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Effect of msa on Antibiotic Resistance and Allelic Replacement of pKOR1 in *Staphylococcus aureus*

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Effect of *msa* on antibiotic resistance and allelic replacement of pKOR1 in *Staphylococcus aureus*

by

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Abstract

Staphylococcus aureus is an important human pathogen that causes hospital and community-acquired infections (52). These infections are difficult to treat due to resistance to a wide range of antibiotics and spread of antibiotic-resistant strains (13, 52). *S. aureus* causes infection by regulation of accessory genes encoding for expression of factors contributing to virulence (9, 11, 12, 29, 34, 43, 45), including severe infection, biofilm formation, autolysis, and antibiotic resistance (4, 5, 7, 27, 56). Actually, extracellular DNA and nutrient release during autolysis has been shown to contribute to the ability of bacteria to form and maintain a biofilm as well as to the prevalence of resistant strains within a bacterial species (2). The purpose of this study was to determine the effect *msa*, a global virulence regulator recently discovered in *S. aureus* (49, 50), has on resistance to different cell-wall-active antibiotics. This study also attempted to generate an allelic replacement vector causing antibiotic susceptibility, confirming the previous finding by Bae and Schneewind (2008). Population analysis profiles (PAPs) of twelve different cell-wall-active antibiotics in wild-type and *msa* mutant versions of USA300 LAC strain showed *msa* conferred higher resistance to six antibiotics tested. This finding supported the presentation of *msa* as a novel regulation mechanism for autolysis and antibiotic resistance within *S. aureus* (47). Also, after transduction of LAC with the pKOR1 plasmid encoding for chloramphenicol (CAM) resistance, homologous recombination into the bacterial chromosome, and chromosomal excision and loss of pKOR1, successful growth of one colony susceptible to CAM was observed. According to our knowledge, this was only the second time allelic replacement without selection by antibiotic markers has been produced within the bacterial species.

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Chapter I: Problem

Staphylococcus aureus, named for its characteristic golden yellow, spherical colonies (52), occurs as normal flora in the nasal passages, skin, digestive tract, and mouth of humans and is considered the main pathogen responsible for nosocomial, or hospital-acquired, and community-acquired infections. It is a gram-positive bacterium able to grow between 15°C and 45°C and also at salt concentrations as high as 15%. Abilities such as these to survive in the presence of a variety of environmental conditions explain how *S. aureus* functions as an increasing pathogen. In strains whose genes encode for the production of various proteins and toxins that contribute to the capacity to cause disease within a host system, also known as virulence factors, escalation of disease-causing cases has been observed. In a study taken of children in south Texas, cases of community associated-methicillin resistant *Staphylococcus aureus* (CA-MRSA) infection increased 14-fold in a two year period up to 2001, and in 2007, CA-MRSA was shown to be the most prevalent infection of skin and soft tissues in United States emergency rooms. Diseases caused by *S. aureus* range from mild, such as boils and styes, to more severe, such as pneumonia, meningitis, and urinary tract infections, to potentially fatal, such as osteomyelitis and endocarditis. Treatment of diseases caused by the bacterium has proven difficult because *S. aureus* exhibits strong resistance to many antibiotics, including beta-lactam antibiotics such as methicillin and penicillin (52). From 2002 to 2010, the Centers for Disease Control (CDC) have recorded 11 cases of vancomycin-resistant strains of *S. aureus* (VRSA) causing infection (13), an important finding in that vancomycin is often used to treat methicilliin-resistant strains. Therefore, understanding the mechanisms by which genes regulate expression of factors that contribute to

increased virulence in the species, especially characterizing the pathways of regulation between these genes, is important for the discovery of new methods to combat the increasing antibiotic-resistant strains of *S. aureus*.

S. aureus causes diseases in humans by modulation of a complex regulatory network of accessory genes (43) that encode for the expression of a variety of virulence factors (9). *SarA* (Staphylococcal accessory regulator A), which regulates a number of genes in *S. aureus*, is a principal global regulator protein of virulence in the species (11). Experimentational analysis has shown *sarA* regulates virulence in *S. aureus* by binding to several loci specific as gene promoters, including *agr*, *hla*, *spa*, and *fnbA*, and thus controlling specific gene transcription through regulation of *agr* or even through *agr*-separate mechanisms (12). The function of *sarA* is controlled by the global regulatory gene modulator of *sarA* (*msa*), which has been shown to be crucial for complete expression of *sarA* and for the function of virulence factors affected by *sarA* (49, 50). In *S. aureus*, *sarA* has been shown to negatively regulate autolysis (39), which is an important process in the cell wall synthesis of gram positive bacteria and also significant to bacterial virulence by causing higher susceptibility to cell-wall-active antibiotics (13, 25, 26). Therefore, I hypothesize that *msa* may play a role in the resistance or susceptibility to antibiotics targeted toward cell wall synthesis in *S. aureus*.

Chapter II: Literature Review

The principal global regulator *sarA*, along with *agr*, another main global regulator protein of virulence factors in *S. aureus* (9, 29, 34, 45), has been shown to function in varied pathogenesis of *S. aureus*, including the ability of *S. aureus* to cause severe infection in host cells. In an experiment determining the functions of *sarA* and *agr*, the inoculation of *sarA* and *agr* negative mutant strains into neonatal mice followed by the quantification of *S. aureus* strains in the lung and spleen of the mice showed strains containing *sarA* and *agr* loci were essential for fatal pulmonary infection in the host, and *sarA* was specifically crucial in order for bacteremia to occur (27). *SarA* has also been proven to be essential for pathogenesis of the species in other anatomical sites, as proven in an experiment infecting the tail vein of neonatal mice with strains of *S. aureus* containing *sarA* and *agr* as well as *sarA* and *agr* mutant versions of the strains, collecting the bacterial colonies, and analyzing the inflammation response that resulted in the knee joint, tibia, and femur from the appropriate strains in the infected mice. *S. aureus* strains lacking *sarA* and *agr* were determined to cause osteomyelitis and septic arthritis less efficiently than strains containing the two loci (7).

SarA has also been shown to be necessary in the capacity of *S. aureus* to form biofilm in six of eight wild-type *S. aureus* strains tested, UAMS-1, UAMS-601, SA113, SC-01, S6C, and DB. However, Newman strain was unable to effectively produce a biofilm that was not significantly changed by mutation of *sarA* or *agr*, and RN6390 was able to form a biofilm even after mutation of *sarA*. Mutation of *agr* did not significantly affect the capacity of RN6390 strain to produce biofilm, yet double mutants of *sarA* and *agr* in RN6390 were unable to produce biofilm. This finding showed the effect of *sarA*

on the production of biofilm is independent of the interaction of *sarA* and *agr* (4). Characterizing this epistatic relationship between *sarA* and *agr* in regards to biofilm formation has therapeutic importance by direct correlation with antibiotic susceptibility within *S. aureus* (5). When specifically tested in the presence of a biofilm, *sarA* mutants show increased susceptibility to such antibiotics as daptomycin, linezolid, and vancomycin (56). After induction of *agr* expression, however, *S. aureus* cells were observed to detach from an established biofilm, causing increased susceptibility to a number of diverse antibiotics, including rifampicin and levofloxacin (8, 37, 38). *Agr* mutants have also been observed to accumulate during biofilm formation, becoming the dominant subpopulation within the biofilm (57), and one report found that loss of *agr* function may explain, in part, the selective advantage of clinical glycopeptide-intermediate *S. aureus* (GISA) and clinical MRSA isolates in response to vancomycin therapy (48). Therefore, *sarA* expression has been linked to antibiotic resistance in the context of a biofilm, and *agr* expression has been correlated with biofilm-associated susceptibility (5). In relation to its role in the regulation of *sarA* and *sarA*-associated virulence factors, in an experiment testing the ability of *msa* mutant strains of *S. aureus* to form biofilm, use of microtiter plate assay and flow cells to test this ability supported *msa* regulates virulence in the species through modulation of *sarA*. Binding assays and initial adherence assay supported *msa* regulates *sarA* during the accumulation phase of biofilm formation (49).

Differences observed in vancomycin heteroresistance between several *agr* prototype strains has been hypothesized to be related to autolysis since the *agr* operon has been found to regulate expression of a number of murein hydrolases involved in this

process (19). Murein hydrolases are a ubiquitious class of enzymes among bacteria (21) found to be involved in many important cellular processes, including growth and division, daughter cell separation, cell wall synthesis, and turnover and recycling of peptidoglycan (23, 25, 26, 54). The enzymes function by cleaving the covalent bonds that make up bacterial cell peptidoglycan (murein), thus priming the peptidoglycan for expansion of the cell wall. Actually, the break down and build up of cell wall peptidoglycan has been found to be a dynamic process, in which the murein net is constantly being hydrolysed and subjected to the addition of new material (28). Therefore, some murein hydrolases act as autolysins for destruction of the cell wall, potentially resulting in cellular death, and their expression and activity must be tightly regulated to ensure proper function and cell survival (28, 19).

As a gram-positive bacteria, *S. aureus* cell walls are composed of peptidoglycan cross-linkages. More specifically, short peptide chains cross-link alternating β-1,4-linked amino sugars made up of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), and these short peptides are bound to one another by an amide bond. The three dimensional structure and rigidity of the cell wall is formed by the peptides linking to interpeptide bridges having glycan strands. Biosynthesis of the cell wall occurs in several stages, and specific enzymes catalyze the addition of appropriate peptide to the growing peptidoglycan chain (51). Figure 1 illustrates the process of peptidoglycan biosynthesis, including the transglycosylation, or transfer of one glycosidically linked sugar molecule to another glycoside bond, and transpeptidation, or transfer of amino acids between peptide bonds, steps and the enzymes involved at each stage. Antibiotics take advantage of the need for control of the autolysis and cell wall synthesis processes

by inhibiting cell wall biosynthesis at a variety of enzymatic steps or through targeting specific cell-envelope structures involved, as shown in Figure 2 by the mechanism of inhibition of specific cell-wall-active antibiotics in *S. aureus*. (13).

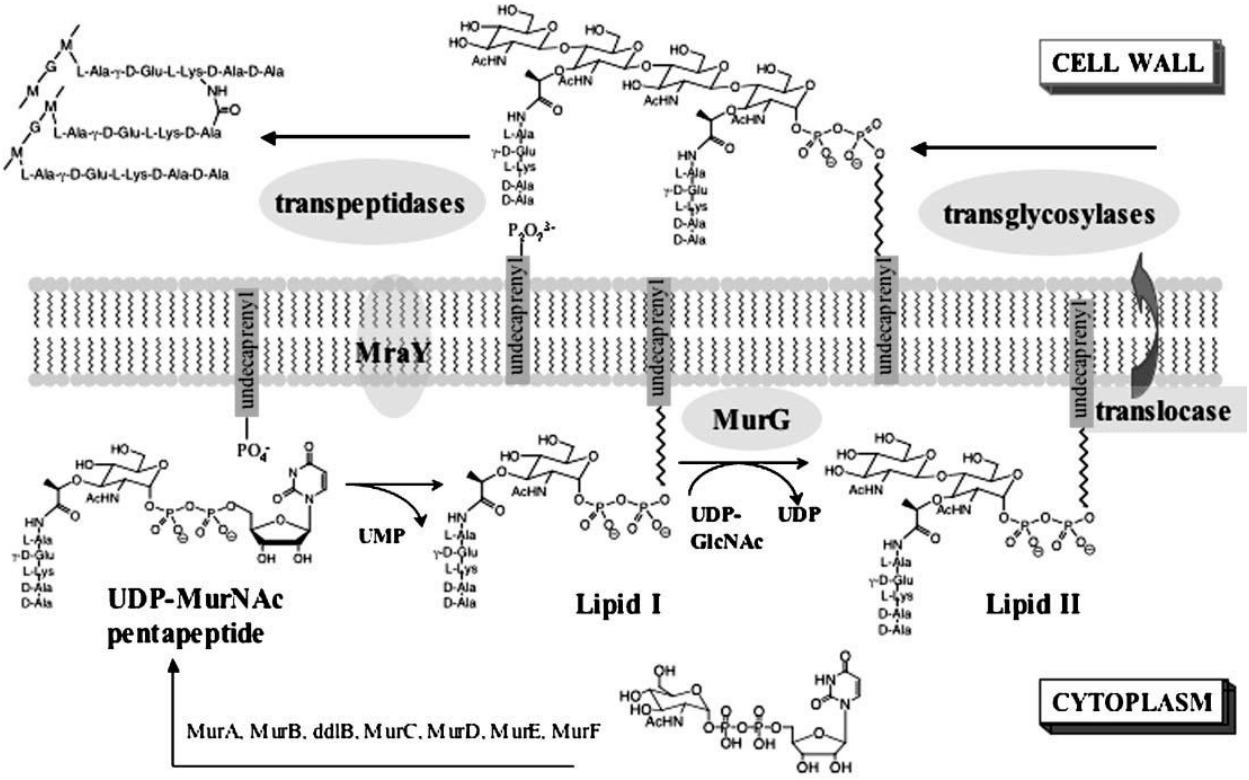

Figure 1. Schematic representation of peptidoglycan biosynthesis, including the enzymes and binding sites of the transglycosylation and transpeptidation steps (17).

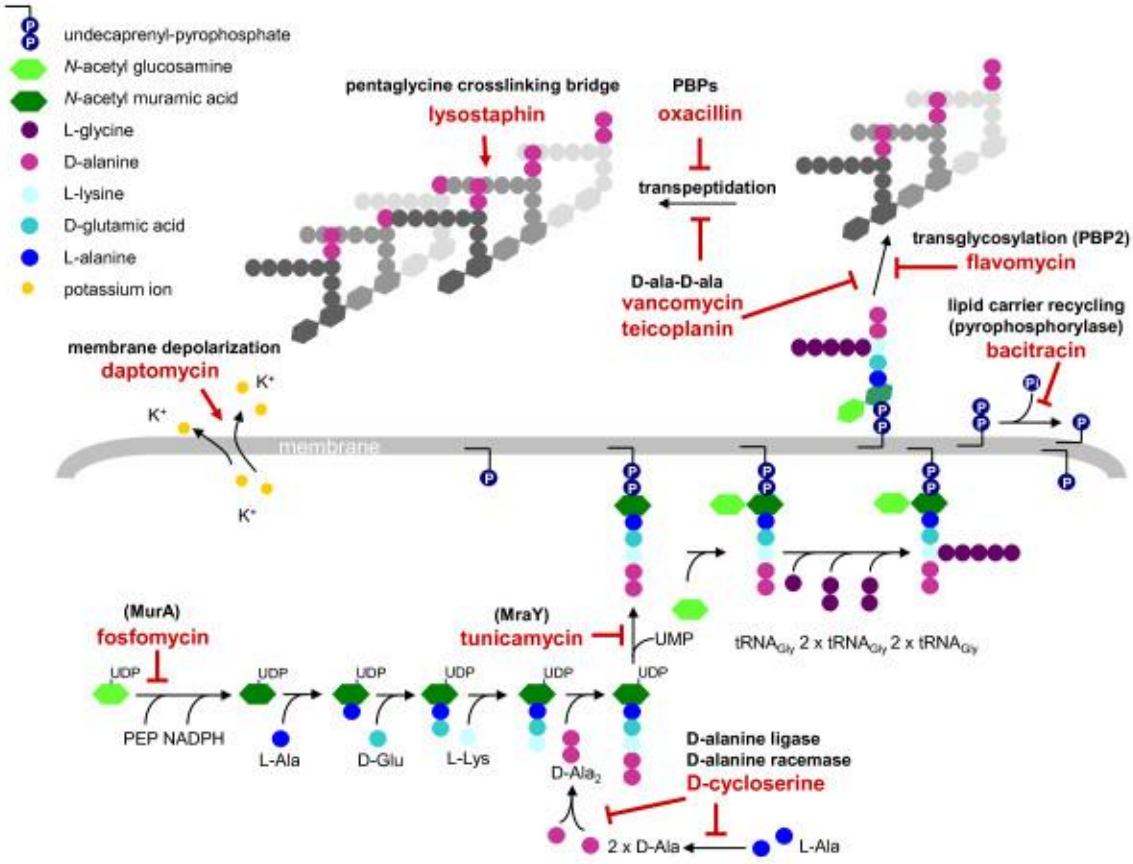

Figure 2. Diagram of the steps and enzymes involved in cell wall synthesis of *S. aureus* as well as indication of cell-wall-active antibiotic targets (14).

In response to challenge with cell-wall-active antibiotics, a number of specific genes are upregulated in expression in *S. aureus*, which has led to the identification of a cell wall stress stimulon (CWSS), known as the VraSR two-component system, within *S. aureus* (20, 35, 53). This group of genes functions to allow higher tolerance of *S. aureus* to cell wall stressors caused by stress-induced genetic signaling events, such as hydrolysis, synthesis inhibition, and antibiotic disruption of cell wall synthesis (14). VraSR-TCS is believed to protect against further damage to the cell envelope by inducing genes that promote peptidoglycan synthesis, including *pbp2*, the single staphylococcal penicillin binding protein (PBP) having both transpeptidase and transglycolase activity

(44), *mgt*, a gene encoding for monofunctional glycosyltransferase (55), *fmtA*, a penicillin-binding protein interacting specifically with beta-lactam antibiotics (16), *murZ*, which encodes for transglycolase activity in the first irreversible step of peptidoglycan biosynthesis (6), and several other genes whose role in stress response is currently unknown (32, 33, 35, 40).

In an experiment to determine the effect of *sar* and *agr* genes on autolysis within *S. aureus*, Triton-X-100 induced and penicillin-induced autolysis of wild type compared to *sar* and *agr* mutants showed decreased autolysis rates among the *agr* mutants and increased rate of autolysis among the *sar* mutants (19). *SarA* has been determined to negatively regulate autolysis by repression of *sarV*, a gene encoding for murein hydrolase activity (39). Autolytic activity resulting in release of extracellular DNA (eDNA) and nutrients from dead cells plays a role in the ability of bacteria to produce an effective biofilm as well as contributes to antibiotic resistance by promotion of horizontal gene transfer (2), which, in part, explains the effect of *sarA* on virulence by its regulation of autolysis. In relation to its regulation of *sarA*-associated virulence factors, *msa* gene has recently been shown to negatively regulate autolysis in a glucose-dependent manner independent of the *cid* and *lrg* murein hydrolase regulatory pathways (46), and this was related to the inability of the *msa* mutant to effectively form a mature and established biofilm (47). This finding presented a novel regulatory mechanism for autolysis regulation in *S. aureus*, which therefore has an effect on biofilm formation and antibiotic resistance within *S. aureus* (47).

Chapter III: Methods

Population analysis profiles (PAPs). In order to study the effect *msa* has on resistance to different cell-wall-active antibiotics in *S. aureus*, population analysis profiles for wild type and *msa* mutant versions of USA300 LAC strain were performed. Twelve different cell-wall-active antibiotics, oxacillin, cloxacillin, cephradine, phosphomycin, cefoxitin, cefotaximine, D-cycloserine, tunicamycin, vancomycin, teicoplanin, ramoplanin, and flavomycin, were tested. Based on the MICs (minimum inhibitory concentrations) for each antibiotic tested, TSA (tryptic soy agar) and MHA (Mueller-Hinton agar) with 2% NaCl agar cultures containing serial dilutions of the appropriate antibiotic were made. The wild type, *msa* mutant, and complement to the wild type strains were grown at 37˚C for 24 hours in TSB (tryptic soy broth) or MHB (Mueller-Hinton broth) with 2% NaCl, respectively, then diluted to 10^{-6} and inoculated onto the appropriate agar cultures. After incubation at 37˚C for 24 hours, the resulting colonies were counted and antibiotic concentration was compared to amount of growth.

Allelic Replacement of pKOR1. Allelic replacement of pKOR1 plasmid into *S. aureus* USA300 LAC strain was also performed as a means to generate a susceptible strain without the need for antibiotic marker selection. After transduction of the pKOR1 plasmid, which codes for chloramphenicol (CAM) resistance, into LAC, the strain was grown on TSA with 10 µg/mL of CAM at 30ºC for 24 hours. Several colonies were then transferred to a broth culture of TSB with 10 µg/mL CAM and grown at 43ºC, a non-permissive temperature for pKOR1 replication (18), for 24 hours. The broth culture was then inoculated onto a TSA with 10 µg/mL CAM agar plate and incubated at 43ºC for 24 hours, and the resulting colonies were grown in TSB broth culture at 30ºC for 24 hours.

Each overnight culture was diluted to 10^{-4} with sterile H_2O and spread onto TSA with 100 ng anhydro-tetracyclin (ATc) as well as TSA as a positive control and incubated at 30ºC for 24 hours. Colonies were transferred from the ATc plate onto TSA with 10 µg/mL CAM as well as TSA and grown at 37ºC for 24 hours. Chromosomal DNA from the colonies sensitive to CAM was harvested, and a specific region surrounding the *msa* gene was amplified by PCR with terminal attB forward and reverse primers.

Chapter IV: Results

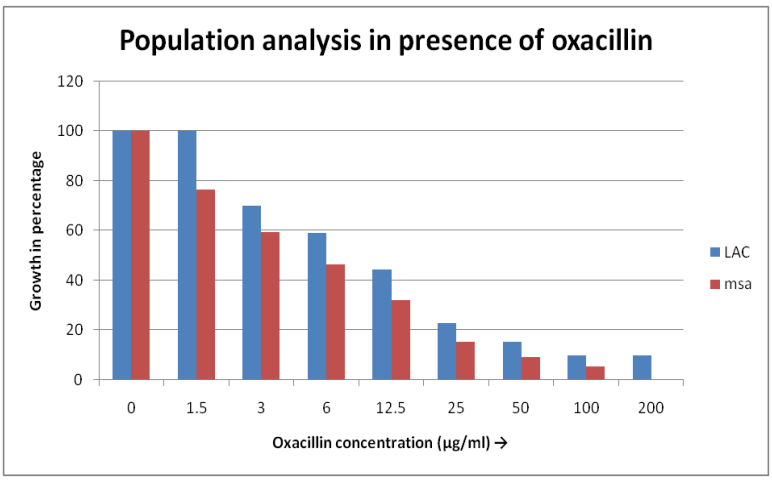

Figure 3.1. Graph comparing the growth percentage of wild-type USA300 LAC and *msa* mutant strains under exposure to serial concentrations in µg/mL of oxacillin.

In the presence of oxacillin ranging from 1.5 µg/mL to 200 µg/mL, the *msa* mutant strain had a lower growth percentage than the LAC strain as observed from the number of resulting colonies after population analysis. The number of colonies observed at 0 µg/mL of oxacillin was considered 100% growth.

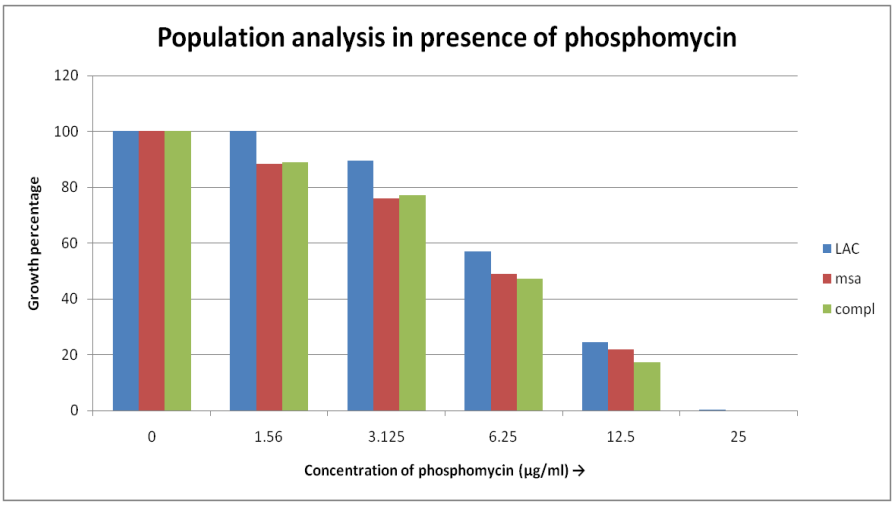

Figure 3.2. Graph comparing the growth percentage of wild-type USA300 LAC and *msa* mutant strains under exposure to serial concentrations in µg/mL of phosphomycin.

Upon exposure to 1.56 µg/mL to 12.5 µg/mL serial concentrations of phosphomycin, the *msa* mutant had a lower growth percentage than wild-type LAC and complement to wild-type LAC as observed from the number of resulting colonies after population analysis. No growth was observed in LAC, complement to LAC, or the *msa* mutant in serial concentrations above 12.5 µg/mL. The number of colonies observed at 0 µg/mL of phosphomycin was considered 100% growth.

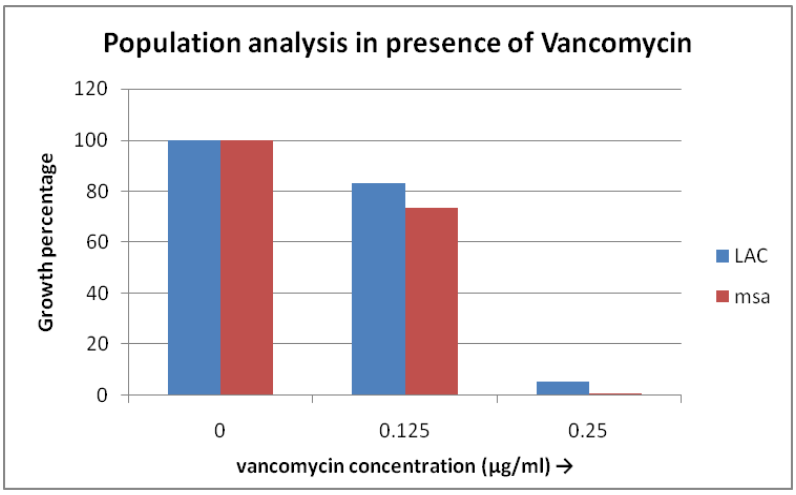

Figure 3.3. Graph comparing the growth percentage of wild-type USA300 LAC and *msa* mutant strains under exposure to serial concentrations in µg/mL of vancomycin.

After population analysis in the presence of vancomycin at the serial concentrations of 0.125 µg/mL and 0.25 µg/mL, the *msa* mutant had a lower growth percentage than the wild-type LAC as observed from the number of resulting colonies after population analysis. No growth was observed for either strain at serial concentrations above 0.25 µg/mL. The number of colonies observed at 0 µg/mL of vancomycin was considered 100% growth.

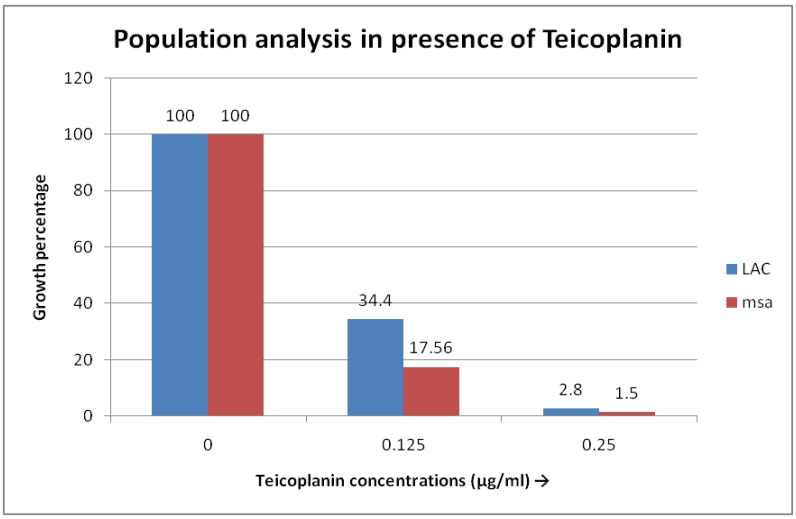

Figure 3.4. Graph comparing the growth percentage of wild-type USA300 LAC and *msa* mutant strains under exposure to serial concentrations in µg/mL of teicoplanin.

Similar to vancomycin, the *msa* mutant had a lower growth percentage compared to wild-type LAC at 0.125 µg/mL and 0.25 µg/mL serial concentrations of teicoplanin after population analysis, and no growth was observed in either strain at serial concentrations of teicoplanin above 0.25 µg/mL. The number of colonies observed at 0 µg/mL of teicoplanin was considered 100% growth.

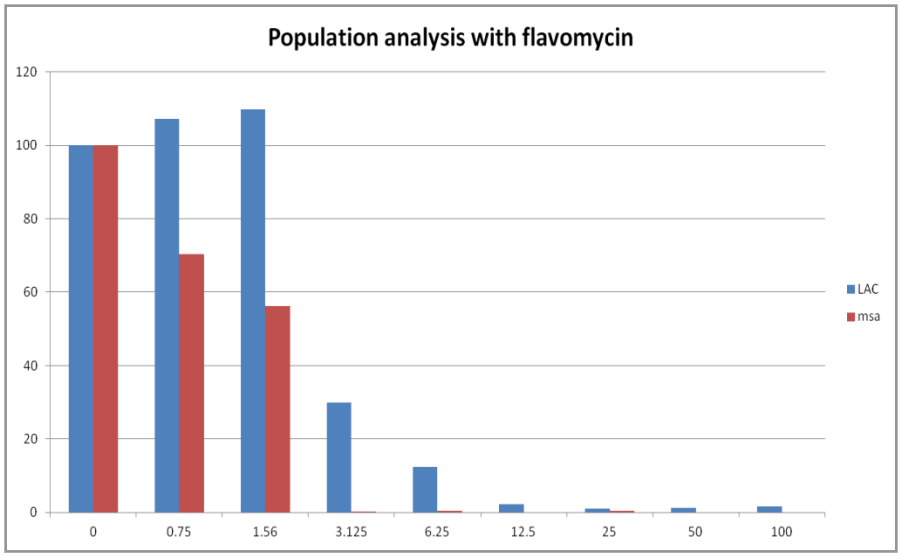

Figure 3.5. Graph comparing the growth percentage of wild-type USA300 LAC and *msa* mutant strains under exposure to serial concentrations in µg/mL of flavomycin.

Population analysis of the *msa* mutant and wild-type LAC strains with flavomycin resulted in lower growth percentage of the *msa* mutant strain compared to the wild-type LAC strain at serial concentrations ranging from 0.75 µg/mL to 100 µg/mL of flavomycin. The number of colonies growing at 0 µg/mL was considered 100% growth.

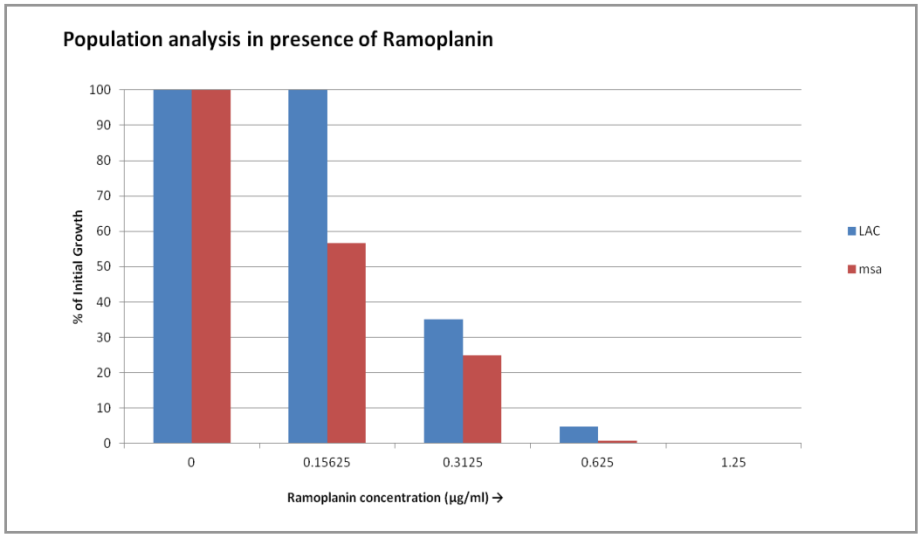

Figure 3.6. Graph comparing the growth percentage of wild-type USA300 LAC and *msa* mutant strains under exposure to serial concentrations in µg/mL of ramoplanin.

In the presence of ramoplanin at serial concentrations of 0.15625 µg/mL, 0.3125 µg/mL, 0.625 µg/mL, and 1.25 µg/mL, the *msa* mutant strain had a lower growth percentage than the wild-type LAC strain as observed from the number of resulting colonies after population analysis. No growth resulted for either strain at serial concentrations above 1.25 µg/mL of ramoplanin. The number of colonies observed at 0 µg/mL of ramoplanin was considered 100% growth.

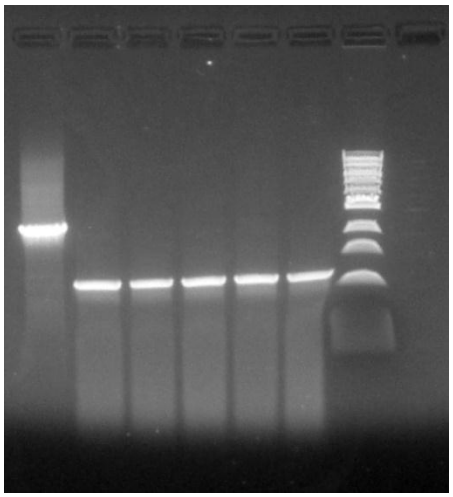

Figure 4. Gel electrophoresis of *msa* operon and the 1.5 kb region surrounding *msa* operon after deletion of *msa* gene by allelic replacement using pKOR1 in wild-type USA300 LAC strain. The first lane represents the *msa* operon, and the next five lanes represent the region containing the deleted *msa* gene. The seventh lane contains the 1 kb DNA Ladder.

Regarding the allelic replacement of pKOR1 in LAC strain, the screening of more than 200 colonies on TSA with 10 µg/mL of CAM and TSA resulted in one colony that was sensitive to CAM but was able to grow on TSA. PCR with terminal attB primers of the chromosomal DNA and electrophoresis on a 1% agarose gel showed a 1.5 kb band, verifying the *msa* gene had been deleted in the specific region of DNA amplified by attB primers. Using terminal attB primers, the *msa* operon in LAC strain was also amplified by PCR as a control, and the presence of a band in the 3 kb region of the gel verified presence of the *msa* operon.

Chapter V: Discussion

As indicated by Figures 3.1-3.6, the *msa* mutant strain showed higher susceptibility than the wild-type LAC strain to oxacillin, phosphomycin, vancomycin, teicoplanin, ramoplanin, and flavomycin when the growth percentage was compared to the antibiotic concentration each culture was exposed to after population analysis. Therefore, presence of the *msa* gene was determined to be correlated with antibiotic resistance to certain cell-wall-active antibiotics in *S. aureus*. The specific mechanism by which *msa* confers this resistance is still unknown at this time. It is known that oxacillin blocks cell wall synthesis by binding the transpeptidase active domain of PBPs (penicillin-binding proteins) (22), and flavomycin binds the transglycosylase domain of PBP2 for inhibition of transglycosylation (24). Also, phosphomycin inhibits MurA activity (30), which is an enzyme encoded by *murA*, an isozyme of *murZ* (6). Vancomycin and teicoplanin have been determined to function as glycopeptide antibiotics by binding to Lipid II of the cytoplasmic membrane and preventing transglycosylation and transpeptidation (3), and ramoplanin has also been found to prevent transglycolysation by binding to Lipid II (17). By future studies on the genes involved in stress response to cell-wall-active antibiotics and their correlation to *msa*, the function of *msa* as a regulator of antibiotic resistance within *S. aureus* could be better understood.

Since pKOR1 is unable to replicate at 43˚C, growth of the LAC strain carrying this plasmid selected for homologous recombination of pKOR1 into the bacterial chromosome (18). Following this step, growth of the strain on ATc selected for colonies lacking the plasmid since ATc induces the Pxyl/tetO promoter of pKOR1 to encode for

secY antisense transcripts, a condition which does not permit staphylococcal growth (1). Growth of the resulting colonies on TSA with CAM as well as TSA verified loss of the plasmid by selection of colonies sensitive to CAM. As further verification that pKOR1 allowed for allelic replacement of the *msa* gene within the strain, the *msa* operon was amplified by PCR using terminal attB primers (15). The gel electrophoresis as shown by Figure 4 resulted in presence of a band in the 1.5 kb region, indicating the *msa* gene had been deleted in the DNA region amplified. PCR amplification of the *msa* operon without the deleted *msa* gene in the LAC strain served as a control, verifying PCR using terminal attB primers of the *msa* operon successful by observed presence of a band in the 3 kb region of the gel after electrohporesis. This experiment confirmed the former finding by Bae and Schneewind (2005) that allelic replacement can be accomplished in low frequencies using pKOR1 in *S. aureus*, and to our knowledge, presents for the second time use of an allelic replacement vector to create antibiotic-susceptible strains without the need for antibiotic marker selection. Also, according to our knowledge, this experiment presented for the first time allelic replacement of the *msa* gene in *S. aureus* using an allelic replacement vector without the need for antibiotic markers to select for susceptibility. Bae and Schneewind (2005) used attB primers to amplify 1 kb regions upstream and downstream of the *rocA* gene, a gene encoding for an enzyme involved in proline degradation in *S. aureus* (15), in order to confirm successful allelic replacement.

It was concluded from this project that *msa* plays a role in antibiotic susceptibility within *S. aureus* by conferring a higher resistance to certain cell-wall-active antibiotics. Since it has previously been determined that *msa* affects the ability of *S. aureus* to produce a mature biofilm through its regulation of *sarA* (49), and since the ability of *S.*

aureus to effectively form a biofilm has been directly correlated with specific antibiotic resistance or susceptibility through *agr*-dependent and *sar*-dependent regulation (5, 8, 37, 38, 48, 56), future studies on the effect of *msa* on antibiotic susceptibility or resistance in the context of a biofilm could determine the relationship between *msa* and the *sar* and *agr* global virulence regulators. This could have therapeutic importance by determination of the genetic mechanism by which *S. aureus* produces a biofilm for increased resistance as well as shows a selective advantage to certain antibiotic treatments.

By inserting DNA into pKOR1 through recombination without the need for plasmid inserts to clone the DNA, the cloning process for a specific DNA region was made more rapid (1). This experiment also confirmed that pKOR1 can be used as an allelic replacement vector in order to counter-select for the plasmid replication marker in other *S. aureus* strains as well as select for allelic replacement of specific genes in *S. aureus*. Since ATc was found to successfully inhibit growth of strains carrying pKOR1, this experiment provided a possible treatment for antibiotic-resistant strains created through allelic replacement by plasmid recombination. Actually, ATc was found to select against growth of strains carrying pKOR1 even at 50 ng/mL concentrations of ATc (1). Future experiments using pKOR1 to induce counter-selection of strains carrying virulence genes in *S. aureus* could thus provide a novel possible treatment for certain *S. aureus* strains of clinical importance.

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