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Deletion of Putative *msa* Operon Gene Fragment and Its Phenotypic Characterization in Community Acquired-Methicillin Resistant *Staphylococcus aureus* USA300 Strain LAC

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The University of Southern Mississippi

Deletion of Putative *msa* Operon Gene Fragment and Its Phenotypic Characterization in
Community Acquired-Methicillin Resistant *Staphylococcus aureus* USA300 strain LAC

by

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A Thesis

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Abstract

Staphylococcus aureus is an important human pathogen that causes a wide variety of diseases. Many *S. aureus* strains have emerged which are resistant to the penicillin class of antibiotics. Of primary importance is methicillin-resistant *S. aureus* (MRSA), which has caused frequent hospitalizations due to infections. In the past, MRSA was typically confined to hospital settings, but recently, community-associated MRSA (CA-MRSA) have been reported. CA-MRSA poses a major public health threat because of increased virulence and success in infecting otherwise healthy individuals. Previously we discovered a gene, *msa*, which plays a critical role in biofilm formation and regulation of the disease process. Recent studies indicate that *msa* is part of a three open reading frame operon and that the upstream neighboring genes may play a role in the regulation of the *msa* operon. In this study, we investigated the possibility that genes *1294-1298* regulate virulence factors of *S. aureus*. We constructed a *1294-1298* mutant in CA-MRSA USA300 strain LAC using the allelic replacement vector pKOR1 and found that it produced a weaker biofilm in addition to increased autolysis, protease production, pigmentation production, hemolysin production and lipase production—all indicators that genes *1294-1298* play a role in the virulence of *S. aureus*. We hope to explore the possibility of exploring the regulatory network of the *msa* operon and its neighboring genes and exploit them as a target for therapy for recalcitrant staph infections.

Key words: *Staphylococcus aureus*; CA-MRSA; *msa*; biofilm

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Introduction

Staphylococcus aureus emerged as an important human pathogen in the nineteenth century and continues to be a major cause of hospital acquired infections worldwide. *S. aureus* are gram positive, facultative anaerobes that can metabolize carbohydrates (glucose, xylose, lactose, sucrose, maltose and glycerol) to produce a white to deep yellow pigment on solid media (1,2,4). *S. aureus* is differentiable from other Staphylococci by its ability to metabolize the sugar mannitol, the production of protein A and the enzymes coagulase-which converts fibrinogen to fibrin-and deoxyribonuclease (DNase) (1,2,4).

Humans are a natural reservoir of *S. aureus*, with about 25-50% of healthy individuals having been persistently colonized, usually on the skin, nasal or vaginal passages however *S. aureus* is capable of surviving on nonliving surfaces (1,2,3,4). *S. aureus* is able to adhere and grow on nearly any surface in multicellular communities called biofilm (4). Certain factors can predispose individuals to colonization: diabetes, indwelling medical devices, intravenous drug use and AIDS, for example (3,4). Local infection occurs from the inoculation of organisms on the skin or hair follicles and the subsequent inflammatory response results in skin abscesses or furuncles (2,3). Infections commonly occur from an individual's own colonies but *S. aureus* can be directly transmitted through contact with other colonized individuals or environmental surfaces (2,4). Invasive infections can occur if *S. aureus* gains access to the bloodstream or surrounding tissue and spreads to other organ systems of the body, causing organ specific infections such as endocarditis, osteomyelitis, pneumonia and septicemia (2,3,4). Finally, certain syndromes, such as food poisoning, toxic shock syndrome and scalded skin

syndrome, can result from secreted *S. aureus* toxins without the need for an established colony (2,3,4). Although it is usually extracellular, *S. aureus* can survive inside a variety of cell types such as osteoblasts, endothelial cells and phagocytes, and replicate in their cytoplasm (5). This survival strategy is thought to contribute to *S. aureus*' success in evading host immune responses and anti-microbial effects (5).

In 1941 penicillin, one of many β -lactam antibiotics, was introduced to the mass market for *S. aureus* treatment (4). β -lactams operate by disrupting with the biosynthesis of bacterial cell walls, specifically the cross linkage of the rigid macromolecule peptidoglycan catalyzed by transpeptidase (9). β -lactams are able to bind to transpeptidase and form a stable intermediate, thereby disrupting cell wall synthesis (9). As early as 1944, penicillinase-producing strains of *S. aureus* had already been identified, although from hospitalized patients, and had become commonplace in hospitals by the early 1950's (4). The first epidemiological study of drug resistant strains of *S. aureus* was performed with 2,000 isolates collected between 1957 and 1969 in the Statens Serum Institute in Copenhagen, Denmark (4). It supported the high prevalence of penicillinase-producing *S. aureus* in hospitals at 85%-90% but surprisingly, reported that 65%-70% of strains were also prevalent in the community. By 1970, penicillinase-producing strains of *S. aureus* were prevalent at 70%-85% in and out of the United States (US) from rural to urban areas, effectively equaling nosocomial rates (4).

This pattern of resistance closely follows that of methicillin-resistant *S. aureus* (MRSA). Methicillin was introduced in 1961 as the first penicillinase-resistant manufactured form of penicillin but MRSA strains were isolated from hospitals in the United States sooner than one year after its' introduction (4). Since then, reports from all

over the world indicate that the prevalence of MRSA is growing at an alarming rate. In various health centers in the US, reports showed that MRSA prevalence increased from 30%-50% from 1988 to 1990 and that prevalence of MRSA out of all bacterial isolates increased from 6%-50% from 1998 to 2002 (6). In France, Mangeney et al. reported that an increase of MRSA prevalence from 33%-62% in their hospital wards, with similar trends being reported elsewhere in Europe (6). Studies from Asia show a high increase in the prevalence of MRSA with up to 70% in South Korea and 54% in Japan (6). Although there are poor surveillance systems in place for MRSA in Africa, studies carried out in the sub-Saharan countries of Nigeria, Cameroon and Kenya reported prevalence rates of 21%-30% among them (6). Additionally, hospital-acquired MRSA (HA MRSA) has had an increasing economic impact on both healthcare and patients. Outbreaks of HA MRSA between 1971 and 1980 were investigated by the CDC in the US and reported that infection of HA MRSA was associated with longer hospital stays (approximately twice as long) and other studies showed an association with higher hospital costs (250% increase) and treatment with antibiotics (7).

However, in the early 1990's, reports detailing patients who acquired MRSA in the community or were outside traditional definitions for at risk individuals began surfacing and prompted the recognition of community-acquired MRSA (CA MRSA) (4,6,8). CA MRSA strains were reported to be much more aggressive than hospital-acquired MRSA i.e. severe skin infections, large and/or recurring abscesses and necrotizing pneumonia were typical (8). Their resistance profiles were also unique: CA MRSA strains appeared to be resistant to only β -lactam antibiotics and susceptible to other classes of antibiotics, whereas HA MRSA was resistant to multiple classes of

antibiotics (8). Initially, patients were athletes, members of the military, prisoners and those individuals who lived in close communities (8). However, surveys of daycare centers in Dallas, Texas in 1998 found up to 25% of children were colonized and a survey of children admitted to hospital in Chicago found a 25 fold increase in colonization (4). In 1999, the deaths of four children in the US brought increased awareness to the issue (4,8).

The β -lactam resistance of MRSA is due to the production of penicillin-binding protein 2a (PBP2a) which has a much lower affinity for β -lactams, allowing MRSA to continue synthesizing cell wall even in the presence of β -lactams (9). In 1987, the *mecA* gene was isolated and sequenced in Japan and found to encode for PBP2a. Additionally, the staphylococcal chromosomal cassette *mec* (*SCCmec*) was identified as a genomic island within which *mec* is embedded (10). Six major subtypes of *SCCmec* have been identified (*SCCmec* I-VI). Types I, II and III are larger and found in HA MRSA and the smaller types IV, V and VI are found in CA MRSA (8,9). Common to all subtypes is the insertion sequence *IS431mec* and cassette chromosomal recombinase gene complex (*crr*), responsible for the excision and integration of the transposon into the target site of the chromosome (8). Unique to *SCCmec* type II is the transposon Tn544, which encodes multiple antibiotic resistance to the macrolide-lincosamide-streptogramin antibiotics and spectinomycin, and the *mec* complex, composed of *mecA* and the regulatory genes *mecR1-mecI*, which is found as a truncated form in *SCCmec* type II (8).

The diversity and virulence of *S. aureus* infection can be attributed to the production of a variety of virulence factors that are involved in every stage of the infection process, from initial binding to host cells, invasion of host tissue and evasion of

the host immune system (2, 3, 12). Virulence factors include surface-associated proteins, which are mainly expressed during the exponential growth phase of the culture, and extracellular proteins, which are produced as the culture enters the post-exponential growth phase (2). The expression of virulence factor genes is tightly controlled by global regulators that can adapt to changing environments, thereby allowing organisms to survive and express virulence factors at an appropriate time (2,3). Several regulators are produced in response to quorum sensing: the perception of the relative density of secreted signaling molecules by nearby bacterial cells and the adjustment of gene expression accordingly (11). The accessory gene regulator (*agr*) is one such system, regulating the expression of several virulence genes as a result of quorum sensing by way of a regulatory RNA molecule called RNAIII that can serve as both a repressor and as an activator (5). RNAIII production is also controlled by another global regulator, staphylococcal accessory regulatory locus (*sar*), among its other functions as a regulator of biofilm formation and systemic infections (5,12,13). Three promoters generate transcripts that terminate at the same point downstream of the *sarA* open reading frame and together, encode *sarA* which can bind to enhancer elements of *agr* and has been shown to have regulatory functions independently of *agr* (5,12).

As previously mentioned *S. aureus* can exist as planktonic cells or as an aggregate of microorganisms within an extracellular matrix called biofilm (4). Biofilms can arise from a single cell or as phenotypically distinct populations due to differences in gene expression as the biofilm develops (15). Biofilms contribute to the ubiquity of bacteria as they can form anywhere and recently, biofilm formation on medical instruments has contributed to difficult-to-treat infections (15). The extracellular matrix can be composed

of various components such as polysaccharides, DNA and proteins which contribute both a structural barrier to unfavorable environmental conditions and can confer antibiotic resistance (15). Biofilm formation is also composed of several steps which begin with the initial attachment and adhesion of the microorganism to a surface, the accumulation of cells within the extracellular matrix, maturation of the biofilm and the detachment of cells that can travel to other sites of infection (15,17,20). Microbial surface components recognizing adhesive matrix molecules (MSCRAMM) represent a class of adhesin proteins that bind tightly to specific host proteins to initiate attachment to surfaces including fibrinogen, fibronectin and collagen (16). Most strains of *S. aureus* have either a polymer of the polysaccharide N-acetyl glucosamine (PNAG) or otherwise known as polysaccharide extracellular adhesion (PIA) to form biofilm and/or biofilm associated proteins (Bap) that attach to neighboring cells within the biofilm (15). Also, the controlled cell lysis of some cells within the biofilm releases genomic DNA into the biofilm (15,17). This extracellular DNA (eDNA) has been shown to contribute stability to the biofilm and confer antibiotic resistance by inducing expression of antibiotic resistance genes (15,17). In *S. aureus*, the *icaADBC* operon encodes for the enzymes that are required for the synthesis of PIA and whose mutation results in a reduced ability to form biofilm (15,16). Two such regulators of the *icaADBC* operon are *agr* and *sarA* (16). Beenken et al. demonstrated that mutation of *sarA* of *S. aureus* results in a decreased binding to fibronectin, increased production of proteases and a biofilm-deficient phenotype that has a reduced capacity to form biofilm in both plates and when exposed to shear forces in a flow cell (16). On the other hand, mutation of *agr* resulted in an

enhanced capacity to bind to fibronectin and little effect in biofilm formation in most strains tested (16).

For now, the reasons for the effect of *sarA* on biofilm formation are unclear but nevertheless, new genes are characterized that modulate the expression of *sarA*. Previously, Sambanthamoorthy et al. identified and characterized a novel gene, *msa*, that modulates the expression of *sarA* along with several other virulence factors (18). It has been postulated that *msa* is a membrane protein with three transmembrane regions and 3 phosphorylation sites, two outside the membrane and one in the cytoplasm, suggesting that *msa* is involved with signal transduction if those sites are phosphorylated by kinases (19). Sambanthamoorthy et al. demonstrated that mutation of *msa* resulted in a twofold and 2.85-fold decrease in *sarA* expression in *S. aureus* strains RN6390 and UAMS-1, showing that *msa* mutation of *sarA* is not strain-dependent (18). Furthermore, mutation of *msa* resulted in altered expression of genes that are regulated by *sarA* including those that are essential for biofilm formation, such as decreased expression of genes encoding fibronectin-binding protein A (18). Sambanthamoorthy et al. also studied the role of *msa* in biofilm formation by constructing an *msa* mutant in the *S. aureus* strain COL (20). Under both steady-state and shear force conditions, the *msa* mutant was unable to form as thick and complete of a biofilm as the wild-type COL strain and the complemented *msa* mutant and those biofilms that were formed by the *msa* mutant did not persist and dispersed quickly (20). Although there was no discernible difference in the initial attachment to surfaces of test strains, the absence of a thick biofilm in the *msa* mutant suggested a defect in the accumulation stage of biofilm formation (20). Whether *msa* plays a role in the maturation or detachment stage is yet to be studied. In addition,

preliminary results show that *msa* is a part of an operon that may play role in biofilm formation, cell death and antibiotic resistance.

In this study, we attempt to delete a putative gene fragment containing the *msa* gene using a method adapted from Bae et al. in which a new allelic replacement vector was constructed, pKOR1 (21). pKOR1 is a *Escherichia coli*/*S. aureus* shuttle vector that uses antisense *secY* RNA expression and the lambda recombination cassette to generate allelic replacement(21). *secY* is a membrane protein that functions as a subunit of the preprotein translocase pathway of gram-negative bacteria and is essential for gram-negative growth so transcription of antisense *secY* RNA would inhibit growth (21). The lambda recombination cassette is comprised of several genes that function to permit rapid cloning of mutant genes (21). Some key components include the gene sites of *attP* and *attB* which when transformed inhibits growth of cells that lack the recombinant plasmid using *ccdB*, which encodes for a gyrase inhibitor, which blocks relaxation of supercoiled DNA, a requirement for transcription and translation (21). The plasmid is electroporated into *S. aureus* strain RN4220 and grown at 43°C, a non-permissive temperature for pKOR1 replication, to select for plasmid integration into the cells chromosome (21). Then growth at 30°C with anhydrotetracycline (ATC), which induces antisense *secY* transcription via the P_{xyl}/tetO promoter, selected for cells that had removed the plasmid (21). If successful, further work can study the effect of deletion of the *msa* operon on biofilm formation, cell death and antibiotic resistance as a potential target for therapy.

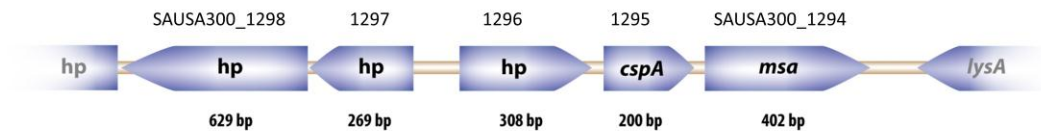


Figure 1 Gene map showing deletion sites.

Materials and Methods

Bacteria and growth conditions: The community-associated methicillin – resistant *S. aureus* (CA-MRSA) strain USA300 LAC was used in this study. Five genes were deleted from the USA300 LAC strain, designated as 1294-1298. Strains were grown on tryptic soy agar (TSA) and in tryptic soy broth (TSB) at 37 °C with constant aeration and shaking. For electro transformation, strains were inoculated in B2 broth: 1% casein hydrolyzate (Sigma Inc.), 2.5% yeast extract (Difco Inc.), 0.5% glucose, 0.1% KHPO₄, 0.5% NaCl at pH 7.5. Agar was occasionally supplemented with chloramphenicol (10 µg/ml) and used to grow the complemented *1294-1298* mutant to ensure maintenance of the plasmid carry the *1294-1298* genes.

Electro-transformation of pKOR1 construct into *S. aureus* RN4220: Electro-competent *S. aureus* RN4220 cells were first prepared. Briefly, RN4220 was inoculated in 5mL of B2 broth at 37°C for 24 hours with shaking. Then, the culture is inoculated with 100mL of fresh B2 broth and incubated at 37° with shaking and the absorbance is monitored until mid-exponential phase is reached (OD₅₆₀ of 0.6-1.2). Cells are then centrifuged at 10,000 rpm for 5 minutes then resuspended in 100 mL of cold deionized water and centrifuged at 10,000 rpm for 5 minutes. This wash is repeated 3 times then the

cells are washed again in 20 mL of deionized water containing 15% glycerol and finally resuspended in 10 mL of deionized water containing 15% glycerol. 100 μ L aliquots are made for storage at -80°C . For the transformation, one aliquot for 100 μ L is thawed on ice for 15 minutes then 50 μ L of competent cells are mixed with 2 μ L of DNA in a microcentrifuge tube and left to incubate for 30 minutes at room temperature. On ice, place one 2mm eletroporation cuvette (bio-rad) and 1 mL of B2 broth that has been aliquoted into a microcentrifuge tube. After incubation, the DNA and cell mixture was transferred to the cold cuvette, placed into the micropulser holder and electroporated using the *S. aureus* settings. At the end of the pulse, quickly take out the cuvette and resuspend with 1 mL of B2 broth and transfer the mixture to a 15 mL culture tube and incubate it at 30°C with shaking for 1 hour. Spread 50 μ L, 100 μ L and 500 μ L of the mixture on both TSA and TSA with 10 $\mu\text{g}/\text{mL}$ of chloramphenicol and incubate at 30°C for 24 hours. Finally, isolate the plasmid using the Spin MiniPrep Kit (Qiagen Inc.) and verify by electrophoresis on agarose gel.

Deletion of 1294-1298 in *S. aureus* strain USA300 LAC: A previous described mutagenesis protocol (21) was used to construct a nonpolar, in-frame deletion of 1294-98 in USA300 strain LAC. Briefly, the flanking regions of the *1294-1298* genes were amplified by polymerase chain reaction using the *Taq* DNA Polymerase Mix (Qiagen Inc.) and ligated together at an introduced *Bam*HI restriction site (New England Biolabs Inc.). This PCR product was inserted into the temperature-sensitive plasmid pKOR, which confers chloramphenicol resistance, using Gateway BP Clonase Enzyme Mix (Invitrogen Inc.). The pKOR1 plasmid was transduced into LAC using phage lysate previously prepared and the transduced strain was grown on TSA with 10 $\mu\text{g}/\text{mL}$ of

CAM at 30°C, the permissive temperature for pKOR1 replication, for 24 hours. Then, colonies were transferred to culture tubes with 5mL TSB with 10 µg/mL CAM and grown at 43°C, a non-permissive temperature for pKOR1 replication, for 24 hours with 220rpm shaking (21). A loopful of the broth culture was then inoculated on TSA plates 10 µg/mL CAM and incubated at 43°C for 24 hours and afterwards, colonies were transferred to 4 culture tubes with 5mL TSB and grown at 30 °C for 24 hours with 220 rpm shaking. Then the cultures were diluted to 10⁻⁴ with sterile H₂O and 50 µL was spread onto TSA plates with 100 ng anhydro-tetracyclin (ATc), which induces antisense secY RNA expression and promotes loss of plasmid. Another 50 µL was spread onto plain TSA as a positive control. Both sets of plates were incubated at 30 °C for 24 hours. Colonies were then streaked to a TSA plate and using the same swab, streaked on TSA with 10 µg/mL of CAM and incubated at 30 °C for 24 hours. CAM sensitive colonies were spread onto TSA plates and incubated 37 °C for 24 hours. Chromosomal DNA from said colonies was harvested, and regions surrounding *1294-1298* were amplified by PCR with primers containing attB sites for upstream and downstream sequences and visualized using gel electrophoresis with a 1kb ladder for verification.

Microtiter plate biofilm assay: Microtiter plate analysis of biofilm formation.

Wells were coated with human plasma and incubated overnight at 4°C. The human plasma was carefully removed by pipetting and the wells were then inoculated with 1mL of 1:200 diluted overnight *S. aureus* cultures that were grown in TSB with 0.5% dextrose and 3.0% NaCl. Control wells contained only the described media. Plates were incubated overnight at 37°C. Bacterial cultures were then removed by pipetting and washed three times with sterile phosphate buffered saline (PBS). The wells were then fixed with 100%

ethanol, which was then immediately aspirated and left to air dry in a sterile hood. The biofilm was then stained with crystal violet, then removed and washed three times with PBS then left to dry overnight. Then, the crystal violet was eluted with 100% ethanol for 10 minutes then the eluted stain was gently transferred to a new microtiter plate and the absorbance was measured at OD₅₉₅ using an ELISA plate reader.

Autolysis assay. Autolysis assays were performed as described by (22).

Overnight cultures of *S. aureus* were diluted to an OD₆₀₀ of 0.05 in TSB containing 1 M NaCl and allowed to grow at 37°C with shaking until an OD₆₀₀ of 0.7 was reached. Cells were harvested by centrifugation, washed twice with ice-cold water, and then resuspended in the same volume of 0.05 M Tris-Cl (pH 7.2) containing 0.025% Triton X-100. Cells were then incubated at 30°C with shaking. Absorbance (OD₅₈₀) was measured every 30 minutes to quantify lysis.

Protease assay: Assays were performed to quantify protease activity. Overnight cultures of *S. aureus* were diluted to an OD₅₆₀ of 0.05. Cells were harvested and separated by centrifuging at 10,000 rpm for 5 minutes. The supernatant was collected; filter sterilized using a 0.45µm syringe filter, and inoculated with 3 mg/mL solution of azocasein in Tris buffered saline (pH 7.5). TSB was used as a negative control. Solutions were incubated at 37°C with shaking in the dark for 24 hours. Un-degraded azocasein was precipitated by addition of 50% trichloroacetic acid solution. Solution was centrifuged for 10 minutes at 10,000 rpm and absorbance (OD₃₄₀) was measured.

Pigmentation assay. Assays for pigment production were performed. Overnight cultures of *S. aureus* were harvested and separated by centrifugation at 10,000 rpm for 1 minute. Cells were washed in water and suspended in 200 µL of methanol. The solution

was heated at 55°C for 3 minutes and centrifuged at 10,000 rpm for 1 minute to remove cell debris. Supernatant was collected and methanol washing was repeated. Finally, the final volume was adjusted to 1 mL with methanol and absorbance (OD₄₀₅) was measured.

Hemolytic assay: Assays for hemolytic activity were performed. Overnight cultures of *S. aureus* were diluted to an OD₅₆₀ of 0.05. Cells were harvested and separated by centrifuging at 10,000 rpm for 5 minutes. The supernatant was collected; filter sterilized using a 0.45 µm syringe filter, and inoculated with 2% rabbit blood in 10mM Tris-HCl (pH 7.5) with 0.9% NaCl. TSB was used as a negative control and 1% sodium dodecyl sulfate was used as a positive control. Solutions were incubated at 37°C with shaking for 15 minutes. The unlysed blood cells were precipitated by centrifugation for 10 minutes at 10,000 rpm and absorbance (OD₄₀₅) was measured.

Lipase assay. Overnight cultures of *S. aureus* were diluted to OD₅₆₀ of 0.10. Cells were then inoculated on tributyrin agar plates with 1 mL tributyrin and incubated overnight at 37°C.

Results and Discussion

Deletion of the five open reading frames, *I294-I298*, was successful and confirmed by electrophoresis using primers containing *attB1* and *attB2* sites for upstream and downstream sequences (Fig.2). The mutant's biofilm was analyzed by growth on a microtiter plate and measurement by an ELISA plate reader and the mutant was found to be defective in biofilm formation (Fig.3). To further examine this phenotype, the rate of autolysis was measured in the presence of Triton-X-100. The *I294-98* mutant lysed at significantly higher rate than the wild type, *S. aureus* strain USA300 LAC, and the complemented mutant showed a rate of autolysis comparable to the wild type (Fig.4). Extracellular proteases were also measured and at four hours, the *I294-I298* mutant had significant higher levels of proteases compared to the wild type and the complemented mutant, both of which had comparable protease levels (Fig. 5). Pigment production was also analyzed by measuring absorbance and the *I294-I298* mutant was found to produce significantly less pigment than the wild type (Fig. 6). Hemolysin production was also analyzed by measuring absorbance and although the *I294-I298* mutant produced less hemolysin than the wild type, it wasn't significantly less, and the complemented mutant showed a level of hemolysin production comparable to the wild type (Fig. 7). Finally, lipase production was measured by growth on tributyrin agar plates and there was no significant difference in lipase production between the mutant, wild type and the complemented mutant (Fig. 8).

Conclusion

The results presented here demonstrate that mutation of *1294-1298* genes in USA300 strain LAC of *S. aureus* results in weaker biofilm formation. This defect is likely due to the reduced expression of *sarA* resulting from the mutation of *msa*, which is among the five open reading frames deleted. The *1294-1298* mutant also results in increased production of proteases and rates of autolysis, which could contribute to a reduced capacity to form biofilm. However, there was not an observed difference in the production of hemolysins and lipases. These proteins could be subject to protease-mediated degradation, which has been demonstrated in *sarA* mutants in USA300 strain LAC (23). Previous studies show that *msa* is part of a 3 open reading frame operon and the upstream neighboring genes (*1297-1298*), which is another operon and spans in the opposite orientation, may also play a role in the regulatory functions of the *msa* operon. In order to understand how the *msa* operon works, genes *1294-1298* were deleted and phenotypically studied. Further experiments could be performed using a flow cell assay for obtain a more representative model of biofilm formation. Experiments could also be performed with protease mutants, to mitigate the effect of protease mediated degradation. Finally, looking at the effect of genes *1294-1298* in an *in vivo* model would be the next step in determining whether these genes would be viable targets for vaccines.

Acknowledgement

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Figures

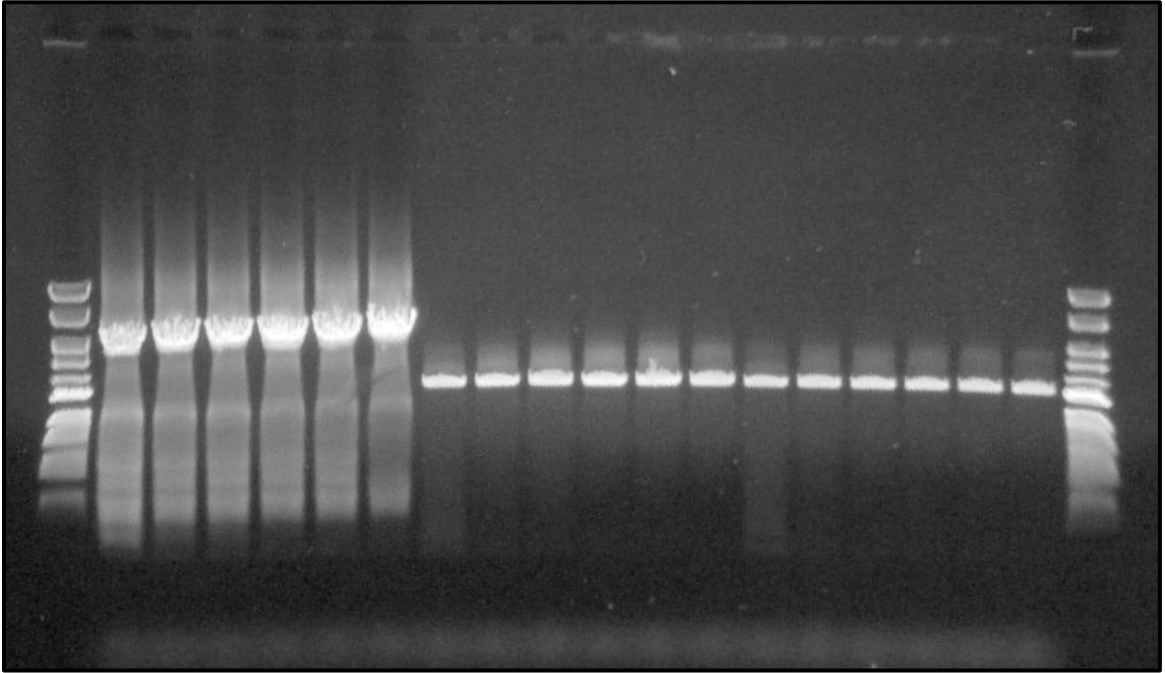


Figure 2. Conformation of 1294-1298 mutant. Lanes 2-7 are wild type USA300 strain LAC *S. aureus*. Lanes 8-19 are mutants. Lanes 1 and 20 are 1kb ladders.

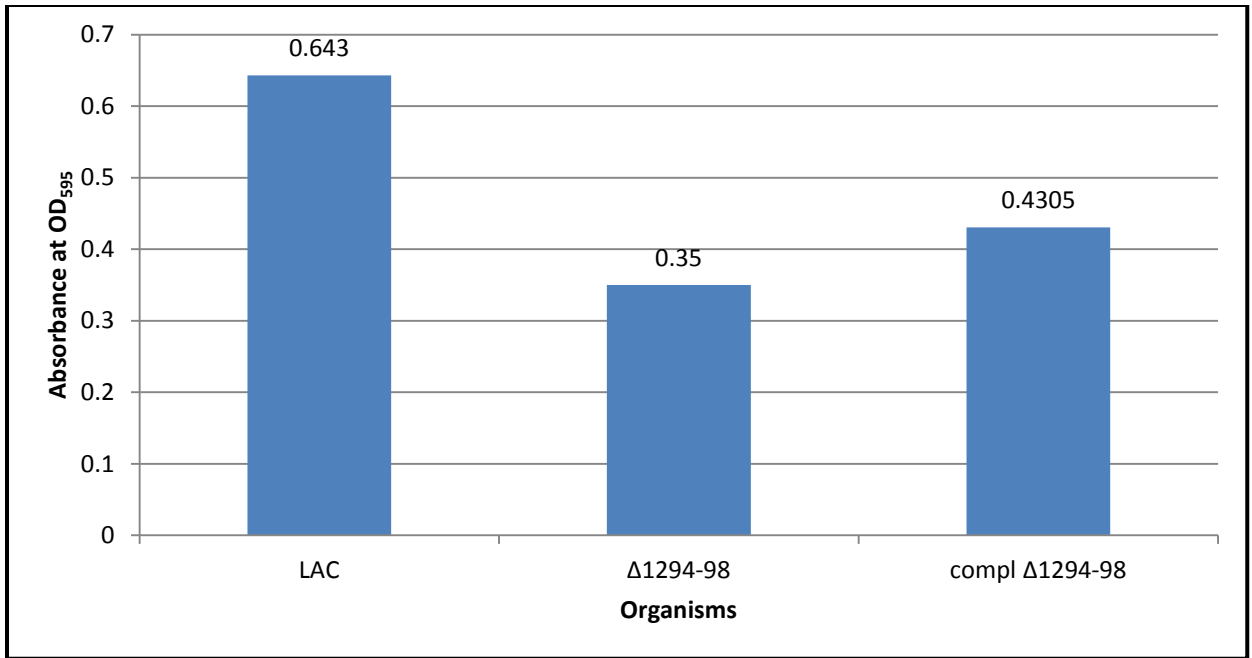


Figure 3. Microtiter plate analysis of biofilm formation. Wells were coated with human plasma and incubated overnight at 4°C. The human plasma was carefully removed and the wells were then inoculated with 1mL of 1:200 diluted overnight *S. aureus* cultures that were grown in TSB with 0.5% dextrose and 3.0% NaCl. Plates were incubated for 96 hours at 37°C. Bacterial cultures were then removed and washed with sterile phosphate buffered saline (PBS). The wells were then fixed with 100% ethanol, and then stained with crystal violet. The crystal violet was eluted with 100% ethanol then the eluted stain was gently transferred to a new microtiter plate and the absorbance was measured at OD₅₉₅ using an ELISA plate reader.

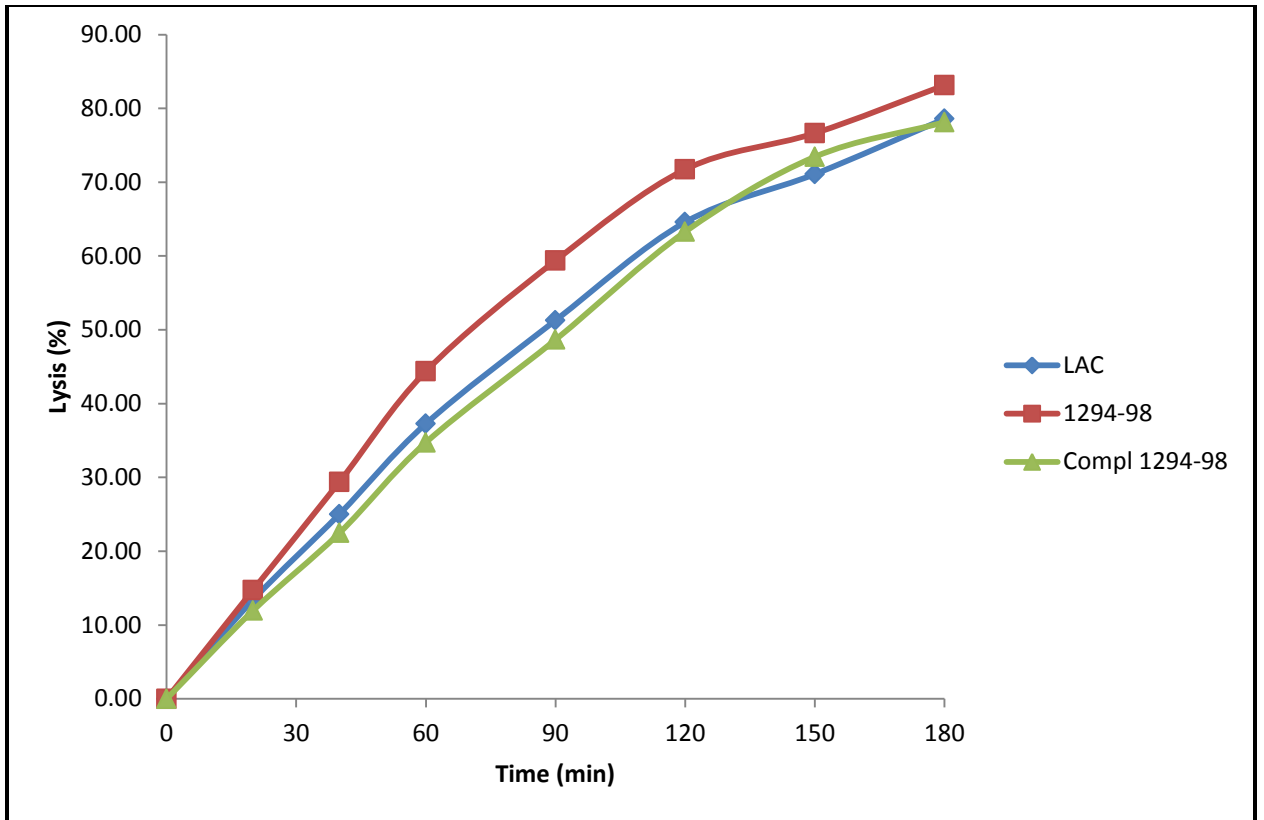


Figure 4. Autolysis assay. Overnight cultures of cells were diluted to an OD of 0.05 and grown to an OD of 0.7 at 37°C shaking at 220 rpm. Cells were harvested and washed twice with ice cold water and then resuspended in lysis buffer, 0.05M Tris (pH-7.5) with 0.025% Triton-X 100 and rate of autolysis were measured as a rate of decrease in OD₅₈₀ every 30 minutes.

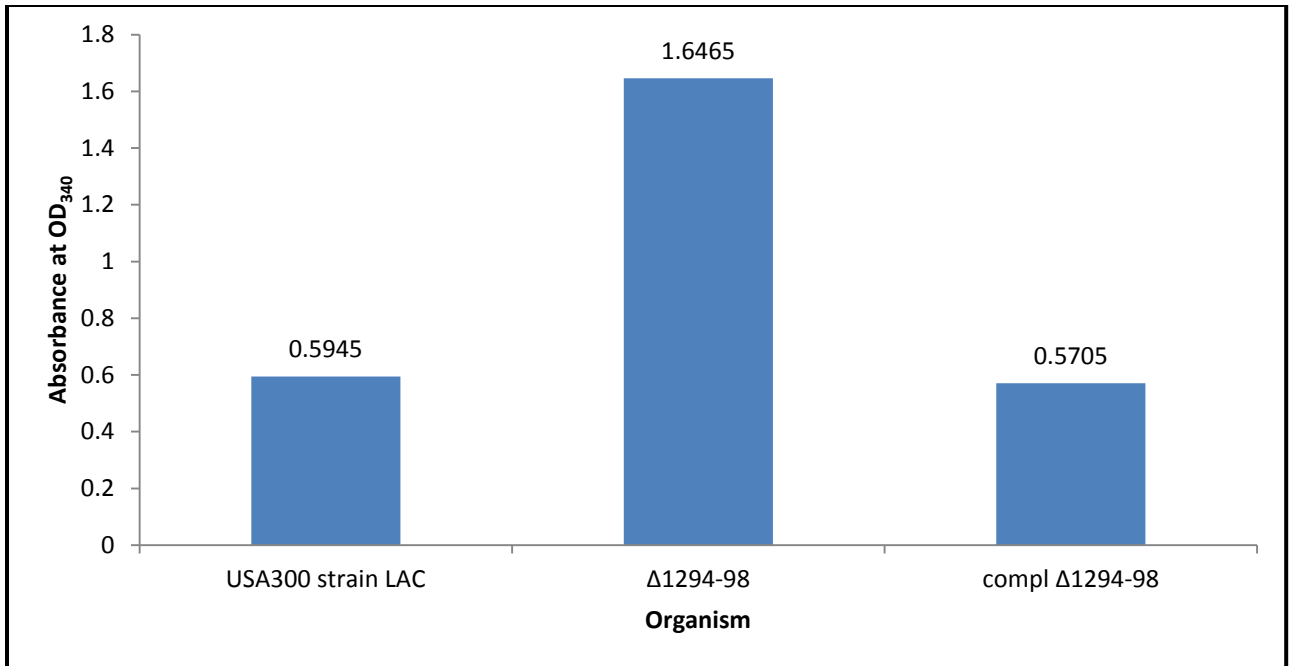


Figure 5. Protease assay. 4 hour cultures of *S. aureus* were diluted to an OD₅₆₀ of 0.05. Cells were harvested and separated. The supernatant was collected, filter sterilized, and inoculated with 3mg/mL solution of azocasein in Tris-buffered saline (pH 7.5). Solutions were incubated at 37°C with shaking in the dark for 24 hours. Un-degraded azocasein was precipitated by addition of 50% trichloroacetic acid solution. Solution was centrifuged for 10 minutes at 10,000 rpm and absorbance (OD₃₄₀) was measured.

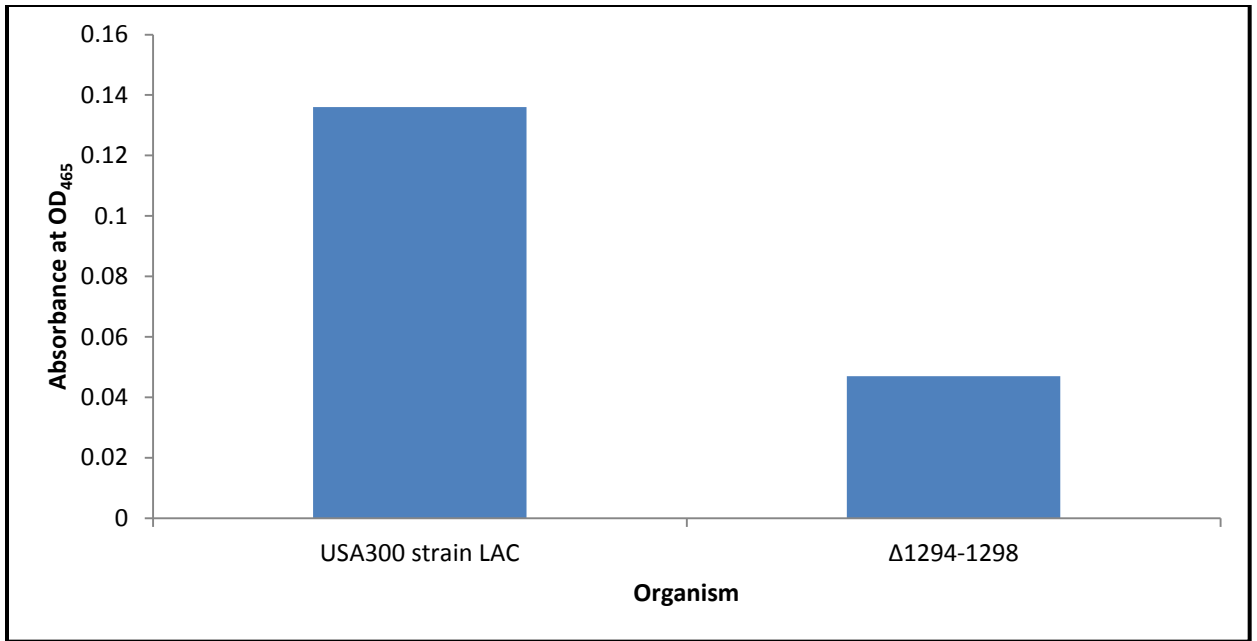


Figure 6. Pigmentation assay. Overnight cultures were harvested and washed with water then methanol and heated in 55°C water for 3 minutes. Methanol wash was repeated then final volume was adjusted to 1mL with methanol and absorbance (OD₄₆₅).

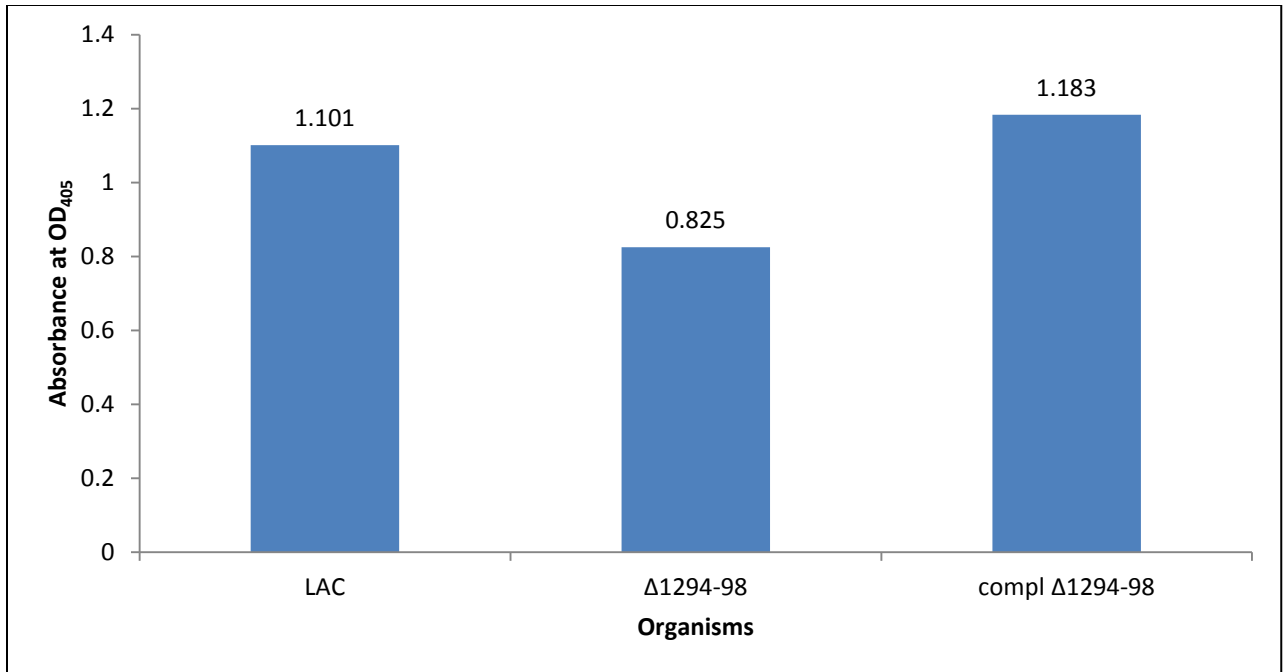


Figure 7. Hemolysis assay. Overnight cultures of *S. aureus* were diluted to an OD₅₆₀ of 0.05. Cells were harvested and separated by centrifuging at 10,000 rpm for 5 minutes. The supernatant was collected, filter sterilized, and inoculated with 2% rabbit blood in 10mM Tris-HCl (pH 7.5) with 0.9% NaCl. Solutions were incubated at 37°C with shaking for 15 minutes. The unlysed blood cells were precipitated by centrifugation for 10 minutes at 10,000 rpm and absorbance (OD₄₀₅) was measured.

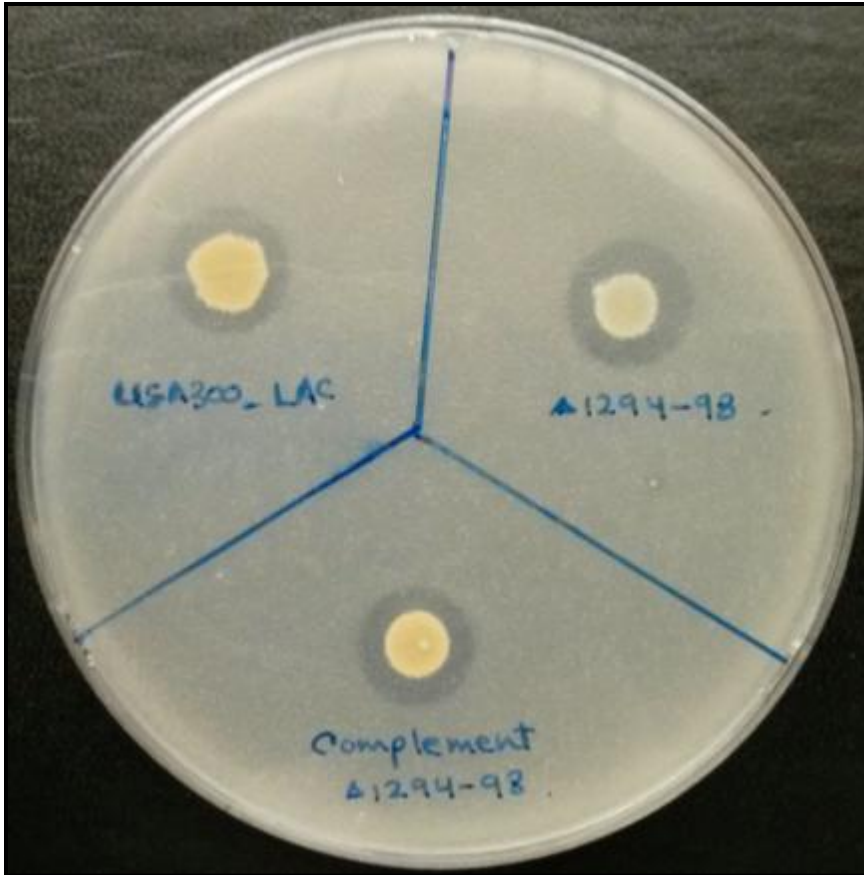


Figure 8. Lipase assay. Overnight cultures of *S. aureus* were diluted to OD_{560} of 0.10. Cells were then inoculated on tributyrin agar plates with 1mL tributyrin and incubated overnight at 37°C.

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