SELECTION OF PHAGE DISPLAYING PEPTIDES SPECIFIC FOR STAPHYLOCOCCUS AUREUS

BY

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SELECTION OF PHAGE DISPLAYING PEPTIDE SPECIFIC FOR *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is a notorious Gram positive bacterium that has been implicated in causing hospital-acquired and community-associated infections. The goal of this project was to retest previously selected *S. aureus*-specific phage clones from the phage library PhD-7 (New England BioLabs) and identify phage with a peptide that is able to bind to the capsular polysaccharide of *S. aureus*. The phage clones were amplified and PEG-purified prior to testing for specificity by performing ELISAs and sequencing. Whole cell bacterium ELISA using PEG-purified phage MMT5-2, 4, 5, 6 and 7 showed that all phage clones tested bound preferentially to *S. aureus*, except MMT5-7. Using purified carbohydrate as antigen, phage clone MMT5-4 showed high background but preferential binding to the capsule of *S. aureus*, while MMT5-6 showed non-specific binding. MMT5-2, 4, 5, 6 and 7 were selected for sequencing and MMT5-4 DNA sequence was translated into peptide sequence. The sequence revealed that the peptide displayed by the phage was SARXLLK and that the peptide was positively charged and hydrophobic in nature. Sequencing of our selected phage clone MMT5-4 agreed to Rao's work with respect to the polar nature and hydrophobicity of the amino acid. However, our peptide did not show the LQX motif, as reported by Rao and coworkers. Nevertheless, it did show binding specificity against *S. aureus* as a whole cell bacterium, like Rao's peptide and most importantly it showed specific binding to the capsule of *S. aureus,* type 5. This work is the first to report a peptide specific for *S. aureus* capsular carbohydrate. In future studies we hope to combine a toxin to the peptide to eradicate *S. aureus* infections.

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LIST OF ABBREVIATIONS

ELISA – Enzyme-Linked Immunosorbent Assay

CP5 – Capsular Polysaccharide type 5

MMT5 – Martin Maratani type 5

LQX – Leucine (L), glutamine (Q) and any amino acid (X)

PhD – Phage Display

DNA – Deoxyribonucleic Acid

RF – Replication Form

M13KE – Filamentous Phage

SA5 – *Staphylococcus aureus* type 5

PEG – Polyethylene Glycol

ZKST5 – Zak *Staphylococcus aureus* type 5

PBS – Phosphate Buffered Saline

pH – Hydrogen Potential

IPTG – Isopropyl β- d-1-thiogalactopyranoside

Xgal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

LB – Luria Bertani

NaCl – Sodium Chloride

TBS – Tris-buffered Saline

E. coli – Escherichia coli

BSA – Bovine Serum Albumin

CA-MRSA – Community-associated Methicillin Resistant *Staphylococcus aureus*

HA-MRSA – Hospital-associated Methicillin Resistant *Staphylococcus aureus*

ddNTP – Dideoxynucleoside triphosphate

bp – Base pair

MW – Molecular Weight

Pfu – Plaque forming unit

TMB – 3,3’,5,5’-Tetramethylbenzidine

PO - Peroxidase

SD – Standard Deviation

Tet - Tetracycline

OD – Optical Density

TBE – Tris/Borate/EDTA (Ethylenediaminetetraacetic acid)

ON – Overnight

dsDNA – Double stranded Deoxyribonucleic acid

Ig - Immunoglobulin

Rpm – Revolution per minute

HRP – Horesradish peroxidase

Nm - Nanometer

RIP – RNA (Ribonucleic acid) inhibiting peptide

Selection of phage displaying peptides specific for *Staphylococcus aureus*

Problem Statement

S. aureus can become extremely dangerous and harmful if it crosses the skin and enters the dermis of the skin (Kobayashi et al., 2015). Staphylococcal infections were largely treated with antibiotics until the emergence of antibiotic-resistant strains. These antibiotic-resistant strains have not only been discovered in hospitals and clinics but, more importantly, they have also been well-established in the community (Kim et al., 2012). *S. aureus* strains resistant to penicillin and methicillin are well-known. Methicillin-resistant *S. aureus* is well-established in clinics (Hospital-Acquired MRSA) and communities (Community-Associated MRSA), and diseases caused by such strains, especially CA-MRSA, can be very devastating. A recent study has revealed that MRSA strains have developed resistance to majority of traditionally used antibiotics, such as penicillin; methicillin and now vancomycin, a significant anti-microbial drug for treatment of severe MRSA infections. (Kobayashi et al., 2015). *S. aureus* is also resistant to daptomycin or linezolid, antibiotics used for the treatment bacterial skin infections due to severity of infection (Kim et al., 2012). Considering the resistance of *S. aureus* strains to antibiotics, persistence in their hosts is causing more infections. This study investigates the development of a *S. aureus* specific phage-displayed peptide that in the future, may be conjugated to a toxin and used to kill *S. aureus* strains.

Staphylococcus aureus and associated infections

S. aureus is a gram-positive bacterium that is also considered a commensal bacterium – causes no harm or benefit to its host- which lives on the skin and upper respiratory surfaces of approximately 20-30% of humans (Nanra et al., 2013). Despite its harmless relationship with the host, *S. aureus* can cause great havoc in the tissues and organs of its host if it penetrates the skin and finds itself the dermal layer of the skin. Usually, staph infections occur when there is damage to the skin. In the United States and other developed countries, *S. aureus* is one of the major causes of bacterial infections (Kobayashi et al., 2015). Infections caused by *S. aureus* include bacteremia, sepsis, endocarditis (Kim et al., 2012) and bone infections, also called osteomyelitis (Rao et al., 2013).

Staphylococcus aureus toxins and their roles in disease

The persistence of infections and the spread of *S. aureus* strains across the globe results from the fact that these strains developed tactics that enabled them to evade host's immune cells (Kim et al., 2012). *S. aureus* secretes Staphylococcus protein A (SpA), Staph binder of immunoglobulin (Sbi) and Adenosine synthase A (AdsA). SpA binds to the Fc (constant) region of immunoglobulin, preventing phagocytes from being able to phagocytose pathogen coated with immunoglobulin. SpA is also involved in binding to immunoglobulin on the surface of responding B cells, causing proliferation and programmed cell death. Sbi performs a similar function to that of SpA, as it binds to the constant region of immunoglobulin, preventing phagocytic killing. However, Sbi does

not cause B cell death. Sbi does decrease B cell responses by binding to complement component C3 and removing it from serum. This results in decreased B cell activation, as a fragment of C3 bound to immune complexes helps activate B cell responses. AdsA interrupts phagocytic killing of *S. aureus* by decreasing the inflammatory response needed to bring white blood cells to the site of infection. AdsA is an enzyme that produces adenosine in the extracellular fluid. Adenosine binds to receptors on cells, resulting in platelet aggregation, decreased neutrophil bacteriocidal activity, decreased T cell activation, and decreased IL-1 synthesis (a cytokine that causes inflammation). It also increases Il-10 synthesis (a cytokine that decreases inflammation). This results in suppression of the immune response (Kim et al., 2012).

CA-MRSA and HA-MRSA produce toxins that enable *S. aureus* strains to be persistent in their harmfulness against their hosts. CA-MRSA produces PVL (Panton Valentin Leukocidin) (ref), a pore-forming protein that is cytotoxic, resulting in skin infections and lesions. (Cooke and Brown., 2010). *S. aureus* is also well-known to produce a variety of toxins and enzymes that enable them to escape host immune cells to thrive and spread in the host's tissues, causing more infections (Kong et al., 2018). These harmful proteins secreted by *S. aureus* are hemolysin, leukotoxin, exfoliative toxin, enterotoxin, toxic-shock-syndrome-toxin-1 (TSST-1) and coagulase. Hemolysin is primarily responsible for bursting red blood cells releasing needed iron for bacteria enzymes. Leukotoxin is implicated in lysing white blood cells, thus decreasing the immune response. Exfoliative toxin cuts desmoglein-1, a desmosomal glycoprotein which plays an important role in maintaining cell-to-cell adhesion in the superficial

epidermis. This causes skin blisters and loss of skin layers. Enterotoxin is a superantigen which causes activation of T cells resulting in the release of cytokines that cause inflammation of the gastrointestinal tract. This causes frequent passage of watery stools and vomiting which increases dissemination of the pathogen, Toxic Shock Syndrome Toxin (TSST-1) is another superantigen which causes inflammation and shock of the blood circulatory system leading to death in severe cases. Coagulase is responsible for causing blood clots (Heilmann et al., 2002) in the host's blood circulatory system, leading to severe health condition. Coagulase is also important in the formation of abscesses, in which the pathogen is surrounded by layers of fibrin. The formation of abscesses results in a protective environment for the pathogen, as antibiotics and immune proteins and cells cannot penetrate the abscess.

S. aureus also produces capsular polysaccharides which form a coat that surrounds bacterial cell. *S. aureus* serotype 5 capsular polysaccharide is anti-phagocytic (Thakker et al., 1998). Capsules play a major role in determining the harmfulness of a *S. aureus* strain. These molecules enable the bacterial strain to avoid being ingested by bacteria-eating white blood cells of the host immune system. The capsular molecule shields both the complement and antibody effector portion from receptors of phagocytes. Ninety percent of *S. aureus* strains have been isolated and divided into eleven different types of capsule-coated strains. Most of the strains that cause hospital acquired infections are the types 5 and 8. The study further revealed that culture medium influenced the synthesis of capsule molecules as strains grown in agar medium (simulating bacteria bound to tissues) had maximum production of capsule-coated types

5 and 8 which escaped destruction by specific antibodies that were exposed to them while strains grown in broth medium (simulating growth in the blood) had a smaller amount of capsule molecules produced. These strains with small capsule coats were taken up by specific antibodies against them. The authors suggested that capsule-coated strains with maximum production of capsule molecules in agar medium evaded the attack of host white blood cells. This data reflects how these capsular bacterial strains in the host's tissues could avoid being phagocytized and persist in the host. On the other hand, strains with small capsule molecule production were killed by phagocytes, which could also indicate that bacterial strains in the lymph and blood stream would be readily cleared by phagocytes.

Resistance to Antibiotics

Resistance to antimicrobial drugs such as penicillin and ampicillin, among others, became obvious as *S. aureus* infections could no longer be treated and remained persistent. Methicillin and Vancomycin, antibiotics that once proved efficacious in disrupting the growth of *S. aureus*, were later reported to have lost their potency so that resistant strains now emerged to be popularly known as MRSA (Methicillin-resistant *S. aureus*) and VRSA (Vancomycin-resistant *S. aureus*) (Fattom et al., 2004).

Alternative Treatments for *S. aureus* Infections

CP5 and CP8 (where CP is Capsular Polysaccharide) specific antibodies were initially reported to be efficacious in promoting phagocytic killing of *S. aureus.* The vaccine was produced by combining capsular polysaccharide types 5 and 8 plus a carrier protein (*Pseudomonas aeruginosa* exotoxin A was covalently bound to the carbohydrate and used as a strong antigen to elicit a stronger immune response) in what is known as a conjugate vaccine (Fattom et al., 2004). Moreover, they reported that the anti-conjugate vaccine antibodies (Immunoglobulin G) which were isolated and purified and later administered in animal models conferred protection against *S. aureus* infection after healthy animal models were injected with it. Also, they observed in their trial that animal models with heart disease were protected against *S. aureus* due to the administration of passive vaccine (anti-CP5+CP8 plus Carrier protein antibodies). The combined CP5 and 8 plus carrier protein vaccine, known as StaphVAX, was potent in both healthy and unhealthy animals and humans throughout the trials. In addition, their data showed that individuals with kidney infections, receiving long-term treatments, needed to receive higher amount of anti-conjugate vaccine to have long-term immunity against *S. aureus* than healthy individuals (Fattom et al., 2004). The authors concluded that the combinatorial vaccine was harmless and potent in eliciting strong immune response in individuals and most especially in those hospitalized individuals who are at a higher risk of *S. aureus* infections. The antibodies generated, after administering the vaccine in kidney disease patients, were involved in significant clearance of *S. aureus* infections in blood. This suggests that there was an increase in white blood cell killing of

S. aureus. *S. aureus* strains with no capsules were killed by neutrophils in the presence of cow serum without complement (heat-inactivated cow serum), whereas capsule-coated strains (types 5 and 8) were not significantly killed (Kampen et al., 2005). Serum with specific antibodies against capsule-coated strains, either type 5 or 8, increased white blood cell killing, resulting in significant reduction the strains. Capsule-coated strains had an advantage by shielding either complements or antibodies from neutrophils. Strains without capsules attracted killing by neutrophils. However, the authors failed to explain how serum without complements would attract neutrophils for bacteria uptake.

An overview of the various non-antimicrobial therapies highlighting their developments, current position and failures in clinical testing was reported by Schaffer and Lee (Schaffer and Lee, 2008). Preformed antibodies have been tested (passive immunization) with varied success. Purified polyclonal human Immunoglobulin G, also known as Altastaph, with high specificity for *S. aureus* strains CP5 and CP8 did not demonstrate effectiveness in immunity against *S. aureus* after passive immunization in Phase II clinical trials. As a result, therapy with Altastaph has been halted. Veronate, human polyclonal antibodies to surface adhesins extracted from blood plasma from *S. aureus* immunized donors, also failed to be potent against *S. aureus* in the third phase of a recently completed study. However, a combination of human and mouse monoclonal antibody, also known as Pagibaximab (Weisman, 2007), a humanized mouse chimeric mAb against lipoteichoic acid found in Gram-positive *S. aureus* showed positive signs of efficacy against *S. aureus* when given to low-birth-weight infants and is currently

undergoing improvement for future study. Due to the failures of the antibody to *S. aureus* capsule in clinical trials, the authors proposed the employment of vaccines stemming from two or more sources combined for efficacy purpose and treatment of *S. aureus* infections.

Many different proteins have been tested in animal models for their effectiveness in Staphylococcal vaccines. *S. aureus* produces several toxins and extracellular surface molecules, such as capsular polysaccharides and attachment proteins that enable the bacterium to avoid the host's immune cells, attack host's tissues and establish infection. Many of these have been tested as vaccine candidates in animal models but have not progressed to clinical testing (Schaffer and Lee, 2008). A target that would affect the production of multiple toxins and adhesins has been Identified, the accessory gene regulator (agr). Agr regulates the expression of many virulence factors. Agr is a quorum sensor that detects the presence of small cyclic autoinducing peptides (AIPs) secreted by *S aureus*. AIPs are responsible for regulating the genetic machinery (through agr) in response to increased bacterial density and subsequently initiating the expression of the transcriptional units (RNAII and RNAIII) producing toxins. The development of monoclonal antibodies against the AIPs could be potent in quenching the toxic effect of the entire bacterium's regulatory system (Schaffer and Lee, 2008). While vaccines and passive immunization with antibodies show promising results in animal models, there is still much work to do before these are effective treatments for humans.

An alternative to the production of vaccines or antibodies is the production of antibody fragments or specific peptides using phage display technology (Willats, 2002; Mullen et al., 2006). The advent of this novel technology has brought in its wake an alternative approach to drug discovery and delivery. A phage or bacteriophage is simply defined as virus that solely feeds on bacteria. In this new technology, a gene of interest is inserted into the genome of bacteria-eating virus and subsequently the expected protein of interest is expressed on the surface of the phage. A phage library can be produced which has a vast repertoire of proteins expressed on individual phage. The phage producing a peptide specific for a particular molecule can be selected by panning. In panning, the ligand of interest is bound to a plate. Phage are placed in the well and non-binding phage are washed off. The phage containing peptides or antibody fragments specific for the desired ligand isF then eluted and the virus propagated by growth in bacteria. This technique is an inexpensive, rapid method that can be used for research purposes, leading to the discoveries of vaccines, and drugs based on known protein-targets. The author highlighted the significance of adopting phage display technology by first describing its mode of construction and then its remarkable application especially how it could be used to express portions of antibodies as a way of targeting foreign molecules in order to neutralize their toxins. Expression of peptide genes which are inserted into the genetic material of bacteria-eating virus and subsequent screening and selection of these surface peptides gives us a snapshot of what phage display is like.

There is still more room for improvement of finding out the best preventative therapy or cure for this devastating Gram-positive bacterial infection. A completely developed single chain variable fragment of Immunoglobulin G from human source produced using phage display technology (Development and Identification of fully human scFv-Fcs against *S. aureus*, 2016). In this report, the single variable portion of Ig had strong affinity to *S. aureus* and with the help of phage display technology the variable portion of Ig was screened and the peptide with the strongest affinity was selected (Nian et al., 2016). The scFv fragment includes the antigen binding portion of an antibody but does not include the effector portion (constant region) of the antibody needed to bind to phagocyte receptors. In this study, the scFv gene was inserted into a plasmid adjacent to an inserted Fc region (constant region) gene. This produced a whole, functional antibody molecule that could be shown to stimulate phagocytosis of *S. aureus*. This was the first time a full variable portion of Ig coupled to the effector portion, Fc, was initiated and developed. However, there are limitations associated with the novel anti-*S. aureus* antibody Ig: short lifespan and binding to only one epitope of an antigen. This molecule would not have multiple interactions between an antibody and its cognate antigen, which are needed for high affinity binding. These limitations may compromise the efficiency of the single binding variable portion of the synthetic antibody.

Platelet-binding domains in two fibrinogen-binding proteins of *S. aureus* were also identified by phage display of the bacterial peptides and selecting for phage that bound to fibrinogen (Heilmann et al., 2002). The authors adopted a phage display

technique of identifying *S. aureus* proteins responsible for promoting blood clot via binding to thrombocytes in a host. Their aim was to find out via phage display the binding specificity of bacterial enzymes that caused blood clot, to the terminal regions of thrombocytes. From their result, *S. aureus* secreted clotting enzymes coagulase and fibrinogen-binding protein did bind to the fibrinogen site of platelet even after coagulase was stripped of its fibrinogen-binding site and they hypothesized that perhaps coagulase might have relied on fibrinogen as a carrier to bind to platelet. Since coagulase is one of the several enzymes secreted by *S. aureus* and often associated with virulence by causing abnormal clotting of host blood cells there is therefore the need to study the pattern of binding coagulase to its cognate platelets (Platelet-binding domains in two fibrinogen-binding proteins of *S. aureus* identified by phage display, 2002). This will provide a guide to how to target coagulase by an anti-coagulase peptide preventing it from binding to platelet minimizing or preventing *S. aureus* infection.

Phage display technology is a technique for producing proteins that can be used to detect the process of infections and targets for the development of vaccines (Mullen et al., 2006). The manner of development of *S. aureus* infection was investigated using peptide library of bacteriophage displaying *S. aureus* peptides which was then panned against RNA-III activating protein, a protein that activates the production of toxins in *S. aureus*. The selected peptide from the phage library could decrease the infectivity of *S. aureus* in an animal model. This mechanism for selection of a peptide important in infectivity serve as a mechanism for discovering potential vaccine therapeutic agents. The authors provided a list of phage displayed peptides that have binding specificity

against peptides of microbes of interest or against man-made antibodies that could serve as vaccine targets.

Rao and coworkers (Rao et al., 2013) also demonstrated that a random peptide library could be used to select for peptides with specificity for an infectious organism. They compared the sequences of the selected clones to develop a consensus peptide sequence that could be used to bind to the pathogen. The peptide developed in this study bound to *Bacillus anthracis*, a pathogen which produces spores that are often used as a bioterrorism weapon. The peptide developed through phage display could be used in a biosensor for the detection of *B. anthracis*. Rao and coworkers also used phage display to select for a peptide that bound specifically to *S. aureus* (Identification and Evaluation of a novel peptide binding to the surface of *S. aureus*, 2013). By binding to cell surfaces of *S. aureus* the selected peptide can serve as a biomarker in detection of bacteria. However, they failed to mention if this study could be carried out in vivo.

Knowing how peptides of microbes interact with host's peptides via phage display is a great step that leads to the development of vaccines effective in treating microbial infections, especially *S. aureus* infections. Nevertheless, there are still some gaps that are needed to be closed. One possible avenue of investigation is to bind bacteria specific peptides to toxins that can kill the targeted pathogen.

Antimicrobial peptides, special molecules produced by immune cells of skin, have been investigated by some researchers to treat *S. aureus* infections. Defensins are an example of an antimicrobial peptide. Defensins are amphipathic proteins that cause

osmotic lysis of bacteria. Beta-defensin 3 (Zanger et al., 2010) is increased in expression in *S. aureus* infected tissues from the onset while a decrease in expression of the antimicrobial peptide is related to the advanced stage of *S. aureus* infection. Zhu and coworkers (Zhu et al., 2013) also reported that human beta-defensin 3, plays a significant role in inhibiting the clumping of *S. aureus*- in the formation of biofilms, one of the major causes/mechanisms of infection in the host (Human beta defensin-3 inhibits antibiotic resistant staphylococcus biofilm formation, 2013). In their study there was a significant reduction of antibiotic-resistant strains of *S. aureus* and *S. epidermidis* found on the surface of metallic implanted devices. The authors made their observation throughout the various phases of biofilm formation of the bacteria and found out that human beta-defensin 3 was very effective in reducing the number of strains found on the metallic implant device in comparison to control samples.

A second antibacterial peptide RNA-inhibiting peptide (RIP) was investigated for its effect on *S. aureus* infections (Gov et al., 2001). RIP is a peptide of seven amino acids produced by coagulase negative *Staphylococcus*. It is a potent inhibitor of *S. aureus* infections that are related to the skin, cells, skin immune cells, bones and breasts of cows. RIP works by inhibiting agr, which is necessary for activating synthesis of toxins from the transcriptional units, RNAII and RNAIII. RIP also inhibits adhesion and has been shown to work effectively on the tissues of host cell models and in animal models. Thus, RIP has a great prospect of being a treatment tool against *S. aureus* infections (RNAIII inhibiting peptide (RIP), a global inhibitor of *S. aureus* pathogenesis: structure and function analysis, 2001).

Peptides can also act synergistically with antibiotics in the treatment of *S. aureus* infection. Rishi and coworkers (Rishi et al., 2018) produced synthetic peptides with structural similarity to the amphipathic peptides discussed previously (defensins) and tested these for their direct effect on bacteria and their ability to improve the effect of antibiotics. This work highlighted the significant role of peptides acting as substances that can aid antibiotics in their efficacy against *S. aureus* infections by interacting with the cellular wall of the pathogen. The authors suggested that the peptides block molecular pathways either facilitating the entry of antibiotics into the cell or preventing the movement of antibiotics out of the pathogen's intracellular component. The antibiotics are thus retained in the pathogen to cause death of the bacterium.

The hypothesis of this thesis is that phage display technology can be used to produce peptides specific to Staphylococcus aureus, type 5 capsule. In previous studies by this laboratory, phage-displayed peptides specific for *S. aureus* capsule type 5 were selected and their binding specificity was confirmed via bio-panning and ELISA assays. The aim of this study is to retest the phage clone peptides for their binding specificity against *S. aureus* carbohydrate coated type 5 strain and detect the peptide that could bind to the strain. Our future goal is to combine the selected peptide with a toxin that could treat *S. aureus* infections. The *Staphylococcus* specific peptide could then target the toxin directly to the bacterium.

Materials and Methods

Materials

Bacto-tryptone, yeast extract, agar, sodium chloride, xgal, IPTG, PEG, TMB, DMSO, TBS, PBS, Tetracycline, Ammonium sulfate, Sulfuric acid, Sodium bicarbonate, Tween-20, Casein, phage library, Bovine serum albumin were purchased from Sigma-Aldrich, St. Louis, Missouri, United States. The Wizard® *Plus* SV Minipreps DNA Purification System was purchased from Promega Corporation (Madison, WI). GenomeLab Dye Terminator Cycle Sequencing with Quick Start Kit was purchased from Beckman Coulter® (Brea, CA). The anti-M13 monoclonal antibody conjugated to Horse Radish Peroxidase (HRP) was purchased from GE-Healthcare (Chicago, IL).

Preparation of Overnight Culture of Bacteria

LB (Luria Bertani) broth (20 ml) was measured and dispensed into 125 ml Erlenmeyer and 20ul of tetracycline was added to it. Using a sterile loop, a colony of bacteria was added to the medium and incubated overnight, with a shaking of 210-150 rpm (revolution per minute) at 37 degrees Celsius.

ELISA using HRP/Anti-M13 Monoclonal Conjugate (phage recombinant antibodies)

A 96-well microtiter plate was filled at the periphery with 200 µl of water to prevent evaporation of samples in the wells. One well had positive control, blank (in duplicate), negative control (in duplicate), No *S. aureus* and Test wells (in duplicate). The positive control well was coated with 100ul of $5.7x10^9$ pfu/ml M13KE phage diluted in

10 µl of Na_2CO_3 (50 mM carbonate/bicarbonate buffer, pH 9.6); both blank and negative control wells had 100 µl of *Staphylococcus aureus* (SA, see bacteria preparation below); No SA and Test wells had Blocking buffer (1% casein-TBS) and SA, respectively. The plate was incubated overnight at 37^0C in a humidified chamber, centrifuged at 400xg for 15 mins. The coating solution was aspirated from the wells and the wells were washed once with wash buffer (PBS or PBS + 0.05% Tween-20). Each well was later filled with 200 µl of blocking buffer and incubated for 37^0C for 30 mins.

The blocking buffer was aspirated; the test and no SA wells were coated with 100 µl of MMT5-1 to MMT5-10 phage; the negative control wells were coated with 100 µl of $5.7x10^9$ pfu/ml M13KE; the positive control and blank wells were TBS (Tris-Buffered Saline). The microtiter plate was incubated at room temperature for 30 mins at 37^0C. After incubation, the wells were washed six times with wash buffer and later filled with 100 µl of diluted (0.1% BSA in PBS or 0.1% casein in PBS + 0.05% Tween-20 = sample buffer) HRP/anti M13 monoclonal antibody peroxidase conjugate (also known as secondary antibody). The secondary antibody was dissolved in sample buffer in a 1:5000 ratio. The well was incubated for 30 mins at 37^0C and later washed six times with wash buffer. 100 µl TMB substrate solution was pipetted into the wells and incubated at room temperature for 20-60 mins. When the positive control well turned blue, 50 µl of stop solution, H_2SO_4, was added to the wells and absorbance was read at 450 nm wavelength.

ELISA for S. aureus Capsular Polysaccharide

Wells of microtiter plates were coated with 100 μl of antigen (1 μg/ml of purified carbohydrate in PBS) by incubating the plates at 37^0C for 30 min. Solution in the plates was removed by flicking the plates over the sink and blotting on paper towel. Blocking buffer (200 μl 1% BSA in PBS) was added to the wells and incubated at 37^0C for 30 mins. After incubation, the plates were washed three times with PBS-0.05% tween 20 (wash buffer) and the solution was removed from wells by flicking the plates over and blotting on paper towel, after each wash. M13 in sample buffer (2%BSA in PBS and 0.05% Tween-20) was added to the wells and was left to bind for 30 minutes at 37^0C. Antibody control wells contained 100 μl hybridoma culture supernatant. The wells were washed three times with wash buffer. Anti-mouse Ig-PO (diluted in a 1:1000 sample buffer containing 2% BSA and 0.05% Tween-20, or 1:1000 anti-Ig-PO for antibody controls) will be added to the antibody positive control well and was allowed to bind for 30 minutes at 37^0C.

After 30 minutes, the wells were washed three times and solution blotted from the wells as described in previous steps. 100ul of TMB was added to the wells, incubated for 30 minutes at 22^0C or until color develops. H_2SO_4 (2N Sulfuric acid) was added to each well as a stopping solution and absorbance was read at 450 nm.

Bacteria Preparation for ELISA

S. aureus was grown in Columbia broth plus 2% NaCl (Sodium Chloride) as an overnight culture at 37^0C in shaking incubator; at 210 rpm (revolution per minute). Cells

were washed twice with PBS + 1% BSA and once with PBS – cells were centrifuged at 13,000 xg at 4^0C for 30 minutes, supernatant poured off. Pellets are vortexed into a smooth suspension and re-suspended in 10 ml PBS or PBS + BSA. Cells were treated with 3% formalin overnight on rotator at 4^0C. Cells were washed three times, as described previously, and then treated with trypsin (1 mg/ml) at 37^0C overnight with rpm of 200. Cells were washed 3 times as described previously. Cells were brought up to an optical density of 1.0 at 550 nm, or highest possible with all samples the same. 100ul of cell suspension were added to the wells of microtiter plate and incubated overnight at 37^0C. The plate was centrifuged at 2000 xg.

Phage Amplification for ELISA or Sequencing

E. coli ER2738 were incubated in LB-tetracycline media overnight at 37°C shaking at 250 rpm. Terrific Broth with tetracycline was inoculated with 800ul of the overnight culture and incubated for one hour at 37°C shaking at 250 rpm. The shaking speed was then decreased to 100 rpm for 10 min to regenerate sheared pili. Phage (4×10^9) was added to 20 ml of the prepared cells and incubated at 37°C shaking at 250 rpm until OD reaches 0.4-0.5 at 560-595 nm (4 hr). The sample was centrifuged at 4500 x g for 10 min to remove bacteria, the supernatant collected and centrifuged a second time. Thirty-two ml of supernatant was pipetted off and added to 8 mL of 2.5 M NaCl/20 % PEG-8000 (w/v). The sample was mixed by inverting and phage precipitated overnight at 4°C. Phage were pelleted by centrifugation at 12000 x g at 4°C for 15 min. The pellet was resuspended in 2 ml TBS and added to 200 µL of 2.5 M NaCl/20% PEG-8000. This was

incubated on ice for 1 hr and centrifuged at 14000 rpm in a benchtop centrifuge for 10 min. The pellet was resuspended pellet in 200 µL TBS and stored at -20°C.

Phage Titer

LB tet plates were pre-warmed in an incubator at 37^0C for at least one hour, one plate per expected dilution. Top agar was melted in microwave and 3 ml was dispensed into sterile culture tubes, one tube per expected phage dilution. Culture tubes are maintained at 45^0C, in water bath. 10^{-2}-fold serial dilutions of phage were prepared in PBS (phosphate buffered saline). Aerosol resistant pipette tips should be used to prevent contamination of dilutions and for each dilution a new pipette tip should be used. Overnight culture of ER2738 (200 µl) was dispensed into sterile microfuge tubes, one for each phage dilution. Each phage dilution (20 µl) was added to each microfuge tube containing the bacteria culture. The microfuge tube was vortexed and incubated at room temperature for 1-5 minutes (maximum).

The infected cells were transferred to culture tubes containing 45^0C top agar, 40 µl 2% X-gal and 40 ul 2% IPTG. The tubes were vortexed quickly and immediately poured onto previously warmed LB tet plate. The plates were gently tilted and rotated to spread the top agar evenly. The plates were then allowed to cool for 5 minutes, inverted and incubated overnight at 37^0C. The plaques were then counted the following day. Plates that have approximately 100 plaques should be counted. Phage number should be multiplied by the dilution factor for that plate to obtain phage titer in plaque forming units per 10 µl.

Wizard plus SV minipreps (DNA Purification System)

Production of cleared lysate: 5-10ml of overnight culture is pelleted by centrifugation at 10,000 xg for 5 minutes. The pellet is thoroughly re-suspended with 250 µl of cell resuspension solution. 250ul of cell lysis solution is added to each sample, inverted thoroughly for 4 times to mix. The samples are incubated at room temperature for 5 minutes. 10 ul of alkaline protease is added to the sample, inverted thoroughly for four times to mix and incubated at room temperature for 5 minutes. Neutralization solution (350 ul) is added to the sample and inverted four times to mix. The samples are centrifuged at a 10,000 xg for 10 minutes at room temperature.

Binding of plasmid DNA: A spin column is inserted into a collection tube and the cleared lysates were decanted into a spin column. The spin column was centrifuged at 10,000 xg for one minute at a room temperature. The flowthrough was discarded, and the column was reinserted into collection tube.

Washing: 750 µl of wash solution was added to the sample, centrifuged at 10,000 xg for 1 minute at room temperature. The flowthrough was discarded, and the column reinserted into a collection tube. 250ul of wash solution was added to the sample, centrifuged at 10,000 xg for 1 minute at room temperature. The flowthrough was discarded, and the column reinserted into collection tube.

Elution: The spin column was transferred into a sterile 1.5 ml microcentrifuge. 100 µl of nuclease-free water is added to the spin column, centrifuged at a 10,000 xg for

1 minute at room temperature. The spin column was discarded, and the DNA stored at -20^{0}C or below.

Nanodrop for measurement of DNA concentration

A nanodrop microvolume spectrophotometer was used to determine the DNA concentration of samples. The nucleic acids tab was selected from the home screen button and dsDNA was selected. A baseline correction is specified and 1-2ul of blanking solution is pipetted onto the lower pedestal and lower arm. The arm is lifted and both pedestals are cleaned with a new laboratory wipe. 1-2 μl of sample solution was pipetted onto the pedestal, the arm is lowered, and the measure button is tapped. After the sample measurement is completed, values of interest are displayed. The END EXPERIMENT button is tapped after all samples are measured.

DNA Electrophoresis

Agarose gel (1%), which contained gel green dye, was put into the electrophoresis apparatus with the wells on the cathode end, facing the power supply. 1X TBE buffer was poured carefully into the tray of the gel apparatus covering the wells at a designated mark. Wells should be flushed with running buffer if there are air bubbles in the wells. DNA loading dye (2 μl) was mixed with 5 μl of DNA samples and loaded onto the gel. One well with ladder of 1 kb x 10 was added. The lid was placed on the tank, the electrodes and plug attached in their units. By selecting the voltage, 100V, and pressing the ON button, the gel was run at 100V for 30 minutes or until the first dye

ran off. After completion of electrophoresis, the OFF button was pressed, and results were visualized using the PrepOne Illuminator. The gel was placed on a viewing area tray (by taking the gel out of the electrophoresis tray). The hood with camera was placed over the gel and light turned on and a picture taken. The data was transferred to computer using cord with connectors for a flash drive port.

DNA Sequencing

In a 0.2 ml microfuge tube, the following volumes of DNA (195 ng needed for 50 fmole) were pipetted: MMT5-2= 1.3 μl; MMT5-4= 1.5 ul; MMT5-5=8.8 ul; MMT5-6= 1.6 ul; MMT5-7= 8.8 ul and the following volumes (in μl) of nuclease-free water added: 7.5, 7.3, 0, 7.2, and 0 (each sample solution was brought up to 10 μl by the addition of nuclease-free water). Two tubes were made for forward and reverse reactions (and one tube for control). Using a thermocycler, samples were heated at 96^{o}C for 1min and let cooled at room temperature. Two mixes for each plasmid forward and reverse reactions were made – 2ul of 16ul M13 forward primer and 8ul Master Mix. For forward and reverse primers for library (1 pm/ul primers, need 3.2 pm), 3.2ul of primer plus 8 μl master mix (x6= 19.2 ul primer plus 48 ul master mix). All samples were mixed and centrifuged. Following centrifugation, 10ul of the Forward mixture was added to one tube and 10ul of the reverse mixture was added to another tube. The master mix contained DNA polymerase, 1,4, deoxyribonucleotides and four fluorescently labelled ddNTPs. The tubes (one forward and one reverse) were loaded in the thermocycler and run for 96^{0}C for 20secs (to denature DNA); 50^{0}C for 20secs (primer annealing); 60^{0}C for

4 mins (extension). The first three steps were repeated 29 times to get a total of 30 cycles. Samples were sent to Genomics/Proteomics lab for sequencing.

Results

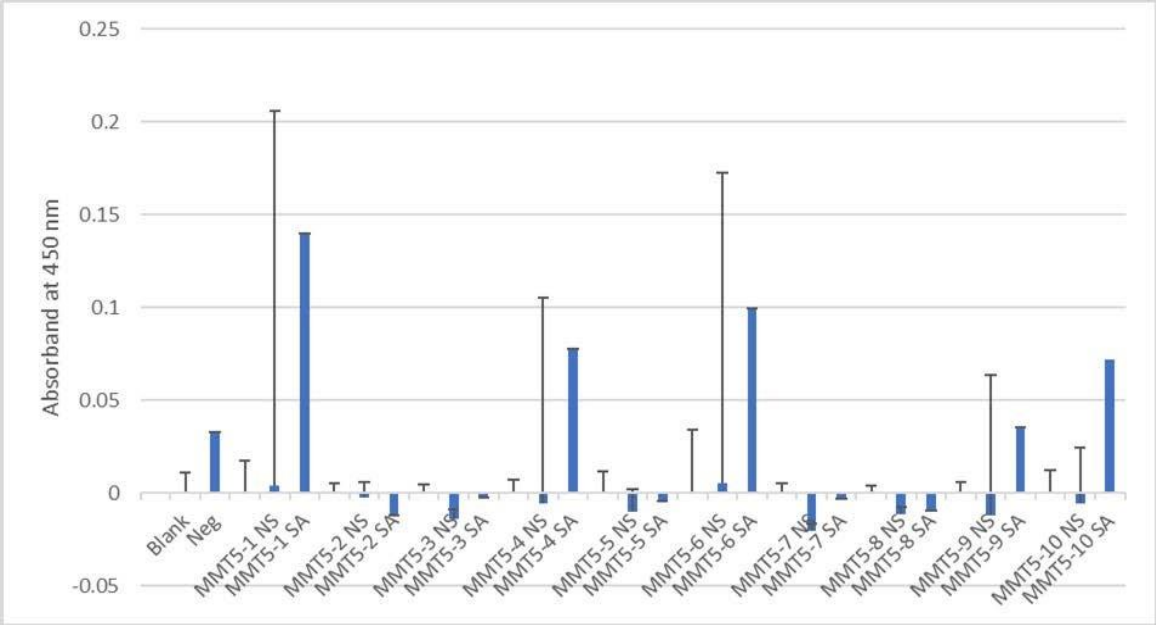
ELISA – Whole cell bacteria, *S. aureus*.

Phage display technology was previously used to select for phage specific for *S. aureus* capsule (Maritani M, 2017), using the PhD-7 library (New England BioLabs) which has random peptides containing 7 amino acids displayed on pIII capsid protein of M13 phage. Panning was performed against formalinized and trypsinized *S. aureus*. Formalin kills the bacterium and fixes the molecules on the surface of the bacterium. The enzyme trypsin is used to digest surface proteins, including Staph protein A. Staph protein A is removed, as it binds to the constant region of antibodies, resulting in non-specific attachment of all antibodies to the bacterium. Trypsinization also removes most proteins from the surface, while leaving capsular carbohydrates intact. The whole bacterium was attached to a poly-L-lysine coated plate through charge interactions, as the capsule is negatively charged while the plate is positively charged. Panning was performed by incubating the plates containing bacteria with the phage library. Non-specific binders are washed off and specific binders were eluted by decreasing the pH of the wash solution. Phage were titered by plating in soft agar and individual plaques picked from the plate and grown as clones. In previous studies, clones MMT5-2, MMT5-4, MMT5-5, MMT5-6 and MMT5-7 were shown to bind specifically to whole cell *S.*

aureus, but not to *S. epidermidis* or *E. coli*. These clones were tested further in these studies.

Supernatants from all phage clones (1 through 10) were grown in *E. coli* overnight and the supernatants tested by ELISA to determine whether the clones were still viable and able to bind to *S. aureus (*Figure 1). M13KE dissolved in 10 µl of Na_2CO_3 and bound to the plate was the positive control; The negative control contained M13KE (the phage used to make the library) that did not display a specific peptide. The ligand in the wells was formalinized and trypsinized whole cell *S. aureus*. All wells were blocked with 1% casein in PBS and incubated with phage in sample buffer (PBS containing 0-01% casein and 0.05% Tween-20). All tests, except for positive control, were carried out in duplicate. The Phage clones were tested for binding in the presence (SA) and absence (NS) of whole cell *S aureus*. Bound phage was detected by antibodies against M13 covalently bound to the enzyme peroxidase. TMB substrate was acted upon by the enzyme to cause the development of color in wells containing phage. Color development was measured with a multi-well spectrophotometer. Blank (no anti-M13 antibody) was subtracted from the average of any two data points to give resulting data of average – blank. Both graphs had good positive controls. For graph 1 (Figure 1) the negative control was not detectable (as expected). For graph A, clones MMT5-1, 4, 6, 9 AND 10 demonstrated specific binding, although variability was seen in the replicas. In graph B MMT5-1, 4, 6 and 10 showed increased binding in the presence of *S. aureus*.

A.



B.

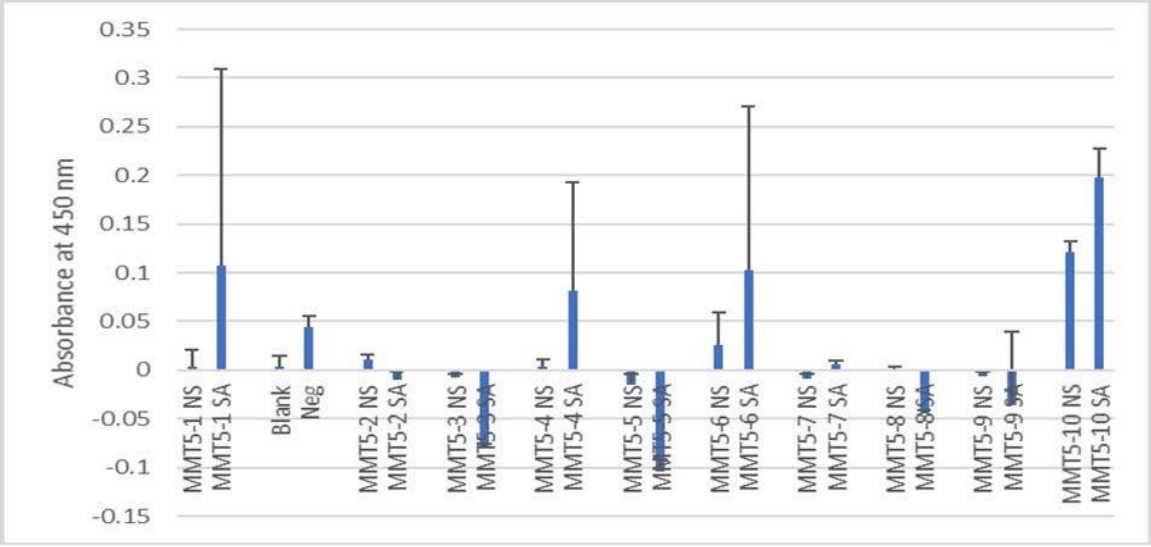


FiGURE 1. ELISA graph highlighting the binding specificity of phage clones in culture supernatants to *S. aureus* (two replicas). The positive control is 100ul M13KE (the phage used to produce the library) of $5.7x10^9$pfu/ml in 0.05M sodium carbonate (Na_2CO_3). Positive control: A = 2.6, B = 2.6. Formalinized and trypsinized *S. aureus*, type 5 (100 ul, OD 1 at 550 nm) was

added to blank, negative and SA wells. Plates were incubated at 37^{o}C overnight and centrifuged at 400 xg for 15 min. After washing, 1% casein-PBS (block was added to all wells for 30 min at 37^{o}C. Phage (50 ul) were added to 50 ul of sample buffer (PBS + 0.1% BSA) was The positive control and the blank received 100 ul sample buffer. M13KE (diluted 1:2 in sample buffer) was added to the negative controls. After washing wells, peroxidase conjugate anti-M13 antibody was added to all wells, except for the blanks. Tetramethylbenzidine substrate solution was added, stopped after color developed with 2N H_2SO_4 (sulfuric acid) and the absorbance at 450 nm measured using a microplate reader. All tests, except for positive control, were carried out in duplicate. The Phage clones were tested for binding in the presence (SA) and absence (NS) of whole cell *S. aureus*. Blank was subtracted from the average of any two data of absorbance read to give resulting data of Avg-Blk (Average –Blank). This data was used to plot the graph. Data represents the mean +/- SD. The experiment was repeated once (A and B).

Not all clones that were positive previously were positive in these ELISAs. One possible difference is the bacteria preparation used. To check for effects resulting from different bacteria preparations, Figure 2 shows the data from MMT5-4 and 10 tested against two new preparations of formalinized and trypsinized *S. aureus*. MMT5-4 did not show significant binding in either assay and MMT5-10 had increased non-specific binding to the well not having bacteria.

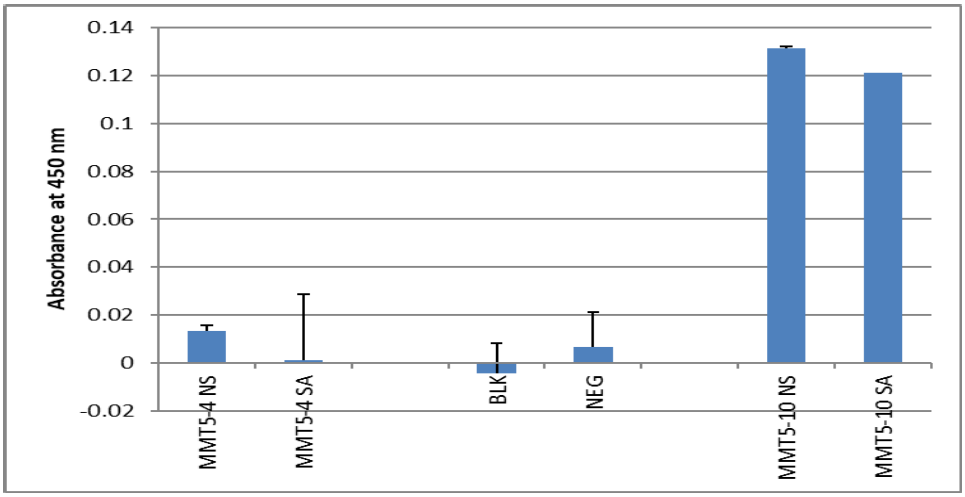
Amplification of phage

Phage clones, MMT5-2, 4, 5, 6 and 7 were amplified and PEG purified to increase their number and purity for ELISA and Sequencing. Following PEG purification, purified phage pellets were resuspended in 200 µl of TBS. A titer was performed to determine the number of phage in the purified sample (Table 1). Phage concentration in supernatants prior to amplification and PEG purification ranged from 2.08×10^{10} pfu/ml to 1.1×10^{12} pfu/ml. Concentrations of PEG purified phage was lower, ranging from 5×10^{3} pfu/ml to 4×10^{10} pfu/ml. Phage clones 2 and 6 were at much lower concentrations than the other clones, with clone 2 being 10^{7}-fold lower than other clones and clone 6 being 10^{3}-fold lower phage concentrations than other clones.

Whole Cell ELISA of Amplified and PEG Purified Phage

The whole cell ELISA was repeated using amplified and PEG purified phage preparations. Conditions were unchanged from previous whole cell ELISAs, however the phage concentration used was lower. Fifty microlites of phage was used mixed with 50

A



B

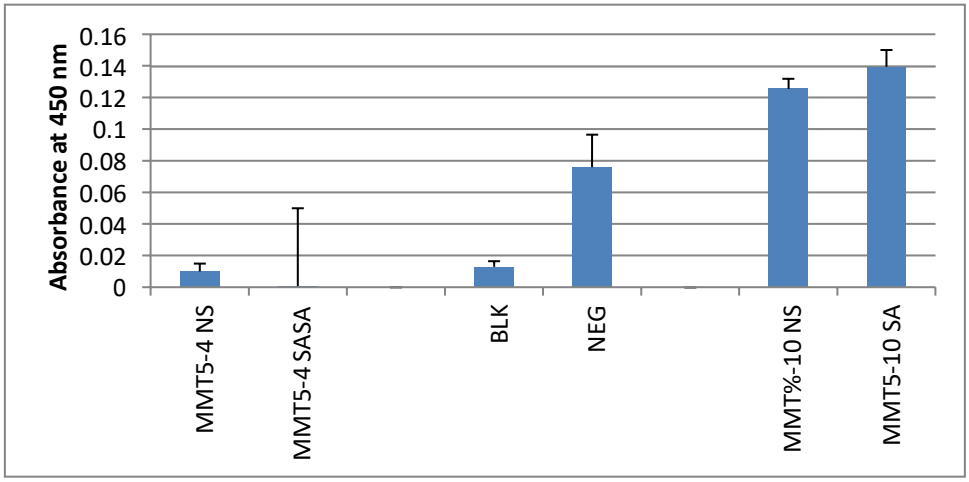


FIGURE 2. **Two ELISAs showing the binding of phage clone 10 using MM and ZK T5 Vaccine respectively.** The ELISAS were identical to those in Figure 1, except that we used two different preparations of *S. aureus* antigen: MMT5 (graph A) and ZKT5 (graph B). Phage clone 4 was PEG-purified while phage clone 10 was supernatant phage. Data represents the mean +/- SD. Positive control: A. 1.6, B. 2.0.

Table 1: Titer of *S. aureus*, type 5 clones before and after amplification

Phage clones MMT5[a]	Titer Before Amplification[b]	Titer After Amplification
MMT5-2	1.1×10^{12}pfu/ml	5×10^{3}pfu/ml
MMT5-4	1.850×10^{11}pfu/ml	1×10^{9}pfu/ml
MMT5-5	2.4×10^{11}pfu/ml	1×10^{10}pfu/ml
MMT5-6	2.3×10^{11}pfu/ml	5×10^{7}pfu/ml
MMT5-7	2.08×10^{10}pfu/ml	4×10^{10}pfu/ml

[a]MMT5* -- Martin Maratani S. aureus, type 5 specific phage clones: MMT5-2 to MMT5-7.

[b] 10^{-2} -fold serial dilutions of phage (20 ul) in PBS was incubated 1-4 min with 200ul of an overnight culture of ER2738 *E. coli*. The infected cells were transferred to culture tubes containing 3 ml 45^{0}C top agar, 40ul 2% X-gal and 40ul 2% IPTG. The tubes were vortexed quickly and immediately poured onto previously warmed LB tet plate, then incubated overnight at 37^{0}C. The plaques were then counted the following day. Plaque number was multiplied by the dilution factor for that plate to obtain phage titer in plaque forming units per 20ul.

μl of sample buffer (PBS + 0.1% BSA), except for MMT5-2 (20 μl + 80 ul sample buffer) and MMT5-4 (10 μl + 90 ul sample buffer). Due to the limited amount of purified phage available, the phage concentrations used were low: MMT5-2 = 2.5 x 10^2, MMT5-4 = 1.0 x 10^5, MMT5-5 = 5.0 x 10^6, MMT5-6 = 2.5 x 10^3, MMT5-7 = 2 x 10^6. Optimal phage suggested by the protocol is 2 x 10^{10} phage per well. Samples MMT5-2, MMT5-4, MMT5-5, and MMT5-6 showed specific binding to *S. aureus*, however MMT5-6 had high background binding to the no *S. aureus* wells (Figure 3). Clone MMT5-7 did not show specific binding to *S. aureus*.

ELISA- Using Purified carbohydrate of *S. aureus*

To determine the binding specificity of phage clones against purified carbohydrate of S. aureus, purified capsular carbohydrate prepared previously (Maratani, 2017) was used as the ligand in ELISAs. To produce the purified carbohydrate, a *S. aureus* culture was autoclaved and sonicated prior to digestion with DNase, RNase and protease. The remaining carbohydrate preparation was dialyzed and subjected to DEAE column chromatography. This procedure results in a pure preparation of capsular carbohydrate. The ELISA was performed similarly to the whole cell ELISA, with the exceptions that the antigen was purified carbohydrate and the wash contained 0.05% Tween-20. Due to limited amounts of purified capsular carbohydrate, only MMT5-4 and MMT5-6 were tested. Fifty microliters of phage (MMT5-4 = 5 x 10^5 phage, MMT5-6 = 2.5 x 10^3 phage) in 50 μl of sample buffer was used. MMT5-4 showed specific binding to capsule but had

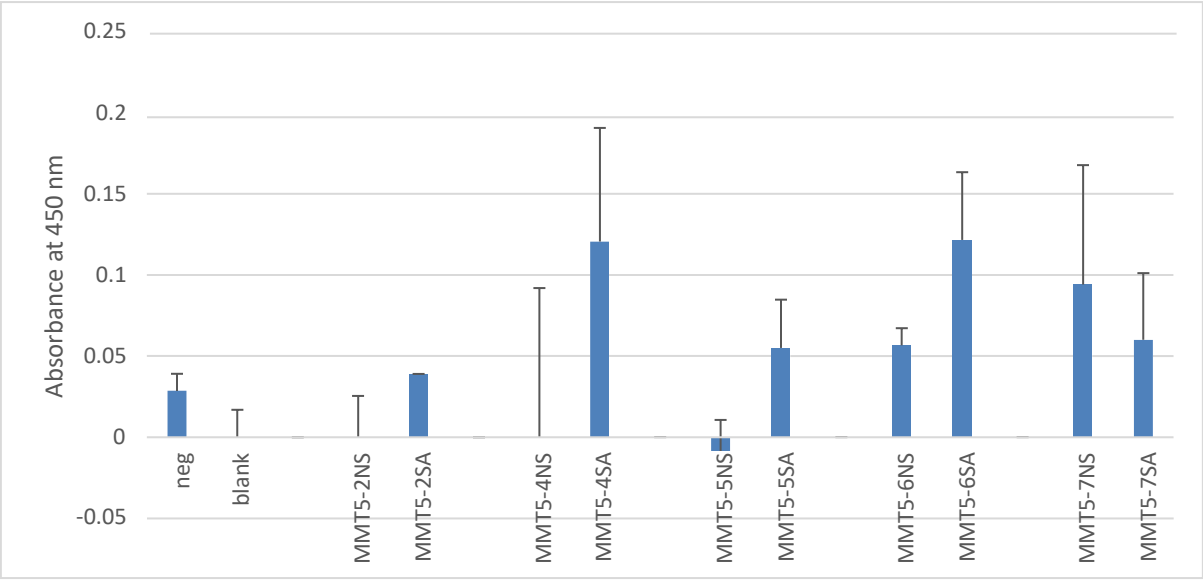


FIGURE 3. Demonstration of Binding Specificity of Amplified and PEG purified Phage Clones Against *S. aureus* as a Whole Cell Bacterium. This graph demonstrates an ELISA of *S. aureus*, type 5 whole cell bacteria, using newly amplified and PEG purified phage clones. The ELISA was performed identically to Figure 1, with the exception that the sample buffer used was PBS + 0.1% BSA + 0.05% Tween-20. The phage clones tested were MMT5-2,4,5,6, and 7. The phage concentrations are shown in Table 1. Fifty microliters of phage sample plus 50 ul sample buffer were tested, except for MMT5-2 (20 ul) and MMT5-4 (10 ul). The positive control was a single sample with an absorbance of 2.509. All other samples were performed in duplicate. Data represents the mean +/- SD.

high background binding to the no capsule wells (NS) (Figure 4). MMT5-6 did not show specific binding to capsule.

Nanodrop (DNA Concentration)

DNA purification of RF phage from clones MMT5-2,4,5,6, and 7 was performed using the *Wizard® Plus SV Minipreps DNA Purification System.* Following DNA purification, the concentration of phage clone replicative form DNA was measured (Table 2) using absorbance at 260 and 280 nm wavelength ultraviolet light (A260 dsDNA = 50 µg/ml). The DNA concentration of the samples ranged from 160-204 ng/ul. Unfortunately, samples for MMT5-5 and MMT5-7 the nanodrop calculations indicated the presence of protein contamination (for pure DNA, $A_{260/280}$ is expected to be approximately 1.8). These samples were purified again and the absorbance at 260 and 280 nm wavelength repeated (Table 3). MMT5-5 purity improved but the concentration decreased to 17 ng/ul. MMT5-7 purity improved, and the concentration decreased to 19 ng/ul.

Electrophoresis of RF phage DNA

The DNA samples were electrophoresed on a 1% agarose gel containing green gel dye. Loading dye was mixed with 5 µl of DNA samples and loaded onto the gel. Well 2 contained a 1 Kb DNA ladder, MMT5-2 was in well 3, MMT5-4 was in well 4, MMT5-5 was in well 5, MMT5-6 was in well 6 and MMT5-7 was in well 7. The gel was run for 30 minutes at 100 V or until the first the gel ran off. The samples were visualized using a

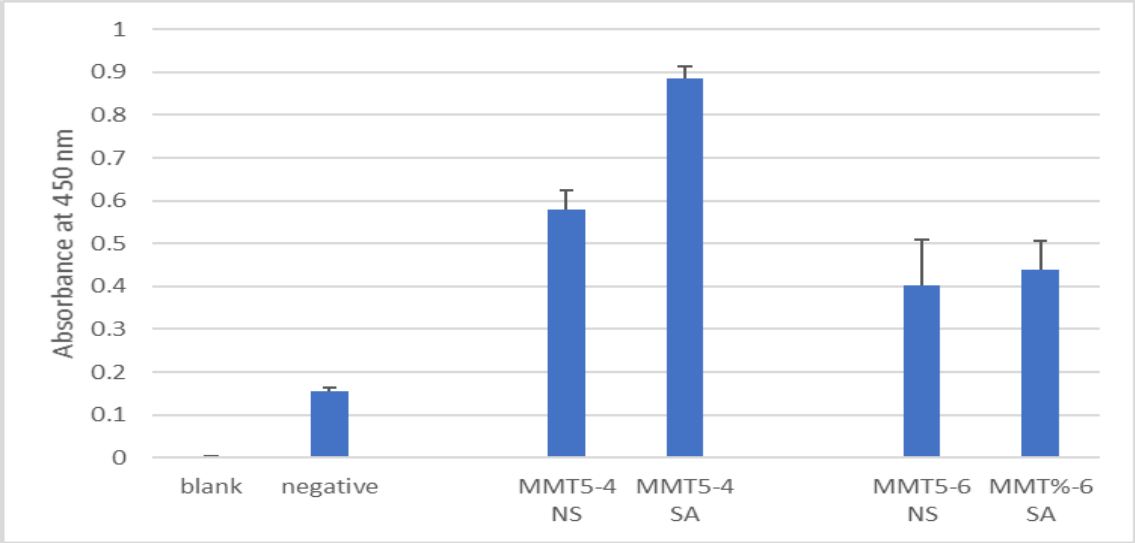


FIGURE 4. ELISA Graph Showing Binding of Phage Clones against Capsular Polysaccharide of *S. aureus*. To determine the binding specificity of phage clones against purified carbohydrate of *S. aureus*, wells of 96-microtiter plate were coated with 100 µl of 1 µg/ml purified capsular carbohydrate in PBS by incubating the plates at room temperature for 2 hr at room temperature. Solution in the plates was removed by flicking the plates over the sink and blotting on paper towel. Blocking buffer (200 ul of 1% Casein in PBS) was added to the wells and incubated at 22^0C for 30 mins. The plates were washed three times with PBS-0.05% Tween 20 (wash buffer). The phage clones tested were MMT5-4 and MMT5-6. The phage concentrations are shown in Table 1. Fifty microliters of phage sample were tested. The phage were added to the wells in sample buffer (PBS + 0.1% BSA + 0.05% Tween-20) up to 100 µl. The samples were incubated at 37^0C for one hour. M13KE was added to the negative control and sample buffer was added to the blank. The wells were washed and anti-M13-PO diluted 1:5000 in sample buffer was added to each well and allowed to bind for 30 minutes at 37^0C. The wells were washed three times and solution blotted from the wells as described in previous steps. 100ul of TMB was added to the wells, incubated for 30 minutes at 22^0C or until there was color formation. 50ul of H_2SO_4 (Sulfuric acid) was added to each well as a stopping solution and absorbance was read at 450nm. The positive control is 100ul M13KE (the phage used to produce

the library) of $5.7x10^9$pfu/ml in 0.05M sodium carbonate (Na_2CO_3) which had an absorbance of 2.212. Standard deviations of data were obtained and values were used to plot the graph.

Table 2: Measurement of DNA Concentration of Phage Clones

Sample[a] Name	Nucleic Acid(ng/uL)	A260/A280	A260/A230	A260	A280	Nucleic Acid Factor	Baseline Correction (nm)	Baseline Absorbance
MMT5-2	200.818	1.809	1.491	4.016	2.22	50	340	0.07
MMT5-4	180.783	1.776	1.296	3.616	2.036	50	340	0.077
MMT5-5	165.492	1.737	1.223	3.31	1.905	50	340	0.046
MMT5-6	159.598	1.682	1.036	3.192	1.898	50	340	0.041
MMT5-7	204.114	1.641	0.954	4.082	2.487	50	340	0.071

Sample Name	Corrected (ng/uL)	Corrected %CV	Impurity 1
MMT5-5	157.75	1.82	Protein
MMT5-7	188.5	1.88	Protein

[a]RF DNA from phage clones MMT5-2,4,5,6,7 was purified using a Wizard® *Plus* SV Minipreps DNA Purification System. DNA concentration and purity was determined using a A nanodrop microvolume spectrophotometer.

Table 3: Second Nanodrop experiment for DNA concentration measurement

Sample[a] Name	Nucleic[b] Acid(ng/uL)	A260/A280	A260/ A230	A260	A280	Nucleic Acid Factor	Baseline Correction (nm)	Baseline Absorbance
H2O	-1.155	0.937	0.289	-0.023	-0.025	50	340	0.015
MMT5-2	214.809	1.822	1.483	4.296	2.358	50	340	0.093
MMT5-4	188.671	1.792	1.314	3.773	2.105	50	340	0.103
MMT5-5	17.324	1.903	1.098	0.346	0.182	50	340	0.107
MMT5-6	170.831	1.709	1.035	3.417	1.999	50	340	0.128
MMT5-7	18.953	1.875	1.092	0.379	0.202	50	340	0.126

[a]RF DNA from phage clones MMT5-2,4,5,6,7 was purified using a Wizard® *Plus* SV Minipreps DNA Purification System. DNA concentration and purity were determined using a a nanodrop microvolume spectrophotometer. Methods were identical to those in Figure 1, except that the nuclease free-water was changed and the device was blanked again to obtain accurate values.

[b]Table 3 shows the concentration of phage DNA 5 and 7 following their purification a second time to remove protein contaminants.

PrepOne Illuminator. The clone RF molecular weights corresponded to the weight of M13 KE = 6407 bp (Figure 5).

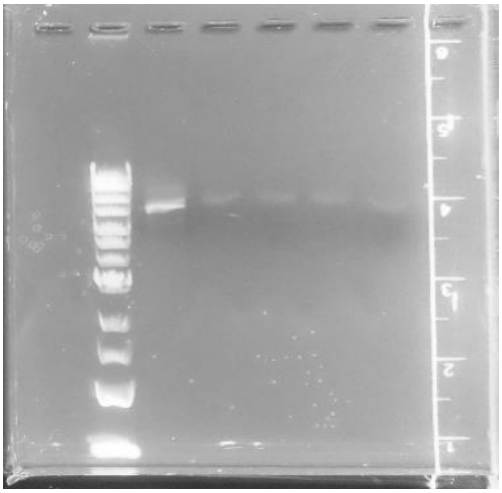
DNA Sequencing

DNA sequencing was performed using the GenomeLab™ Dye Terminator Cycle sequencing with Quick Start kit. Primers were supplied with the PhD-7 library. DNA analysis was performed by Ed Budde, a Molecular Biology Instrumentation Specialist in the Molecular Biology Analytical Core Laboratory at Youngstown State University, OH. Following sequencing, phage clone 4 was selected for translation. After translating the DNA sequence 4, it appeared the amino acids of the peptide are hydrophobic or polar, with two of the amino acids having a positive charge. The translated sequence for the seven amino acid peptide displayed on MMT5-4 is Ser-Ala-Arg-X-Leu-Leu-Lys (Figure 6).

DISCUSSION

Our data support the hypothesis of this thesis is that phage display technology can be used to produce peptides specific to Staphylococcus aureus, type 5 capsule. Staph specific peptides may be useful in the treatment of *S. aureus* infections. *S. aureus* infections have become persistent both in hospital and community settings (Kim et al., 2012) and finding the best therapy to either minimize or eradicate them is like finding a needle in a haystack. *S. aureus* infections have been treated by antibiotics such as penicillin, methicillin, and vancomycin. As effective as these antimicrobial drugs are, strains of *S. aureus* have developed resistance to them, thus rendering them ineffective

A



B

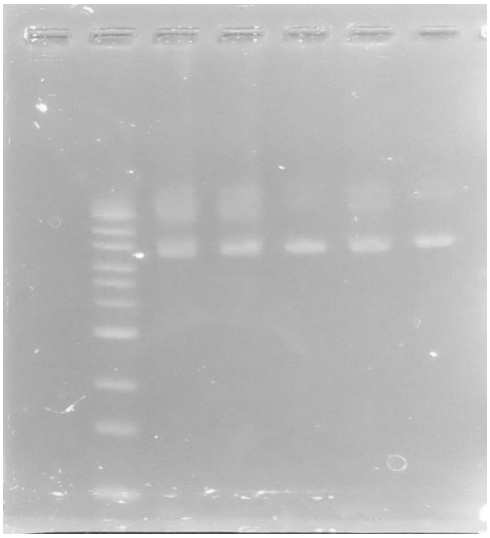


FIGURE 5. ELECTROPHORESIS OF PHAGE REPLICATION FORM OF DNA.

Two examples of DNA from our phage clones run on an agarose gel to determine their molecular weights and to show the nucleic acids were not degraded during purification. Briefly, the RF DNA was produced using Wizard plus SV minipreps. A 1% agarose gel (which contains gel green dye) was put into the electrophoresis apparatus with the wells facing the cathode. TBE Buffer was added to the wells until gel was covered. 2ul 6X DNA loading dye was mixed with 5ul

of DNA samples and loaded onto the gel. Our gel had 6 wells: 2 (which was the well with ladder of 1 kilo-base pair), 3 (phage clone 2), 4 (phage clone 4), 5 (phage clone 5), 6 (phage clone 6) and 7 (phage clone 7). The gel was run for 30 minutes at 100 V or until the first the gel ran off. After 30 minutes of electrophoresis, the apparatus was turned off and result was visualized using PrepOne Illuminator. The phage RF molecular weights corresponded to the weights expected for M13KE = MW 6407 bp.

Sequence MMT5-4 Consensus

GGAATACATATAAGTGATAACATTTCAGGCCCATTTACTACTCGTCTTGTGTTCTCGTCG
GGGCAGCCTATTCACTGAATGAGCAGCCTTGTTACGTTGATTTGGGTAATGAATATCCGG
TTCTTGTCAAGAATACTCTGATGAAGGTCAGCCAGCCTATGCGCCTGGTCTGTACACCGK
TCATCKGTCCTCTTTCAAAGTTGGTCAGTTCGGTTCCCTTATGATTGACMGTCTGCGCCT
CGTTCCGGCTAAGTAACATGRAGCAGGTCGCGGATTTCGACACAATTTATCAGGCGATGA
TACAAATCTCCGTTGTACTTTGTWTCGCGCTTGGTATAATCGCTGGGGGTCAAAGATGAG
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GTATTTTACCCGTTTAATGGAAACTTCCTCATGAAAAAGTCTTTAGTCCTCAAAGCCTCT
GTAGCCGTTGCTACCCTCGTTCCGATGCTGTCTTTCGCTGCTGAGGGTGACGATCCCGCA
AAAGCGGCCATTTAACTCCCTGCAAGCCTCAGCGACCGAATATATCGGTTATGCGTGGGC
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GAAAGCAAGCTGATAAACCGATACAATTAAAGGCTCCTTTTGGAGCCTTTTTTTTGGAGA
TTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACT
CTGGTCATTGTGGTTCGAATACTGGTGGAGGTTCGGCACGGWAACTGTTGAAAAGTGMTT
TAGCAGAATCCCATACAGCCCCATCTCATAAGTAAGCCTGCGTCAGACAGCACAAAATAG
TT

Translation of seq 4

PLVVPFYSHSGHCGSNTGGGSARXLLKSXLAESHTAPSHK-ACVRQHKIV

Characteristics of amino acids

Ser Ala Arg X Leu Leu Lys = 0H+XHH+ (H = hydrophobic, + = polar with positive charge, 0 = polar uncharged)

FIGURE 6: DNA Sequencing of Phage Clones. Using the GenomeLab™ Dye Terminator Cycle sequencing with Quick Start kit, DNA sequencing was performed. The volumes of DNA were pipetted into a 0.2 tube as follows: MMT5-2= 1.3ul; MMT5-4= 1.5ul; MMT5-5=8.8ul; MMT5-6= 1.6ul; MMT5-7= 8.8ul and the volumes brought up to 10 µl with nuclease-free water and heated at 96°C for 1 min. Forward and reverse reactions were performed using primers supplied by the library. The master mix (8 µl) contained DNA polymerase1,4, deoxyribonucleotides and four fluorescently-labelled ddNTPs. The tubes (one forward and one reverse) were loaded in the thermocycler and run for 96^0C for 20secs (to denature DNA); 50^0C for 20secs (primer annealing); 60^0C for 4 mins (extension). First three steps were repeated 29

times to get a total of 30 cycles. Samples were sent to Genomics/Proteomics lab for sequencing. The highlighted sequence represents the 7 amino acid peptide displayed on the phage.

(Kobayashi et al., 2015). Christensen and coworkers (Christensen et al., 2001) also reported that many methicillin-resistant *S. aureus* (MRSA) strains have developed resistance genes to other antibiotics, such as erythromycin, gentamycin and quinolones. They further reported that vancomycin, which is considered to be antibiotic of the last resort, has become ineffective against most strains of *S. aureus*. Therefore, antibiotic therapy against *S. aureus* is proven to be unreliable. To develop a more efficient therapy, scientists have tried using *S. aureus*-specific antibody. Capsular polysaccharide types 5 and 8 have been discovered to play a major role in phagocytosis evasion, since the capsule covers complement deposited on the cell wall, preventing phagocytosis (Kampen et al., 2005). *S. aureus* capsule-specific antibodies bind to the bacterium in the blood stream and stimulate white blood cell phagocytosis of the bacterium (Kampen et al., 2005). *S. aureus*-specific antibody can be given to treat infection; however, it only works for a short time and patients will not produce their own immune response (Nian et al., 2016). Moreover, data from clinical tests did not show any improvement with only antibody treatment.

Identifying a peptide from a phage library via phage display technology will allow identification of *S. aureus* in the blood (Rao et al., 2013) and, if coupled to a toxin, could potentially bring about a more effective and long-lasting treatment (Mullen et al., 2006), as the peptide would be too small to stimulate an immune response. The identification of a surface molecule of microbial pathogens is crucial in the development of therapeutic treatments. Thus, phage display technology is needed to direct phage-displayed peptides against epitopes – molecular structures found on bacterial agents

(Mullen et al., 2006). Phage display involves inserting the gene of a peptide of interest into the phage genome and direct display of such peptide on the surface of phage (Williats, 2002). We hope, in future studies, to insert our selected peptide plus toxin into the phage genome, to be produced and used against *S. aureus*.

In a previous study, carried out in Fagan's lab, Martin Maratani identified phage clones that could bind to whole *S. aureus* bacteria (Maratani, 2017). The PhD.7 phage library (New England BioLabs) was used to select for peptide that could bind to *S. aureus*. Bovine serum albumin (BSA) was used as a block to prevent non-specific binding of phage. BSA was also important in preventing non-specific binding during the ELISA. Amplification and titering of phage were done after each round of biopanning to increase the number of phage. Plaques were selected during titering after three rounds of panning and grown in *E. coli* to produce phage clones. ELISAs were performed against whole cell formalinized and trypsinized *S. aureus* and showed specific binding to S. aureus, with little binding to *S. epidermidis* and no binding to *E. coli*. Since MMT5-2, 4, 5, 6 and 7 bound preferentially in the previous studies, we isolated them for our work. These clones were not tested for specific binding to capsule.

The selected phage clones were tested for viability and specificity in this study, before and after amplification and PEG purification. Amplification was performed by infecting *E. coli* with phage, the bacteria removed by centrifugation and the amplified phage precipitated using a PEG/NaCl solution on ice and pelleted by centrifugation. The phage pellet was resuspended in TBS prior to performing titers to quantitate the phage numbers (Table 1). Phage amplification is a method of increasing the number of phage

clones selected in order to get sufficient number of phage for ELISA and sequencing, while PEG purification removes impurities from the broth culture.

Phage titering is used to determine the number of viable phage after amplification and this process was performed similarly to the methods used previously (Maratani, 2017). To perform a titer, we prepared an overnight culture of *E. coli* by inoculating LB medium containing tetracycline. Tetracycline is added to the LB medium to select for F^+ cells, since the F factor gene is what makes the bacteria strain resistant to tetracycline and is also needed for the expression of pili. The pIII capsid protein of M13 phage binds to the pili to facilitate entry into the bacterium. The overnight culture was then inoculated with PEG-purified phage and the mixture transferred to a glass tube containing top agar plus Xgal and IPTG. IPTG is an inducer that enables phage expression of beta galactosidase (an enzyme that helps breakdown beta galactosides into simple sugars) and Xgal is a colorimetric lactose substrate which is acted upon by beta galactosidase to produce blue plaques in the top agar. The top agar, which is a soft agar, enables the phage clones become localized at a fixed point, thus restricting their free movements. Plaque forming units was obtained by counting the blue plaques and multiplying the number by the phage dilution.

ELISAs were performed testing for phage in culture supernatant's ability to bind to whole cell *S. aureus* (formalinized and trypsinized bacteria) to determine if phage previously cloned in the lab (Maratani, 2017) were viable and retained the peptide and thus the specificity for binding to *S. aureus* that was demonstrated previously. Maratani was able to show specific binding to *S. aureus* for clones MMT5-2, MMT5-4, MMT5-5,

MMT5-6, and MMT5-7 (Maratani, 2017). Initial experiments in this study tested culture supernatants from all ten clones (Figure 1). While considerable variability in binding was seen, specific binding to *S. aureus* was demonstrated for clones MMT5-1, MMT5-4, MMT5-6, and MMT5-10. Clones MMT5-1 and MMT5-10 had very high binding to the wells that did not contain bacteria, so much of our subsequent work was focused on clones MMT5-4 and MMT5-6.

Reasons for the variability seen in the replicas could be inaccurate measurements of sample volumes, improper adjustments of pipette tips and pipettors, the presence of foreign particles in test samples during preparation. Low sample availability limited the number of replicas tested. In future studies, larger phage preparations will be needed to increase the number of replicas. More replicas will be needed to determine the significance of the data. In addition, Rao and coworkers (Rao et al., 2013) reported that a change in pH was a factor in determining the binding specificity of *S. aureus* specific peptide. The peptide was suspended in phosphate buffered saline of pH ranging from 3.0 and 10.0 and, it turned out that the binding specificity of the peptide was between 6.0 and 8.0 but binding was strongest at 7.0. We used PBS (Tween-20) in our ELISAS during the washing steps without determining its pH. While the buffer should be a neutral pH, if the pH were more acidic it might be another cause for poor binding of phage clones.

The formalinized and trypsinized bacteria for the ELISAs is prepared as needed and we were not using the same preparation used by Maratani (Maratani, 2017). To determine if our T5 bacterium we were using for the ELISA experiment was causing us to

have fewer specific clones, we tested two types of T5 preparations: MMST5 and ZKST5. Our phage clones for this test were culture supernatant MMT5-4 and MMT5-10. MMT5-4 bound specifically to the previous bacteria sample, while MMT5-10 had a large amount of non-specific binding to the well without bacteria (Figure 1). In these experiments (Figure 2) MMT5-10 bound to *S. aureus* with high background; MMT5-4 showed no binding against any of the bacteria antigen. Both preparations were prepared previously and stored frozen. It is possible that in future tests a fresh preparation of bacteria would produce more consistent results.

Phage were amplified and PEG purified to improve the phage preparation tested. PEG purified phage were tested for binding to whole cell bacteria (Figure 3) and to purified capsular carbohydrate (Figure 4). In these experiments only clones MMT5-2, MMT5-4, MMT5-5, MMT5-6, and MMT5-7 were tested. In the whole cell ELISAs, all the tested clones bound to *S. aureus*, however, MMT5-6 and MMT5-7 had high nonspecific binding to wells that did not contain bacteria. Due to limited availability of purified carbohydrate, only clones MMT5-4 and MMT5-6 were tested for binding to purified carbohydrate. MMT5-4 bound specifically to *S. aureus* but had high background. It is likely that the background could be remedied by changing the wash conditions in future studies. MMT5-6 had high nonspecific binding in both the whole cell and carbohydrate ELISAs. It is possible that this clone is binding to some component in the block or to exposed plastic in the wells.

This study was primarily centered on finding the phage peptide, a 7-amino acid peptide, that could bind to the capsular polysaccharide of *S. aureus*. In other studies by

Rao and coworkers (Rao et al., 2013), phage containing a 12-amino acid peptide (SA5-1), selected from phage library via bio-panning, was taken through an ELISA test to confirm its binding specificity to the whole cell *S. aureus*. SA5-1, bound strongly to *S. aureus* relative to other bacteria. Ten clones from SA5-1 were sequenced and a consensus sequence for binding to *S. aureus* was determined. To determine the peptide sequence for our clones, phage clones MMT5-2, 4, 5, 6 and 7 were selected for DNA purification. M13 DNA purification was done to isolate the phage RF DNA from the bacterium. We followed the manufacturer's protocol (*Wizard® Plus SV Minipreps DNA Purification System*) to purify our phage DNA and further proceeded to carry out their measurements using nanodrop technology (Table 2). A repeat of this experiment was made because there was protein contamination in MMT5-5 and MMT5-7. The reason for such impurity could be that the MMT5-5 and 7 DNA preparations were performed using a too large sample volume (10 ml) and the columns were overloaded. Following the second purification, the DNA concentration of MMT5-5 and 7 were measured and accurate values were obtained (Table 3). The results displayed in both Tables 2 and 3 indicate the absorbance of ultraviolet light and the concentration of the double stranded RF DNA of the phage clones. We analyzed our purified DNA samples using agarose electrophoresis to determine their molecular weights and to show whether their nucleic acids were not degraded during purification (Figure 5). The results show that the molecular weight of the nucleic acids corresponded to that expected for M13KE, the phage used to construct the library.

The selected phage clones MMT5-2, 4, 5, 6 and 7 were sequenced using the GenomeLab™ Dye Terminator Cycle sequencing with Quick Start kit. Forward and reverse primers were supplied with the PhD-7 library. DNA analysis was performed by Ed Budde, a Molecular Biology Instrumentation Specialist in the Molecular Biology Analytical Core Laboratory at Youngstown State University, OH. Following sequencing, Most of the clones did not produce translatable sequence and a repeat experiment will be needed using new primers. The library primers were only oriented in one direction. We will develop new primers in both directions for future studies. Phage clone 4 produced good sequence and was selected for translation. After translating the DNA sequence 4, it appeared the amino acids of the peptide are hydrophobic or polar, with two of the amino acids having a positive charge. The translated sequence for the seven amino acid peptide displayed on MMT5-4 is Ser-Ala-Arg-X-Leu-Leu-Lys. The consensus sequence found by Rao and coworkers (Rao et al., 2013) was VPHNPGLISLQG containing the LQX motif. Our peptide did not show the LQX motif, however the Rao consensus sequence contained primarily hydrophobic or positively charged amino acids, as was seen in our peptide sequence for MMT5-4.

In conclusion, a phage clone was identified with a peptide that could bind to the purified carbohydrate of *S. aureus*. Phage clone MMT5-4 was selected and sequenced. MMT5-4 had both hydrophobic and polar nature with an amino acid sequence of SARXLLKS. CP5 capsule is well-known for its ability to prevent phagocytosis and plays an important role in *S. aureus* pathogenesis (Kampen et al., 2005). Developing a CP5 specific peptide is an

important step toward identifying *S. aureus*. In future studies a toxin could be coupled to this peptide and used as a method for targeting and killing *S. aureus.*

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