Cloning Strategies Toward the Development of a Single Chain Variable Fragment (scFv) Specific for *S. aureus* Type 5

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ABSTRACT

Staphylococcus aureus (S. aureus) is an opportunistic pathogen that is frequently encountered in hospital settings. It is a gram-positive, cluster forming bacteria that causes a variety of infections in humans, including bacteremia. The control of *S. aureus* infections has become complicated because bacteria have become resistant to a wide range of antibiotics. In these studies we attempted to create a single chain variable fragment (scFv) specific for *S. aureus* Type 5 capsular polysaccharide (CP) using phage display technology. Hybridomas specific for type 5 *S. aureus* were grown and total RNA was isolated. cDNA was synthesized and amplified by PCR. Mouse IgM light chain primers (with *Sac*I and *Hind*III restriction sites) and heavy chain primers (with *Xho*I and *Spe*I restriction sites) were used to PCR antibody cDNA and the resulting DNA was purified. Restriction digests of purified TOPO plasmid and PCR products were ligated together, which produced a band about 350-450 base pairs in length. Sequence analysis was performed and the resulting sequence matched a ribosomal protein, not the antibody fragment we were attempting to clone. We believe that the primers that were used to sequence the antibody fragments were too degenerate and thus led to this nonspecific sequence being primed. Future studies will include purifying IgM protein from different hybridomas to determine the amino acid sequence. Using this sequence, more specific primers will be designed to PCR amplify the cDNA from the heavy and light chain of different clones creating a scFv. The scFv will be amplified, cloned into a phagemid vector, and transformed into competent *E.coli*. Helper phage infection should yield recombinant phage expressing the scFv fused to phage g3p (pIII). These recombinant antibodies have the potential of being used to treat *S. aureus* infections.

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LIST OF ABBREVIATIONS

AMP	Ampicillin
BLAST	Basic Local Alignment Search Tool
β-ME	β-Mercaptoethanol
BSA	Bovine serum albumin
CAM	Chloramphenicol
CHO	Carbohydrate
CP	Capsular polysaccharide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethyl alcohol
HAT	Hypoxanthine Aminopterin Thymidine
IgM	Immunoglobulin M
KCl	Potassium chloride
LB	Luria-Bertani
MOPS	3-(N-morpholino) propanesulfonic acid
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
PCR	Polymerase chain reaction
scFv	single chain variable fragment
TAE	Tris-Acetate-EDTA

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I. INTRODUCTION

Statement of the Problem

Staphylococcus aureus (*S. aureus*) is an opportunistic pathogen that is frequently encountered in hospital settings (Durand *et al.*, 2006, Peschel *et al.*, 2001, Ribeiro *et al.*, 2005, Smith and Jarvis, 1999). It is a gram-positive, cluster forming bacteria that causes a variety of infections in humans, including bacteremia (Fattom *et al.*, 1990). These infections are common in humans that are immunocompromised, meaning they have weak immune systems and trouble fighting antimicrobial resistant strains. The control of *S. aureus* infections has become complicated because bacteria have become resistant to a wide range of antibiotics, including vancomycin (Lowy, 1998, Peschel *et al.*, 2001, and Senior, 2002), which is a glycopeptide antibiotic produced by *Streptomyces orientalis* that is used in severe cases of staphylococcal infections when all other antibiotics fail to kill the bacteria. Heavy use of antibiotics accelerates the onset of mutations in bacteria leading to antibiotic resistance and making it more difficult to rid the body of infections (Smith and Jarvis, 1999).

Most gram-positive bacteria secrete a layer of polysaccharides called the glycocalyx, which serves as a buffer between the cell and the external environment. When the glycocalyx is thick and tightly connected to the cell it is called a capsule (Pommerville, 2004). Clinical isolates of *S. aureus* have been shown to possess more than 11 capsular polysaccharide (CP) types, with types 5 and 8 being the most abundant (Arbeit *et al.*, 1984, Fattom *et al.*, 1998, Lowy, 1998, Thakker *et al.*, 1998). Previous studies have shown that the capsule of pathogenic strains of *S. aureus* diminishes phagocytosis of the bacteria (Cunnion *et al.*, 2003). They established the importance of complement for mouse survival of CP Type 5 bacteremia and also confirmed that the binding of opsonic fragments (which enhance the action of phagocytes on foreign cells) to mice was inhibited by the Type 5 capsule. This inhibition of opsonin binding caused by the presence of the capsule dramatically decreased phagocytosis and increased the virulence of the pathogen.

Type-specific monoclonal antibodies that specifically bind to the capsular polysaccharide of serotypes 5 and 8 *S. aureus* have been produced (Nelles *et al.*, 1985). These anti-Type 5 and anti-Type 8 monoclonal antibodies were tested against clinical isolates of homologous capsular *S. aureus* to see if they would react and bind to the appropriate capsule and also against the heterologous capsular type and a number of gram-negative bacteria to see if they would cross-react with other gram-positive *S. aureus* or other non-specific bacteria. They found that these anti-type specific monoclonals reacted with isolates possessing the homologous capsular type of *S. aureus* and did not cross react with the heterologous capsular type or the gram-negative bacteria. This led to the conclusion that these monoclonal antibodies are specific for Type 5 and Type 8 *S. aureus* CP.

Monoclonal antibodies are antibodies that are specific for only one epitope (part of an antigen recognized and bound by an antibody) on a complex antigen (Alberts *et al.*, 2002). They are produced from a single clone by isolating mouse spleen cells and fusing them to myeloma cells creating hybrid cells called hybridomas (Ingraham, 2004). These hybridomas express the antibody genes of normal B-cells or T-cells and also continue to grow indefinitely like cancer cells. B-cell hybridomas that have been cloned (one cell grown to produce a homogenous population) secrete monoclonal antibodies that are used as diagnostic, imaging, and therapeutic reagents in clinical medicine (Goldsby *et al.*, 2003). Our lab has produced hybridoma clones that are specific for *S. aureus* Type 5 CP and Type 8 CP in mice (Slusher, 2005). HAT selection (Morgan and Darling, 1988) was used to kill the myeloma cells that were not fused to spleen cells. MRC 5 feeder cells were used to grow the hybridoma cells and the hybridomas were cloned by rapid limiting dilution (Harlow and Lane, 1988). These hybridomas have the capability of binding specifically to *S. aureus* (Type 5 CP and Type 8 CP). The disadvantages of using these and other hybridomas as therapeutic tools are that they are difficult and expensive to grow, structurally unstable, and easily contaminated. Because of these problems associated with monoclonals, different methods of antibody production are needed. One such method is phage display technology (Hombach *et al.*, 1998), which uses genes that encode the antigen binding site of monoclonal antibodies to produce a phage display library of anti-capsular antibody fragments.

Significance of the Research

Phage display is an alternative method to monoclonal antibodies that results in the production of high affinity binding antibodies. It is a molecular technique that was introduced by G.P. Smith in 1985 that involves the expression of proteins, including antibodies, on the surface of filamentous phage (Viti *et al.*, 2000). It is an efficient way of producing large numbers of diverse proteins and peptides and isolating molecules that perform specific functions (Willats, 2002). The specific DNA sequences of interest are inserted into a location in the genome of filamentous bacteriophage so that the protein is expressed on the surface of filamentous phage as a fusion product with one of the phage coat proteins (Figure 1). The phage consists of circular single stranded DNA that is surrounded by the major coat protein g8p (pVIII) (Azzazy and Highsmith, 2002). The genes that encode the variable domains of the protein of interest are fused to gene III (g3). The coat protein, g3p (pIII), one of the minor coat proteins (encoded by g3), is located at the tip of the phage and is where the protein of interest is displayed as a fusion product with g3p (pIII) (Pistillo *et al.*, 1997, Arap, 2005). A phage display library is a collection of antibody-covered phage containing several billion peptide variants in which each phage expresses only one antibody-like molecule (Yan *et al.*, 2004). These libraries can be used to purify and select specific phage particles that have sequences of interest with desired binding specificities (Willats, 2002).

Fv fragments are the smallest antibody fragments that still retain the entire antigen binding site (Maynard and Georgiou, 2000). A scFv is a single chain form of the variable region of an antibody. It is composed of a light chain variable region and a heavy chain variable region connected by a linker peptide (Willats, 2002) (Figure 2). ScFv's are produced using phage display technology. Genes of the light and heavy variable chains of antibodies are prepared by reverse transcription of messenger RNA (mRNA) from B-lymphocytes (Brichta *et al.*, 2005). The resulting gene products are then amplified and made into a single gene (scFv) using a peptide linker fragment (Maynard and Georgiou, 2000). This assembled scFv is then amplified by PCR, cloned into a phagemid vector, and introduced into competent *E.coli* by transformation (Viti, 2000). Phagemid containing bacterial cells are then infected with a helper phage, such as M13, by a process called phage rescue (Kramer *et al.*, 2003). This yields recombinant phages that express the scFv. The expression of a scFv results in recombinant antibodies fused to the g3p (pIII) protein at the tip of a phage (Hombach *et al.*, 1998). These recombinant antibodies are antigen specific and have the potential of being used to treat a number of diseases and infections in humans, including *S. aureus*.

Figure 1: Filamentous phage displaying scFv molecules.

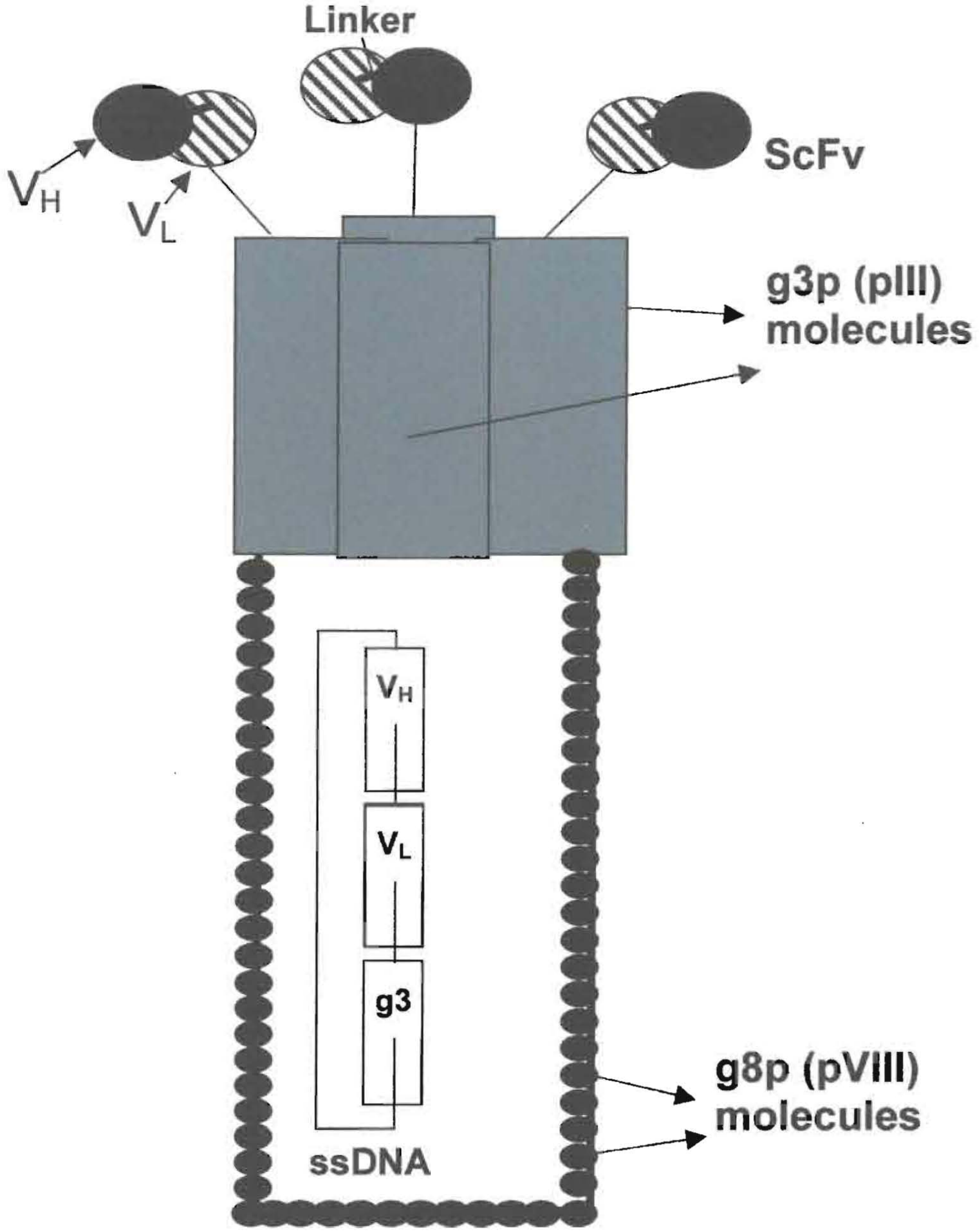
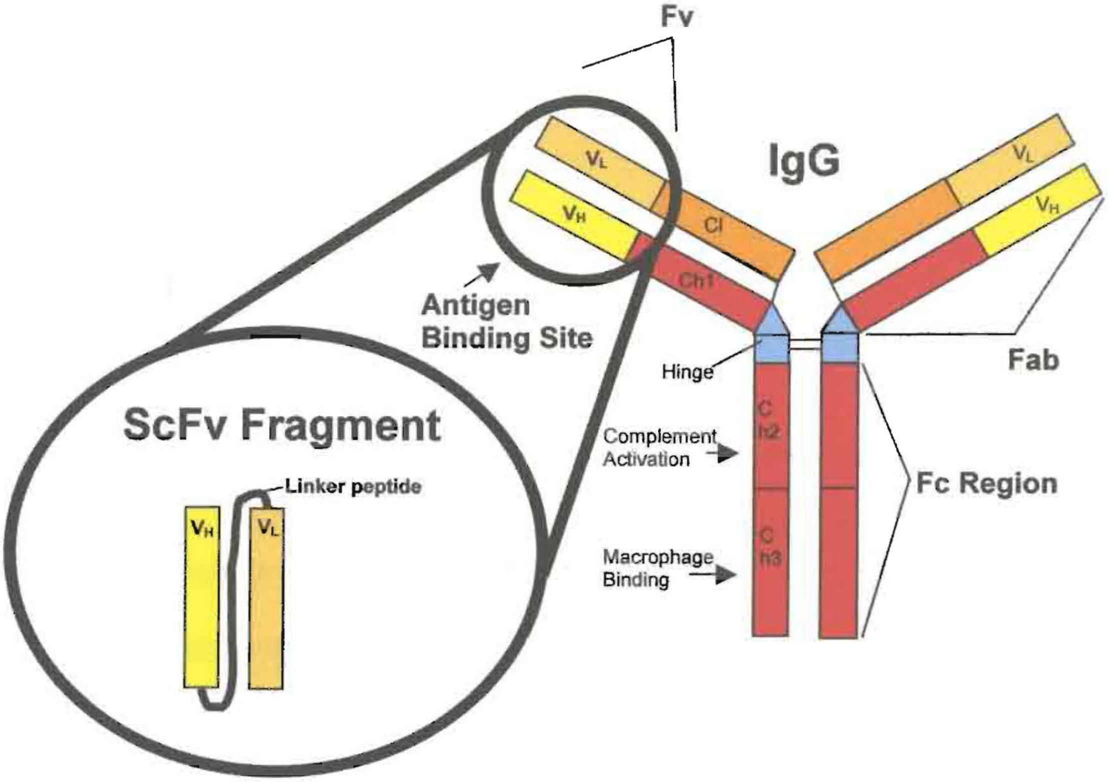

Linker
V_H
V_L
ScFv
g3p (pIII) molecules
V_H
V_L
g3
ssDNA
g8p (pVIII) molecules

Figure 2: Shows a scFv fragment of an IgG. It is composed of a light and heavy chain variable region connected by a linker peptide.


Fv
IgG
V_L
V_H
Cl
Ch1
V_L
V_H
Antigen Binding Site
Hinge
Fab
ScFv Fragment
Linker peptide
V_H
V_L
Complement Activation
Ch2
Macrophage Binding
Ch3
Fc Region

Purpose and Objectives

Purpose:

Produce a scFv that will bind tightly to *Staph. aureus* Type 5 capsular polysaccharide.

Objectives:

- Purify plasmids pcomb3-C and pSCL.
- Isolate RNA from hybridoma clones of SM.T5.B2.A12.C9 cells.
- Convert the RNA into cDNA
- PCR amplify the light and heavy chain variable regions
- Ligate the light and heavy chains into phagemid pSCL
- Analyze fragments by sequencing

II. MATERIALS

Hybridoma clone SM.T5.B2.A12.C9 was obtained from a previous graduate student in Dr. Fagan's laboratory (Slusher thesis, 2005). Phagemids pSCL and pComb3-C were obtained from Dr. Jeff Smiley, Professor of Chemistry, Youngstown State University (Smiley and Benkovic, 1994). Chemically competent XL1 Blue *E. coli* cells (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]) were purchased from Stratagene (La Jolla, CA). Tryptone and yeast extract were purchased from Difco Laboratories (Detroit, MI). Molecular weight marker (1 kb) was purchased from Amresco Inc. (Solon, OH). TOPO cloning kit, P3X media, fetal bovine serum, L glutamine, and RPMI were purchased from Invitrogen/GIBCO BRL Corporation (Carlsbad, CA). Heavy chain, light chain, and sequencing (*lac*P, -28 gIII, and -96 gIII) primers were purchased from Integrated DNA Technologies (Coralville, IA). CEQ 2000 Dye Terminator Sequencing Quick Start kit was purchased from Beckman Coulter, Inc. (Fullerton, CA). Agarose, NaCl, glucose, EDTA, Tris, and glycerol were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). RNeasy isolation kit, QIAquick PCR purification kit, and plasmid preparation kit were purchased from Qiagen, Inc. (Valencia, CA). DNA ligase T4, ligation buffer, 100 bp molecular weight marker, *Taq* polymerase and *Taq* polymerase buffer, cDNA First Strand Synthesis kit, and restriction enzymes *SacI*, *HindIII*, *SpeI*, and *XhoI* were purchased from New England Biolabs (Ipswich, MA). Agar, Chloramphenicol (CAM), Ampicillin (AMP), glacial acetic acid, ethanol, ethidium bromide, gel loading buffer, and NaOH were purchased from Fisher Scientific, Inc. (Fair Lawn, NJ). Plasmid miniprep kit was purchased from Eppendorf, Inc. (Westbury, NY).

III. METHODS

A. LB plates with Cloramphenicol (CAM)

LB media (0.5 L) was made containing 5g of tryptone, 2.5g of yeast extract, and 2.5g of NaCl (Ausubel *et al.*, 1997). The media was adjusted to pH 7.4 with NaOH. This LB media (200 mL) was put into a 1L Erlenmeyer flask and the other 300 mL was put into six 250 mL Erlenmeyer flasks with 50 mL in each. Using one of the flasks containing 50 mL of LB media, ten tubes was made with each tube containing 5 mL of LB media. All of these flasks were sterilized by autoclaving for 20 minutes. Glucose stock (20%) was made, autoclaved, and stored at -20°C. Chloramphenicol (CAM) (20mg/mL) was dissolved in EtOH with vortexing. Finally, LB plates were made. Agar (1.5%) was added to an Erlenmeyer flask, autoclaved, and cooled to 50°C. CAM (0.1%) and glucose (2%) were added to the LB media-agar mixture and plates were poured.

B. Transformation of Competent Cells

Frozen chemically competent XL1 Blue cells (Stratagene, La Jolla, CA) (100µl), treated with CaCl, were thawed and transferred immediately into test tubes containing DNA from pSCL and pComb3-C (Smiley and Benkovic, 1994). The tubes were gently swirled to mix and then placed on ice for 10 minutes. The cells were then heat shocked by placing the test tubes into a 42°C water bath for 2 minutes. LB media (1 ml) was added to each tube and each tube was placed onto a roller drum incubator at 250 rpm for 1 hour at 37°C. Aliquots of the transformation culture were plated on the LB/CAM containing plates. Transformation mixture was spread on 2 plates (100µl or 20µl of each). After the plates dried, they were incubated overnight at 37°C. Colonies were then counted for each of the eight LB plates used to demonstrate growth of bacteria transfected with CAM resistant plasmids.

C. Purification of Plasmids pComb3-C and pSCL

Plasmids were purified using Qiagen's (Valencia, CA) Plasmid Midi DNA Purification Kit (Birnboim and Doly, 1979). A single colony from a freshly streaked selective plate was used to inoculate a starter culture of 2-5 ml of LB medium containing the appropriate selective antibiotic. This was incubated for 12-20 hrs. at 37°C with vigorous shaking (~300 rpm). The starter culture was then diluted 1/500 to 1/1000 into 50 ml of selective LB medium and grown at 37°C for 12-16 hr. with vigorous shaking (~300 rpm). The bacterial cells were harvested by centrifugation at 6000 X *g* for 15 min. at 4°C.

The pellet that is produced was resuspended in 6 ml of Buffer P1 [resuspension buffer (Qiagen kit)], which contains 50 mM Tris-Cl, pH 8.0, 10 mM EDTA, and 100 μg/ml RNase A. Buffer P2 (6 ml of lysis buffer), which contains 200 mM NaOH; 1% SDS (w/v), was added to the mixture and mixed thoroughly by inverting 4-6 times. This mixture was incubated at room temperature for 5 min. Chilled buffer P3 (6 ml of neutralization buffer), containing 3.0 M potassium acetate, pH 5.5, was added to the lysate and mixed immediately by gently inverting 4-6 times. The lysate was then poured into the barrel of the QIAfilter cartridge and incubated at room temperature for 10 minutes.

A HiSpeed Midi Tip was equilibrated by applying 4 ml of buffer QBT (Equilibration Buffer), which contains 750 mM NaCl; 50 mM MOPS, pH 7.0; 15%

isopropanol (v/v); 0.15% Triton X-100 (v/v). The cap from the QIAfilter outler nozzle was removed. The plunger was then gently inserted into the QIAfilter Midi Cartridge and the cell lysate was filtered into the equilibrated HiSpeed Tip entering the resin by gravity flow. The HiSpeed Midi Tip was washed with 20 ml (2 X 10 ml) Buffer QC (Wash Buffer) containing 1.0 M NaCl; 50 mM Tris-Cl, pH 7.0; 15% isopropanol (v/v). Then the DNA was eluted with 5 ml of Buffer QF (Elution Buffer), which contains 1.25 M NaCl; 50 mM Tris-Cl, pH 8.5; 15% isopropanol (v/v), and precipitated by adding 3.5 ml (0.7 volumes) of room-temperature isopropanol to the eluted DNA. This was mixed and incubated at room temperature for 5 minutes.

The QIAprecipitator was placed over a waste bottle and the eluate/isopropanol mixture was transferred into a 20 ml syringe and filtered through the QIAprecipitator using constant pressure. The DNA was then washed by adding 2 ml of 70% ethanol through the QIAprecipitator. Then the membrane and the outlet nozzle of the QIAprecipitator was dried to prevent ethanol carryover. Buffer TE (1ml) containing 10 mM Tris-Cl, pH 8.0, and EDTA (1 mM) was added to a 5 ml syringe to elute the DNA into a collection tube again using constant pressure. Absorbancies (A_{260} and A_{280}) of the purified plasmids, pSCL and pcomb3-C, were taken and an agarose gel was run using electrophoresis.

D. Agarose Gel Electrophoresis

Powdered agarose (1%) was added to 50 ml of Tris-acetate (TAE) electrophoresis buffer (containing 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) and heated until the agarose dissolved. The solution was cooled to 50°C. The edges of a clean, dry, glass plate were sealed with autoclave tape to form a mold. When the seal is set, the warm agarose solution was poured into the mold and the comb was immediately clamped into position near one end of the gel, the teeth of which will form the sample wells. After the gel was completely set (about 30-45 minutes at room temperature), the comb and the autoclave tape was carefully removed and the gel was put into the electrophoresis tank. Electrophoresis buffer was then added to cover the gel to a depth of about 1 mm. Samples were mixed with loading buffer (containing 5-10% glycerol, 7% sucrose, and 0.025% bromophenol blue) and loaded into the slots of the submerged gel. Loading buffer was made up as a 6-fold to 10-fold concentrated solution, which was mixed with the sample and then slowly applied to the gel using a disposable micropipette, an automatic micropipettor (Eppendorf or Gilson). Plasmid (5μL) and loading buffer (5μL) were mixed, loaded on the gel, and the gel was run at 50-60 mV for 1-1.5 hours. After electrophoresis the gel was placed into a pan containing water and Ethidium bromide (50μl from a stock solution of 10 mg/ml in water, stored at 4°C in a light-proof bottle) for 20 minutes to stain the gel so the bands appear. Finally a photograph of the gel was taken using transmitted UV light.

E. Growing Clones of Monoclonal Antibody SM.T5.B2.A12.C9

Clones of mouse hybridomas producing monoclonal antibodies specific for Type 5 *Staph. aureus* were grown. The first clone that was used, SM.T5.B2.A12.C9, tested positive for binding to T5 CHO and T5 bacteria and negative for binding to T8 CHO and T8 bacteria. This clone was put into a six well culture plate and fed every other day with 2 ml of P3X medium containing 80% RPMI 1640, 20% heat-inactivated fetal bovine serum, and 2 mM glutamine. These clones were expanded and fed until 3 x 10^8 cells were produced. Then they were centrifuged for 10 minutes at 1200 rpm (400 X g) at 4°C. The pellet was frozen at -70°C for future use.

F. Isolation of Total RNA from SM.T5.B2.A12.C9 Cells

Total RNA was isolated using an RNeasy isolation kit (Qiagen Inc., Valencia, CA). SM.T5.B2.A12.C9 cells (1 X 10^8) were harvested and then centrifuged for 5 min. at 300 X g in an RNase-free glass or polypropylene centrifuge tube. The supernatant was removed completely by aspiration and the pellet was dislodged by tapping the tube. The cells were then disrupted by the addition of Buffer RLT (4.0 ml) containing 14.0M β-ME (40 μl). The sample was homogenized by vortexing for 10s and passing the lysate 5-10 times through an 18-20 gauge needle fitted to an RNase-free syringe. One volume (4.0 ml) of 70% ethanol was added to the homogenized lysate and mixed thoroughly by vigorous shaking. The sample was then loaded into an RNeasy midi column placed in a 15 ml centrifuge tube and centrifuged for 5 min. at 3000 – 5000 X g. The flow-through containing Buffer RLT was discarded. Buffer RW1 (4.0 ml) was added to the RNeasy column and centrifuged for 2 min. at 3000 – 5000 X g to wash the column. Again, the flow-through was discarded. Next, Buffer RPE (2.5 ml), containing ethanol, was added to the RNeasy column and centrifuged for 2 min. at 3000 – 5000 X g to wash the column. The flow-through was discarded and another 2.5 ml of Buffer RPE was added to the column and centrifuged for 5 min. at 3000 – 5000 X g to dry the RNeasy silica-gel membrane. To elute the RNA, the RNeasy column was transferred to a new 15 ml collection tube. RNase-free water (250 μl) was pipetted directly onto the RNeasy silica-gel membrane and let to stand for 1 min. Then it was centrifuged for 3 min. at 3000 – 5000 X g. This elution step was repeated using the eluate to obtain a higher concentration of RNA. The absorbance (A_{260} and A_{280}) of the RNA was taken using a spectrophotometer to obtain the concentration.

G. First Strand cDNA Synthesis

A First Strand cDNA Synthesis Kit from New England Biolabs (Ipswich, MA) was used to produce the first strand of cDNA. Total RNA (5 µl) from the SM.T5.B2.A12.C9 cells, primer $dT_{23}VN$ (2 µl), dNTP's (4 µl), and nuclease-free water (5 µl) were mixed and heated for 5 minutes at 70°C. This mixture along with 10X RT buffer (2 µl), RNase inhibitor (1 µl), and M-MuLV reverse transcriptase (1 µl) were mixed and incubated at 42°C for one hour. After incubation the mixture was heated at 95°C for 5 minutes to inactivate the enzyme. RNase H (1 µl) was added and the mixture was incubated again for 20 minutes at 37°C to degrade the RNA. Then, the reaction mixture was heated at 95°C for 5 minutes to inactivate the enzyme. Finally, the reaction was diluted to 50 µl with water and amplified by Polymerase Chain Reaction (PCR).

H. Primers specific for IgM mouse antibody light and heavy chain variable regions

Primers were designed that are complementary to the consensus antibody sequences (Okamoto, *et al.*, 2004) at the 3' ends and contained the desired restriction sites at the 5' ends. Primers contained three parts: the first part is made up of insignificant nucleotides, the second part contains the restriction site, and the third part is the sequence encoding protein that is complementary to the antibody sequence of the cDNA. All primers contained the first five bases AAGGG at the 5' end that ensured that the restriction enzymes would be able to digest the DNA. Light chain 5' primers contained the restriction site GAGCTC for the restriction enzyme *Sac*I, while light chain 3' primers contained the restriction site AAGCTT for the restriction enzyme *Hind*III (Table 1). Heavy chain 5' primers contained the restriction site CTCGAG for the restriction enzyme *Xho*I, while 3' heavy chain primers contained the restriction site ACTAGT for the restriction enzyme *Spe*I (Table 2).

I. PCR of heavy and light chain variable regions of antibody cDNA

PCR was done to amplify the antibody cDNA from SM.T5.B2.A12.C9 cells using the light chain and heavy chain primers listed in Table 1 and Table 2. The first step in the PCR process was done one time for 2 minutes at 95°C. The second step was done at 95°C for 30 seconds (DNA strands separate), 50°C for 60 seconds (primers bind), and 72°C for 90 seconds (polymerase makes DNA) for 35 cycles. The final step, the extension step, was done one time for 5 minutes at 72°C. An Eppendorf thermocycler was used for all PCR reactions. Ten light chain PCR reactions were done using a combination of the 5' light chain primers and the 3' light chain primers in each reaction, while eighteen heavy chain reactions were done using combinations of the 5' and 3' heavy chain primers. Each reaction was composed of sterile water (14.5 µl), *Taq* buffer with Mg2+ (10 mM KCL, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCL, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 @ 25°C), dNTP's (2.5 mM dATP, 2.5 mM dGTP, 2.5 dTTP, 2.5 mM dCTP, in water titrated to pH 7.0 with Tris-HCl), cDNA from

SM.T5.B2.A12.C9 cells (.25 µl), *Taq* polymerase (1.25 Units), 5' primer (4 nM), and 3' primer (4 nM) for a total volume of 25 µl.

J. Purification of DNA from PCR reactions

The DNA from the PCR reactions was purified using a microcentrifuge and a PCR purification kit (Qiagen, Valencia, CA). Buffer PB (binding buffer containing guanidine hydrochloride and isopropanol) (5 volumes) was added to the PCR sample (1 volume) and mixed. A QIAquick spin column was placed in a 2 ml collection tube. To bind the DNA of the PCR sample, the sample was placed in the QIAquick column and centrifuged for 60 seconds. The flow-through was discarded and the QIAquick column was placed back into the same tube. Buffer PE (0.75 ml) was then added to the QIAquick column and centrifuged for 60 seconds to wash the sample. Again the flow-through was discarded and the QIAquick column was placed back in the same tube and centrifuged for an additional 60 seconds. The QIAquick column was then placed in a clean 1.5 ml microcentrifuge tube and the DNA was eluted by adding buffer EB (50 µl), which contains 10 mM Tris-Cl, pH of 8.5 to the center of the QIAquick membrane and centrifuging for 60 seconds.

K. Restriction digests of plasmid pSCL and PCR products

Purified plasmid pSCL and light and heavy chain PCR products were cut with restriction endonucleases so that the light and heavy chain cDNA could be ligated into the vector of pSCL. Light chain restriction digestions contained the PCR products (43 µl), Buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9 @

Table 1: Mouse antibody light chain variable region primers[1]

VL-5' primers
(LC5A) 5'-aag ggG AGC TCa tgg ccG AYA TTG TDH TVW CHC AGT C-3'
(LC5B) 5'-aag ggG AGC TCa tgg ccG AYA TTN WKM TVA HDC AGT C-3'
(LC5C) 5'-aag ggG AGC TCa tgg ccG AYR TYB WRM TSA CMC ARW C-3'
(LC5D) 5'-aag ggG AGC TCa tgg ccG AYA TYS WGM TGA CNC ARB C-3'
(LC5E) 5'-aag ggG AGC TCa tgg ccG AYR YTG TKR TRM YYM RGD W-3'

VL-3' primers
(LC3A) 5'-aag ggA AGC TTC CGT TYN AKY TCC ARC TTD G-3'
(LC3B) 5'-aag ggA AGC TTM CST WBN ABH KYC AVY YTD G-3'

*Note: S = C/G, R = G/A, K = G/T, M = A/C, Y = C/T, W = A/T, H = A/C/T, B = C/G/T, V = A/C/G, D = A/G/T, and N = A/T/G/C.

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[1] Okamoto, *et al.*, 2004

Table 2: Mouse antibody heavy chain variable region primers[1]

VH-5' primers
(VH-51) 5'-aag ggC TCG AGS AKG TBM AGC TBM AGS AST C-3'
(VH-52) 5'-aag ggC TCG AGS AGG TYC ARC TBC ARC ART C-3'
(VH-53) 5'-aag ggC TCG AGS AVG TSM WSB TGR WGS ART C-3'
(VH-54) 5'-aag ggC TCG AGG AKG TGM AVS KGR TGG ART C-3'
(VH-55) 5'-aag ggC TCG AGG ARG TRM ARS TTS WBG AGT C-3'
(VH-56) 5'-aag ggC TCG AGS AKG TBM MNY TVV WVS WRY S-3'

VH-3' primers
(VH-37) 5'-aag ggA CTA GTY GAR GAR ACD STG ASM RKR GT-3'
(VH-38) 5'-aag ggA CTA GTY GAR GAR RMS KKK ASW GWG RT-3'
(VH-39) 5'-aag ggA CTA GTY GAG GAG ACK GTG ASH GDG GH-3'

*Note: S = C/G, R = G/A, K = G/T, M = A/C, Y = C/T, W = A/T, H = A/C/T, B = C/G/T, V = A/C/G, D = A/G/T, and N = A/T/G/C.

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[1] Okamoto, *et al.*, 2004

25°C) from New England Biolabs (NEB) (5 µl), and restriction enzymes *Sac*I and *Hind*III (20 Units each). Heavy chain digestions contained heavy chain PCR products (43 µl), Buffer 2 from NEB (5 µl), and restriction enzymes *Spe*I and *Xho*I (10 Units each). The purified plasmid pSCL was cut with *Sac*I and *Hind*III for light chain ligation and *Spe*I and *Xho*I for heavy chain ligation. pSCL digestions contained pure pSCL (14 µl), sterile distilled H_20 (72 µl), Buffer 2 (10 µl), and restriction enzymes *Sac*I and *Hind*III (40 Units each) or *Spe*I and *Xho*I (20 Units each). All restriction digestions were incubated for 2 hours at 37°C. After incubation, digestions were cleaned up with the same PCR purification kit as mentioned earlier. pSCL was eluted with 40 µl of Buffer EB and the PCR products were eluted with 20 µl of Buffer EB. An agarose gel was used to see if the DNA was purified with the restriction cuts.

L. Ligation of Heavy and Light Chain Fragments into Plasmid pSCL

The light chain and heavy chain sequences of DNA cut with the restriction enzymes were ligated into the plasmid pSCL (Figure 3) using an enzyme called DNA ligase T4 (NEB, Ipswich, MA). This was done in two steps, with the light chain being inserted into pSCL first, then the heavy chain. The reactions contained 10 X concentrated ligation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM Dithiothreitol, 25 µg/ml BSA, pH 7.5 @ 25°C), cut pSCL (4 µl), insert from either the light or heavy chain DNA (3 µl), sterile distilled H_2O (10 µl), and DNA ligase T4 (400 Units). Everything except the DNA ligase was mixed and heated at 37°C for 2 minutes, then the enzyme was added. This reaction occured at room temperature for 1 hour. These ligation reactions were then transformed into competent XL1 Blue cells.

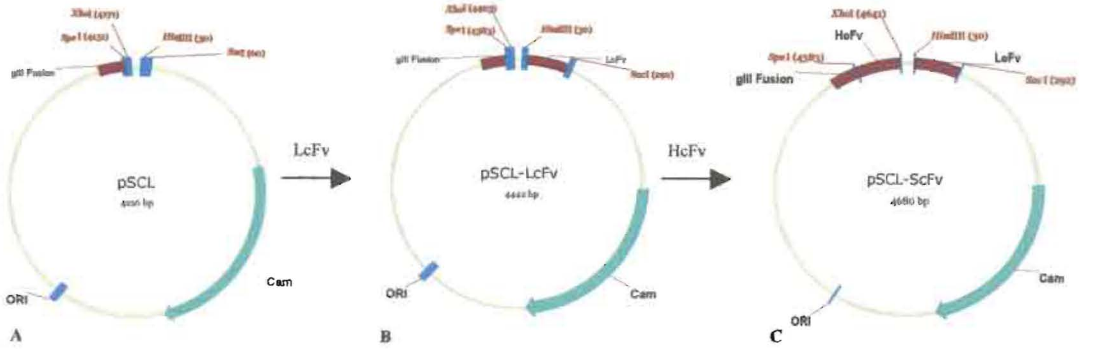
M. Transformation of Ligation Reactions into Competant XL1 Blue Cells

Competent XL1 Blue cells (100 µl) were placed into test tubes containing ligated reactions (10 µl). These tubes were gently swirled to mix the contents and then placed on ice for 10 minutes. After that the cells were heat shocked by placing the tubes into a 42°C water bath for one minute. Then LB media (1 ml) containing glucose (100 µl) was added to each tube as recovery media. Next, the test tubes were placed on a roller drum incubator at 250 rpm for one hour at 37°C. Aliquots (200 µl) of the transformation culture were then plated on LB/CAM containing plates. After the plates dried they were incubated at 37°C overnight. Individual colonies were inoculated into LB media (5 ml) containing CAM (50 µl) and 20% glucose (500 µl) and incubated overnight at 37°C with shaking. Plasmid minipreps were performed and the purified plasmids were cut with restriction enzymes (either *Sac*I and *Hind*III for the light chain or *Xho*I and *Spe*I for the heavy chain) and run on an agarose gel to determine if the light and heavy chain fragments of DNA were inserted into plasmid pSCL. A negative control ligation reaction (without inserts), a positive control of purified uncut pSCL, and XL1 Blue cells alone (no plasmid or ligation reaction) were also be tested to compare to the results of the ligation reaction with inserts.

N. Sequencing

Sequencing reactions were performed to see if the heavy and light chain fragments of SM.T5.B2.A12.C9 were inserted into pSCL. A CEQ 2000 Dye Terminator Sequencing with Quick Start Kit from Beckman Coulter (Fullerton, CA) was used for this process. Preparation of the sequencing reactions was done in a 0.2 mL thin-walled tube.

Figure 3: Construction of a scFv (Drawn in Vector NTI, Invitrogen, Carlsbad, CA). **(A)** plasmid pSCL. **(B)** pSCL with the light chain variable region inserted. **(C)** pSCL with the light and heavy chain inserted creating an scFv.

XhoI (4370)
SpeI (4130)
HindIII (30)
SacI (60)
gIII Fusion
pSCL
4150 bp
ORI
Cam
A
LcFv
XhoI (4403)
SpeI (4383)
HindIII (30)
gIII Fusion
LcFv
SacI (290)
pSCL-LcFv
4442 bp
ORI
Cam
B
HcFv
XhoI (4641)
HcFv
HindIII (30)
SpeI (4383)
LcFv
gIII Fusion
SacI (292)
pSCL-ScFv
4680 bp
Cam
ORI
C

Each reaction was composed of distilled H_20 (0-9.5 µl), DNA template (0.5-6.0 µl), a sequencing primer diluted to 1.6 µM (2.0 µl), and DTCS quick start master mix (DNA polymerase, reaction buffer, deoxynucleotide triphosphates, and all four dye labeled dideoxynucleotide triphosphate terminators) (12 µl) for a total of 20.0 µl. Reactions were then put in a thermocycler for 30 cycles of: 96°C for 20 seconds, 50°C for 20 seconds, and 60°C for 4 minutes. After that, ethanol precipitation took place in a new sterile 0.5 mL microfuge tube for each sample. To each tube 4 µl of Stop solution (1.5 M NaOAc + 50mM EDTA prepared fresh daily by mixing equal volumes of the 3M NaOAc and 100mM EDTA) and 1 µl of 20 mg/mL glycogen were added. Then, the sequencing reaction was transferred and thoroughly mixed. Next, 60 µl of cold 95% (v/v) ethanol/H2O was added and this mixture was centrifuged at 14,000 rpm at 4°C for 15 minutes. After centrifugation the supernatant was removed leaving only the pellet. The pellet was rinsed 2 times with 200 µl of 70% (v/v) ethanol/dH2O and centrifuged at 14,000 rpm at 4°C for 2 minutes. Again after centrifugation the supernatant was removed leaving only the pellet. This was vacuumed dried for 40 minutes and then the sample was resuspended in 40 µl of the Sample Loading Solution and frozen at -20°C. Finally, the sample was loaded and run on the Beckman Coulter CEQ 2000 XL DNA Analysis System sequencing machine.

O. TOPO Cloning of Light Chain and Heavy Chain into TOPO Vector

A pBAD-TOPO vector (TOPO cloning kit, Invitrogen, Carlsbad, CA) (Shuman, 1994) was used to clone the heavy and light chain fragments into to be sequenced. The TOPO cloning reaction uses the following reagents: Fresh PCR product, either light chain

I or heavy chain XV (0.5 to 4.0 µl), salt solution (1.2 M NaCl and 0.06 M $MgCl_2$) (1 µl), sterile water (add to a final volume of 5 µl), and pBAD-TOPO vector (10 ng/µl plasmid DNA in: 50% glycerol, 50 mM Tris-HCl, pH 7.4 @ 25°C, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 100 µg/ml BSA, and phenol red) (1 µl). These reagents were mixed gently and incubated for 5 minutes at room temperature and then placed on ice until needed for transformation. The chemically competent TOPO10 *E. coli* cells (supplied in the TOPO cloning kit) were thawed on ice and the transformation was performed. The TOPO cloning reaction (2 µl) was added to a vial of One Shot TOP10 chemically competent *E. coli* and gently mixed. This was then incubated on ice for 30 minutes to allow the DNA to bind to the cells. The cells were then heat shocked for 30 seconds at 42°C and then immediately transferred to ice. Room temperature S.O.C. medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) (250 µl) was added to the mixture and the tube containing the mixture was shaken horizontally at 200 rpm at 37°C for 1 hour. The transformation (25 and 50 µl) and LB medium (75 µl) were spread onto pre-warmed LB/AMP plates and incubated overnight at 37°C. Colonies were then counted from each plate and recorded.

P. Preparation of Samples Sent to Cleveland Genomics to be Sequenced

Competent cells were prepared for transformation of the minipreps to make more DNA. *E. coli* (LM6194) was streaked out on LB plates and then colonies were grown in LB broth. This culture was diluted (1/50) and grown to an O.D. of 0.792. These cells were pelleted, resuspended in NaCl (0.15M), pelleted again, resuspended in transformation buffer (contained $CaCl_2$, $MgCl_2$, Tris-HCl, and glycerol), incubated on ice, and frozen at -80°C. Minipreps were transformed into these competent cells and plated on LB/AMP plates. Colonies were grown in LB/AMP/glucose media, minipreps were performed, and the DNA was quantified and then sent to Cleveland Genomics (Cleveland, OH) to be sequenced.

IV. RESULTS

Phagemids pSCL and pComb3-C (Smiley and Benkovic, 1994) were transformed into competent XL 1 Blue *E. coli* cells. Aliquots (20 µl and 100 µl) of these transformation cultures were plated on LB/CAM containing plates and colony counts were performed (Table 3). Colonies from phagemids pSCL and pComb3-C were then grown in LB/CAM medium and purified using a plasmid purification kit (Qiagen, Valencia, CA). Gel electrophoresis was used to show that these phagemids were in fact purified and did not contain any debris, RNA, or other impurities (Figure 4). Since they were found to be pure phagemids, the absorbance of each was determined and the concentration calculated. The absorbance of pComb3-C was 0.251 (260 nm) and 0.140 (280 nm) giving a 260/280 absorbance ratio of 1.792. The concentration was then calculated to be 125 ng/µl. The absorbance of pSCL was 0.285 (260 nm) and 0.155 (280 nm), which resulted in a 260/280 absorbance ratio of 1.839. The concentration of pSCL was found to be 143 ng/µl.

Phagemid pSCL was digested with restriction enzymes *Sac*I and *Hind*III for the light chain and with *Spe*I and *Xho*I for the heavy chain to determine if the phagemid contained any inserts. When digested with *Sac*I and *Hind*III no inserts were present (Figure 5). When digested with *Spe*I, the resulting band produced was around 4,300 base pairs in length (Figure 6, lane 2), compared to the 3,000 base pair length of the undigested pSCL (Figure 6, lane 3).

Clone SM.T5.B2.A12.C9 was fed every other day with P3X media until 10^8 cells were present (20 days). These cells were centrifuged for 10 minutes at 1200 rpm (400 x g) at 4°C and then frozen at -70°C. Total RNA was then isolated from these cells using

Table 3: Colony count of different phagemids transformed into XL1 Blue *E. coli* cells.[1]

Phagemid	**pSCL**	**pComb3-C**	**Negative control**[2]
20 µl	915	90	0
100 µl	4,628	456	13

[1] Phagemids pSCL and pComb3-C were transformed into XL1 Blue *E. coli* competent cells by mixing the phagemids with the competent cells and incubating them on ice for 10 minutes. Then these mixtures were heat shocked at 42°C for 1 minute, added to LB media containing glucose, and incubated on a roller drum incubator at 250 rpm for 1 hour at 37°C. Finally aliquots (20µL and 100 µL) of the transformation were plated on LB/CAM plates and colony counts were performed.

[2] Competent XL1 Blue *E. coli* cells that did not contain any phagemids were used as a negative control.

Figure 4: Purified phagemids pSCL and pComb3-C. Lane 1: 1 kb molecular weight marker. Lanes 2-3: Phagemids pComb3-C (lane 2) and pSCL (lane 3) purified using a plasmid purification kit (Qiagen).

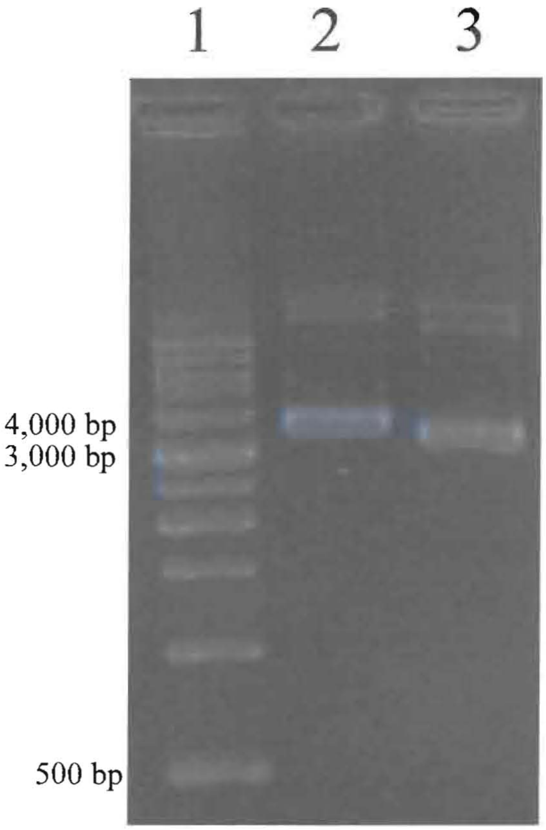

1
2
3
4,000 bp
3,000 bp
500 bp

Figure 5: Phagemid pSCL digested with restriction enzymes *Sac*I and *Hind*III. Lane 1: 1 kb molecular weight marker. Lane 2: Purified phagemid pSCL (undigested). Lane 3: Phagemid pSCL digested with *Sac*I. Lane 4: Phagemid pSCL digested with *Hind*III. Lane 5: Phagemid pSCL digested with *Sac*I and *Hind*III. Lane 6: Control (undigested).

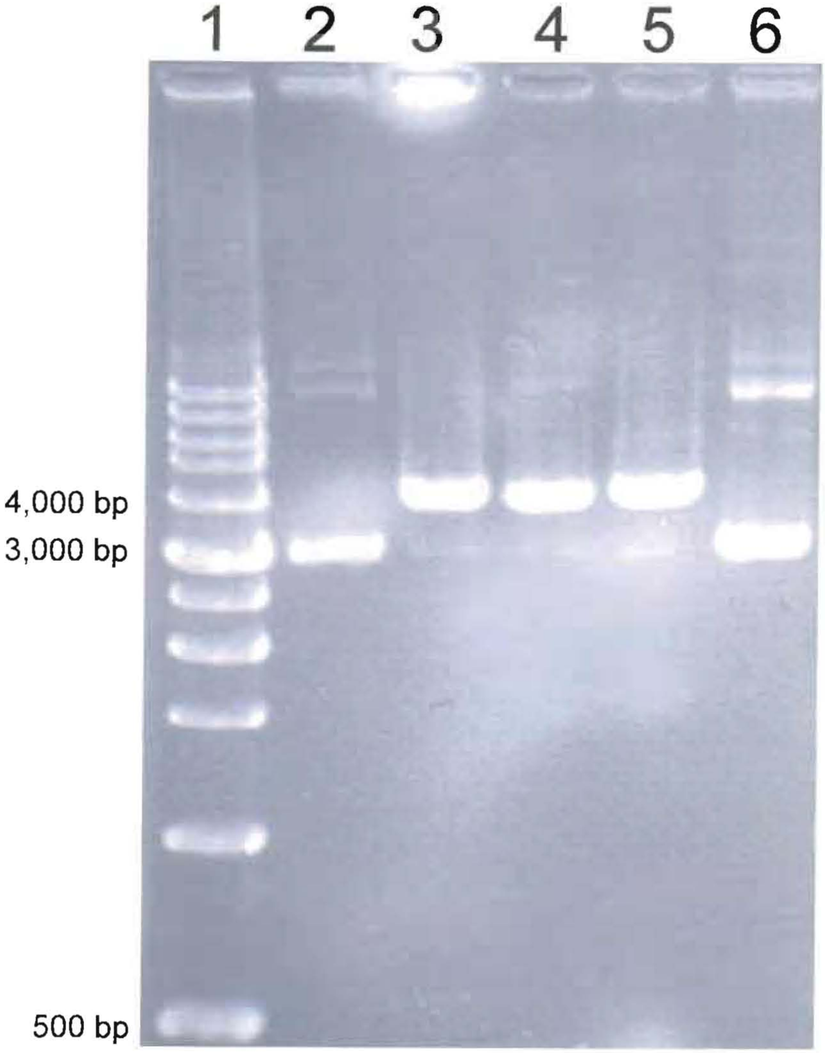
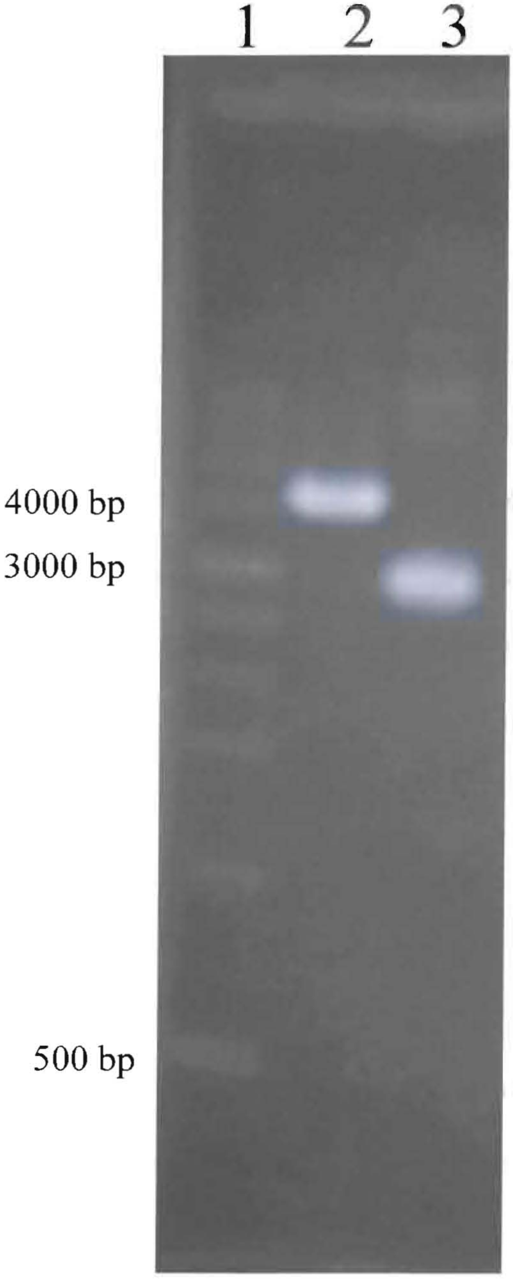

1
2
3
4
5
6
4,000 bp
3,000 bp
500 bp

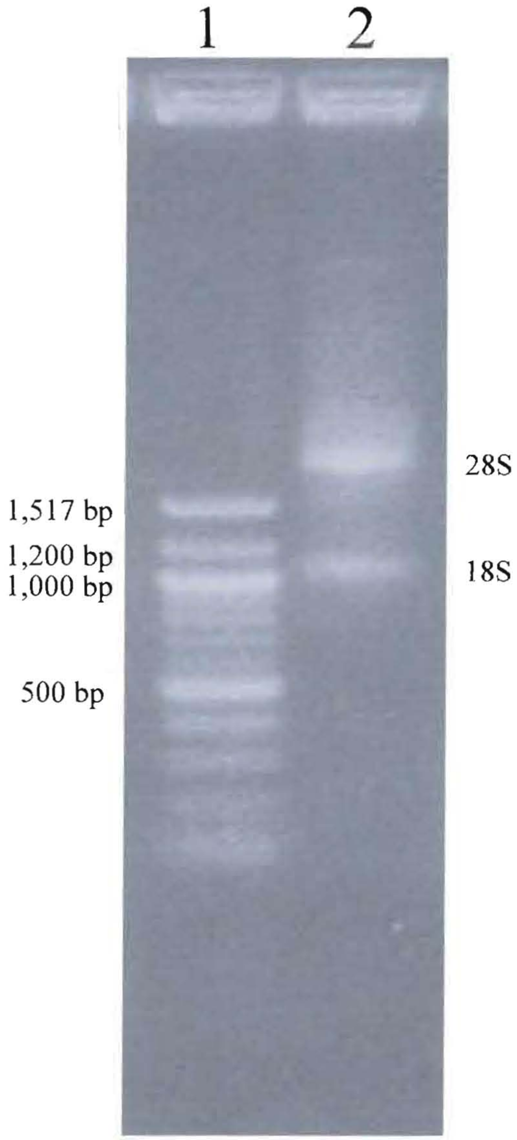
Figure 6: Phagemid pSCL digested with restriction enzyme *Spe*I. Lane 1: 1 kb molecular weight marker. Lane 2: Phagemid pSCL digested with restriction enzyme *Spe*I. Lane 3: phagemid pSCL (undigested).


1
2
3
4000 bp
3000 bp
500 bp

Eppendorf (Hamburg, Germany) Biophotometer and RNase-free cuvettes. The absorbance at 260 nm was 8.932, while the absorbance at 280 nm was 4.256 giving a 260/280 ratio of 2.04 nm. The concentration of the RNA was calculated to be 357 μg/ml and the total yield of RNA isolated was 0.178mg. As shown in Figure 7, the sizes of the ribosomal RNA (rRNA) are approximately 1,100 bp for the 18S rRNA and about 1,700 bp for the 28S rRNA confirming that the total RNA isolated was purified.

Total RNA was then used to synthesize the first strand of cDNA (Figure 7, lane 4). This was done in an Eppendorf (Hamburg, Germany) thermocycler using a first strand cDNA synthesis kit (New England Biolabs, Ipswich, MA). Primers (Tables 1 and 2) that are complementary to the consensus antibody sequences (Okamoto, *et al.*, 2004) at the 3' ends and contain restriction sites at the 5' ends were designed. Different combinations of 5' and 3' light chain primers (Figure 8) and 5' and 3' heavy chain primers (Figure 10) were used to produce the second strand of DNA by PCR. Positive results for light chain amplification were shown in LC5A/LC3A, LC5B/LC3A, LC5C/LC3A, and LC5D/LC3A to be around 450 bp in length (Figure 8, lanes 2, 4, 6, and 8). Two of these potential light chain fragments, LC5A/LC3A and LC5B/LC3A (Figure 8, lanes 2 and 4) were PCR amplified even further, purified (using a QIAquick PCR purification kit, Qiagen), and digested with restriction enzymes *Sac*I and *Hind*III (Figure 9, lanes 4-5). Figure 9 also shows a comparison between phagemid pSCL undigested and phagemid pSCL digested with *Sac*I and *Hind*III (Figure 9, lanes 2-3). These digested light chain fragments were then ligated together with the digested phagemid pSCL in a ligation reaction and transformed into competent XL1 Blue *E.coli* cells. Aliquots of the transformation culture were plated on LB/CAM containing plates (200 μl on all plates)

Figure 7: Synthesis of cDNA from SM.T5.B2.A12.C9 clones. Lane 1: 100 bp molecular weight marker. Lane 2: RNA from SM.T5.B2.A12.C9 clones (*Note: A DNA molecular weight marker was used, so the corresponding weights are different than what they would be had an RNA molecular weight marker been used). Lane 3: 1 kb molecular weight marker. Lane 4: cDNA synthesized from RNA of SM.T5.B2.A12.C9 clones, amplified using GAPDH control primers (NE Biolabs).


1
2
28S
1,517 bp
1,200 bp
1,000 bp
18S
500 bp

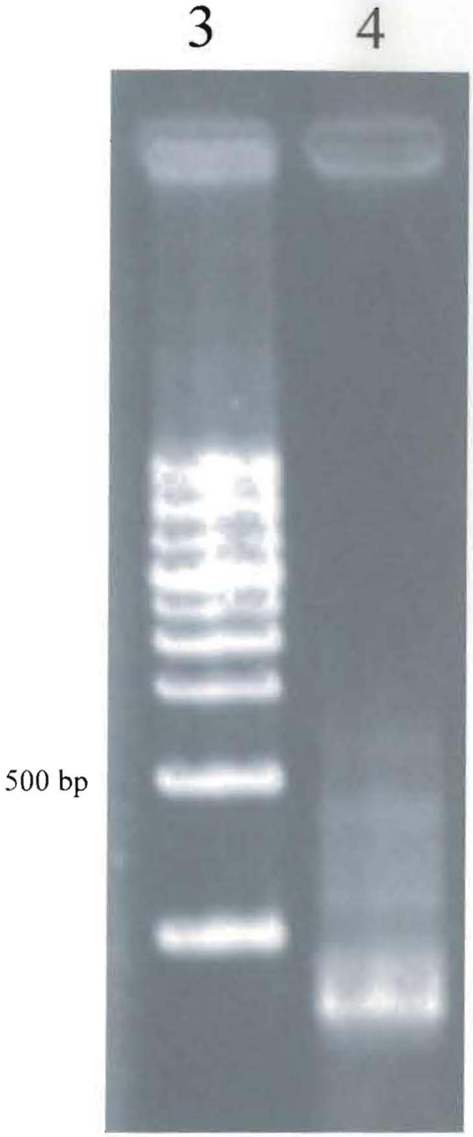
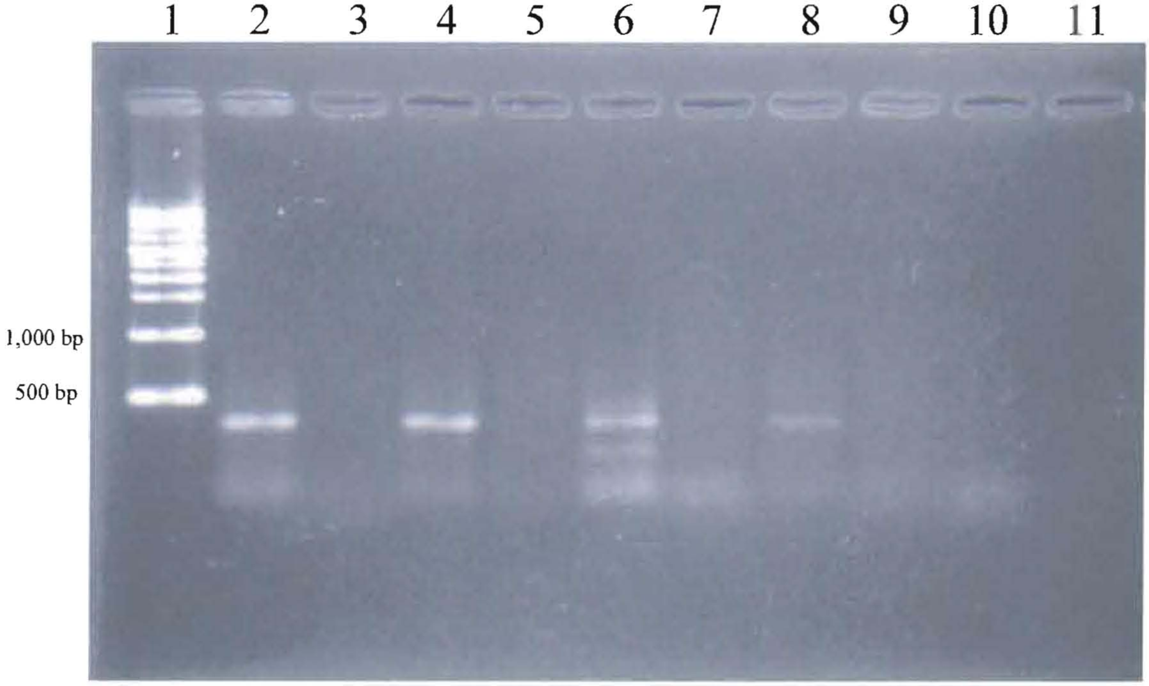

3
4
500 bp

Figure 8: Isolation of light chain from cDNA of SM.T5.B2.A12.C9 clones. Different combinations of light chain 5' and light chain 3' primers (see Table 1) were used to PCR the variable light chain region of the antibodies. PCR was done at 95°C for 2 minutes (one-time), 95°C for 30 seconds, 50°C for 60 seconds, and 72°C for 90 seconds (35 cycles), and 72°C for 5 minutes (one-time). Lane 1: 1 kb molecular weight marker. Lane 2: primers LC5A and LC3A. Lane 3: primers LC5A and LC3B. Lane 4: primers LC5B and LC3A. Lane 5: primers LC5B and LC3B. Lane 6: primers LC5C and LC3A. Lane 7: primers LC5C and LC3B. Lane 8: primers LC5D and LC3A. Lane 9: primers LC5D and LC3B. Lane 10: primers LC5E and LC3A. Lane 11: primers LC5E and LC3B.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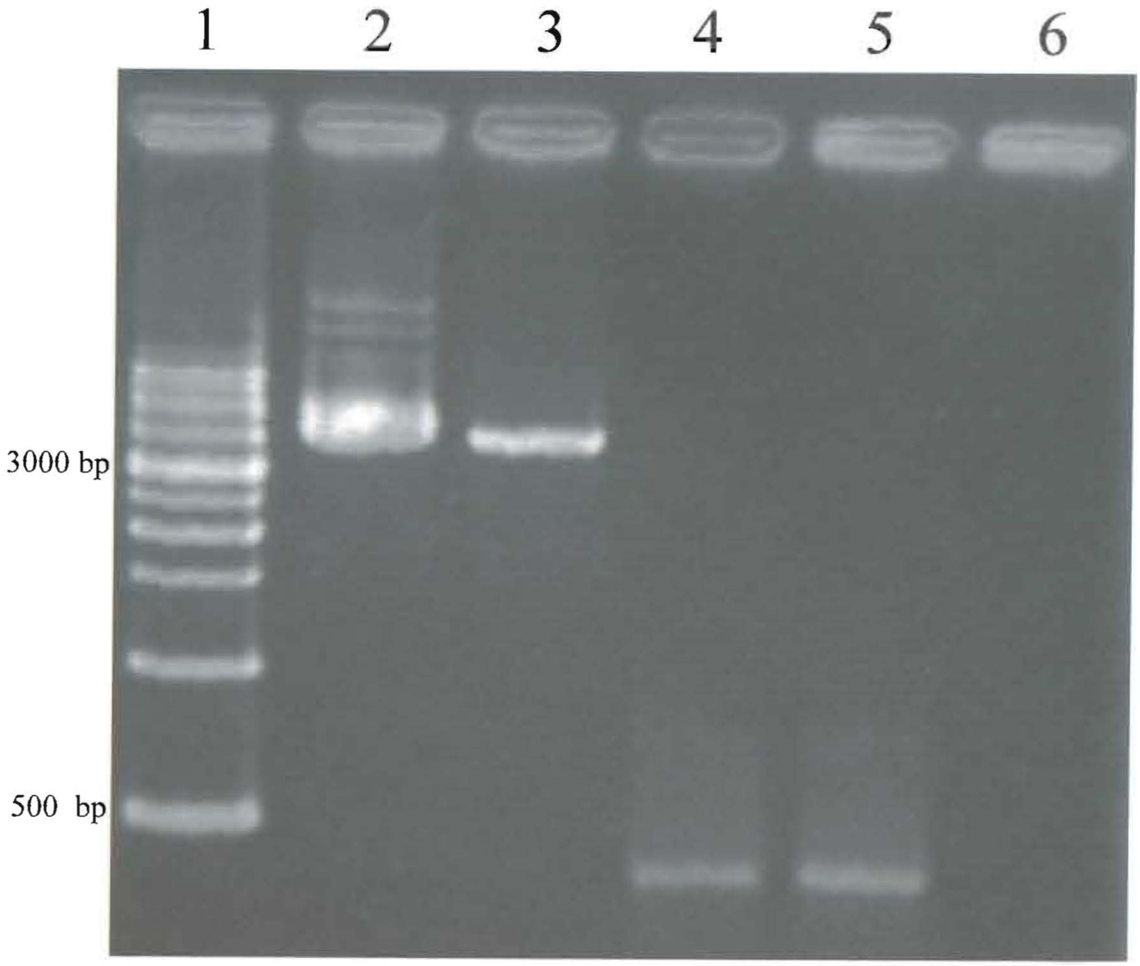
10
11
1,000 bp
500 bp

Figure 9: Light chain PCR products 5A3A and 5B3A digested with both *Sac*I and *Hind*III. Purified PCR products LC5A/LC3A and LC5B/LC3A (refer to Figure 6) were digested with both *Sac*I and *Hind*III. Lane 1: 1 kb molecular weight marker. Lane 2: Phagemid pSCL (undigested). Lane 3: Phagemid pSCL digested with *Sac*I and *Hind*III. Lane 4: Light chain PCR product 5A3A digested with *Sac*I and *Hind*III. Lane 5: Light chain PCR product 5B3A digested with *Sac*I and *Hind*III.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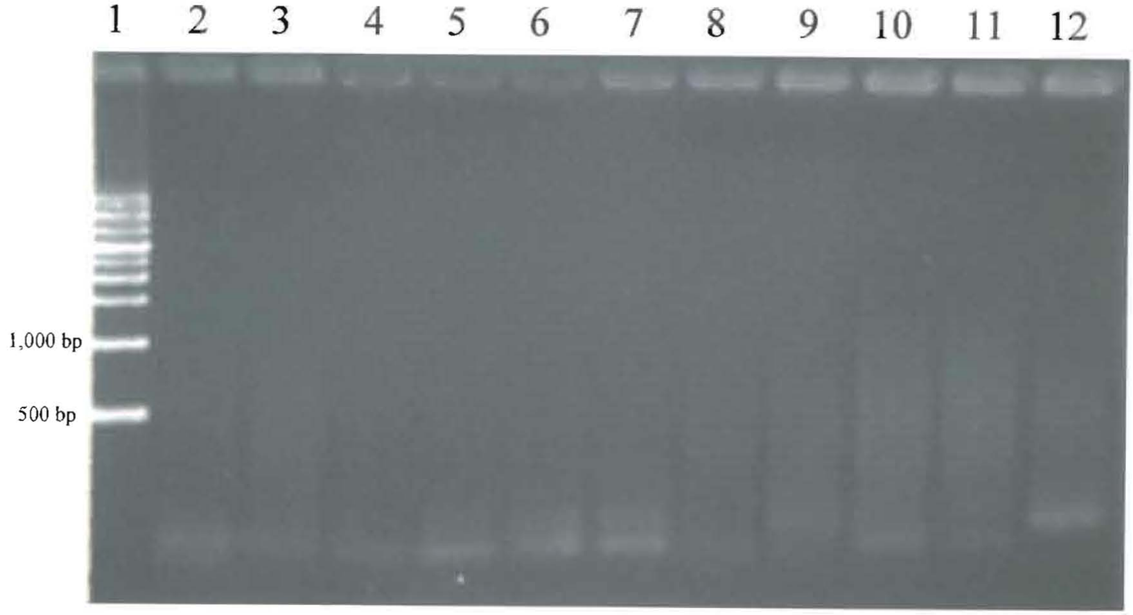
5
6
3000 bp
500 bp

Figure 10: Isolation of heavy chain from cDNA of SM.T5.B2.A12.C9 clones. Different combinations of heavy chain 5' and heavy chain 3' primers (see Table 2) were used to PCR (using the same PCR times and temperatures as in Figure 8) the variable heavy chain region of the antibodies. **A:** Lane 1: 1 kb molecular weight marker. Lane 2: primers VH-51 and VH-37. Lane 3: primers VH-51 and VH-38. Lane 4: primers VH-51 and VH-39. Lane 5: primers VH-52 and VH-37. Lane 6: primers VH-52 and VH-38. Lane 7: primers VH-52 and VH-39. Lane 8: primers VH-53 and VH-37. Lane 9: primers VH-53 and VH-38. Lane 10: primers VH-53 and VH-39. Lane 11: primers VH-54 and VH-37. Lane 12: primers VH-54 and VH-39. **B:** Lane 1: 1 kb molecular weight marker. Lane 2: primers VH-54 and VH-39. Lane 3: primers VH-55 and VH-37. Lane 4: primers VH-55 and VH-38. Lane 5: primers VH-55 and VH-39. Lane 6: primers VH-56 and VH-37. Lane 7: primers VH-56 and VH-38. Lane 8: primers VH-56 and VH-39.


1
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7
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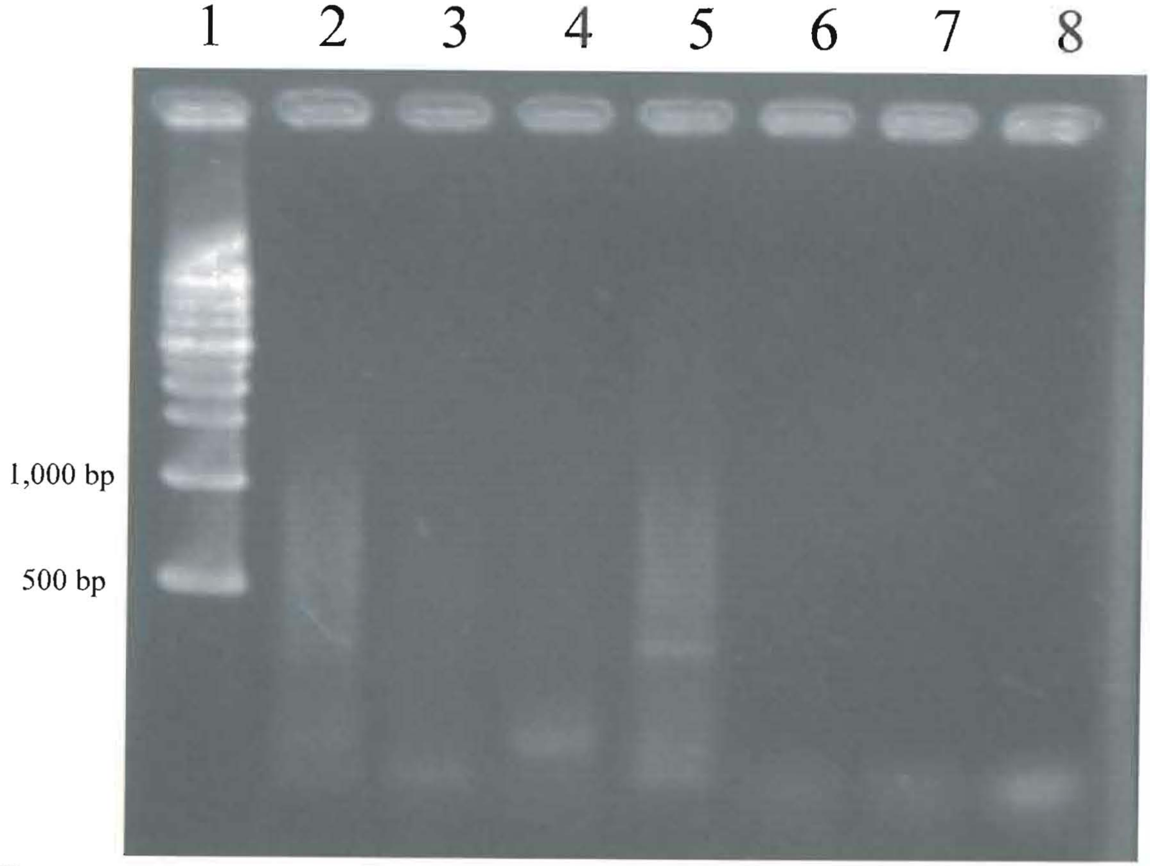
10
11
12
1,000 bp
500 bp

A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1,000 bp
500 bp

B

colony counts were performed (Table 4). Individual colonies were then grown and plasmid minipreps were performed (Figure 11). Miniprep 1.2.1 (Figure 11, lane 4) and miniprep 1.5.1 (Figure 11, lane 6) were purified and digested with restriction enzymes *Sac*I and *Hind*III (Figure 12). Inserts were shown in miniprep 1.2.1 when digested with *Sac*I only, *Hind*III only, and with both *Sac*I and *Hind*III (Figure 12, lanes 3-5), while no inserts were shown in miniprep 1.5.1 (Figure 12, lanes 7-9). Since the presence of inserts was shown in miniprep 1.2.1 when digested with *Sac*I only, *Hind*III only, and with both *Sac*I and *Hind*III, a second round of minipreps was done to see if similar results would occur. Six individual colonies from transformation #1 (LC5A/LC3A, pSCL, and XL1 Blue cells) and transformation #2 (LC5B/LC3A, pSCL, and XL1 Blue cells) were grown, purified, and run on a gel (Figure 13). Two of these minipreps, 1.1.2 and 1.2.2 (Lanes 5 and 6, Figure 13) were larger in size than the other minipreps (~400-500 bp larger) and were chosen for further investigation. They were purified and digested with *Sac*I and *Hind*III to detect the presence of inserts. Miniprep 1.2.2 did not contain any insert bands (data not shown). As shown in Figure 14 (lanes 7-9), miniprep 1.1.2 contained bands when digested with *Sac*I only and both *Sac*I and *Hind*III, but not with *Hind*III only. Miniprep 1.2.1 (Figure 14, lanes 3-5) contained inserts when digested with *Sac*I only, *Hind*III only, and with both *Sac*I and *Hind*III just as was shown in round one with 1.2.1 (Figure 12, lanes 3-5). These two minipreps (1.1.2 and 1.2.1) were compared to light chain PCR product 5B/3A and the bands were shown to be similar in sizes (Figure 15). Since these minipreps were comparable in size with the light chain PCR product produced and they were showing bands when digested with only one restriction enzyme, it was decided to try to sequence miniprep transformations 1.1.2 and 1.2.1.

Table 4: Colony counts of transformations of ligation reaction of phagemid pSCL with LC5A/LC3A or LC5B/LC3A into XL1 Blue *E.coli* cells.

	Colony count[1]
LC5A/LC3A #1	374
LC5A/LC3A #2	254
LC5B/LC3A #1	265
LC5B/LC3A #2	146
Negative control[2]	44
Positive control[3]	1424
XL1 Blue cells (only)[4]	0

[1] Light chain PCR products LC5A/LC3A or LC5B/LC3A were ligated with phagemid pSCL using DNA ligase T4. These ligation products were then transformed into competent *E. coli* XL1 Blue cells by mixing the ligation products with the competent cells and incubating them on ice for 10 minutes. Then these mixtures were heat shocked at 42°C for 1 minute, added to LB media containing glucose, and incubated on a roller drum incubator at 250 rpm for 1 hour at 37°C. Finally aliquots (200 μL on all plates) of the transformation were plated on LB/CAM plates and colony counts were performed.

[2] Negative control did not contain any light chain PCR inserts.

[3] Pure pSCL was used as a positive control.

[4] XL1 Blue cells alone without any phagemid or light chain PCR inserts was used as another negative control.

Figure 11: Phagemid minipreps of pSCL-LC PCR products (5A3A or 5B3A) transformed into XL 1 Blue *E.coli* cells. Purified phagemid pSCL and light chain PCR product (LC5A/LC3A or LC5B/LC3A) were ligated together using DNA ligase. These ligations were then transformed into competent XL 1 Blue *E. coli* cells. Lane 1: 1 kb molecular weight marker. Lane 2: Phagemid pSCL (purified). Lane 3: pSCL-LC 1.1.1. Lane 4: pSCL-LC 1.2.1. Lane 5: pSCL-LC 1.4.1. Lane 6: pSCL-LC 1.5.1. Lane 7: pSCL-LC 2.1.1. Lane 8: pSCL-LC 2.2.1. Lane 9: pSCL-LC 2.3.1. Lane 10: pSCL-LC 2.4.1.

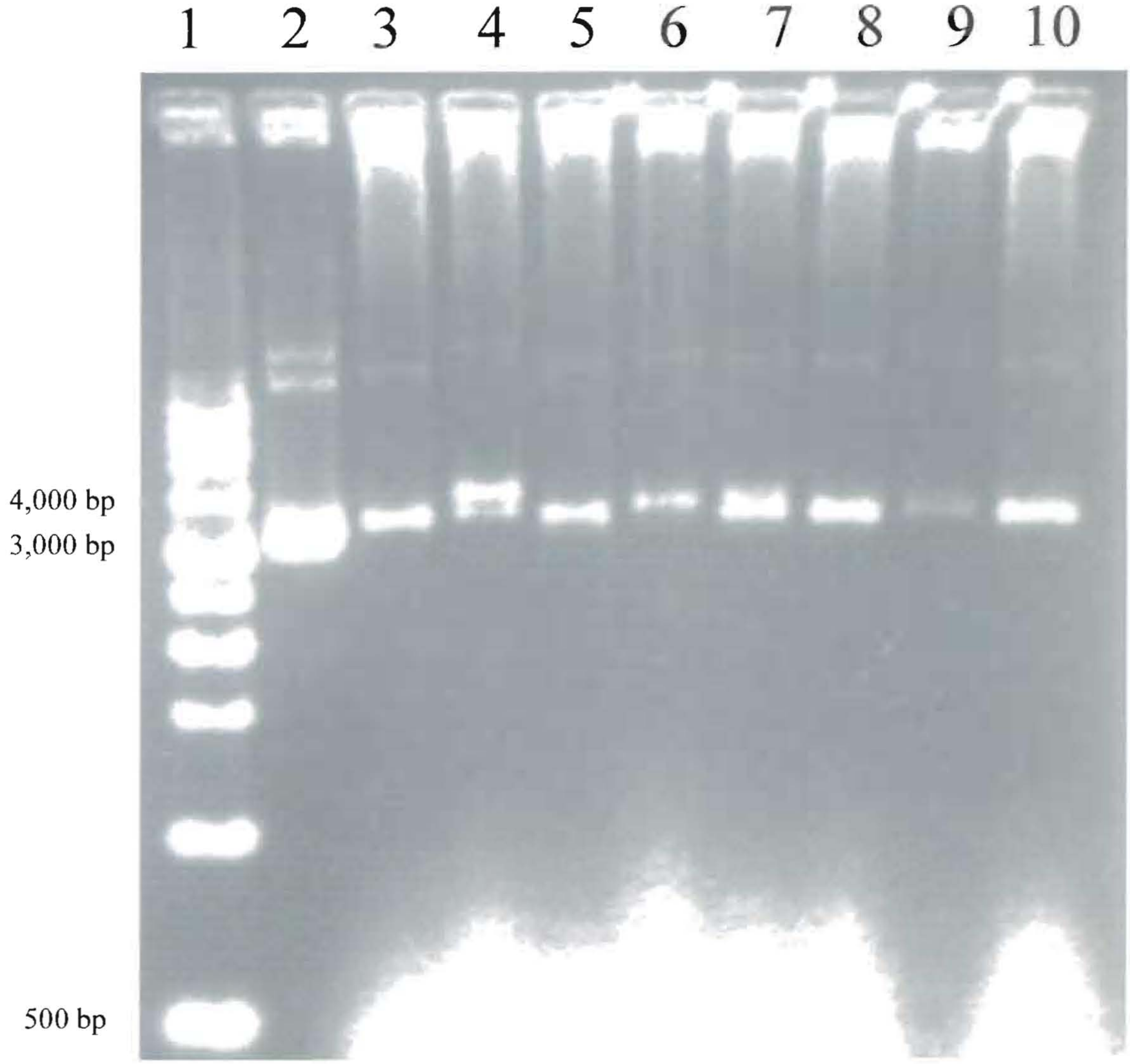

1 2 3 4 5 6 7 8 9 10
4,000 bp
3,000 bp
500 bp

Figure 12: Purified phagemids 1.2.1 and 1.5.1 digested with *Sac*I and *Hind*III. Lane 1: 1 kb molecular weight marker. Lane 2: 1.2.1 (undigested). Lane 3: 1.2.1 digested with *Sac*I. Lane 4: 1.2.1 digested with *Hind*III. Lane 5: 1.2.1 digested with *Sac*I and *Hind*III. Lane 6: 1.5.1 (undigested). Lane 7: 1.5.1 digested with *Sac*I. Lane 8: 1.5.1 digested with *Hind*III. Lane 9: 1.5.1 digested with *Sac*I and *Hind*III. Lane 10: 100 bp molecular weight marker.

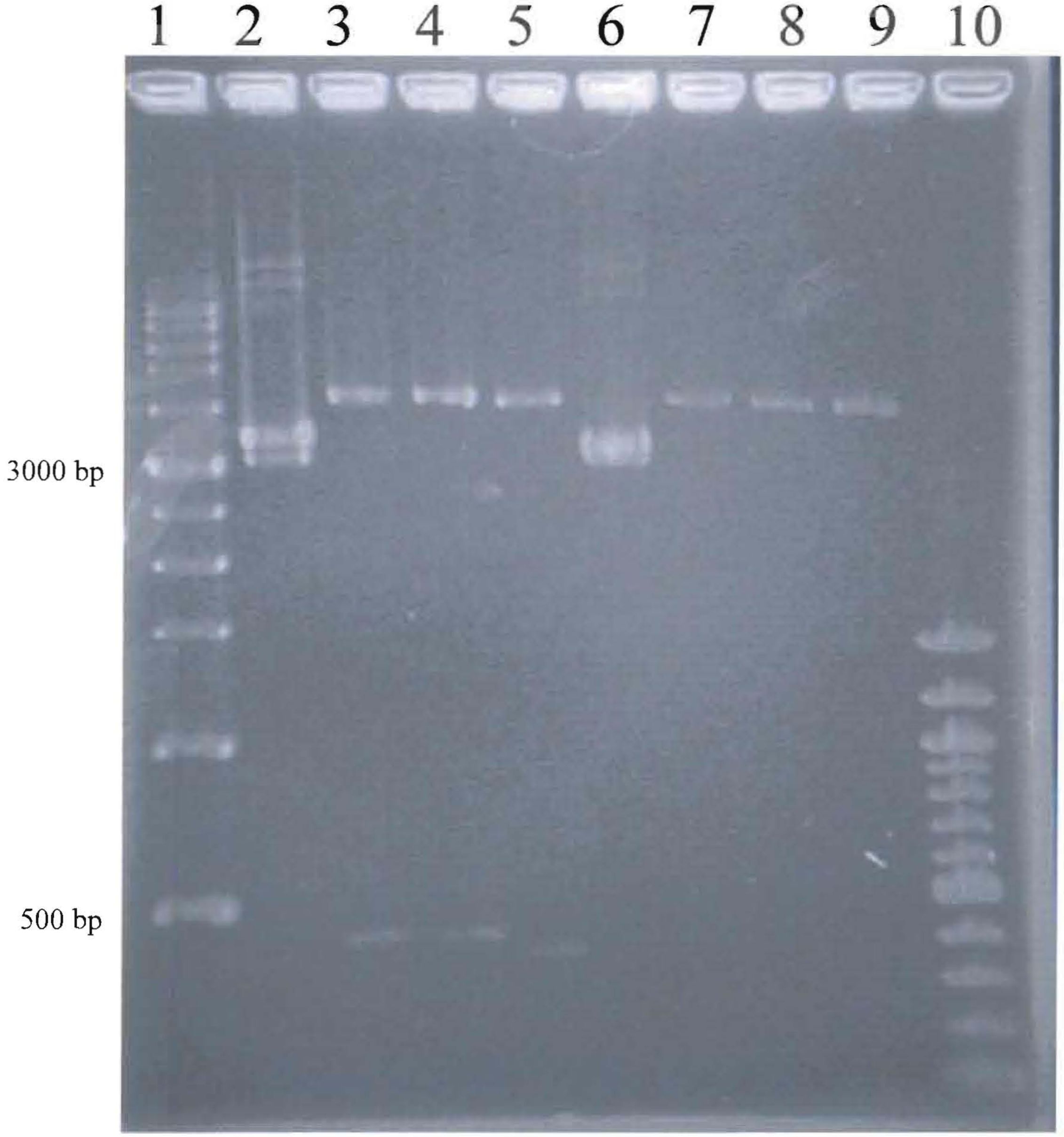

1
2
3
4
5
6
7
8
9
10
3000 bp
500 bp

Figure 13: Second round of minipreps of pSCL-LC-XL 1 Blue cells. Lane 1: 1 kb molecular weight marker. Lane 2: Phagemid pSCL (undigested). Lane 3: Phagemid pSCL digested with *Sac*I and *Hind*III. Lane 4: LC PCR 5A3A digested with *Sac*I and *Hind*III. Lane 5: 1.1.2. Lane 6: 1.2.2. Lane 7: 1.3.2. Lane 8: 1.4.2. Lane 9: 1.5.2. Lane 10: 100 bp molecular weight marker.

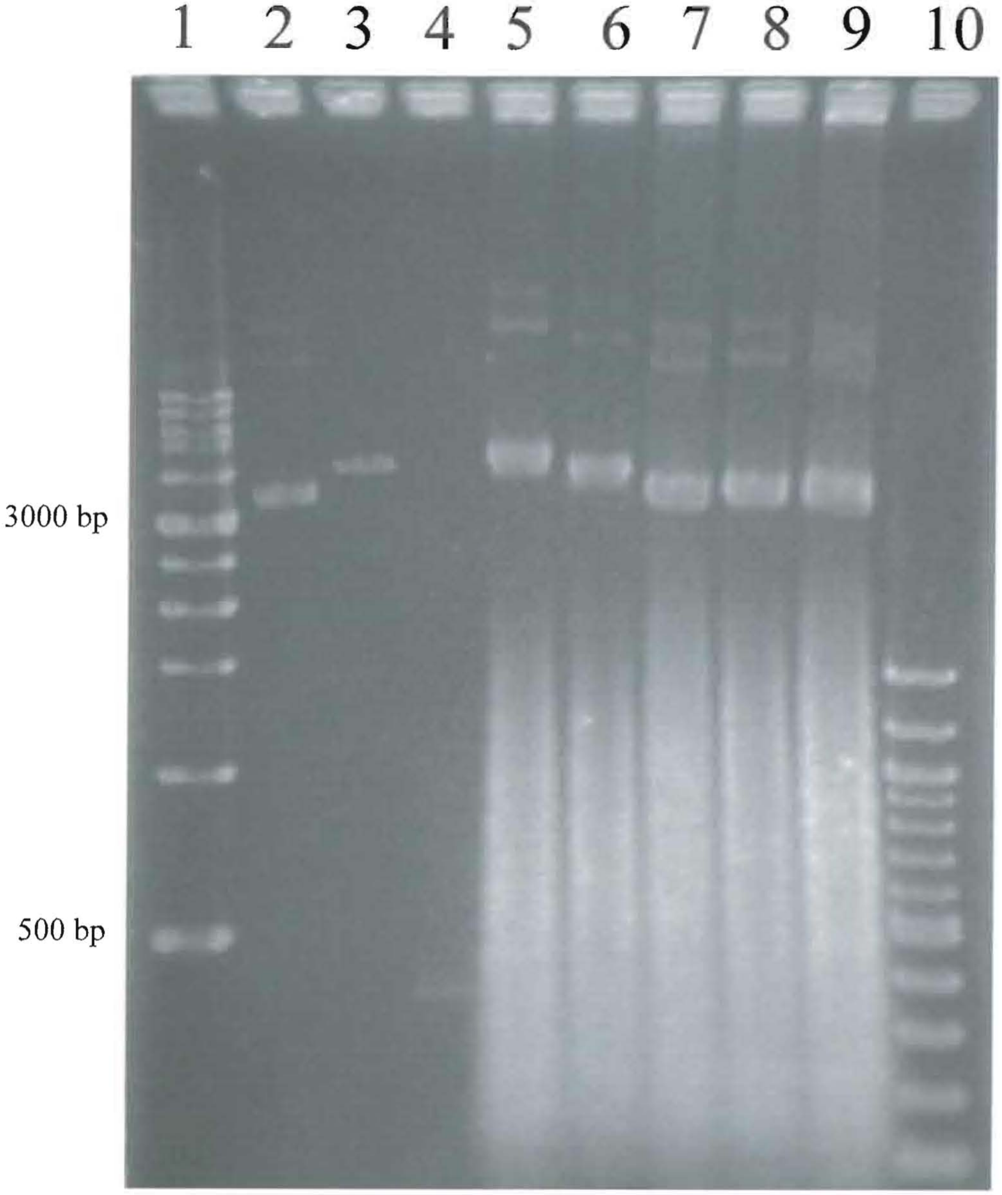

1 2 3 4 5 6 7 8 9 10
3000 bp
500 bp

Figure 14: 1.2.1 and 1.1.2 digested with *Sac*I and *Hind*III. Lane 1: 1 kb molecular weight marker. Lane 2: Purified, undigested 1.2.1. Lane 3: 1.2.1 digested with *Sac*I. Lane 4: 1.2.1 digested with *Hind*III. Lane 5: 1.2.1 digested with *Sac*I and *Hind*III. Lane 6: Purified, undigested 1.1.2. Lane 7: 1.1.2 digested with *Sac*I. Lane 8: 1.1.2 digested with *Hind*III. Lane 9: 1.1.2 digested with *Sac*I and *Hind*III. Lane 10: 100 bp molecular weight marker.

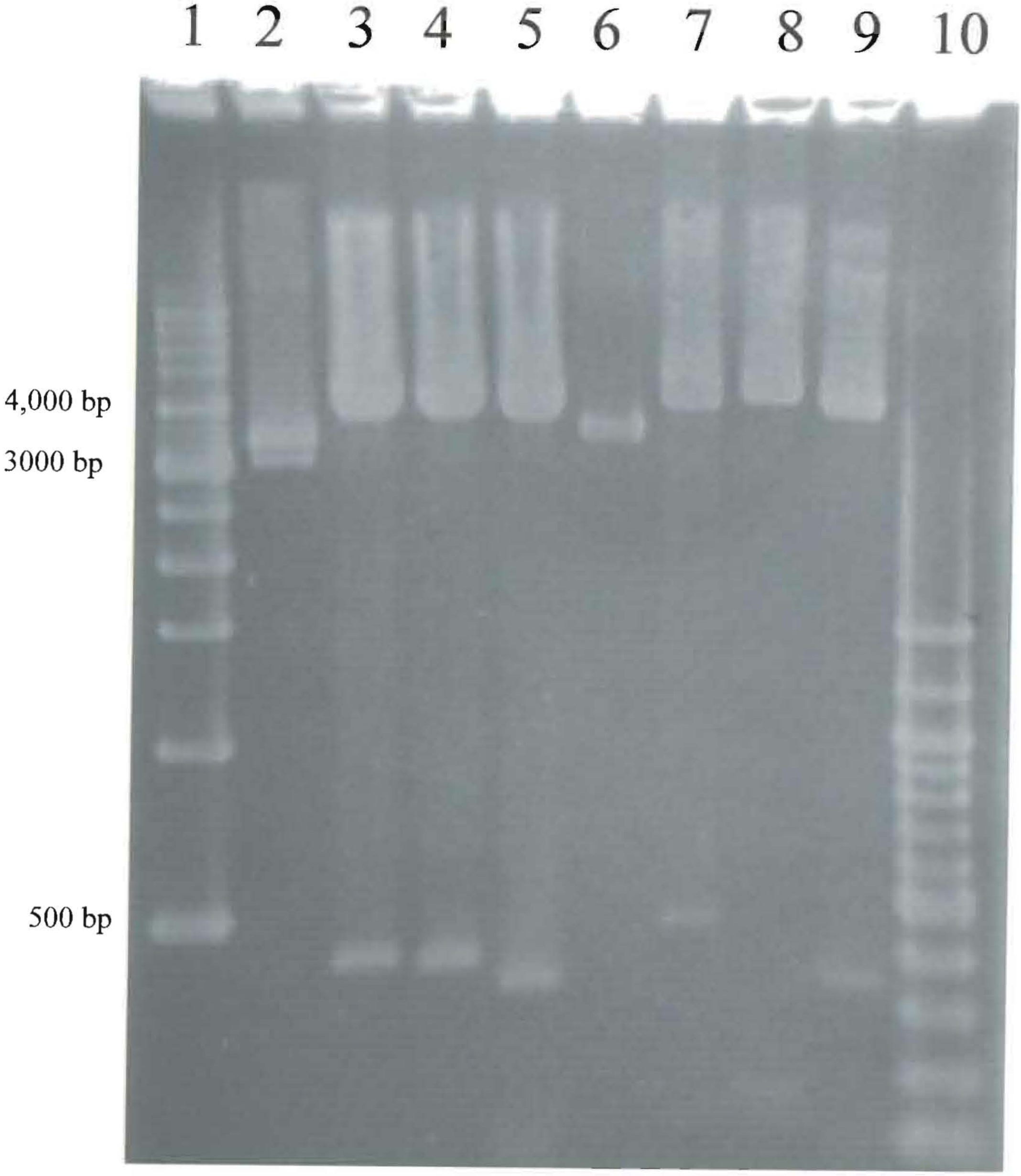
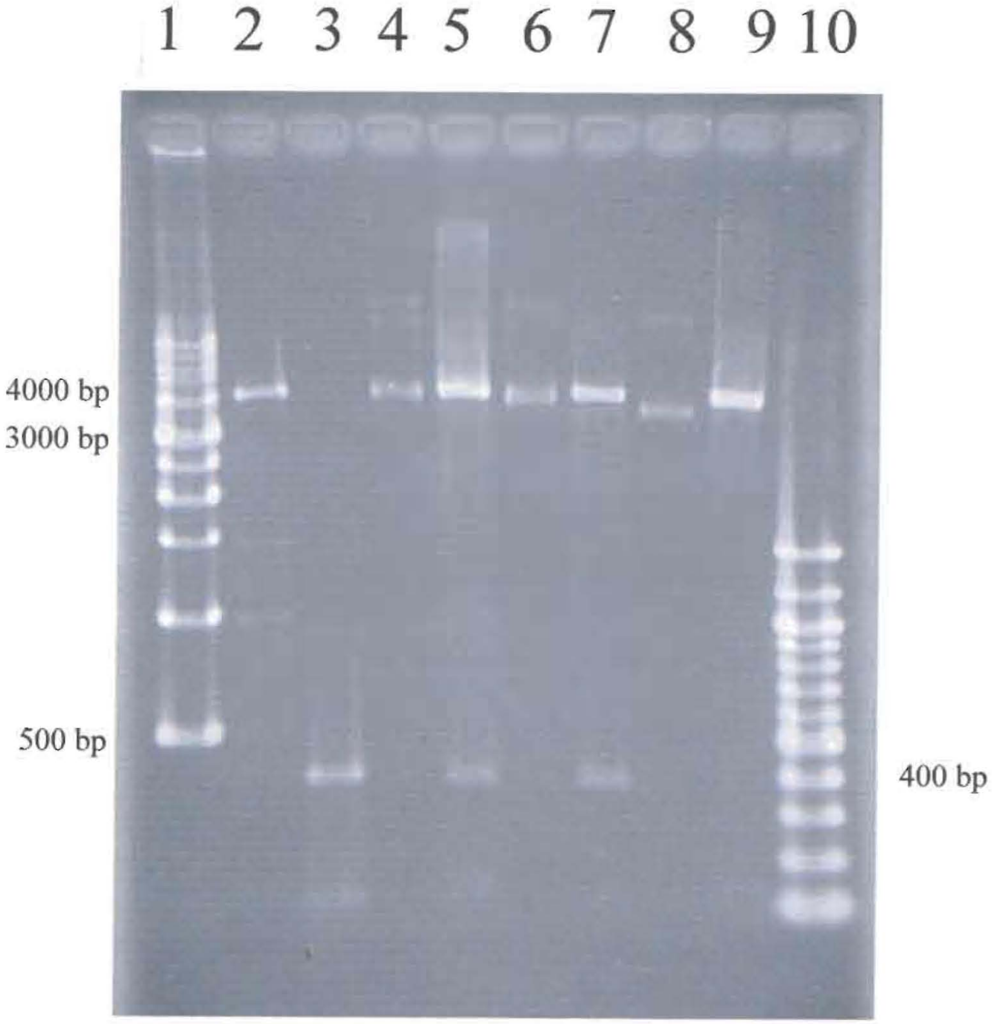

1 2 3 4 5 6 7 8 9 10
4,000 bp
3000 bp
500 bp

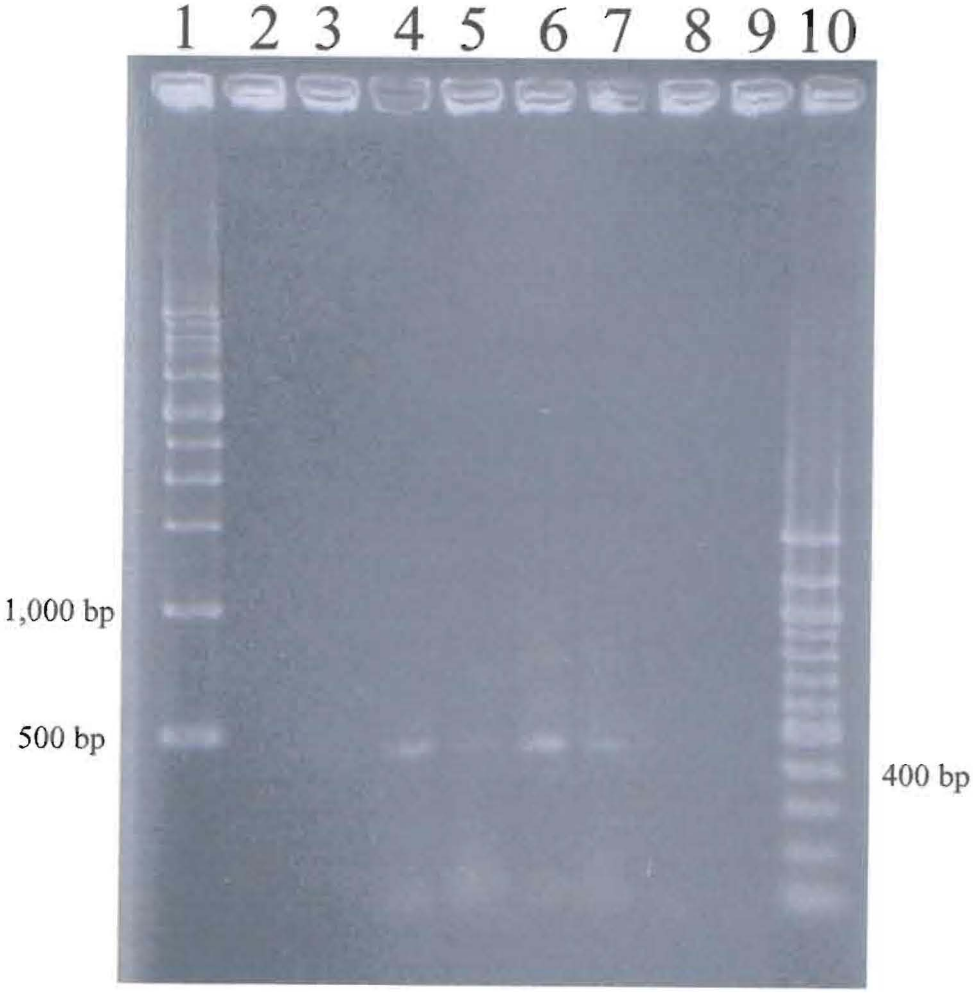
Figure 15: Comparison of light chain PCR product (5B/3A) with minipreps 1.1.2 #3, 1.2.1 #3, and 1.2.2 #1. Lane 1: 1 kb molecular weight marker. Lane 2: pSCL digested with *Sac*I and *Hind*III. Lane 3: Light chain PCR product 5B/3A. Lane 4: Purified, undigested 1.1.2 #3. Lane 5: 1.1.2 #3 digested with *Sac*I and *Hind*III. Lane 6: Purified, undigested 1.2.1 #3. Lane 7: 1.2.1 #3 digested with *Sac*I and *Hind*III. Lane 8: Purified, undigested 1.2.2 #1. Lane 9: 1.2.2 #1 *Sac*I and *Hind*III. Lane 10: 100 bp molecular weight marker.


1
2
3
4
5
6
7
8
9
10
4000 bp
3000 bp
500 bp
400 bp

DNA from minipreps 1.1.2 and 1.2.1 was quantified using an Eppenforf (Hamburg, Germany) Biophotometer. The absorbance at 260nm was found to be 0.132 and the absorbance at 280nm was found to be 0.070 giving a 260nm/280nm ratio for 1.1.2 of 1.886. The concentration of 1.1.2 was calculated to be 66 μg/ml. The absorbance of 1.2.1 was 0.274 at 260nm and 0.147 at 280nm giving a 260nm/280nm ratio of 1.864. The concentration of 1.2.1 was calculated to be 137 μg/ml. Phenol extraction was performed to prepare these transformations for sequencing.

Sequencing was attempted using the CEQ 2000 Dye Terminator Sequencing with Quick Start Kit from Beckman Coulter (Fullerton, CA). DNA template from minipreps 1.1.2 (4.5μl) and 1.2.1 (5.6 μl) were used in the reaction along with the *lac* promoter primer (5'- GCACC CCAGGCTTTACACTT-3') (Miller, 1992) (diluted to 1.6 μM). Good sequencing data was sequenced for the control (Beckman Coulter Kit), but no DNA data was shown for 1.1.2 and 1.2.1 (data not shown). To try to determine the problem with the sequencing of 1.1.2 and 1.2.1, the *lac* promoter primer and gIII primers (-28 gIII sequencing primer: 5'-GTATGGGATTTTGCTAAACAAC-3' and -96 gIII sequencing primer: 5'-CCCTCATAGTTAGCGTAACG-3') (Integrated DNA Technologies, Coralville, IA) were used to amplify by PCR the 1.2.1 transformation. The gIII primers both showed positive amplification, but the *lac* promoter primer did not (Figure 16), suggesting that the *lac* promoter is not present in phagemid pSCL as was first thought to be. The gIII primers were then used in the next attempt at sequencing 1.1.2 and 1.2.1, but yet again no DNA sequence was observed. Since no sequencing data was able to be obtained using phagemid pSCL, a different vector (TOPO vector, Invitrogen, Carlsbad, CA) was used to try to obtain sequencing results.

Figure 16: PCR analysis of phagemid pSCL. Lane 1: 1 kb molecular weight marker. Lane 2: 1:10 dilution with no primers. Lane 3: 1:100 dilution with no primers. Lane 4: -28/5A 1:10 dilution. Lane 5: -28/5A 1:100 dilution. Lane 6: -96/5A 1:10 dilution. Lane 7: -96/5A 1:100 dilution. Lane 8: *Lac*P promoter/-28 1:10 dilution. Lane 9: *Lac*P promoter/-28 1:100 dilution. Lane 10: 100 bp molecular weight marker.


1 2 3 4 5 6 7 8 9 10
1,000 bp
500 bp
400 bp

The complete sequence of the TOPO vector is known and the kit contains sequencing primers specific for cloning PCR products. Light chain PCR product I (Figure 8, lane 2) and heavy chain PCR product XV (Figure 10B, lane 5) were gel purified, amplified by PCR, and transformed into TOP10 competent *E.coli* cells. Aliquots of the transformation culture were plated on LB/AMP plates (25 and 50 μl) and colony counts were performed (Table 5). Fifty colonies (40 light chain colonies and 10 heavy chain colonies) were placed in ten tubes with each tube containing five colonies. These cultures were grown overnight, purified, and digested with restriction enzymes *Pme*I and *Nco*I (Figure 17). No inserts were observed for the light chain colonies (Figure 17A), but positive results were shown for heavy chain colonies 41-50 (Figure 17B, lanes 2 and 3). Minipreps were performed on these ten colonies and then the minipreps were digested with restriction enzymes *Pme*I and *Nco*I. Promising bands were seen in six of the ten colonies (Figure 18A and B). These six minipreps were then digested with *Xho*I and *Spe*I to confirm the presence of the heavy chain fragment (Figure 19). All but one of the minipreps digested with *Xho*I and *Spe*I displayed appropriately sized bands. So, to explore this even further and know for sure that these bands were indeed the heavy chain fragment that was desired, sequence analysis was performed.

Sequence analysis was again unsuccessful with the Beckman Coulter kit (data not shown), so these potential heavy chain fragments were sent out to Cleveland Genomics (Cleveland, OH) to be sequenced. Two of the minpreps, 41 and 42 (Figure 19, lanes 3 and 4), were transformed into these competent cells and plated on LB/AMP plates. Colonies were counted (Table 6), grown in LB/AMP/glucose media, minipreps were

Table 5: Colony counts of light chain I and heavy chain XV TOPO transformants.

	Colony count[1]
Light chain I (25 µl)[2]	36
Light chain I (50 µl)	66
Heavy chain XV (25 µl)[3]	35
Heavy chain XV (50 µl)	35
TOPO control[4]	7
H_20 control	0

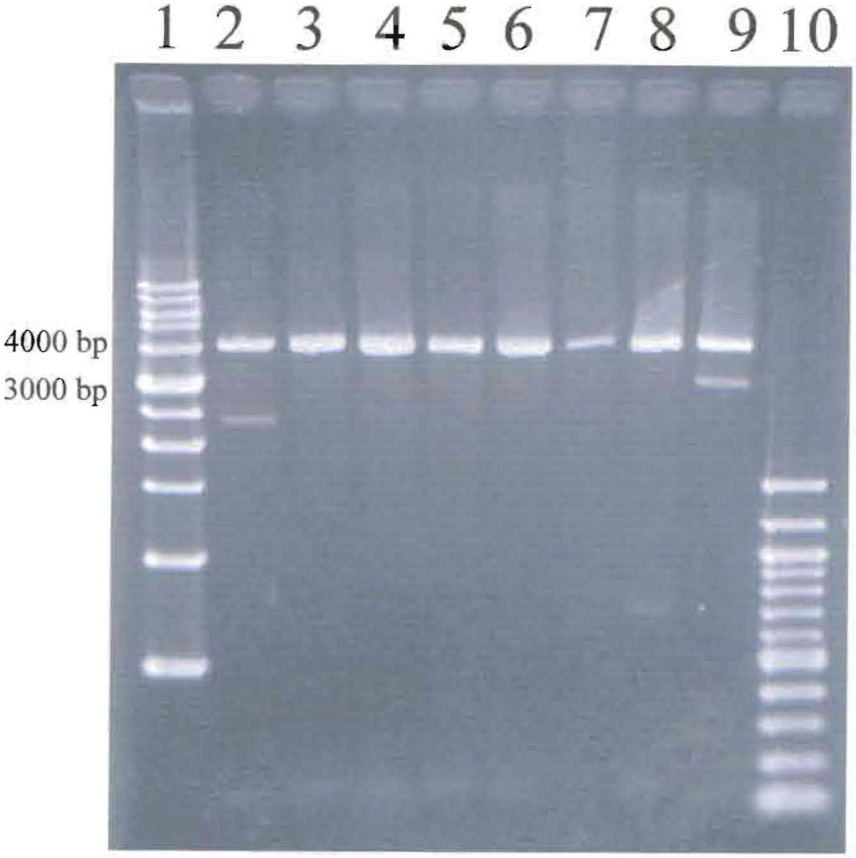
[1] PCR products from light chain I and heavy chain XV (2 µl) were mixed in individual reactions with salt solution (1 µl), sterile water (2 µl), and TOPO vector (1 µl) and transformed into One Shot TOP10 chemically competent *E. coli* cells. These reactions were incubated on ice, heat shocked for 30 seconds at 42°C, transferred to ice and S.O.C. medium (250 µl) was added. This mixture was then shaken at 200 rpm (37°C) for one hour, spread on LB/AMP plates and incubated overnight at 37°C. Colony counts were then performed on each plate.

[2] Light chain I is the transformants using PCR product (Figure 8, lane 2) using primers LC5A and LC3A (Table 1).

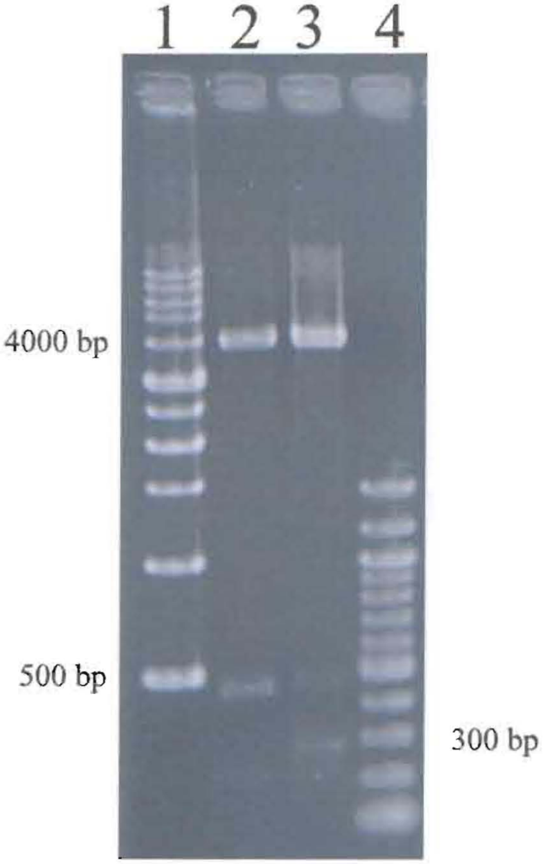
[3] Heavy chain XV is the PCR product (Figure 10B, lane 5) using primers VH-55 and VH-39 (Table 2).

[4] pUC19 control plasmid from the TOPO cloning kit (Invitrogen, Carlsbad, CA).

Figure 17: Light chain PCR I and heavy chain PCR IX digested with *Pme*I and *Nco*I. A: Light chain PCR I digested with *Pme*I and *Nco*I. Lane 1: 1 kb molecular weight marker. Lanes 2-9: Minipreps from light chain PCR I digested with *Pme*I and *Nco*I. Lane 10: 100 bp molecular weight marker. **B:** Heavy chain PCR IX digested with *Pme*I and *Nco*I. Lane 1: 1 kb molecular weight marker. Lanes 2 and 3: Heavy chain PCR product IX digested with *Pme*I and *Nco*I. Lane 4: 100 bp molecular weight marker.

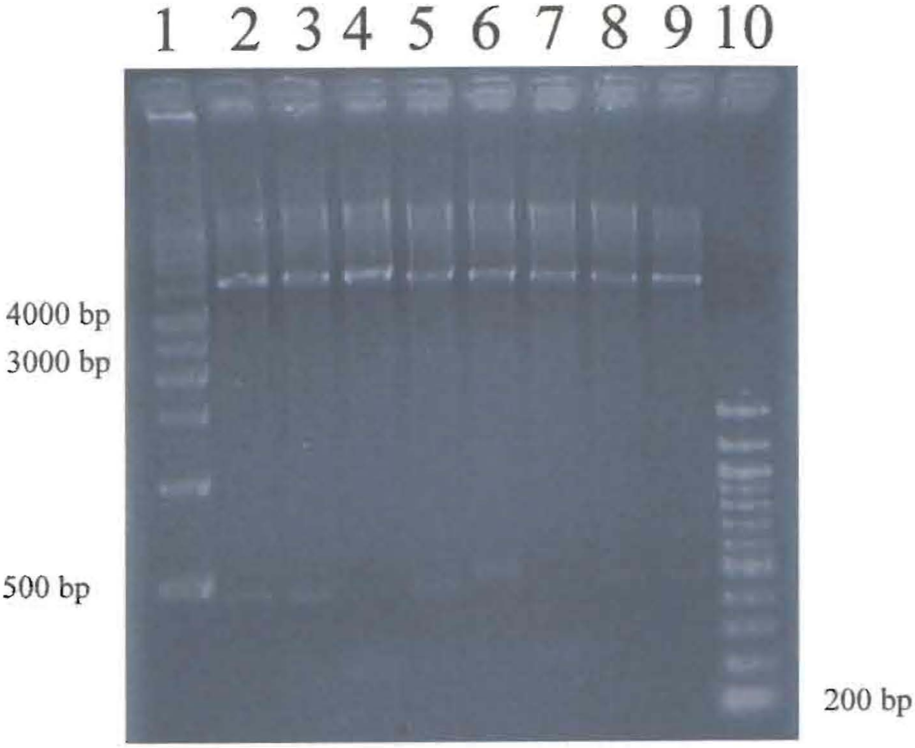

1 2 3 4 5 6 7 8 9 10
4000 bp
3000 bp

A

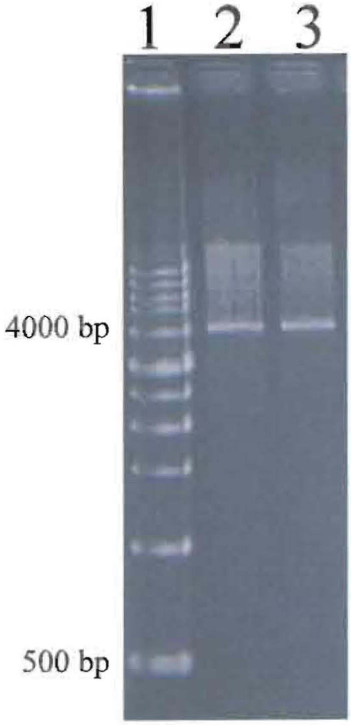

1
2
3
4
4000 bp
500 bp
300 bp

B

Figure 18: Minipreps of heavy chain colonies 41-50. Minipreps were performed on heavy chain colonies 41-50 (Figure 17B, lanes 2 and 3) and digested with restriction enzymes *Pme*I and *Nco*I. **A**: Lane 1: 1 kb molecular weight marker. Lane 2: miniprep 41. Lane 3: miniprep 42. Lane 4: miniprep 43. Lane 5: miniprep 44. Lane 6: miniprep 45. Lane 7: miniprep 46. Lane 8: miniprep 47. Lane 9: miniprep 48. Lane 10: 100 bp molecular weight marker. **B**: Lane 1: 1 kb molecular weight marker. Lane 2: miniprep 49. Lane 3: miniprep 50.


1 2 3 4 5 6 7 8 9 10
4000 bp
3000 bp
500 bp
200 bp

A


1 2 3
4000 bp
500 bp

B

Figure 19: Individual heavy chain PCR products (from Figure 17B) digested with *Pme*I and *Nco*I. Lane 1: 1 kb molecular weight marker. Lane 2: Colony 41 undigested. Lanes 3-8: Individual heavy chain colonies (41-48) digested with *Pme*I and *Nco*I. Lane 9: Nothing. Lane 10: 100 bp molecular weight marker.

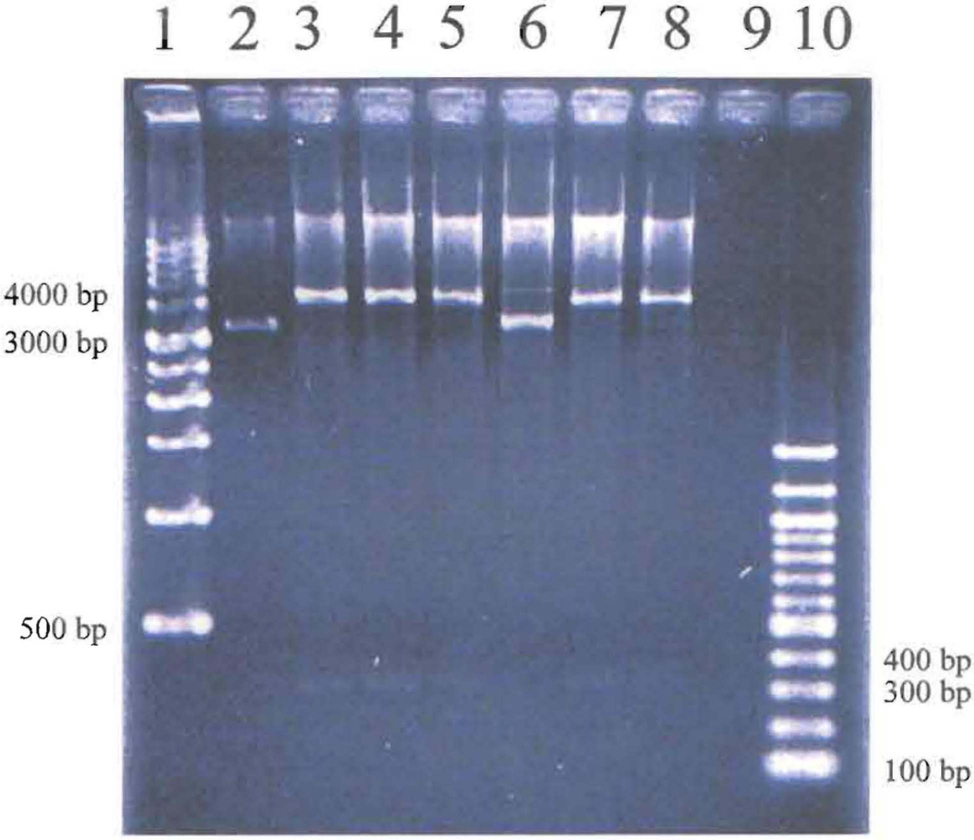

1 2 3 4 5 6 7 8 9 10
4000 bp
3000 bp
500 bp
400 bp
300 bp
100 bp

Table 6: Colony count of heavy chain minipreps 41 and 42.

	Colony count[1]
HC 41	312
HC 42	231
Control[2]	0

[1] Competent *E. coli* cells were prepared for transformation. TOPO minipreps (heavy chain 41 and 42) were added to the competent cells and incubated on ice for 30 minutes. This mixture was then heat shocked at 42°C for 50 seconds, cooled on ice, LB broth was added, and this all was incubated at 37°C for one hour. Transformation mixtures were plated on LB/AMP agar plates and colony counts were performed.

[2] Control was a pUC19 control plasmid included in the TOPO cloning kit (Invitrogen, Carlsbad, CA)

Table 7: DNA quantification of heavy chain minipreps 41 and 42[1].

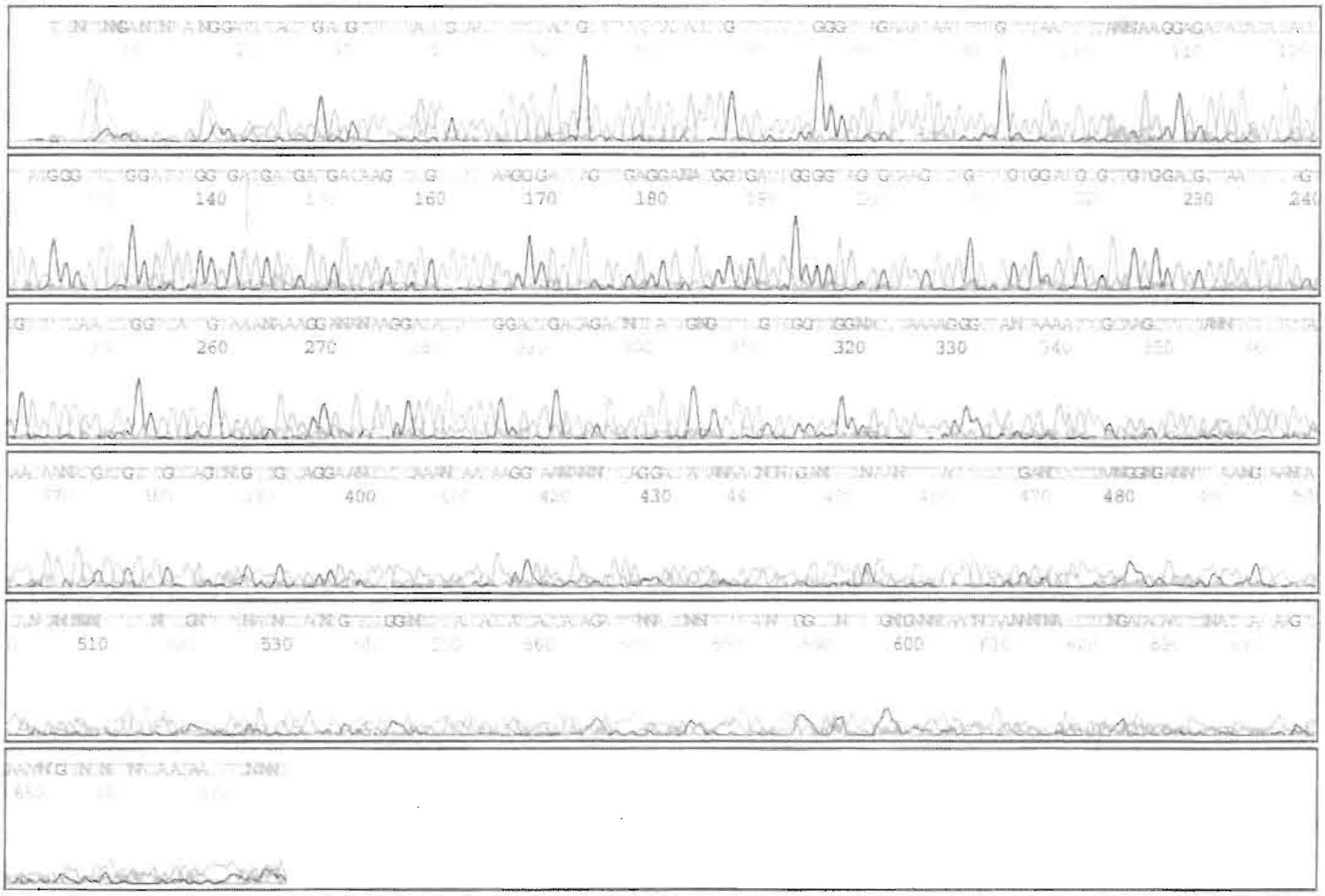
	concentration	260/280	260	280
42 #1.1[2]	188 ng/µl	1.81	.094	.052
41 #1.1[2]	50 ng/µl	1.95	.050	.026
42 #2.1	134 ng/µl	1.42	.134	.094
41 #2.1	448 ng/µl	1.15	.448	.390

[1] Colonies (Table 6) were picked from plates and grown overnight at 37°C with shaking in LB media (6 mL)/AMP (100 µg/mL)/glucose (60µL). Minipreps were performed, the DNA was quantified, and the concentrations were calculated.

[2] Miniprep 42 #1.1 and miniprep 41 #1.1 were both sent to Cleveland Genomics to be sequenced.

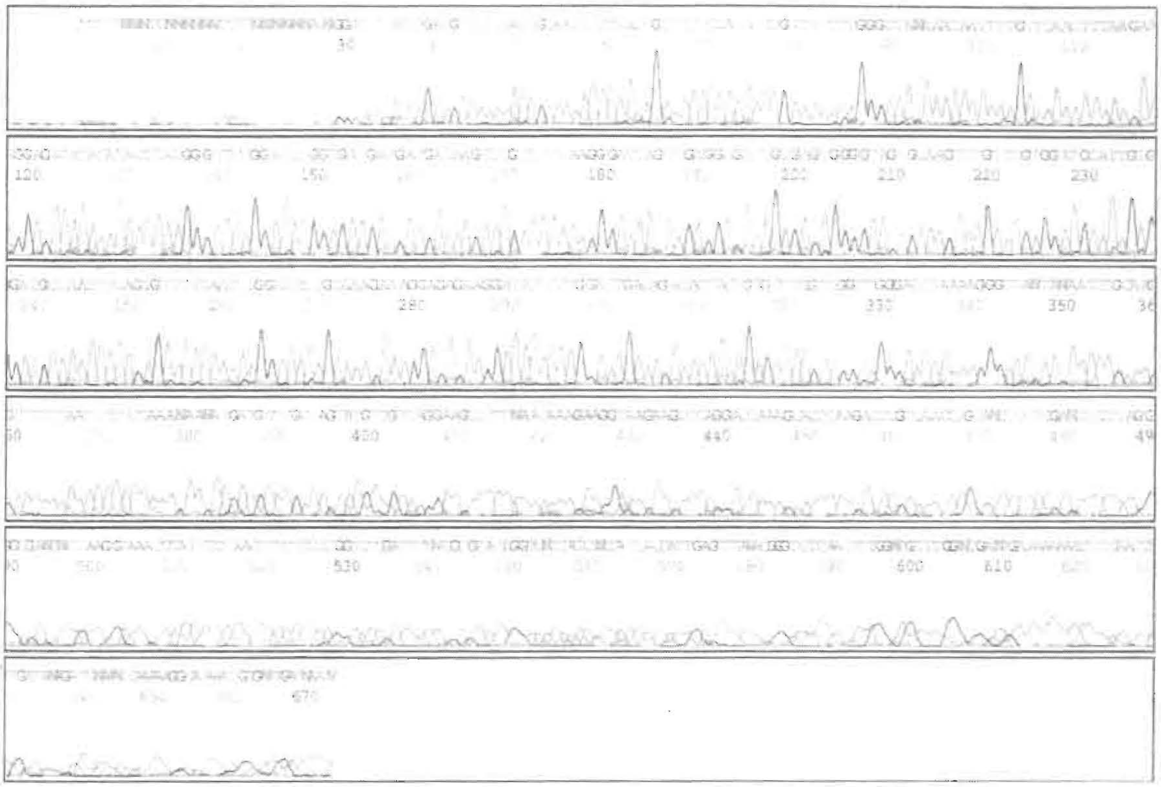
performed, and the DNA was quantified using a Eppendorf Biophotometer (Table 7). Minipreps 41 #1.1 and 42 #1.1 were sent to Cleveland Genomics to be sequenced. Figures 20 and 21 show the sequencing data received for these two potential heavy chain sequences. A BLAST (Altschul *et. al*, 1997) search was performed on these two sequences and they were shown to match up with the sequence of a mouse ribosomal protein, instead of the heavy chain fragment that was desired (Figure 22).

Figure 20: Sequence analysis of heavy chain miniprep 41.



41-PBAD-F

Figure 21: Sequence analysis of heavy chain miniprep 42.



42-PBAD-F

Figure 22: BLAST search analysis of heavy chain miniprep 41.

```
gi|14714896|gb|BC010604.1|  Mus musculus ribosomal protein S6, mRNA
(cDNA clone MGC:6573
IMAGE:3481640), complete cds
Length=833

 Score =  428 bits (216),  Expect = 9e-117
 Identities = 244/249 (97%), Gaps = 0/249 (0%)
 Strand=Plus/Plus

Query  179
AGCGCAAGTCTGTTCGTGGATGCATTGTGGACGCTAATCTCAGTGTTCTCAACTTGGTCA  238

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305
AGCGCAAGTCTGTTCGTGGATGCATTGTGGACGCTAATCTCAGTGTTCTCAACTTGGTCA  364

Query  239
TTGTAAAGAAAGGAGAGAAGGATATTCCTGGACTGACAGACACTACTGTGCCTCGTCGGT  298

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  365
TTGTAAAGAAAGGAGAGAAGGATATTCCTGGACTGACAGACACTACTGTGCCTCGTCGGT  424

Query  299
TGGGACCTAAAAGGGCTANTANAATCCGCAAGCTTTTTAATCTCTCCnnnnnnnnTGATG  358
            |||||||||||||||||| || |||||||||||||||||||||||||||| ||
||||||
Sbjct  425
TGGGACCTAAAAGGGCTAGTAGAATCCGCAAGCTTTTTAATCTCTCCAAAGAAGATGATG  484
```

V. DISCUSSION

S. aureus is becoming resistant to most common antibiotics, including methicillin (Talon *et al.*, 2002) and vancomycin (Lowy, 1998, Peschel *et al.*, 2001, Senior, 2002, and Smith and Jarvis, 1999). As a result, other methods of treatment are needed. Our lab has produced hybridomas that secrete monoclonal antibodies that are specific for binding to *S. aureus* Type 5 CP. Monoclonal antibodies could be used to treat *S. aureus* infections, but this method of antibody production produces proteins that are seen as foreign when injected into a human model. In addition, monoclonal antibodies are unstable and technically difficult to produce in large quantities. Phage display technology is a more efficient method of producing a library of antibody fragments (scFvs) that are expressed on the surface of the phage and are easily selected for high affinity binding to an antigen of interest. When coupled to a toxin, these scFvs could bind to the capsule of the bacterium and rid the body of the infection. When compared to monoclonal antibodies, scFvs are easier to produce, have less contamination problems, and it is easy to manipulate the genes encoding the product to increase the affinity of binding to antigens.

The purpose of this work was to produce a scFv that would have high affinity binding that was specific to Type 5 *S. aureus* capsular polysaccharide. Clones of hybridoma SM.T5.B2.A12.C9, which produced monoclonal antibodies that tested positive for Type 5 CHO/bacteria and negative for Type 8 CHO/bacteria (Slusher thesis, 2005), were grown and used to produce total RNA. The method of producing scFvs from hybridoma mRNA has been used by other labs. Krebber *et al.* used this same method to clone scFvs directed toward tumor cell lines by reengineering a flawed phage display system. They eliminated aberrant light and heavy chain mRNAs by creating scFvs that bound to the specific antigen of interest (Krebber *et al.*, 1997). While most labs utilize random phage display libraries and screen the library for the desired clone that binds to the antigen of interest, our lab has chosen to use hybridomas specific for the antigen of interest to create a scFv. The advantage of this method is that all scFvs produced should bind to the antigen of interest, making it easier to identify clones with low binding affinity than when screening a library with a large number of potential variants. The integrity and size distribution of the total RNA that was isolated was checked by agarose gel electrophoresis. Mouse RNA contains a 18S rRNA band (1.9 kb) and a 28S rRNA band (4.7 kb) and the 28S band should be double the intensity of the 18S band when denatured and stained on an agarose gel. This was shown in Figure 7, lane 2, where the intensity of the 28S rRNA band is almost twice that of the 18S rRNA band and the size of the 28S band is more than double that of the 18S band, thus confirming the presence of pure RNA. This pure total RNA was then used to make the first strand of cDNA using a cDNA first strand synthesis kit (NEBiolabs, Ipswich, MA).

The resulting cDNA and primers specific for the light chain variable region and the heavy chain variable region (Okamoto, *et al.*, 2004) were used to make the second strand of DNA by PCR. These primers were chosen because they were IgM specific and they were, according to the authors, diverse enough to cover the whole variable light chain and variable heavy chain gene repertoires, which is crucial to amplifying all the possible sequences. This however has some drawbacks because of the degenerative nature of the different primer sets used (Tables 1 and 2). Each non-A, C, G, or T nucleotide represents several different possibilities for that position, which results in an enormous amount of possible sequences that have the potential of being primed.

Restriction sites (*Sac*I and *Hind*III for the light chain and *Xho*I and *Spe*I for the heavy chain) were added to these primers so that they could be ligated into phagemid pSCL and then transformed in competent *E.coli* cells. The light chain primers were used first to make the light chain variable fragment. Ten different 5'/3' primer combinations were used in a PCR to amplify the cDNA. No bands were seen on an agarose gel the first time PCR was performed on the cDNA using the light chain primers (data not shown). The PCR reactions were tried again using a lower annealing temperature (50°C instead of 58°C that was used in the first round of PCR) and smaller total reaction volumes (25μl instead of 100μl). This second round of PCR reactions produced four reactions that all showed positive results for amplification of the light chain variable region (Figure 8). The DNA from two of these reactions (LC5A/LC3A and LC5B/LC3A) was amplified further by PCR and purified using a QIAquick spin column (Qiagen, Valencia, CA) (data not shown).

These two reactions and phagemid pSCL were digested with restriction enzymes *Sac*I and *Hind*III (Figure 9) and then cleaned up using a QIAquick kit (Qiagen, Valencia, CA). Once it was determined that the light chain PCR reactions and phagemid pSCL were pure, each reaction was separately ligated together with the phagemid using DNA ligase (data not shown). Phagemid pSCL was chosen because it previously produced successful results (Barbas *et al*., 1991) and was thought to contain the appropriate gIII protein, restriction sites, and linker sequence ideal for creating and cloning scFv fragments. It was designed and used previously by Barbas and coworkers to convert functional antibody (Fab) genes into scFv genes. It was produced from plasmid pComb3-C by deleting the light chain cDNA cloning region and the *lac* promoter, and replacing the heavy chain cloning region with a synthetic oligonucleotide that encodes two cloning regions plus a 19-codon linker to connect the light and heavy chain variable region genes.

The ligation reactions were transformed into chemically competent XL1 Blue *E. coli* cells (Stratagene, La Jolla, CA) that were endonuclease deficient (which improves the quality of miniprep DNA) and recombination deficient (which improves the insert stability). Tranformations were then plated on LB/CAM containing agar plates to isolate only the colonies containing cells with the phagemid (containing insert) that have resistance to CAM. Transformant counts of the different reactions were all good compared to the negative control (Table 4). Minipreps were performed on five transformants from each ligation reaction and then analyzed by agarose gel electrophoresis (Figure 11). Two of these minipreps, 1.2.1 and 1.5.1 (both from ligation reaction #1, which used primers LC5A and LC3A), showed positive results. DNA having the correct molecular weight for the light chain was inserted into the vector of phagemid pSCL. These minipreps were chosen to be analyzed further. When digested with restriction enzymes *Sac*I alone, *Hind*III alone, and *Sac*I and *Hind*III together, various results were seen. Restriction digests demonstrated the presence of unexpected restriction sites (more than one band on gel) or missing restriction sites or inserts (no bands on gel) on a few of the clones (Figure 12). These restriction enzymes should only produce an insert band when both *Sac*I and *Hind*III are used together. These varied results led us to believe that the restriction sites are found in the phagemid twice and when cut with one enzyme it cuts at both sites producing a length of DNA from between the two sites. Krebber *et al.* hypothesized that vector systems that use a set of four enzymes to clone variable light and variable heavy chains separately may be at risk of cutting in antibody genes and incorporate internal restriction sites in the variable regions that cause mismatches with the antibody template and non-specific amplification by poor primer hybridization. They suggested that *Sac*I cuts in the majority of mouse λ chains and is not suitable for simultaneous cloning of λ and κ light chains (Krebber *et al.*, 1997). This problem remains unresolved, though we suspect that more than one insert may be found in these clones. The DNA from minipreps that produced inserts similar in molecular weight (~400 bp) to the light chain PCR product were sequenced to see if the inserts were in fact light chain inserts.

Sequencing reactions were performed using a *lac* promoter primer and a CEQ 2000 Dye Terminator Sequencing with Quick Start Kit (Beckman Coulter, Inc., Fulleron, CA) and ran on a Beckman Coulter CEQ 2000 XL DNA Analysis System. Good, clear DNA base pair data was obtained for the kit control DNA, but the data obtained for minipreps 1.1.2 and 1.2.1 was not readable (data not shown). Different primer combinations [primer LC5A/-28gIII primer (which begins priming 28 nucleotides back from the gIII protein), primer LC5A/-96gIII primer (which begins priming 96 nucleotides back from the gIII protein), *lac*P primer (which begins priming at the *lac* promoter) (Miller, 1992)/-28gIII primer] were used to try to amplify miniprep 1.2.1 to see if phagemid pSCL contained any of these sites. It was found that the two gIII primers worked, but the *lac*P primer did not, leading us to believe that this phagemid contained the gIII sites but not the *lac* promoter. Sequencing reactions were repeated using the gIII primers instead of the *lac*P primer. Sequencing reactions with these primers were unsuccessful and no sequence was obtained.

Since the sequence of phagemid pSCL was not known and we were unsuccessful on many attempts to sequence what was inserted into pSCL, we decided to switch vectors and try using a vector that had a known sequence and specific sequencing primers. The vector chosen was a TOPO vector that was part of a kit containing everything needed to clone the insert. Using this vector, light chain PCR reactions I (using primers LC5A and LC3A) and III (using primers LC5B and LC3A) (Figure 8, lanes 2 and 4) and heavy chain PCR reaction XV (using primers VH-55 and VH-39) (Figure 10B, lane 5) were cloned and transformed into TOP10 competent *E. coli* cells. Minipreps were then done on the resulting transformants and digested with restriction enzymes *Pme*I and *Nco*I (restriction sites in the TOPO vector). Ten minipreps were performed (8 light chain and 2 heavy chain) with only the heavy chain inserts showing an appropriate size band when digested with the appropriate enzymes (*Spe*I and *Xho*I). None of the light chain minipreps produced a band of any kind when digested with their specific enzymes (*Sac*I and *Hind*III). We sequenced the heavy chain minipreps that showed a positive band. Two heavy chain minipreps, HC-41 and HC-42, were sent to Cleveland Genomics (Cleveland, OH) to be sequenced. Upon receiving the results of our sequencing data, it was found that we did get an actual readable sequence, but it was not what we expected to see. The DNA sequence that we obtained from Cleveland Genomics was put into a BLAST search and it matched a mouse ribosomal protein, not the heavy chain IgM mouse sequence that we were looking for. A reason for this may be due to the fact that the primers that were used to amplify the heavy chain and light chain sequences were degenerative and primed the wrong mRNA.

Since the methods used to culture the clones, produce pure RNA, produce cDNA, amplify the cDNA, ligate pieces of DNA together with different phagemids and plasmids, purify phagemids, etc. were shown to be efficient and able to produce an insert and sequence (although not the sequence desired) and the sequencing results obtained from the heavy chain inserts were not the heavy chain fragments that were expected, IgM protein from the cell culture of clone SM.T5.B2.A12.C9 will be purified to determine the amino acid sequence. Using this sequence, primers will be designed to PCR amplify the cDNA from the heavy and light chain of this clone. These fragments will then be digested with the appropriate restriction enzymes, ligated into a suitable vector, and sequenced to confirm the presence of a scFv. Three or four scFvs will be produced and gene shuffling will be performed between these clones. These new clones will be tested for increased binding affinity by panning and then the clones that had the highest binding capabilities will be coupled to a toxin and tested in mice for their ability to bind to and kill the pathogen.

While phage display is still a relatively new technique, this form of antibody production (scFv) has enormous therapeutic potential. It has been used by other labs to isolate antibody fragments towards viral infections (human immunodeficiency virus type 1, herpes simplex virus, respiratory syncytial virus, and coronavirus) (Maynard and Georgiou, 2000), blood clots (Yan *et al.*, 2004), and various forms of cancer cell-surface tumor antigens (melanoma, ovarian, prostate, esophageal, lymphoma, hepatocellular carcinoma, lung, etc.) (Chowdhury *et al.*, 1998, Hombach *et al.*, 1998, Lee *et al.*, 2002, Pavlinkova *et al.*, 2000, and Yu *et al.*, 2005). This technique shows promise for other diseases and infections, including bacterial infections like *S. aureus*. If the scFvs produced are found to be efficient at killing *S. aureus in vitro*, then they can be tested for their ability to treat *S. aureus* infections in mice. This technique has the potential to produce much needed alternative therapies for antibiotic resistant *S. aureus* infections.

VI. WORKS CITED

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