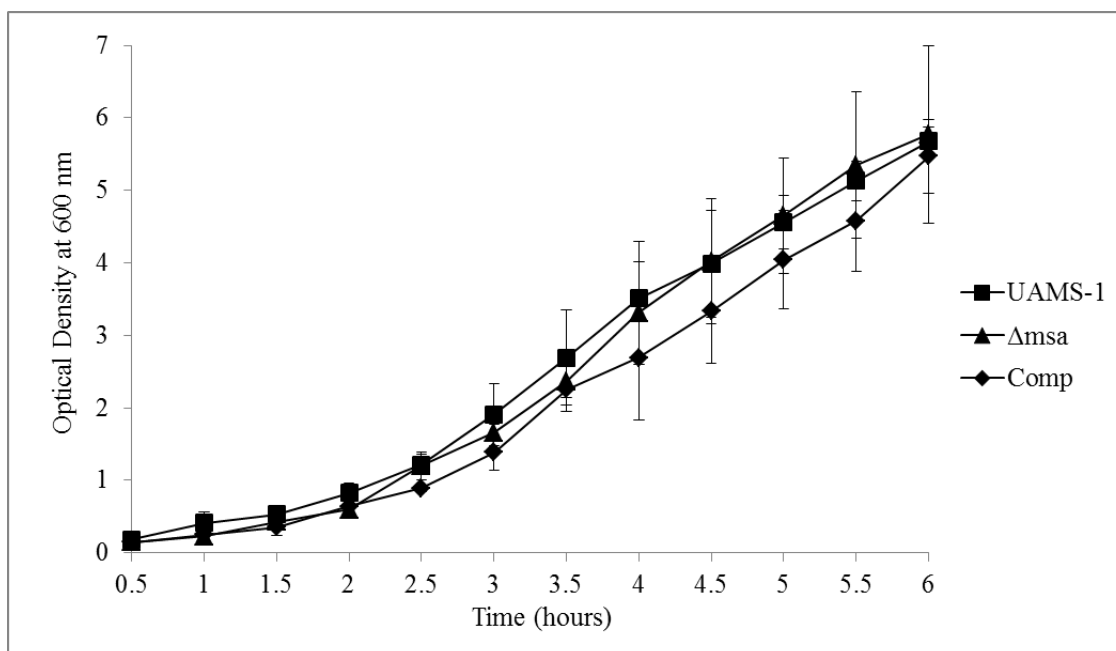


Figure 25. Growth Curve of UAMS-1 strains showing no effect on the growth of UAMS-1 strains on deleting the *msa* gene.

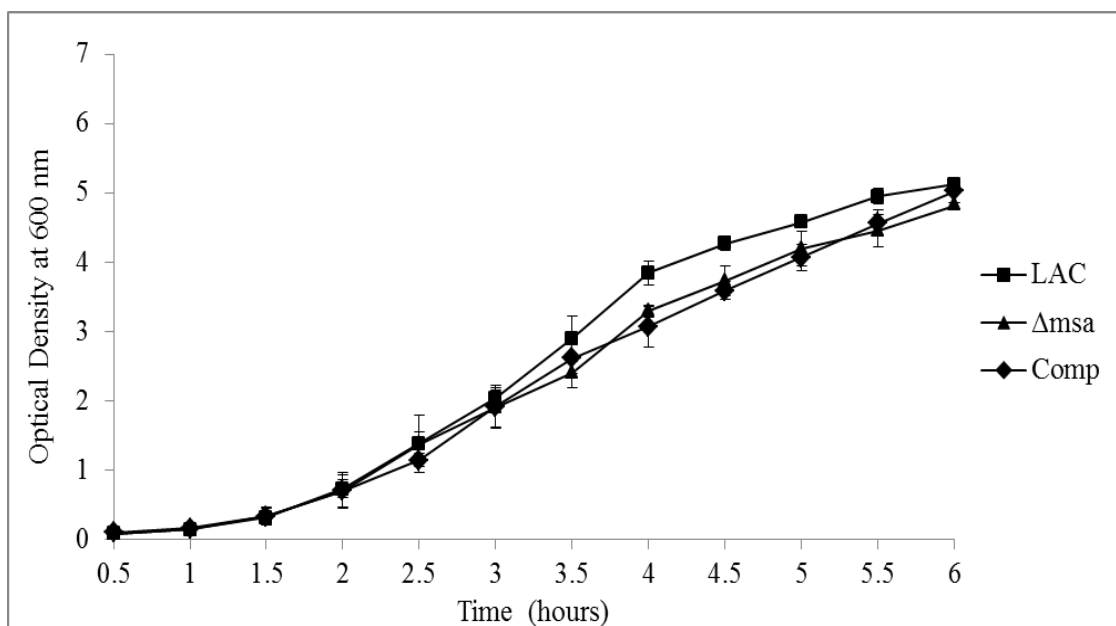


Figure 26. Growth Curve of LAC strains showing no effect on the growth of LAC strains on deleting the *msa* gene.

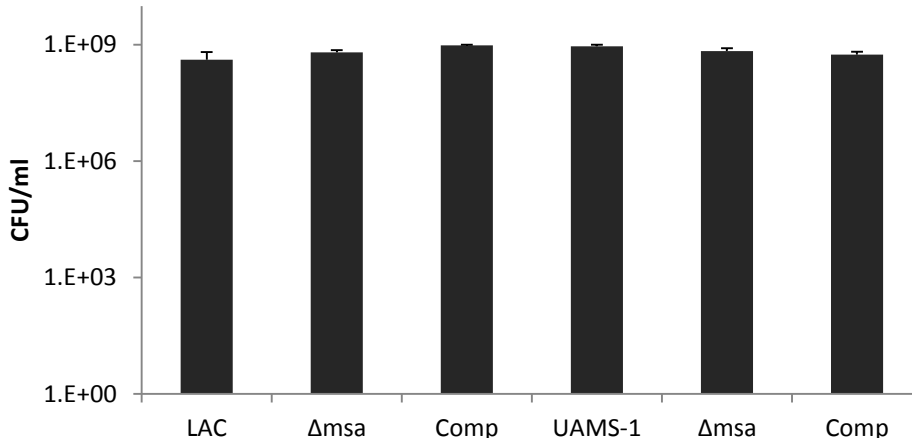


Figure 27. Viability Assay of the strains *S. aureus* LAC and UAMS-1 showing no change in viability on deleting the *msa* gene.

The effect of *msa* on growth and viability was also tested (Figure: 25-27). Neither strain showed a difference between the wild type and mutant. This, ruled out the possibility of a growth defect caused by the mutation and confirmed that the survival rate of the mutants in blood was influenced by the interactions of the bacteria with the components of the whole blood. In the presence of blood, the *msa* mutant of UAMS-1 was unable to survive as well as the wild type or the strains of LAC either. These results suggest that *msa* plays an important role in determining clearance or persistence of *S. aureus* in the bloodstream of the host. However, this role was observed to differ across the two genetic backgrounds tested.

msa Does Not Contribute to Preventing Chemotaxis

Chemotaxis assay using the supernatant of strains of LAC and UAMS-1 did not reveal any significant difference in attracting PMNs as seen in Figure 28. Thus, we have no evidence of *msa* preventing chemotaxis.

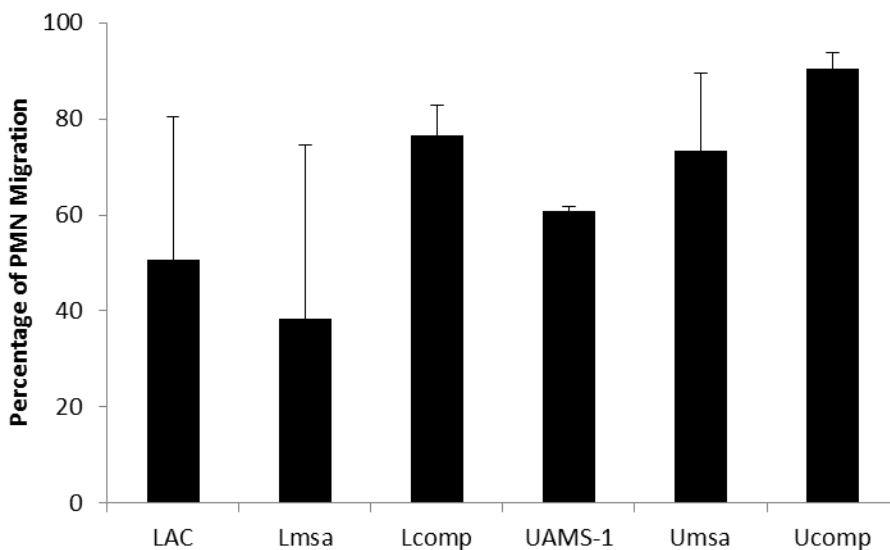


Figure 28. Percentage of PMN migrating in response to the supernatant of the strains of LAC and UAMS-1. No significant change was observed in the chemotaxis of neutrophils in response to the supernatants of *S. aureus* strains.

msa Prevents Neutrophil Activation

Neutrophils are spherical in shape until they are activated by the presence of a pathogen, then they branch out to form pseudopodia and invagination cups to engulf the pathogen. After incubating the neutrophils for 10 minutes with the strains of LAC and UAMS-1, only the neutrophils in the presence of *msa* mutants of UAMS-1 showed extensive branching, and the UAMS-1 complements showed shorter branching. Thus, based on the morphological change, we report that the mutants increased the activation of neutrophils. All other strains retained their spherical shape.

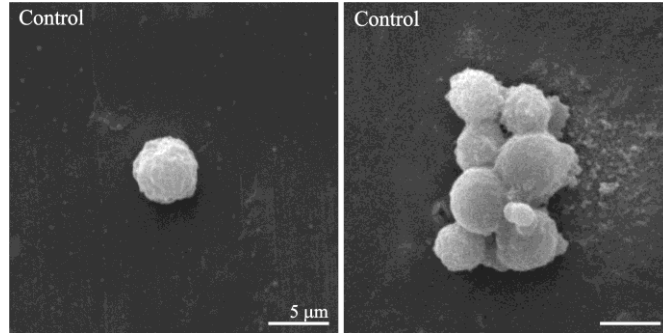


Figure 29 a. Scanning Electron Microscopy showing the spherical neutrophils in the absence of *S. aureus*. Neutrophils in the absence of *S. aureus* were used as a negative control to reveal the spherical shape of inactive neutrophils.

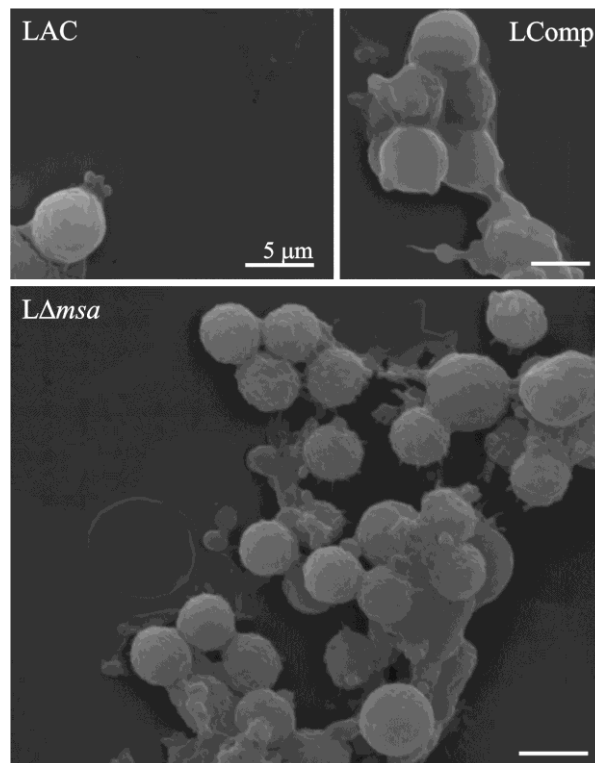


Figure 29 b. Scanning Electron Microscopy of neutrophils showing no change in shape in the presence of *S. aureus* LAC strains

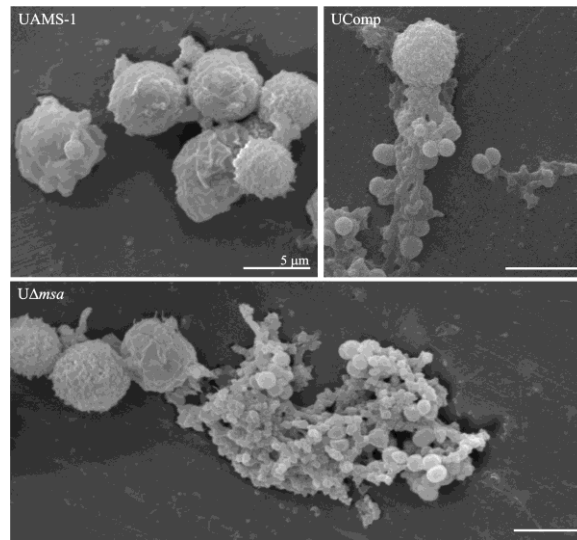


Figure 29 c. Scanning Electron Microscopy of neutrophils comparing the extent of branching of neutrophils in the presence of UAMS-1 strains.

msa contributes to the outcome of PMN attack

Neutrophils are the primary components of the innate immune system whose rapid, un-biased response clears pathogens from the host system. We tested the effect of this non-specific clearance by neutrophils on the mutant and wild type strains of USA300 LAC and UAMS-1 on engulfing the cells (Figure 30). The mutant in UAMS-1 showed decreased intracellular counts when rescued from the neutrophils (Figure 32). In some experimental runs (not shown here), the UAMS-1 mutant showed zero CFUs reflecting death and eradication of the mutant by the neutrophils. Thus, mutation of *msa* in the UAMS-1 strains somehow made these strains susceptible to immunological clearance by neutrophils compared to the wild type UAMS-1. The same mutation in LAC strains showed no change in the intracellular survival (Figure 31). The mutant and wild type strains of USA300 LAC survived equally well when exposed to phagocytosis by human neutrophils.

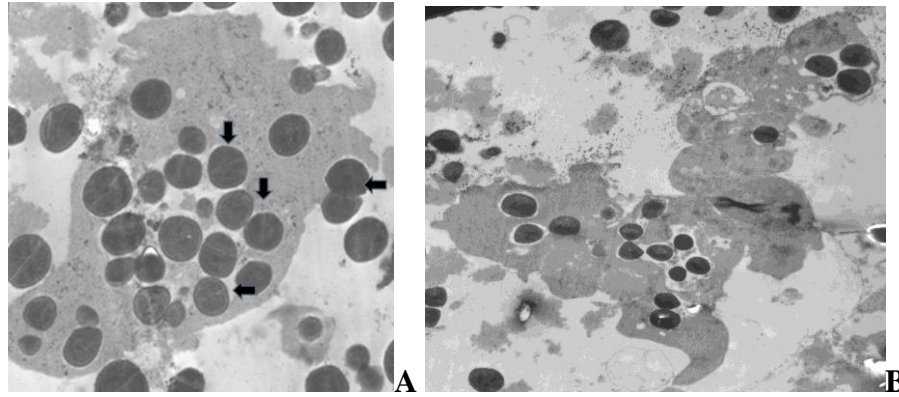


Figure 30. TEM image showing the intracellular presence of *S. aureus* (A) LAC strains and (B) UAMS-1 in human neutrophils.

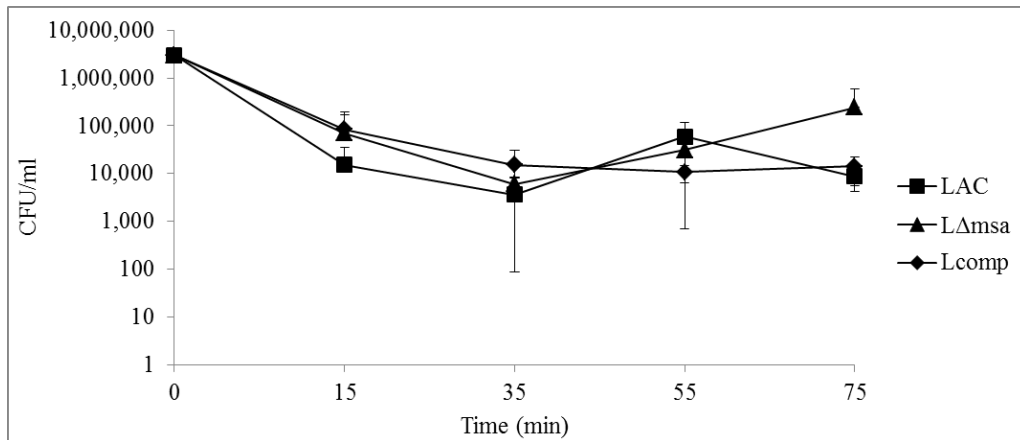


Figure 31. Phagocytosis Survival Assay of *S. aureus* LAC strains showing no difference in intracellular survival on deleting the *msa* gene.

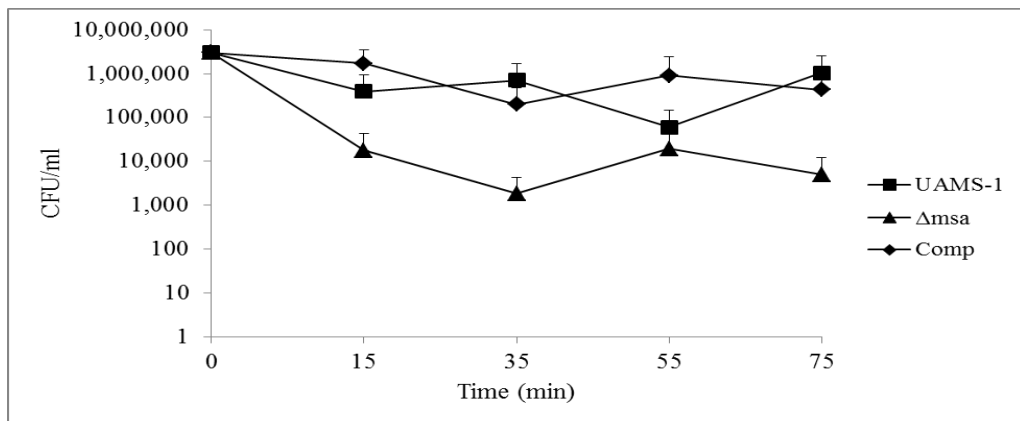


Figure 32. Phagocytosis Survival Assay of *S. aureus* UAMS-1 strains showing a decrease in intracellular survival on deleting the *msa* gene

msa regulates the Immune Evasion Cluster

USA300 strains of *S. aureus* were observed to carry bacteriophage-encoded immune evasion cluster (IEC) type B (Highlander et al., 2007). The IEC type in UAMS-1 was unknown. Based on the classification described by van Wamel (2006) and our results from the detection of the *sea*, *sak*, *chp* and *scn* by PCR (Figure 33), we report UAMS-1 to be IEC type A. RT-qPCR analysis showed that deletion of *msa* reduced the expression of *scn* and *sak* in UAMS-1 (Table 12). Thus, we show that *msa* regulates IEC, a mobile genetic element of UAMS-1 strains.

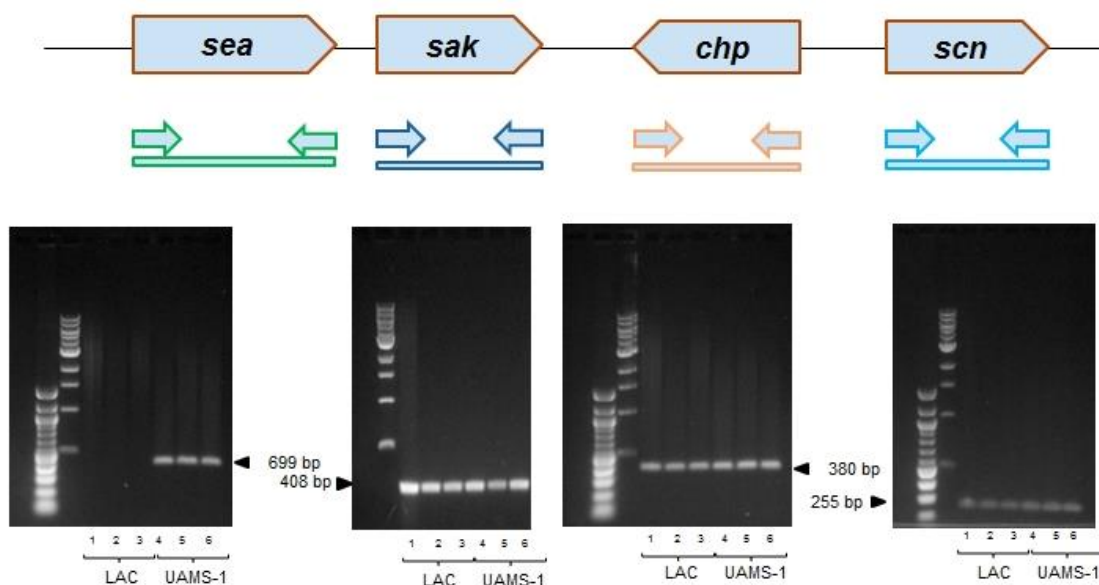


Figure 33. Detection of *sea*, *sak*, *chp* and *scn* genes in *S. aureus* LAC wild type (1), *LAC*Δ*msa* (2), LAC complement (3), UAMS-1 (4), UAMS-1Δ*msa* (5) and UAMS-1 Comp (6). *S. aureus* LAC strains bear the IEC type A and UAMS-1 the IEC type B.

msa Regulates Phagocytic Evasion Factors

Transcript analysis revealed several evasion factors to be regulated by the *msa* gene (Table 12) and, interestingly, the regulation differed between the staphylococcal strains of LAC and UAMS-1. In LAC, deletion of *msa* resulted in decreased expression

of *clfA* and increased expression of *aur*. In UAMS-1, there was a decrease in expression of *clfA*, *aur*, *scn*, and *spa*.

Table 12

Relative fold change in the expression of the genes required for phagocytic evasion on deleting the msa gene.

Gene	LAC	UAMS-1
<i>chp</i>	- 2.56 [¥]	3.23
<i>scn</i>	- 1.36	- 6.06 [¥]
<i>sak</i>	1.89	- 5.5 [¥]
<i>clfA</i>	- 10.96 [¥]	- 41.75 [¥]
<i>spa</i>	1.56	- 4.04
<i>aur</i>	8.12 [¥]	- 4.44 [¥]
<i>hla</i>	- 0.6	*
<i>sarA</i>	- 6.54 [¥]	- 6.03 [¥]
<i>agrB</i>	-2.52 [¥]	- 6.28 [¥]

Note. [¥] - p-value less than 0.05; * - Naturally mutated.

msa Prevents Opsonization

We tested several factors previously known to be employed by *S. aureus* to prevent opsonization and/or opsonophagocytosis to study if *msa* regulated any of them. The factors we tested were ClfA (Josefsson, Hartford, O'Brien, Patti, & Foster, 2001; Palmqvist et al., 2004, McAdow et al., 2011), protein A (Peterson, Verhoef, Sabath, & Quie, 1977; Gemmell, Tree, Patel, O'Reilly, & Foster, 1991; Palmqvist, Foster, Tarkowski, & Josefsson, 2002; Patel, Nowlan, Weavers, & Foster, 1987; Uhlen et al.,

1984), and capsular proteins (Nilsson et al., 1997; Thakker, Park, Carey, & Lee, 1998;).

The regulatory effect of *msa* on the expression of these factors was studied by analyzing the transcripts and assaying for each of these factors.

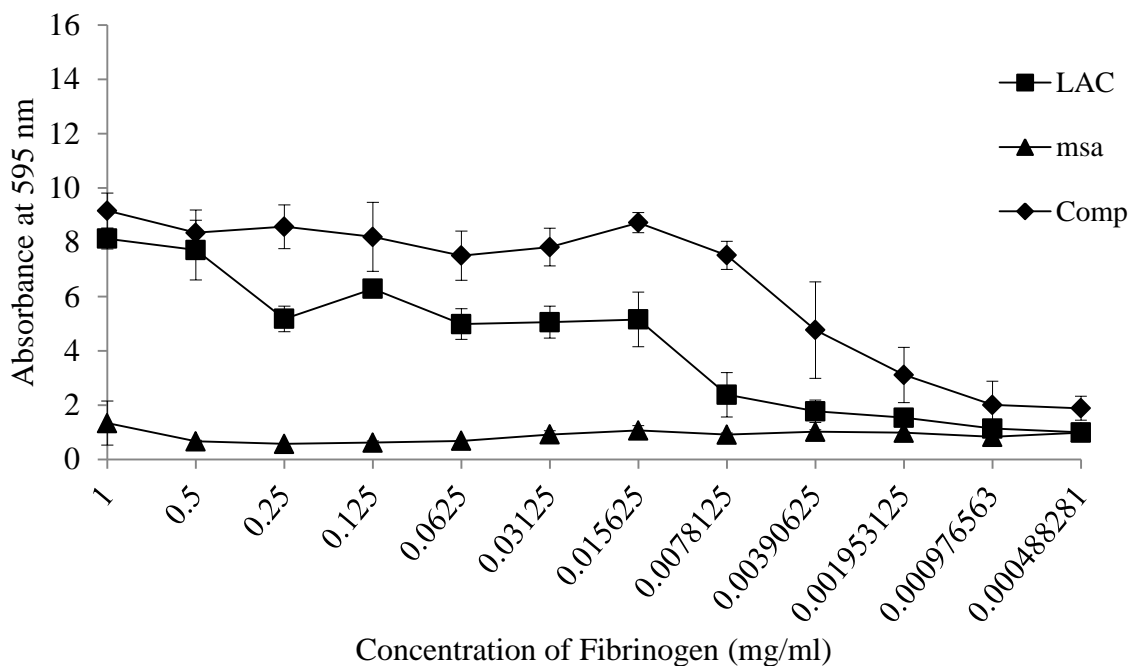


Figure 34. Quantification of ClfA levels based on the ability to bind to fibrinogen revealed reduced ClfA levels in the mutants of *S. aureus* LAC

Owing to the ability of ClfA to bind to fibrinogen, levels of surface exposed ClfA was measured by quantifying the cells bound to the range of fibrinogen concentrations. Deletion of *msa* in LAC and UAMS-1 showed a significant drop in fibrinogen-bound cells (Figure 34 and 35) indicating reduced levels of surface associated ClfA proteins. This observation was further supported by the reduced transcription of *clfA* seen in transcript analysis (Table 12). Thus, we have evidence that the *msa* gene is required for preventing opsonization by maintaining the levels of *ClfA* in *S. aureus*. Analyzing the *spa* transcripts and Spa protein levels using western blots showed no difference between wild

type and *msa* mutant suggesting that in both LAC and UAMS-1 strains (Figure 36) *msa* does not regulate the protein A.

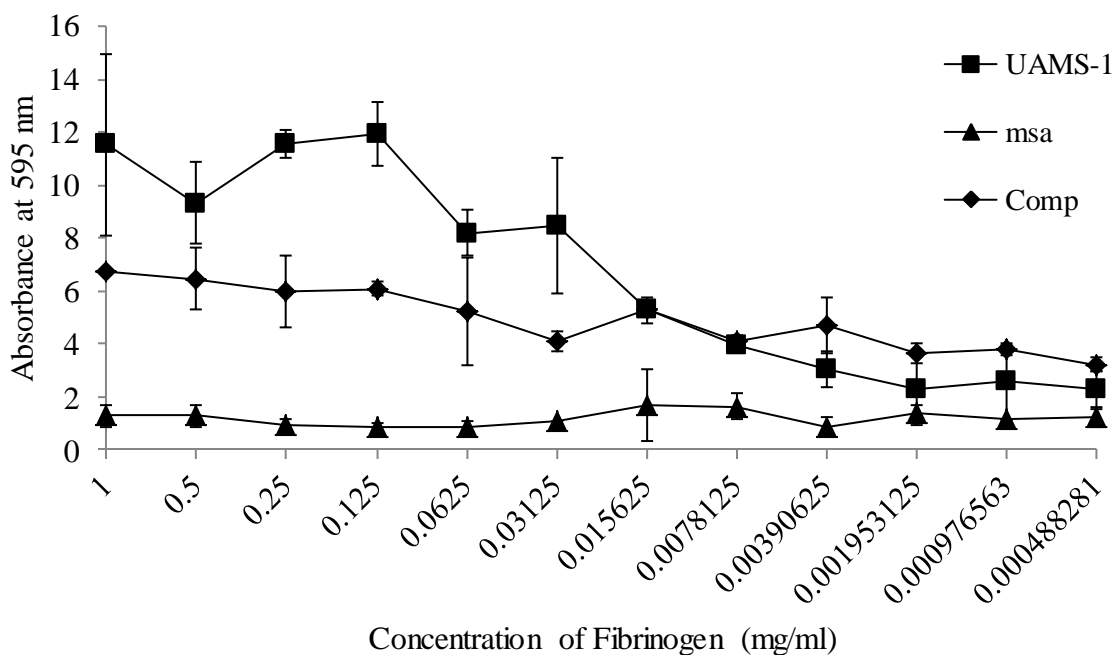


Figure 35. Quantification of ClfA levels based on the ability to bind to fibrinogen revealed reduced ClfA levels in the mutants of *S. aureus* UAMS-1.

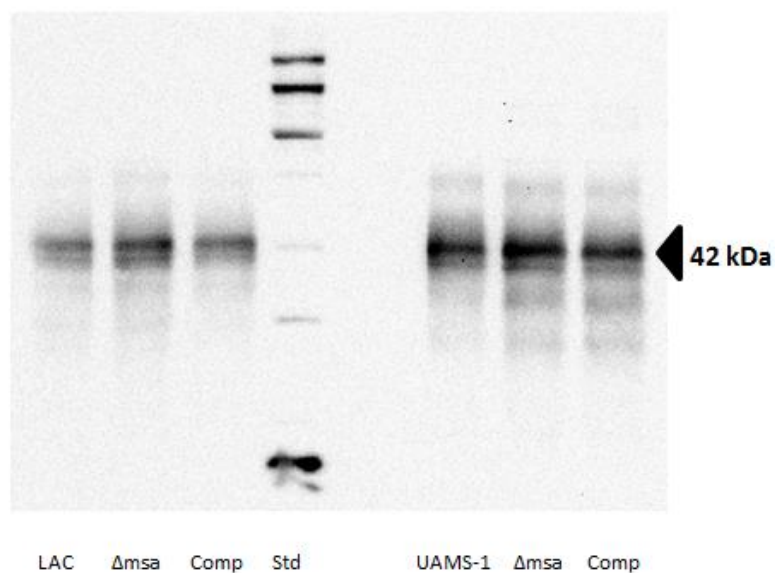


Figure 36. Western Blot with anti-Spa antibodies showing no difference in Spa levels between the wild type and mutant strains of LAC and UAMS-1.

When testing staphylokinase transcript levels in mutant and wild type strains, there was a -5.5 decrease in *sak* expression in UAMS-1 strains whereas in USA300 LAC there was no significant increase or decrease in *sak* levels (Table 12).

Taken together, these results indicate that of the factors tested *msa* regulates evasion of opsonization by regulating the *clfA* levels.

msa Does Not Aid in Escaping from the Phagocyte

Strains of *S. aureus* LAC express the α -hemolysin (encoded by *hla*) when phagocytized which aids in the escape of the bacteria by the lysis of PMN (Kubica et al., 2008, Pang et al., 2010). Hemolysin expression and toxicity was assayed. Cells were collected at an early stationary phase of about OD600 nm = 3.5 to analyze the transcript levels. Deletion of *msa* in LAC strains showed no significant change in *hla* transcription. The supernatant collected from the same time point was used for hemolytic assay, which showed no difference in activity between the wild type and mutant (Figure 37). UAMS-1 has an inherent mutation in the *hla* gene, which rules out the possible contribution it could have to lysis and phagocytic escape (Weiss et al., 2009).

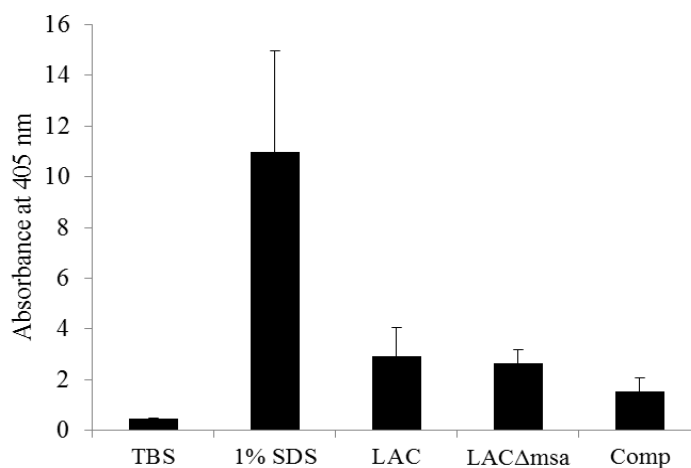


Figure 37. Hemolytic activity showing no difference between the mutant and wild type strains of *S. aureus* LAC strains

msa Contributes to the Intracellular Phagocytic Survival

Deletion of *msa* in *S. aureus* LAC increased the levels of extracellular proteases (Figure 38). Differential assaying to identify individual classes of proteases revealed aureolysin to be the most abundantly increased on deleting the *msa* gene. Supporting this finding, RT-qPCR also revealed an increase in *aur* expression (Table 12) on deleting the *msa* gene. In UAMS-1 deletion of *msa*, showed reduced transcripts (Table 12). This decrease in protease activity although was not seen in the protease assay (Figure 39). This suggested that *msa* contributes to regulation of intracellular survival via the protease levels although the extent of influence on the intracellular survival differs among strains of *S. aureus*.

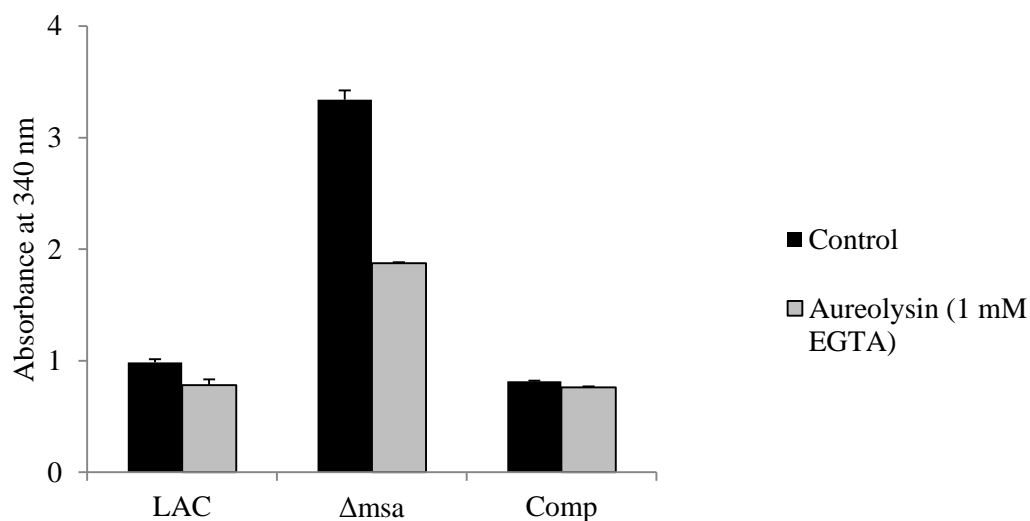


Figure 38. Protease activity of *S. aureus* LAC strains using azocasein as a substrate showed increased protease activity on deleting the *msa* gene in *S. aureus* LAC strains.

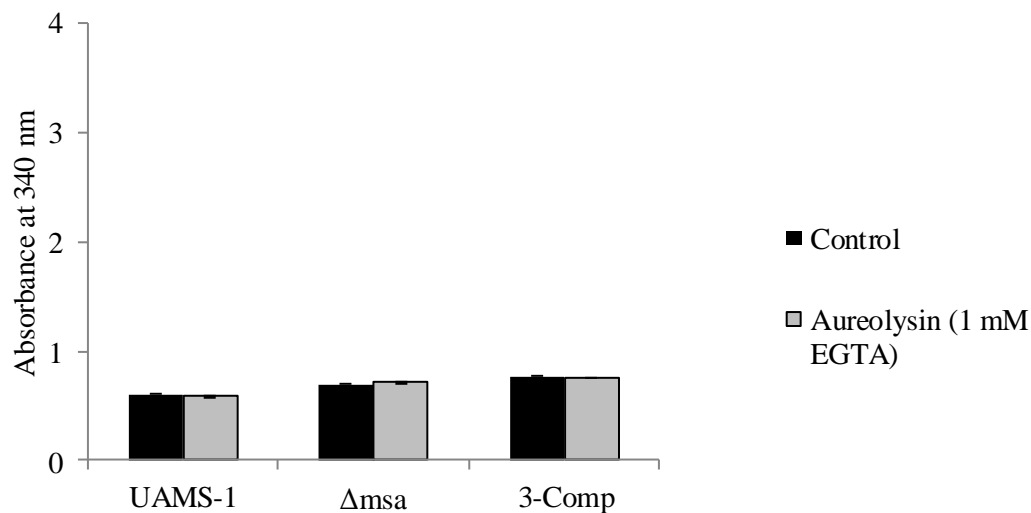


Figure 39. Protease activity of *S. aureus* UAMS-1 strains using azocasein as a substrate showed no difference on deleting the *msa* gene in *S. aureus* LAC strains.

As shown earlier, the deletion of the *msa* gene decreased transcript levels of staphylokinase by a – 5.5 fold in UAMS-1 but did not influence the same in LAC strains (Table 12). Jin et al (2004), showed the neutralizing effect of the staphylokinase on human defensin peptides. Hence, the susceptibility of the strains LAC and UAMS-1 were assessed using human defensin peptides. Mutants of *msa* did not show an increase or decrease in susceptibility to HNP (Figure 40-41).

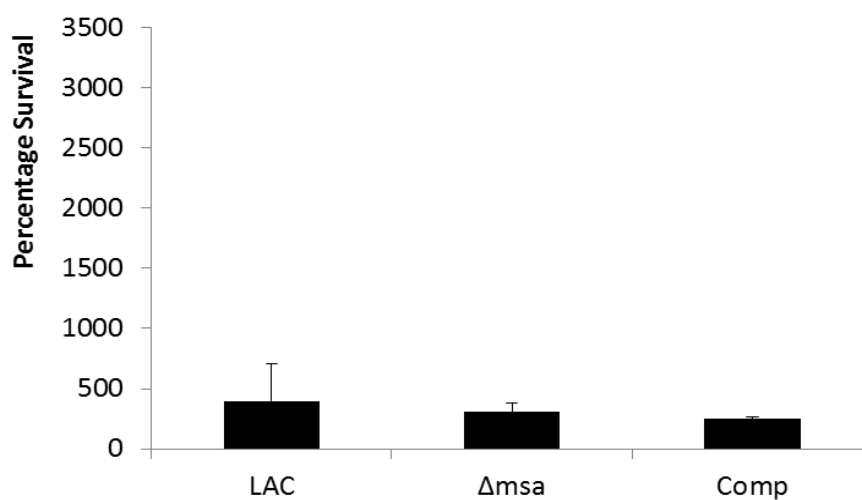


Figure 40. Mutation of *msa* in LAC did not influence susceptibility of *S. aureus* LAC strains to HNP-1.

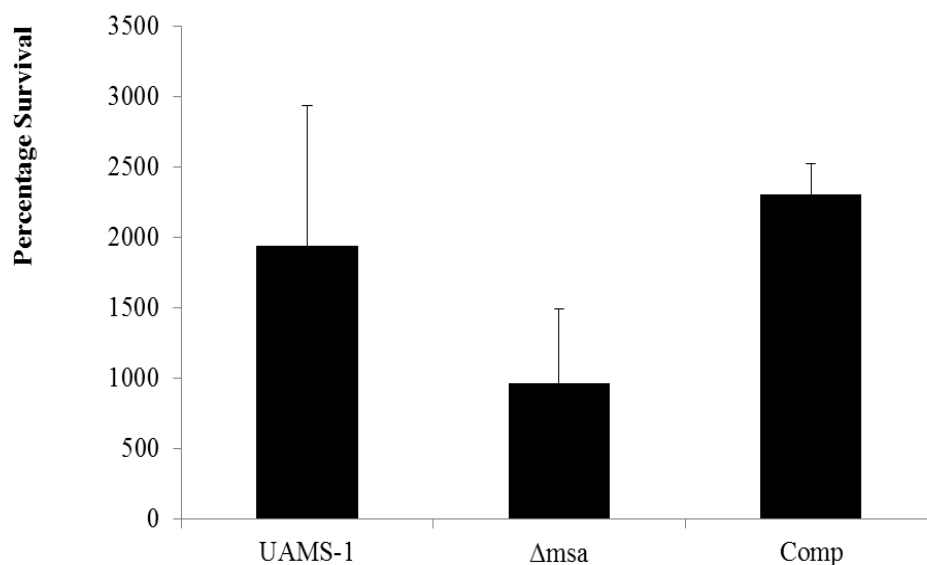


Figure 41. Mutation of *msa* in UAMS-1 did not influence susceptibility of *S. aureus* UAMS-1 strains to HNP-1.

Discussion

Most invasive infections of *S. aureus* have been observed to be accompanied by sepsis (Liu et al., 2011). Klevens et al., in 2008 reported that less than half of the sepsis patients under intensive care succumbed to death (Klevens et al., 2008). Infection in the blood provides a convenient route of infection dissemination of bacteria like *S. aureus*. In order to disseminate to and colonize the various organs of the host, *S. aureus* should be able to share the path of the hematogenous route with phagocytes, complement proteins, and other members of the immune system that are constantly clearing the host system of such foreign entities. Thus, in order to study the role of *msa* in host persistence and infection dissemination a sepsis model was employed.

Organs have their own organ-specific immune factors that defend the organs from an infection. The liver plays an important role in bacterial clearance (Benacerraf,

Sebestyen, & Schlossman, 1959; Brandborg & Goldman, 1990; Mackaness, 1962; Mims, 1987). When septicemic blood flows through the liver, the bacteria adhere on to the hepatocytes via an interaction between their carbohydrate receptors and lectins respectively. Immigrant polymorphonuclear neutrophils (PMNs) attracted to these hepatocyte bound bacteria engulf and phagocytize them (Ofek & Sharon, 1984; Perry & Ofek, 1984, Perry, Keisari, & Ofek, 1985). PMNs are important and abundant players of the innate immune system that prevent the propagation of bacteria in the liver (Gregory et al., 1996). Spleen also plays an important role in bacterial clearance and detoxification of toxins (Altamura et al., 2001). Phagocytosis is one of the most important modes of bacterial clearance, which is accomplished by the splenic macrophages, marginal zone macrophages of the spleen, and alveolar macrophages of the lungs. The spleen receives 200-300ml/min/100-150g organ of blood flow in human and at the site of its red pulp it purifies the blood of old and damaged RBCs along with any cell debris (Timens, 1991). Blood also flows through the B- and T-lymphocyte containing white pulp of the spleen and exposes the antigens carried by the blood to antibody production. These antibodies aid in opsonization of bacteria and its subsequent clearance from the blood (van den Dobbelen et al., 1993).

The murine sepsis model used to study the regulation of infection dissemination by *msa* showed varying results in the two strains used: LAC and UAMS-1. Deleting the *msa* gene in the genetic background of LAC did not alter the dissemination of infection via the blood stream to the organs. Bacterial load in the lungs although was reduced in the murine models infected with the mutant strains of UAMS-1 as compared to the wild type. This finding suggests several possibilities. Firstly, in the strains of LAC the regulation of infection dissemination by the *msa* gene is overpowered by a stronger

global regulator or this regulator may not be completely under the regulation of the *msa* gene. In UAMS-1 the same possibilities could be the case although the extent of the influence of the regulation by *msa* could be more than that seen in LAC strains. Second and most importantly, the sepsis model is that a murine organism. Hence, the findings of the murine sepsis model may not truly reflect the true behavior of the strains, as the virulence factors of *S. aureus* are human specific (Rooijackers et al., 2005c; Serruto et al., 2010).

The blood stream, which is the carrier of the infection, also harbors the factors that kill microbes. We assessed the ability of the wild type and mutants to survive in gender-pooled whole human blood. When the strains of *S.aureus* were co-incubated with whole human blood, the mutant of LAC survived better than the wild type (Figure 23), while in the case of UAMS-1, the wild type survived better than the mutant (Figure 24). These results were in agreement with the results obtained from the phagocytosis survival assay (Figure 31 and 32).

Neutrophils in the presence of chemotactic factors undergo changes in shape like blebbing and branching of extensions. According to Guowy et al., (2004), the chemotactic factor interleukin -8 was responsible for blebbing while IL-8 and monocyte chemotactic protein – 3 (MCP-3) was responsible for the extensions from the neutrophils. Neutrophils exposed to *S. aureus* strains for 10 minutes showed blebbing in all UAMS-1 strains but the highest level of extensions were observed in UAMS-1 mutants. This indicated that UAMS-1 mutants induced the highest levels of chemotactic factors. Comparing our observations with that of Guowy et al., (2004) we suggest that these factors could be IL-8 and MCP-3. Although we observed increased chemotactic response

from the neutrophils exposed to UAMS-1 mutants, the chemotaxis assay did not show an increase in chemotaxis as compared to the wild type.

In the phagocytosis survival assay, the mutant in UAMS-1 showed increased susceptibility to phagocytosis (Figure 32), but in LAC the mutation showed no change in the susceptibility to phagocytosis. Thus, we have evidence of the regulatory influence of the *msa* gene in immunological clearance by neutrophils and persistence in the bloodstream. This observation also indicates the importance of the *msa* gene in combating the attack of the innate immune system on *S. aureus*.

The susceptibility or resistance observed in the blood and phagocytosis assay, however, was not in congruence with the results of the sepsis model. Decreased bacterial counts were observed only in the lungs when infected with UAMS-1 mutants. There was no significant decrease in survivors of the UAMS-1 mutants in the other organs that utilize phagocytosis for clearing the blood supplied microbes. This we assume is due to the high number of phagocytes and efficient organ-specific phagocytosis that clears the wild type and the mutant without any significant discrimination. Another important factor to take into consideration is that the animal model used was that of a murine species. These results may not coincide with the results obtained from the experiments conducted with blood and neutrophils obtained from humans.

Sambanthamoorthy et al. in 2006 showed that the *msa* gene regulated other global regulators like *sarA* and *agr* (Sambanthamoorthy, Smeltzer, & Elasri, 2006). When analyzing the effect of the *msa* mutation on the expression of these global regulators in the strains of LAC, RNA III levels decreased to a smaller extent (- 2.52 fold) as compared to the decrease seen in the UAMS-1 strains (- 6.5 fold). Intracellular survival and escape of the *S. aureus* USA300 strains from the PMN is strongly regulated by the

agr system (Pang et al., 2010). Thus, there is a possibility that the down-regulation of RNA III observed in LAC on deleting the *msa* gene, may not have reduced the *agr* levels to a degree that will make the LAC strains susceptible to immunological clearance. Thus, the *msa* mutant in LAC retained the ability to survive undeterred in the host. This reasoning could explain the findings of the animal model (ignoring the difference between organisms), blood survival, and neutrophil assays.

On the other hand, in UAMS-1 there have been no previous studies indicating the key global regulator involved in intracellular survival in PMNs. The results obtained in our study, revealing increased susceptibility and clearance in the phagocytosis and blood survival assays, indicate a possibility of *agr* also being involved in PMN survival of UAMS-1 strains. Since, the mutation of *msa* reduced the RNAlIIII levels in UAMS-1 more than it did in LAC, we could assume that this is the reason why mutation of *msa* weakened the strain of UAMS-1 but not LAC.

The *msa* gene also regulates the *sarA* levels in *S. aureus* (Sambanthamoorthy, Smeltzer, & Elasri, 2006). Deleting the *msa* gene reduced the *sarA* levels to a -6.54 and a -6.03 fold in the LAC and UAMS-1 strains. Owing to the fact that in UAMS-1, the key regulator required for PMN survival has not been shown yet, the 6 fold reduction seen in *sarA* levels could also contribute to PMN survival as *sarA* is an important global regulator. Until the key regulator of UAMS-1 required for surviving a PMN attack is identified, we cannot safely place our assumptions on the regulation of *msa* through by either *agr* or the *sarA* systems.

The staphylococcal feat of phagocytic evasion can be divided into four levels: prevention of chemotaxis, prevention of opsonization, phagocytic escape (post-engulfment), and intracellular survival (Figure 42). Testing for the various factors

contributing to the illustrated levels of preventing phagocytosis, *msa* was observed to regulate the levels of clumping factor A (ClfA), staphylococcal complement inhibitor (SCIN), staphylokinase (Sak), protein A (Spa), and aureolysin (Aur) in the staphylococcal strains. However, depending on the strain, the combination of factors influenced by the gene regulation by *msa* differs.

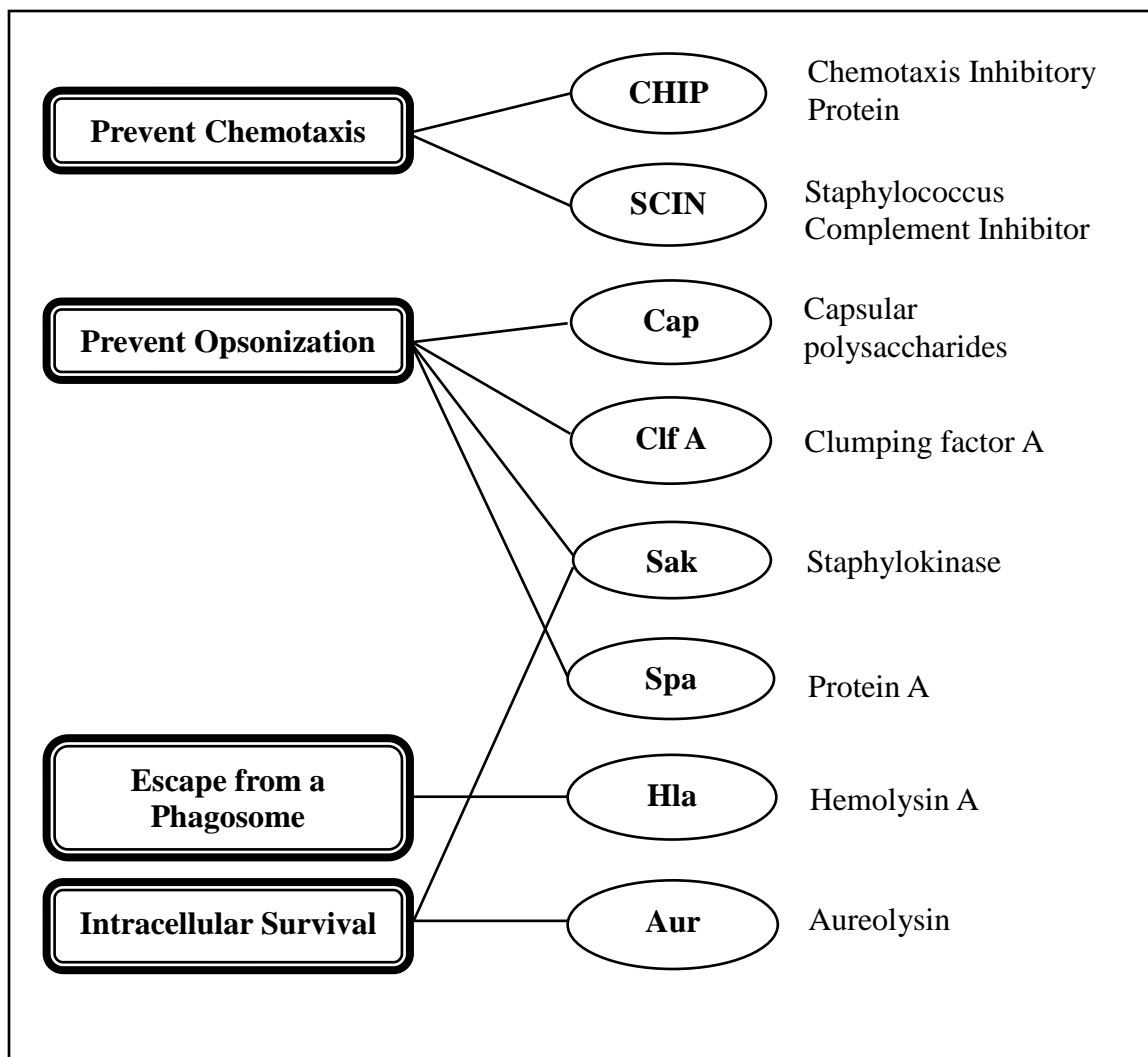


Figure 4.2. Schematic representation of the four levels of phagocytic evasion by *S. aureus*. Staphylococcal factors of immune evasion that contribute to the various stages are also enlisted in this representation.

In the strains of LAC, deletion of the *msa* gene decreased the ClfA levels and increased the Aur levels, which seems conducive to the resistance to immunological clearance. The decrease in the ClfA levels could have increased the probability of opsonophagocytosis. Phagocytic uptake appears to be instrumental to resisting the immune system as the staphylococcal cells, once engulfed, have the ability to survive within the phagocyte owing to the increased levels of aureolysin. Thus, the mutation in LAC provided a safe haven from the non-phagocytic and phagocytic attacks of the immune system. In addition, the intracellular survival in the circulating neutrophils could have also served as a vehicle of dissemination. These assumptions based on the ClfA and Aur levels explain the undeterred survival of the mutants of LAC in the sepsis model, blood survival, and phagocytosis assays. Thus, *msa* in the strains of LAC is required for maintaining the levels of ClfA and Aur to evade the innate immune system.

In UAMS-1, deleting the *msa* gene decreased the transcription of SCIN, ClfA, Spa, Sak and Aur. Assaying for ClfA levels clearly showed a decrease in surface associated levels of ClfA on mutating the *msa* gene. However, phenotypic assays for studying levels of Spa and Aur showed no difference. We, thus, suggest that the significant reduction in *clfA* levels in the mutant could have contributed to the increased probability of opsonophagocytosis. However, unlike in the case of the *msa* mutant in LAC strains, phagocytized mutants of UAMS-1 do not have high levels of Aur or an undeterred level of Sak that support intracellular survival in PMN. Thus, the mutant of *msa* in UAMS-1 is susceptible to clearance by the neutrophils. This, we propose, could have contributed to the increased susceptibility seen in blood survival and phagocytosis assays. Reduced transcription of SCIN was observed in the mutants of UAMS-1, although no difference was observed in the chemotaxis assay. Thus, in UAMS-1 the

regulation by the *msa* gene is required for upregulating the *clfA*, *spa*, *sak*, *aur* and *scn* levels to resist the innate immune system and to disseminate via intracellular survival in neutrophils.

Thus, we have evidence of the role of the *msa* gene in the regulatory network governing the susceptibility or resistance to immunological clearance and exploitation of PMNs for infection dissemination—a remarkable characteristic of *S. aureus*. The extent of regulation by the *msa* gene, however, seems to differ across strains. In addition, we report for the first time the regulatory influence of *msa* on the IEC, a mobile genetic element. There are several factors that we suggest could hold a plausible explanation to the strain-dependent behavior of *msa*. Some of them include (a) the sequence variation of a gene (virulence factor or gene regulator) across the strains of *S. aureus* that could influence the regulatory effect of *msa* on that particular gene, (b) inherent single nucleotide polymorphisms that could render a gene inactive or weak, (c) acquisition of mobile genetic elements, and (d) redundancy of virulence factors that could mask the effect of *msa* mutation as seen in the case of LAC.

CHAPTER V
REGULATION OF PROTEASES BY *MSA* IN BIOFILM
AND NON-BIOFILM CONDITIONS

Abstract

Staphylococcal biofilms have been a growing problem in the healthcare sector due to the resistance to antimicrobial treatment options, repetitive surgical interventions and containment of infection. Detachment of cells from a biofilm not only plays an important role in biofilm development, but also in the dissemination of infection. The *msa* gene of *S. aureus* has been previously observed to down-regulate proteases. Extracellular proteases of *S. aureus* are very important virulence factors that play an important role in several stages of the staphylococcal infection and especially in dissemination. Hence, we studied the effect of *msa* on the staphylococcal proteases of the community-acquired MRSA strain USA300. Interestingly, deletion of *msa* increased protease levels when the cells were growing under non-biofilm, planktonic conditions, but in biofilm conditions the mutation had no effect on the proteases. This revealed an environment-dependent variation of the regulation by the *msa* gene. Thus, the *msa* gene might play a more significant role in non-biofilm infections than in biofilm infections of *S. aureus*.

Introduction

Proteases are extracellular enzymes released by *Staphylococcus aureus* during the post exponential phase of growth. *S. aureus* produces three important classes of proteases namely metalloprotease (Aureolysin), serine protease (V8 protease) and the cysteine or thiol proteases (Arvidson, 2000).

These proteases are translated as proenzymes, which are cleaved in order to activate them. The *ssp* operon contains *sspA*, *sspB*, and *sspC*. Aureolysin cleaves the proenzyme form of serine protease (Drapeau, 1978). The SspA thus active in turn is required for cleavage and activation of SspB, a cysteine protease (Karlsson & Arvidson, 2002; Rice, Peralta, Bast, deAzavedo, & McGavin, 2001). SspC (also known as staphostatin B) formed is a specific inhibitor of *aur*. This *ssp* operon is positively regulated by *agr* and negatively regulated by σ B and the regulator *sarA* (Blevins et al., 2002; Karlsson & Arvidson, 2002; Shaw et al., 2004; Tsang, Cassat, Shaw, Beenken, & Smeltzer, 2008). These serine proteases play important roles in processing cell surface proteins like fibronectin binding protein (FnBP) and protein A (Karlsson & Arvidson, 2002; McGavin, Zahradka, Rice, & Scott, 1997) and in immune evasion (Burlak et al., 2007). Several such surface proteins, namely the biofilm-associated protein Bap (Cucarella et al., 2001), protein A (Merino et al., 2009), FnBP (O'Neill et al., 2008), and SasG (Corrigan, Rigby, Handley, & Foster, 2007) play vital roles in biofilm formation.

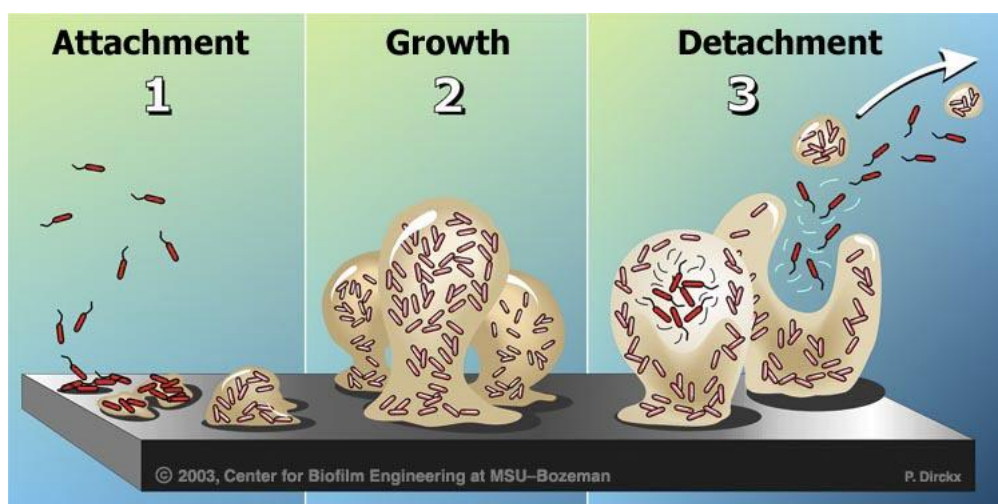


Figure 43. Stages of biofilm development of *S. aureus*. *S. aureus* form biofilms through the stages of attachment onto a surface, growth and formation of biofilm towers and detachment of biofilm (Dirckx & Stoodley, 2003).

Analyzing the constituents of biofilms, several studies showed that along with the conglomeration of cells, biofilms had an extracellular matrix consisting of proteins, DNA (Rice et al., 2007), and/or polysaccharide intercellular adhesion PIA/PNAG encoded by the *icaADBC* operon (Cramton, Gerke, Schnell, Nichols, & Gotz, 1999). To weaken these recalcitrant biofilms, degrading agents specific for these individual constituents were added (Boles & Horswill, 2008; Izano, Amarante, Kher, & Kaplan, 2008; Mann et al., 2009). These biofilms formed by several clinical isolates have been observed to be polysaccharide dependent or even independent (Boles, Thoendel, Roth, & Horswill, 2010; Lauderdale, Boles, Cheung, & Horswill, 2009; Lauderdale, Boles, Morcuende, & Horswill, 2010; O'Neill et al., 2007).

As seen previously in *S. aureus* COL, deletion of the *msa* gene in the new and virulently emerging strain USA300 LAC increased the protease levels (Sambanthamoorthy, Smeltzer, & Elasri, 2006). Deletion of the *msa* gene was also observed to weaken the biofilm formed by *S. aureus* COL (Sambanthamoorthy, Schwartz, Nagarajan, & Elasri, 2008). This led us to question if the weakening of biofilms formed by the *msa* mutant was due to the increased protease levels. Here, in this study, we tested the effect of protease regulation by the *msa* gene in biofilm and non-biofilm conditions, to understand the relationship of *msa* and proteases.

Materials and Methods

Protease Assay

Protease activity was quantified as described in Chapter III. In order to quantify the individual classes of proteases, the respective inhibitors were added. The protease activity for each class was assessed by adding specific protease inhibitors. EGTA (1 mM), PMSF (200 μ M), and E-64 or 20 μ M L-trans-epoxy-succinyl-leucylamido (4-

guanidino) butane was employed to inhibit metalloproteases, serine proteases, and thiol proteases, respectively. Proteases from biofilm effluents were also assessed similarly.

RNA Isolation and Real-time PCR

RNA was isolated from cells grown in planktonic and biofilms conditions using Rneasy kit (Qiagen Inc.). Transcript levels of the proteases were determined using real-time quantitative PCR (RT-qPCR) with the primers described in Table 13. As an endogenous control *gyr* (gyrase) primers were used.

Table 13

Primers for RT-qPCR

Gene	Primer Sequence (5' to 3')	Reference
<i>aur FP</i>	GCA CTT TAT CAC CAG CAG CA	(Sambanthamoorthy, Smeltzer, & Elasri, 2006)
<i>aur RP</i>	TTG ACC GCA TCA CTC TTT TG	
<i>ssp FP</i>	TCA AGC AAA CAG CAA ACA CC	(Sambanthamoorthy, Smeltzer, & Elasri, 2006)
<i>ssp RP</i>	TTT GCG TGT TCA CGT TGT TC	
<i>gyr FP</i>	CAT TGC CAG ATG TTC GTG AC	(Sambanthamoorthy, Smeltzer, & Elasri, 2006)
<i>gyr RP</i>	CCG GTG TCA TAC CTT GTT CA	

Biofilm Detachment Assay

Biofilms were grown in flow cells (Figure 44) following the methods described by Beenken, Blevins, & Smeltzer, (2003) and Beenken et al. (2004). Briefly, microtiter plates (Thermo Fisher Scientific Inc.) or flow cells (Stovall Life Science Inc.) were coated with human plasma and incubated at 4°C overnight before inoculating the biofilm systems. Inoculums were prepared by normalizing the overnight cultures and added or

injected into the microtiter plate and flow cell respectively. Incubation was conducted at 37°C. Flow cells were pumped with TSB (supplemented with NaCl and glucose) at a flow rate of 0.5 ml/min (Figure 44). Samples were collected periodically and analyzed for protease activity and detachment.

Effluents from flow cells were collected, serially diluted, and plated on TSA plates to enumerate the cells and, thus, determine the rate of detachment of biofilms cells. For quantifying protease activity, effluents were filter sterilized and subjected to the protease assay as described above.

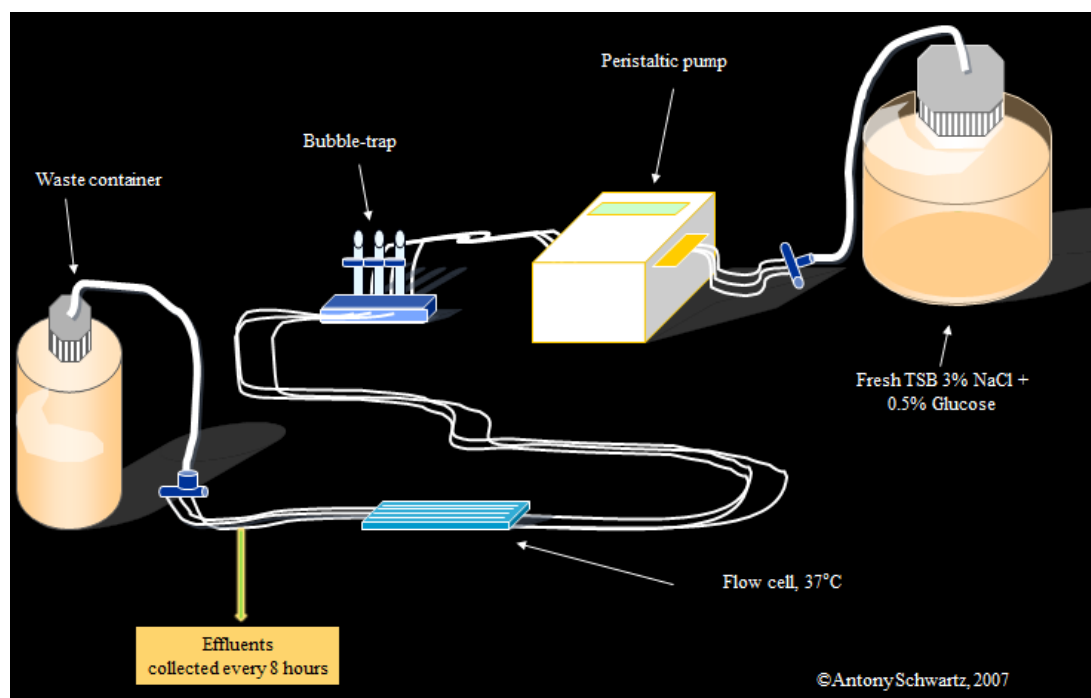


Figure 44. Flow cell set up employed for *in vitro* growth of biofilms of *S. aureus*. This set up consists of media pumped into the flow cell system using a peristaltic pump, a flow cell unit for the growth of biofilms, and a waste container.

Results

msa Does Not Contribute to Biofilm Detachment

Deletion of the *msa* gene did not affect the detachment of biofilm cells as seen in Figure 45. The number of cells detaching from the biofilms of wild type and mutant were equal at all the time points tested. This also revealed that the detached cells were all viable and hence the degree of metastatic infection was not influenced by the *msa* gene.

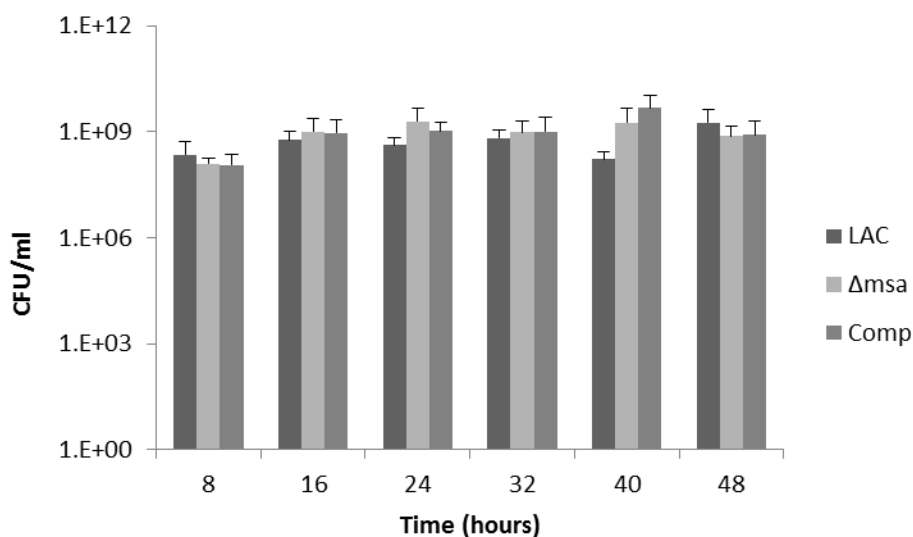


Figure 45. Biofilm Detachment Assay showing no difference in biofilm detachment on deleting the *msa* gene from *S. aureus* LAC strains.

Differential Regulation of Proteases

Deletion of the *msa* gene increased the protease levels as observed by the protease assay (Figure 46) and the transcript analysis (Table 14). Clearly, this indicates the vital role of *msa* in maintaining the protease levels in *S. aureus* LAC strains. However, when the very same strains were grown under biofilm conditions, the deletion of the *msa* gene did not increase protease levels (Figure 46 and Table 14-16). Deletion of *msa* showed no change in the transcript levels of proteases in cells of the biofilms and that of the detaching cells.

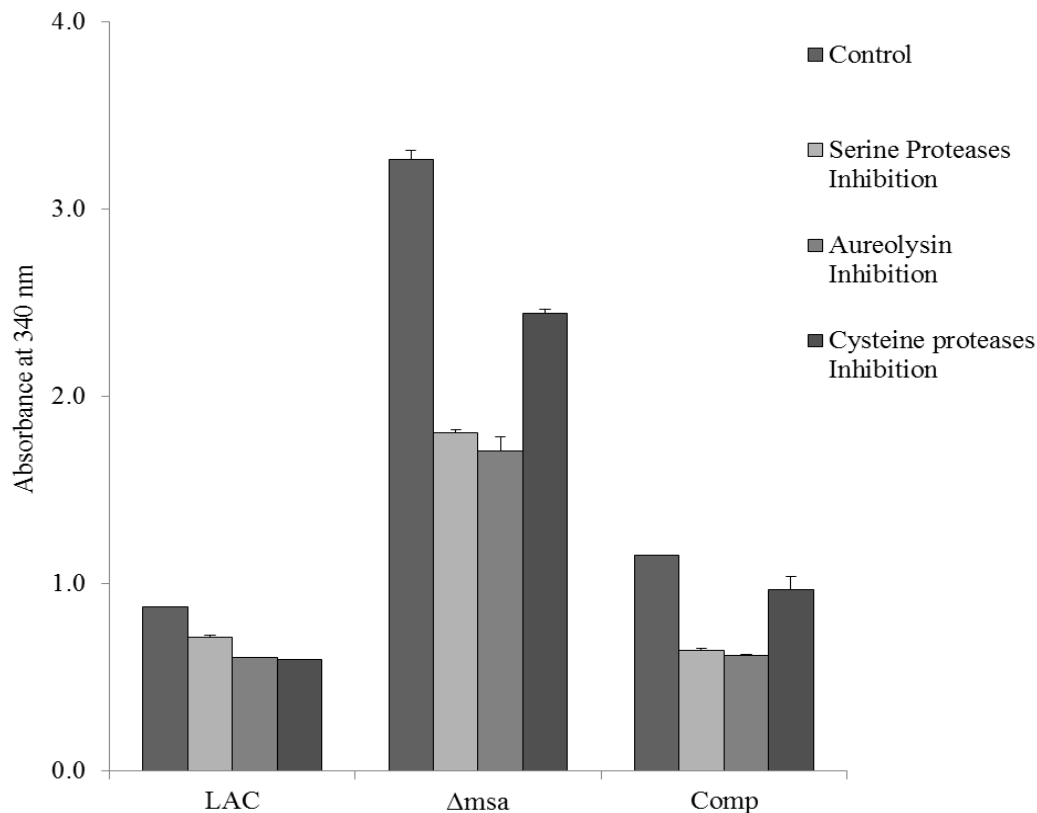


Figure 46. Protease activity of non-biofilm cells of *S. aureus* LAC strains using azocasein. Individual classes of proteases were assessed by the addition of specific inhibitors to the sample. Serine protease was inhibited by PMSF, aureolysin by EGTA and cysteine protease by E-64.

Table 14

Relative Expression of proteases

Gene	Class of Proteases	Fold Change
<i>aur</i>	Metalloprotease	8.12
<i>ssp</i>	Serine Protease and Cysteine Protease (Co-transcribed)	8.28

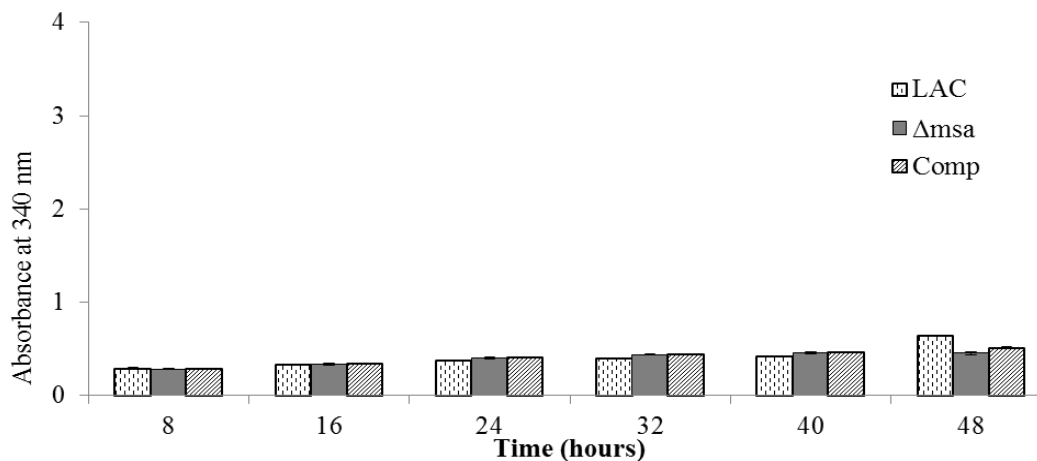


Figure 47. Protease Activity of the biofilm effluents of LAC strains of *S. aureus*

Table 15

Relative Expression of proteases in 50 hr mature biofilm.

Gene	Class of Proteases	Fold Change
<i>aur</i>	Metalloprotease	1.15
<i>ssp</i>	Serine Protease and Cysteine Protease (Co-transcribed)	1.17

Table 16

Relative Expression of proteases in detached cells from 50 hr mature biofilm.

Gene	Class of Proteases	Fold Change
<i>aur</i>	Metalloprotease	-1.07
<i>ssp</i>	Serine Protease and Cysteine Protease (Co-transcribed)	1.26

Discussion

Proteases play a very vital role in the virulence of *S. aureus*. They are not only virulence factors by themselves but also agents of regulation. In this work, it is evident that *msa* regulates these proteases, as deletion of the *msa* gene increased the overall protease activity and the transcript levels of aureolysin and serine proteases. Three classes of proteases were individually quantified to study the role of *msa* on each of them.

The *msa* gene as seen in the detachment assay does not regulate the detachment of biofilms of LAC strains. Our observations have shown that under the biofilm conditions, the protease levels did not increase on deleting the *msa* gene, suggesting that although *msa* regulates proteases, under biofilm conditions the scenario of regulation might be different. The *msa* gene may not have control over proteases under biofilm conditions and/or there is another regulating factor that controls and maintains the protease production under biofilm conditions. This regulator may or may not be under the regulation of the *msa* gene. Clearly, the regulation of proteases under biofilms seems more complex than it appears under non-biofilm conditions. If proteases played a role in biofilm detachment in LAC strains, it would have possibly hinted at the constituents of the biofilms formed by LAC to be proteinaceous. At this point, we have no evidence of the nature of the constituents of the biofilms formed by the staphylococcal strains of LAC.

The effects of the regulation by the *msa* gene seem to be influenced by the environment in which the staphylococcal cells are present. This could explain why we see an effect on the protease regulation in non-biofilm (planktonic) cells and not in cells of the biofilm. Thus, we conclude that the *msa* gene regulates proteases and might be

important in non-biofilm infections, but in biofilm infections the *msa* gene might be over-powered or insignificant in regulating the proteases.

APPENDIX

IRB APPROVAL LETTER

**INSTITUTIONAL REVIEW BOARD**

118 College Drive #5147 | Hattiesburg, MS 39406-0001
Phone: 601.266.6820 | Fax: 601.266.4377 | www.usm.edu/irb

NOTICE OF COMMITTEE ACTION

The project has been reviewed by The University of Southern Mississippi Institutional Review Board in accordance with Federal Drug Administration regulations (21 CFR 26, 111), Department of Health and Human Services (45 CFR Part 46), and university guidelines to ensure adherence to the following criteria:

- The risks to subjects are minimized.
- The risks to subjects are reasonable in relation to the anticipated benefits.
- The selection of subjects is equitable.
- Informed consent is adequate and appropriately documented.
- Where appropriate, the research plan makes adequate provisions for monitoring the data collected to ensure the safety of the subjects.
- Where appropriate, there are adequate provisions to protect the privacy of subjects and to maintain the confidentiality of all data.
- Appropriate additional safeguards have been included to protect vulnerable subjects.
- Any unanticipated, serious, or continuing problems encountered regarding risks to subjects must be reported immediately, but not later than 10 days following the event. This should be reported to the IRB Office via the "Adverse Effect Report Form".
- If approved, the maximum period of approval is limited to twelve months.
Projects that exceed this period must submit an application for renewal or continuation.

PROTOCOL NUMBER: 13010901

PROJECT TITLE: **Role of the MSA Gene in Phagocytic
Clearance of Staphylococcus Aureus**

PROJECT TYPE: **New Project**

RESEARCHER(S): **Maria D. S. Basco**

COLLEGE/DIVISION: **College of Science & Technology**

DEPARTMENT: **Biological Sciences**

FUNDING AGENCY/SPONSOR: **National Institutes of Health**

IRB COMMITTEE ACTION: **Expedited Review Approval**

PERIOD OF APPROVAL: **02/22/2013 to 02/21/2014**

Lawrence A. Hosman, Ph.D.
Institutional Review Board

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