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Î?-Lactam-Induced Changes In Cell Wall Composition And Autolytic Properties Of Daptomycin Resistant Staphylococcus Aureus

Michael Krzyskowski
Illinois State University, mkrzysk@ilstu.edu

β-LACTAM-INDUCED CHANGES IN CELL WALL COMPOSITION AND AUTOLYTIC PROPERTIES OF DAPTOMYCIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

Michael A. Krzyskowski

40 Pages

Staphylococcus aureus is a major human pathogen that is well known for its ability to develop resistance to antimicrobial therapies. Recent studies have identified a combination of β-lactam antibiotic and daptomycin (DAP) to be highly effective at killing single and multi-drug-resistant *S. aureus*. Of particular interest is this drug combination's efficiency with which it kills strains classified as DAP-resistant methicillin-resistant *Staphylococcus aureus* (DAP-R MRSA). The goals of the present study were two-fold: to provide a physiological profile of a DAP-R *S. aureus* strain undergoing beta-lactam treatment by observing changes in autolytic rate, susceptibility to lytic enzymes, levels of peptidoglycan O-acetylation, release of lipoteichoic acid, and localization of the major cellular autolysin Atl. Additionally, the manner by which β-lactam+DAP therapy was bactericidal was explored by examining cell viability and culture optical density in response to DAP+Nafcillin (NAF). The strains used in this study were a well-characterized DAP-R MRSA D712 and its isogenic DAP-Susceptible MRSA precursor (DAP-S MRSA) D592. Growth of DAP-S and DAP-R MRSA in the presence of the nafcillin increased whole cell and crude cell wall (CCW) autolysis.

Interestingly, while D712 initially exhibited decreased whole cell autolysis both strains responded remarkably similarly to triton X-100 stimulated autolysis. CCWs of both D592 and D712 were almost entirely resistant to autolysis, lysing only ~10% over four hours. After NAF treatment a significant increase in autolysis was observed, however, the increase was more pronounced in D592. NAF exposure also increased purified cell wall (PCW) susceptibility lysoszyme and lysostaphin. Similar to CCW autolysis, nearly complete resistance to lysozyme was reversed upon growth in NAF, in contrast, lysostaphin was effective at lysing PCW, and NAF exposure produces a pronounced effect. Interestingly, D712 exhibited slight lysostaphin resistance, a phenotype that may be worth investigating. Despite the dramatic increase in lysoszyme susceptibility, growth in NAF did not result in attenuation in the level of O-acetyl groups, a factor that has been previously implicated in lysozyme resistance. In addition, NAF exposure appeared to alter Atl processing. Cell wall protein extracts in equal amounts displayed a highly similar ability to lyse suspended *Micrococcus luteus* cells, providing evidence that Atl may be delocalized upon NAF treatment. Together, these data suggest that changes in cell wall structure caused by β-lactams that may render cells more susceptible to daptomycin, and elucidation of these factors provided clues to the complicated nature of DAP resistance and will guide future research.

KEYWORDS: Antibiotics, Beta-lactam, Daptomycin, Resistance, Staphylococcus

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MICHAEL A. KRZYSKOWSKI

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MICHAEL A. KRZYSKOWSKI

COMMITTEE MEMBERS:

Brian J. Wilkinson, Chair

John C. Sedbrook

Tom M. Hammond

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M. A. K.

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CHAPTER I

INTRODUCTION

Since the introduction of penicillin in the early part of the twentieth century antibiotics have revolutionized the medical world, enabling practitioners to treat previously fatal infections with compounds that are relatively harmless to the patient. However, a significant problem with antibiotic therapy is the emergence of resistance to a particular compound within the organism being treated. The gram-positive pathogen *Staphylococcus aureus* is known to rapidly evolve resistance to novel antibiotics: in 1960-only one to two years after the introduction of methicillin--a penicillinase-resistant member of the β-lactam antibiotic family, methicillin-resistant *Staphylococcus aureus* (MRSA) strains began to emerge (1).

Today, MRSA infections number approximately 75,000 annually (2). Treatment for MRSA bacteremia and infective endocarditis typically consists of antimicrobial monotherapy with vancomycin (VAN) or daptomycin (DAP) (3). Treatment with VAN or DAP as first and second line treatments can in many cases lead to the emergence of resistance phenotypes (4), leaving practitioners with limited therapeutic options. The emergence of resistance phenotypes is a common and expected phenomenon. Traditional practice when treating resistant infections has been to change therapy to an antibiotic to which the organism remains susceptible; however, this inevitably leads to resistance evolving to the novel compound. Continued use ultimately leads to a dwindling arsenal of new antimicrobials with which to treat infections. One strategy to combat the emergence

of drug resistance is to develop combination therapies that utilize currently available compounds to more effectively treat drug resistant infections. Although there is currently no approved combination therapy for the treatment of MRSA bacteremia it has been shown in a number of cases that a combination of an antistaphylococcal β-lactam and DAP is an extremely effective combination (4), having cleared infections that had persisted for up to three weeks surviving and developing resistance to vancomycin and daptomycin monotherapy (5). Although the effectiveness of the β-lactam+DAP combination therapy is interesting in its own right it is especially fascinating that daptomycin resistant (DAP-R) strains exhibited a daptomycin susceptible (DAP-S) phenotype when challenged with this drug combination (4).

β-lactam Antibiotics

Penicillin, the most well known of the β-lactams, was discovered by Alexander Fleming in 1928. Following this discovery was a torrent of research that determined that strain, media, and culture conditions resulted in several penicillin derivatives (6), However, the presence of a characteristic "β-lactam" ring remained constant, hence the drug class name. Despite the initial efficacy of penicillin, resistant strains of *S. aureus* developed quickly, and within twenty years more than 80% of strains were found to be penicillin-resistant (7). In response to increasing prevalence of penicillin-resistant strains, considerable work was put into developing penicillin derivatives that would remain effective. One highly successful derivative is methicillin, the first penicillinase-resistant β-lactam which was approved for clinical use in the United States in 1960 (6, 7). Synthesis of β-lactam derivatives has proven invaluable in modern medicine, however, natural product discovery has led to the development of compounds with notably

different β-lactam rings. For example, cephalosporins possess an entirely different "β-lactam nucleus" to that of penicillin (6-APA vs. 7-ACA, respectively), while carbapenems and monobactams are significantly different from both penicillin's 6-APA ring and the cephalosporin 7-ACA ring (6).

β-lactam resistance can develop in several ways. Penicillin resistance is rooted in the secretion of penicillinase, an enzyme that breaks down penicillin. To combat penicillinase-based resistance, methicillin, a penicillinase-resistant β-lactam was introduced. Despite methicillin's initial effectiveness resistant strains were quickly found, some as early as the year of its clinical approval (1, 6, 7). Although β-lactamases are an effective mechanism of resistance, they are not the only mechanism. Other critical sources of β-lactam resistance lie in the penicillin-binding proteins (PBPs), the binding target of β-lactams that are integral in the construction of the cell wall. *S. aureus* typically encodes four PBPs (PBP1-4). Methicillin resistance is rooted in the acquisition of Staphylococcal Chromosomal Cassette *mec* (SCC*mec*) which includes the *mecA* gene that encodes a fifth PBP, PBP2a—a PBP with low affinity for β-lactams allowing unperturbed cell wall synthesis in the presence of β-lactams (7). Although the presence of PBP2a is pivotal in the development of β-lactam resistance, previous work has reinforced the important roles of other PBPs in the context of alternative therapies, demonstrating that β-lactams that selectively target PBP1 enhance DAP activity to a greater extent than those that non-selectively inhibit PBPs such as nafcillin (8).

Daptomycin

Daptomycin is a 1.6 kDa anionic lipopeptide antibiotic that associates with Ca^{2+} in its active form giving it a net positive charge. It is isolated from *Streptomyces*

roseosporus and was approved for clinical use in 2004 (9, 10). Current models for the DAP mechanism of action identifies the target of DAP as the cell membrane, where it binds phospatidylglycerol and aggregates, causing the leakage of ions, membrane depolarization, disruption of protein, DNA and RNA synthesis and ultimately cell death (9, 11). To bind its target, DAP requires Ca^{2+}, the binding of which results in conformational changes that account for membrane insertion and oligomerization potentially as a result of increased exposure of hydrophobic residues in the molecule (9). Daptomycin resistance is complex, involving a diverse range of genes ranging from those responsible for membrane lipid biosynthesis to cell wall regulation (12, 13). Interestingly, gain of function mutations in the gene *mprF* – responsible for synthesis of lysyl phosphatidylglycerol, a positively charged membrane phospholipid –have been found to be relatively common in clinical DAP-R isolates (14). Due to the increased presence of positively charged phospholipids in clinical DAP-R strains a charge repulsion mechanism has been proposed as part of the DAP resistance phenotype, while for *in vitro* derived strains charge repulsion cannot always explain resistance (14). Previous work has demonstrated that exposure to β-lactams results in increased DAP binding (4) suggesting that a decrease in cell surface positive charge is associated with β-lactam treatment. It is speculated that release of membrane-bound lipoteichoic acid (LTA), which is often modified with D-alanine, thereby increasing the overall net positive charge of the membrane, could play a role in the increased ability of DAP to bind its target. This is supported by research demonstrating the exceptionally increased binding ability of mammalian group IIa phosphpolipases A_2, which are similarly charged and bind similar targets as DAP, to *S. aureus* strains with mutations in *dltA*, rendering them unable to add

D-alanine to LTA (15). Exposure to β-lactam antibiotics has been shown to cause the release of LTA from the cell membrane (16); however, this has yet to be directly demonstrated in a study examining this synergy. Further evidence for the significance of charge-based repulsion has been provided by Sakoulas et. al. (5), where enhanced binding and killing due to host cationic defense peptides was observed upon treatment with sub-MIC concentrations of the β-lactam nafcillin (NAF).

Unlike β-lactam antibiotics that primarily cause cell lysis due to a weakened cell wall, DAP can be bactericidal without lysis (17). When challenged with daptomycin, early exponential phase cells exhibit rapid loss of viability with little to no concomitant drop in optical density, implying a lack of lysis, which was further confirmed by electron microscopy (17). Interestingly, previous work has demonstrated an ability of daptomycin to remain lethal against stationary-phase cultures. Although daptomycin does affect cell division machinery, and thus the cell wall (12, 17, 18), it remains to be determined whether cells undergoing β-lactam+DAP therapy are preferentially killed by one antimicrobial mechanism or another.

Autolysins

Previous work has demonstrated that growth of MRSA in β-lactam-containing media causes whole cells to exhibit increased autolysis, and crude and purified cell wall preparations are more susceptible to degradation by lysozyme (17). One potential source of increased autolysis could be a result of delocalization of the major cellular autolysin Atl. In addition to charge-based repulsion, simple size exclusion is another potential source of DAP resistance. Previous studies of vancomycin-resistant strains have found that VAN resistance and DAP resistance are correlated with increased cell wall thickness

(10). It could be possible that delocalized Atl sufficiently weakens the cell wall and "opens" it to penetration by DAP molecules.

Autolysins, named for their capacity to cause rapid cell lysis unless tightly regulated are also commonly referred to as peptidoglycan hydrolases and play significant roles in the regulation of cell wall growth/turnover and the separation of daughter cells during cell division (19, 20). AtlA is the most prominent peptidoglycan hydrolase in *S. aureus* with implications in cell wall maintenance and cell division as well the initial stages of biofilm formation (19, 20). AtlA is expressed as a 137.5 kDa pro-protein that is cleaved into four distinct parts, two of which are amidase and glucosaminidase domains that are responsible for much of the previously discussed function of Atl (20). Loss of the *atl* gene has been shown to decrease the effectiveness of lytic antimicrobials such as β-lactams, due to the cell's inability to divide (21). In addition to Atl, Sle1 is another important hydrolase involved in cell septation, although it has not been determined to be implicated in autolysis of *S. aureus* (22).

In addition to autolysins, several antibacterial lytic enzymes are classified as peptidoglycan hydrolases, two of these enzymes are lysozyme and lysostaphin. Lysozyme is an antimicrobial enzyme naturally produced by many organisms, and one of the most common sources of lysozyme is hen egg white (7). Lysozyme targets the bond joining the N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) subunits of peptidoglycan (PGN) to cause rapid lysis, although many pathogenic organisms, *S. aureus* included, are highly resistant to lysozyme. Currently, the O-acetylation of C-6 on NAM is proposed as the reason for this resistance, since many non-pathogenic organisms (*Staphyococcus carnosus* and *Staphyococcus xylosus* for example) lack O-acetyl groups

(23, 24). Lysostaphin is a potent lytic enzyme that is isolated from *Staphylococcus simulans* biovar *staphylolyticus*, and is effective against most species due to its targeting of the *staphylococcal*-specific pentaglycine cross-bridge that cross-links individual strands of PGN (7, 25, 26).

Lipoteichoic Acid

The staphylococcal cell envelope consists primarily of PGN and teichoic acids (TAs) (27). While PGN is well characterized, the functions of teichoic acids are not fully known (27). Teichoic acids are of two types: wall teichoic acids (WTA) and lipoteichoic acids (LTA), and determining the specific function of each has been made complicated due to their multiple functions, several of which overlap (27). LTA are embedded in the cell membrane and composed of a polyglycerolphosphate backbone, while WTA are covalently linked to cell wall PGN and composed of a polyribitolphosphate backbone (24, 27). An important modification to teichoic acids is D-alanylation, which occurs via proteins specified by the *dlt* operon. D-alanine serves a general function as a counterion to determine net anionic charge of a teichoic acid (24). The *dlt* operon consists of four genes (*dltABCD)* that slightly overlap (24). *dltA* and *dltC* encode Dcl and Dcp, respectively, while *dltB* encodes a transport protein and *dltD* a membrane protein. Together, the general model of LTA D-alanylation consists of attachment of D-alanine to Dcp by Dcl and transport to the extracellular surface through a channel (DltB), with DltD serving as a binding site for both Dcp and Dcl (24). Interestingly, it has been determined that D-alanine esters on WTA are derived from LTA, however, the process by which this happens is not clear (24).

In *B. subtilis*, inactivation of *dltABC or D* results in both wall and lipoteichoic acids lacking D-alanine; additionally, strains deficient in D-alanine exhibited increased autolysis and methicillin sensitivity (24). *S. aureus dlt* mutants are unable to alanylate their teichoic acids and exhibit increased susceptibility to cationic host defense peptides due to the increased net anionic charge on TAs (24). D-alanyl TAs have had a multitude of functions ascribed to them depending on the species. These functions include general cation homeostasis, cell wall electrochemical composition, protein folding, antibiotic resistance, and biofilm formation (24). As previously mentioned, D-alanine can migrate between LTA and WTA with relative ease, and it has been proposed that the location of D-alanine may serve as a signal for proteins that require specific ionic environments to function (24). One class of proteins that fit this description are autolysins, thus, a loss of LTA leads to hypo-alanylated WTA, which could lead to autolysin delocalization.

Specific Aims and Hypotheses

Cell wall physiology and autolytic properties are essential knowledge when understanding mechanisms of resistance and DAP-R cells are relatively uncharacterized in the context of β-lactam+DAP therapy. To remedy this, we aimed to document the physiological changes in the bacterial cell wall and cell wall-associated elements pre- and post-β-lactam treatment. We hypothesized that growth in media containing NAF would result in a wide range of effects including increased rates of autolysis of whole cells and crude cell walls in addition to cells with walls with a decreased level of O-acetylation that are more prone to lysis by lytic enzymes. In addition, we expected to see increased liberation of LTA into the culture medium, a phenomenon that may be implicated in increased autolysis due to delocalization of the major cellular autolysin Atl.

Our second objective was to examine the loss of cell viability in response to β-lactam+DAP treatment. We expected that exponentially growing cells would exhibit cell lysis as the main mode of killing during β-lactam+DAP therapy, likely due to the compounding physiological defects in the cell wall induced by β-lactams as well as daptomycin. We expected that this combination would not remain effective against stationary-phase cells, despite DAP's potential to kill stationary-phase cells by depolarizing the membrane and disrupting protein, DNA, and RNA synthesis due to an inability to reach and/or bind its target at the cell membrane.

Determination of the cell wall physiological properties of DAP-R cells in response to beta-lactam treatment as well as clarification of the mode of killing during the course of β-lactam+DAP therapy is crucial to further elucidation of the mechanism of DAP resistance, and may prove useful for the development of future therapies for the treatment of DAP-R isolates.

CHAPTER II

MATERIALS AND METHODS

The strains of *S. aureus* utilized in this study are D712, a daptomycin-resistant, methicillin-resistant, vancomycin-intermediate *Staphylococcus aureus* (DAP-R VISA) and D592, its daptomycin-susceptible, methicillin-resistant, heterogeneously vancomycin-intermediate (DAP-S hVISA) isogenic precursor. Both strains were provided by Dr. George Sakoulas (University of California, San Diego). Experiments were performed in triplicate and averaged unless stated otherwise. Statistical significance of lysis assays was determined by Kolmogorov-Smirnov (K-S) test while bar charts were subjected to t-test. All calculations were performed in Microsoft Excel.

Whole Cell Autolysis Assays

Overnight cultures of *S. aureus* D592 and D712 were prepared in Tryptic Soy Broth (TSB) and then diluted 1:50 into TSB or TSB containing 20 µg/ml NAF and grown to mid-exponential phase (OD_{600} 0.6-0.7). Cells were then harvested by centrifugation (10 minutes, 13,000 xg) at 4°C. Following two washes with cold dH_2O cell pellets were resuspended to OD_{600} 0.5-0.7 in pH 7.5 lysis buffer containing 0.05 M Tris-HCl and 0.05% wt/vol Triton X-100. Cells were then incubated in spectrophotometer cuvettes at 37°C for four hours, and the OD_{600} was observed every thirty minutes. Data are presented as a percentage of initial OD.

Isolation of Crude and Purified Cell Wall Fragments

Isolation of cell walls was carried out essentially the same as Bera et. al (24) with some modifications. Overnight cultures of *S. aureus* D592 and D712 were prepared in TSB and then diluted 1:50 into plain fresh TSB or fresh TSB containing 20 µg/ml NAF and grown to mid-exponential phase (OD_{600} 0.6-0.7) at 37°C with 200 RPM shaking. Cells were then harvested by centrifugation at 4°C for 10 minutes at 13,000 xg and washed twice in an excess cold 0.9% NaCl for 10 minutes. Following washing, pellets were resuspended in cold 0.9% NaCl and mechanically disrupted using 0.1mm glass beads in five two minute intervals for a total of ten minutes of disruption. Glass beads were separated from disrupted cell material by vacuum filtration through a 40µm filter, and the cell fragments transferred to centrifuge tubes for harvesting (as done previously). After harvesting, a portion of the crude cell wall fraction (CCW) was resuspended in cold dH_2O and saved for use in CCW autolysis experiments. The remaining material was washed twice in cold dH_2O before boiling in 2% SDS for 30 minutes. After boiling in SDS, peptidoglycan was harvested by centrifugation at 20°C and then washed twice in room temperature dH_2O. After washing, remaining material was incubated overnight at 37°C in 0.1M Tris-HCl solution (pH 6.8) containing 100 µg/ml Proteinase K. Following proteinase K treatment the solution was harvested by centrifugation for 10 minutes at 13,000 xg, washed four times in room temperature dH_2O to remove as much left over SDS as possible, and then lyophilized and saved as purified cell wall (PCW) fragments.

CCW Autolysis

CCWs obtained from mechanical disruption using glass beads were diluted in 0.05M K_2HPO_4 buffer pH 7.2 to OD_{580} 0.4-0.6, incubated at 37°C for four hours and the

OD_{580} was monitored every thirty minutes. Data are presented as a percentage of the initial OD.

PCW Lysis

Lyophilized PCWs were resuspended in Tris-HCl pH 7.5 to OD_{580} 0.5-0.6 and incubated with 100 µg/ml lysozyme at 37°C for four hours and the OD_{580} was monitored every thirty minutes. A lysostaphin lysis assay was carried out alongside the lysozyme assay with a few modifications: a concentration of 5 µg/ml lysostaphin was used, and OD_{620} was monitored instead of OD_{580} in accordance with established lysostaphin lysis assay protocols (25). Data are presented as a percentage of the initial OD.

Degree of O-acetylation

Taking care to keep pH below 7.0 during the isolation of PCW ensures that the highly alkali-labile O-acetyl group remains present on the wall. To liberate O-acetyl groups from PCW, lyophilized PCW fragments (5-10mg) were incubated in either 400µl 80mM NaOH or pH 6.8 PBS (negative control) for 3 hours at 30°C. After incubation NaOH was neutralized with an appropriate amount of H_2SO_4. Following centrifugation for 10 minutes at 13,000 xg the supernatant was harvested to determine the degree of O-acetylation, which was analyzed using the K-ACET Acetic Acid Analysis Kit (Megazyme Inc.). Addition of reagents was carried out according to kit protocol. O-acetyl groups were quantified against a standard curve constructed using several concentrations of acetic acid standard provided in the kit (0.1 mg/mL), and presented as nano-moles of acetic acid per mg of dry PCW.

Quantification of Released Lipoteichoic Acid

In order to quantify the amount of LTA released due to exposure to β-lactams, bacterial supernatants were analyzed via ELISA as described by Van Langevelde *et al.* (16) with some modification. An LTA standard curve was produced using purified *S. aureus* LTA (Sigma-Aldrich) in concentrations ranging from 0-500 ng. Bacterial supernatants were incubated undiluted and diluted 10x alongside LTA standard concentrations with an anti-mouse gram-positive LTA monoclonal antibody (Thermo Fisher) overnight at 4°C. Following washing the plate was blocked for one hour using a 1:1 mixture of pH 7.2 PBS and low-fat soy milk (28). Detection was facilitated by observation of OD_{410} upon the addition of 1-step ABTS (Thermo Scientific) to a goat-α-mouse IgG-peroxidase conjugate (Thermo Scientific).

Freeze/Thaw Extraction and Zymographic Analysis of Peptidoglycan Hydrolases

Peptidoglycan hydrolases were extracted from the cellular surface of D592/712 strains grown in the presence or absence of 20 µg/ml NAF by the freeze/thaw method and analyzed using a zymographic method essentially as described by Koehl *et al*. (29): After growing to an OD_{600} of approximately 0.4, 100ml of culture was harvested at 13,000 xg at 4°C then washed once with cold water and once with 0.01 M phosphate buffer (pH 7.0) before being resuspended in 1 ml of buffer. The suspension was then subjected to three freeze/thaw cycles, each cycle consisting of incubation for 1 hour at -80°C followed by 10 minutes at 37°C. Following three cycles the suspension was centrifuged at 13,000 xg at 4°C for 10 minutes, and the supernatant saved as the freeze/thaw autolysin extract. 10 µl extracted protein was electrophoresed in a 10% SDS gel containing 0.2% wt/vol *Micrococcus luteus* cells. Following electrophoresis the gel

was incubated overnight in renaturation buffer containing 25 mM Tris-HCl (pH 8.0) and 1% Triton X-100. After renaturation the gel was stained with 1% methylene blue in 0.01% KOH, and destained with dH_2O. Peptidoglycan hydrolases (autolysins) are visualized as clear bands in the gel.

Autolysin Localization Assay

Following freeze/thaw autolysin extraction the protein concentration of extracts was determined by Bradford assay. Equivalent concentrations of protein extracts were added to cuvettes containing *Micrococcus luteus* cells suspended to OD_{600} = 0.5-0.6, and the optical density monitored for three hours. Data are presented as a percentage of initial OD_{600}.

Mode of Killing

Mode of killing experiments were performed essentially according to Cotroneo *et al.* (17). Cells were grown to mid-log phase in the presence and absence of 20 µg/ml NAF and then exposed to 10 µg/ml DAP. Cell viability was quantified as CFU/ml calculated via broth serial dilution and plating on TSA plates. Alongside cell viability tests the OD_{600} of the culture was monitored; a decrease in cell viability without significant decrease in OD_{600} implies the killing of cells without lysis, while a decrease in OD_{600} implies lysis. The antibiotic concentrations utilized here were derived from Dhand *et al.* (4), where a treatment of 20µg/ml NAF and 10µg/ml DAP provided the most effective bactericidal action.

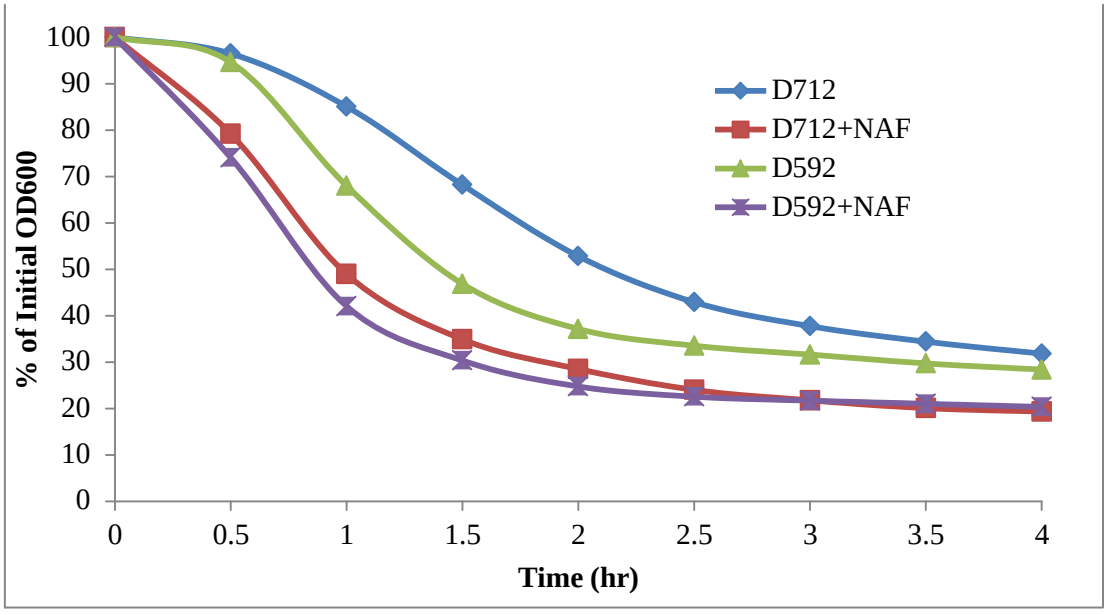
CHAPTER III

RESULTS

Growth in the Presence of Nafcillin Increases Whole Cell and CCW Autolysis Rates

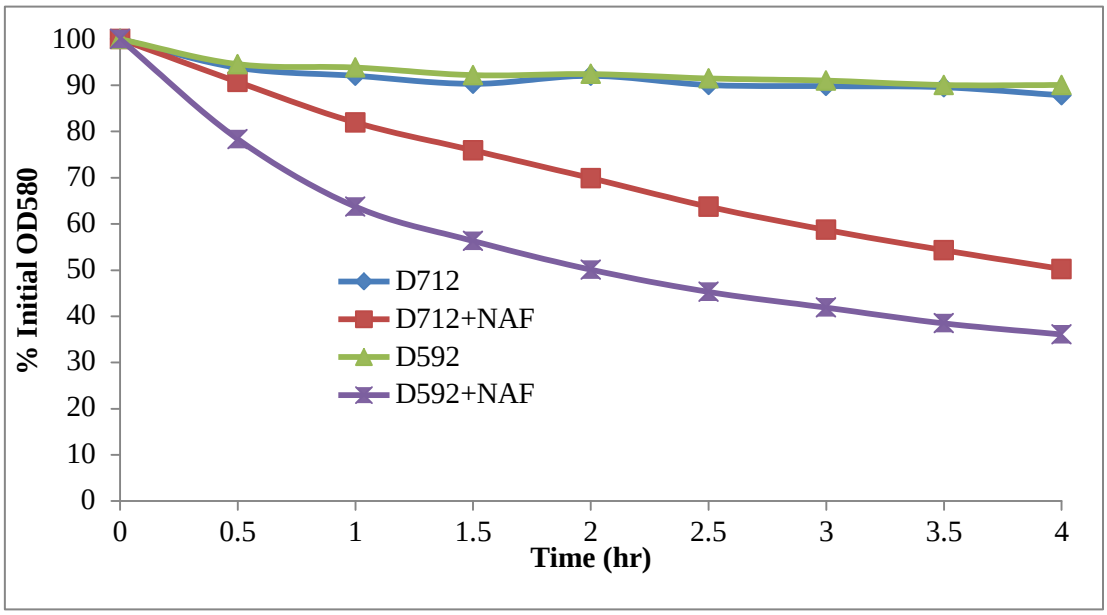
Both whole cell and crude cell wall preparations from cells grown in the presence of nafcillin autolyse at a faster rate (Fig. 1, 2). While unexposed CCW preparations fail to autolyze almost entirely, losing only about 10% of the initial OD, CCW of exposed cells clear to approximately 50% and 35% in D712 and D592 respectively.

Figure 1. Triton X-100 Stimulated Whole Cell Autolysis



Triton X-100 stimulated whole cell autolysis in S. aureus strains D592 and D712 unexposed or exposed to 20µg/ml NAF represented as % decrease of optical density over 4 hours. Significance was determined by K-S test of NAF treated vs. NAF untreated strains, in each case $D > D_{\alpha}$

Figure 2. Crude Cell Wall Autolysis

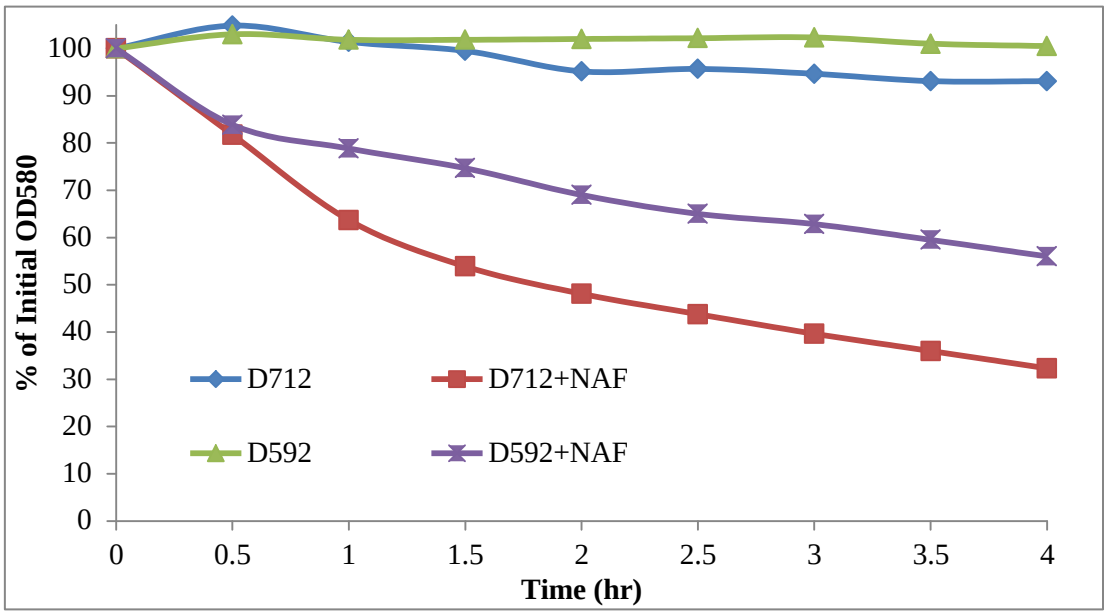


Crude cell wall autolysis of S. aureus strains D592 and D712 unexposed or exposed to 20μg/ml NAF represented as percent decrease of OD over 4 hours. Significance was determined by K-S test of NAF treated vs. NAF untreated strains, in each case $D > D_\alpha$

Growth in the Presence of NAF Sensitizes PCW to Lytic Enzymes

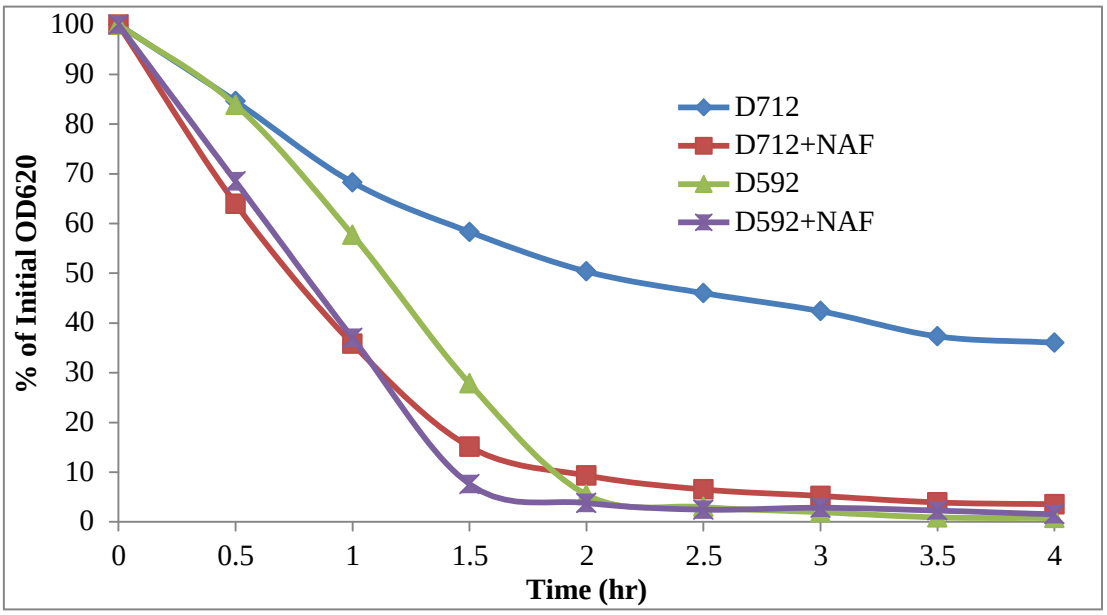
Treatment with β-lactam antibiotics is likely to affect the composition of the overall cell wall with respect to cross-linking or peptidoglycan chain length. To evaluate cell wall integrity crude cell wall preparations were further purified by boiling in 2% SDS and incubation with proteinase K. Similar to whole cell and CCW autolysis studies, NAF+ purified cell wall preparations are much more susceptible to degradation by lytic enzymes (lysozyme and lysostaphin) than untreated preparations (Fig. 3, 4). Interestingly, untreated D712 displays slight lysostaphin resistance (Fig. 4); however, this resistance was negated after growth in NAF.

Figure 3. Purified Cell Wall Lysis with Lysozyme



Purified cell wall lysis with lysozyme (100µg/ml) in S. aureus strains D592 and D712 unexposed or exposed to 20µg/ml NAF. Data represented as percent decrease in initial OD. Significance was determined by K-S test of NAF treated vs. NAF untreated strains, in each case $D > D_\alpha$

Figure 4. Purified Cell Wall Lysis with Lysostaphin

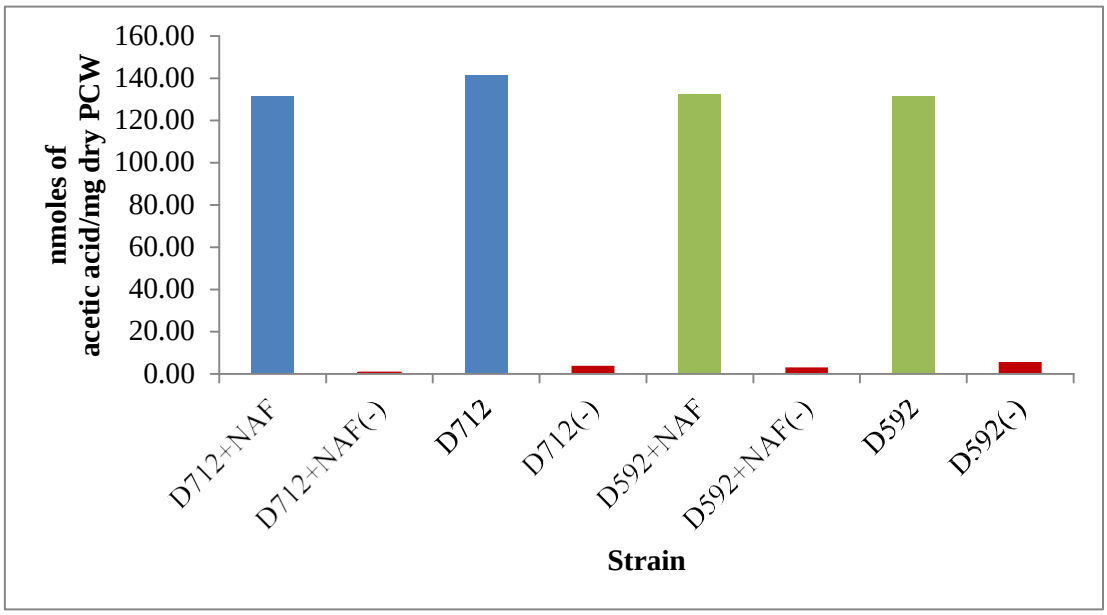


Purified cell wall lysis with lysostaphin (5μg/ml) in S. aureus strains D592 and D712 unexposed or exposed to 20μg/ml NAF. Data represented as percent decrease in initial OD. Significance was determined by K-S test of NAF treated vs. NAF untreated strains, in each case $D > D_\alpha$

O-acetylation of Cell Walls is not Significantly Affected by Growth in NAF

O-acetylation has previously been shown to aid in immune system evasion (23). Estimation of O-acetylation levels was performed using the MegaZyme acetic acid assay kit as reported elsewhere (30) and the total released acetate is reported. While O-acetylation levels do vary by about 7% between treated and untreated strains of D712 this trend does not hold true for D592 (Fig. 5). In addition, while high levels of O-acetylation have been demonstrated in previous studies (23) to contribute to lysozyme resistance, we did not consider this relatively small difference a to be a significant source of increased lysozyme resistance, and did not pursue it further.

Figure 5. Cell Wall O-acetylation

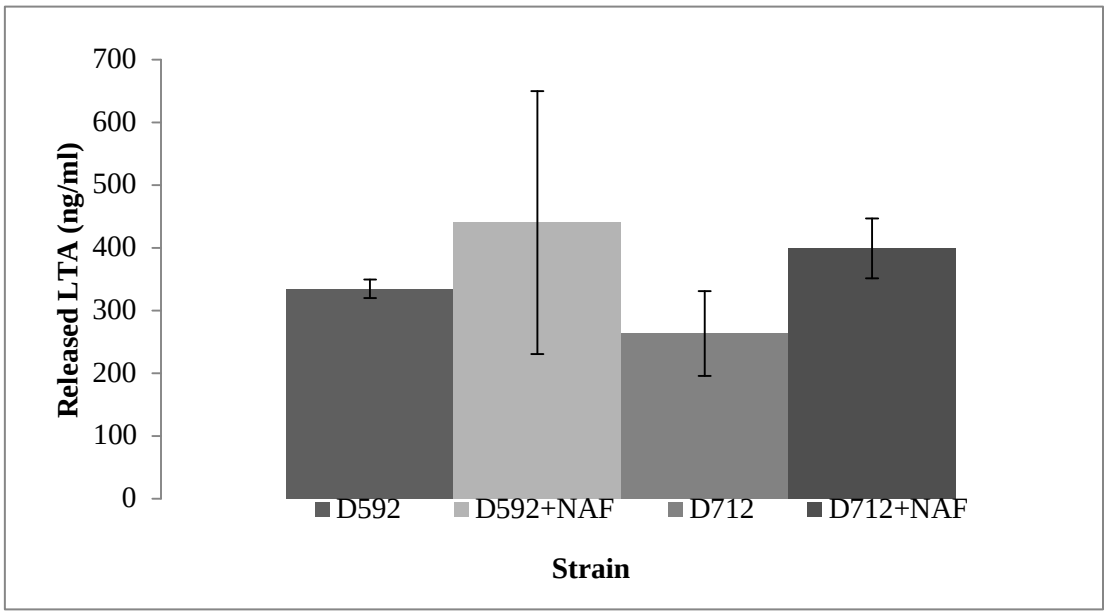


Cell Wall O-acetylation of S. aureus strains D592 and D712 grown with or without NAF (20µg/ml). Samples marked "(-)" are negative controls incubated in pH 6.8 PBS to assure no release of O-acetyl groups.

LTA Release is Increased in Response to NAF Treatment

To determine and quantify the amount of LTA released into culture medium ELISA was performed. Cells grown in sub-MIC concentrations of nafcillin exhibited an increase in the amount of LTA released into culture medium by an average of approximately 120 ng/ml (Fig. 6), interestingly, significance was detected in strain D712 via a paired sample t-test but not strain D592.

Figure 6. Quantified Release of Lipoteichoic Acid



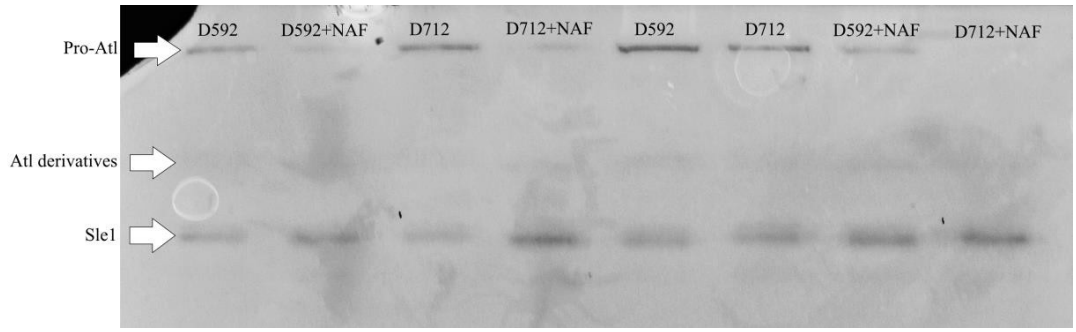
LTA release as a result of NAF exposure. Growth in NAF results in an approximate 30% increase in LTA release. Significance was detected in strain D712 upon growth in nafcillin ($p < 0.05$) while no significance was found for D592.

Growth in NAF Alters Atl Processing

S. aureus strains grown in nafcillin were hypothesized to undergo a delocalization of the major cellular autolysin Atl. A method was developed to infer the location of Atl in the cell wall during exponential phase. If increased rates of autolysis upon beta lactam treatment are due to delocalized surface autolysins, and not an increased or decreased amount of enzyme, there should be no significant difference in autolysis rates between treatments within the same strain. Cells were harvested in exponential phase (OD_{600} 0.4-0.5) and subjected to freeze/thaw extraction. To verify lytic activity of extracts each extract was subjected to zymographic analysis (Fig 7). 10 µl of freeze/thaw extract from each treatment was electrophoresed on a polyacrylamide gel containing 0.2% lyophilized *Micrococcus luteus* cells and clearing of stained cells was observed. Following

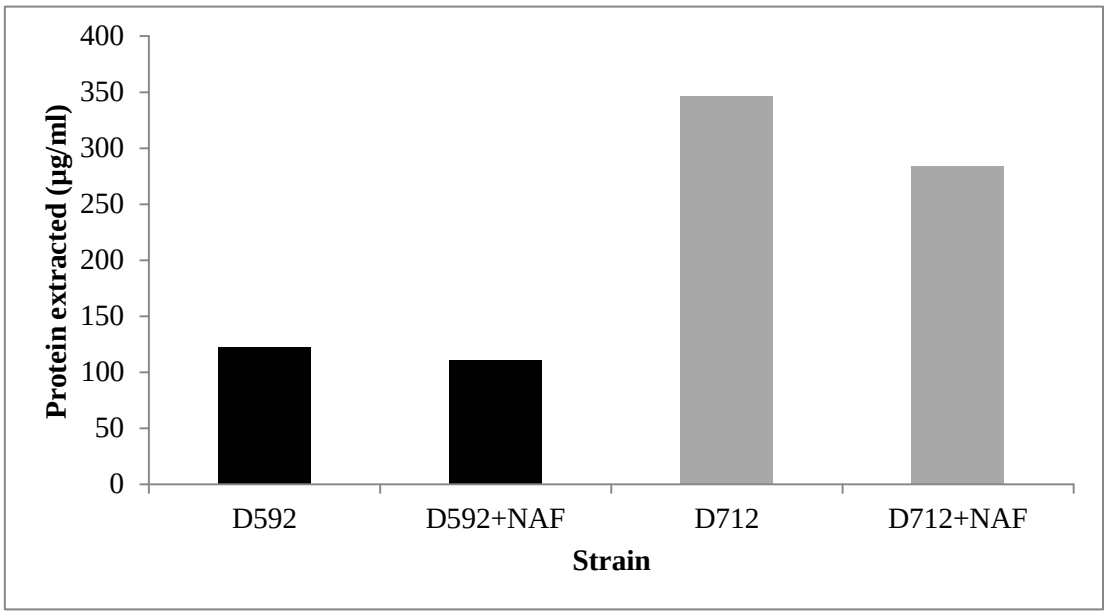
confirmation of lytic activity the protein concentration of each extract was determined by Bradford assay (Fig. 8), and suspensions of *M. luteus* were challenged with equivalent amounts of protein from each extract. Differences in band intensity observed during zymographic analysis are due to differences in total protein concentration of extracts. Differences in protein extracted per strain and treatment may be a result of differences in extraction efficiency. Both 1 µg (data not shown) and 10 µg (Fig. 9) treatments display little to no activity in suspension.

Figure 7. Zymographic Analysis



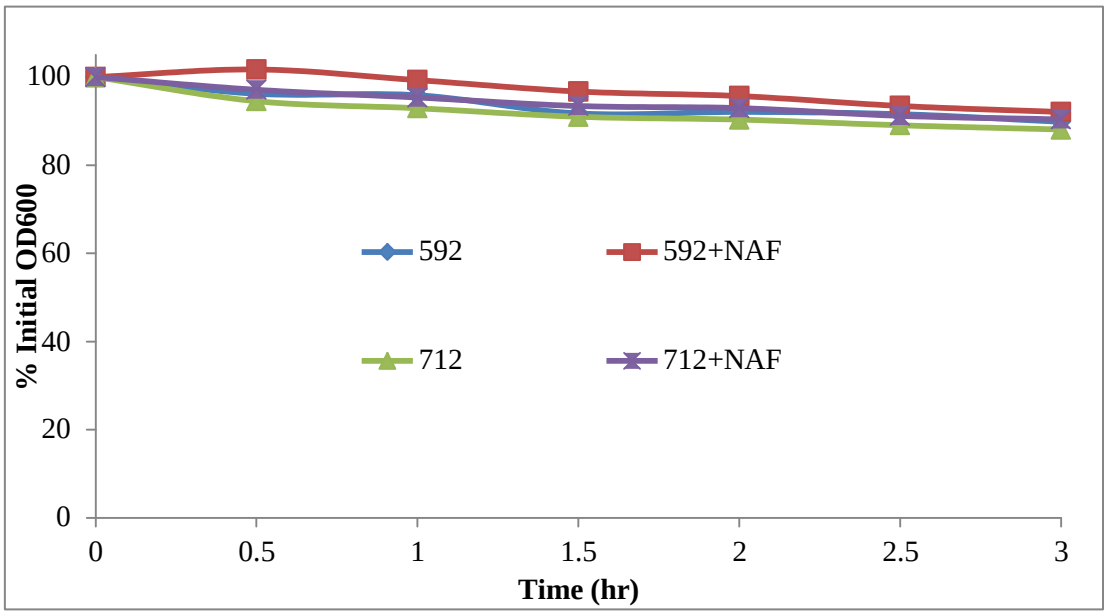
Zymographic analysis for verification of lytic activity of freeze/thaw protein extracts. Zymogram was prepared pre-Bradford assay to verify activity of extracts. 10 µl of each extract was added per well; differences in band intensity are due to different overall protein concentration of extracts.

Figure 8. Quantification of Extracted Protein



Protein extracted via freeze/thaw as quantified by Bradford assay.

Figure 9. Lysis of *M. luteus* Cells by 10 µg Extracted Protein



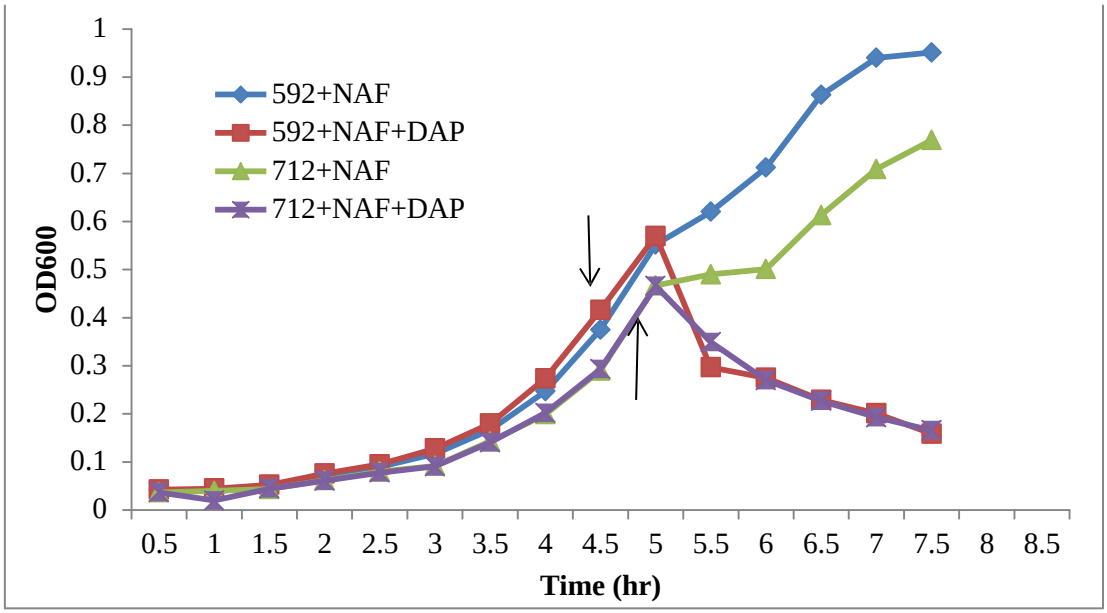
Lack of lysis of M. luteus cells by 10 µg freeze/thaw protein extract. 10 µg of protein extract was added to cuvettes containing M. luteus cells at OD_{600} *~0.6. Data represented as a percent decrease in initial OD.*

NAF+Dap is Lytic in Action and Ineffective against Stationary Phase Cells

NAF and DAP exhibit distinct mechanisms that result in loss of cell viability in different ways. While NAF induces cell lysis through a weakening of the cell wall, DAP is able to cause loss of viability without lysis by disrupting cell membrane polarization affecting protein, DNA, and RNA synthesis. To determine if cells undergoing NAF+DAP treatment are dying preferentially to one antimicrobial mechanism, its potency was tested against exponential and early stationary phase cells. Exponential phase cells exhibited lysis as a result of NAF+DAP treatment as is indicated by the dramatic decrease in optical density upon addition of DAP (Fig. 9), while those untreated with DAP continue to grow normally. Stationary phase cells growing in the presence of

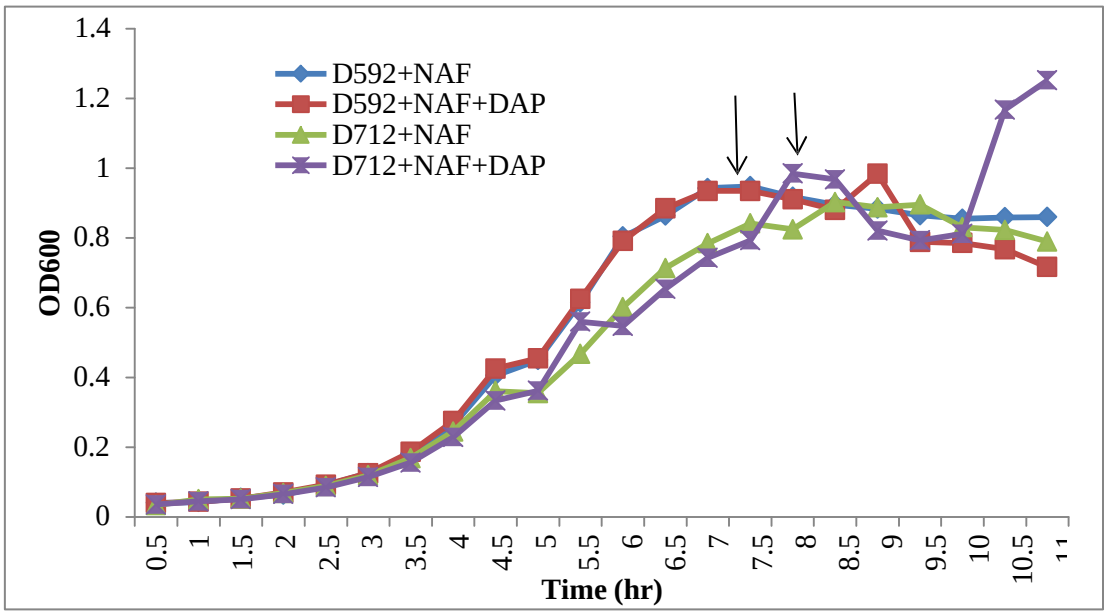
NAF were largely unaffected by DAP, and in some cases continued to grow (D712+NAF+DAP) (Fig. 10).

Figure 10. Growth Curve of D592 and D712 DAP Added at Exponential phase



DAP 10 µg/ml added to each culture after first reading OD_{600} > 0.4 (arrows)

Figure 11. Growth Curve of D592 and D712 DAP Added at Stationary phase



DAP 10 µg/ml added to each culture after first reading of plateaued growth (arrows)

CHAPTER IV

DISCUSSION

MRSA infections are a serious but treatable problem. Unfortunately, in the case of *S. aureus* resistance is an inevitable outcome. Evolution of resistance phenotypes leaves practitioners with the necessity of using novel drugs that have extensive development time and may be cost prohibitive to the patient. An alternative treatment in cases with particularly stubborn infections is the use of combination therapy. Daptomycin+Nafcillin is an extremely potent combination with well documented effectiveness (4). While the potency of this combination has been known for some time, its mechanism of action has remained unclear. A necessary first step in fully elucidating the mechanism of this antibacterial combination is knowledge of multiple physiological markers present in the bacterial cell wall when undergoing beta-lactam therapy to provide clues for future research.

Here we have examined a number of physiological traits present in DAP-R *S. aureus* cells to provide a more complete physiological profile of the DAP-R phenotype in response to β-lactam treatment. Whole cells and crude cell wall preparations become much more susceptible to autolysis upon growth in β-lactam containing media, suggesting an overall weakened cell wall. Cell wall disruption by β-lactams is due to interruption of the PBPs, and in *S. aureus* a significant degree of cell wall integrity is derived from cross-linking. Cross-linking in MRSA has previously been shown to be decreased due to inhibition of PBP4 (17, 31), a phenomenon exhibited by PBP non-

specific β-lactams such as NAF. The ability of lysostaphin to rapidly lyse PCW from cells grown in the presence of NAF supports the presence of hypo-crosslinked cell walls. Lysostaphin targets the pentaglycine cross-bridge of *S. aureus* to significantly disrupt cell wall integrity and cause lysis. Interestingly, we detected notable lysostaphin resistance in strain D712 when grown in the absence of NAF. Lysostaphin resistance is not well understood, however, lysostaphin resistance A (*lyrA*) has been determined as a major factor (32). Interestingly, *lyrA* does not significantly modify the pentaglycine crossbridge to produce an un-bindable substrate for lysostaphin (32). Should this phenotype be pursued in future work, sequencing is first required to examine this strain for any *lyrA* mutations, along with other mutations that may be compounding factors such as *femAB,* which produces significant alterations in pentaglycine crossbridges (32).

Additionally, PCW from cells grown in NAF exhibited increased susceptibility to lysozyme. O-acetylation has been implicated as a major effector of lysozyme resistance in cells with O-acetylated NAM subunits, and while levels of O-acetylation do decrease slightly upon growth in nafcillin the difference in this case is negligible in the case of D712 while being almost entirely absent in the case of D592. O-acetyl groups are highly alkali-labile, and extensive care must be taken to keep all pH conditions under 7.0 when preparing PCW to ensure the retention of O-acetyl groups. PCW lysis assays were performed in a pH 7.5 Tris-HCl buffer, which has potential to liberate O-acetyl groups from PCW samples. Since we see a significant effect of β-lactam treatment on lysozyme susceptibility under conditions that should remove a main factor in lysozyme resistance there should be another factor affecting lysozyme's inability to lyse non-β-lactam treated PCW. If we wish to explore this phenomenon further, a necessary next step will be to

extract O-acetyl groups from PCW samples as done when determining O-acetylation levels and treat these newly O-acetyl group free PCW samples with lysozyme. In addition, further work should be done to definitively determine O-acetylation levels by using previously established methods (28).

An additional source of disrupted cell wall integrity could lie in inhibition of PBP1. Recent work suggests that β-lactams that selectively bind PBP1 potentiate DAP to a greater extent than those that specifically target other PBPs (6). PBP1 of *S. aureus* is homologous to PBP3 of *E. coli*, which interacts with important cell division machinery (6), and recent studies suggest that interruption of PBP1 activity is implicated in construction of aberrant cell walls (33). NAF is a nonselective β-lactam and exhibits a degree of binding to all PBPs (33), thus, in addition to inactivation of PBP2. Repetition of these experiments using β-lactams specific for certain PBPs may help clarify this phenomenon.

We observed a significant increase in the release of LTA into culture medium from cells undergoing growth in β-lactam containing media. Teichoic acids play numerous roles in the cell and their full function is not yet understood, however, previous work has determined that, while the absence of either LTA or WTA is non-lethal, the cell cannot survive the absence of both (27). LTA and WTA are implicated in many cellular processes, and an important modification for that function is the D-alanylation of LTA (24). Enhanced DAP binding has already been observed upon β-lactam treatment, and the exact mechanism by which this happens has yet to be determined (4). One suggestion for increased DAP binding in the presence of nafcillin is that the release of D-alanyl LTA

results in a less positive membrane environment that lessens the impact of lysyl-phosphatidylglycerol in a charge repulsion mechanism (4).

We aimed to examine the localization Atl as a possible source of increased whole cell and crude cell wall autolysis. In addition to autolysis assays, we extracted Atl from cell walls as bulk protein extract by the freeze/thaw method. When *M. luteus* cells were exposed to equivalent amounts of cell wall protein extracts we hoped to observe similar lysis rates, implying that the fraction of Atl in any given treatment is similar, and that the increased rates of autolysis are not due to an increase in Atl number or activity, but rather could be due to the location of the enzyme. However, we did not observe any degree of lysis in either 1 µg or 10 µg treatments, which may be due to the amount of protein used in the assay. While cells also fail to lyse when exposed to 10 µg of extracted protein, there are slightly more pronounced differences. This particular experiment needs to be repeated with higher concentrations of extracted protein to fully determine if there is any significant difference in cell wall protein extracts of these strains when treated with NAF.

Atl is a tightly regulated enzyme due to its ability to degrade peptidoglycan, and it is likely that delocalized and unregulated Atl activity could cause significant weakening of the cell wall. D-alanyl WTA has been proposed as a method of Atl sequestration. As reported earlier, previous work has determined that D-alanine on WTA are derived from LTA. Earlier studies have suggested that the ease with which D-alanine moves throughout the envelope could provide an essential environment for proteins that require specific ionic conditions, some of which are autolysins (24). If D-alanine plays a significant role in autolysin sequestration, and β-lactam treatment results in the release of LTA from the cell membrane, then upon β-lactam treatment there is likely to be little D-

alanine present on WTA and thus, delocalized Atl. Assuming delocalization of Atl upon β-lactam treatment suggests a range of other effects. Delocalized Atl in this case is likely not enough to cause cell death on its own, however, it is likely to weaken the cell wall. Atl delocalization as a result of the loss of D-alanyl LTA could cause holes in the wall that are big enough to allow the relatively large DAP molecule (~1.6 kDa) to better penetrate the wall and find its target. While these experiments suggest a delocalization of Atl they are far from final. Construction of a fluorescent Atl mutant, quantification of Atl transcription by qPCR, or other methods to identify the exact location of Atl on the cell wall during beta-lactam therapy are necessary steps to definitively demonstrate this phenomenon. The effects of β-lactam treatment on cell wall thickness and cross-linking described here could contribute, along with delocalization of Atl to an enhanced ability of DAP to penetrate the cell wall and reach its binding target at the cell membrane.

DAP has been previously shown to be able to kill without lysis, demonstrating an ability to kill stationary phase cells (17). We aimed to determine the main mode of killing by examining culture optical density and corresponding CFU/ml. Exponentially growing cells show a clear decrease in OD_{600} and are thus overwhelmingly lysing. Stationary phase cells challenged with NAF+DAP do not appear to exhibit lysis, and, in the case of D712, continue growing. Since stationary-phase cells are not actively growing and dividing the effect of NAF on cell viability will be insignificant even in stationary-phase NAF-susceptible cells. What remains to be determined, however, is if β-lactam-mediated increased DAP binding persists in stationary-phase cells, and whether DAP binding under those conditions is sufficient to cause cell death. Future studies may seek to perform

experiments similar to those done in previous work (4) on stationary phase cells exposed to this drug combination.

Future Directions

Given the complex nature of DAP resistance it is unlikely for the phenotype to be completely understood, however, the work done for this thesis could support several areas of future work. Avenues for future research could involve examination of the resistance of PCW to lysozyme in the apparent absence of O-acetyl groups. As mentioned previously, fully removing O-acetyl groups from PCW preparations and treating with lysozyme should provide some clarity. In addition to examining PCW lysozyme resistance, it may be more intriguing to determine the cause of lysostaphin resistance in untreated D712 since this phenotype is unexpected, relatively unresearched, and especially interesting due to the reversion to a susceptible state upon β-lactam treatment. Cell wall thickness/crosslinking should be examined pre and post-β-lactam treatment to determine if any significant decrease is present, and, whether that decrease plays a role in more significant DAP binding. Additionally, it is still unclear if stationary-phase cells are sensitive to NAF+DAP therapy. Though this is unlikely, experiments similar to those performed by Dhand et. al (4) could provide clarity.

The inferential process used here to determine Atl localization is imperfect, and definitive confirmation of delocalized Atl requires additional techniques. One way to support this work is through the use of qPCR to determine if the Atl gene is up/down regulated upon beta-lactam treatment. Similar levels of transcription among strains growing exposed and unexposed to NAF would support the data presented here. In addition to qPCR, construction of a fluorescent protein labeled Atl strain for direct

microscopic visualization is essential, however, while this process is possible, it may be prohibitively cost or time-consuming. What also may be possible is to design an immunofluorescent system using monoclonal antibodies specific to the amidase and glucosaminadase domains of Atl. Recent work has proposed the creation and use of monoclonal antibodies to the glucosaminidase domain to disrupt MRSA cell growth during osteomyelitis (34). Visualization of Atl during beta-lactam treatment is a necessary next step in elucidation of this mechanism.

Conclusions

Daptomycin resistance remains a complex problem with several genes implicated and significant differences among those strains deriving resistance *in vitro* versus those *in vivo*. Despite this complexity here we have provided clarification of the physiological profile of an *in vivo*-derived DAP-R strain and its isogenic DAP-S precursor undergoing beta-lactam therapy. Specifically, we have found that Growth of DAP-S and DAP-R MRSA in the presence of the nafcillin increases whole cell and crude cell wall (CCW) autolysis, and Growth in NAF increases susceptibility to lysozyme and lysostaphin. These effects are likely due to an overall weaker cell wall. Interestingly, increases in PCW susceptibility to lysozyme appear to be independent of the levels of O-acetylation. In addition, we found apparent alteration in Atl processing, and observed NAF+DAP to kill exponentially growing cells via lysis and have no apparent effect on stationary phase cells. Future work may include direct visualization of Atl, more in-depth examination of cell wall thickness/crosslinking, and exploration of the lysostaphin resistance exhibited by strain D712.

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APPENDIX A

SUPPLEMENTAL FIGURES

Figure S1. MegaZyme Acetic Acid Kit Acetic Acid Standard Curve

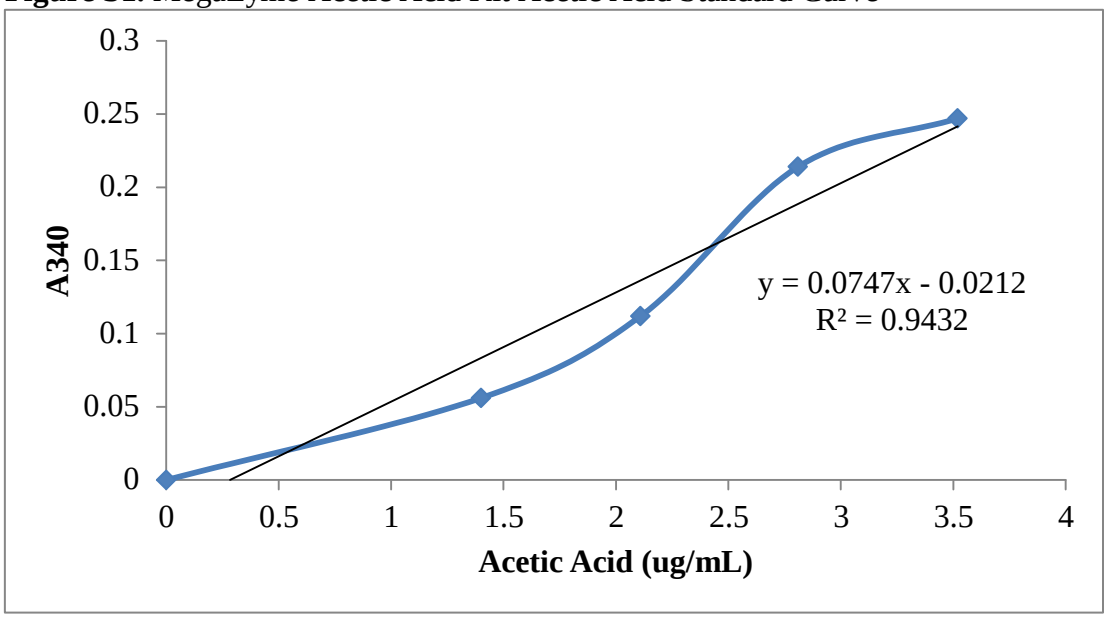

Figure S2. BSA Standard Curve for Protein Concentration Determination (Bradford assay)

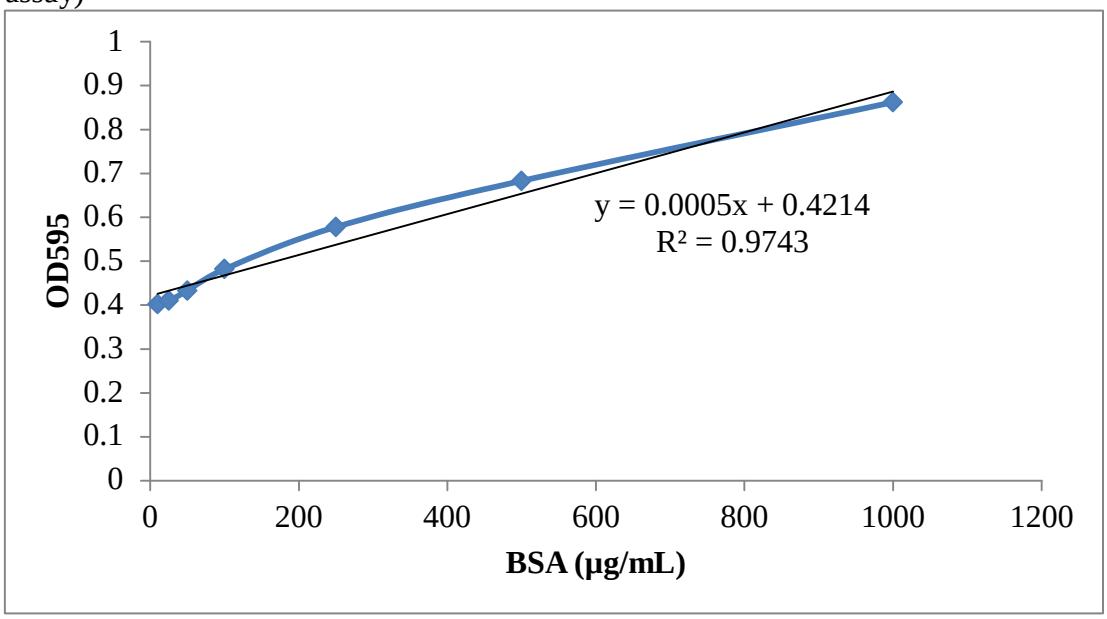

Figure S3. LTA ELISA Standard Curve