

Using Phage Display to Select Peptides Binding to Type 8 Capsular Polysaccharide of
Staphylococcus aureus

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Staphylococcus aureus
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Abstract

S. aureus aureus (*S. aureus*) is a major bacterial pathogen causing an array of critical infections. Increasing the problem of *S. aureus* infections is the fact that forty to sixty percent of *S. aureus* infections have now become antibiotic resistant, making it difficult to treat. *S. aureus* is protected by a polysaccharide capsule, which contributes to its virulence. The goal of this project is to identify a peptide that will bind to *S. aureus* type 8 capsular polysaccharide using phage display technology. Phage display is a technique used to display proteins or peptides on the surface of a phage (a virus that infects a bacterium). If a peptide displayed on the phage surface binds to a molecule of interest, the DNA for that peptide can be sequenced and used to synthesize the pure peptide. In this study, *S. aureus* capsular carbohydrate was purified and the presence of carbohydrate was confirmed using a red tetrazolium carbohydrate assay and an ELISA using anti-capsular hybridoma antibodies. The sample was determined to be free of contaminants using a Bradford assay for protein, phosphate assay for teichoic acid, and a DNA concentration assay for nucleic acid contamination. Using a phage display library, biopanning was performed to identify peptides that bind to whole bacteria. A series of ELISAs were performed to prove binding of the peptide to type 8 capsular polysaccharide of *S. aureus*. These ELISAs concluded specific clones 1, 2, 3, and 6 showed the most significant amount of binding to the type 8 capsular polysaccharide of *S. aureus*. Future studies will include further testing of the specific clones, and DNA sequencing of the most specific clone. The peptide can then be purchased and tested for specificity. The clone DNA may then be transferred to expression vectors and coupled to toxins.

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Table of Contents

Chapter	Page
1.0 Introduction	1
1.1 <i>Staphylococcus aureus</i>	1
1.2 Monoclonal Antibodies	13
1.3 Phage Display	23
2.0 Materials	27
3.0 Methods	28
3.1 Purification of Type 8 Capsular Polysaccharide of <i>S. aureus</i>	28
3.2 Dialysis of Purified Type 8 Capsule	29
3.3 Lyophilization	29
3.4 Carbohydrate Assay: Red Tetrazolium	30
3.5 Tests for Impurities	30
3.5.A Protein Assay: Bradford Assay	30
3.5.B Phosphate Assay: Test for Teichoic Acid	31
3.5.C DNA concentration Assay	31
3.6 Carbohydrate ELISA	31
3.7 Surface Panning with Amplification	32
3.7.A Biopanning	32
3.7.B Titering	33
3.7.C Amplification	34
3.8 Growth of Whole Cell <i>S. aureus</i>	35
3.9 M13 ELISA	35

4.0 Results	37
4.1 Purification of Type 8 Capsular Polysaccharide of <i>S. aureus</i>	37
4.2 Carbohydrate Assay: Red Tetrazolium.....	37
4.3 Contaminants in Carbohydrate Sample.....	38
4.3A Protein Assay: Bradford Reagent.....	38
4.3B Phosphate Assay: Test for Teichoic Acid.....	42
4.3C DNA concentration Assay.....	42
4.4 Carbohydrate ELISA.....	45
4.5 Biopanning.....	45
4.6 M13 ELISA of Phage to Whole Cell Type 5 and Type 8 <i>S. aureus</i> Bacteria.....	47
4.7 M13 ELISA of Phage to Purified Type 8 Capsular Polysaccharide of <i>S. aureus</i>	47
4.8 M13 ELISA of Phage Clones to Whole Cell Type 8 <i>S. aureus</i> Bacteria.....	52
4.9 M13 ELISA of Phage Clones to Purified Type 8 Capsular Polysaccharide of <i>S. aureus</i>	52
5.0 Discussion.....	59
6.0 References.....	67

Table of Figures

Figure	Page
<i>Figure 1: Red tetrazolium Carbohydrate Assay of DEAE Column Fractions</i>	42
<i>Figure 2: Red Tetrazolium Carbohydrate Assay of Pooled DEAE Column Fractions</i>	43
<i>Figure 3: Bradford Reagent Protein Assay of pooled carbohydrate fractions</i>	44
<i>Figure 4: Phosphate Assay of Carbohydrate sample</i>	46
<i>Figure 5: Carbohydrate ELISA</i>	46
<i>Figure 6: M13 ELISA of P3T8 binding to whole cell S. aureus bacteria</i>	50
<i>Figure 7: M13ELISA of P3T8 binding to whole cell S. aureus bacteria.</i>	51
<i>Figure 8: ELISA of Type 8 S. aureus carbohydrate to P3</i>	53
<i>Figure 9: Phage clone ELISA to whole cell S. aureus bacteria</i>	54
<i>Figure 10: Phage clone ELISA to Type 8 S. aureus Carbohydrate</i>	57

Table of Tables

Table	Page
<i>1: The Percent Concentration of Nucleic Acids within the Purified Carbohydrate Samples</i>	44
<i>2: Plaque Forming Units before and after each panning round</i>	48
<i>3: Showing PFU/mL of selected titering plaques from before and after amplification</i>	49
<i>4: Fold increases of each plaque with and without S. aureus from the ELISA to whole cell bacteria. P-values of each plaque with and without S. aureus, as determined by a student two-tailed t-test</i>	55
<i>5: Fold increases of each plaque with and without S. aureus from the ELISA to type 8 capsule of S. aureus. P-values of each plaque with and without S. aureus, as determined by a student two-tailed t-test.</i>	58

Abbreviations

Staphylococcus *aureus* – *S. aureus*

Methicillin resistant staphylococcal aureus – MRSA

Penicillin-binding proteins – PBPs

Vancomycin resistant staphylococcal aureus – VRSA

Polymerase chain reaction - PCR

Capsule polysaccharides - CPs

Hypoxanthine-aminopterin-thymidine – HAT

Fragment crystallization - Fc

Surface plasmon resonance – SPR

Complementarity determining region – CDR

Very low birth weight - VLBW

Iron surface determinant B – IsdB

Filamentous phage family - Ff family

Bovine serum albumin - BSA

Hydrochloric acid – HCl

Double stranded DNA - dsDNA

Single stranded DNA – ssDNA

Single stranded RNA – ssRNA

Enzyme – Linked Immunoassay – ELISA

Phosphate buffered saline - PBS

Optical Density – OD

3,3', 5,5'-Tetramethylbenzidine - TMB

Diethylaminoethyl cellulose - DEAE

Fetal calf serum – FCS

Plaque forming units – PFU

Polyethylene Glycol –PEG

Too numerous to count - TNC

Chapter 1: Introduction

1.1 Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) is a gram-positive bacterial strain that has become a leading problem among infectious diseases (Holtfreter, 2009). *S. aureus* infections are seen universally. Newborns usually come into contact with *S. aureus* soon after birth, and become colonized within the first year of life. The colonization rate decreases with age. Twenty percent of adults are persistent *S. aureus* carriers; thirty percent of adults carry *S. aureus* intermittently; while fifty percent of adults are never carriers. Carriers of *S. aureus* usually have colonization of the nasal cavity. Although it appears that the *S. aureus* infection of the host is non-threatening, it has been seen that it is a balancing act. When there is a decline in the immune system or the staphylococcal infection invades a new site in the host, the infection can become serious (Holtfreter, 2009).

Genome sequencing of 13 *S. aureus* strains shows a remarkable degree of variability between strains (Holtfreter, 2009). These variations include surface-associated genes, many virulence factors and resistance factors. The diversity of the *S. aureus* genome allows it to adapt to different microenvironments through the regulation of gene expression and protein synthesis mechanisms. These mechanisms make it possible for *S. aureus* to attach to the nasal septum, survive inside epithelial cells, grow in hostile environments and survive the bacterial killing conditions within the blood. The diversity of *S. aureus*, as well as, the diversity of the human host is a complicating factor in research. Some hosts are more susceptible to infection, the mode or location of bacterial

entry, the virulence potential and symptoms and severity are all diversifying conditions that complicate research (Holtfreter, 2009).

There are a variety of factors that can predispose a person to developing a *S. aureus* infection, including newborns, nursing mothers, patients with influenza, cystic fibrosis, pulmonary emphysema, leukemia, neoplasms, transplants, prostheses or other foreign bodies, burns, chronic skin disorders, surgical incisions, diabetes mellitus and intravascular plastic catheters (Fattom et al, 2004). *S. aureus* can also cause a variety of infections, including, but not limited to, neonatal infections, mastitis, post-operative infections, furuncles and carbuncles, pneumonia, bacteremia, endocarditis, osteomyelitis or enterocolitis. Other infections related to *S. aureus* are toxin-mediated, for example ingesting heat-stable *S. aureus* enterotoxins causes food poisoning. Another example is toxic shock syndrome, which can be associated with the use of vaginal tampons (Gotz 2004). The most prevalent *S. aureus* infection in hospitals is bacteremia, with *S. aureus* accounting for 22% of all blood infections. That is followed by respiratory infections accounting for 23.2% of all respiratory infections and 39.2% of all skin and soft tissue infections (Fattom .et al., 2004).

S. aureus is not only a problem for humans; it is also a problem in the dairy industry (Lee, 1998). *S. aureus* causes mastitis in dairy cows, preventing the production of milk. Mastitis is the inflammation of the breast or udder tissue. Unfortunately, attempts to prevent mastitis encourage antibiotic resistance. By supplying the cows with antibiotics to reduce the possible infection, the meat processed from these cows contains antibiotic resistant bacteria that will be consumed by the population (Lee, 1998).

The most common site of *S. aureus* colonization in humans is within the anterior nares (Williams, 1963). Other sites that are common for colonization include the skin, perineum and pharynx. There are three identified carriage patterns in healthy individuals, persistent carriage, intermittent carriage and non-carriage. Although, there is not a current rule to determine carriage, a “culture rule” has been implemented that designates two nasal swabs taken a week apart can accurately classify the persistence of *S. aureus* infection of a particular individual (Nouwen JL, 2004). There are differences among individuals, with persistent carriage versus intermittent carriage (Armstrong-Esther CA, 1976). Usually, persistent carriers are normally colonized by a single strain of *S. aureus* for a long period of time, whereas intermittent carriers may carry different strains over time. Persistent carriers are at higher risk of infection than intermittent carriers, due to the fact that persistent carriers having a higher load of *S. aureus*. This increased load of *S. aureus* also leads to a higher dispersal rate of the infection. Nasal carriage mechanisms are multifactorial, based on a study the researchers (Nouwen, 2004) concluded that host characteristics influence the *S. aureus* carriage state. In this particular study, persistent or non-carrier volunteers were inoculated intranasally with a mixture of *S. aureus* strains. Non-carriers were inoculated with four different *S. aureus* strains, persistent carriers were inoculated with the same four strains as non-carriers, but the persistent carriers inoculation also included the strain they are a carrier for. The inoculated *S. aureus* strains were quickly eliminated in non-carriers, with the exception of one volunteer. However in, seven of the 11 persistent carriers, the participant remained a persistent carrier, four with their original strain. Three were persistent carriers of a genetically unique strain that was not included in the inoculation mixture. Only one persistent carrier reverted to being a

non-carrier. This data indicates that, given the opportunity, persistent carriers will continue to be infected by the strain that is adapted best to their particular nasal cavity environment. The host characteristics that lead to different carriage patterns are still unknown. However, according to this study, there must be some differences within the host, as the strains that grew well in an individual did not vary when the subject was inoculated with multiple different bacteria (Nouwen, 2004). According to a review by Wertheim *et al.* (Wertheim *et al.*, 2004), colonization within the nasal cavity can be seen as the result of attractive and repulsive forces. This review cited that there are four prerequisites for nasal colonization. First, *S. aureus* has to come into contact with the epithelium of the nasal cavity, and then it must adhere or bind to certain receptors located within the epithelium. Once it has bound within the nasal cavity, it must survive the host immune system, and then needs to be able to grow and thrive within the nasal cavity (Wertheim *et al.*, 2004).

Environmental factors also influence the *S. aureus* carriage state. Hospitalization greatly increases the risk for developing colonization. This is due to the easy transmission of *S. aureus*. Health care professionals who carry the bacteria on their skin as a commensal organism can pass it to a patient. A health care professional may come into contact with another patient who has a staph infection and spread the infection to other unaffected patients. In addition, it can also be spread from patient to patient. It has also been shown that household members can pass on their carriage state to the rest of their housemates. Close proximity as a mode of transmission has been shown between mother and children who carry the same strain of *S. aureus* (Reviewed by Wertheim *et al.*, 2005).

The virulence potential of an infection is the key factor in predicting the severity or outcome of a particular infection. *S. aureus* virulence results from toxins that cause specific tissue damage to the infected host, or by adhesions, which facilitate invasion of the host by the infectious organism. Various sites on the *S. aureus* chromosome contribute to the virulence, due to many regulatory proteins that control the expression of individual toxins. *S. aureus* also has adhesions that confer the ability to bind to host glycoproteins such as fibrinogen and fibronectin. Fibrinogen will form a fibrin coat around the bacteria, which is impermeable to the immune system. This causes an abscess, which is protected from the immune response and antibiotics by the fibrin coat. Fibronectin binding also allows the infectious agent to be taken up by the host cells. Staphylococcal fibronectin binding protein A is able to facilitate colonization of heart valves and dissemination into the spleen. The fibronectin binding protein A contains 11 binding repeats that are non-identical to each other. It is hypothesized that these repeats allow fibronectin-binding protein to bind multiple fibronectin molecules, leading to bacterial uptake (Edwards, 2010).

The virulence factors of *S. aureus* are coordinately expressed during different stages of infection. Due to the diverse expression of these factors, it has been hypothesized that global regulators coordinate the expression of these virulence factors. Two major families of global regulators have been determined through the use of genomic scans: 1) Two-component regulatory systems and 2) *sarA* homologs. Active synthesis of cell-wall proteins, also called microbial surface components recognizing adhesive matrix molecules, including protein A, fibrinogen, fibronectin and collagen binding proteins, occurs during the exponential phase of an infection. When the organism transitions from

the exponential phase to the post-exponential phase, extracellular enzymes and toxins are predominately synthesized. The transition between these two phases has been shown to be actuated by the global regulators, such as *sarA* and *agr* (Cheung et al, 2004). An understanding of whether an isolate is highly adhesive or highly toxic will help to determine a better course of treatment by using chemotherapeutics that can target either the adhesive or toxic nature of the strain (Laabei et al, 2014).

A problem associated with the treatment of staphylococcal infections is the steady increase of antibiotic resistant organisms. There are many underlying reasons for the development of antibiotic resistance. Overuse of antibiotics in many parts of the world drives bacterium to develop resistance. For example, minor infections treated with antibiotics or food-producing animals being treated with antibiotics prior to developing infections allow for bacteria to become resistant to the antibiotics. If a patient is prescribed an antibiotic for a viral infection, the antibiotic will not affect the virus infection, instead the antibiotic attacks commensal bacteria in the patients body. This can cause harmless bacteria to develop resistance that they can pass onto other, possibly harmful bacteria. Also, misuse or failure to complete the course of treatment with antibiotics can lead to resistance. Not completing a course of antibiotics can lead to needing repeat courses of the antibiotic, which can lead to increased resistance among harmful bacteria. Microbial characteristics also play a role in resistance. Different strains of bacteria are more susceptible to antibiotics, whereas others are able to develop resistance. For example, *Streptococcus pyrogenes* strains are susceptible to penicillin, while *S. aureus* strains are resistant to penicillin. Modern societal and technological attributes also contribute, such as the availability of antibiotics and the ability to travel

within and between countries, which can subsequently lead to microbial resistance (Schito, 2006).

Up to 60% of hospital-acquired infections are due to drug-resistant infections. Pathogenic *S. aureus* is now regarded as an antibiotic resistant “superbug” (Review in Gotz, 2004). It is established that nearly 95% of *S. aureus* isolates are resistant to penicillin or ampicillin and more than 50% are now resistant to methicillin (Fattom et al, 2004). The first methicillin resistant staphylococcal aureus (MRSA) strain was identified in 1961. MRSA is resistant to an entire class of penicillin-like antibiotics called beta-lactams. This includes resistance to penicillin, amoxicillin, oxacillin, methicillin, and others. While MRSA infections are most common in the hospital setting, there are recent reports of community acquired MRSA infections, or infections without any predisposing risk factors that are causing disease (Fattom et al, 2004).

Prior to the discovery of antibiotics, the mortality rate of patients with an *S. aureus* bacteremia was over 80% (Lowy, 2003). In the early 1940's penicillin was introduced. This introduction improved the prognosis of patients with *S. aureus* dramatically. Unfortunately, only two years later the first penicillin-resistant *S. aureus* strains were identified. Not only were these resistant strains observed in the hospital setting, but they were also prevalent among community-acquired infections. Penicillin belongs to a family of antibiotics called β -lactams. β -lactams all share a common four-atom ring. A class of enzymes called β -lactamases breaks this ring, rendering antibiotics inactive. Penicillinase, a specific β -lactamase to penicillin, is now produced by more than 90% of staphylococcal strains. The gene that encodes β -lactamase, *blaZ*, mediates the penicillin resistance seen in strains of *S. aureus*. When penicillin comes into contact with a resistant strain of *S.*

aureus, the Penicillinase will cleave the four-carbon atom of the β -lactam through a hydrolysis reaction causing inactivation of the penicillin (Lowy, 2003).

Methicillin is a Penicillinase-resistant β -lactam antibiotic. Penicillinase is unable to cleave the β -lactam ring of methicillin, due to the bulky side chain processes on the drug, so methicillin is the common antibiotic of choice for a penicillin-resistant *S. aureus* infection. Methicillin works by preventing bacterial cell wall synthesis by inhibiting penicillin-binding proteins (PBPs). PBPs (bacterial transpeptidases) are responsible for crosslinking the glycopeptides that form the peptidoglycan cell wall. Methicillin resistance is conferred by the activation of the *mecA* gene in staphylococcal strains, which encodes PBP2a. PBP2a is different from other PBPs in that its active site will competitively inhibit the binding of all β -lactams, which allows for cell wall synthesis to continue (Lowy, 2003).

The ability of MRSA clones to cause a world- wide infectious threat stems from variations in the toxicity and adherence. The treatment for a MRSA infection is to prescribe vancomycin plus a second antibiotic, such as clindamycin or linezolid to reduce toxin expression. Vancomycin is a bactericidal antibiotic. In contrast clindamycin or linezolid are bacteriostatic antibiotics that will stop protein synthesis by inhibiting ribosomal translocation.

Alarmingly, vancomycin resistance is now being seen in clinical isolates. Vancomycin is a glycopeptide and previously was the drug of last resort. The first clinical isolate to display intermediate sensitivity to vancomycin was discovered in Japan in 1996. After the first report, clinical isolates with intermediate vancomycin resistance were reported worldwide. Intermediate vancomycin resistant strains develop resistance

by thickening their cell walls (Gemmell, 2004). The first vancomycin resistant staphylococcus aureus (VRSA) strain was identified more recently in 2002 in the United States. It developed resistance by acquiring the *vanA* gene that is identical to the gene found in vancomycin resistant enterococci. This leads researchers to believe that the *vanA* gene can be transferred through horizontal gene transfer from enterococcus faecalis via a plasmid. The first VRSA infection was isolated from a catheter tip of a male patient undergoing renal dialysis. The *vanA* gene was confirmed in this patient by polymerase chain reaction (PCR). The DNA sequence was identical to the *vanA* gene from an infected foot ulcer caused by an isolate of Enterococcus faecalis of the same patient. This showed evidence of transfer from vancomycin resistance from E. Faecalis to *S. aureus* in vivo (Gemmell, 2004).

There are two mechanisms for vancomycin resistance that have been identified. Normally vancomycin will bind to D-alanine-D-alanine residues at the C-terminus of peptidoglycan near the cytoplasmic membrane of the bacterium. This renders the carboxypeptidase unable to cleave the D-alanine residues, inhibiting proper cell wall synthesis of the bacterium. The two ways *S. aureus* avoids inhibition via vancomycin is 1) substituting a D-alanine with a D-lactate, and 2) by increasing the amount of D-alanine-D-alanine residues to trap the vancomycin, which will act to impede further vancomycin molecules from reaching their target on the cytoplasmic membrane. In the first case, when the bacterium substitutes D-lactate for D-alanine an ester bond is now formed. Vancomycin cannot recognize this bond, and is unable to bind and inhibit cell wall synthesis. This form of resistance is caused by the *VanA* gene acquisition (Lowy, 2003). In the second case, there are newly synthesized peptidoglycans that will result in

thickened cell walls. There is also a notable decrease in the peptidoglycan cross-linking due to a decrease in the amidation of mucopeptides of the cell wall, which results in the exposure of free D-alanine-D-alanine residues. This causes increased binding of vancomycin, and the vancomycin will become trapped to inhibit binding of vancomycin molecules near the cell membrane (Lowy, 2003).

Due to the ever-changing genome and the opportunity for antibiotic resistance, the need for a vaccine against *S. aureus* is at an all time high. Now that *S. aureus* has become vancomycin resistant, there are very few options for the treatment of an infection. Identification of different staphylococcal capsule types has been a highly important research prospect for vaccine development.

Strains of *S. aureus* produce different and distinct types of capsule. Capsules are composed of extracellular polysaccharides and they are seen at the surface of nearly 90% of natural *S. aureus* bacterial isolates. Capsules are an important virulence factor in many bacteria species. *S. aureus* produces a variety of capsule polysaccharides (CPs). Currently 13 different CPs have been identified, but type 5 and type 8 CPs are most commonly isolated in animal and human infections (Kampen et al, 2005). The structure of type 5 CP and type 8 CP are nearly identical, consisting of two N-acetyl-L-fucosamines (FucNAc) and one N-acetylmannosaminuronic acid (ManNAcA). The only differences are in their glycosidic bonds and the site of the O-acetyl groups. Despite the similarity of the two capsules, there is very little cross-reactivity between them (Jones, 2005). In general, CPs are poor immunogens, meaning they do not elicit a strong immune response. The immunogenicity is also related to age of the host. Very young and elderly patients display

significant decreases in the immunogenicity of the *S. aureus* strains they are infected with (Fattom, 1995).

Although it has been difficult to define the role of type 5 and type 8 CP in the virulence of *S. aureus*, it has been shown that the *S. aureus* CP5 capsule has antiphagocytic properties (Kampen et al, 2005). In a study conducted by Kampen et al, the respiratory burst and opsonophagocytic killing of *S. aureus* by bovine neutrophils was studied and the influence of the expression of CP 5 and CP 8 was investigated. Bovine antiserum was obtained from cows immunized either with C5-human serum albumin conjugate vaccine or whole bacteria of a CP8-producing *S. aureus* strain. Attaching a polysaccharide antigen to a carrier protein creates a conjugate vaccine. For the bactericidal assay, neutrophils were isolated and added to a suspension of live *S. aureus*, and heat-inactivated serum. Heat inactivated serum is added to degrade complement to prevent activation of the complement system in the serum. In the bactericidal assay, 73.4% of capsule-negative bacteria were killed, indicating they had been phagocytized by the neutrophils. However, the capsule-positive bacteria showed much less killing by neutrophils. However, the addition of serotype-specific antisera greatly increased the killing of capsule-positive strains. Similarly, the flow cytometric respiratory burst assay showed that capsule-negative bacteria were able to stimulate respiratory burst within bovine neutrophils but the capsule-positive strains stimulated very little or no respiratory burst activity. The respiratory burst was also increased when capsule specific antisera was added. In both assays the anti-CP5 antisera had little effect on the CP8-positive strains. Likewise, the anti-CP8 antisera had little effect on the CP5-positive strains. This study demonstrates that the capsule is protective against killing by bovine neutrophils in

vitro, and that the addition of antisera to a specific CP can greatly increase the incidence of killing by neutrophils (Kampen et al, 2005).

In a mouse bacteremia model of staphylococcal infection, the parental strain, serotype 5 strain Reynolds, was found to be more virulent when compared to capsule-defective mutants (Thakker et al, 1998). This increase in virulence could be attributed to the antiphagocytic properties contributed by the capsule of the parental strain. It was shown *in vitro* that the parental strain resisted phagocytic killing by immune cells. The capsule-defective mutants were opsonized by complement, indicating complement binding to the bacterium and will initiate an innate immune response by the activation of complement. Complement consists of many small blood proteins that will initiate a series of events leading to one or all of the following outcomes: opsonization, enhanced phagocytosis, chemotaxis of macrophages and neutrophils to the site of infection, cell lysis, and/or agglutination (Thakker et al, 1998).

Vaccines containing capsules of *S. aureus* have shown some effectiveness in decreasing the virulence of subsequent infections in rat models (Jones, 2005). There are a few current therapies in production that are designed to contain or recognize the CPs of staphylococcal strains: StaphVAX, Altastaph, Veronate and Pagibaximab.

StaphVax is a bivalent vaccine that is designed to stimulate an immune response to type 5 and type 8 capsular polysaccharides (CPs) of *S. aureus*. It is designed for hospitalized patients with an increased risk of *S. aureus* infection (Fattom et al, 2004). The vaccine contains type 5 and type 8 CPs conjugated to a carrier protein, exoprotein A of *Pseudomonas aeruginosa*. The CPs of *S. aureus* have been proven to contribute to the ability of *S. aureus* to evade the immune system. By developing vaccines containing the

CPs, antibodies could be made against the capsule that could activate the immune system. In an initial clinical trial involving 76 humans, the recipients received two injections of either type 5 or type 8 conjugate vaccines. Following a single dose of the conjugate vaccine, there was a 10-20-fold increase in antibodies against specific CPs, including IgG and IgM. The second vaccination did not increase the levels of type-specific antibodies, indicating that the first vaccine resulted in a near maximum response in these test patients. A subsequent blood draw was performed 33 months after the type 8 vaccination and 47 months after the type 5 vaccinations. The antibody levels were 57% and 42% of the levels measured at six months post-vaccination, respectively. In this early trial, the researchers believed that StaphVax could provide long-term immune responses with a slow decline in antibody levels. Unfortunately, after phase three clinical trials, it was shown that StaphVax did not meet the primary goal of preventing *S. aureus* infections in patients with kidney disease (Fattom et al, 2004). However, it was said that the vaccine did increase the levels of type specific antibodies against CPs.

1.2 Monoclonal Antibodies

Although, a vaccine has not been successful, new treatments and protocols are being implemented in the hospital setting. Many of these new treatments target bacterial protein synthesis and peptidoglycan synthesis. Monoclonal antibody therapies are now being implemented as a new source of treatment. Monoclonal antibodies used for passive immunization will specifically identify and mark the unwanted cells so the immune system can identify and destroy the target cells. This is so far thought to be the best way to identify the *S. aureus* cells for destruction by the immune system.

Monoclonal antibodies can be used in the detection and treatment of a variety of diseases. Monoclonal antibodies are derived from a single clone and are specific for a single molecule found on its target. The production of hybridoma cells is essential for monoclonal antibody synthesis. Hybridoma cells are the combination of a plasma cell and a myeloma cell. Plasma cells alone will not grow in culture. Hybridoma cells are used in order to have a cell that produced antibodies, which will continue to proliferate in culture.

Kohler and Milstein first introduced the procedure that is still used today to produce monoclonal antibodies (Kohler, 1975). In order to produce an antibody-secreting hybrid line, in which one of cells is an antibody-producing spleen cell, a myeloma cell line from BALB/c origin was used. A fusion experiment was conducted between a mouse myeloma cell line, P3-X6Ag8 (P3-X) and spleen cells from a mouse immunized with sheep red blood cells. The cells were fused using inactivated Sendai virus and incubated in Hypoxanthine-aminopterin-thymidine (HAT) medium. P3-X cells produce the antibody MOPC21, an IgG1, and are resistant to 8-azaguanine, which is responsible for killing cells with HGPRT. Incubation in HAT medium, which contains hypoxanthine, aminopterin and thymidine selects for hybridomas, spleen cells fused with a myeloma cell. Aminopterin inhibits the *de novo* pathway of DNA synthesis. The hypoxanthine and thymidine in the HAT medium provide the necessary components for the cells to perform the salvage pathway of DNA synthesis. The myeloma cell only produced one of the two enzymes needed for the salvage path (HGPRT), so only the fused cells would contain both enzymes required for growth in the HAT medium (Kohler, 1975).

The hybrid lines were confirmed by a number of qualifications. The hybrid lines were capable of growing in HAT media, the karyotype of the fused cells were smaller

than the sum of the two parental lines but twice the size of the normal BALB/c cells, showing the resulting cells were not a result of fused spleen cells. The fused cells showed equal chromosome arm length as the parental line, and the secreted immunoglobulin's contained the MOPC21 protein made by the myeloma cells (Kohler 1975).

Antibody secreting cells were identified using a plaque assay, with sheep-anti-red blood cells as the antigen. A direct plaque technique showed that the hybrid lines were likely producing IgM antibodies, as only IgM is able to activate complement without a secondary antibody. To confirm, inhibition of sheep-anti-red blood cells lysis by a specific anti-IgM antibody was shown (Kohler, 1975). These experiments proved that cell fusion techniques could be used to produce specific antibody producing cells by fusing spleen cells from an animal immunized with a predetermined antigen (Kohler, 1975).

Monoclonal antibodies have been produced previously against purified *S. aureus* CPs. In a study by Nelles, clinical isolates of non-typable, type 5 and type 8 capsules were utilized. Reynolds (type 5) and Becker (type 8) were the prototype strains for their respective capsular types. BALB/c mice were immunized three times with formalin fixed *S. aureus* bacterial vaccines, and allowed enough time to create plasma cells producing antibodies against the bacteria. Two groups of BALB/c mice were immunized with formalin-fixed vaccines of *S. aureus*. One group of mice was immunized with the Reynolds strain (type 5) and the other group was immunized with Becker (type 8). Spleen cells taken from the immunized mice were fused with a mouse myeloma cell line, P3-X63-Ag8.653. HAT medium, as previously described, was utilized to select hybrid cell lines and limiting dilutions were used to clone the hybridomas. All of the type specific

anti-*S. aureus* monoclonal antibodies were either IgM or IgG3. In order to test the reactivity patterns of type specific anti-*S. aureus* monoclonal antibodies with clinical isolates, agglutination studies were performed. The agglutination procedures were as follows: bacterial cell suspensions were diluted and was mixed with hybridoma cell suspensions, the mixture was incubated for 1 hour at 37°C, and then overnight at 4°C. The results were then scored on a scale of 0-4 from the complete absence of agglutination to full agglutination. The data showed that all 19 of the monoclonal antibodies from fusion 17 (anti-type 5) reacted with each of the 4 different type 5 clinical isolates, but did not react with any of the type 8 isolates. Similarly, all 5 monoclonal antibodies from fusion 18 (anti-type 8) reacted with the type 8 isolates but did not react with any of the type 5 isolates (Nelles et al, 1985). Unfortunately, these monoclonal antibodies are no longer available.

There are many problems associated with the use of monoclonal antibodies, including the problem of losing clones. Other problems associated with the use of monoclonal antibodies have more recently been investigated (Ober *et al*, 2001). Preclinical therapeutic antibodies are tested for their pharmacokinetics and efficiency. These preclinical testings are normally performed in an animal model, often mice. As stated before, the production of monoclonal antibodies is usually done in a mouse model, using mouse myeloma cells, as described by Kohler. It has been shown that mouse IgG is cleared from the human circulation much faster than it is cleared from mouse circulation, suggesting that a mouse model for preclinical testing may be misleading (Ober et al, 2001). This can also be substantiated by the poor performance of mouse antibodies in human clinical trials. The substantial difference in clearance of the IgG, lead researchers

to believe there must be a difference in an Fc receptor of mice and humans. The Fc receptor is a protein found on the surface of phagocytic cells. The Fc receptor will bind to a specific part on the antibody, called the Fc (fragment crystallizable) region, when the antibody is attached to an antigen, or infectious agent. The binding of the Fc region will then trigger the body's immune system to stimulate phagocytic, or cytotoxic cells to destroy the targeted cell. The Fc receptor that causes differential retention of immunoglobulin between mice and humans is hypothesized to be specifically the FcRn. The FcRn is responsible for regulating serum levels of IgG in mice, and likely has a similar mechanism in humans (Ober *et al*, 2001). In mice and humans, FcRn has a known function of transporting IgG across the placenta, transferring IgG antibodies from mother to child. The suggested mechanism by which FcRn can regulate IgG serum levels, as well as transport antibodies across the placenta is due to its ability to bind to the Fc region on the IgG, and then transfer the bound IgG within and between cells. IgG molecules that do not bind to FcRn have a shortened persistence in serum. FcRn can have a protective effect by binding the IgG and interfering with degradation of the IgG molecule. A direct correlation has been observed between the binding affinity of the FcRn and the IgG/Fc fragment and serum half-life. In humans, less is known about the mechanism of action for the FcRn. FcRn is found within the human placenta and is able to transport antibody across the placenta *in vitro*. FcRn has also been reported to be within the endothelial tissue, suggesting a role in serum IgG regulation (Ober *et al*, 2001). Previously, interactions of human FcRn and IgG from heterologous species had yet to be identified. In this study, researchers investigated the differences between the interactions of the FcRn, of mice and humans, and of IgG obtained from a variety of species using surface

plasmon resonance (SPR). Surface plasmon resonance is a spectroscopy method that is used to study ligand-binding interactions with membrane proteins. This method is preferred because it has the ability to give a real-time analysis of binding affinities. Unlike other immunoassays, SPR is useful as it does not require labeling the molecule to be studied. In order to determine the binding affinities, one binding element is immobilized on a sensor chip while the other binding component is prepared within a solution to be poured over the chip. Small changes are detected in the refractive index of the sensor surface when binding occurs (Patching, 2014). The study conducted by Ober and coworkers compared the binding specificity of mouse and human FcRn to human, mouse, rabbit, guinea pig sheep and rat IgG (Ober *et al.* 2001). It was discovered that unlike mouse FcRn, human FcRn is highly specific. Mouse FcRn was capable of binding all of the IgG provided from other species, whereas human FcRn had little reactivity with some of the heterospecies IgG. Human FcRn did bind to human, rabbit, and guinea pig IgG but did not bind specifically with rat, bovine, sheep, or mouse IgG. This study was able to provide insight into the mechanism of human FcRn, and questioned the validity of pharmacokinetic studies performed in a mouse model (Ober *et al.* 2001). In order to accurately predict the half-life of antibody containing therapeutic agents, Ober et al suggested using human FcRn binding studies complemented with the use of preclinical mouse studies.

New studies are now focusing on developing a method of making human monoclonal antibodies. In order to make humanized-mouse monoclonal antibodies, mouse Fc regions on the antibody are replaced with human components. Antibodies containing both mouse and human elements are called chimeric antibodies. Of the current

FDA approved monoclonal antibodies, five are chimeric antibodies (Duvall *et al.* 2011). Some antibodies have now been fully humanized, by complementarity determining region (CDR) grafting. The CDR region found on antibodies is part of the variable region. This is the binding site of the antibody that is specific for the antigen. As the name implies, this is the most variable region of the antibody. By placing a mouse CDR in a human antibody, the specificity of the antibody would be identical to the mouse monoclonal antibody, but the ability of the antibody to interact with human receptors would be improved. Mouse monoclonal antibodies also elicit an immune response in humans, limiting their effectiveness. Placing the mouse CDRs in a human antibody would produce antibodies that would not stimulate an immune response and would extend the effective life of the antibody. The production of human monoclonal antibodies has been studied to deter many of the problems associated with mouse monoclonal antibodies.

Weisman recently published a review of three antibody preparations designed for low birth weight neonates with high risk for *S. aureus* infections. This review covered three passive immunotherapy treatments, Altastaph, produced by North American Biologics Inc. (the same producer of StaphVAX), Veronate, produced by Inhibitex Inc., and Pagibaximab, produced by Biosynexus Inc. and Glaxo Smith Kline Inc.

Altastaph is a *S. aureus* serotype 5 and serotype 8 hyperimmune polyclonal antibody (Weisman, 2007). The antibodies are obtained from plasma donations from healthy patients who have been vaccinated with StaphVAX. The mechanism of action for Altastaph is similar to the mechanism of StaphVAX. The specific antibodies included in the vaccine will bind to the polysaccharide capsule of the bacteria and allow recognition

by the host's immune system (Rupp et al, 2007). However, in the case of Altastaph, the patients were not required to form their own immune response, as the antibodies were passively provided to the patient. In a phase two clinical trial of Altastaph in very low birth weight neonates, neonates were randomly selected for two identical injections of either placebo, 0.45% NaCl, or 1000mg of Altastaph (Benjamin, 2006). Each neonate was monitored for 28 days after the second injection or until they were discharged. In order to determine if the injections were sufficient to produce anti-CP IgG, serum levels of *S. aureus* type 5 and type 8 CPs IgG were measured pre-infusion and at various times after each infusion. The results of this study showed that Altastaph was well tolerated, and there were high levels of *S. aureus* anti Type 5 and Type 8 antibodies, but the incidence of *S. aureus* bacteremias were the same for both groups, about 3% incidence (Benjamin et al, 2006). Due to the lack of evidence supporting that Altastaph is protective against *S. aureus* further, production has been put on hold (Reviewed in Weisman, 2007).

Veronate is a polyclonal antibody obtained via plasmaphoresis of patients with high levels of Abs against microbial surface components recognizing adhesive matrix molecules (MSCRAMMS). The goal of this antibody was to reduce *S. aureus* frequency (Capparelli et al, 2005). In a study conducted by Capparelli et al, infants of 3 to 7 days old received intravenous injections of Veronate at 500mg/kg of body weight. The first infusion was given between 3-7 days postnatal, the second dose was given 7 days following dose 1, the third dose was given 14 days after dose two, and the fourth dose was given 14 days after dose 3. Infants were then monitored for 70 days. This study was

an early trial that could not accurately describe the protective effects of Veronate due to low study population and the lack of a placebo comparison.

A second study was performed by Dejonge *et al.*, looking at differences between placebo injections and injections of Veronate in very low birth weight (VLBW) infants (Dejonge et al, 2006). Infants enrolled in the study were randomized to receive four doses of either Veronate or placebo. The study was trying to determine if Veronate injections would decrease late-onset sepsis in VLBW infants. This study showed no differences between the placebo group and the group that received the Veronate, which implied no protective effect against late onset sepsis. The company has since then placed production of this vaccine on hold (Reviewed in Weisman, 2014).

Pagibaximab is a humanized mouse chimeric monoclonal antibody against lipotechoic acid. Lipotechoic acid is found in the cell walls of gram-positive bacteria. Pagibaximab is the only product that was covered in the review by Weisman that has been proven to have a protective effect against *S. aureus* and was well tolerated in phase two clinical trials (Reviewed in Weisman, 2007). A randomized study of three once a week intravenous injections of either 60mg/kg dosage of Pagibaximab paired with a placebo group or 90mg/kg Pagibaximab dosage paired with a placebo group was studied to determine the effectiveness of Pagibaximab. The subjects were of 2-5 days old and weighed between 700 and 1300g. The subject group who received the 60mg/kg dosage still had a 20% occurrence of infection where as the group that received 90 mg/kg showed no occurrence of infection. From this study, it was concluded that a higher dosage of Pagibaximab had protective effects against *S. aureus* infections (Reviewed in Weisman 2007).

A recent review by Fowler et al (Fowler, *et al*, 2014) determined that murine models of infection and immunization do not correlate with success in a human host. To date, active and passive immunizations for *S. aureus* have not passed a stage three clinical trial. Of the treatments reviewed, all of them completed phase one and two clinical trials and were all based upon increasing opsonic antibodies to surface antigens of *S. aureus*. They also all failed to meet pre-trial endpoints in phase three clinical trials.

A monoclonal antibody to CiFA, named tefibazumab, is used in conjunction with standard therapy for *S. aureus* related bacteremia. Phase three clinical trials failed to show significant differences in the endpoints of death, relapse, or development of an *S. aureus* related complication that was not present at baseline.

Altastaph, a polyclonal antibody against type 5 or type 8 *S. aureus* capsular carbohydrate failed to show significantly different clinical outcomes in patients with *S. aureus* bacteremia. However, the length of stay of patients was decreased with Altastaph.

Pagimaximab was another compound studied. It is a humanized mouse chimeric monoclonal antibody against lipotechoic acid of gram-positive bacteria. It did show promising results in phase 2 clinical trials but a following registration trial did not show the same results.

StaphVAX, a bivalent vaccine of capsular carbohydrates 5 and 8, failed to show a protective effect after 54 weeks, the pre-specified endpoint. A second trial also failed to show a protective effect after doubling the sample size and changed the endpoint to six months.

The most alarming of the compounds reviewed was V710. V710 is a mouse monoclonal antibody targeting iron surface determinant B (isdB). IsdB promotes *S.*

aureus adherence and internalization by human cells that are non-phagocytic. The murine models showed high efficiency, however the human trial was terminated based on increased mortality rates of recipients. Post study testing showed recipients were five times more likely to die of multi-organ failure than control recipients that developed an *S. aureus* infection.

As demonstrated by these studies, monoclonal antibodies may not be the best option for production of an *S. aureus* vaccine. Therefore, a novel selection technique, phage display, has become a more promising way of selecting a molecule that will bind to *S. aureus*.

1.3 Phage Display

Phage display technology is a process by which proteins or peptides that bind to specific biological targets are selected via a process termed biopanning. The peptides or proteins are expressed on the surface of a phage particle, while the DNA sequence, that encodes the peptide, is linked within the genome to a phage coat protein. Bacteriophage, or a virus that infects bacteria, is the vehicle by which this process is carried out (reviewed in Willats 2002). As well as being inexpensive, fast, and not requiring any special equipment, phage display has many advantages. One, there is diversity of peptides that may be displayed. A variety of 10^{10} is normal. Phage display could also be performed in vitro or in vivo. This allows for variation in the targets being tested (reviewed in Willats 2002).

For the purposes of our experiment, our biological target will be the type 8 capsular polysaccharide of *S. aureus*. The goal of this project is to identify a peptide that will bind

to *S. aureus*. In the future, it is anticipated that a toxin may be coupled to the peptide as an alternative form of treatment for the infection.

The M13, of the filamentous phage family (Ff family) is the bacteriophage used for these studies comes from. This family is advantageous, due to the phage having no size constraints on the amount of DNA that may be inserted. The addition of foreign sequences is accommodated by longer phage particles. However, the Ff family of phage uses non-lytic propagation. This means that before the assembly of the mature phage particle, the components of the phage coat must be exported through the bacterial inner membrane. If the proteins cannot withstand this mechanism, other phage, such as Lambda or T7 lytic phage may be used (Ph.D. Phage Display Instruction Manual). The Ff filamentous bacteriophages specifically infect *E. Coli*. The minor coat protein PIII of the phage interacts with F-pilus of *E. Coli* cells. The structure of the M13 bacteriophage is about 1 μ M in length and less than 10 nm in diameter. The DNA is single stranded and surrounded by a protein coat. The phage coat contains one major coat protein, PVIII, and four minor coat proteins, PVII, PIX, PIII, and PVI. Coat protein PVIII covers the length of the phage particle; PIII and PVI are on one end of the phage, while PVII and PIX are found on the other end. Coat protein PIII is responsible for recognition of pilli and the infection into the host cell. The PIII protein is composed of 3 domains, each with their own function. The N-terminal domain is responsible for transporting the viral DNA into the host cell cytoplasm. The second domain binds to the F-pilus of the host cell and is also responsible for recognition of the host cell. The final C-terminal domain is involved in the interaction of PIII with the other coat proteins (Sindu, 2001).

Binding of the N-terminal domain of PIII to the F-pilus of E.coli initiates phage infection. The F-pilus will then retract pulling the virion inside the E. Coli inner membrane (Deng 1998). Once the viral single-stranded DNA is inside the cytoplasm, it is converted to double-stranded DNA. This double-stranded DNA is then used as the template for phage-protein replication. Utilizing the “rolling-circle” mechanism of replication, new single-stranded DNA is created from the double-stranded DNA. The coat proteins are embedded within the inner membrane of the host cell, while other phage proteins form a pore, to allow the passing of the viral DNA through the membrane. The coat proteins will again surround the phage particle during the assembly process. Assembly begins with the incorporation of PVII and PIX, which is followed by the elongation process where PVIII molecules are assembled along the length of the particle. Assembly is terminated by the addition of PIII and PIV. The assembled phage is then released via a non-lytic process by which infected cells will continue to release M13 phage particles and grow and divide (Sindu, 2001).

A phage library is a library of billions of variant peptides expressed on a phage particle. These various peptides can then be screened. The specific library used for our experiment is Ph. D- 7 (New England Biolabs), which displays five heptapeptides on the minor coat protein PIII. This library can have up to 10^9 independent clones. With this high diversity, it is likely that the majority of the possible peptide sequences that could bind to *S. aureus* capsule are encoded within the library, making this library suitable for our project (Ph.D. Phage Display Instruction Manual).

In order to select for phage that bind to *S. aureus*, we performing an affinity selection process termed biopanning that allows us to screen the variable peptides

displayed on the phage to the desired biological target, and to select the peptides with high binding affinities to the target. The biopanning process is a combination of binding, washing, elution, and amplification experiments. First the phage library is exposed to the target, non-binding phage are washed away, and binding phage are eluted. The binding phage are then amplified and prepared for another round of panning. After three rounds of panning, plaques will be selected from the titers of the panning 3 phage. The plaques will then be tested by ELISA to select the plaque with the highest binding affinity (Reviewed in Willats 2002).

Our hypothesis is that by utilizing phage display technology, we will be able to identify a peptide ligand that binds specifically to type 8 capsular polysaccharide of *S. aureus*. By identifying this ligand, future work could include coupling a toxin to the peptide to be used as a form of treatment.

To summarize, *S. aureus* infections are a major problem among hospital and community acquired infections. With the increase of antibiotic resistance *S. aureus* strains, the diversity of *S. aureus* virulence, and the lack of a therapeutic agent, the number of *S. aureus* mortalities could increase. As of yet, a therapeutic agent has not been identified. However, the use of phage display technology could be successfully used to produce tools that could identify and treat *S. aureus* infections, including those infections that are antibiotic resistant.

Chapter 2: Materials

Phage display peptide library kit (cat. # E8100S) was purchased from New England Biolabs (Beverly, MA; www.neb.com). Bovine serum albumin (BSA) (cat. #A-1009), fetal calf serum (cat. # N4762), DNase (cat. # DN-25), RNase (cat. # R-4875), Protease K (cat. # P-6556), red tetrazolium (cat. # T-8877), Isopropyl- β -D-thiogalactopyranoside (IPTG) (cat. # 16758), Anti-mouse polyvalent immunoglobulin peroxidase (cat # A0412), tetramethylbenzidine (TMB) (cat. #T0440), tetracycline hydrochloride (cat. #T3383) were purchased from Sigma Chemical Company (St. Louis, MO). Casein in TBS blocking buffer (cat. #37532), EZ-Link[®] Hydrazide-PEG₄-Biotin (cat. #21360), and Bradford reagent (cat. # 1856210) were purchased from Thermo Scientific (Rochester, NY). 96 well Polyvinyl chloride plates (cat. #353912) was purchased from Corning (NY). 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) (cat. #0428) Amresco (Solon, OH). Dialysis tubing (45 mm wide, 12,000-14,000 molecular weight cut off) (cat. # 2115214) was purchased from Fisher scientific (Pittsburgh, PA.). Whole cell Type 8 *S. aureus* Wright bacterium (cat. # 49525) was purchased from American Type Culture Collection (Rockville, Maryland).

Chapter 3: Methods

3.1 Purification of type 8 capsular carbohydrate of *S. aureus*

The *S. aureus* cells were grown in Columbia broth containing 2% NaCl overnight in a shaking incubator (225 rpm at 37 °C). The cells were then centrifuged (13,000 X g at 4 °C for 30 minutes) and the cell paste was collected. This procedure was repeated until 50mL of cell paste was collected. Once 50mL of *S. aureus* cell paste was obtained, the cells were suspended at 0.5g/mL in 0.05M tris-2mM MgSO₄ and autoclaved. To ensure the bacteria had been killed by the autoclave, a control plate was streaked and incubated. Thereafter, 100µL of DNase and RNase was added and incubated with shaking at 37°C for 6 hours, followed by an overnight treatment with 4 units/mL of protease K, to remove any DNA, RNA or protein remaining in the sample. The solution was dialyzed with at least four water changes to remove salts and degraded proteins. The mixture was then centrifuged at 13,000 xg for 30 minutes at 4 °C and the supernatant was filter sterilized (Lee et al., 1998). Following this, 0.05M sodium periodate was added to the sample and incubated overnight at room temperature in the dark to remove teichoic acid (Tzianabos et al., 2001) (Tollersrud et al., 2001). The sample was then dialyzed again and lyophilized. The powder was dissolved in 0.05M sodium acetate and 0.05M sodium chloride (starting buffer). The sample was added to a DEAE-Sephacel column equilibrated in the same buffer. The column was washed with 600 mL starting buffer and the carbohydrate eluted with 600mL of 0.05M sodium acetate and 0.15M sodium chloride (Fattom et al., 1990). Following elution of *S. aureus* capsule, a carbohydrate assay was performed on the fractions. Carbohydrate positive fractions were pooled,

dialyzed and lyophilized. The carbohydrate was resuspended in water and tested for protein and phosphate contamination (Lee et al., 1998).

3.2 Dialysis of purified type 8 capsule

The dialysis tubing (45 mm wide, 12,000-14,000 molecular weight cut off) was previously prepared following the method used by Maniatis. The tubing was cut into 15 inch pieces, then boiled in sodium bicarbonate (2%) and EDTA (1mM), then rinsed in distilled water. The tubing was boiled for an additional 10 minutes in EDTA (0.001 M). Before the tubing was used, it was rinsed thoroughly with distilled water (Maniatis et al, 1982). The tubing was then filled with the previously purified type 8 capsule and placed into a 1 L Erlenmeyer flask that was filled with autoclaved millieq water. The flask containing the water and dialysis tubing was then placed on a stir plate in a cold room. Every six hours the autoclaved water was discarded and new autoclaved water was added. This was done four times total. Upon completion of the dialysis, the samples were replaced in 50mL conical tubes and placed in the -80 degrees Celsius freezer in preparation for Lyophylization (Maniatis et al, 1982).

3.3 Lyophylization

Samples were frozen at a slant, to maximize surface area in a -20 degrees Celsius freezer overnight, then moved to the -80 degree Celsius freezer for an additional overnight cycle. Upon removing the samples from the -80 degree Celsius freezer, they were immediately placed in a bucket of dry ice for transport to the lyophilizer. The tops of the tubes were replaced with a kimwipe secured with a rubber band. The samples were in the lyophilizer for approximately 48 hours. When the process was completed, the

dry samples were suspended in 5mL of MilliQ water and stored in the -20 degree Celsius freezer.

3.4 Carbohydrate test: Red Tetrazolium

In order to ensure that the samples obtained did contain carbohydrate, a red tetrazolium test was performed. Carbohydrate (200 μ L) to be tested were added to a clean, glass, 13 X 100mm test tube. Serial two fold dilutions of 0.1mg/mL glucose solution was also added to glass, 12 X 100mm test tubes, to act as a positive control. Red tetrazolium (1 mL of 0.5 aqueous solution) and 200 μ L of 10% NaOH solution were added to the carbohydrate solution. At this time, the test tubes were placed in boiling water until a red color developed. After noting the time and development of the color change, 100 μ L of the samples were placed in a 96 well plate. The positive control is 0.1mg/mL glucose and the negative control was water. The absorbance was read at 490nm. The absorbance of the sample carbohydrate was compared with the standard curve produced by the serial two-fold dilution of glucose. By utilizing a linear regression line, the amount of carbohydrate within the sample could be calculated (Fieser et al, 1987).

3.5 Tests For impurities

3.5.A Protein Assay: Bradford assay

To ensure our samples did not contain protein, a Bradford assay was performed. A standard curve was created by placing 160 μ L of bovine serum albumin (BSA) (40.0 μ g/mL) into the first well of a 96 well plate. Serial 1:2 dilutions were performed to the next 5 wells that already contained 80 μ L of water. To the sixth well, add 80 μ L of water to act as the negative control or blank. The sample (60 μ L and 20 μ L water) was added to the 96 well plate. Bradford reagent (20 μ L) was then added to each well. The plate was

placed on a mixer for 5 seconds, or agitated gently. The absorbance was measured after 10 minutes at 595nm (Bradford, 1976).

3.5.B Phosphate test: Test for teichoic acid

Reagent C was prepared by combining 1 volume of 6N sulfuric acid with two volumes of distilled water and 1 volume of 2.5% ammonium molybdate. One volume of 10% ascorbic acid was added and mixed well. Water and elution buffer acted as the negative controls and the positive control was PBS. For the procedure, a small amount of sample was dissolved into 0.5 mL of 2M hydrochloric acid (HCL). The sample and HCL mixture will be placed into a boiling hot water bath for 30 minutes. The volume was adjusted to 4 mL with distilled water. Reagent C (4mL) was added to each tube. The tube was mixed well. The test tube rack was placed in a 30°C oven for 1.5-2 hours and the color change was measured by absorbance at 820nm (Chen et al, 1956).

3.5.C DNA concentration test

To determine the DNA concentration of the samples, the absorbance of the buffer and sample were measured at 230nm, 280nm, and 320nm on an ultraviolet spectrophotometer (Maniatis et al, 1982). MilliQ water was used as the blank. The optical densities obtained from the spectrophotometer were converted into concentration ($\mu\text{g/mL}$). For the conversions, the OD at 260nm was multiplied by 50 $\mu\text{g/mL}$ for double stranded DNA (dsDNA), 37 $\mu\text{g/mL}$ for single stranded DNA (ssDNA) and by 40 $\mu\text{g/mL}$ for single stranded RNA (ssRNA). These concentrations were then divided by the concentration of carbohydrate in our samples and multiplied by 100 in order to determine the percent concentration of nucleic acid contamination (Sambrook and Russell, 2001).

3.6 Carbohydrate Enzyme-Linked Immunoassay (ELISA)

To prove that the purified type 8 capsule is binding to previously made anti-capsular antibodies, an ELISA for *S. aureus* capsular polysaccharide was performed. A 96 well tissue culture treated microtiter plate was coated with 100µL of carbohydrate sample or formalinized and trypsinized *S. aureus* type 8 for the positive control. The plate was incubated for 2 hours at room temperature. After incubation of the plates, the solution was removed from the plates. The unreacted protein-binding sites were blocked with 150µL of blocking buffer (1%BSA in PBS) for 30 minutes at room temperature. The plates were washed once with phosphate buffered saline (PBS). Mouse antisera to *S. aureus* type 8 (100µL) for the positive control, or sample buffer (to act as the negative), or AnT8B1C5T9 hybridoma supernatant (for the test wells) were added to the wells and incubated for two hours at 37 °C. After binding, the wells were washed three times with PBS. Anti-mouse Immunoglobulin Peroxidase Conjugate (100 µL) diluted 1:1000 in sample buffer (PBS) was added to each well, and incubated for 30 minutes at 37 °C. The wells were washed with PBS three times. After adding TMB (100µL), the plate was incubated until a color change developed. The reaction was stopped by the addition of 2N H₂SO₄ (50µL) and the absorbance was read at 450 nm (Thakker et al, 1998) (Sinha et al, 1999).

3.7 Surface Panning with Amplification

3.7.A Biopanning method

Formalinized and trypsinized *S. aureus* (100µL) was added to the bottom of 6 wells in a 96 well tissue culture treated microtiter plate. Casein-block was added to the two pre-adsorption wells. The plate was incubated overnight at 37°C in a humidified chamber to coat the wells. The plate was then centrifuged at 400 X g for 15 minutes. The

solution was aspirated from all wells containing bacteria. Blocking buffer (1% Casein in TBS for the first and third panning steps, fetal calf serum for the second panning step) (230 μ L) was added to each bacterial well. The blocking solution from the two casein-only pre-adsorption wells was aspirated and those wells were washed 6 times with PBS. The phage library (2×10^{11} in 100 μ L of PBS + 0.01% casein) was added to the casein only pre-adsorption wells, followed by incubation at room temperature for 2 hours while rocking. The phage was then collected from the pre-adsorption wells and stored at 4 °C until the first panning step. To begin the first panning step, two panning wells containing bacteria were washed 10 times with PBS. The pre-adsorption phage was then added to the first panning wells and the plate was incubated with rocking for two hours at room temperature. The non-binding phage was discarded and the binding phage was eluted with 100 μ L of elution buffer (500 mM KCl and 10 mM HCl, pH2). The eluate was pipetted into a microcentrifuge tube and neutralized with 50 μ L of 2 M Tris-HCl, this was labelled as panning 1 phage. The next day the specific phage was transferred to the next panning wells and the steps were repeated for panning two and panning three. Intermediate titers and amplification was performed in between each panning round (Dennis, *et al.* 2002).

3.7.B Titers

LB-tet plates were pre-warmed for at least an hour. The top agar was melted and 3mL was dispensed into sterile culture tubes (1 per dilution), the tubes were then maintained at 45 degrees Celsius. Serial 100-fold dilutions of phage in PBS were performed. Subsequently, 200 μ L of ER2738 (Overnight culture of E.coli) was dispensed into sterile microfuge tubes (1 per dilution). Phage dilution (10^{-1}) was added to the

microfuge tube containing the overnight culture, the tube was vortexed and then allowed to incubate for 1-5 minutes at room temperature. Following the incubation, the infected cells were transferred to the culture tubes containing top agar, 2% X-gal and 2% IPTG. The tube was then vortexed and poured onto a pre-warmed LB-tet plate. The plates were cooled at room temperature for 5 minutes, then inverted and incubated overnight at 37 °C. The following day, the plaques were counted (Barbas CF, *et al.* 2001.)

3.7.C Amplification

Amplification was performed following each round of biopanning. LB-tet media was inoculated with ER2738 E. Coli and incubated overnight at 37 °C, shaking at 250 rpm. The next day, 20 mL of terrific broth with Tetracycline (20 µg/mL) was inoculated with 800 µL of the overnight culture. This was then incubated for 1 hour shaking at 250 rpm at 37 °C. The speed was then reduced to 100 rpm for 10 minutes to allow for regeneration of the sheared F-pili. Phage (4×10^9) were added to 20 mL of cells and were incubated for 4-5 hours shaking at 250 rpm at 37 °C. The cells were removed by centrifugation at 4500 x g for 10 minutes, the supernatant was transferred to a fresh tube and the centrifugation was repeated. The top 16 mL of supernatant was transferred to a new tube and 4mL of 2.5M NaCl/20%PEG-8000 was added and mixed. The phage were precipitated overnight at 4 °C. The phage was pelleted by centrifugation at 12000 X g for 15 minutes, the supernatant was aspirated and the pellet was resuspended in 2mL of TBS. The solution was transferred to eppendorf tubes (1mL/tube), and spun briefly to remove cell debris. The supernatant was transferred to a fresh tube and 200 µL of 2.5 M NaCl/20% PEG-8000 was added. The tubes were incubated on ice for 30 minutes. The tubes were centrifuged at 12,000-14,000 X g for 10 minutes and the supernatant was

subsequently discarded. The tubes were centrifuged for 2 minutes and the remaining supernatant was removed with a pipette. The pellet was resuspended in 200 μ L TBS and stored at 4 °C. For long term storage 200 μ L of glycerol was added and the phage was stored at -20 °C. Following each amplification step a subsequent titering step was performed. This method was also performed on the 10 picked plaques from the final round of biopanning (Ph.D. Phage Display Libraries Instruction Manual).

3.8 Growth of whole cell *S. aureus*

S. aureus was grown in Columbia broth and 2% NaCl overnight at 37 °C shaking at 200rpm. The cells were then washed with 10mL PBS and 1% BSA twice and once with PBS. The cells were centrifuged at 13,000 X g at 4 °C for 20 minutes. The supernatant was poured off and the cell pellet was vortexed to a smooth suspension and re-suspended in 10mL of PBS (this was done three times). The cells were treated with 3% formalin overnight on a rotator at room temperature. The cells were washed as described previously. The cells were treated with Trypsin (1mg/mL) at 37 °C overnight with shaking at 200 rpm. The cells were washed as described previously. The cells were then brought up to an Optical Density of 1.0 at 550nm (Thakker M *et al.* 1998.).

3.9 M13 ELISA

Using a polyvinyl chloride, cell-culture treated plate, the *S. aureus* wells were coated with 100 μ L of formalinized and trypsinized *S. aureus* (3 each for the blank, M13KE negative control and *S. aureus* wells). The positive control wells were coated with 100 μ L of 1×10^{10} pfu/ml phage in 50 mM sodium carbonate buffer, pH 9.6. To the no *S. aureus* wells, 100 μ L of blocking buffer (1% casein in PBS) was added. The plate was then incubated overnight in a humidified chamber at 37 °C. Following the overnight

incubation the plate was centrifuged at 400 X g for 15 minutes. The coating solution was aspirated off and the wells were washed once with wash buffer (PBS). The wells were filled with blocking buffer and the plate was incubated at room temperature for 1 hour. The blocking buffer was then aspirated and the wells were coated as follows: the test and no *S. aureus* wells were coated with 100 μ L of 2×10^{11} pfu/ml phage in PBS, the negative control wells were coated with 100 μ L of 2×10^{11} pfu/ml M13KE phage in PBS, the blank and positive control wells were coated with PBS. The plate was then incubated for 1 hour at room temperature. Following the incubation, the wells were washed six times with PBS. Subsequently, the HRP/anti-M13 monoclonal peroxidase conjugate was diluted 1:5000 in PBS. The diluted HRP/anti-M13 monoclonal antibody peroxidase conjugate (100 μ L) was added to all wells, and the plate was incubated at room temperature for 1 hour. The wells were washed six times with PBS. TMB substrate (100 μ L) was added to each well and the plate was incubated for a maximum of 10-60 minutes (until a blue color has formed). Once the color change occurs, 50 μ L of the stop solution, 2N H₂SO₄ was added and the absorbance was read at 450 nm. This method will be used to test binding of whole bacteria and also to test the binding of purified type 8 capsule with our identified peptide. (HRP/Anti-M13 Monoclonal Conjugate, ELISA screening of phage recombinant antibodies, protocol leaflet.)

Chapter 4: Results

Goals and Objectives

The goal of this project is to identify a peptide ligand that binds to *S. aureus* type 8 capsule to find a novel approach for treatment or detection of *S. aureus* infections. *S. aureus* infections are a leading problem among infectious disease due to a variety of factors. In order to identify a specific peptide ligand, the type 8 capsule was purified and tested by ELISA to confirm the presence of the carbohydrate. Biopanning was the selection technique implemented to select a peptide to bind to the target. Following biopanning, 10 clones were picked and tested by ELISA to show their binding affinity to the target.

4.1 Purification of type 8 Capsular Polysaccharide of S. aureus

Bacteria cell paste was collected, and suspended in 0.05 M tris- 2 mM MgSO₄ and autoclaved. DNase and RNase were added to the cells and incubated, followed by the addition of protease K. The solution was then allowed to incubate at room temperature over night. Sodium periodate was then added to the mixture, and incubated overnight in the dark. The mixture was dialyzed extensively against autoclaved MilliQ water, followed by lyophilization. The sample was then dissolved in sodium acetate and NaCl and applied to a DEAE column. The column was washed, the carbohydrate was eluted and 7mL fractions were collected. Following fraction collection, a red tetrazolium assay was performed to determine the carbohydrate concentration in the fractions. The positive samples were pooled and tested for impurities using a protein assay, a phosphate assay and a DNA assay (Lee, *et al.*).

4.2 Carbohydrate Assay of DEAE column fractions

A red tetrazolium carbohydrate assay for reducing sugars was performed on the 80 7mL fractions collected following DEAE column chromatography (Fieser et al, 1987). The results were used to determine the fractions that contained carbohydrate, which were pooled and used for further testing. The fractions that were pooled were 4-7, 13-22, 37-45, and 50-57, for a total off approximately 700mL of pooled purified carbohydrate (Figure 1). In order to determine the concentration of carbohydrate within our sample, a red tetrazolium carbohydrate assay was performed on the pooled fractions. The red tetrazolium assay measures reducing sugars within a sample. The positive control, a solution of known glucose concentration (.1mg/mL) was serially diluted (1:2) in MilliQ water. A linear regression line was formed using the serial dilutions of the glucose solution. This linear regression line gave an equation of: $y = 0.6087x - 0.0339$. The data shows a strong correlation ($R^2 = 0.8692$). Using this concentration and the absorbance of our sample at 450 nm, the concentration of carbohydrate in our sample is approximately equal to 0.1mg/mL resuspended to 50mL (Figure 2).

4.3 Contaminants in Carbohydrate Sample

4.3A Bradford Reagent Protein Assay

To determine the protein contamination of the carbohydrate sample, a Bradford reagent protein assay was performed (Bradford, 1976). The positive control, Bovine Serum Albumin (BSA) (40mg/mL), was serially diluted (1:2). The negative control used was MilliQ water. Using the equation from the linear regression line produced from the dilutions of BSA ($y = 0.0007x + 0.0826$), the protein concentration of the carbohydrate samples was determined to be negligible (equal to less than the negative control) (Figure 3).

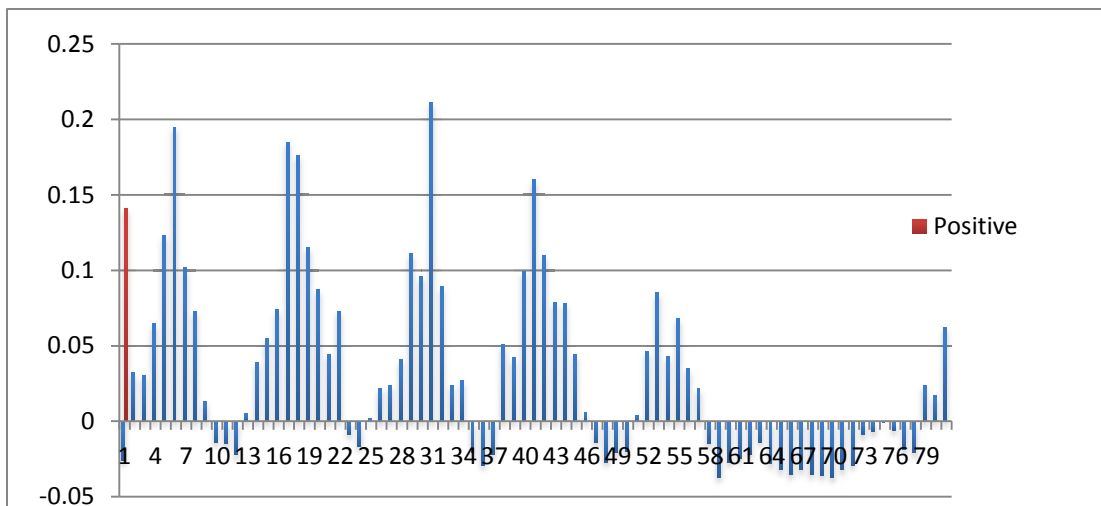


Figure 1: Red tetrazolium Carbohydrate Assay of DEAE Column Fractions. Fifty mL of *S. aureus* cell paste was autoclaved in Tris-MgSO₄ then incubated with DNase, RNase, protease and NaIO₄ to remove contaminants. The dialyzed and lyophilized carbohydrate preparation was dissolved in 0.05M sodium acetate and 0.05M sodium chloride and applied to a DEAE column equilibrated in the same buffer. The carbohydrate was eluted with 0.05M sodium acetate and 0.15M sodium chloride and collected in 7mL fractions. Each fraction (200μL) was added to a glass tube. Red tetrazolium (1mL) and NaOH (200μL) were then added to each tube containing the fractions. The tubes were then boiled and 100μL of each sample was added to a microtiter plate and the absorbance of the samples was then read at 490nm using a microplate reader. The absorbances of the individual carbohydrate fractions were compared with the positive control (0.1mg/mL) to determine the fractions to be pooled. The blank was MilliQ water with an absorbance of 0.079. The absorbance was read at 490nm on a microplate reader. The fractions pooled were as follows: fractions 4-7, 13-22, 28-34, 37-45, 50-57, resulting in 700mL of pooled fractions to be further tested and concentrated by lyophilization.

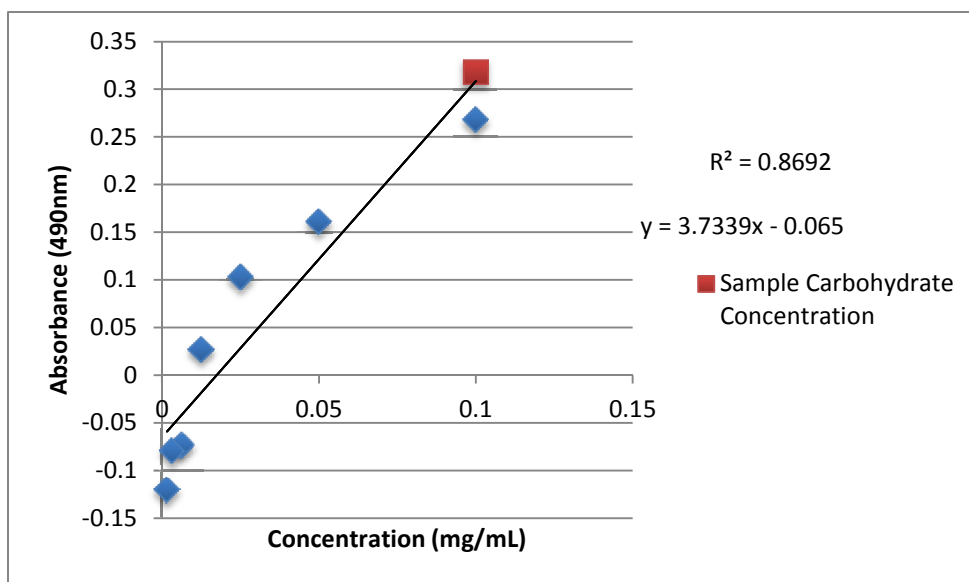


Figure 2: Red Tetrazolium Carbohydrate Assay of Pooled DEAE Column Fractions.

In order to determine the concentration of carbohydrate within our sample, a red tetrazolium carbohydrate assay was performed on the pooled fractions. The positive control, a solution of known glucose concentration (.1mg/mL) was serially diluted (1:2) in MilliQ water. The negative control was MilliQ water. Red tetrazolium and NaOH were added to each tube containing the glucose solution, the negative controls, and the purified carbohydrate sample. The samples were boiled until a red color developed and added (100 μ L) to a 96 well plate. The plate was then read at 490nm using a microplate reader. A linear regression line was formed using the serial dilutions of the glucose solution. A linear regression line was determined using a known concentration of carbohydrate, 0.1mg/mL glucose solution (concentration range of 0.1mg/mL - 0.00156mg/mL). Using the formula provided by the line, the concentration of carbohydrate (done in triplicate, n=3) in our samples was calculated to be 0.1mg/mL.

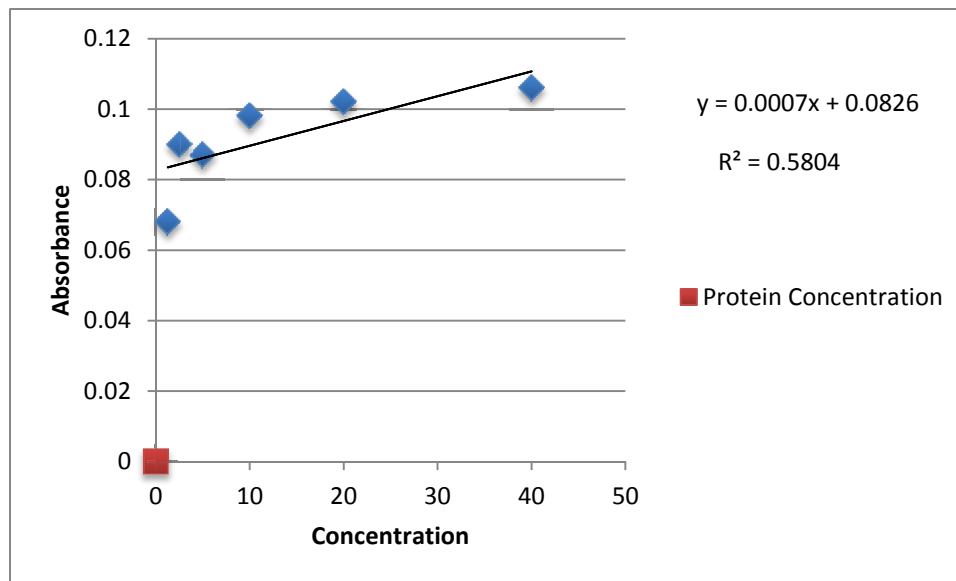


Figure 3: Bradford Reagent Protein Assay of pooled carbohydrate fractions.

Bradford reagent was added to the BSA wells, the negative control wells and the wells containing the carbohydrate sample. After mixing the plate, the absorbance was read at 595nm with a microplate reader. A linear regression line was constructed using a protein of known concentration (BSA 40mg/mL – 1.25mg/mL). The assay was conducted in triplicate, n=3. Using the equation from the linear regression line, the concentration of protein was found to be lower than the lowest concentration tested.

4.3B Phosphate Assay: Test for teichoic acid

Teichoic acid is a sugar, ribitol phosphate, found bound to the cell wall or cell membrane of *S. aureus*. Teichoic acid could possibly be found in the DEAE eluate and would test positive for carbohydrate using the red tetrazolium test. The teichoic acid should have been destroyed by the addition of NaIO_4 treatment (Tzianabos et al., 2001) (Tollersrud et al., 2001). In order to confirm the absence of teichoic acid within the purified carbohydrate sample, a phosphate assay was conducted to determine the teichoic acid concentration of our purified carbohydrate (Chen et al, 1956). A positive control of Phosphate buffered saline (PBS) at 10X and 1X concentrations was used. The negative control was milliQ water. A two-tailed T-test was used to determine the p-value (0.14) between the negative control and the carbohydrate samples. (Figure 4).

4.3C DNA concentration Assay

To determine the nucleic acid contamination of the samples, the absorbance of the purified carbohydrate samples was read at 230nm, 260nm, 280nm, and 320nm using a spectrophotometer (Table 1) (Maniatis et al, 1982). The absorbances were 0.118 at 230nm, 0.052 at 260nm, 0.035 at 280nm and 0.005 at 320nm. the absorbance was 0.035, and at 320nm the absorbance was 0.005. To determine the percent nucleic acid content of our purified carbohydrate samples, the absorbances were first converted to concentration in $\mu\text{g/mL}$. This was done by taking the OD at 260nm (0.052) and multiplying by 50 $\mu\text{g/mL}$ for double stranded DNA (dsDNA), for single stranded DNA (ssDNA) the OD at 260nm was multiplied by 37 $\mu\text{g/mL}$, and for single stranded RNA (ssRNA) the OD at 260nm was multiplied by 40 $\mu\text{g/mL}$ (Sambrook and Russell, 2001). From these conversions the samples were found to contain 2.6 $\mu\text{g/mL}$

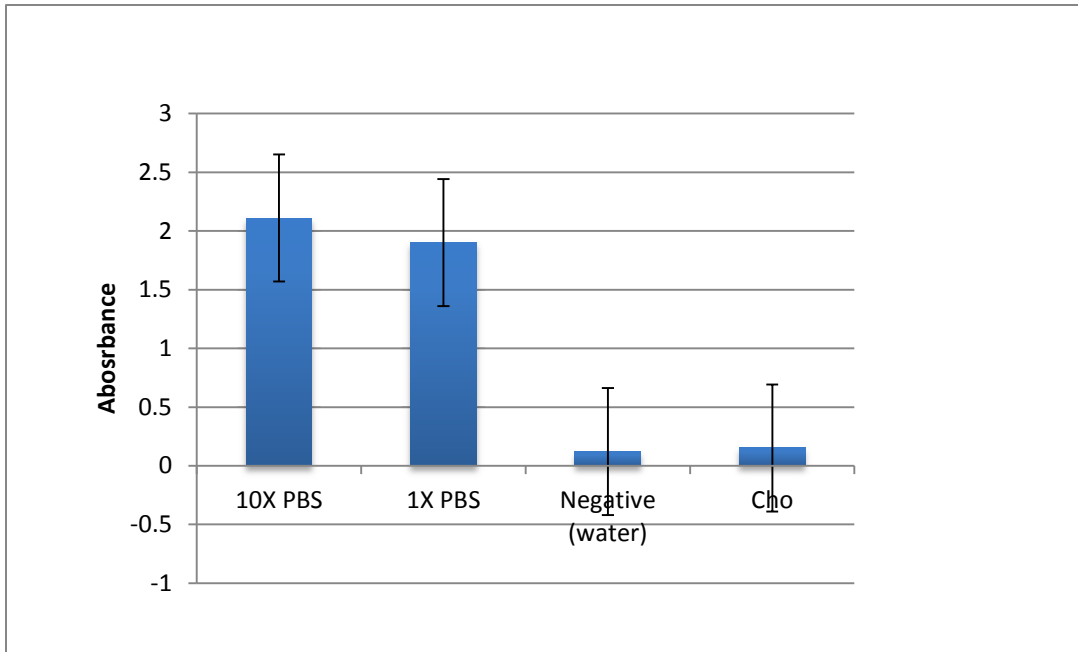


Figure 4: Phosphate Assay of Carbohydrate sample. Phosphate was released from teichoic acid by dissolving the sample in 2M HCl and boiling for 30 minutes. One volume of 6N sulfuric acid with two volumes of distilled water and 1 volume of 2.5% ammonium molybdate was added to the tubes containing the positive control (10X PBS and 1X PBS), negative control (MilliQ water) and the purified carbohydrate sample dissolved in 2M HCL. The tubes were then placed in a 30 °C oven and the color change was measured by spectroscopy at 820nm. The carbohydrate samples were read in triplicate, n=3. The student t-test comparing the carbohydrate samples and the negative control gave a p-value of 0.14, indicating there is not a significant difference between the negative controls and the carbohydrate samples.

Table 1: The Percent Concentration of Nucleic Acids within the Purified Carbohydrate Samples

	DS DNA ^b	SS DNA ^c	SS RNA ^d
% Nucleic acid in ^a sample	2.6%	1.9%	2.1%

a) The absorbance of the purified carbohydrate samples were read at 260nm. The concentration was then divided by the concentration of carbohydrate within the sample (100 µg/mL) and multiplied by 100 to give a percentage of dsDNA.

b) The OD at 260nm was then converted to concentration by multiplying by 50 µg/mL. This gave a concentration of dsDNA to be 2.6 µg/mL.

c) The OD at 260nm was then converted to concentration by multiplying by 37 µg/mL. This gave a concentration of ssDNA to be 1.9 µg/mL.

d) The OD at 260nm was then converted to concentration by multiplying by 40 µg/mL. This gave a concentration of ssRNA to be 2.1 µg/mL.

dsDNA, 1.9 µg/mL ssDNA and 2.1 µg/mL ssRNA. These concentrations were then divided by the concentration of the carbohydrate sample (100 µg/mL) and multiplied by 100 to determine the percentages within the purified carbohydrate samples. This showed 2.6% dsDNA, 1.9% ssDNA and 2.1% ssRNA contamination (Table 1).

4.4 Carbohydrate ELISA

To confirm the presence of carbohydrate we used an ELISA to demonstrate the binding of the purified type 8 capsule to previously made anti-capsular antibodies ANT8.B1.C5.G9. The absorbance was read at 450nm on a microplate reader. The test wells were tested in triplicate and averaged. A two-tailed student t-test determined a p-value of 0.023 showing a significant difference between the test wells and the negative control (Figure 5) (Thakker et al, 1998) (Sinha et al, 1999).

4.5 Biopanning of Ph.D 7 phage library

Three rounds of biopanning of the Ph.D. 7 phage library against *S. aureus* whole bacteria (Wright Type 8) were performed to select clones with specific binding to *S. aureus*. A preadsorption step was included to increase the specificity of binding to *S. aureus*. The preadsorption step was to casein blocking buffer, this step removed casein specific phage. The non-binding phage following preadsorption were then panned against *S. aureus* using 1% casein block for panning rounds 1 and 3, and fetal calf serum (FCS) for the second panning round. The use of two different blocking buffers increases the specificity of binding to the target, by removing block-specific phage (Dennis, *et al.* 2002). Intermediate amplification was performed between each panning rounds (Ph.D. Phage Display Libraries Instruction Manual). The phage titers were determined before and after

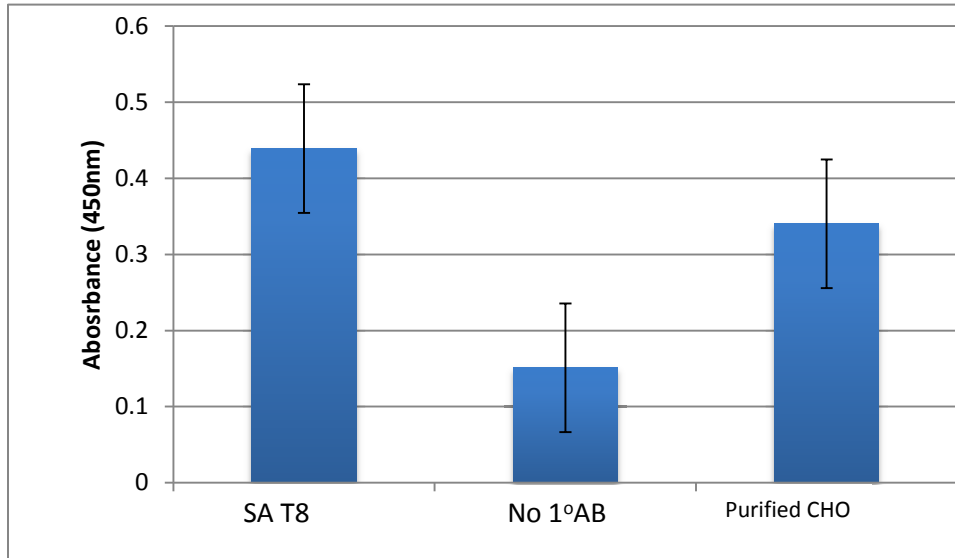


Figure 5: Carbohydrate ELISA. To confirm the presence of capsular carbohydrate within the pooled samples, a carbohydrate ELISA was performed. The wells of a 96 well microtiter cell culture treated polyvinyl plate were coated with the purified carbohydrate samples, or whole cell bacteria for the positive control. The negative control included everything but the primary antibody. Following an overnight incubation, the wells were washed and incubated with blocking buffer (1% BSA in PBS). The wells were then coated with 100 μ L of antibody in sample buffer, 100 μ L of PBS (negative control), or 100 μ L of hybridoma supernatant and incubated for 1 hour. The wells were then washed and 100 μ L of 1:1000 goat anti-mouse Ig-peroxidase conjugate (in sample buffer). Following incubation, the wells were washed and 100 μ L of TMB substrate was added and incubated until a blue color formed. Following a color change, 50 μ L of stop solution (H_2SO_4) was added and the absorbance was read at 450nm. The test wells were tested in

triplicate, n=3. A two-tailed student t-test gave a value of 0.023, showing a significant difference between the test wells and the negative control.

each amplification step (Barbas CF, *et al.* 2001.). Following the first round of panning the PFU/mL was 2.4×10^{10} , following the second round of panning the PFU/mL following amplification was 4×10^9 , and following amplification of the third round of panning the PFU/mL was 1.7×10^8 . Table 2 shows the input phage vs. the output phage (PFU/mL) for each round of biopanning. Table 3 shows the PFU/mL pre-PEG purification and post-PEG purification.

4.6 M13 ELISA of Panning 3 Phage to Whole Cell Type 8 and Type 5 *S. aureus* bacteria

Amplification and polyethylene glycol (PEG) purification of the phage was performed following the third round of panning. An M13 ELISA was performed to determine the binding of the phage to whole cell type 5 and type 8 whole cell *S. aureus* bacteria. The wells were seeded Type 8 or Type 5 *S. aureus* bacteria and blocked with 1% casein in TBS. Phage was then added to the wells. Anti-M13 monoclonal peroxidase conjugate was subsequently added. The wells were washed to remove non-specific phage and the color changing substrate, TMB was added (HRP/Anti-M13 Monoclonal Conjugate, ELISA screening of phage recombinant antibodies, protocol leaflet.). Figures 6 and 7 show absorbances at 450nm. A t-test was used to compare the binding of the phage with *S. aureus* and without *S. aureus*. The t-test showed significance difference ($p=0.0087$) indicated more binding of phage to wells with *S. aureus* than to those without *S. aureus* (Figure 6, Figure 7).

4.7 M13 ELISA of Phage to Purified Type 8 Capsular Polysaccharide of *S. aureus*

Table 2: Plaque Forming Units before and after each panning round.

	Panning 1 ^a	Panning 2	Panning 3
Input phage ^b	Phage library	2.4 X 10 ¹⁰ PFU/mL	2.2 x10 ¹³ PFU/mL
Output phage ^c	2.4 X 10 ¹⁰ PFU/mL	4.65 X 10 ⁹ PFU/mL	1.7 X10 ⁸ PFU/mL

a) Biopanning was the affinity selection technique used to select high affinity ligands for *S. aureus*. The phage library was added to the first panning round, non-binding phages were aspirated and bound phage were eluted. The eluted phage were then added to the next panning round. This was repeated three times for three rounds of biopanning.

b) Plaque forming units were counted using a blue-white screening process. Dilutions of phage were added to an overnight culture of *E. coli* (ER2738) vortexed and allowed to incubate for 1-5 minutes. The mixture was then added to top agar containing IPTG and X-gal and poured onto a pre-warmed LB-tet plate. The plates incubated overnight at 37 °C and the plaques were counted the next day. Input phage was calculated before each round of biopanning following amplification.

c) Output phage was calculated following each round of biopanning prior to amplification.

Table 3: Showing PFU/mL of selected titering plaques from before and after PEG purification.

	^a Plaque1	Plaque2	Plaque3	Plaque4	Plaque5	Plaque6	Plaque7	Plaque8	Plaque9	Plaque 10
^b Pre-PEG purification	9.5×10^1_2	4.6×10^4	2.6×10^1_3	1.0×10^1_3	6.0×10^1_3	2.6×10^1_3	5.0×10^{13}	8.0×10^{12}	2.0×10^{12}	5.5×10^1_4
Post-PEG purification	TNC*	3.8×10^1_2	3.4×10^1_3	5×10^{11}	2.5×10^1_2	5.6×10^1_4	1.26×10^1_5	1.58×10^1_4	4.68×10^1_2	9.7×10^1_4

a) Specific plaques were selected from the titers performed on the panning 3 phage.

Plaque forming units were determined using the method described in Table 2.

b) PEG purification was used to concentrate the samples for use in an ELISA. Phage clones were added to an overnight culture of *E. coli*. The mixture was incubated and the cells were removed by centrifugation. NaCl/PEG-8000 was added to the supernatant and the phage clones were precipitated overnight. The cell pellet was resuspended in TBS and cell debris were removed. NaCl/PEG-8000 was added and incubated then centrifuged.

(*TNC = too numerous to count)

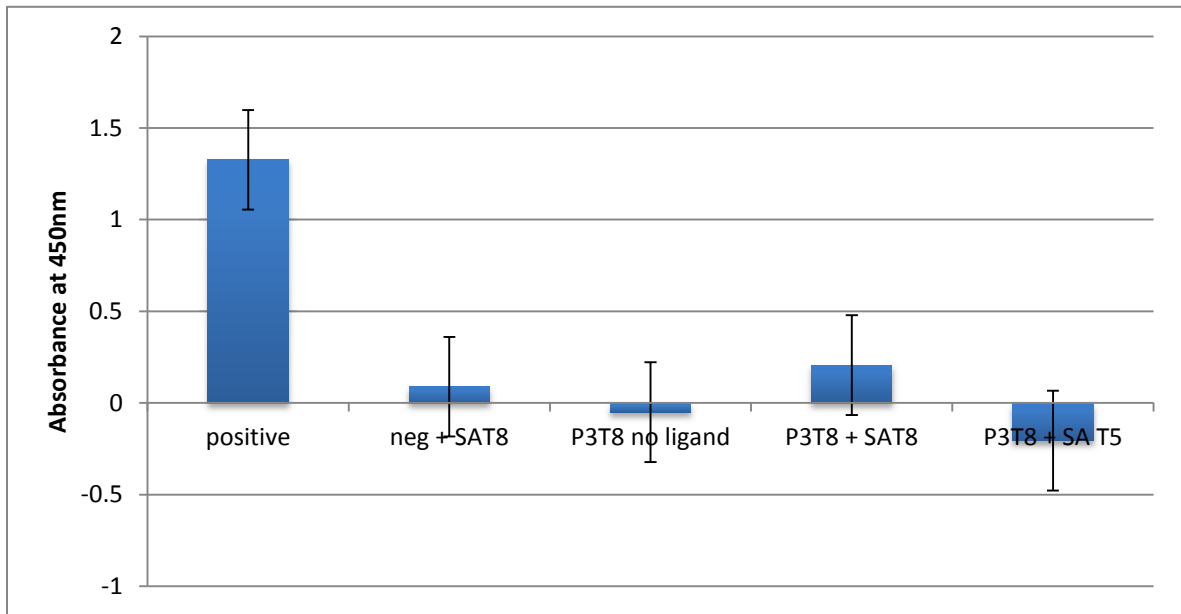


Figure 6: M13 ELISA of P3T8 binding to whole cell *S. aureus* bacteria. An ELISA was performed to compare the binding of the panning 3 phage to whole cell *S. aureus* bacterium type 8 and type 5 vs. without *S. aureus*. Positive control was amplified phage (100 μ L of 1×10^{10} pfu/ml) in PBS. The negative control is M13KE phage (100 μ L of 2×10^{11} pfu/ml) in PBS. All conditions were performed in triplicate, (n=3). Following seeding of the wells with type 8 or type 5 whole cell *S. aureus* (OD=1), the wells were filled with blocking buffer (1% Casein in TBS). Following incubation, 2×10^{11} pfu/mL of panning 3 phage were added to the wells. The wells were washed with PBS and the Anti-M13 Monoclonal Peroxidase conjugate was added to the wells and incubated overnight. 3,3',5,5',-Tetramethylbenzidine substrate solution (TMB) was added to the wells as a color changing substrate and stopped with 2N H_2SO_4 . A two-tailed T-test determined a P-value of 0.0087 between no *S. aureus* and *S. aureus* type 8. The test was performed in triplicate with n=3.

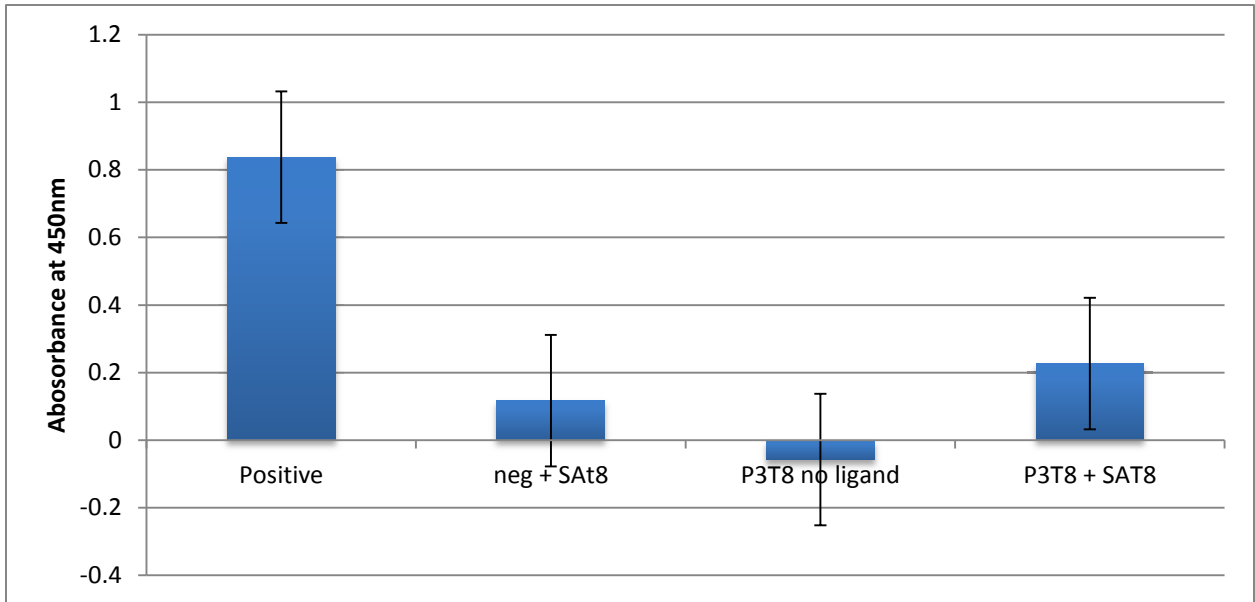


Figure 7: M13ELISA of P3T8 binding to whole cell *S. aureus* bacteria. An ELISA to compare the binding of panning 3 phage to whole cell *S. aureus* bacterium type 8 vs. without *S. aureus*. The procedure was described previously in Figure 6. Positive control was amplified phage (100 μ L of 1×10^{10} pfu/ml) in PBS. The negative control is M13KE phage (100 μ L of 2×10^{11} pfu/ml) in PBS. All conditions were performed in triplicate, n=3). The ELISA was conducted in triplicate with n=3. A two-tailed student T-test determined a P-value of 0.08286 comparing no *S. aureus* and *S. aureus* type 8.

The phage was tested by ELISA for binding to the purified type 8 capsular carbohydrate of *S. aureus*. The wells were seeded with the purified type 8 capsular carbohydrate, blocked with 1% casein in TBS, the phage was added, and the anti-M13 monoclonal peroxidase conjugate was used to determine phage binding following the addition of the TMB substrate (Figure 8) (HRP/Anti-M13 Monoclonal Conjugate, screening of phage recombinant antibodies, protocol leaflet.). Binding of phage to *S. aureus* was not significantly different from phage binding to the no *S. aureus* wells ($p=0.067$).

4.8 M13 ELISA of 10 Phage Clones to Whole Cell Type 8 S. aureus

Following the third round of panning, 10 plaques were picked and then tested for binding to whole cell type 8 *S. aureus*. Each plaque was amplified and PEG purified. The ELISA was performed as described for the M13 ELISA of pooled phage from panning round 3 to whole cell type 8 *S. aureus*. Each phage clone was added to the wells that were seeded with type 8 *S. aureus*, and blocked with 1% casein in TBS. The M13 peroxidase conjugate was added followed by the addition of TMB. The absorbances were read at 450nm with a microplate reader (Figure 9) (HRP/Anti-M13 Monoclonal Conjugate, ELISA screening of phage recombinant antibodies, protocol leaflet.). The fold increase of each plaque binding to *S. aureus* was determined and recorded in Table 4. All phage clones showed at least a two-fold increase with the exceptions of clones 6, 8, and 10. T-tests were also conducted for each plaque with and without *S. aureus*, the p-values are listed in Table 4. Phage clone 9 showed the most significance ($p=0.023$).

4.9 M13 ELISA of 10 Phage Clones to Purified Type 8 Capsular Polysaccharide of S. aureus

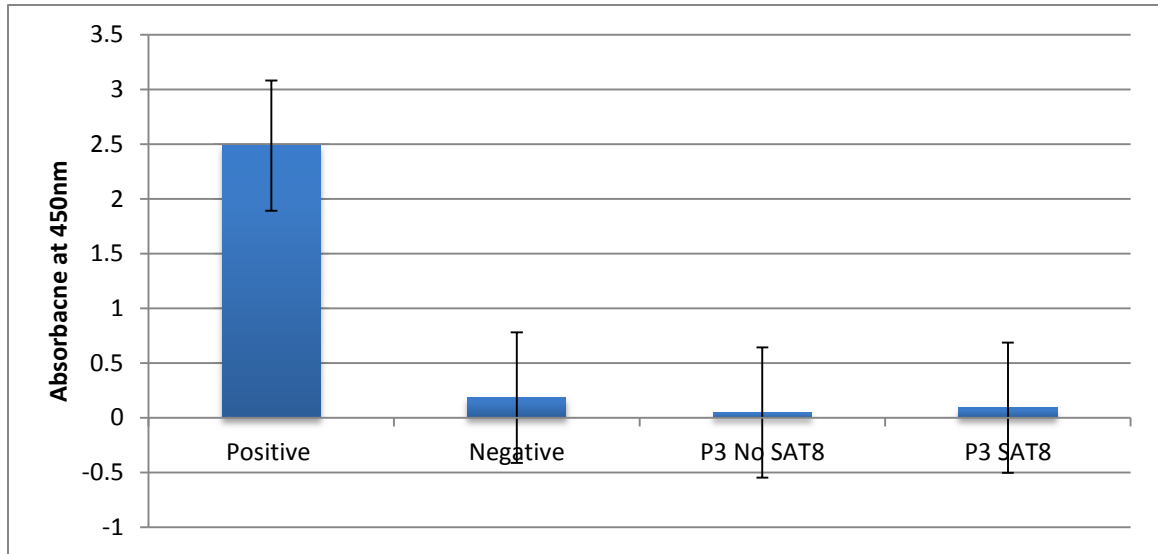


Figure 8: ELISA of Type 8 *S. aureus* carbohydrate to P3. ELISA of panning 3 phage to purified type 8 *S. aureus* capsular carbohydrate (n=) The same procedure as Figure 6 was followed except the wells were seeded with purified type 8 carbohydrate from *S. aureus* (100 μ L of 0.51mg/mL in MilliQ water). Positive control was amplified phage (100 μ L of 1×10^{10} pfu/ml) in PBS. The negative control is M13KE phage (100 μ L of 2×10^{11} pfu/ml) in PBS. All conditions were performed in triplicate, n=3). A student two-tailed t-test determined a p-value of 0.06799 between.

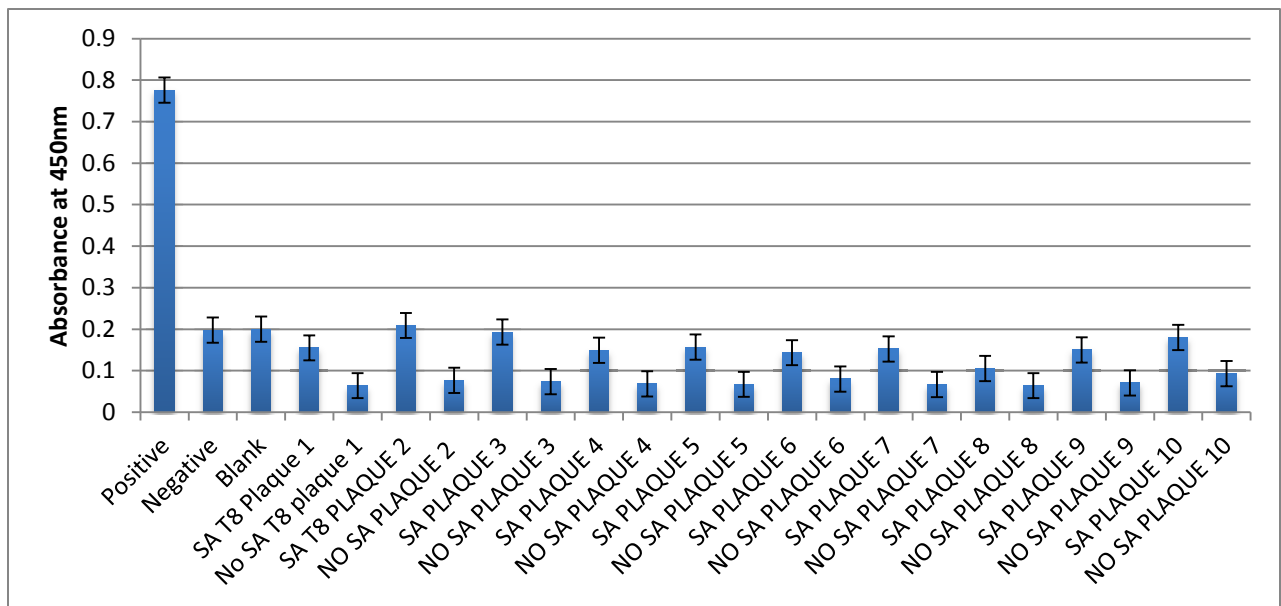


Figure 9: Phage clone ELISA to whole cell *S. aureus* bacteria. Following the third round of biopanning, 10 plaques were chosen to be tested for binding to *S. aureus*. A phage ELISA to whole cell *S. aureus* bacteria was used to determine the binding of individual plaques to whole cell type 8 *S. aureus* bacteria. The procedure is similar to the procedure cited in figure 6. Positive control was amplified phage (100 μ L of 1×10^{10} pfu/ml) in PBS. The negative control is M13KE phage (100 μ L of 2×10^{11} pfu/ml) in PBS. Student two-tailed t-tests were run on the individual plaques and can be seen in table 4.

Table 4: Fold increases of each plaque with and without *S. aureus* from the ELISA to whole cell bacteria. P-values of each plaque with and without *S. aureus*, as determined by a student two-tailed t-test.

	Plaque 1 ^a	Plaque 2	Plaque 3	Plaque 4	Plaque 5	Plaque 6	Plaque 7	Plaque 8	Plaque 9	Plaque 10
Fold ^b Increase	2.42	2.73	2.63	2.19	2.343	1.79	2.29	1.64	2.13	1.94
P-values ^c	0.141	0.192	0.112	0.196	0.065	0.211	0.163	0.065	0.023	0.073

a) Following the third round of panning, 10 specific clones were chosen to be tested by ELISA.

b) The fold increase of phage binding was determined by dividing the absorbance of binding to *S. aureus* from the wells without *S. aureus*.

c) The p-values were determined using a student t-test comparing the wells with *S. aureus* and those without *S. aureus*.

The 10 amplified and PEG purified plaques picked from the third round of panning, were tested for binding to purified type 8 capsular carbohydrate of *S. aureus*. The ELISA was performed as described for the M13 ELISA of picked plaques to purified type 8 capsular carbohydrate. The wells were seeded with the purified type 8 carbohydrates and blocked with 1% casein in TBS. The phage plaques were added and incubated. The anti-M13 monoclonal peroxidase conjugate was added and the substrate solution, TMB, was added. Using a microplate reader, the absorbance was read at 450nm (Figure 10) (HRP/Anti-M13 Monoclonal Conjugate, ELISA screening of phage recombinant antibodies, protocol leaflet.). The fold increase of each plaque binding to type 8 carbohydrate was determined and recorded in Table 5. T-tests were used to determine the p-values of each plaque with *S. aureus* and without bacteria (Table 5). Phage clones 1 (p= 0.004), 2 (p=0.016), 3 (p=0.05), and 6 (p=0.022) showed significance.

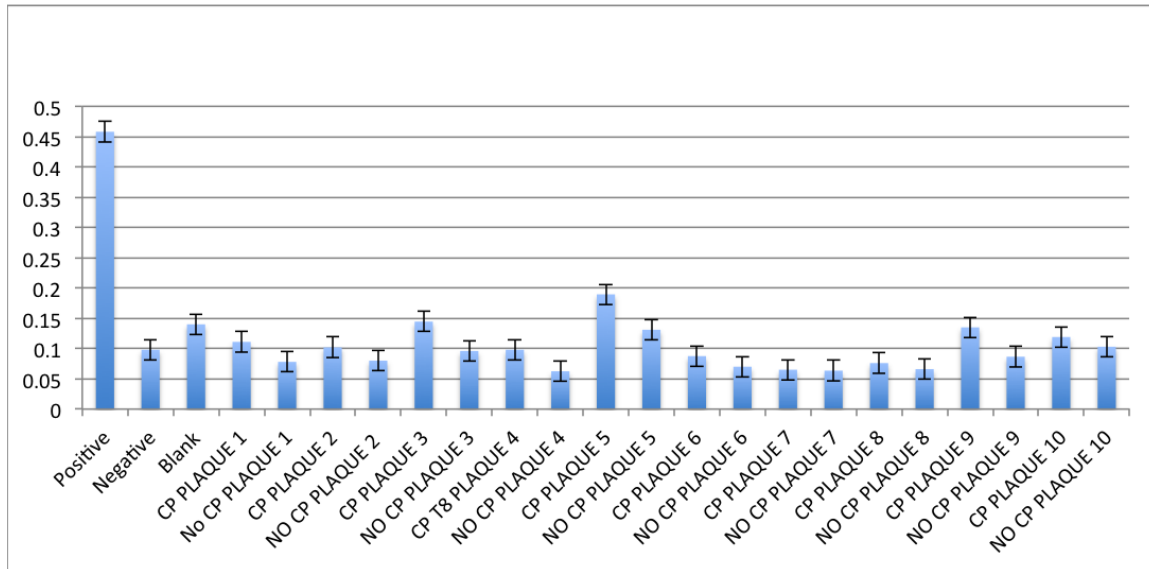


Figure 10: Phage clone ELISA to Type 8 *S. aureus* Carbohydrate. A Plaques ELISA was used to compare the binding of the 10 phage clones to type 8 capsular polysaccharide of capsule vs. no capsule. The method was identical to that described in figure 9, with the following modifications: the wells were coated with type 8 capsule instead of whole cell bacteria. Positive control was amplified phage (100 μ L of 1×10^{10} pfu/ml) in PBS. The negative control is M13KE phage (100 μ L of 2×10^{11} pfu/ml) in PBS. Student two-tailed t-tests were used to compare each plaque with and without type 8 capsule, the p-values of comparisons between capsule vs. no capsule are listed in found in table 5.

Table 5: Fold increases of each plaque with and without *S. aureus* from the ELISA to type 8 capsule of *S. aureus*. P-values of each plaque with and without *S. aureus*, as determined by a student two-tailed t-test.

	Plaque 1 ^a	Plaque 2	Plaque 3	Plaque 4	Plaque 5	Plaque 6	Plaque 7	Plaque 8	Plaque 9	Plaque 10
Fold ^b Increase	1.42	1.28	1.51	1.56	1.45	1.25	1.02	1.15	1.20	1.15
P-values ^c	0.004	0.016	0.05	0.096	0.134	0.022	0.887	0.550	0.455	0.401

a) Following the third round of panning, 10 specific clones were chosen to be tested by ELISA.

b) Fold increases of each plaque with and without *S. aureus* from ELISA to Type 8 carbohydrate. The fold increase of phage binding was determined by dividing the absorbance of binding to *S. aureus* from the wells without *S. aureus*.

c) P-values of each plaque with and without *S. aureus* as determined by a student two-tailed t-test. The p-values were determined using a student t-test comparing the wells with *S. aureus* and the wells without *S. aureus*.

Chapter 5: Discussion

In this study, phage display was used to identify a peptide ligand that binds to purified type 8 capsular carbohydrate of *S. aureus*. Phage display technology employs a selective technique, termed biopanning, to select proteins, peptides or antibody fragments that bind to a biological target (Reviewed in Willats, 2002). After successful identification of a peptide that binds to the type 8 carbohydrate, the peptide could then be used in the detection and treatment of *S. aureus* infections.

S. aureus is an opportunistic infection that is becoming a major problem with increased antibiotic resistance. The infection that used to be seen primarily among hospital-acquired infections has become a community-acquired infection. Due to these factors, a treatment for *S. aureus* needs to be identified to decrease the rate of infections.

S. aureus produces capsular polysaccharides which protect the bacterium from destruction by white blood cells. Of the 13 identified capsular polysaccharides, type 5 and type 8 are the most commonly isolated in human and animal infections (Kampen *et al.* 2005). Despite type 5 and type 8 strains having similar structures, there is very little cross-reactivity of antibody binding between them (Jones, 2005). The capsule has protective effects for the bacteria, including protection from phagocytosis by the hosts' immune system.

Monoclonal antibodies were formally thought to be an ideal vaccine candidate for *S. aureus* infections; however, a recent review determined that active and passive immunizations to date have not passed stage three clinical trials. The three treatments reviewed did not show significant differences in endpoints of death, relapse or

development of complications, failed to show a protective effect or showed multi-organ failure.

Type 8 capsular polysaccharide to *S. aureus* was purified following the modified procedure of Lee et al (Lee et al., 1998). The last step of purification was the addition of the dialyzed and lyophilized type 8 carbohydrate to a DEAE-Sephacel column equilibrated in 0.05M sodium acetate and 0.05M NaCl. The column chromatography method used was developed by Fournier and coworkers (Fournier et al. 1984) and Fattom and coworkers (Fattom et al., 1990). In Fournier's method a linear gradient of NaCl was employed to elute the type 8 carbohydrate (Fournier et al. 1984). Fattom and associates modified the linear gradient to a stepwise gradient in which the column was equilibrated with 0.05M sodium acetate and 0.05 NaCl, and then eluted with 0.15M NaCl in a sodium acetate buffer (Fattom et al., 1990). For this purification procedure we followed the stepwise gradient used by Fattom and coworkers.

Tzianabos *et al* and Tollersrud *et al* incorporated the use of sodium periodate (NaIO_4), to remove the teichoic acid from the purified capsule (Tzianabos et al., 2001) (Tollersrud et al., 2001). The teichoic acid of *S. aureus* is composed of ribitol phosphate (Sanderson et al., 1962). Teichoic acid binds to the DEAE column, along with the capsular carbohydrate. In order to remove the teichoic acid, Tzianabos and Tollersrud, used sodium periodate to liberate the phosphate from the purified sample. Previous studies (Fattom and Fournier) used a sephadex s-300 size exclusion chromatography method to remove the teichoic acid. In the size exclusion chromatography method, the larger capsule will come off the column first due to its size and the smaller teichoic acid will become trapped within the beads of the column effectively separating the teichoic

acid from the capsule. In this work the method of Tzianabos and Tollersrud was used to remove the teichoic acid. The use of a sephadex size exclusion column was eliminated. The sample tested to determine if using sodium periodate alone was sufficient for eliminating the teichoic acid from the capsule

To determine the carbohydrate concentration within the purified samples, using a red tetrazolium carbohydrate assay. Although previous studies (Fournier et al., 1984)(Fattom et al., 1990) (Tollersrud et al., 2001) (Tzianabos., 2001) used capillary diffusion or rocket immunodiffusion to determine the carbohydrate concentration, in this study a red tetrazolium carbohydrate assay was used in addition to an ELISA with a known anti-type 8 capsule *S. aureus* antibody developed in this laboratory. Although Fehling and Tollen's carbohydrate tests are more common carbohydrate assays, a red tetrazolium assay was chosen because of the sensitivity of the reagent for reducing sugars (Feisner et al., 1987).

In order to select a peptide that binds to *S. aureus* type 8 capsule, biopanning was employed as the selection technique. The PhD 7 library was panned against whole cell *S. aureus* type 8. This library contains bacteriophages that contain five heptapeptides on the minor coat protein PIII. This library can have up to 10^9 independent clones. The biopanning procedure was conducted in polyvinyl tissue culture treated microtiter plates to ensure the bacteria bound to the plates. As reviewed in Willats, biopanning consists of five fundamental steps. First, the phage particles are exposed to a target, non-binding phage are washed away and the bound phage are eluted. The eluted phage are then amplified in host bacterium. This biopanning procedure is repeated three to six times to increase specific binding (Reviewed in Willats, 2001).

The washing procedure must be done with care. If the washing solution is too harsh, the weak binders with high specificity may be lost. Conversely, if the washing solution is not harsh enough, selected clones may have low specificity. It is important to adjust washing times and the stringency of the washing solution. The washing procedure employed in our experiment, used PBS alone as the washing solution (Adey et al. 1995). The phage display manual recommends using PBS and Tween-20 (Ph.D. Phage Display Libraries Instruction Manual). However, Hakami and coworkers found that including Tween-20 within the wash and blocking buffers could promote non-specific binding to plastic (Hakami et al. 2015). To avoid this issue, we did not include Tween-20 in either our wash or blocking buffers. Another method employed to decrease nonspecific binding was to change the blocking buffers between rounds of panning (Hakami et al., 2015). We used casein in TBS for the first and third panning rounds and fetal calf serum for the second biopanning step.

Plastic-binding phage is a common problem, resulting in the recovery of non-specific phage. Adey and associates found through multiple experiments that to suppress plastic-specific phage a few techniques can be employed, including increasing the target concentration, using a non-fat milk block and elution techniques that are specific for the binding phage of interest. The first experiment was designed to determine experimental conditions that increase plastic binding phage. In short, the wells of polystyrene or polyvinyl chloride plates were blocked with BSA, non-fat milk, or left unblocked. Plastic binding phage or phage library particles diluted in PBS, PBS with BSA, non-fat milk or tween-20 were then added to the wells. The wells were subsequently washed with the same coating solution. The results, as determined by ELISA, showed that plastic binding

phage bind to wells blocked with non-fat milk, BSA and unblocked wells of either type of plate. These results confirm that the phage are binding to plastic and not to the blocking buffers. In addition this experiment showed that non-fat milk is the most ideal blocking buffer tested. In fact, the plastic-specific phage only bound to milk-blocked wells when the phage were diluted with tween-20. These results also confirms the idea that tween-20 increases plastic binding. The next experiment conducted aimed to determine the effects of the target concentration on specific binding. Due to the observation that plastic-binding phage is not always obtained, or that it is rarely the predominant phage isolated, Adey and coworkers compared monoclonal antibody binding phage to plastic binding phage when subjected to various amounts of immobilized antibody within one round of biopanning. The ELISA results showed that recovery of the antibody specific phage increased with increasing amounts of target, while plastic-specific phage recovery remained stable. These results indicate that the recovery of plastic binding phage occurs when either a target-specific phage clone is absent from the library or when there is an insufficient amount of target adding during the screening process. The final experiment conducted by Adey and coworkers looked at the effects of elution buffers on phage recovery. The researchers compared monoclonal antibody specific phage binding and plastic specific phage at the highest concentration of target with and without an acid elution step, similar to that used in this study. The results showed that antibody specific phage were more efficiently removed by acid elution. The researchers implied that other methods of phage elution might increase plastic-binding phage recovery (Adey et al., 1995).

Despite intermediate amplification being suspected to increase non-specific binding, we performed intermediate amplification after each of the three rounds of biopanning following the phage display manual's instructions (Ph.D. Phage Display Libraries Instruction Manual, Reviewed in Willats, 2001, and Sang *et al*, 2014). The problems with intermediate amplification can be that phage with decreased binding specificity, but preferential growth characteristics will be amplified. (Reviewed in Willats).

We did include a pre-adsorption step prior to beginning the biopanning process. We exposed the library to casein block to eliminate phage that bound to the plastic or to the casein block. We removed the non-binding phage from the pre-adsorption wells and transferred them to the first panning well to begin our selection technique. Rao et al., (Rao et al., 2013), also employed a subtractive method of biopanning where an initial depletion step is used to remove unwanted non-specific binding phage. Rao depleted the phage library by binding to *E. Coli* to remove phage that bound to gram-negative bacteria, and to *S. aureus epidermis*, which removes phage clones that bind to a close relative of *S. aureus*. The researchers found that by implementing the subtractive method, there was a decrease in non-specific binding phage (Rao et al., 2013).

Following the third round of biopanning the phage was tested by ELISA for binding of the phage to whole cell *S. aureus* bacteria, and to the purified type 8 carbohydrate of *S. aureus*. A two-tailed student t-test was used to determine the significance of binding to the different targets. When testing the binding of the phage to whole cell bacteria, we found significance ($p=0.0087$) indicating the phage does bind to the bacterium (Figure 7). When comparing the phage to binding to the type 8 capsular

polysaccharide of *S. aureus*, significance was not found ($p=0.067$). This could be due to binding of the phage to other surface elements of *S. aureus* or to non-specific binding (Figure 8). Although it is not significant ($p<0.05$), it does indicate some binding of the phage to the capsular carbohydrate. Following the third round of biopanning, the specific phage were amplified and titered. From the titers, 10 plaques were picked, amplified and PEG purified. An ELISA was conducted to test the specificity of binding of the 10 picked plaques to whole cell *S. aureus* and to the type 8 capsular carbohydrate of *S. aureus*. A two-tailed student t-test was conducted for each ELISA comparing each plaque with and without *S. aureus* (Figures 9 and 10). The p-values for binding of the plaques against whole cell bacteria showed only one significant value for plaque 9 ($p= 0.023$) (Table 4). The ELISA testing binding to the carbohydrate showed significance binding with 4 clones, 1, 2, 3, and 6, with plaque 1 showing the most significance ($p=0.004$) (Table 5). As plaque 1 had the highest significance, it will be further tested to confirm its specificity, and then will be used in future studies.

Future work will be conducted to decrease nonspecific binding in the ELISAs. . Modifications of the pre-adsorptions step, washing and blocking buffers, eliminating intermediate amplification and increasing the rounds of biopanning could all contribute to higher specific binding. When the ELISAs have been optimized to decrease nonspecific binding, the plaque ELISA to demonstrate binding of the phage to carbohydrate will be repeated to confirm which clone has the most specific binding. The DNA from specific clones can then be sequenced and a peptide having that amino acid sequence purchased and further tested for binding specificity. When a specific peptide has been confirmed,

the DNA for that peptide may be transferred to expression vectors coupled to toxins that may then be targeted to *S aureus* type 8 using the specific peptide.

Chapter 6: References

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