Purification and Characterization of Type 5 *Staphylococcus aureus*

by

Thomas Rudnicki

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Masters of Science

in the

Chemistry

Program

YOUNGSTOWN STATE UNIVERSITY

August, 2010

Purification and Characterization of Type 5 *Staphylococcus aureus*

Thomas Rudnicki

Signature:

Thomas Rudnicki, Student Date

Approvals:

Dr. Diana Fagan, Thesis Advisor Date

Dr. Peter Norris, Committee Member Date

Dr. Nina Stourman, Committee Member Date

Peter J. Kasvinsky, Dean of School of Graduate Studies and Research Date

THESIS ABSTRACT

Staphylococcus aureus (S. aureus) infections are notoriously difficult to treat due to antibiotic resistance and virulence. One virulence factor employed by *S. aureus* is a protective polysaccharide coating called a capsule. This structural component plays a significant role in immune response evasion. Drugs that target bacterial capsule circumvent the mechanisms utilized by traditional antibiotics and create a novel way to treat *S. aureus* infections. A systematic purification and characterization of the capsule can further drug development by elucidating the immunological interaction of this element. Purification of the capsule was achieved by implementing ion-exchange, size exclusion chromatography, and enzymatic digestion. The purified sample was then characterized by NMR spectroscopy.

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. Diana Fagan for all of her help throughout my research and writing. I would also like to thank my committee members, Dr. Nina Stourman and Dr. Peter Norris for improving my work by taking the time to contribute comments and revisions. I would also like to thank my fellow graduate students for all of their support and friendship for the past two years. I would like to thank the Youngstown State University Chemistry and Biology departments for facilitating my education. Finally, I would like to thank my parents for their unwavering support of my education as well as other life endeavors.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Nosocomial infections caused by *Staphylococcus aureus (S. aureus)* have become an increasingly large problem due to antibiotic resistance. *S. aureus* has developed resistance to many drugs including methicillin and the "antibiotic of last resort," vancomycin. This alarming trend has lead to a surge in the development of new antibiotics to combat *S. aureus* infections, as well as the establishment of passive or active immunity. A recently developed conjugate vaccine, StaphVAX ™ has conferred immunity but only transiently (Fattom et al., 2004). Due to this limited success, the Fagan and Norris research groups at Youngstown State University sought to develop either a glycomimetic drug or monoclonal antibodies to eradicate antibiotic resistant strains of *S. aureus*.

GENERAL BACKGROUND:

S. aureus is a staphylococcal bacterial strain that is a member of the Micrococcaceae family. Visually, the bacteria are spherically shaped and approximately one micrometer in diameter. They are distinguishable by their gold color and their formation of grape-like clusters. Their gold color is due to the presence of triterpenoid carotenoids in their cell membranes (Kuroda et al., 2001). The formation of the cluster pattern is attributed to their mode of cellular division. The bacteria also test positive for the catalase and coagulase enzymes (Casey et al., 2007). Catalase protects the cell from the damaging effects of hydrogen peroxide by catalyzing its decomposition to water and oxygen. Coagulase facilitates fibrin production. It has been speculated that bacterium associated with fibrin are able to resist phagocytosis. *S. aureus* is classified as facultatively anaerobic and Gram-positive. Gram-positive bacteria lack an outer

membrane and feature a thick, cross-linked peptidoglycan wall (Fischetti, 2000). The cell wall is composed of an A3α-type peptidoglycan with pentaglycine oligopeptide units joining two muropeptides through the ε-amino group of the lysine of one to a penultimate D-alanine of another (Tomasz, 2000). The peptidoglycan consists of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid cross-linked by peptides attached to the *N*-acetylmuramic acid. Teichoic acid provides rigidity to the cell wall via intermittent attachment to the 6-hydroxyl group of *N*-acetylmuramic acid residues in the glycan chain. In addition to providing structural integrity, the cell wall also interacts with the host's innate immune system. The cell wall may resemble an endotoxin which stimulates a cascade of immune responses such as cytokine release by macrophages (Lowy, 1998). Additionally, the cell wall provides an attachment site for host surface proteins.

B-LACTAM INTERACTION:

Penicillin, a commonly used broad spectrum antibiotic, targets the bacterial cell wall. Penicillin belongs to a family of antibiotics designated β-lactams because their structures share a β-lactam ring. The β-lactam family targets a key enzyme in bacterial cell wall synthesis, transpeptidase (Tomasz, 2000). β-lactam antibiotics mimic the D-Ala-D-Ala dipeptide, the substrate for the transpeptidase. The following figures show the structural similarities between β-lactam antibiotics and the D-Ala-D-Ala dipeptide which are emphasized in red.

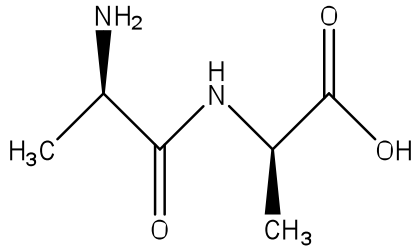


Figure 1: D-alanyl-D-alanine

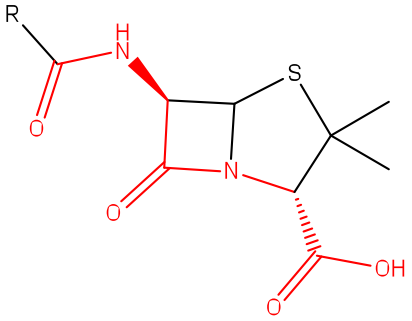


Figure 2: β-lactam antibiotics

The β- lactam substrate acts as a suicide inhibitor, when a substrate analog irreversibly complexes covalently with the enzyme. When the β-lactam is in the active site it forms a stable acyl-enzyme intermediate allowing it to be slowly hydrolyzed (Zapun et al., 2008, Sauvage et al., 2008). The occupation of the active site prohibits further reactions with the intended bacterial substrate. The inhibition of this enzyme disrupts the biosynthetic pathway which results in bacteria without a cell wall. The lack of a cell wall makes the organism vulnerable to death by osmotic lysis.

S. AUREUS CAPSULE:

A capsule has evolved in bacteria to protect the cell wall. The capsule is a polysaccharide coating of the bacteria which confers virulence by resisting phagocytosis (engulfment of bacteria by white blood cells). Capsule is produced by 90% of *S. aureus* and exists as 11 different serotypes encoded by the *cap* gene cluster (C. Lee and J. Lee, 2000). The capsular serotypes one and two exhibit thick mucoid capsules whereas serotypes 3-11 feature nonmucoid microcapsules. These microcapsules are so small that they require electron microscopy to visualize them. Type 5 and 8 microcapsules produced by *S. aureus* are sensitive to a plethora of environmental factors. Type 8

capsule exhibits a 300 fold increase of growth on agar plates along with a 4-8 fold increase on iron limited medium (C. Lee and J. Lee, 2000). Type 5 capsule has shown enhanced growth under high oxygen tension. Conversely, alkaline and high carbon dioxide conditions result in reduced capsule synthesis (C. Lee and J. Lee, 2000).

The genes found in the *cap5* and *cap8* loci are responsible for producing type 5 and 8 capsule respectively. Each gene cluster consists of 16 genes, labeled A through P, with genes A-G and L-P being identical for the two capsule types. The common genes found between the two strains suggest that the cap5 and cap8 loci are allelic. The H, I, J, and K genes are unique to the capsular type. Both gene loci are responsive to genetic regulators, *agr* and *mgr*. *Agr*, accessory gene regulator, is a global regulator in *S. aureus* which produces an auto-inducing peptide. When this peptide is activated, transcription of capsular polysaccharide is induced (Dassy et al., 1993). *Mgr*, multiple gene regulator, is a global regulator that affects the transcription of many *S. aureus* genes responsible for nuclease, coagulase, protease, and capsular polysaccharide expression. Deletion mutagenesis and genetic complementation have revealed that *mgr* has a significant role in the induction of capsular polysaccharide production in type 8 *S. aureus* strain Becker (Luong et al., 2003). Figure 3 is modified from O'Riordan and Lee and describes the roles of the *cap5* locus in the biosynthetic pathway of *Staphylococcus aureus* type 5 (O'Riordan and Lee, 2004).

The *cap5H* gene has been predicted to encode an *O*-acetyltransferase (O'Riordan and Lee, 2004). This enzyme is responsible for *O*-acetylation at the third carbon in the ManNAcA found in type 5 capsule. The *cap5P* gene encodes an epimerase and *cap5O* a dehydrogenase enzyme (J. Lee and C. Lee 1999, Portoles et al., 2001). Both of these

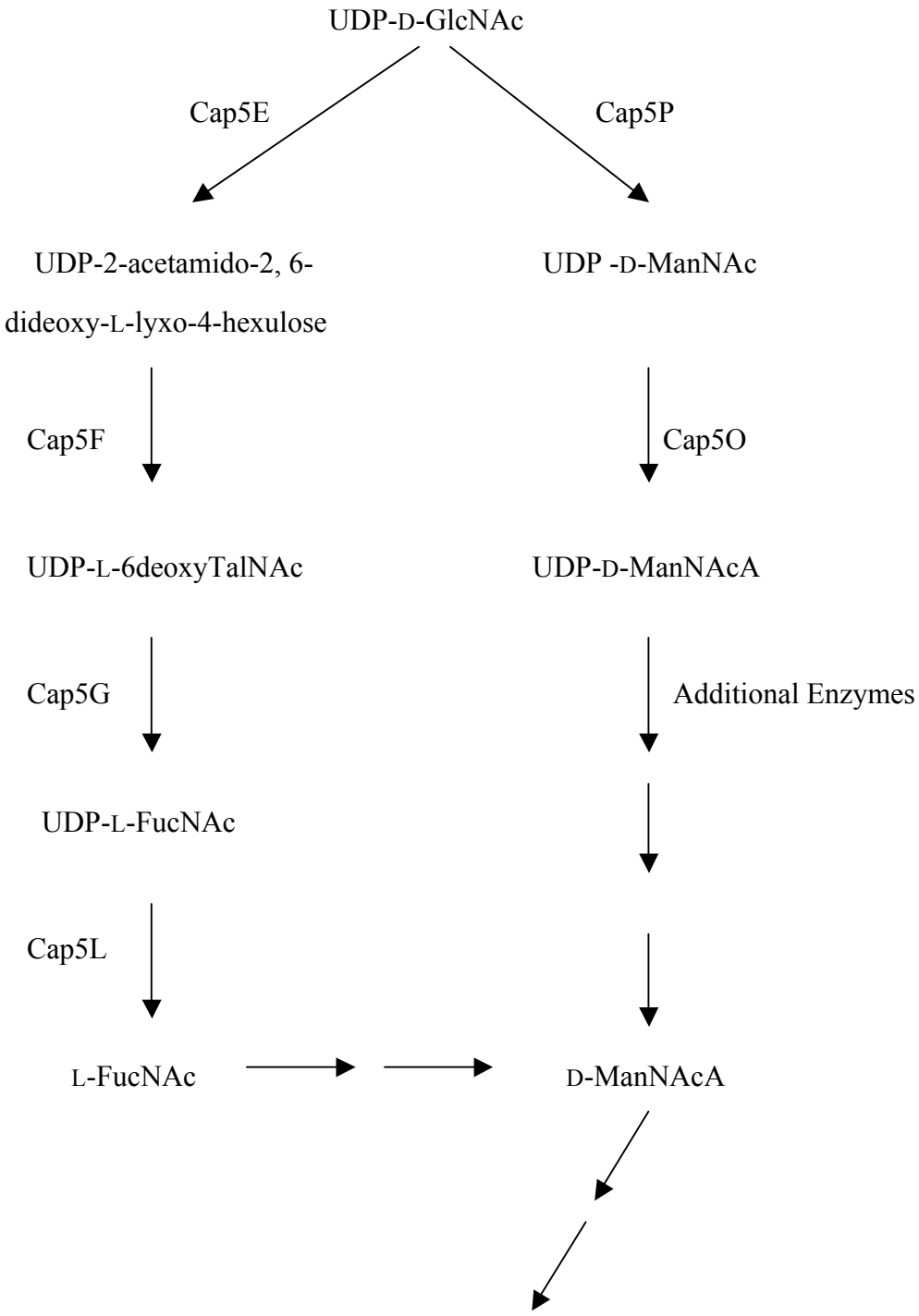


Figure 3: Biosynthetic Pathway for *S. aureus* Capsule

enzymes have been implicated in the synthesis of UDP-*N*-acetylmannosaminuronic acid from UDP-*N*-acetylglucosamine (O'Riordan and Lee, 2004). Purification of *cap5P* protein has confirmed its role as a UDP-GlcNAc epimerase (Kiser et al., 1999). The gene product of *Cap5B* is implicated in the determination of chain length of capsular polysaccharide units (Sau et al., 1997). The *cap5E* gene has been shown to encode an enzyme that catalyzes the 4,6-dehydration and 3,5-epimerization of UDP- *N*-acetylglucosamine (O'Riordan and Lee, 2004). *Cap5F* and *cap5G* act as a reductase and a 2-epimerase respectively to make *N*-acetyl-L-fucosamine (Kneidinger et al., 2003).

The products from the genes D-I and L-P work in concert to synthesize type 5 capsular polysaccharide, which is composed of the repeating trisaccharide: (→4)-β-D-ManNAcA-(1→4)-3-*O*-Ac-α-L-FucNAc- (1→3)-β-D-FucNAc-(1→)$_n$ (Portoles et al., 2001,Jones, 2005). This can be compared to the repeating unit in type 8 capsular polysaccharide: (→3)-4-*O*-Ac-β-D-ManNAcA-(1→3)-α-L-FucNAc-(1→3)-β-D-FucNAc-(1→)$_n$ (O'Riordan and Lee, 2004, Jones, 2005).

The structures for types 5 and 8 capsule are show in Figure 4:

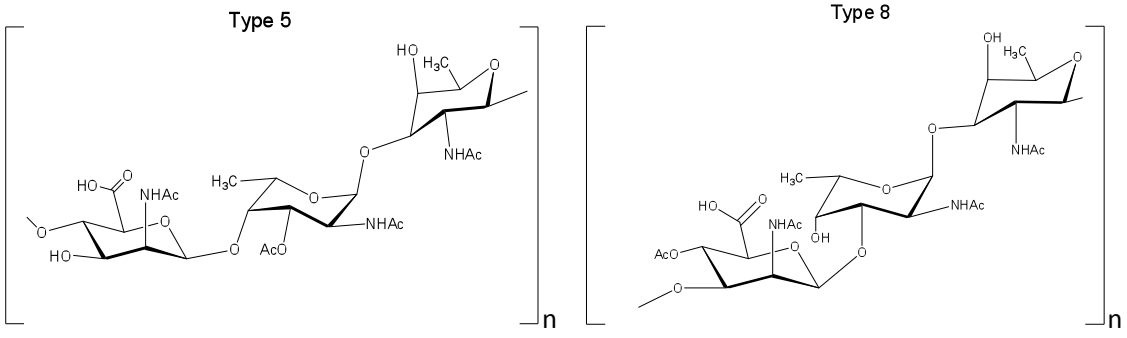


Figure 4: Repeating Trisaccharide Units for Type 5 and 8 Capsule

The structures for the two capsular types are similar. They both contain a repeating trisaccharide unit comprised of *N*-acetyl-D- mannosaminuronic acid, *N*-acetyl-L-fucosamine, and *N*-acetyl-D-fucosamine (Moreau et al., 1990). The main differences are in the position of the *O*-acetylation between the mannose and the fucose sugar and the glycosidic linkages. The uniqueness of the *O*-acetylation site in type 8 *S. aureus* makes it a significant antigenic target. Antibodies specific for *O*-acetylation of type 8 *S. aureus* more effectively induce phagocytosis (Karakawa et al., 1988). Another difference between the two serotypes is the degree of *N*-acetylation. Watts and coworkers showed that type 5 capsular polysaccharide was 98% *N*-acetylated whereas type 8 was 89% *N*-acetylated (Watts et al., 2005).

Studies have indicated that encapsulated types 5 and 8 strains resisted phagocytic killing by polymorphonuclear leukocytes (Karakawa et al., 1988, O'Riordan and Lee, 2004). Nilsson also proposed that macrophages ingest and kill nonencapsulated *S. aureus* more easily than those which expressed type 5 capsule (Nilsson et al., 1997). Opsonization is a crucial process required for killing by phagocytes. Antibodies and complement act as opsonins in serum and facilitate opsonophagocytic killing. Both antibodies and complement have sites which bind to the antigen as well as a site recognized by the phagocyte, initiating phagocytosis of the bacterium by the phagocyte. A study by Thakker and coworkers showed that mutant bacteria which lacked capsules were opsonized more easily by complement, leading to a greater rate of phagocytosis (Thakker et al., 1998). Similarly, Cunnion and coworkers showed mortality in 64% of mice with depleted C3 complement levels and *S. aureus* infections whereas 8% of control mice with normal complement levels died (Cunnion et al., 2003). In addition to

preventing opsonization, *S. aureus* capsule may also act as an adhesin to allow binding to host tissues and increase bacterial virulence (Soell et al., 1995).

When comparing the isogenic serotypes 5 and 8, studies by Watts and coworkers demonstrated that serotype 5 is more virulent in a murine model and survives better in opsonophagocytic killing assays (Watts et al., 2005). Through immunoelectron microscopy, they also showed that complement molecules bind to the cell wall which is masked beneath the capsule. The masking of complement molecules interferes with their ability to be recognized by receptors on polymorphonuclear leukocytes.

ADDITIONAL *S. AUREUS* VIRULENCE FACTORS:

In addition to capsule, *S. aureus* possess other virulence factors, such as Protein A, fibronectin-binding and collagen-binding proteins. Protein A is a surface protein which increases virulence by decreasing opsonization and inhibiting phagocytosis. Protein A binds IgG molecules by their Fc region, the opposite end from the antigen binding site, and orients them in the wrong direction (Fischetti, 2000). This improper orientation impairs the immune system's ability to opsonize and phagocytize cells. Another surface protein is fibronectin binding protein. The suggested role of fibronectin binding protein is to bind bacterial cells to immobilize elastin peptides in host tissues. Adherence allows the organism to colonize the host more easily (Roche et al., 2004). Another example of a virulence determinant on the cell surface is a collagen-binding protein called collagen-adhesin protein encoded by the gene, *cna*. This protein has been shown to promote adhesion and contribute to osteomyelitis and septic arthritis (Roche et al., 2004). *S. aureus* posses other adhesive molecules called microbial surface components recognizing

adhesive matrix molecules (MSCRAMMS). Examples of MSCRAMMS are clumping factors A and B which are responsible for attachment to a host target cell. Host attachment sites include fibrinogen, fibronectin, vitronectin, laminin and elastin (Otto, 2008). These factors have shown promise for passive and active immunization due to their ability to stimulate the production of opsonizing antibodies (Holtfreter et al., 2010 Vernachio et al., 2003). Despite the variety of disease causing factors, many individuals exhibit a stable colonization of *S. aureus* which does not manifest disease.

DISTRIBUTION OF *S. AUREUS* STRAINS:

Tenover and Gaynes have estimated that 20% of the population is asymptomatic carriers of *S. aureus* (Tenover and Gaynes, 2008). Additionally, 30 to 50% of the population exhibit transient colonization of the nose, perineum, or vagina (Tenover and Gaynes, 2008). Colonization of the nose is problematic because it leads to hand carriage of the bacteria. Colonization of the hands affords the bacteria the ability to establish an opportunistic infection by entering tissue through breaches in skin barriers. Once infection is established *S. aureus* can cause a variety of diseases.

Among the 11 capsule serotypes, type 5 accounts for 26% of human isolates and type 8 accounts for 55% (Hochkeppel et al., 1987). Studies by von Eiff and coworkers have indicated that of the 11 serotypes, type 5 accounts for 45.3% of methicillin-resistant *Staphylococcus aureus* (MRSA) infections, and type 8 accounts for 41.9% of MRSA infections (von Eiff et al., 2007). There are two distinct types of MRSA infections, hospital-acquired (HA) and community-acquired (CA). Hospital-acquired infections

occur during a hospital stay. Community-acquired infections are present in individuals who have not recently been hospitalized or subjected to a medical procedure.

Community-acquired *S. aureus* infections have only been recently detected. There are several unique characteristics of CA-MRSA isolates, including the production of Panton-Valentine leukocidin (PVL), patterns in antibiotic susceptibility and the presence of the mobile genetic element SCC*mec* type IV or V (Leclercq, 2009 Popovich et al., 2008). Infections due to CA-MRSA cause characteristic diseases, such as pediatric infections and epidemic furunculosis. Furunculosis, a hallmark of CA-MRSA infection, is recognized by the formation of a crusted plaque on the skin resulting from primary necrotic lesions. The manifestation of furunculosis is attributed to the necrotizing effect PVL has on tissue (Zetola et al., 2005). The most susceptible populations include children, Native Americans, military personnel, prison inmates, homosexual men, and athletes (Campbell et al., 2004 Anon, 2003 Groom et al., 2001 Herold et al., 1998 Main et al., 2005).

GENOMIC DIFFERENCE AMONG *S. AUREUS* STRAINS:

There are a wide range of interactions between *S. aureus* strains and human hosts. Analysis of genomic differences between strains can help elucidate these unique interactions and provide insight into specialized treatments. Developments with multilocus sequence typing (MLST) schemes have made genomic characterization of individual *S. aureus* isolates possible (Enright et al., 2000). Whole genome sequencing of MRSA can also locate novel antibiotic resistance genes. Additional information can be derived from genomic data, such as the determination that most MRSA resistance

genes were carried by either plasmids or by mobile genetic elements (Kuroda et al., 2001). Full genomic typing of *S. aureus* isolates will establish information about common surface antigen expression. Elucidation of universal antigens will allow for the development of broad strain vaccines which exploit these targets (Schaffer and Lee, 2008).

DISEASES MANIFESTED BY *S. AUREUS*:

Bacteremia, or the presence of bacteria in the blood has become increasingly problematic in both community and hospital settings. Tenover and Gaynes have estimated that *S. aureus* causes 11 to 38% of community-acquired bacteremia (Tenover and Gaynes, 2008). In 10 to 40% of bacteremia cases the disease progresses to endocarditis (Tenover and Gaynes, 2008). Endocarditis can present itself in three ways: right sided, left-sided native valve, and prosthetic valve. Left side native valve endocarditis is the most severe infection and can lead to embolization and heart failure.

Osteomyelitis, or infection of the bone is another infection caused by *S. aureus*. Osteomyelitis manifests in two forms, acute and chronic. The acute form typically affects children and infects the long bones of the lower extremity (Tenover and Gaynes, 2008). Acute infections can be cured if they are treated early. The chronic form commonly affects adults and is typified by an infection of six months or longer. Chronic infections are often hard to eradicate and resist antimicrobial therapy (Tenover and Gaynes 2008).

S. aureus is also responsible for a variety of toxin-mediated diseases. Impetigo is one example of an *S. aureus* toxin mediated disease. Impetigo is a skin infection which is

highly contagious and prevalent among infants and young children. Food poisoning is another example of disease caused by *S. aureus*. Symptoms of food poisoning include gastrointestinal discomfort and diarrhea. The disease is mediated by several staphylococcal toxins including enterotoxin A (Tenover and Gaynes, 2008). More severe toxin mediated diseases include scalded skin syndrome and toxic shock syndrome.

Scalded skin syndrome is caused by exotoxins called exfoliatins. Exfoliatins travel through the blood stream causing the upper skin layers to separate and peel off like sheets. Bacteremia (bacteria in the blood) commonly leads to death. Toxic shock syndrome is another disease which is mediated by a toxin. Toxic shock syndrome toxin-1 is a potent superantigen which elicits the production of cytokines that produce the majority of the symptoms (Tenover and Gaynes 2008). Symptoms include fever, low blood pressure, and a red rash that later peels.

Although many of these diseases are severe, the most challenging infections are nosocomial infections, which are diseases contracted in a hospital setting. There are a variety of diseases caused by nosocomial infections, such as urinary tract infections, mediastinitis which is swelling of the area between lungs, and nosocomial toxic shock syndrome. Pneumonia can also result from *S. aureus* infection and is commonly seen in elderly patients with influenza. Pneumonia can lead to more serious conditions such as empyema or an infection of the pleural space due to a collection of pus.

Surgical site infections are also caused by *S. aureus* and account for 15% of hospital infections (Tenover and Gaynes, 2008). *S. aureus* is a common cause of surgical site infections because bacteria are present on the skin or mucous membranes of patients.

Increasing commonality is garnered by the use of prosthetic devices. As discussed previously, *S. aureus* possesses a glycocalyx (capsule), a sugar coating which protects the organism from host defenses on the surface of prosthetic devices. Surgical site infections pose a major problem because they are associated with high mortality rates. The prevalence of surgical site infections can be attributed to a variety of factors. The most important is the degree of microbial contamination of the operating room. Second is the duration of the operation. The last major factor is the intrinsic susceptibility of the patient to contract *S. aureus* infections. Examples of such susceptibility include old age and level of nutrition.

ANTIBIOTIC RESITANCE:

Treatment of surgical site infections and other nosocomial infections are difficult due to the antibiotic resistance of bacteria. Initially, penicillin was the main treatment for *S. aureus* infections. Resistance became a problem in 1946 when about 6% of *S. aureus* produced penicillinase (Livermore, 2000). Penicillinase, a β-lactamase breaks the β-lactam ring of β-lactam antibiotics, thereby decreasing their activity. Resistance to other antibiotics soon arose. Treatment with chloramphenicol, erythromycin, and streptomycin soon became ineffective due to transposon and plasmid mediated resistance (Livermore, 2000). This widespread resistance led to a surge in antibiotic development. Gentamicin, an aminoglycoside drug was introduced in the 1960s and was effective against *S. aureus* infections. The effectiveness of gentamicin was limited and resistance became widespread in the 1970s and 80s (Livermore, 2000). Gentamicin resistance is associated with plasmid mediated production of a single protein which has both aminoglycoside acetyltransferase and aminoglycoside phosphotransferase activities that inactivate

aminoglycosides (Rouch et al., 1987). Another noteworthy drug produced in the 1960s was a semisynthetic β–lactam antibiotic, methicillin. Methicillin is a β–lactam antibiotic that has dimethoxybenzoyl group in place of acyl group. This bulky group sterically hinders the activity of penicillinase, making penicillin resistant *S. aureus* vulnerable to methicillin. Alarmingly, the first cases of methicillin resistant *Staphylococcus aureus* (MRSA) were documented soon thereafter in 1961.

Antibiotic resistance is largely due to the incorporation of the mobile genetic element SCC*mec* into the *S. aureus* genome. This element is considered an antibiotic resistance island which consists of a 27-76kb fragment of DNA and is incorporated near the origin of replication (Hiramatsu et al., 2001). This segment of DNA contains genes necessary for antibiotic resistance, such as *mec* encoding for penicillin binding proteins (PBPs) as well as mobility genes such as *ccr* encoding for cassette chromosome recombinases (CCRs) (Ito et al., 1999). There are three distinct types of SCC*mec*, I, II, and III in nosocomial infections. SCC*mec* I contains a type 1 *ccr* complex and a class B *mec* complex. SCC*mec* II and III contain a class A *mec* complex and types 2 and 3 *ccr* complexes (Hiramatsu et al., 2001). SCC*mec* I also exclusively contains only the β-lactam antibiotic resistance gene *mecA* whereas SCC*mec* II and III contain additional resistances genes. The additional resistance genes are given in Table 1 (Hiramatsu et al., 2001).

Resistance element:	Characteristic:
Tn544	Resistance to Erythromycin
ψTn544	Resistance to Cadmium
IS431	Facilitates acquisition of resistance
pUB110	Resistance to Tobramycin and Bleomycin
pT181	Resistance to Tetracycline

Table 1: Antibiotic Resistance Elements in SCC*mec* II and II

Normal *S. aureus* possess four penicillin binding proteins numbered one through four and exhibit a high binding affinity for the antibiotic, penicillin. These proteins act as bifunctional enzymes which catalyze transglycosylation and transpeptidation, necessary for synthesis of the cell wall. These activities assemble the glycan strands and cross-link peptidoglycan, respectively (Sauvage et al., 2008). The N-terminal domain of the penicillin binding protein contains glycosyltransferase activity and the C-terminal domain contains the transpeptidase domain (Kuroda et al., 2001). MRSA strains have an additional protein PBP2a which possesses the biosynthetic activities of all four PBPs. PBP2a has a low affinity for β-lactam antibiotics unlike the other four PBPs which have high affinities for β-lactams (Livermore, 2000). This reduced affinity is due to a modified active site which lowers its acylation rate. (Lim and Strynadka, 2002) Thus, in the presence of β-lactam antibiotics, PBPs 1-4 are inhibited by β-lactams, and PBP2a independently biosynthesizes the cell wall. The potential presence of an additional monofunctional glycosyltransferase in *S. aureus* has been reported by Kuroda and coworkers (Kuroda et al., 2001). The presence of an additional cell wall synthetic enzyme may provide *S. aureus* a mechanism of circumventing the activity of penicillin.

As described previously, *S. aureus* are categorized as hospital-acquired (HA) or as community-acquired (CA). The two strains differ genetically and have different profiles of drug resistance. HA-MRSA contains type I, II, and III staphylococcal cassette chromosome *mec* (Casey et al., 2007). HA-MRSA is more resistant to the drug ciprofloxacin than CA-MRSA. CA-MRSA genetically differs from HA-MRSA because they possess type IV staphylococcal cassette chromosome *mec* (Casey et al., 2007). CA-MRSA infections are exclusively resistant to β-lactam antibiotics and are susceptible to other antibiotic treatments. CA-MRSA cases have been on the rise primarily due to increased nasal colonization rate. Studies by Popovich indicate that CA-MRSA is nasally colonized by 9.2% of the population compared to HA-MRSA which colonizes only 0.8% (Popovich et al., 2008). Current treatments have been established for both CA-MRSA and HA-MRSA.

PROFILE OF TREATMENTS:

Quinupristin and dalfopristin are drugs implemented which compare to vancomycin, but exhibit a decrease in biocidal activity (Casey et al., 2007). Linezolid, a member of the oxazolidinone family is another compound used to treat *S. aureus* infections. Linezolid inhibits protein synthesizing bacteria by blocking the formation of the ribosomal initiation complex (Casey et al., 2007). Daptomycin, from the lipopeptide class, causes insertion into the bacterial cell membrane leading to depolarization. This effect inhibits DNA, RNA, and protein synthesis (Casey et al., 2007). Tigecycline is a glycylcycline antibiotic that inhibits protein synthesis by binding to the 30s ribosomal subunit and blocking the addition of amino acids to the growing polypeptide chain (Casey et al., 2007). Tigecycline is susceptible to antibiotic resistance through

chromosomally encoded multidrug efflux systems. An additional treatment involves the administration of antimicrobial peptides in conjunction with lysostaphin, an endopeptidase. One example of a promising antimicrobial peptide is Ranalexin, which is isolated from the North American Bullfrog. A combination of Ranalexin and lysostaphin in animal models showed efficacy in reducing wound and systemic infections with little toxicity (Desbois et al., 2010).

VANCOMYCIN AND RESISTANCE:

Vancomycin, which was produced in 1958, has become a hallmark in MRSA treatment (Perl, 1999). Vancomycin is a glycopeptide antibiotic that inhibits bacterial cell wall synthesis. The structure of vancomycin is given by Figure 5.

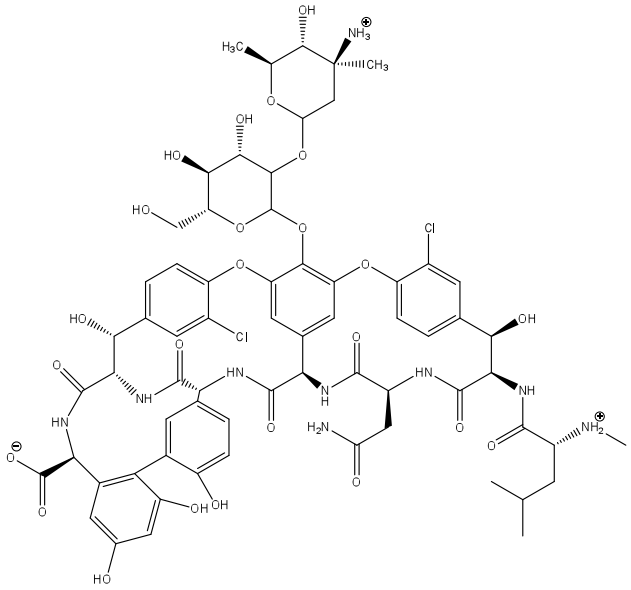


Figure 5: Structure of Vancomycin

Vancomycin contains a cleft which forms hydrogen bonds with D-alanyl-D-alanine, a substrate for peptidoglycan formation (Reynolds, 1989). This bonding pattern

is modified from Wright and is shown in Figure 6 with the bonding portion of vancomycin isolated (Wright, 2007).

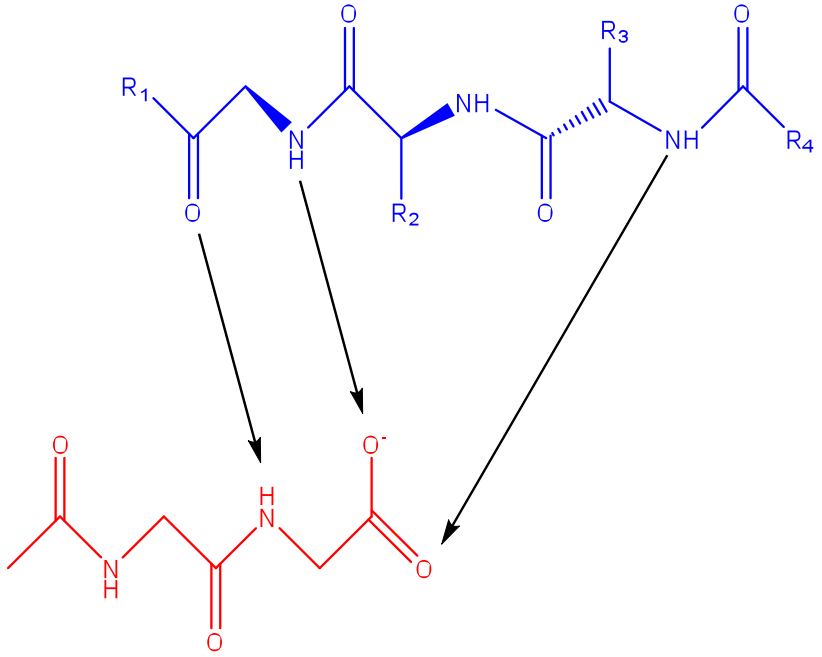


Figure 6: Hydrogen Bonding Between D-alanyl-D-alanine and Vancomycin

D-alanyl-D-alanine is represented in red and the bonding portions of vancomycin are colored blue. Vancomycin forms three hydrogen bonds with D-alanyl-D-alanine and their locations are indicated by the arrows. This bonding sterically misaligns the normal transpeptidases employed by the bacteria and drastically lowers substrate availability. The subsequent reduction in cell wall synthesis decreases structural stability and makes the bacteria susceptible to osmotic lysis. Additionally, this effect on transpeptidation makes development of resistance to vancomycin difficult (Reynolds, 1989).

Regarded as the "drug of last choice," vancomycin is used on a limited basis due to fear of the development of vancomycin resistant *S. aureus*. Alarmingly, the first case of vancomycin resistance was reported in 1996 (Hiramatsu, 2001). The isolated bacteria

exhibit a cell wall that is twice as thick and has a threefold increase in production of PBP2 and PBP2' which confers resistance. *S. aureus* strains that are resistant to glycopeptides are classified either as intermediate resistant (VISA/GISA) or high-level vancomycin-resistant (VRSA). VISA strains typically have vancomycin minimum inhibitory concentrations of 8-16 mg/l. (Hiramatsu, 2001). The thickened cell wall exhibited by these strains allows the cell wall to bind to and sequester glycopeptide molecules (Cui et al., 2006). Pereira and coworkers have suggested that resistance may also be conferred by a decreased rate of drug diffusion (Pereira et al., 2007). Alternatively, VRSA strains possess the *van* operon from *Enterococci*. Two genes *vanX* and *vanY* encoded by this operon produce enzymes which eliminate the D-ala-D-ala ends of the cell wall precursors (Arthur et al., 1998). The elimination of this structure reduces the vulnerability of the cell wall to β-lactam antibiotics.

This alarming trend in antibiotic resistance has spurred the development of new antibiotic treatments as well as the need for a vaccine to prevent infection with antibiotic resistant *S. aureus*. According to Gotz, promising targets for drug development are enzymes which are involved in cell wall biosynthesis or compounds which inactivate toxins (Gotz, 2004). Cell wall components which make good targets are the capsule, slime layer, and teichoic acids. The capsule has been a target for many vaccine developers. Possible drawbacks of such a vaccine include the fact that there are many different serotypes of the capsule and many *S. aureus* strains feature non-typeable capsule (Gotz, 2004).

Figure 7 shows an estimation of the overall hospitalizations for the years 1999-2005 due to MRSA infections (Klein et al., 2005).

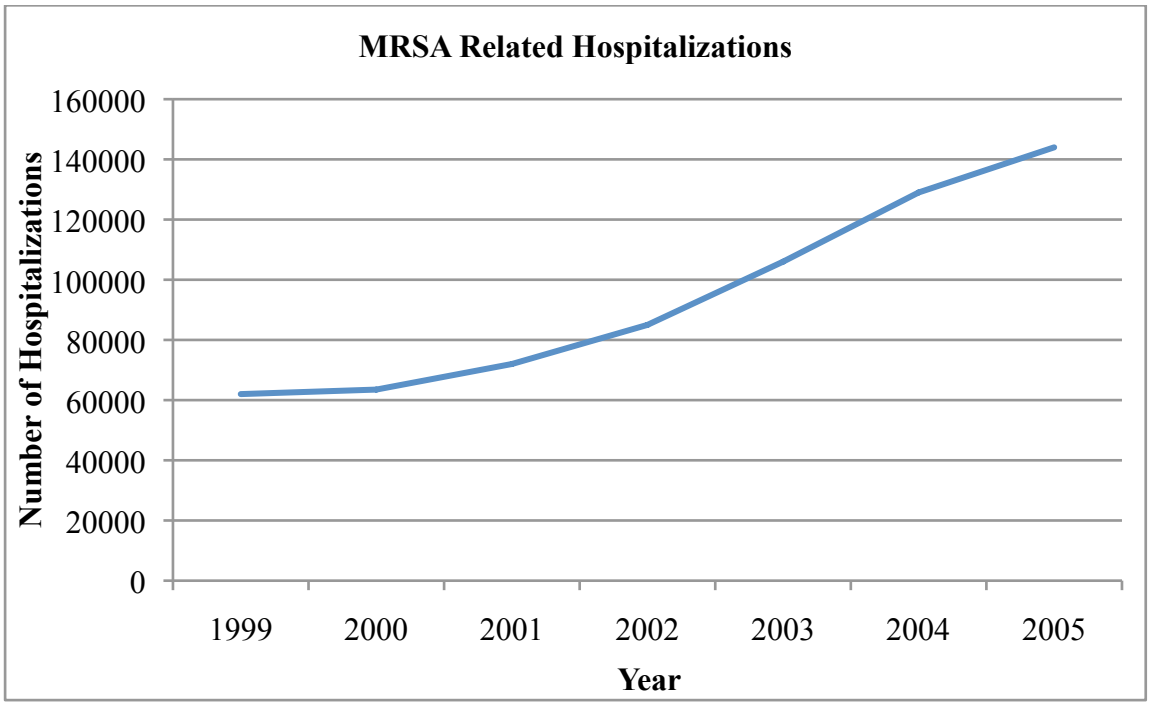


Figure 7: Hospitalizations due to MRSA from 1999-2005

Hospitalizations collectively consisted of primary diagnoses of cellulitis, abscess, post operative infections, device associated infections, osteomyelitis, and diabetes mellitus. As evidenced by Figure 7, MRSA related hospitalizations are on the rise due to lack of effective treatment. Conventional treatments including β- lactam antibiotics and vancomycin are no longer effective. Cessation of this trend must therefore rely on novel methods of MRSA therapy.

PROSPECTIVE TREATMENTS:

In addition to antibiotic administration, the establishment of passive or active immunity is also promising. Active immunity is typically conferred by a vaccine and is a primary immune response to an antigen. Passive immunity is achieved by the direct administration of antibodies not naturally produced by the patient's immune system.

Vaccine development is advantageous because prophylactic treatment reduces the use of antibiotics and therefore the risk of drug resistance. Optimal antigens are selected for vaccines based on their location, and their role in virulence. Otto has suggested that surface antigens are often more antigenic due to their extracellular location (Otto, 2008). *S. aureus* capsule provides for a good vaccine target because similar vaccines targeting other bacterial capsules have been successful. StaphVAX^{TM}, a vaccine created by Nabi Pharmaceuticals consists of capsular polysaccharide types 5 and 8 conjugated to *Pseudomonas aeruginosa* exoprotein A. In a 54 week trial of this vaccine in dialysis patients, there was a 26% reduction in bacteremia (Shinefield et al., 2002). Despite this early success, additional studies have failed to reproduce reduction in bacteremia. Schaffer and Lee have speculated the cause of this reduction in efficacy is due to a reduced role of capsule in *S. aureus* proliferation (Schaffer and Lee, 2008). Additionally, studies have shown that staphylococci are often acaspular while in the growth phase and may be unaffected by treatment focused on capsule formation (Pohlmann-Dietze et al., 2000). Vaccine efficacy may also be hindered by the fact that the human immune system has an established interaction with commensal *S. aureus* organisms (Otto, 2008). In such a scenario, the immune system will have antibodies formed against *S. aureus* and therefore be unresponsive towards active immunization.

S. aureus capsule has also been targeted by antibody administration to establish passive immunity. Early studies conducted by Fattom and coworkers have shown that administration of capsule specific antibodies in murine models has decreased morbidity following a lethal challenge dose of *S. aureus* (Fattom et al., 1996). Further studies by Lee and coworkers have found a reduction in catheter-associated endocarditis when a

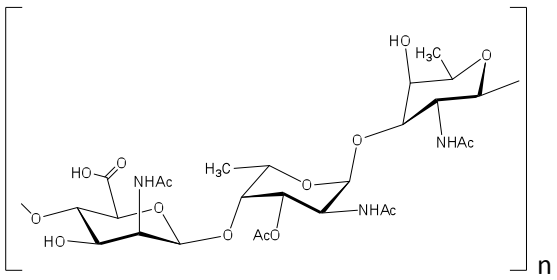
therapeutic dose of type 5 capsular polysaccharide specific antibodies were administered to rats (Lee et al., 1997). AltaStaph, a polyclonal antibody treatment created from the antibodies produced by individuals immunized with StaphVAX™ targets capsular polysaccharides 5 and 8 of *S. aureus*. Special target populations such as patients undergoing emergency surgery, prosthetic implantation, and those in intensive care units are strong candidates for passive immunization (Schaffer and Lee, 2008). Despite insignificant efficacy of standalone AltaStaph administration, it has a possible role in adjunctive therapy along with antibiotics for severe infections such as endocarditis (Schaffer and Lee, 2008).

Monoclonal antibody therapy also exists as a possibility for treatment. Monoclonal antibodies are highly specific and can be engineered to increase specificity (Kokai-Kun and Mond, 2004). Large quantities of antibodies can be made which allows for a widespread dissemination of the therapy. Exact control of antibody production environment also allows for the elimination of any possible contaminants (Kokai-Kun and Mond, 2004). Another advantage to monoclonal antibody therapy is that medical practitioners are familiar with it and feel comfortable utilizing it. One potential drawback to antibody therapy is that since the antibody is highly specific, one mutation can eliminate the target epitope (Kokai-Kun and Mond, 2004). Also, many of the antibodies are produced from non-human organisms and will need to be humanized before therapeutic use. Testing of monoclonal effectiveness can be achieved by purification of the target epitope. In the Fagan lab, monoclonal antibodies have shown efficacy in eliminating type 5 and 8 *S. aureus*. A purification of the target epitope, capsular polysaccharide will elucidate antibody-antigen binding.

GLYCOMIMETICS:

Another avenue of research is the development of a mimetic drug which resembles the capsular polysaccharides type 5 and 8. A mimetic is a saccharide which is structurally similar to the capsular polysaccharide. This similarity would allow for the mimetic to be incorporated by enzymes in the capsular polysaccharide biosynthetic pathway, such as glycosyltransferase and inhibit capsule production. Carbohydrate biosynthetic pathways are often susceptible to inhibition from small synthetic molecules (Bertozzi and Kiessling 2001). Mimetic incorporation can lead either to a dysfunctional capsule or the cessation of capsule synthesis altogether. Successful inhibition of a glycosyltransferase, alpha-2, 6-sialytransferase by a small polar synthetic oligosaccharide has been shown by Muller and coworkers (Muller et. al., 1998). Despite this success, one major drawback to this glycomimetic treatment involved the lack of membrane permeability to the synthetic compound (Bertozzi and Kiessling, 2001). One major consideration for establishing a synthetic oligosaccharide is its polarity and potential permeation through a lipid bilayer. Permeating is possible and was achieved in a whole cell model by Platt and coworkers (Platt et al., 1997). They have shown the inhibition of a glycosyltransferase by a synthetic sugar, *N*-butyldeoxynojirimycin.

The Norris group has synthesized a mimetic drug named TC-I-019. We will investigate its ability to inhibit capsule production in type 5 *S. aureus*. A comparison between the structure of this compound and the structure of the repeating trisaccharide unit in *S. aureus* type 5 bacteria is given below.



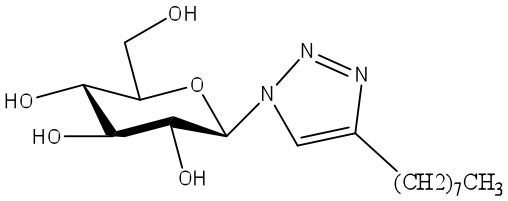


Figure 8: Type 5 Capsule **Figure 9: TC-I-019**

In theory, the enzymes responsible for synthesizing capsule will incorporate the glycomimetic, TC-I-019 instead of the natural substrate. If the mimetic is incorporated either capsule biosynthetic inhibition will be achieved or a dysfunctional capsule will be produced. The Fagan group will test efficacy of this compound in inhibiting bacteria proliferation. If successful, an NMR analysis of the *S. aureus* capsular polysaccharides type 5 and 8 when treated with the carbohydrate mimetic TC-I-019 will be conducted. This analysis should support or reject the ability of the mimetic drug to affect capsular polysaccharide synthesis.

MATERIALS

The sodium chloride, magnesium sulfate, formaldehyde, TRIS, and dialysis tubing were all purchased from Fisher Scientific (Fairlawn, NJ). DNAse, RNAse, Pronase, sodium acetate, sodium hydroxide, 2,3,5 - triphenyltetrazolium chloride, glucose, bovine serum albumin, tetramethylbenzidine solution, deuterium oxide, goat anti-mouse immunoglobulin conjugated to peroxidase, trypsin from porcine pancreas, polyoxyethylene sorbitan monolaurate, and sodium periodate were purchased from Sigma (St.Louis, MO). Hydrochloric acid, ascorbic acid, and ammonium molybdate were purchased from J.T. Baker (Phillisburg, NJ). The bacteria used, *Staphylococcus aureus* strain Rosenbach serotype 5, were purchased from the American Type Culture Collection (Manassas, VA). Sulfuric acid was purchased from Pharmaco-AAPER (Brookfield, CT). Columbia broth was purchased from Becton, Dickinson and Company (Sparks, MD). The DEAE Sephadex A-25 was purchased from GE Healthcare (Piscataway, NJ). Bottle top filters (0.45 μm) were purchased from Corning (Corning, NY). Lysozyme was purchased from Amresco (Solon, OH).

METHODS

1. Purification of Bacteria and Carbohydrate Preparation

Bacteria were prepared and capsular carbohydrate was purified using a modified form of the procedure by Tzianabos and coworkers (Tzianabos et al., 2001). Strain Rosenbach *Staphylococcus aureus* was grown in Columbia broth containing 2% NaCl by shaking at 225 rpm at 37 ^{o}C for 22 hours. The solutions were centrifuged at 13,000 x *g* for 30 minutes at 4 ^{o}C. The cell pellet was then suspended at 0.5 g of bacterial paste per ml in buffer (0.05M Tris- 2mM $MgSO_4$, at pH =7.5) and autoclaved for 20 minutes at 121 ^{o}C. DNAse and RNAse were added in 100 μg/ml concentrations and the mixture was incubated for 6 hours at 37 ^{o}C with shaking at 225 rpm. Pronase was then added in a concentration of 4 units/ml and the incubation continued overnight. The solution was then dialyzed using 12 K – 14 K μw tubing against water, which was changed a minimum of four times. The remaining bacteria fragments in the solution were then pelleted by centrifugation at 25,000 x *g* for 30 minutes at 4 ^{o}C. The pellet was discarded and the solution was filtered using a 0.45 μm sterile filter. The sample was then treated with 0.05 M sodium periodate and dialyzed using the procedure as described above. The sample was then lyophilized and dissolved at 0.015 g/ml in 0.05 M sodium acetate containing 0.01 M NaCl at a pH 6.0.

2. Diethylaminoethyl (DEAE) Sephadex Column

    a. Regeneration

The regeneration procedure was based on previous work done by Lopez and coworkers (Lopez et al., 1997). The column was purchased from GE healthcare and had a total volume of 565 cm^3. To remove any bound molecules the column bed was washed with one column volume of 1M sodium acetate with a pH of 3.0, 0.5M sodium hydroxide, 1.5 column volumes, was then added to the column overnight. The column was then recharged by the addition of 1.5 column volumes of 1.0 M sodium acetate (pH of 3.0). The column was then re-equilibrated using five column volumes of starting buffer (0.05 M sodium acetate with 0.05 M NaCl at pH 6.0).

    b. Sample Elution

The sample was dissolved in 10 ml of the starting buffer and was applied to the column. The sample was eluted with two column volumes of the elution buffer (0.05 M sodium acetate with 0.15 M NaCl at pH 6.0). This buffer was shown by Moreau and coworkers to elute capsular polysaccharide (Moreau et al., 1990). Fractions were collected in 7 ml portions.

3. S-300 Column

    a. Preparation

        The S-300 column with a total volume of 150 cm^3 was regenerated as described by GE Healthcare (GE Healthcare). The column was first washed with one column volume of 0.5 M NaOH solution. The contact between the solution and the gel was maintained for two hours. The column was then immediately re-equilibrated by washes with two column volumes of the eluent buffer (0.05 M sodium acetate).

    b. Sample Elution

        Approximately 6 ml of the carbohydrate sample in eluent buffer was loaded onto the top of the column. The sample was then loaded onto the column and additional buffer was added to the top of the column. Next the column was washed with three column volumes of eluent buffer to elute the sample. The eluent buffer was shown to effectively purify capsular polysaccharide by Fournier and coworkers (Fournier et al., 1984). Fractions were collected as a result of buffer elution in approximately 7 ml portions.

4. Red Tetrazolium Test

    Red tetrazolium (2,3,5-triphenyltetrazolium chloride) is a substance which forms an insoluble red product, diformazan in the presence of reducing sugars. One ml of aqueous red tetrazolium solution (0.5%) was added to test tubes containing 200 µl of each fraction of the column eluate. This same quantity of red tetrazolium was also added to

200 µl of the negative control, water, and the positive control, 0.1mg/ml glucose. Sodium hydroxide (10%) was added (200 µl) to each tube, which was then placed in a boiling water bath. The time required to develop color was recorded. The relative absorbance at 490 nm was determined for each of the fractions (Fieser and Williamson, 1987).

5. Phosphate Test

The phosphate test determines the presence of teichoic acid in a sample. Several column fractions were pooled, 200 µl of each pool were diluted with 0.5 ml of 2 M HCl in a test tube. The samples were then placed in a water bath for 30 minutes. Distilled water (4 ml) was added to each tube. Reagent C (4 ml) was added to each tube. Reagent C consisted of 20 ml of 6 N sulfuric acid with 40 ml of distilled water, 20 ml of 2.5% ammonium molybdate and 20 ml of ascorbic acid. The tubes were capped with parafilm and placed in a 37 ^{o}C incubator for 1.5 to 2 hours. Relative absorbance was observed in a spectrophotometer at 820 nm. The negative control was distilled water and the positive control was phosphate buffered saline (Chen et al., 1956).

6. Preparation of Bacterial Antigen for ELISA

The following method was adapted from Thakker et al. and Sinha et al. (Thakker et al.,1998 Sinha et al., 1999). *S. aureus* cells were grown in Columbia broth plus 2% NaCl overnight at 37 ^{o}C with shaking at 225 rpm. The cells were then treated with 10 ml of phosphate buffered saline (PBS) and centrifuged at 13,000 x *g* at 4 ^{o}C for 30 minutes. The pellet

was suspended in 10 ml of PBS containing 1% BSA. This procedure was repeated three times with the third containing PBS alone. The pellet was then treated with 3% formalin overnight at 4 ^{o}C and washed an additional three times with PBS with or without BSA, as described above. The pellet was then treated overnight with 1 mg/ml trypsin at 37 ^{o}C. The cells were then washed with PBS as described above. They were then brought up to an optical density of 1.0 at 550 nm. The cell suspension was added (100 µl) to a microtiter plate and incubated overnight at 37 ^{o}C. The plate was then centrifuged at 400 x *g* for 15 minutes.

7. Enzyme Linked Immunosorbent Assay (ELISA)

A modified procedure from Thakker et al. and Sinha et al. was implemented (Thakker et al.,1998 Sinha et al., 1999). Tissue culture 96 well plates were coated with 100 µl of capsular polysaccharide solution, water, or whole cell bacteria. The plate was then incubated overnight at 37 ^{o}C. Plates containing whole cells were centrifuged prior to the next step, as described above. Excess solution was removed through aspiration. Blocking buffer (200 µl of 1% bovine serum albumin in PBS) was added to each well and incubated at 22 ^{o}C for 30 minutes. The plates were then washed three times with wash buffer (PBS-containing 0.05% tween-20). Excess solution was aspirated off after each washing. Antibody diluted 1:100 to 1:1000 in sample buffer (PBS containing 0.1% BSA and 0.05% tween-20) was added to each well. The plate was then incubated at 37 ^{o}C for 2 hours. The plate was washed with wash buffer

three times. Anti-mouse immunoglobulin conjugated to peroxidase was diluted at 1:1000 in 100 µl and added to each well. The plate was then incubated for 2.5 hours at 37 ^{o}C. The substrate, tetramethylbenzidine solution (100 µl) was added to each well and the plate was incubated at 22 ^{o}C until the positive control was blue. The reaction was stopped with 50 µl of 2 N H_2SO_4 and the absorbance at 450 nm was recorded for each well.

8. Bradford Assay

A Bradford assay was used to determine protein concentration. A standard curve was first generated using 1.2, 2.4, 3, 6, 8, and 10 µg/ ml concentrations made from stock bovine serum albumin (BSA). Each of these six concentrations were individually added in 800 µl quantities and mixed with 200 µl of Bradford reagent in a test tube. Similarly, the pooled carbohydrate fractions after separation by S-300 column chromatography were added in 800 µl quantities to 200 µl of Bradford reagent. After 10 minutes, the absorbencies of all samples were then recorded at 595 nm using a spectrophotometer (Bradford, 1976).

9. DNA Test

The concentration of DNA in each sample was quantified using UV absorbance at 260 nm based on the method of Kabat and Mayer (Kabat and Mayer 1961). A standard 50 µg/ml solution of DNA had an optical density of 1.0 at 260 nm using a UV spectrophotometer. The DNA concentration of each of the pools was determined by comparing their absorbance at 260 nm relative to the DNA standard solution.

10. Sample Preparation for Nuclear Magnetic Resonance Spectroscopy (NMR)

The sample was prepared in a method similar to Jones (Jones, 2005). The purified capsule was first dialyzed three times with sterile water. The sample was then lyophilized. About 2 mg of polysaccharide was deuterium exchanged three times by lyophilization with deuterated water. The sample was then resuspended in 0.35 ml of deuterated water in an NMR tube for analysis.

11. Sonication Procedure

The sample was placed in a sterile 125 ml Erlenmeyer flask and dissolved in 10 ml of 0.05 M sodium acetate. The sample was then sonicated continuously for 72 hours.

12. Lysozyme Digestion

A lyophilized, 5mg sample of the bacteria was dissolved in 20 ml of 0.85% NaCl buffer. A total of 60 mg of lysozyme was then added to obtain a concentration of 3 mg of lysozyme per 1 ml of buffer. The sample was then incubated at 37 ^{o}C and shaken for two hours at 200 rpm (Kern et al. 1957).

RESULTS

Monoclonal antibodies produced in the Fagan lab have shown reactivity to whole cell bacteria, but further studies with purified capsule are needed to confirm its identity as the specific epitope the antibody binds to. Strain Rosenbach *S. aureus* cells (0.5 g) were grown in Columbia broth and subjected to enzymatic digestion with DNAse, RNAse and pronase. These enzymes degraded DNA, RNA and protein, respectively. Next, the sample was separated on a DEAE column in order to purify the negatively charged carbohydrates. The sample was equilibrated with 0.05 M sodium acetate with 0.05 M sodium chloride. The salt concentration was then increased in the eluting buffer to 0.05 M sodium acetate with 0.15 M sodium chloride, which eluted the bound carbohydrate. In order to test if the capsular polysaccharide eluted from the column, a red tetrazolium test was conducted. This test determines the presence of reducing sugars; a positive absorbance would therefore indicate the presence of the carbohydrate capsule. Eluted 6 ml fractions of sample were collected as a result of chromatographic separation. Overall, there were 80 fractions collected and tested, which were plotted against their respective absorbance at 490 nm in Figure 10. The fractions showed a cyclical increase and decrease in absorbance approximately every 10 fractions. These peaks were pooled together in the following fashion: fractions 1-10 in pool 1, 11-22 in pool 2, 23-33 in pool 3, 34-45 in pool 4, 46-57 in pool 5, 58-68 in pool 6, 69-80 in pool 7. As the number of fractions collected increased, so did the absorbance, up until pool 4, at which point the absorbance decreased. The corresponding fluctuation in absorbance reflects the amount of reducing carbohydrate in each pool.

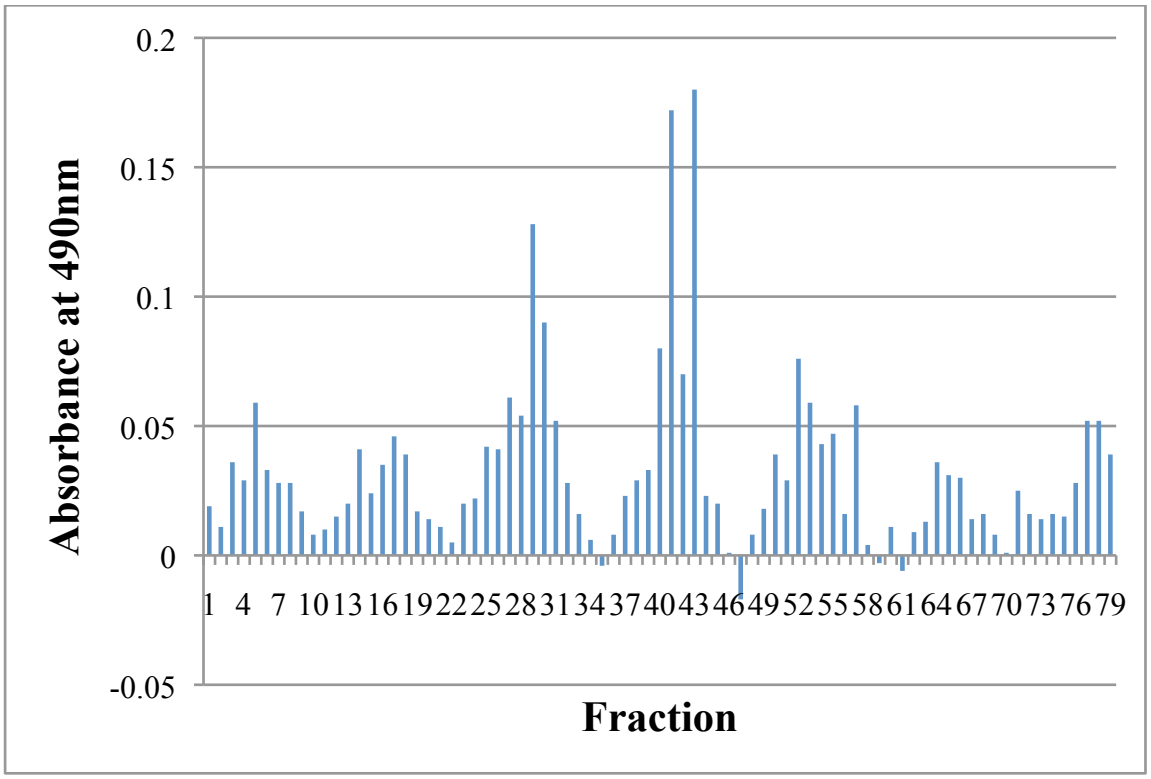


Figure 10: Red Tetrazolium Test of Rosenbach Carbohydrate after Purification with DEAE Column Chromatography: Strain Rosenbach *S.aureus* was treated with DNAse, RNAse and pronase and centrifuged. The sample was dialyzed, centrifuged and filter sterilized. The precipitate was resuspended and teichoic acid removed using sodium periodate. The remaining powder was dissolved in 0.05M sodium acetate + 0.01M NaCl at a pH of 6.0. The sample was then added to a DEAE sephadex column. The sample was then eluted using 0.05 M sodium acetate + 0.15 M NaCl at pH 6.0. Approximately 7 ml fractions were then collected and combined with 0.5% red tetrazolium. Fractions were then pooled together based on similarity, approximately every 10 fractions.

In order to ensure that all carbohydrate had been eluted through the column, additional fractions were collected. These fractions are illustrated in Figure 11 and show some positive absorbance across the 69 fraction span. The absorbance and subsequent amount of carbohydrate was deemed irrelevant and was therefore not included in the remainder of the study. Similarly, pool 7 was discarded due to lack of significant presence of reducing carbohydrate. Next, the remaining 6 pools were tested for the presence of phosphate. This test was conducted because a phosphate containing sugar, teichoic acid, is associated with the outer structure of *S. aureus* and may potentially remain affiliated with the capsular polysaccharide. Each of the pools was combined with reagent C and their absorbance was detected at 820 nm. A positive absorbance indicates the presence of phosphate, which will be found in teichoic acid. In Figure 12 only the positive control, phosphate buffered saline, shows a positive absorbance at 820 nm, which was expected. Pools 1-6 showed a negligible amount of phosphate indicating that they are relatively free of teichoic acid contamination.

An ELISA was conducted in order to confirm the presence of capsular polysaccharide in the pools (Figure 13). The primary antibody used in the assay was antiserum (αT5) which showed reactivity against type 5 *S. aureus*. A positive absorbance indicates that the antibody was reacting to an antigen present in the sample, most likely the capsular polysaccharide. The absorbencies of the 6 pools at 450 nm are shown in Figure 13, along with the absorbance of the positive control consisting of whole type 5 bacteria. All of the 6 pools showed reactivity to the antibody. The most significant reactivity was observed in pools 3, 4, 5 and 6 which had higher absorbencies than the

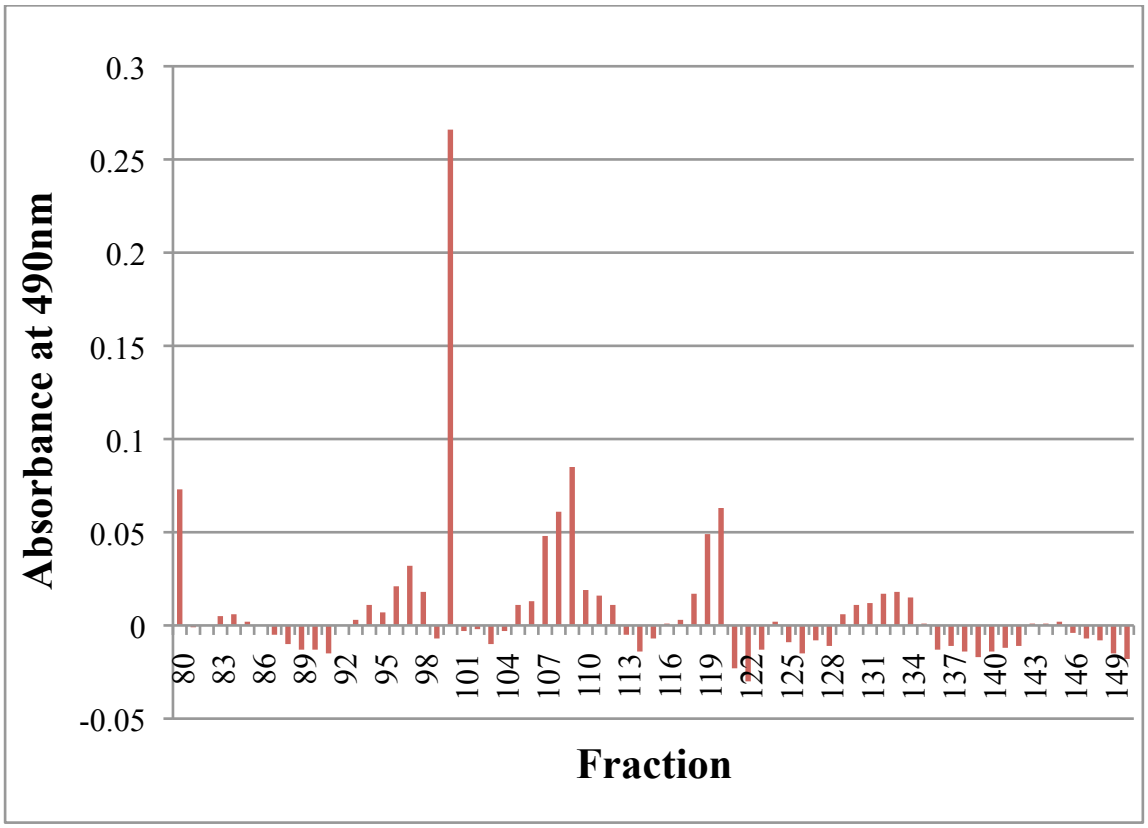


Figure 11: Additional Red Tetrazolium Test of Rosenbach Carbohydrate after Purification with DEAE Column Chromatography: The bacteria were prepared and analyzed as described by Figure 10.

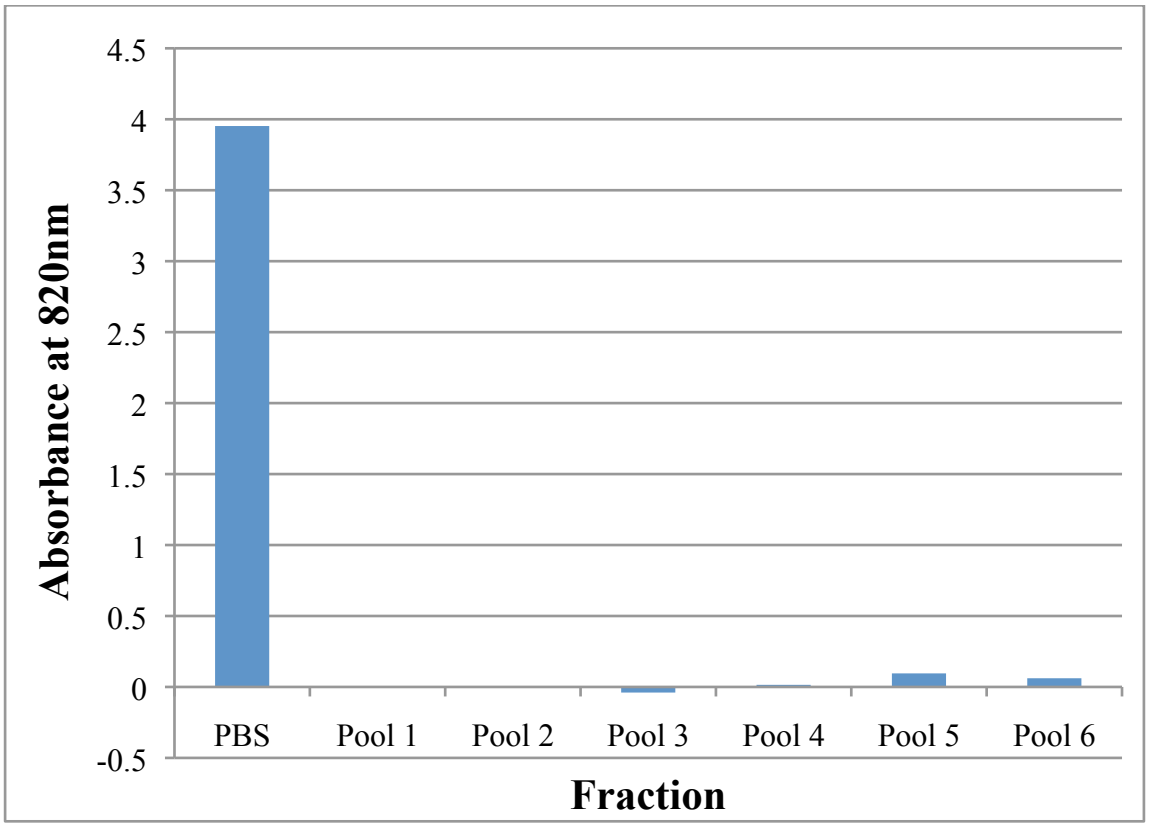


Figure 12: Phosphate Test of Fractions from Rosenbach Carbohydrate after DEAE Column Chromatography: The phosphate test was administered to ensure that there was no teichoic acid in the purified sample. Reagent C was mixed with each of pools and the absorbance at 820 nm was recorded. Phosphate buffered saline was used as a positive control and distilled water was used as a negative control.

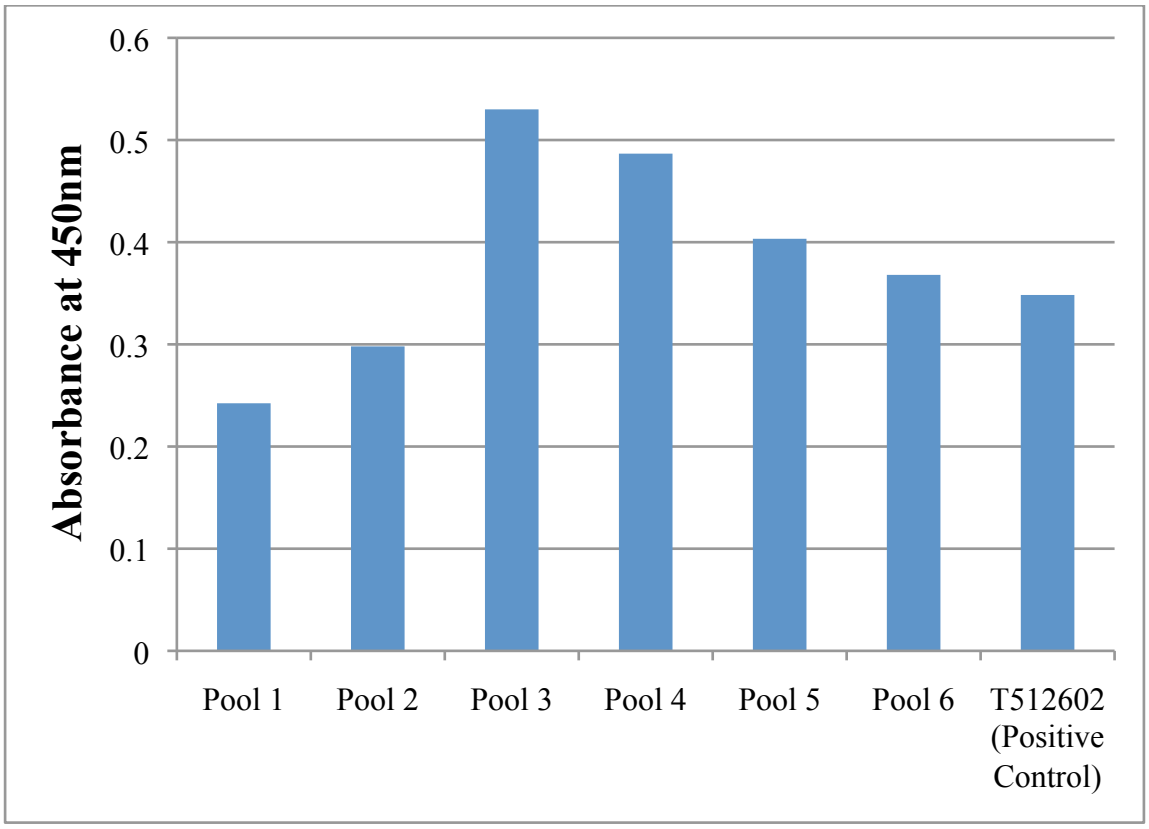


Figure 13: Enzyme Linked Immunosorbant Assay (ELISA) of Purified Rosenbach Carbohydrate utilizing anti-type 5 *S. aureus* antibody (adsorbed with type 8 *S.aureus*): The pooled fractions were added to a 96 well plate along with whole Rosenbach bacteria which served as a positive control and distilled water which served as a blank. αT5 ads containing antibody was added as the primary antibody. Next, a secondary antibody (anti-mouse polyvalent immunoglobulin) conjugated to peroxidase was added and the absorbances were recorded at 450 nm.

positive control. These positive results indicate that each pool still has the target epitope that the antibodies react to.

The sample was further purified by size using an S-300 size exclusion column. In theory, the capsule should elute first because of its larger size compared to any smaller molecules which still may be contained within the sample. The sample was eluted from the column using a buffer containing 0.05 M sodium acetate at pH 6 and 7 ml fractions were collected. A red tetrazolium test was then performed to detect reducing sugars on each of the fractions. In Figure 14, the fractions show the same cyclical peaking pattern as was observed in Figure 10. These fractions were then pooled together as follows: 1-10 in pool 1, 11-20 in pool 2, 21-33 in pool 3, 34-38 in pool 4, 39-46 in pool 5, 47-58 in pool 6, 59-70 in pool 7, 71-83 in pool 8, 84- 94 in pool 9, 95-107 in pool 10 and 108-143 in pool 11. The highest absorbance was seen in the first 4 pooled fractions indicating the most reducing carbohydrate present.

Each of the 11 pools obtained were tested with an ELISA to determine reactivity with anti-type 5 capsular antibodies. A positive result indicated by a significant absorbance at 450 nm reflects capsular polysaccharide in the sample. The antibody used in the assay was monoclonal antibody MS.T8.B2.G2.F11, which showed binding to S. *aureus* type 5, the positive control. Figure 15 shows the absorbance of each of the pools at 450 nm. In Figure 15, pools 1-7 and 10-11 show significant reactivity against the anti-type 5 antibodies. These positive results indicate the presence of the capsular antigen. Pools 8 and 9 do not show reactivity indicating a lack of any antigen in the sample.

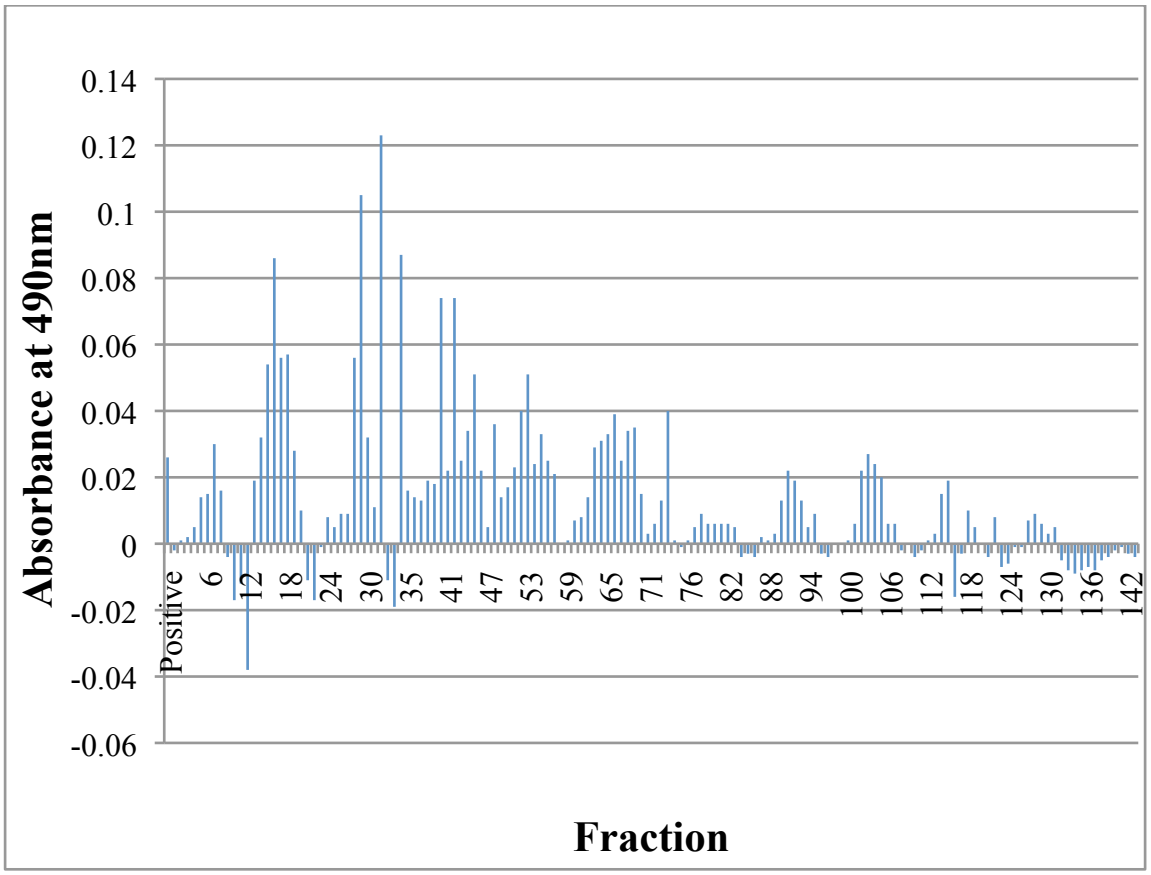


Figure 14: Red Tetrazolium Test of Rosenbach Carbohydrate after Purification with S-300 Column Chromatography: All of the fractions were combined and added to the column which were eluted using 0.05M sodium acetate at pH 6. Approximately 7 ml fractions of the resultant eluate were collected and tested with red tetrazolium.

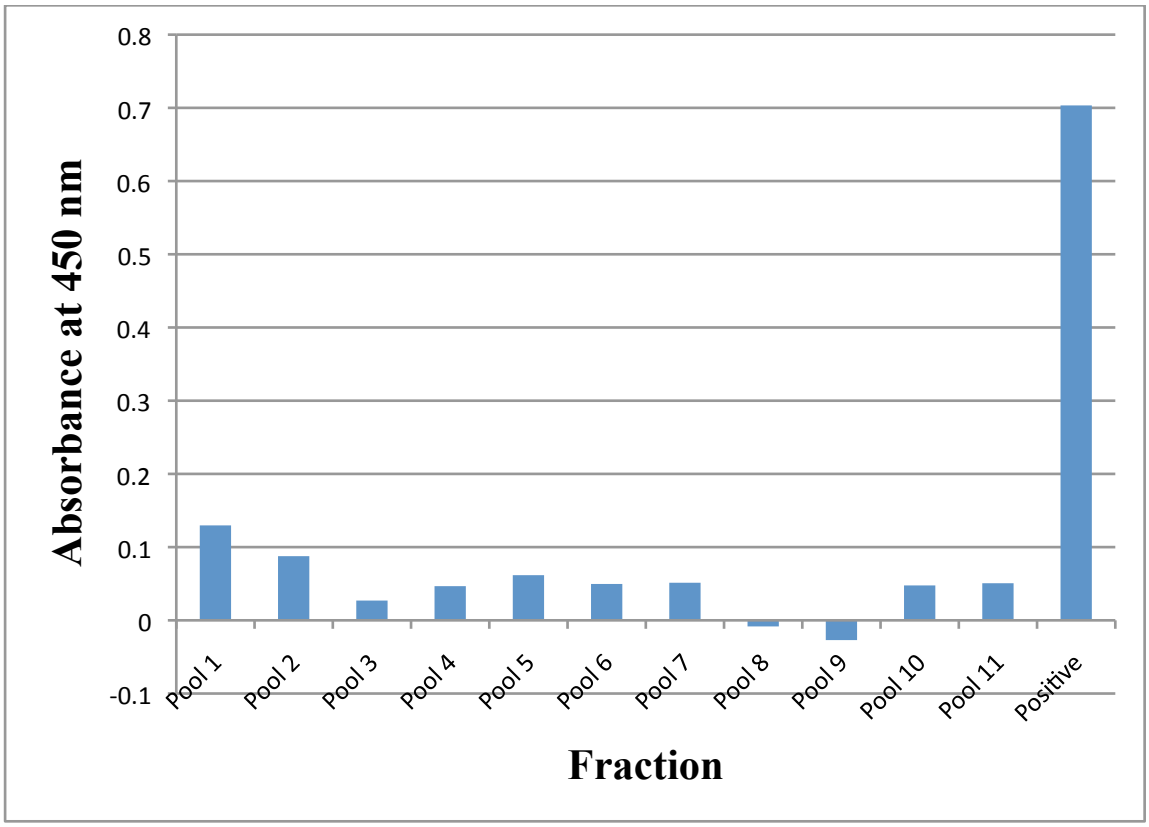


Figure 15: ELISA of Purified Rosenbach Carbohydrate after Purification with S-300 Column Chromatography: The ELISA on pooled carbohydrate fractions was performed as described by Figure 13 with slight modification, the primary antibody utilized was MS.T8.B2.G2.F11, a monoclonal antibody against type 8 *S. aureus*.

The sample was further tested for the presence of teichoic acid via a phosphate test. All 11 pools were combined with reagent C which produces an absorbance at 820 nm in the presence of phosphates. The results are shown in Figure 16 which used PBS as a positive control. None of the 11 pooled fractions showed any significant absorbance. This indicates that there is insignificant phosphate in any of the pooled fractions.

Next, pools 1-6 were chosen for further analysis, as they contained significant amounts of carbohydrate as evidenced by the red tetrazolium test and the ELISA. Pools 1-6 were tested for purity by testing for the presence of DNA and protein. In order to test for protein, a Bradford assay was performed using bovine serum albumin as a positive control. To each of the 6 pools, Bradford reagent was added and the absorbance was recorded at 595 nm. In the presence of protein a positive absorbance is observed. Using the standard curve generated from the bovine serum albumin, the relative concentration of protein in each pool was determined. Based on this relative concentration and the overall mass of the sample, the percentage of protein in the sample could be expressed. In Figure 17, the percentage of protein in the sample is indicated by the red bar. The highest protein percentages, 0.55% and 0.57% were found in pools 2 and 3 respectively. Pool 1 and the higher numbered pools, 4, 5, and 6 show lower protein percentages. Overall the percentages of protein, less than 0.60% was insignificant when considering the purity of the sample. In order to test for DNA, the absorbance of the sample was recorded at 260 nm. Based on standard data, the relative concentration of DNA could be estimated and expressed as a percentage of the overall mass of the pool. The percentage of DNA in each pool is represented by the yellow bar in Figure 17.

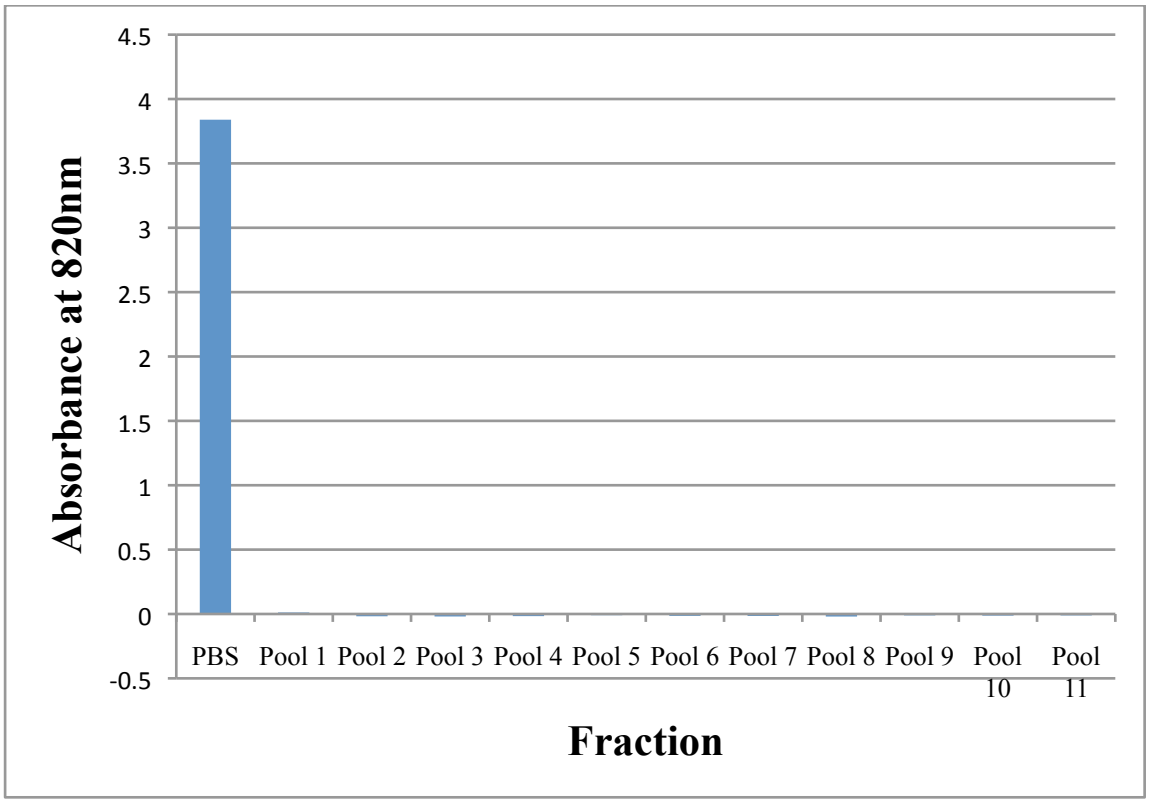


Figure 16: Phosphate Test of Purified Rosenbach Carbohydrate after S-300 Column Chromatography: A phosphate test was performed on the carbohydrate pools formed as a result of S-300 size exclusion chromatography. The method for this test was described in Figure 12.

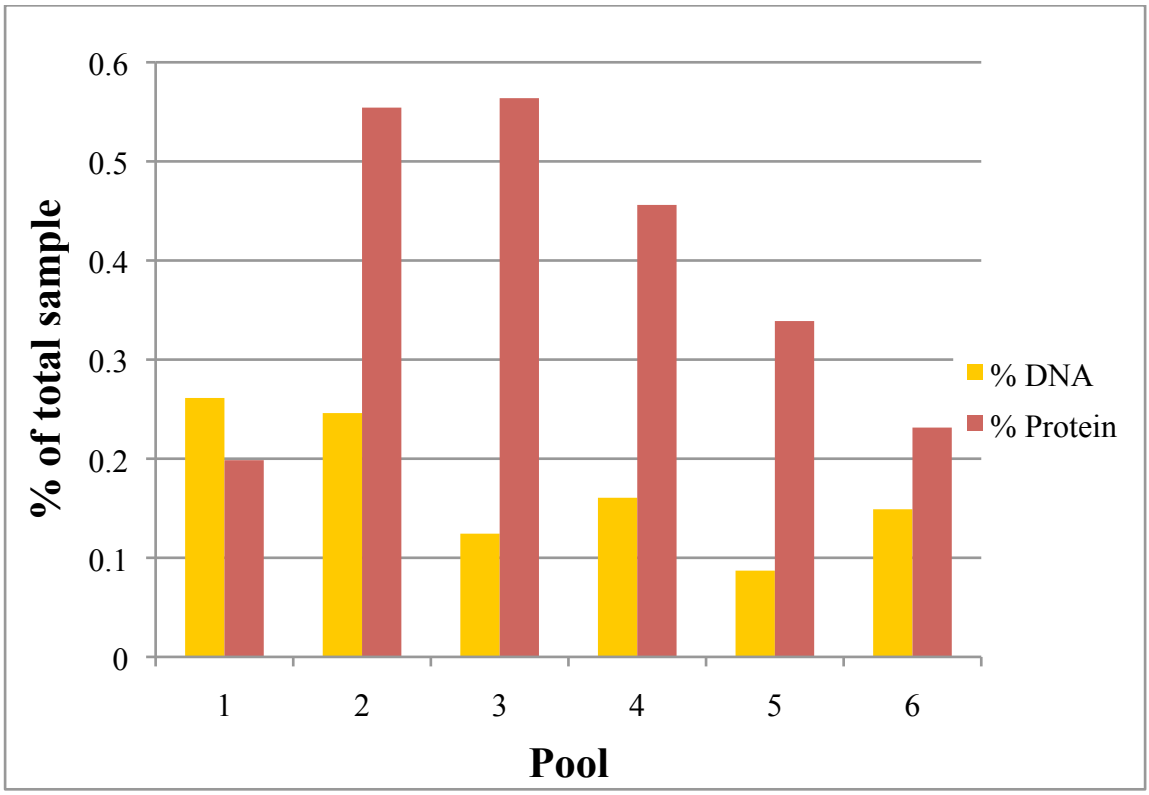


Figure 17: DNA and Protein Concentrations in Purified Rosenbach Carbohydrate after DEAE and S-300 Chromatography: A Bradford assay was then conducted on each of the fractions to test for the presence of proteins. The amount of DNA was determined by observance of sample absorbance at 260 nm. Each concentration of DNA and protein was expressed as a percentage by dividing it by the overall mass of sample the respective fraction.

The highest amount of DNA was found in the earlier pools, such as pool 1 with 0.26% DNA. The percentages of DNA are so small that they are insignificant in regards to contamination of the overall sample. As evidenced by Figure 17, there is an insignificant amount of both DNA and protein in pools 1-6.

Pool 3 from Figure 14 was studied further because it showed the strongest absorbance at 490 nm of any of the peaks. Approximately 3.8 mg of carbohydrate was obtained from pool 3 which was analyzed using NMR spectroscopy. Figure 18 shows the results of a ^{1}H NMR spectrum conducted on pool 3. Noticeable carbohydrate was not observed due to the presence of a strong water peak with a shift of approximately 5 ppm. Carbohydrate may be present in the sample, but characterization was inconclusive due to the contamination by water potentially masking peaks attributed to capsule.

The sample was then further separated in order to obtain more resolvable data via NMR spectroscopy. Additional chromatography alone was deemed ineffective in further separating the carbohydrate. All fractions were therefore pooled together and then sonicated for a total of 72 hours. Sonication was performed to break carbohydrate polymers into smaller monomer units. It was hoped that this would allow elution of the sample into one peak containing molecules with a more homogenous composition necessary for the NMR analysis. The sample was then subjected to size separation via an S-300 column to isolate aforementioned smaller units. The fractions eluted through the column were then subjected to a red tetrazolium test. Figure 19 shows the results of the red tetrazolium test of the 135 fractions eluted from the column. A similar cyclical pattern was observed as a result of the test as was seen from Figures 5 and 1. The highest

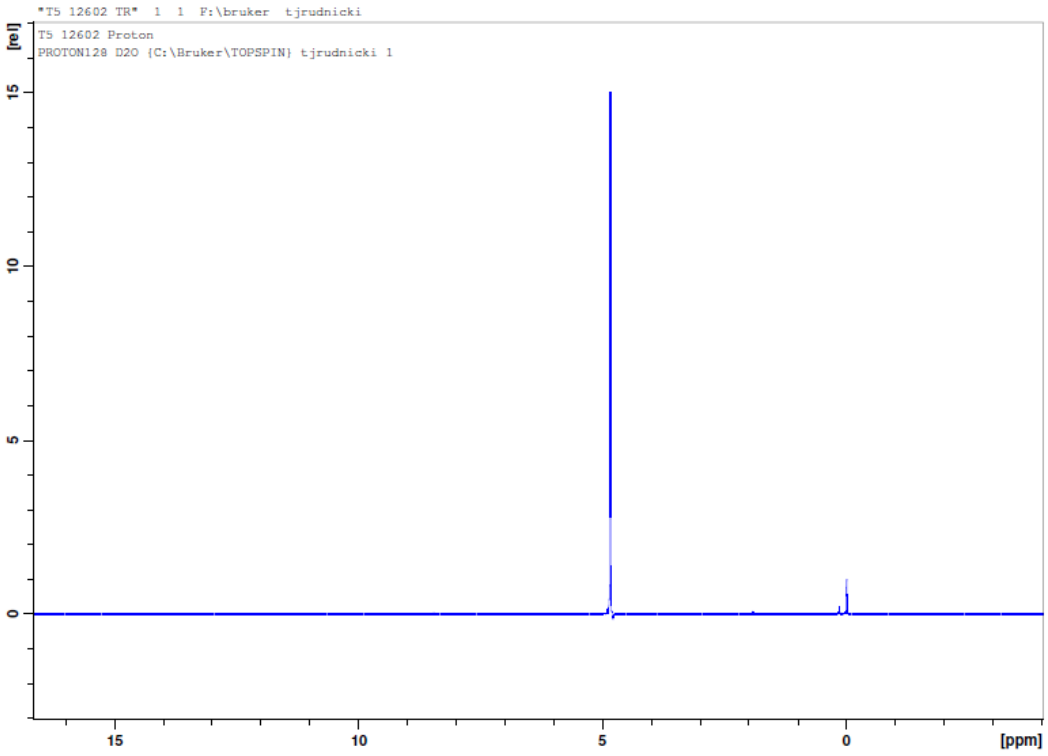


Figure 18: Proton NMR of Purified Rosenbach Carbohydrate: Purified carbohydrate obtained as described in Figure 14 was dialyzed using Deuterium exchanged water. The sample (3.8 mg) was then lyophilized and mixed with deuterium exchanged water. An H^1 proton NMR analysis was then performed using a Bruker Advance II 400 MHz spectrophotometer with an indirect detection probe.

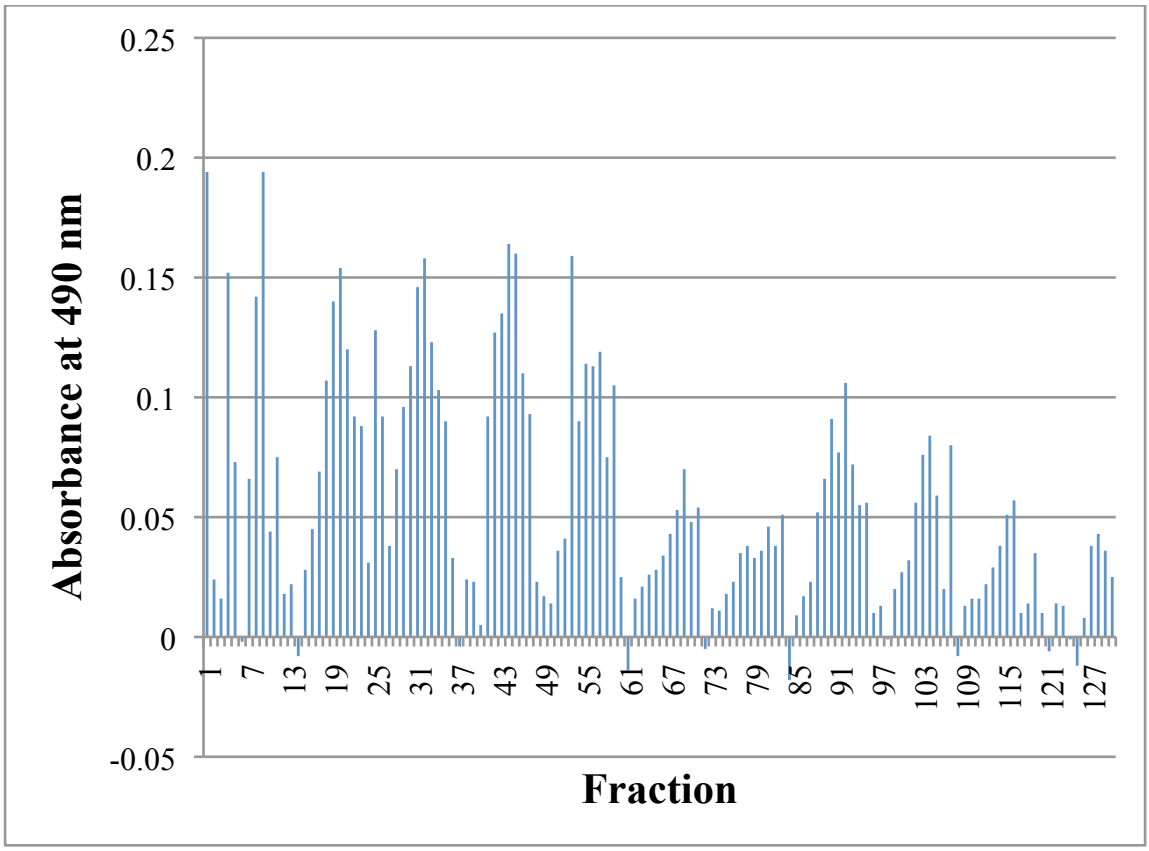


Figure 19: Red Tetrazolium Test of Purified Carbohydrate after Second S-300 Column Chromatography and Sample Sonication: Fractions were obtained as a result of size exclusion chromatography using an S-300 column similar to the method employed in Figure 14. The resultant fractions were combined into one vessel and were subjected to sonication for 72 hours. After sonication, the carbohydrate sample was then eluted through the S-300 column a second time utilizing the method described in Figure 14.

absorbing peaks notably came off first from the column indicating the most reducing sugar. As fraction number increased, the absorbance in the group peak decreased indicative of less reducing sugar.

The fractions were then pooled together and treated with the enzyme, lysozyme (3 mg/ ml) to generate more resolvable NMR data. After incubation with the enzyme, the solution was dialyzed as previously described. Next, the sample was separated on an S-300 column in order to validate the efficacy of the digestion. A red tetrazolium test for reducing sugars was then performed and is shown in Figure 20. The same cyclical pattern was observed with four elution peaks; fractions 1-24 in pool 1, 25-35 in pool 2, 36-48 in pool 3 and 49-59 in pool 4. There was a decrease in the amount of reducing carbohydrate as more fractions were collected. Pool 1 had the most carbohydrate present whereas pool 4 had the least amount.

Testing of the glycomimetic TC-I-019 was performed using a whole cell ELISA. The ELISA utilized primary antibodies which reacted against type 5 bacteria (MS.T8.B2.G2.G2). High absorbance at 450 nm would therefore correspond to more of the antibody antigen, the capsular polysaccharide. An effective chemical would show decreased absorbance at 450 nm when compared to the positive control. This trend would indicate that the chemical could successfully halt or reduce the synthesis of functional capsule, resulting in reduced antibody reactivity. Type 5 bacteria, T5 49521 were treated with various concentrations of TC-I-019 (1 μg/ml, 0.1 μg/ml, and 0.01 μg/ml). The absorbance of each of the concentrations, along with a methanol control was recorded at 450 nm and was represented in Figure 21. As the chemical and the control were diluted by 1:2 serial dilutions less reactivity was seen, as expected. When the four

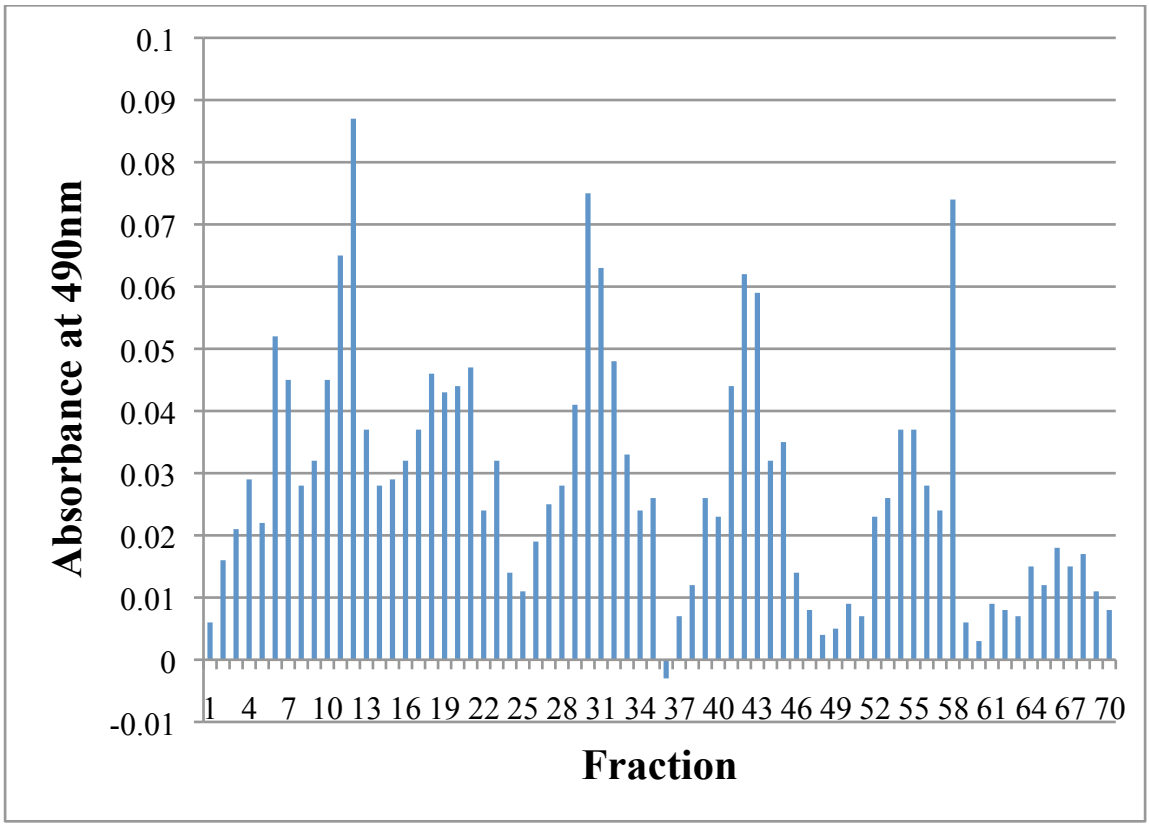


Figure 20: Red Tetrazolium Test of Purified Carbohydrate after Lysozyme Digestion and Third S-300 Column Chromatograhpy: The fractions collected as a result of the sonication and S-300 separation were consolidated into one pool. The sample was dissolved in 0.85% NaCl buffer containing lysozyme. The sample was then loaded onto the column using 0.05 M sodium acetate pH=6 and eluted using the same buffer.

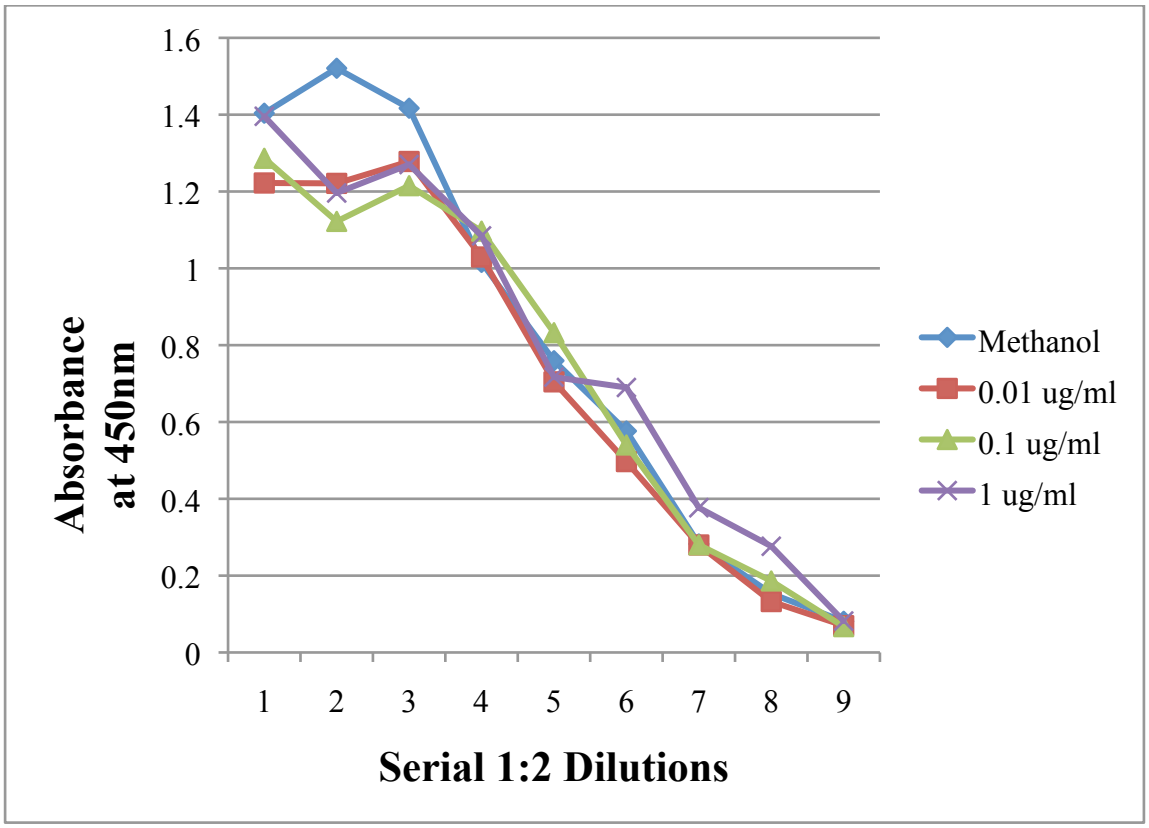


Figure 21: ELISA of Rosenbach *S.aureus* treated with TC-I-019: *S. aureus* strain Rosenbach was treated with various concentrations of TC-I-019 (1 µg/ml, 0.1 µg/ml and 0.01 µg/ml) along with methanol as a negative control. Primary antibody, MS.T8.B2.G2.G2 was 1:2 serial diluted in each of the wells. The secondary antibody, peroxidase conjugated anti-mouse immunoglobulin was also added and the absorbance was recorded at 450 nm.

conditions (with and without treatment with the mimetic) were compared, there was no change among the samples incubated with various concentrations of the chemical. No reproducible difference between the capsule production or reactivity with the antibody was seen in the treated and untreated cells. The data indicates that the chemical, TC-I-019, was ineffective at preventing capsule synthesis.

DISCUSSION

The *S. aureus* infections have devastated the medical community by necessitating the reevaluation of clinical procedure and application of pharmaceuticals. Resistance to β lactam antibiotics and vancomycin has created a vacuum in *S. aureus* therapies that can potentially be filled by monoclonal antibody and glycomimetic therapy. In a 7.5 year study conducted by Wisplinghoff and coworkers, there was a 25% morbidity rate of individuals diagnosed with a nosocomial *S. aureus* bloodstream infection (Wisplinghoff et al., 2004). Alarmingly, over this same period there was a rise in total MRSA isolates from 22% to 57% (Wisplinghoff et al., 2005). These infections are difficult to treat because of an acquired antibiotic resistance and innovative treatments must therefore be produced. One possible treatment for *S. aureus* infections is the administration of monoclonal antibodies specific to type 5 capsular polysaccharide which establishes passive immunity. Monoclonal antibodies have already been developed by the Fagan group and additional testing is needed to confirm efficacy. Isolation and characterization of pure capsular polysaccharide will allow for testing of antibody specificity. A positive reaction between the monoclonal antibody and purified capsule will confirm that the capsular polysaccharide is the antigen rather than another surface epitope of *S. aureus*.

Strain Rosenbach bacteria was grown and enzymatically digested with various enzymes in order to degrade protein, nucleic acid, and to disrupt cell wall interactions. This procedure was conducted with pronase, DNAse, and lysozyme respectively. The negatively charged capsule was separated from other components by a DEAE column. Once separated, the eluent was tested for phosphate, reducing carbohydrate, and nucleic acid. These tests confirmed the exclusive presence of a reducing carbohydrate which still

reacted positively to bacteria specific antibodies via an ELISA. The sample was then subjected to sonication to degrade the carbohydrate polymer into smaller units to allow easier characterization. After dialysis and deuterium exchange through lyophilization, NMR was conducted to characterize the identity of the sample.

For purification, the procedure was modified from Tzianabos and coworkers (Tzianabos et al., 2001). This procedure utilized a two column approach derived from Fournier and coworkers, the DEAE column separated based on charge and the S-300 column separated based on size (Fournier et al. 1984). The main difference between this procedure and the methods previously employed by Fournier and coworkers was the use of the enzyme, pronase instead of lysostaphin (Fournier et al. 1987). Lee and coworkers have shown effective purification using pronase (a collection of proteinases) to degrade cell walls instead of the more costly, lysostaphin. Once the purification procedure was completed, NMR preparation was performed as suggested by Jones (Jones, 2005).

The NMR experiment conducted, ^{1}H was unable to generate any resolvable data. The only resolvable peak from the ^{1}H experiment exhibited by Figure 18 was a shift at approximately 5 ppm due to water. The results of the ^{1}H are unable to characterize the capsular polysaccharide because any peak appearance would be skewed by the strong presence of water at 5 ppm. Possible remedies to generate more resolvable data include: using increased concentration of the sample, using a 500 MHz NMR, and further sample digestion. Lee and coworkers used polysaccharide concentrations of 8 to 20 mg per 0.4 ml of D_2O solvent (Lee et al., 1987) compared to the 3.8 mg of polysaccharide employed in our studies. ^{1}H and ^{13}C spectra successfully characterizing the structure of capsular polysaccharides were also typically obtained at 500 MHz (Lee et al., 1987, Moreau et al.,

1990, Tzianabos et al., 2001, Jones, 2005). Limitations on the spectroscopic sensitivity of NMR facilities and the quanitity of purified capsule require additional enzymatic digestion of the sample to improve NMR data. Sample resolution may be more easily achieved by having a less complex sample. Digestion of the sample will reduce the amount of capsular polymers with different chain lengths and reduce the overall size of these polymers by interrupting glycosidic linkages.

When the purification procedure was executed, we did not see one elution peak as a result of S-300 separation. Instead, many cyclic peaks arose as evidenced by Figure 14. Previous work by other laboratories has shown one large peak eluting early (Fournier et al., 1984). This peak would exclusively contain the capsular polysaccharide trisaccharide. The elution of many peaks throughout fraction collection infers the presence of varying carbohydrate chain lengths. The preferred method for digesting the capsule into trisaccharide subunits is hydrogen fluoride solvolysis, a method not available at this facility (Lee et al., 1987). The usage of hydrogen fluoride solvolysis of polysaccharides showed increased sugar yields during purification (Sanger and Lamport, 1983). One possible method to reduce the carbohydrate size was the sonication of the sample (Szu et al., 1986). Sonication is the application of sound waves to disrupt molecular interactions. This disruption would break up the larger carbohydrate chains into smaller oligosaccharide units which could be resolved into one peak on a red tetrazolium test. Additionally, sonication can reduce broad line width spectra frequently encountered in carbohydrate NMR analysis. The sample was then sonicated for 72 hours and separated using an S-300 column. Figure 12 shows the subsequent results of the red

tetrazolium test and sonication did not significantly reduce the amount of peaks seen. Many cyclical peaks were still seen rather than a single elution peak.

The capsular polysaccharide may be forming aggregates, as it is associated with other cell wall structures such as peptidoglycan. Enzymatic degradation will often separate the capsule from other components of the cell wall. One possible cause for improper digestion was the omission of degradatory enzymes such as lysozyme and lysostaphin in the purification procedure. Lysozyme, an enzyme that degrades peptidoglycan in bacterial cell walls has been shown to successfully cleave purified bacterial capsule from *S.aureus* into smaller oligosaccharides by Wells in this laboratory (Unpublished data). The resultant degradation was evidenced by the appearance of only one eluting peak on a red tetrazolium test for reducing sugars. Lysozyme digestion of the sample was performed based on the method of Kern and coworkers (Kern et al., 1957). After digestion, the sample was eluted using an S-300 column and a red tetrazolium test was performed. The results of the test shown in Figure 20 indicate that lysozyme digestion was ineffective due to the continued presence of many cyclical peaks. These peaks represent many different sized capsular carbohydrates eluting through the column rather than one elution peak representing uniform polymers. The principle difference between the procedure executed by Wells and the one employed in this study was the use of two different strains of type 5 *S. aureus*. Wells used *S. aureus* subspecies Rosenbach designation Lowenstein whereas the bacteria used in this study were subspecies Rosenbach designation NCTC 6133. Unique structural differences between these two strains such as peptidoglycan composition may affect the affinity and efficacy of the

lysozyme enzyme. This phenomenon results in the successful cleavage of one strain while leaving the other strain intact.

Additional digestion of the bacterial structure with lysostaphin, which eliminates pentaglycine cross linkages may refine purification by separating the capsule from other structural elements (M. Huber and T. Huber, 1989). Studies conducted by Schneewind and coworkers show that lysostaphin digestion eliminates the anchor point for many surface proteins in *S. aureus* (Schneewind et al., 1995). Elimination of molecules associated with the capsule will make purification more straightforward. Methods utilized by Lee and coworkers incorporated digestion with pronase, lysostaphin, and lysozyme which collectively generated resolvable ^{1}H and ^{13}C NMR spectra (Lee et al., 1987).

Many purification procedures establish serological activity of purified sample with anti-capsular antibodies (Fournier et al., 1984, Lee et al., 1987 Tzianabos et al., 2001). A modification to the procedure from Lee and coworkers was the use of an ELISA rather than immunological techniques such as immunoprecipitation and bacterial agglutination (Lee et al., 1987). The ELISA was implemented because it is a more sensitive technique compared to double immunodiffusion (Chretien et al., 1994). Increased test sensitivity generates a more reliable validation of capsular polysaccharide in the purified sample.

Testing of glycomimetic compounds was also performed. The efficacy of these inhibitory molecules was tested using a whole bacteria ELISA (Thakker et al., 1998 Sinha et al.,1999). In Figure 12, the results of the ELISA indicate that the glycomimetic,

TC-I-019 did not significantly inhibit synthesis of capsular polysaccharide. This was evidenced by a negligible difference between the amount of capsule produced following incubation with various concentrations of the chemical and the negative control containing methanol. One possible future study might be the testing of the chemical at higher concentrations. More of the chemical interacting with the bacteria may provide an adequate amount mimetic substrate to cause enzymatic inhibition. Also, additional testing of other mimetics which resemble capsule biosynthetic substrates should be performed.

In summary, the purification process was unable to fully isolate the repeating trisaccharide unit in the capsule of type 5 S. aureus. The presence of many cyclical peaks following the red tetrazolium test indicates that the purification procedure generated many different sized carbohydrates. A 72 hour sonication of the sample failed to produce significant depolymerization of the sample as shown in Figure 10. Similarly, treatment with the digestive enzyme, lysozyme was unable to reduce the molecular size of the carbohydrate as evidenced by Figure 20. Additional scrutiny of purified capsule based on molecular size may be achieved by sample dilution into a smaller initial volume and using a taller column during size exclusion chromatography. Modifications to NMR analysis such as performing additional scans during the ^{1}H experiment while suppressing the water peak may also generate interpretable data. Alternatively, the high molecular weight carbohydrates obtained may be too complex for analysis using a 400 MHz ^{1}H spectra. Therefore, additional means can be considered, such as digestion with lysostaphin or solvolysis of the sample with anhydrous hydrogen fluoride. These techniques used together or alone should have a noticeable effect on carbohydrate size,

making analysis possible using a 400MHz NMR. Once NMR analysis is complete, the purified product can be fully characterized and confirmed as *S. aureus* type 5 capsule. The capsular product can then be utilized in immunological studies with monoclonal antibodies formed in the Fagan lab. Reactivity of these antibodies against pure capsule will confirm the hypothesized antigen-antibody reaction. Once this interaction is confirmed, anti-capsular antibodies can be studied in a clinical setting as a potential therapy for cantankerous MRSA infections.

REFERENCES

1. Arthur, M., F. Depardieu, L. Cabanie, P. Reynolds, and P. Courvalin. 1998. Requirement of the VanY and VanX D,D-peptidases for glycopeptide resistance in enterococci. *Molecular Microbiology* 30, (4): 819-30.
2. Bertozzi, C. R., and L. L. Kiessling. 2001. Chemical glycobiology. *Science* 291, (5512): 2357-64.
3. Bradford, M. M. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry* 72, (1-2): 248-54.
4. Campbell, K. M., A. F. Vaughn, K. L. Russell, B. Smith, D. L. Jimenez, C. P. Barrozo, J. R. Minarcik, N. F. Crum, and M. A. K. Ryan. 2004. Risk factors for community-associated methicillin-resistant *Staphylococcus aureus* infections in an outbreak of disease among military trainees in San Diego, California, in 2002. *Journal of Clinical Microbiology* 42, (9): 4050-3.
5. Casey, A. L., P. A. Lambert, and T. S. J. Elliott. 2007. Staphylococci. *International Journal of Antimicrobial Agents* 29: S23-32.
6. Chen, P.S., T. Toribara, H. Warner. 1956. Microdetermination of Phosphorus. *Analytical Chemistry* 28, (11):1756-1758.
7. Chretien, P., E. Soulie, C. Johanet, and N. Abuaf. 1994. Comparisons of double immunodiffusion, elisa, western-blot and cape blot for the detection of anti-ssa antibody - study of anti-ssa prevalence in systemic lupus-erythematosus. *Journal of Autoimmunity* 7 (3): 379-88.

8. Cui, L. Z., A. Iwamoto, J. Q. Lian, H. M. Neoh, T. Maruyama, Y. Horikawa, and K. Hiramatsu. 2006. Novel mechanism of antibiotic resistance originating in vancomycin-intermediate *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* 50, (2): 428-38.
9. Cunnion, K. M., J. C. Lee, and M. M. Frank. 2001. Capsule production and growth phase influence binding of complement to *Staphylococcus aureus*. *Infection and Immunity* 69, (11): 6796-803.
10. Dassy, B., T. Hogan, T. J. Foster, and J. M. Fournier. 1993. Involvement of the accessory gene regulator (agr) in expression of type-5 capsular polysaccharide by *Staphylococcus-aureus*. *Journal of General Microbiology* 139,: 1301-6.
11. Desbois, A. P., Gemmell, C.G and P.J. Coote. 2010. *In vivo* efficacy of the antimicrobial peptide ranalexin in combination with the endopeptidase lysostaphin against wound and systemic methicillin-resistant *Staphylococcus aureus* (MRSA) infections. *International Journal of Antimicrobial Agents* 35, (6): 559-65.
12. Enright, M. C., N. P. J. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt. 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *Journal of Clinical Microbiology* 38, (3): 1008-15.
13. Fattom, A. I., J. Sarwar, A. Ortiz, and R. Naso. 1996. A *Staphylococcus aureus* capsular polysaccharide (CP) vaccine and CP-specific antibodies protect mice against bacterial challenge. *Infection and Immunity* 64, (5): 1659-65.

14. Fattom, A. I., G. Horwith, S. Fuller, M. Propst, and R. Naso. 2004. Development of StaphVAX (TM), a polysaccharide conjugate vaccine against *Staphylococcus-aureus* infection: From the lab bench to phase III clinical trials. *Vaccine* 22, (7): 880-7.

15. Feiser, L. and K. Williamson. (1987). *Organic Experiments.* Lexington: D.C. Heath and Company.

16. Fischetti, V.A.,"Surface Proteins on Gram-Positive Bacteria," *Gram-Positive Pathogens*. 2000, 11-24.

17. Fournier, J. M., W. F. Vann, and W. W. Karakawa. 1984. Purification and characterization of *Staphylococcus-aureus* type-8 capsular polysaccharide. *Infection and Immunity* 45 (1): 87-93.

18. Fournier, J. M., K. Hannon, M. Moreau, W. W. Karakawa, and W. F. Vann. 1987. Isolation of type-5 capsular polysaccharide from *Staphylococcus-aureus*. *Annales De L Institut Pasteur-Microbiologie* 138 (5): 561-7.

19. Gotz, F. 2004. Staphylococci in colonization and disease: Prospective targets for drugs and vaccines. *Current Opinion in Microbiology* 7, (5): 477-87.

20. Groom, A. V., D. H. Wolsey, T. S. Naimi, K. Smith, S. Johnson, D. Boxrud, K. A. Moore, and J. E. Cheek. 2001. Community-acquired methicillin-resistant *Staphylococcus aureus* in a rural American Indian community. *Journal of the American Medical Association* 286, (10): 1201-5.

21. Herold, B. C., L. C. Immergluck, M. C. Maranan, D. S. Lauderdale, R. E. Gaskin, S. Boyle-Vavra, C. D. Leitch, and R. S. Daum. 1998. Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no

identified predisposing risk. *Journal of the American Medical Association* 279, (8): 593-8.

22. Hiramatsu, K. 2001. Vancomycin-resistant *Staphylococcus aureus*: A new model of antibiotic resistance. *Lancet Infect. Dis.* 1, (3): 147-55.
23. Hiramatsu, K., L. Cui, M. Kuroda, and T. Ito. 2001. The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. *Trends in Microbiology* 9, (10): 486-93.
24. Hochkeppel, H. K., D. G. Braun, W. Vischer, A. Imm, S. Sutter, U. Staeubli, R. Guggenheim, E. L. Kaplan, A. Boutonnier, and J. M. Fournier. 1987. Serotyping and electron-microscopy studies of *Staphylococcus-aureus* clinical isolates with monoclonal-antibodies to capsular polysaccharide type-5 and type-8. *Journal of Clinical Microbiology* 25, (3): 526-30.
25. Holtfreter, S., Kolata, J., and B.M. Broeker. 2010. Towards the immune proteome of *Staphylococcus aureus* - the anti-*Staphylococcus aureus* antibody response. *International Journal of Medical Microbiology* 300, (2-3): 176-92.
26. Huber, M. M., and T. W. Huber. 1989. Susceptibility of methicillin-resistant *Staphylococcus-aureus* to lysostaphin. *Journal of Clinical Microbiology* 27, (5): 1122-4.
27. Ito, T., Y. Katayama, and K. Hiramatsu. 1999. Cloning and nucleotide sequence determination of the entire mec DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. *Antimicrobial Agents and Chemotherapy* 43, (6): 1449-58.

28. Jones, C. 2005. Revised structures for the capsular polysaccharides from *Staphylococcus aureus* types 5 and 8, components of novel glycoconjugate vaccines. *Carbohydrate Research* 340, (6): 1097-106.

29. Kabat, E.A. and M.M. Mayer. "Ultraviolet absorption spectra." *Experimental Immunochemistry*. 1961 708.

30. Karakawa, W. W., A. Sutton, R. Schneerson, A. Karpas, and W. F. Vann. 1988. Capsular antibodies induce type-specific phagocytosis of capsulated *Staphylococcus aureus* by human polymorphonuclear leukocytes. *Infection and Immunity* 56, (5): 1090-5.

31. Kern, R. A., M. J. Kingkade, S. F. Kern, and O. K. Behrens. 1951. Characterization of the action of lysozyme on *Staphylococcus -aureus* and on *Micrococcus -lysodeikticus*. *Journal of Bacteriology* 61, (2): 171-8.

32. Kiser, K. B., N. Bhasin, L. Y. Deng, and J. C. Lee. 1999. *Staphylococcus aureus* cap5P encodes a UDP-*N*-acetylglucosamine 2-epimerase with functional redundancy. *Journal of Bacteriology* 181, (16): 4818-24.

33. Klein, E., D. L. Smith, and R. Laxminarayan. 2007. Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999-2005. *Emerging Infectious Diseases* 13, (12): 1840-6.

34. Kneidinger, B., K. O'Riordan, J. J. Li, J. R. Brisson, J. C. Lee, and J. S. Lam. 2003. Three highly conserved proteins catalyze the conversion of UDP-*N*-acetyl-D-glucosamine to precursors for the biosynthesis of O antigen in *Pseudomonas aeruginosa* 011 and capsule in *Staphylococcus aureus* type 5. *Journal of Biological Chemistry* 278, (6): 3615-27.

35. Kokai-Kun, J., and J. J. Mond. 2004. Antibody therapy for treatment or prevention of infectious diseases. *Drug Discovery Today: Therapeutic Strategies* 1, (4): 475-81

36. Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L.Z., Oguchi, A., Aoki, K., Nagai, Y., Lian, J.Q., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N.K., Sawano, T., Inoue, R., Kaito, C.,Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H., Hiramatsu, K. 2001. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* 357, (9264): 1225-40.

37. Leclercq, R. 2009. Epidemiological and resistance issues in multidrug-resistant staphylococci and enterococci. *Clinical Microbiology and Infection* 15, (3): 224-31.

38. Lee, J. C., F. Michon, N. E. Perez, C. A. Hopkins, and G. B. Pier. 1987. Chemical characterization and immunogenicity of capsular polysaccharide isolated from mucoid *Staphylococcus-aureus*. *Infection and Immunity* 55 (9): 2191-7.

39. Lee, C.Y., J.C. Lee., "Staphylococcal Capsule," ASM Press, Washington D.C., 2000 1,361-366.

40. Lee, J.C., C.Y. Lee. *Capsular Polysaccharides of Staphylococcus aureus. In: Genetics of Bacterial Polysaccharides*. 1999. Boca Raton, Florida CRC Press.

41. Lee, J. C., J. S. Park, S. E. Shepherd, V. Carey, and A. Fattom. 1997. Protective efficacy of antibodies to the *Staphylococcus aureus* type 5 capsular polysaccharide in a modified model of endocarditis in rats. *Infection and Immunity* 65, (10): 4146-51.

42. Lim, D., and N. C. J. Strynadka. 2002. Structural basis for the beta-lactam resistance of PBP2a from methicillin-resistant *Staphylococcus aureus*. *Nature Structural Biology* 9, (11): 870-6.

43. Livermore, D. M. 2000. Antibiotic resistance in staphylococci. *International Journal of Antimicrobial Agents* 16: S3-S10.

44. Lopez, Z. K., A. Tejeda, R. M. Montesinos, I. Magana, and R. Guzman. 1997. Modeling column regeneration effects on ion-exchange chromatography. *Journal of Chromatography* 791 (1-2): 99-107.

45. Lowy, F. D. 1998. *Staphylococcus aureus* infections - reply. *New England Journal of Medicine* 339, (27): 2026-7.

46. Luong, T. T., S. W. Newell, and C. Y. Lee. 2003. Mgr, a novel global regulator in *Staphylococcus aureus*. *Journal of Bacteriology* 185, (13) (JUL): 3703-10.

47. Main, C.L., Jayaratne,P., Haley,A., Rutherford, C., Smaill, F., and D. N. Fisman. 2005. Outbreaks of infection caused by community-acquired methicillin-resistant *Staphylococcus aureus* in a Canadian correctional facility. *Can. J Infect. Dis. Med. Microbiol* 16, (6): 343-8.

48. Moreau, M., J. C. Richards, J. M. Fournier, R. A. Byrd, W. W. Karakawa, and W. F. Vann. 1990. Structure of the type-5 capsular polysaccharide of *Staphylococcus-aureus*. *Carbohydrate Research* 201, (2): 285-97.

49. Muller, B., C. Schaub, and R. R. Schmidt. 1998. Efficient sialyltransferase inhibitors based on transition-state analogues of the sialyl donor. *Angewandte Chemie-International Edition* 37, (20): 2893-7.

50. Nilsson, I. M., J. C. Lee, T. Bremell, C. Ryden, and A. Tarkowski. 1997. The role of staphylococcal polysaccharide microcapsule expression in septicemia and septic arthritis. *Infection and Immunity* 65, (10): 4216-21.

51. O'Riordan, K., and J. C. Lee. 2004. *Staphylococcus aureus* capsular polysaccharides. *Clinical Microbiology Reviews* 17, (1): 218-234.

52. Otto, M. 2008. Targeted immunotherapy for staphylococcal infections - focus on anti-MSCRAMM antibodies. *Biodrugs* 22, (1): 27-36.

53. Pereira, P. M., S. R. Filipe, A. Tomasz, and M. G. Pinho. 2007. Fluorescence ratio imaging microscopy shows decreased access of vancomycin to cell wall synthetic sites in vancomycin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* 51: 3627-33.

54. Perl, Trish M. 1999. The threat of vancomycin resistance. *American Journal of Medicine* 106, (5 Part A): 26S-37S.

55. Platt, F. M., G. R. Neises, G. Reinkensmeier, M. J. Townsend, V. H. Perry, R. L. Proia, B. Winchester, R. A. Dwek, and T. D. Butters. 1997. Prevention of lysosomal storage in tay-sachs mice treated with *N*-butyldeoxynojirimycin. *Science* 276 (5311): 428-31.

56. Pohlmann-Dietze, P., M. Ulrich, K. B. Kiser, G. Doring, J. C. Lee, J. M. Fournier, K. Botzenhart, and C. Wolz. 2000. Adherence of *Staphylococcus aureus* to endothelial cells: Influence of capsular polysaccharide, global

regulator agr, and bacterial growth phase. *Infection and Immunity* 68, (9): 4865-71.

57. Popovich, Kyle J., Bala Hota, and Robert A. Weinstein. 2008. Treatment of community-associated methicillin-resistant *Staphylococcus aureus*. *Curr. Infect. Dis. Rep.* 10, (5): 411-20.
58. Portoles, M., K. B. Kiser, N. Bhasin, K. H. N. Chan, and J. C. Lee. 2001. *Staphylococcus aureus* Cap50 has UDP-ManNAc dehydrogenase activity and is essential for capsule expression. *Infection and Immunity* 69, (2): 917-23.
59. Reynolds, P. E. 1989. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *European Journal of Clinical Microbiology & Infectious Diseases* 8, (11): 943-50,
60. Roche, F. M., R. Downer, F. Keane, P. Speziale, P. W. Park, and T. J. Foster. 2004. The N-terminal A domain of fibronectin-binding proteins A and B promotes adhesion of *Staphylococcus aureus* to elastin. *Journal of Biological Chemistry* 279, (37): 38433-40.
61. Rouch, D. A., M. E. Byrne, Y. C. Kong, and R. A. Skurray. 1987. The aaca-aphd gentamicin and kanamycin resistance determinant of Tn4001 from *Staphylococcus-aureus* - expression and nucleotide-sequence analysis. *Journal of General Microbiology* 133 : 3039-52.
62. Sanger, M. P., and D. T. A. Lamport. 1983. A microapparatus for liquid-hydrogen fluoride solvolysis - sugar and amino sugar composition of erysiphe-graminis and triticum-aestivum cell-walls. *Analytical Biochemistry* 128 (1): 66-70.

63. Sau, S., N. Bhasin, E. R. Wann, J. C. Lee, T. J. Foster, and C. Y. Lee. 1997. The *Staphylococcus aureus* allelic genetic loci for serotype 5 and 8 capsule expression contain the type-specific genes flanked by common genes. *Microbiology-Uk* 143, : 2395-405.

64. Sauvage, E., F. Kerff, M. Terrak, J. A. Ayala, and P. Charlier. 2008. The penicillin-binding proteins: Structure and role in peptidoglycan biosynthesis. *FEMS Microbiology Reviews* 32, (2): 234-58.

65. Schaffer, A. C., and J. C. Lee. 2008. Vaccination and passive immunisation against *Staphylococcus aureus*. *International Journal of Antimicrobial Agents* 32, : S71-8.

66. Schneewind, O., A. Fowler, and K. F. Faull. 1995. Structure of the cell-wall anchor of surface-proteins in *Staphylococcus-aureus*. *Science* 268 (5207): 103-6.

67. Shinefield, H., S. Black, A. Fattom, G. Horwith, S. Rasgon, J. Ordonez, H. Yeoh, et al. 2002. Use of a *Staphylococcus aureus* conjugate vaccine in patients receiving hemodialysis. *New England Journal of Medicine* 346, (7): 491-6.

68. Sinha, P., J. Sengupta, and P. K. Ray. 1999. Functional mimicry of protein A of *Staphylococcus aureus* by a proteolytically cleaved fragment. *Biochemical and Biophysical Research Communications* 260 (1): 111-6.

69. Soell, M., M. Diab, G. Haanarchipoff, A. Beretz, C. Herbelin, B. Poutrel, and J. P. Klein. 1995. Capsular polysaccharide type-5 and type-8 of *Staphylococcus-aureus* bind specifically to human epithelial (kb) cells,

endothelial cells, and monocytes and induce release of cytokines. *Infection and Immunity* 63, (4): 1380-6.

70. Szu, S. C., G. Zon, R. Schneerson, and J. B. Robbins. 1986. Ultrasonic irradiation of bacterial polysaccharides - characterization of the depolymerized products and some applications of the process. *Carbohydrate Research* 152 : 7-20.

71. Tenover, F.C, and R, Gaynes. 2000. "The epidemiology of *Staphylococcus* Infections." *Gram-Positive Pathogens*. 414-421.

72. Thakker, M., J. S. Park, V. Carey, and J. C. Lee. 1998. *Staphylococcus aureus* serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. *Infection and Immunity* 66, (11): 5183-9.

73. Tobin-D'Angelo, M., Arnold, K., Lance-Parker, S., and M. LaMarre . 2003. Methicillin-resistant *Staphylococcus aureus* infections in correctional facilities: Georgia, California, and Texas, 2001-2003. *Morbidity and Mortality Weekly Report* 52, (41): 992-6.

74. Tomasz, A., "Staphylococcal Cell Wall." *Gram-Positive Pathogens*. 2000, 351-360.

75. Tzianabos, A. O., J. Y. Wang, and J. C. Lee. 2001. Structural rationale for the modulation of abscess formation by *Staphylococcus aureus* capsular polysaccharides. *Proceedings of the National Academy of Sciences of the United States of America* 98, (16): 9365-70.

76. Vernachio, J., A. S. Bayer, T. Le, Y. L. Chai, B. Prater, A. Schneider, B. Ames, P. Syribeys, J. Robbins, and J. M. Patti. 2003. Anti-clumping factor A immunoglobulin reduces the duration of methicillin-resistant *Staphylococcus aureus* bacteremia in an experimental model of infective endocarditis. *Antimicrobial Agents and Chemotherapy* 47, (11): 3400-6.

77. von Eiff, C., K. L. Taylor, A. Mellmann, A. I. Fattom, A. W. Friedrich, G. Peters, and K. Becker. 2007. Distribution of capsular and surface polysaccharide serotypes of *Staphylococcus aureus*. *Diagnostic Microbiology and Infectious Disease* 58: 297-302.

78. Watts, A., D. B. Ke, Q. Wang, A. Pillay, A. Nicholson-Weller, and J. C. Lee. 2005. *Staphylococcus aureus* strains that express serotype 5 or serotype 8 capsular polysaccharides differ in virulence. *Infection and Immunity* 73, (6): 3502-11.

79. Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond. 2004. Nosocomial bloodstream infections in US hospitals: Analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical Infectious Diseases* 39, (3): 309-17.

80. Wright, G. D. 2007. The antibiotic resistome: The nexus of chemical and genetic diversity. *Nature Reviews Microbiology* 5, : 175-86.

81. Zapun, A., C. Contreras-Martel, and T. Vernet. 2008. Penicillin-binding proteins and beta-lactam resistance. *FEMS Microbiology Reviews* 32, (2): 361-85.

82. Zetola, N., J. S. Francis, E. L. Nuermberger, and W. R. Bishai. 2005. Community-acquired meticillin-resistant *Staphylococcus aureus*: An emerging threat. *Lancet Infectious Diseases* 5, (5): 275-86.