DEVELOPMENT OF IMMUNOLOGICAL REAGENTS FOR DETECTING *Salmonella enterica* SEROVAR TYPHIMURIUM

By

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Salmonella enterica serovar Typhimurium (*S*. Typhimurium) is bacterium responsible for over a million cases of gastroenteritis per year in the United States. Extremist groups have also used this bacterium as an agent of biological terrorism. Salmonella infections cause huge economic losses to the food industry, especially the poultry and diary industries. Effective measures to counter the dangers and economic losses caused by this bacterium should include rapid, sensitive, and specific detection of the bacterium in the samples suspected of contamination.

A biosensor system consisting of an optic fiber probe with antibodies attached to the plastic surface of the probe connected to a photodiode and a computer monitor was developed at the University of South Florida. When the optic fiber probe is placed in the samples, the antibodies on the surface of the probe capture the relevant microbe. Detection antibodies labeled with a fluorescent dye are then added forming a sandwich by the two antibodies with the microbe in between. When light of a certain wavelength is

passed through the optic fiber probe, the fluorescent dye is excited and emits a light of different wavelength. This emitted light is captured by the photodiode and quantified. This entire process can be completed in as little time as 20 min.

We developed an ELISA protocol for using whole bacterial cells as antigens and screened several commercially available murine monoclonal antibodies recognizing surface epitopes of *S.* Typhimurium using the standardized procedure. ELISA was chosen as the method of choice, because it closely resembles the conditions of the biosensor system and has a much higher throughput than the biosensor system. Recombinant antibody phage display libraries offer a powerful, economical, and rapid method of screening libraries of large complexities of recombinant antibody molecules on the order of 10^9 variants. We used the Griffin.1 and Tomlinson I Human Synthetic scFv phagemid libraries to isolate antibodies to surface epitopes of *S.* Typhimurium. Panning was done under varying conditions using whole bacterial cells, purified LPS, or flagella as antigens. Two antibodies recognizing the flagella of *S.* Typhimurium, named SF1 and SF2, were isolated.

We used the SF1, SF2, and the provided anti-BSA positive control phagemids to obtain soluble antibody molecules (scFv) instead of fusion proteins displayed on the phage surface, and genetically modified the antibodies to increase their usefulness. These studies will aid in the continuing development of immunological tools for use with the real time fiber optic biosensor system.

CHAPTER 1
INTRODUCTION

Bioterrorism is a problem of ever-increasing magnitude confronting civil societies all over the world. The cost of development and ease of deployment of agents of bioterrorism make these weapons appealing to extremists (1-3). Mailing of envelopes containing spores of anthrax after September 11, 2001 serves as a good example to illustrate the fact. Fringe extremist groups with limited means to access sophisticated weapons may use agents of bioterrorism to further their interests and cause panic in society. A famous example was the contamination of salad bars with *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) by the Ranjneeshee religious cult in Dulles, Oregon in 1984 to prevent local people from voting in a county election (4,5). The economic and psychological impacts of these attacks can be far more devastating than the direct threat to human health caused by these weapons (6). As the saying goes, prevention of these attacks is the best form of protection that can be offered. Nevertheless, when an attack is suspected, law enforcement officials should be able to verify and confirm actual use of biological agents in a timely manner to take corrective actions. With the increasing incidence of bioterrorism, it is imperative that sensitive, specific, rapid, and reliable systems be available to detect agents of bioterrorism in complex matrices such as food, water, and on surfaces. Such detection systems also facilitate better analysis of clinical samples in a hospital setting leading to a better and quick diagnosis and therapy (7). Such detection systems also increase the efficiency of microbiological labs.

Contamination of food in food processing industries is a problem of huge economic and practical concern. Monitoring food samples at various levels of processing would ensure better control over food quality (8-11). Tests used in such situations, in which multiple samples from multiple sources need to be analyzed quickly, should be easy and rapid to perform, should be sensitive and specific, and should give results in a short time (9,10).

The characteristics of the ideal tests or systems used for detecting contamination with microbes and toxins should be sensitive, specific, rapid, portable, less time consuming, involve minimal handling and processing of the samples, and obviate the need for costly and bulky equipment. The need for skilled technicians should be minimal (12).

Traditional Methods of Bacteriological Detection and Analysis

Some of the traditional methods of determining microbial contamination involve enumeration of coliforms by the Most Probable Number (MPN) method and biochemical tests. Estimating contamination by the MPN method is not very specific and not sensitive (13). Biochemical methods identify the microbes in a very specific manner; however, they are time consuming and not very sensitive. Generally, the detection of microbes involves a pre-enrichment step for 6 to 8 hours, followed by growth in an enrichment medium and then in a selective medium. Several biochemical tests are needed for characterizing the microbes (14-16). Some of the modern techniques used in industry and laboratories for rapid and sensitive detection of microbes involve amplification of specific nucleic acid sequences by Polymerase Chain Reaction (PCR) (17,18); immunomagnetic separation (19); enzyme tests with synthetic chromogenic or fluorogenic substrates (20); real-time PCR (21); and flow cytometry (7,9,22). Many

commercially available kits including API Rapid 20E (bioMerieux, Los Angeles, CA), GN Microplate (Biolog, San Jose, CA), Directigen (BD Diagnostic Systems, Franklin Lakes, NJ) are available for detection of microbes (23). Many of the above-mentioned techniques identify microbes sensitively and specifically; but require costly and sensitive equipment, skilled personnel, and sterile conditions to perform the analysis. Most of these systems are not portable and cannot be used in field conditions.

Antibodies show exquisite specificity toward their target. Murine monoclonal antibodies have been used for many years in research for detecting the antigens and separating the particles from a complex mixture (24). Some of the above-mentioned detection systems employ antibodies in conjunction with some other technique to identify the pathogen. Examples for such techniques using immunological reagents include immunofluorescence (25), fluorescence automated cell sorting (25), immunoprecipitation, immunohistochemistry, and immunomagnetic separation (19).

The Biosensor System

Transportable fiberoptic biosensor systems are being developed that will enable real-time or near–real-time detection of microbes and toxins (26-28). These systems combine the advantage of sensitivity and specificity afforded by antibodies and a robust architecture enabling their use in field conditions. The evanescent wave fiberoptic biosensor works on the principle of a sandwich immunoassay. Monoclonal or polyclonal antibodies are used as capture antibody and reporter antibody. The capture antibodies are attached to the surface of the optical fiber probe. Reporter antibodies are tagged with a fluorescent dye. The evanescent field-sensing region is formed by the final 7.5 cm of the fiber, where the cladding is removed to expose the silica core. When the samples (such as contaminated food and water) that need to be analyzed are brought in contact with the

probe, the capture antibodies bind to the antigen. When the reporter antibodies are added to the solution, they bind to the relevant epitopes of the specific antigen to be detected, forming a sandwich. A 635 nm laser diode provides the excitation light, which is launched into the proximal end of the probe. Fluorescent molecules within approximately 1000 nm of the fiber are excited by the evanescent field; and a portion of their emission energy recouples into the fiber. A photodiode is used to quantitate the collected emission light at wavelengths of 650 nm and above, and the data can be displayed on a computer monitor (26). Similar systems using chemiluminescence are also in use (27).

The advantages of this method over the traditional methods of detection are

- "Dirty" samples can be processed without a need for pre-enrichment or enrichment steps.
- The biosensor is reported to be detecting as low as 3 CFU/mL of *E. coli* O157:H7 in seeded ground beef (26).
- The architecture of the apparatus is robust, enabling the use in field conditions.
- Results are obtained in as little time as 20 min, thereby greatly aiding the law-enforcement officials and health personnel in times of emergencies.

Antigenic Structure of *S.* Typhimurium

Salmonella enterica serovar Typhimurium is a gram-negative bacterium that is responsible for over a million cases of gastroenteritis in the United States every year (29). *Salmonella* spp. are responsible for three classes of diseases: enteric fevers, septicemia and gastroenteritis. Typhoid fevers are caused by *S.* Typhi and *S.* Paratyphi, while *S.* Cholerasuis causes septicemia. However, the most common disease caused by Salmonella is gastroenteritis, the most common causative agent of which is *S.* Typhimurium (30,31). Salmonella gastroenteritis is generally self-limiting. Salmonella infections, with the exception of enteric fevers, are typically food-borne

illnesses that are transmitted to humans by animal products such as poultry, pork, meat, and eggs. *S.* Typhimurium can also be used as an agent of bioterrorism by contaminating food and water supplies with the bacterial culture (3). Salmonella infections cause major economic losses in food industry (32). A biosensor system for detecting *S.* Typhimurium in various matrices would be of great practical use. This thesis describes the efforts done to characterize existing monoclonal antibodies and to isolate recombinant phage display antibodies for use in such a system. Antibodies recognizing the cell surface epitopes were chosen for this study to enable the detection of whole cells, without the need for preparing cell extracts.

The outer membrane of *S.* Typhimurium is composed of lipopolysaccharide (LPS), phospholipids, outer membrane proteins such as porins, and matrix proteins such as OmpA. Lipopolysaccharide is found only in the outer membrane of gram-negative bacteria. Lipopolysaccharide is composed of three components viz., lipid A, core oligosaccharide, and O antigen. Lipid A is the major lipid constituent of the outer membrane and is highly conserved among different gram-negative organisms. Lipid A is responsible for the endotoxic activity of gram-negative bacteria. The core oligosaccharide connects the lipid A moiety to the peripheral O-antigen and is conserved among different bacteria. The outer core region consists of a branched pentasaccharide chain made up of sugars such as glucose, galactose, and 2-acetamido-2-deoxy-D-glucose. The inner core region is made up of L-glycero-D-mannoheptose and 3-deoxy-D-manno-octulosonic acid. Variation in O antigen results from the variation in sugar components and from the variation in the nature of covalent bonds between the sugars and from the order of the sugar molecules in the oligosaccharide. The repeating

units are composed of trisaccharides, pentasaccharides, or branched chain oligosaccharides. The differences in the O side chains are exploited to differentiate between various serogroups by immunological reagents. Thus, O-antigen makes a good target for characterizing gram-negative bacteria (33).

Flagella are another good target for immunological detection of whole cells. Flagella are made up of an external filament, a hook region, and a basal body (34). The hook region, which lies outside the outer membrane, connects the external filament to the basal body, which spans both the outer and inner membranes. The external filament is a polymer of a 60-kD flagellin molecule, which provides many sites for binding of anti-flagellar antibodies (34). Flagellin is encoded by two different genes in *S.* Typhimurium, which are highly homologous, but the two forms of flagellin are antigenically distinct. For every 10^3 to 10^5 generations, there is a reversal in the expression of the genes, but at any given time, only one of the genes is expressed. This is due to a switching in the expression of the promoters for these genes and this phenomenon is called "phase variation" of flagella (35-37)

Murine monoclonal antibodies recognizing various O antigens such as O4 and O12, antibodies of undetermined specificity to the bacterial cell surface, and antibodies recognizing flagella were chosen for this study based on the above-mentioned criteria.

Monoclonal Antibody Production

Kohler and Milstein first described the idea of monoclonal antibodies (38). Monoclonal antibodies have since been used in many varied applications (24). The production of monoclonal antibodies involves immunizing mice with the antigen and boosting the immune response by periodical booster doses of the antigen. Serum is collected periodically, and the response to the antigen is determined by ELISA or

Western blot. When the mice have a satisfactory titer of the antibody, an assay is done to determine the predominant isotype of the antibodies (i.e., IgG or IgM). The mice having good titers and class switching (IgM to IgG) are selected. The spleen of the selected mouse is harvested and separated into single cells. These single cells are combined with mouse cells of the myeloma cell line SP2/0. Polyethylene glycol (PEG) is added to promote the fusion of the cells, so these cells are called hybridomas. The cells are then resuspended in selective medium that kills off nonfused cells and are grown in a 96-well plate. After several days of growth at 37 ºC and intermittent feeding with tumor-conditioned medium, the supernatant is tested for activity by ELISA, and cells in the positive wells are transferred to a 24-well plate and grown further for a week. A secondary screen is done with the supernatant from these wells. Finally, since it is possible that the initial cultures contain different hybridomas, positive cultures are subjected to limiting dilution and reexamined for activity. Antibody-positive cell lines are now called clones and can now be grown in larger cultures for mass production of the antibody from the culture supernatant followed by affinity purification.

Recombinant Phage Display Antibodies and scFv

Phage display is a technique of displaying antibodies or peptide fragments as fusion proteins to one of the coat proteins of the phage. M13 is the commonly used phage for this purpose (39). Single chain variable fragment (scFv) antibody molecules are composed of the variable portions of the heavy and light chains of immunoglobulin linked by a spacer region. The variable portion of the antibody molecule is responsible for the specificity of the antibody. Antibodies can be displayed on the phage particle in either scFv format or in Fab format. The phage antibodies and the scFv fragments can be used in all immunological assays, similarly to a monoclonal antibody (40,41).

M13 Phage Biology

M13 is a nonlytic filamentous bacteriophage of the Ff class of phages, which infect *E. coli* displaying the F conjugative pilus. The phage contains a circular, single-stranded DNA (ssDNA) of approximately 6,400 nucleotides as genomic DNA and is surrounded by a flexible protein coat. pVIII, a 50-amino acid protein present in approximately 2,700 copies, forms the major part of the protein coat. pIII, a 406-amino acid protein, is a minor coat protein present at one end of the phage particle and is present in approximately 5 copies along with another minor coat protein, pVI (39).

pIII is composed of three domains – two amino-terminal domains, N1 and N2, and a carboxy-terminal domain, CT, separated by a glycine-rich region. The first domain, N1, contains the amino-terminal 68 amino acids and is required during infection for the translocation of the phage DNA into the cytoplasm and insertion of coat proteins into the membrane. The second domain, N2, is composed of residues 87 to 217 and is responsible for binding to the F pilus. Both domains contain cysteine molecules that are involved in intramolecular disulfide bonds within each domain. N1 and N2 are exposed on the surface of the phage particles; removal of these domains by protease treatment produces noninfectious phage. The carboxy-terminal 150 residues make up the third domain, CT, which is essential for forming a stable phage particle (39).

Infection is initiated by the binding of the tip of the F pilus to the N2 domain of the phage pIII protein. After the phage binds to the pilus, the pilus retracts bringing the pIII end of the phage particle to the periplasm. N1 interacts with the TolA protein, and pVIII and minor capsid proteins disassemble into the cytoplasmic membrane as the phage DNA is translocated into the cytoplasm.

After the phage ssDNA (+ strand) enters the cytoplasm, the complementary (- strand) is synthesized, and a covalently closed, supercoiled, double-stranded DNA called the replicative form, RF, DNA, is formed. New DNA molecules are synthesized by rolling circle mode of replication. mRNA is synthesized using the (–) strand as the template. The bacteria then synthesize phage proteins. The phage particles are assembled when the concentration of pV reaches a critical concentration. M13 is a non-lytic phage. Therefore, the phage particles bud out of the *E. coli* cell membrane without killing the cell (39).

Phagemid vectors are plasmid vectors that contain both *E. coli* ColE1 *ori* and M13 *ori* sequences. Therefore, phagemids can be propagated as plasmids in bacteria. These vectors do not encode all of the genes necessary for the formation of phage particles by themselves. However, when *E. coli* containing phagemids are superinfected with a helper phage to supply all of the necessary proteins for replication and packaging, the phagemid DNA can replicate and be packaged into viable infectious phage particles (39,42).

Griffin.1 and Tomlinson I + J Human Synthetic $V_H + V_L$ Phagemid Libraries

Griffin.1 and Tomlinson I + J Human synthetic $V_H + V_L$ phagemid libraries are scFv phagemid libraries displaying human antibody sequences as fusion protein to the minor coat protein, pIII. These libraries were constructed by amplifying the genes coding for the variable portions of immunoglobulin molecules from the peripheral blood lymphocytes of unimmunized donors (41,43).

The nucleotide sequence at the 5’ end of the exons coding for V-genes is conserved in human beings. Using universal degenerate primers, the antisense strands of the heavy and light chain V-genes were amplified. Heavy and light chain V-genes were linked by

PCR by using overlap extension technique. The V-genes were amplified by PCR, and the repertoires were combined with linker DNA, which has regions of sequence homology with the 3' end of the V_H gene and 5' end of the V_L gene. Then PCR was done with primers hybridizing to outer flanking sequences of the V_H and V_L genes. The final product contains the V_H and V_L genes linked by the linker region, $(Gly_4Ser)_3$, with restriction sites for facilitating cloning into phagemid vectors (43). The vector used for constructing the Griffin.1 library was pHEN2, and the Tomlinson libraries were constructed in pIT2 vector. Antibody molecules of this form – heavy and light chain regions of the variable portion of the immunoglobulin molecules linked by a peptide linker – are called scFv (single chain variable fragment) antibodies.

For displaying the scFv sequence on the surface of the phage as fusion protein, the scFv gene was cloned in frame with the gIII gene coding for the pIII protein in the phagemid vectors. When *E. coli* harboring these phagemids are superinfected with a helper phage, phage particles are produced which display the scFv-pIII fusion protein.

Some other important features (42) engineered into these libraries are

- The phagemid encodes the *bla* gene, which can be used as a selectable marker. When *E. coli* cells harboring the phagemid are grown on selective media, *E. coli* form colonies rather than plaques because the phagemid cannot form complete phage particles without superinfection by helper phage.
- A bacterial leader peptide sequence, *pelB*, is present at the 5' end of the scFv gene. PelB directs the scFv protein into the secretory pathway, for exporting the protein into the periplasm.
- A c-myc epitope and a hexahistidine tag are present at the 3' end of the scFv gene to allow for affinity purification and detection using appropriate antibodies and nickel matrices, respectively.
- Protein A and protein L binding sites are present in the antibodies produced from Tomlinson libraries. These sites offer additional options for detection and affinity purification. Also, all of the phage antibodies produced from the Tomlinson library are pre-selected for their ability to bind to protein A and protein L to ensure that most of the phage have the scFv-gIII gene, instead of wild type gIII.
- A TAG amber stop codon is present at the junction of the scFv gene and gIII. The

presence of amber stop codon allows the production of scFv molecules as soluble antibody molecules instead of scFv-pIII fusion proteins. When the phagemid is present in a suppressor strain of *E. coli*, the amber stop codon is translated as glutamine and instead of termination of the peptide chain synthesis, and scFv-pIII fusion protein is synthesized. When the phagemid is in a non-suppressor strain of *E. coli*, the synthesis of peptide chain is terminated at amber stop codon, releasing a soluble scFv molecule.

- The scFv-gIII is expressed from the wild type *lac* promoter control of LacI, so that the synthesis of the protein, which could be toxic to the host *E. coli*, can be suppressed by glucose. Alternatively, when scFv antibody molecules are intended to be produced, synthesis can be induced by IPTG.

Phage display offers a powerful, economic, and rapid method of screening libraries of huge complexity in the order of 10^9 library size (41). Short peptides or antibody molecules can be displayed on the surface of the phage. The phage displaying the sequence of interest can be isolated by incubating the library with the antigen to allow for specific binding, and the unwanted phage are washed away. Abundant amounts of the phage of interest can be produced by propagating them in *E. coli*. Antibodies can be obtained as soluble molecules or as fusion proteins. The advantages of this technology can be considered under two aspects – ease of production and ease of manipulation.

The advantages of recombinant phage display antibodies over murine monoclonal antibodies in terms of their production are:

- The Tomlinson I + J libraries and the Griffin.1 library have a complexity of approximately 1.2×10^9, which approximates the complexity of the human immune system.
- Conventional hybridoma technology involves immunizing animals with the antigen and harvesting the spleen cells for creating a hybridoma. There is no need for animal immunization for creating a phage display library and isolating the desired antibodies from the library.
- Isolating a monoclonal antibody takes from 3 to 6 months, while phage display can be as short as a couple of days!
- Isolating antibodies by screening phage display libraries is immensely economical when compared to the production of murine monoclonals.
- Ethical and legal constraints limit injecting toxic substances into mice for antibody production. Antibodies cannot be produced for antigens that are lethal to the host animal. However, antibodies to theoretically any antigen can be

isolated from phage display libraries because the screening is done *in vitro* rather than *in vivo*.

- The recombinant antibodies isolated from phage display libraries can be genetically manipulated relatively easily compared with murine monoclonal antibodies.
- Antibodies obtained by screening human antibody libraries can be used for therapeutic or diagnostic purposes in humans without the fear of eliciting host immune response to the antibodies. Although murine monoclonal antibodies can be humanized by grafting the variable portion of the antibodies to constant region of human immunoglobulins, the antibodies lose their affinity to the antigen (24,44-46). Human monoclonal antibodies can be produced by creating heterohybridomas between human lymphocytes and mouse myeloma cell lines, but success is limited (47,48).
- The gene encoding the antibody is immediately available for manipulation, and effects are immediately observable on the activity of the phage and/or antibody. Therefore, effects of genetic manipulation of the antibody sequence and structure can be studied easily.
- scFv antibodies can be genetically modified to be conjugated to various ligands and reporter molecules, thereby greatly increasing the spectrum of their potential use.
- Affinities of the antibodies can be increased by techniques such as error-prone PCR, shuffling heavy or light chains, or propagating the phagemids in mutator strains of *E. coli* (49-52). These methods mimic the affinity maturation by somatic hypermutatation in B cells.

This thesis describes the efforts to isolate recombinant phage display antibodies to *S.* Typhimurium and the attempts at genetically modifying the antibodies for their potential use in the biosensor system.

The specific aims for this study are:

1. Standardization of a protocol for whole bacterial cell ELISAs: The biosensor is intended to detect whole bacterial cells. However, it is not a very high throughput system for screening antibodies. ELISA closely resembles the conditions of the biosensor system and enables screening large number of antibodies. Therefore, a protocol for whole-cell ELISAs needs to be developed and standardized for screening commercially available murine monoclonal antibodies, recombinant phage display antibodies, or scFv antibodies using whole bacterial cells as antigens.
2. Characterization of commercially available monoclonal antibodies to *S*. Typhimurium: Lim et al. (26) are developing a biosensor at the University of South Florida for the detection of *S.* Typhimurium. We screened commercially available antibodies in a whole-cell ELISA for use in that biosensor system.
3. Isolation of recombinant phage display antibodies to *S*. Typhimurium: As

mentioned above, isolating phage display antibodies is much easier and more efficient than isolating a murine monoclonal antibody. We proposed to isolate phage display antibodies recognizing surface antigens of *S.* Typhimurium from the Griffin.1 and the Tomlinson libraries. We explored the possibility of genetically modifying recombinant antibodies for optimal use.

CHAPTER 2
MATERIALS AND METHODS

Bacterial Strains, Phage Strains, and Growth Conditions

The bacterial strains used and their genotypes are listed in Table 2-1. Griffin.1 and Tomlinson I + J Human Synthetic $V_H + V_L$ phagemid libraries constructed by the Medical Research Council, Cambridge, U.K. were obtained from the Interdisciplinary Center for Biotechnology Research Hybridoma Core, University of Florida. Helper phage M13K07 was obtained from New England BioLabs (Beverly, MA). Hyperphage was obtained from Progen Biotechnik (Heidelberg, Germany).

E. coli TG1 cells were grown in 2xTY broth (16 g tryptone, 10 g yeast extract, 5 g NaCl, and 3 mL of 1 M NaOH in 1 L water) or on 2xTY agar plates containing 1.5% (w/v) agar. *E. coli* TG1 cells infected with phage from the Griffin.1 or Tomlinson libraries or with the phage obtained after elution and neutralization after a round of panning were grown on 2xTY agar plates with 100 µg/mL ampicillin and 1% (w/v) glucose (2xTY AG plates). When the bacteria were superinfected with helper phage or Hyperphage to produce phage particles, they were grown in 2xTY broth with 100 µg/mL ampicillin and 50 µg/mL kanamycin. All of the other bacteria were grown in modified Luria-Bertani broth (LB-N: 10 g tryptone, 5 g yeast extract, 8.5 g NaCl, and 3 mL of 1 M NaOH in 1 L water) or on LB-N plates containing 1.5% (w/v) agar. All of the bacteria were grown as an overnight standing cultures in either LB-N or 2xTY at 37 ºC. The bacteria from the starter culture were diluted 1:10 into fresh medium and grown with aeration at 37 ºC until the optical density at 600 nm (OD_{600}) reached approximately 0.4. The bacteria thus

obtained were in exponential phase of growth. All the experiments were done using exponentially growing bacteria to maintain consistency.

Table 2-1: Strains of bacteria

Strain	Genotype / Antigenic formula	Source / Reference
S. Typhimurium χ3000	1,4,[5],12:i:1,2	(53)
S. Typhimurium (*fliC*)	*fliC*::Tn10	(54)
S. Typhimurium (*flhD*)	*flhD*	(55)
E. coli LE392	F- *hsdR*514 *(r-,m+) supE*44 *supF*58 lac*Y*1	(56)
E. coli χ1918	HfrH *lacZ*-x90 *argEam*-210 *metB*-miss *strA*	Roy Curtis III
E. coli TG1	K12 Δ*(lac-proAB*) *supE thi hsdD*5/F' *traD*36 *proA*$^{+}$*B lacI*^{q} *lacZΔM*15	(42)
E. coli AVB100	K12 MC1061 *araD*139 *Δ(ara-leu*)7696 Δ(*lac*)l74 *galU galK hsdR*2(r_{K-} m_{K+}) *mcrB1 rpsL(Str*^{r})	Avidity
S. Cholerasuis 3246	6,7:c:1,5	Anita C. Wright
S. Enteritids 14213	1,9,12:g,m:-	Anita C. Wright
S. Rubislaw 10717	11:r:e,n,x	Anita C. Wright
S. Worthington	1,13,23:z:l,w	Anita C. Wright
S. Gaminara H0662	16:d:1,7	Anita C. Wright
S. Urbana 9261	30:b:e,n,x	Anita C. Wright
S. Adelaid*e*	35:f,g:-	Anita C. Wright

When biotinylated scFv antibodies were produced in *E. coli* AVB100, d-biotin (Sigma, St. Louis, MO) was added to the culture medium to a final concentration of 50 μM, and *birA* was induced by adding L-arabinose (Sigma) to a final concentration of

0.4% (w/v). The gene encoding the scFv-Avitag was induced by adding IPTG to a final concentration of 1 mM.

ELISA

Enzyme Linked ImmunoSorbent Assay was used for characterizing the activities of antibodies. The procedure consisted of coating the antigen at desired concentration in either carbonate buffer (13 mM Na_2CO_3, 87 mM $NaHCO_3$, pH 9.2) or phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3). For bacterial cell ELISAs, exponentially growing bacteria were centrifuged at 6,000 x g for 10 min at 4 ºC. The cells were washed with PBS by suspending the pellet in 10 mL PBS and centrifuging at 6,000 x g for 10 min at 4 ºC. The pellet was suspended in an appropriate amount of carbonate buffer to give a final concentration of 10^7 CFU/mL, and 8% (w/v) aqueous grade 1 glutaraldehyde (Sigma) was added to a final concentration of 0.05% (w/v) (57). For LPS assays, the antigen was suspended in PBS or carbonate buffer at 5 µg/mL. For protein ELISAs the antigen was suspended in either coating buffer or PBS at 10 µg/mL. One hundred microliters of the antigen suspension was added per well of a 96 well non-tissue culture treated polystyrene microtiter plate (Becton-Dickinson, CA) for coating, and the plate was incubated overnight at 4 ºC.

The wells were washed three times with 200 µL of PBS containing 0.05% (w/v) Tween 20 (Sigma) (PBS-T (0.05)) in EL_X 800 Strip Washer (BioTek, VT). Two hundred microliters of casein blocking buffer (1% (w/v) casein in PBS with 0.05% (w/v) Tween 20) (Sigma) was added to each well for blocking the nonspecific binding sites. The wells were washed as described above after 2 h incubation at 4 ºC.

One hundred microliters of the primary antibody (polyclonal or monoclonal antibodies, phage particles, or scFv antibodies) diluted in casein blocking buffer at

desired concentrations was added per well. The murine monoclonal antibodies used with their specificities are listed in Table 2-2. In ELISAs using PEG-precipitated phage as the primary antibody the concentration of the phage was 10^9 phage/mL. When using phage from culture supernatant, the overnight culture supernatant was diluted 1:2 in casein blocking buffer. After an incubation period of 2 h at 4 ºC, the wells were washed as described above, and secondary antibody was added.

The secondary antibodies were conjugated to either horseradish peroxidase or alkaline phosphatase. The antibody was diluted in casein blocking buffer and used generally at a dilution of 1:2000 at 100 µL per well. The plate was incubated at 4 ºC for 2 h, and the wells were washed as described above.

Substrate for development reaction for HRP-conjugated secondary enzymes was prepared by dissolving one capsule of phosphate citrate buffer with sodium perborate (Sigma) in 100 mL of water (0.05 M phosphate-citrate buffer pH 5.0, 0.03% (w/v) sodium perborate). A 10 mg tablet of 3, 3', 5, 5'-tetramethylbenzidine substrate (Sigma) was added to 10 mL of the buffer to give a final concentration of 1 mg/mL. For alkaline phosphatase-conjugated secondary enzymes, the substrate was prepared by dissolving a 20 mg tablet of p-nitrophenylphosphate (pNPP) substrate (Sigma) in 20 mL of bicarbonate buffer (15 mM $NaHCO_3$, 15 mM Na_2CO_3, 2 mM $MgCl_2$). Two hundred microliters of the substrate solution was added to each well, and the plate was read in an ELx 800 UV plate reader (BioTek, VT) at 405 nm for HRP conjugates or 630 nm for alkaline phosphatase conjugates for 40 min. The data were analyzed with K_C Junior (BioTek, VT) and Microsoft Excel software programs.

Table 2-2: List of monoclonal antibodies

	Company	Clone	Specificity	Antigen
1	US Biologicals	S0060-10	*S.* Typhimurium	O4
2	US Biologicals	S0060-15	*S.* Typhimurium	O4
3	Biospacific	A60580228P	*S.* Typhimurium	O4
4	Biospacific	A60530228P	*S.* Typhimurium	LPS
5	RDI	10D9H	Groups A, B, D Salmonella	O12
6	Virostat	6341	Groups A, B, D Salmonella	O12
7	Virostat	6321	*S.* Typhimurium	Unknown
8	Biodesign	8C11C	Group B Salmonella	O4
9	Virostat	6301	*S.* Typhimurium	Flagellum
10	Accurate	YVS6301	*S.* Typhimurium	Flagellum

Biopanning of the Phage Display Library

Isolation of the recombinant antibodies to the antigens on whole bacterial cells, purified LPS, or flagellar extracts was done by panning the phage display libraries on the antigen either coated on immunotubes or suspended in blocking buffer.

Panning on Immunotubes

The antigen was suspended in the appropriate buffer (whole bacterial cells in coating buffer at 10^9 CFU/mL, flagellar extract or LPS at various concentrations in PBS or coating buffer), and immunotubes (Nunc) were filled with the antigen suspension and incubated at 4 ºC overnight. The antigen suspension was drained, and the tube was washed three times by filling with PBS and aspirating the wash solution without agitation. The nonspecific binding sites on the immunotube were blocked by filling the tube with casein blocking buffer and incubating for 2 h at 4 ºC. The blocking buffer was aspirated, and the tube was washed three times with PBS. Approximately 10^{12} phage were suspended in 4 mL of casein blocking buffer and used for panning. In the first

round of panning, phage from the Griffin.1 or Tomlinson libraries were used. In the subsequent rounds of panning, phage from the previous round of elution and / or amplification were used. The immunotube was filled with the phage suspension and incubated at 4 ºC in the case of whole cells or at room temperature in the case of LPS or flagellar extracts for at least 2 h with continuous rotation. The phage suspension was discarded, and the tube was washed with PBS-T (0.1%) followed by PBS. The number of washes varied depending on the round of panning – 10 times in the first round and 20 times in the subsequent rounds of panning.

The phage bound to the antigen were eluted with either 0.1 M glycine or trypsin depending on the source of phage. Elution was done with 0.1 M glycine, pH 2.8, if the phage were from the Griffin.1 library. One milliliter of glycine was added to the tube and incubated at room temperature for 10 min with continuous rotation. The eluate was transferred to another tube, and the solution was brought to neutral pH by adding 50 µL of 1 M Tris, pH 8.0. Trypsin XIII from bovine pancreas (Sigma) in PBS at a concentration of 1 mg/mL was used for elution if the phage were from Tomlinson I library. For elution with trypsin, 500 µL of 1 mg/mL trypsin solution was added to the tube and incubated for 30 min at room temperature with continuous rotation. The phage were titered and used to infect *E. coli* TG1 for amplification or used in the next round of panning.

Panning in Suspension

Panning on the antigen in suspension was done only for whole bacterial cells. Exponentially growing *S*. Typhimurium cells were suspended in 1 mL of casein blocking buffer in a microcentrifuge tube to a final concentration of 10^9 CFU/mL. Approximately 10^{12} phage from the Griffin.1 or Tomlinson libraries for the first round of panning or

from amplified phage of the previous round were suspended in 200 µL of casein blocking buffer and incubated with the bacteria at 4 ºC for at least 2 h with continuous rotation. The mixture was centrifuged at 5,000 x g for 3 min. The supernatant was discarded, and the bacterial pellet containing cells with the phage bound to the surface antigens were suspended in 1 mL of PBS with 0.1% (w/v) Tween 20 by pipetting. This wash procedure was repeated for at least 10 times in the first round of panning and 15 to 20 times in later rounds. The wash procedure was stopped before all the washes were done if the pellet size seemed to be decreasing. After the final wash the bound phage were eluted by suspending the pellet in 1 mL of 0.5 M glycine, pH 2.8 and incubating for 10 min with continuous rotation. The bacteria were centrifuged, the supernatant was transferred to another tube, and 50 µL of 1 M Tris, pH 8.0 was added to neutralize the solution. When phage from Tomlinson libraries were used elution was done by suspending the pellet in 0.5 mL of 1 mg/mL trypsin and incubating for 30 min at room temperature. The phage thus obtained were used to infect exponentially growing *E. coli* TG1 for titering and amplification (42).

Titering the Phage

The phagemid lacks all the genes necessary for the production of proteins required for phage assembly and packaging, so the phagemid acts as a plasmid in *E. coli*. *E. coli* TG1 infected with the phage, therefore, produce colonies when plated on selective media. The concentration of the phage was determined by counting the colonies produced from infecting *E. coli*. Serial dilutions of phage suspensions were made in PBS, and 10 µL of 10^{-4}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions were used to infect 1 mL of exponentially growing *E. coli* TG1 for 30 min at 37 ºC. One hundred microliters of each

infected culture was plated on 2xTY AG plates and incubated at 37 ºC overnight. The colonies were counted, and the titer was determined.

Amplification of the Selected Phage

The phage obtained after each round of panning and elution were amplified in *E. coli*. This increased the number of the phage bearing the specific antibody sequence relative to the nonspecific phage or the phage having the wild type pIII. To 0.5 mL of the eluted phage, 1.5 mL of exponentially growing *E. coli* TG1 cells at OD_{600} approximately 0.4 were added and incubated at 37 ºC for 30 min. The cells were centrifuged at 6,000 x g for 10 min at 4 ºC. The bacterial pellet was suspended in 0.4 mL of 2xTY, and 0.1 mL each was plated on four 2xTY AG plates and incubated at 37 ºC overnight.

The bacterial mass was gently scraped off the plates with a glass spreader after adding 0.5 mL of 2xTY to loosen the cells. Fifty microliters of cells was added to 50 mL of 2xTY AG, and the culture was grown at 37 ºC with shaking until the OD_{600} was approximately 0.4. Glycerol was added to the remaining cells to a final concentration of 15% (v/v) and stored at -80 ºC after freezing in a dry ice-ethanol bath. Helper phage M13K07 or Hyperphage were added to 10 mL of the 2xTY AG culture with OD_{600} approximately 0.4 at a multiplicity of infection (MOI) of 20. The Hyperphage were at a concentration of 10^{12} phage/mL according to the supplier. However, when titered by plating infected *E. coli* TG1 on LBN-Kan plates, the concentration was only 10^{9} phage/mL. It was impractical to use the measured concentration if a MOI of 20 was to be achieved, so Hyperphage were used at the concentration stated by the supplier. Using Hyperphage at the concentration stated by the manufacturer, however, did not affect the yield of phage particles. The culture was incubated for 30 min at 37 ºC and then centrifuged at 6,000 x g for 10 min at 4 ºC. The bacterial pellet was suspended in

50 mL of 2xTY with 100 µg/mL ampicillin and 50 µg/mL kanamycin and grown with shaking at 30 ºC overnight. The culture was centrifuged at 6,000 x g for 10 min at 4 ºC, and the bacterial pellet was discarded. To precipitate the phage, 10 mL of 20% (w/v) polyethylene glycol (PEG)/2.5 M NaCl was added to 40 mL of the culture supernatant and incubated at 4 ºC for at least 4 h. The suspension was centrifuged at 6,000 x g for 10 min at 4 ºC, and the supernatant was discarded. A brief centrifugation was done to remove any remaining PEG/NaCl, and the pellet was suspended in 1 mL of PBS. The suspended phage were centrifuged at 5,000 x g for 3 min to remove any remaining bacterial debris (42). The phage were titered, as described above.

Panning without Amplification

This method differed from panning on immunotubes in that the amplification of eluted phage in between the rounds of panning was eliminated. The eluted phage from the previous round were suspended in 4 mL of casein blocking buffer and used for panning on the antigen-coated on immunotubes. The scFv-pIII fusion protein has a c-myc site, which is susceptible to trypsin digestion, in between the amino terminal N1 and N2 domains and the carboxy terminal CT domain of the pIII protein. Elution with trypsin cleaves this c-myc site leaving the antibody sequence and N1 and N2 domains of pIII attached to the antigen, while the phage particle is released into the fluid phase. When panning was done without amplification between rounds, elution in the first two rounds was done with 0.1 M glycine, pH 2.8 to maintain the antibody sequence on the phage particle to be able to bind the antigen in the next round. If the phage were from Tomlinson library, elution in the third round was done with 1 mg/mL trypsin. If elution had been done with trypsin in the first and second rounds also, the antibody sequence

would have been cleaved off the fusion protein, and phage could not have bound to the antigen coated on the immunotubes.

Production of Soluble Antibody Fragments (scFv antibodies)

The gene coding for the scFv is in frame with gIII, the gene coding for the coat protein pIII, separated by a TAG amber stop codon. When the phagemid is in an amber suppressor strain of E. coli (*supE*) such as TG1, the TAG codon is translated and glutamine is incorporated into the peptide chain instead of termination. This results in the production of a fusion protein, scFv-pIII. When the phagemid is in a nonsuppressor strain such as *E. coli* χ1918, the translation of the peptide is terminated at the TAG codon releasing the soluble scFv antibody molecule. The phagemid has the *pelB* leader sequence at the 5' end of the scFv-gIII gene, which directs the peptides into secretory pathway. Therefore, when soluble fusion protein is produced, it is directed to the periplasmic compartment and/or released into the culture supernatant.

scFv antibodies were produced from the phage clones testing positive for antibody activity by ELISA by infecting *E. coli* χ1918 with the selected phage. Phage were added to 1 mL of cells at a MOI of 20 and incubated at 37 ºC for 30 min. The cells were added to 9 mL of 2xTY with 0.1% (w/v) glucose and 100 µg/mL ampicillin and were grown with shaking at 37 ºC. When the culture was in exponential phase of growth, IPTG was added to a final concentration of 1 mM to induce the expression of scFv-pIII synthesis, and the culture was grown overnight with aeration at 37 ºC (42).

When biotinylated scFv antibodies were produced from *E. coli* AVB100 the culture was brought to 50 µM d-biotin (Sigma) 1 hr prior to the induction of scFv antibody production with 1 mM IPTG. When the culture is at an OD_{600} of approximately 0.8, *bir*A

was induced with 0.4% (wt/vol) L-arabinose the scFv antibody production was induced with 1 mM IPTG.

The scFv antibodies released into the culture supernatant were harvested by centrifuging the culture at 6,000 x g for 10 min at 4 ºC and discarding the bacterial pellet. The culture supernatant containing the scFv was diluted 1:2 in casein blocking buffer and used in ELISA or Western blot.

DNA Manipulations

Plasmid Extractions

Plasmid extractions were done using QIAprep Spin Miniprep kit (Qiagen) for cultures up to 10 mL and Plasmid Midi kit (Qiagen) for culture volumes of 50 to 100 mL, according to the manufacturer instructions.

Enzyme Manipulations

Restriction enzyme digestions were done with enzymes purchased from Invitrogen, Rockville, MD; New England BioLabs, Beverly, MA; and Promega, Madison, WI, and were used according to manufacturer instructions.

Agarose Gel Electrophoresis

DNA was resolved on 0.7 to 1% (w/v) agarose gels using Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) with 10 µg/mL ethidium bromide. Gel electrophoresis was done at 100 V, and the DNA bands were visualized on a UV transilluminator (58).

Polymerase Chain Reaction (PCR)

PCR was done to verify the presence of scFv insert DNA sequences in phagemids and to ascertain the number of insert-positive phage in the original Tomlinson I library.

The primers used were Lmb3-extend (hybridizing upstream of *pel*B leader sequence) and FDseq1-extend (hybridizing in the 5' region of gIII in the antisense orientation).

Lmb3–extend: CAGGAAACAGCTATGACCATGATTACG

FDseq1–extend: GAATTTTCTGTATGAGGTTTTGCTAAAC

The PCR conditions were: 9 min at 95 ºC; 30 cycles of (45 sec at 95 ºC, 45 sec at 60 ºC, 90 sec at 72 ºC), and 10 min at 72 ºC. The PCR products were resolved on 0.7% (w/v) agarose gels, and the DNA bands were visualized on a UV transilluminator.

Construction of scFv-Avitag Plasmid Vectors

To improve the usefulness of the scFv antibodies, they were biotinylated by cloning the scFv gene into pAC Avitag vectors and expressing them in *E. coli* AVB100 (Avidity). Avitag is a 15-peptide sequence specifically biotinylated by the BirA enzyme of *E. coli.* Plasmid vectors pAC4, pAC5, and pAC6 contain the sequence coding for Avitag peptide distal to a multiple cloning site. The *E. coli* AVB100 strain has *birA* stably integrated into the chromosome under the control of AraC.

The genes coding for anti-flagella scFv and anti-BSA scFv were cloned into the pAC5 Avitag vector. Anti-BSA antibodies were biotinylated and tested as a model for the scFv antibodies.

Plasmid DNA was extracted from *E. coli* TG1 cells containing plasmids encoding the anti-flagella and anti-BSA scFv antibodies. Restriction digestion was done with *Hin*dIII and *Not*I to obtain the fragment containing the ribosome binding site, the *pelB* leader sequence, and the entire scFv gene (Figure 2-1). The 5' overhangs were filled in with Klenow fragment of DNA polymerase I.

The digestion mixture was resolved on a 0.7% (w/v) agarose gel, and the fragment corresponding to *Hin*dIII/*Not*I band was excised. The DNA was extracted from the

agarose gel using GenElute Minus EtBr Spin columns (Sigma). Plasmid vector pAC5 was digested with *Sma*I to linearize the plasmid, and the *Hin*dIII/*Not*I or *Nco*I/*Not*I fragment was blunt end ligated into the vector.

With anti-BSA scFv, another plasmid was constructed without the *pelB* leader sequence by digesting the plasmid with *Nco*I and *Not*I to obtain only the scFv gene. After digestion with *NotI*, the 5' overhang was filled in with Klenow fragment of DNA polymerase I and then digested with *Nco*I, which cuts at the 5' end of the scFv gene. The digestion mixture was resolved on a 0.7% (w/v) agarose gel, and the fragment corresponding to *Nco*I/*Not*I band was excised. pAC5 Avitag vector was digested with *NcoI* and *SmaI*, the gel-purified fragment was ligated into the vector.

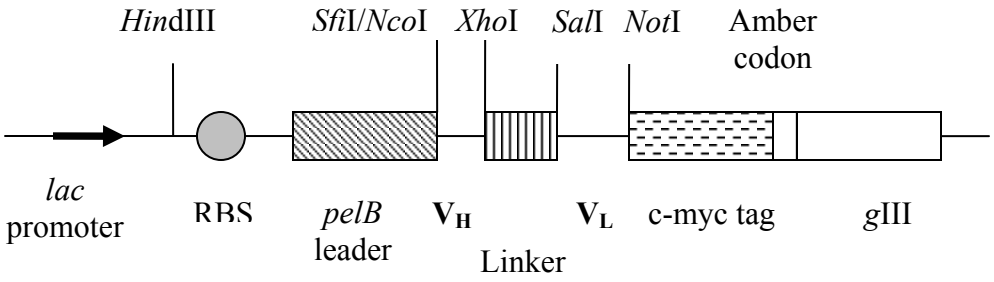

Figure 2-1: Vector map of pIT2 phagemid vector pIT2. The vector map of pIT2 phagemid vector used in constructing the Tomlinson libraries. RBS-Ribosome binding site. pelB-leader peptide sequence. VH and VL-genes coding for heavy and light chains, respectively. Linker- (Gly4Ser)3 linker sequence. Amber stop codon is at the junction of c-myc tag and gIII gene. Not shown in the figure are E. coli ColE1 *ori*, M13 *ori*, and *bla*.

Escherichia coli AVB100 cells were electroporated with the ligation mixture and plated on LBN-Amp plates for selecting the transformed clones. Plasmid DNA was extracted from randomly selected clones and checked for the presence of the insert.

Protein and LPS Manipulations

Extraction of Flagella

Flagella were extracted from *S.* Typhimurium and used for coating immunotubes (34). Briefly, 10 mL of standing overnight culture was added to 1 L of LBN and grown with shaking for 4 h. The cells were pelleted by centrifuging at 6,000 x g for 10 min at 4 ºC. The supernatant was discarded, and the pellet was suspended in 100 mL of 0.5 M Tris-HCl, pH 7.5. The suspension was homogenized in an Osterizer blender at maximum speed for 60 sec to mechanically shear off the flagella. The cells were pelleted by centrifuging at 6,000 x g for 10 min at 4 ºC and were discarded. The supernatant was centrifuged at 100,000 x g for 90 min at 4 ºC to pellet the flagella. The pellet was suspended in 5 mL of 0.5 M Tris-HCl, pH 7.5, and the protein concentration was determined by the Bradford method.

Determination of Protein Concentration

Protein concentration was determined by the Bradford method using the D_C Protein Assay reagent (Bio-Rad, Hercules, CA) according to manufacturer instructions.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

ReadyGel Tris-glycine 10% (w/v) PAGE gels (Bio-Rad) were used with the MiniProtean Electrophoresis system (Bio-Rad) for SDS-PAGE of protein samples. The samples were diluted 1:2 in Laemmli sample buffer (Bio-Rad), and dilutions were made as necessary. When whole bacterial cells were used as antigens, 10^8 CFU were suspended in Laemmli sample buffer and boiled for 10 min. Electrode buffer was 25 mM Tris, 0.17 M glycine, and 0.1% (w/v) SDS. The samples were electrophorosed for 1 hour at 100 V. The gel was stained with Coomassie Blue for visualizing proteins. Alternatively, some gels were used for immunoblotting.

Coomassie Blue Staining for Proteins

Proteins in SDS-PAGE were fixed in 50% (v/v) methanol, 10% (v/v) glacial acetic acid in water for 30 min. The gel was stained with 0.1% (w/v) Coomassie Blue R, 50% (v/v) methanol, 7% (v/v) glacial acetic acid) and agitated gently for 30 min. The gel was destained with 5% (v/v) methanol, 7% (v/v) glacial acetic acid in water. When the protein bands appeared the gel was dried, and a photograph was taken (58).

Immunoblotting

The antigens in the SDS-PAGE gels were transferred onto a nitrocellulose membrane for reaction with monoclonal antibodies, phage, or scFv antibodies according to the procedure of Towbin et al (59). A Mini TransBlotting cell (Bio-Rad) was used for the wet blotting technique. The transfer buffer used was 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. A voltage of 100 V was applied for 1 hour at room temperature. The nonspecific binding sites on the membrane were blocked with casein blocking buffer. Primary antibody diluted in casein blocking buffer was added and incubated for 2 h with continuous rotation. The membrane was washed with 100 mL Tris buffered saline with 0.1% (w/v) Tween 20 with gentle agitation. The membrane was incubated with secondary antibody diluted in blocking buffer for 2 h with continuous rotation followed by another three washes as described above. The development of the reaction was done by placing the membrane in 100 mL of substrate solution and observing the appearance of bands. The substrate used for secondary antibodies conjugated with horseradish peroxidase was 4-chloro-1-naphthol (4CN) (Fisher) prepared according to manufacturer instructions. For alkaline phosphatase the substrate used was nitro blue tetrazolium-5-bromo-4-chloro-3-indoylphosphate (Sigma) prepared according to manufacturer instructions. The development reaction was stopped by removing the

membrane from the substrate and placing in water. The membrane was scanned using an UMax photo scanner (60).

Concentration of scFv Antibodies from Culture Supernatant

scFv antibodies produced from the Tomlinson libraries have protein A and protein L binding sites. scFv antibodies secreted into the culture medium were concentrated by passing the culture supernatant over a protein A-Sepharose column (61). The binding buffer was 0.02 M sodium phosphate, pH 7.0. For elution 0.1 M citric acid, pH 3 was used, and the eluate was neutralized with 1 M Tris-HCl, pH 9.0. The column was first washed with three column volumes of binding buffer, and the sample was passed through the column. The column was washed with ten column volumes of binding buffer, and the bound protein was eluted with three column volumes of elution buffer. The eluate was neutralized with 60 µL of 1 M Tris-HCl, pH 9.0.

Extraction of Soluble Proteins from Periplasmic Space

scFv protein present in the periplasmic compartment of *E. coli* was extracted by the osmotic shock method (62). Briefly, exponentially growing *E. coli* χ1918 cells containing the phagemids were harvested by centrifugation at 6,000 x g for 10 min at 4 ºC. The cells were washed with 40 volumes of cold 10 mM Tris-HCl, 30 mM NaCl, pH 7.1. The pellet was suspended in 40 mL of 33 mM Tris-HCl, pH 7.1. The cells were diluted in 40 mL of 40% (w/v) sucrose in 33 mM Tris-HCl, pH 7.1, and 0.1 M disodium EDTA, pH 7.1 was added to a final concentration of 0.1 mM EDTA. The suspension was shaken in a rotary shaker for 10 min at 24 ºC. The cells were centrifuged at 13,000 x g for 10 min at 4 ºC. The bacterial pellet was then dispersed in 5 mL of ice-cold water. After incubation for 10 min in an ice bath, the cells were centrifuged at 6,000 x g for 10 min. The supernatant containing the scFv antibodies was saved.

CHAPTER 3
RESULTS

Rationale for Study

In a world facing increasing threats of bioterrorism, rapid, sensitive, and specific detection of microbes and toxins would enable the law enforcement agencies to react to the situation effectively and appropriately. Such a detection system would also greatly aid in solving problems of public health importance. A biosensor system consisting of an optical fiber probe with an attached capture antibody and a fluorescently labeled antibody for detection was developed as a step in that direction (26). This thesis describes the efforts made to characterize existing monoclonal antibodies and to isolate new recombinant phage display antibodies to *S.* Typhimurium to be ultimately used in the biosensor system as capture antibodies and detection antibodies for detecting *S.* Typhimurium.

The specific aims for this study were:

1. Standardization of a protocol for whole bacterial cell ELISAs
2. Characterization of commercially available monoclonal antibodies to *S.* Typhimurium
3. Isolation of recombinant phage display antibodies to *S.* Typhimurium and genetically fusing scFv antibodies with ligands or reporter molecules

Specific Aim 1: Standardization of a Protocol for Whole Bacterial Cell ELISAs

A protocol for ELISA using whole bacterial cells as antigens was developed and standardized for characterizing the monoclonal antibodies. The conditions that were

taken into consideration for standardizing the protocol included antigen concentration, coating buffer, blocking buffer, and reaction development time. Each condition was tested by coating the antigen in triplicate. The mean absorbance values were calculated, and the signal to noise ratios were calculated by dividing the mean value of the positive-antigen wells with the mean of the negative-antigen wells. The optimal conditions from these experiments were adapted for characterizing monoclonal antibodies, recombinant phage display antibodies, and scFv antibodies.

Reaction Development Time

Rather than chemically stopping the development reaction at some arbitrary time, we chose to let the development reaction proceed and take multiple reads so that we could use data from the optimal stage of development. The ELx 800 UV microplate reader used for reading the absorbance values in ELISAs takes approximately 1 min for reading all of the 96 wells, so if the reaction time is short enough, the time required to read the plate could significantly add to the reaction time for wells read last. To study the effect of duration of development of the reaction on the signal obtained, bacteria were coated at same concentration in the first three wells of the first row and the last three wells of the last row, and the ELISA was done. Reading the absorbance values 5 to 10 min after starting the reaction resulted in an error because the values obtained in the wells in the last row were higher than the wells in the first row as the reaction was proceeding in the last well while the plate was being read. However, if the reaction was allowed to continue for 40 min there was no difference in the absorbances for the first and last wells. Therefore, the absorbances values in all the subsequent ELISAs were read after 40 min.

Antigen Concentration for Coating

The concentration and amount of the antigen coated in the wells determine the signal obtained and the background activity. If the concentration of the antigen is too high, antibodies might not bind to the antigen because of steric hindrance and also might give false positive values because of nonspecific binding of the antibodies to the antigen (63). The concentration of the antigen needs to be adjusted to maximize the signal and minimize the background activity.

Five-fold serial dilutions of the *S.* Typhimurium cells from 1.0×10^9 CFU/mL to 6.4×10^4 CFU/mL were used for coating wells. Wells coated with either *E. coli* LE392 or coating buffer alone were used as negative antigen controls. Rabbit anti-*S.* Typhimurium serum at a dilution of 1:1000 was used as the primary antibody, and donkey anti-rabbit IgG-HRP conjugate at a dilution of 1:2000 was used as the secondary antibody. The development of color was monitored for 40 min, and the absorbance was read at 630 nm. The peak absorbance value was around 1.72 and stayed the same for antigen concentrations ranging from 1.0×10^9 CFU/mL to 8.0×10^6 CFU/mL (Figure 3-1). Further decreasing the antigen concentration resulted in a significant decrease in the peak signal obtained. The development of the reaction was gradual and linear with respect to time at the antigen concentration of 8.0×10^6 CFU/mL, as opposed to higher antigen concentrations for which the peak signal was reached more rapidly. In all the subsequent experiments for characterizing the monoclonal antibodies, phage antibodies, or scFv antibodies bacteria were coated at a concentration of 1.0×10^7 CFU/mL.

To enhance the binding of bacterial cells to the microtiter plate, glutaraldehyde was added to the antigen suspension to a final concentration of 0.05% (w/v) (57). In an

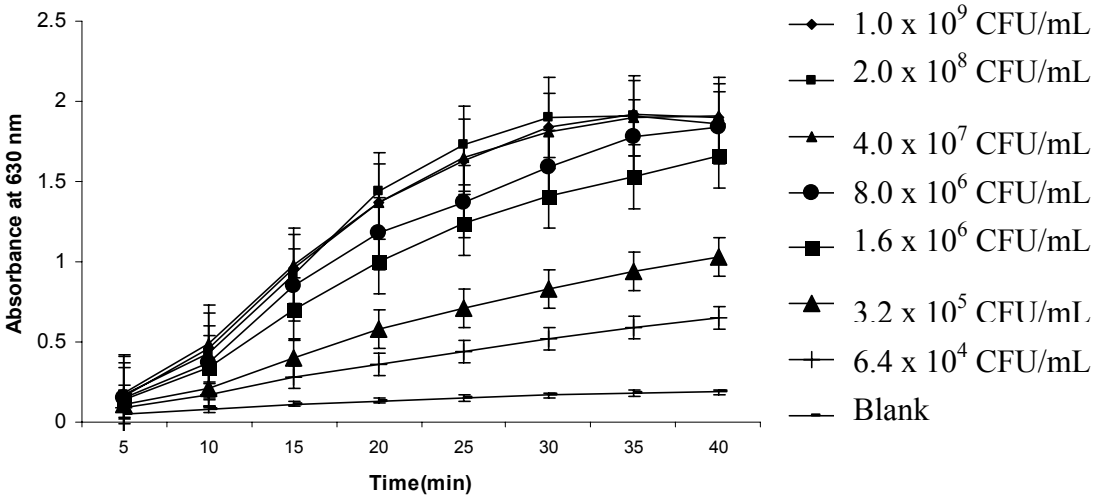

Figure 3-1: Effect of varying antigen concentration on signal. S. Typhimurium cells were serially diluted five-fold from 1.0 x 109 CFU/mL to 6.4 x 104 CFU/mL and were coated in triplicate onto a microtiter plate. ELISA was performed with a 1:1000 dilution of rabbit anti-S. Typhimurium serum and a 1:2000 dilution of HRP-conjugated donkey anti-rabbit IgG antibody as primary and secondary antibodies, respectively. Data are shown as mean and standard deviation.

ELISA done to study the effect of glutaraldehyde on increasing the signal using *S.* Typhimurium whole cells as antigen, goat anti-*S.* Typhimurium serum as primary antibody, and anti-goat IgG conjugated with alkaline phosphatase as secondary enzyme, the absorbance value obtained in wells containing glutaraldehyde was 2.67 while the signal in the wells not containing glutaraldehyde was 2.72, and the difference was not statistically significant. The addition of glutaraldehyde did not significantly enhance the signal obtained. However, we continued adding glutaraldehyde to the antigen suspension.

The effect of coating buffer on the signal obtained was tested by suspending *S.* Typhimurium in either carbonate buffer or PBS. *S.* Typhimurium cells were suspended in either of the buffers at a concentration of 10^7 CFU/mL, and ELISA was done using USB10 as primary antibody. The signal obtained in wells coated with PBS as

the coating buffer was 1.11, while in the wells with carbonate buffer as the coating buffer the signal was 1.14, showing no statistically significant difference. Carbonate buffer was used for suspending the bacteria in all the subsequent experiments.

Comparing the Blocking Efficiencies of Casein and BSA

A common problem in immunological analysis is the nonspecific binding of antibodies to the matrix on which the antigen is bound, such as the wells of a microtiter plate or a nitrocellulose membrane. To overcome this problem all of the sites not coated by the antigen are made inaccessible to antibodies by blocking with agents such as BSA, casein, Tween 20, dextran sulfate, and skim milk (64).

To determine the blocking efficiencies of casein and BSA, 1% (w/v) casein in PBS, 1% (w/v) BSA in PBS, or a mixture of 0.5% (w/v) casein and 0.5% (w/v) BSA in PBS was used as blocking buffer in a whole-cell ELISA. All of the blocking buffers had 0.1% (w/v) Tween 20. The wells of a microtiter plate were coated with *S.* Typhimurium cells at a concentration of 10^7 CFU/mL. Goat anti-*S.* Typhimurium serum at 1:1000 dilution was used as primary antibody, and secondary antibody was donkey anti-goat IgG conjugated with alkaline phosphatase used at 1:2000 dilution. Both primary and secondary antibodies were diluted in the blocking buffer. Wells coated only with coating buffer were used as negative antigen controls. There were no significant differences in the signals obtained in each of the cases, the values ranged from 3.35 ± 0.10 to 3.55 ± 0.07; however, the background activity in the negative-antigen wells was half as much in wells blocked with casein when compared wells blocked with BSA (Table 3-1). Casein mixed with BSA gave background activity similar to that for casein alone. Therefore, for all subsequent experiments 1% (w/v) casein with 0.1% (w/v) Tween 20 was used as the blocking buffer.

Table 3-1: Comparing the blocking efficiencies of casein and BSA

Blocking buffer	Salmonella	Blank	Signal/Noise
1% (w/v) BSA	3.42 ± 0.06	0.34 ± 0.05	10
0.5% (w/v) BSA + 0.5% (w/v) casein	3.55 ± 0.07	0.16 ± 0.04	23
1% (w/v) casein	3.35 ± 0.10	0.13 ± 0.05	26

The wells of a microtiter plated were coated in triplicate either with 10^7 CFU/mL of S. Typhimurium (Salmonella) or with coating buffer (Blank), and ELISA was performed with goat anti-S. Typhimurium serum (1:1000 dilution) and alkaline phosphatase conjugated donkey anti-goat IgG (1:2000 dilution) as primary and secondary antibodies, respectively. The antibodies were diluted in the same buffer that was used as blocking buffer. The data are presented as mean with standard deviation. Signal/Noise value was calculated by dividing the signal obtained in antigen-positive wells with the signal obtained in antigen-negative wells. The absorbance values were not significantly different, but the blank values were significantly different with $p < 0.008$.

In some of the initial experiments to determine the appropriate concentration of bacteria for coating the wells, the secondary antibodies used were conjugated to alkaline phosphatase. For all of the subsequent experiments to characterize the monoclonal antibodies, phage antibodies, and scFv antibodies, the secondary antibodies were conjugated to horseradish peroxidase.

Specific Aim 2: Characterization of Commercially Available Monoclonal Antibodies to *S.* Typhimurium

Murine monoclonal antibodies recognizing various surface epitopes of *S.* Typhimurium including LPS and flagella and monoclonal antibodies of undetermined specificity were tested by ELISA to determine their potential usefulness in the biosensor system as capture antibodies and detection antibodies (Table 2-2). Antibodies recognizing surface epitopes were chosen to facilitate detection of whole bacterial cells using the biosensor system and to obviate the need for preparing cell extracts to identify internal antigens. The monoclonal antibodies were characterized by an ELISA because,

in addition to closely resembling the conditions of the biosensor system, ELISA is a simple, rapid, and economical alternative to other methods such as Western blotting (64). The antibodies were screened by using the standardized conditions determined above. An absorbance value of greater than 1.0 unit was considered satisfactory.

The monoclonal antibodies recognizing LPS or other undetermined surface epitopes were tested by ELISA using whole cells and purified LPS of S. Typhimurium as antigens. LPS was suspended in either PBS or coating buffer at a concentration of 5 μg/mL, and 100 μL per well was used for coating the wells of the microtiter plate. A concentration of 5 μg/mL of LPS gave a signal comparable to the signal obtained when 107 CFU/mL of whole cells were coated. Of all the monoclonal antibodies screened, US Biologicals10 (USB10) monoclonal antibody to LPS of S. Typhimurium performed far superiorly in a whole-cell ELISA, giving absorbance values higher than 1.5 (Table 3-2). USB10 monoclonal antibody gave a similar signal with purified LPS of S. Typhimurium in a LPS ELISA compared with a whole-cell ELISA. USB10 monoclonal antibody was tested by an ELISA at varying concentrations and performed well at concentrations as low as 0.01 μg/mL giving a signal of 0.44 units (Table 3-3). Biospacific53 (Biosp53) monoclonal antibody to LPS performed best among the remaining antibodies and gave a signal higher than 1.3 absorbance units at concentrations of 0.1 μg/mL. Of the remaining antibodies, RDI and Virostat6341 worked moderately well at a concentration of 10 μg/mL giving a signal of 0.7 absorbance units. All of the remaining antibodies to the LPS of *S.* Typhimurium or other surface antigens gave signals less than 0.6 absorbance units and were considered unsuitable for further study.

The anti-flagellar antibodies were tested by ELISA using either whole bacteria or flagellar extracts of S. Typhimurium. Flagella were suspended in PBS at a concentration of 10 μg/mL, and 100 μL was used for coating the wells of a microtiter plate. Of the anti-flagellar monoclonal antibodies, Virostat6321 did not give a signal higher than 0.2 units either with whole cells or with flagellar extracts. AccurateYVS6301 reacted well with flagellar extract giving a signal of 1.0 units, but gave signal less than 0.2 absorbance units with whole cells (Table 3-4). Since it was possible that an inadequate amount of the

Table 3-2: Comparing the Activities of Various Monoclonal Antibodies

Monoclonal Antibody	Antigen Specificity	Salmonella	Blank	Signal / Noise
US Biologicals10	O4	1.19 ± 0.05	0.08	15
US Biologicals15	O4	0.17 ± 0.02	0.08	2
Biospacific53	LPS	1.07 ± 0.02	0.11	10
Biospacific58	O4	0.16 ± 0.04	0.08	2
RDI	O12	0.47 ± 0.09	0.09	5
Virostat6301	Flagellum	0.25 ± 0.02	0.08	3
Virostat6321	Unknown	0.18 ± 0.07	0.13	1
Virostat6341	O12	0.70 ± 0.06	0.09	8
Biodesign	O4	0.18 ± 0.08	0.15	1
AccurateYVS6301	Flagellum	0.12 ± 0.01	0.09	1

The wells of a microtiter plated were coated in triplicate either with 10^7 CFU/mL of *S.* Typhimurium (Salmonella) or with coating buffer (Blank), and ELISA was performed with each of the monoclonal antibodies as the primary antibodies. US Biologicals10 and Biospacific53 were at 1 μg/mL, and all of the remaining antibodies were at 10 μg/mL. Goat anti-mouse IgG antibody conjugated with HRP was used as secondary antibody at a dilution of 1:2000. The data are the mean and standard deviation of the absorbance values at 630 nm and signal / noise values.

antigen was responsible for the weak signal, bacteria were coated at 10^9 CFU/mL, one hundred-fold higher than used in a typical assay, but no higher signal was obtained.

To determine the specificity of USB10 and Biosp53 monoclonal antibodies, whole-cell ELISA was done using *Salmonella* belonging to various serogroups as

Table 3-3: Potency of USB10 and Biosp53 Anti-LPS Monoclonal Antibodies

Concentration of antibody (μg/mL)	USB10	Biosp53
10.00	1.37 ± 0.08	1.35 ± 0.05
1.00	1.36 ± 0.01	1.29 ± 0.11
0.10	1.11 ± 0.08	1.09 ± 0.02
0.01	0.44 ± 0.08	0.35 ± 0.03

S. Typhimurium cells at a concentration of 10^7 CFU/mL were used as antigen, coated in triplicate. USB10 and Biosp53 were diluted in casein blocking buffer at the indicated concentrations and used as primary antibody for the ELISA. The data presented are mean and standard deviation for absorbance values at 630 nm 40 min after the addition of the substrate.

Table 3-4: Activity of anti-flagellar monoclonal antibodies

Antigen	Accurate YVS6301	Virostat6321
Flagella	1.05 ± 0.03	0.20 ± 0.01
Salmonella	0.23 ± 0.02	0.18 ± 0.01

ELISA was done using whole *S.* Typhimurium cells (Salmonella) at a concentration of 10^7 CFU/mL or flagellar extract (Flagella) at a concentration of 10 μg/mL as antigen. Accurate YVS6301 and Virostat6321 anti-flagellar monoclonal antibodies were used as primary antibody at a concentration of 10 μg/mL. The data are presented as the mean values of the absorbances with one standard deviation. The differences are statistically significant with $p < 0.02$.

antigens. The signal obtained with non-group B salmonellae was approximately 0.2 units, while for group B salmonellae it was 0.7 to 1.2 absorbance units (Table 3-5). With *S.* Worthington, which belongs to group G, the signal was approximately 0.4 units.

USB10 and Biosp53 were determined to be suitable antibodies for further study in the biosensor system, as they were giving a satisfactory signal at concentrations as low as 0.1 μg/mL by a whole-cell ELISA and were specific in recognizing *S.* Typhimurium.

Table 3-5: Specificity of USB10 and Biosp53 anti-LPS monoclonal antibodies

Strain	Serogroup	USB10	Biosp53
S. Typhimurium χ3000	B	1.1 ± 0.01	1.2 ± 0.04
S. Typhimurium 13311	B	0.6 ± 0.01	0.7 ± 0.05
S. Cholerasuis 3246	C	0.1 ± 0.01	0.1 ± 0.01
S. Enteritidis 14213	D	0.1 ± 0.01	0.1 ± 0.01
S. Rubislaw 10717	F	0.1 ± 0.01	0.1 ± 0.02
S. Worthington	G	0.4 ± 0.03	0.4 ± 0.04
S. Gaminara H0662	I	0.2 ± 0.02	0.1 ± 0.01
S. Urbana 9261	N	0.1 ± 0.02	0.1 ± 0.01
S. Adelaide	O	0.2 ± 0.01	0.1 ± 0.01

Salmonellae belonging to various serogroups as indicated were used as antigen in a whole-cell ELISA at a concentration of 10^7 CFU/mL. USB10 and Biosp53 were used as primary antibodies at a concentration of 1 μg/mL for the ELISA. The data are presented as mean values of the absorbances at 630 nm after 40 min after the addition of the substrate.

Specific Aim 3: Isolation of Recombinant Phage Display Antibodies to *S.* Typhimurium and Genetically Fusing scFv Antibodies with Ligands or Reporter Molecules

Recombinant phage display antibodies to antigens were isolated from a naïve combinatorial library by panning the library on the antigen either in suspension or immobilized on an immunotube. For isolating phage bearing antibody to surface epitopes of *S.* Typhimurium, panning was done with phage from the Griffin.1 or the Tomlinson I Human Synthetic $V_H + V_L$ libraries. The phage from the Tomlinson I or

Griffin.1 libraries or phage obtained from a previous round of amplification were used for panning on whole cells, LPS, or flagellar extracts. The phage antibodies bound to the antigen were eluted either with 0.1 M glycine-HCl, pH 2.8 if the phage were from Griffin.1 library or with trypsin if the phage were from Tomlinson I library. The phage antibodies were eluted and amplified in a suppressor strain of *E. coli* to increase the proportion of phage displaying specific antibody sequence over the nonspecific phage. Phage were produced by superinfection with either helper phage or Hyperphage. The cycle of panning and amplification was done three or more times to enrich for the desired phage. The culture supernatant was used for immunological analysis or was used for concentrating phage by PEG precipitation. The amplified phage thus obtained were tested by an ELISA either with the phage produced from all the pooled clones of *E. coli* (polyclonal phage ELISA) or with the phage produced from each individual clone (monoclonal phage ELISA).

Preliminary Experiments for Optimizing Production and Analysis of Phage Antibodies

The Griffin.1 and Tomlinson libraries are in a phagemid format. The plasmid has both plasmid ColE1 *ori* and phage M13 *ori* sequences. The phagemid is deficient in all of the genes necessary for phage replication and packaging, except the scFv-gIII coding for the scFv-pIII fusion protein. For this phagemid to be propagated and viable phage particles to be produced, *E. coli* are superinfected with helper phage M13K07, which provides all of the necessary proteins required for phage assembly. However, most of the phage particles produced have wild type pIII rather than the recombinant protein, thereby greatly decreasing the reactivity of the phage population (65,66).

Hyperphage is derived from M13K07 by deleting most of the gIII from its genome (65). Superinfection of *E. coli* with Hyperphage forces the display of the scFv recombinant protein pIII on the phage particles, as that is the only available source of pIII. Although this greatly decreases the phage output, most of the phage produced have the recombinant protein leading to a greatly increased activity.

To test the efficiency of Hyperphage over helper phage M13K07 in improving the potency of the phage antibodies, phage were produced from anti-human thyroglobulin clones, which were included with the Griffin.1 library as a positive control phage, using either Hyperphage or helper phage. The amount of phage obtained with Hyperphage was about two orders of magnitude lower than that obtained with helper phage. The phage were tested by ELISA after normalizing for the titer. The phage derived from superinfection with Hyperphage showed higher activity than those produced with helper phage (Table 3-6). When phage were used at a concentration of 10^9 phage/mL, phage produced with helper phage gave a signal of 1.5 units, while phage produced with Hyperphage gave a signal of 1.7 units. However, when phage were used at a concentration of 10^7 phage/mL the signal obtained with phage produced from Hyperphage was 0.9, twice that of the phage produced with helper phage.

The activity of the phage was primarily measured by ELISA, and the phage giving a signal of greater than one absorbance unit in ELISA were tested in Western blots. Anti-human thyroglobulin phage obtained with the Griffin.1 library and anti-BSA phage obtained with the Tomlinson libraries were used as positive controls, using thyroglobulin or BSA as antigens, respectively. To determine the concentration of phage particles to be used in ELISA, an experiment was done using human thyroglobulin as antigen and

anti-thyroglobulin phage as primary antibody. Human thyroglobulin (Sigma) at a concentration of 10 μg/mL in PBS was used for coating. Anti-thyroglobulin phage were produced by superinfecting anti-thyroglobulin phagemid-bearing E. coli TG1 with helper phage M13K07. Phage were suspended in casein blocking buffer at serial ten-fold concentrations from 10^{11} phage/mL to 10^{6} phage/mL. A concentration of 109 phage/mL gave a good signal of approximately 1.5 absorbance units. In all the subsequent experiments in which PEG precipitated phage were used as primary antibody, a concentration of 10^{9} phage/mL was used.

Table 3-6: Comparing the Activity of Phage Produced Using Hyperphage or helper phage M13K07 by ELISA

Concentration of phage used as primary antibody (phage/mL)	Signal obtained with phage produced using	
	Hyperphage	M13K07
1.0×10^{9}	1.7 ± 0.02	1.5 ± 0.03
1.0×10^{8}	1.3 ± 0.04	0.9 ± 0.04
1.0×10^{7}	0.9 ± 0.03	0.4 ± 0.05

Anti-thyroglobulin phage were produced in E. coli TG1 by superinfection with either Hyperphage or M13K07. Human thyroglobulin at a concentration of 10 μg/mL in PBS was used as antigen. The phage were normalized for the titer, and serial ten-fold dilutions from 1.0×10^{9} phage/mL to 1.0×10^{7} phage/mL were used as primary antibody. HRP-conjugated anti-M13 antibody at a dilution of 1:2000 was used as secondary antibody. The data presented are mean and standard deviation. The differences are statistically significant with $p < 0.0001$.

Isolation of Phage Antibodies Recognizing *S.* Typhimurium

The first phage library we had available was the Griffin.1, the original phage display library. The phage obtained from the Griffin.1 library were used for panning for three rounds, either with or without amplification of phage in between each round, on whole bacterial cells or LPS. For whole cells, panning was done with the antigen either

suspended in casein blocking buffer or coated on immunotubes in coating buffer. LPS was coated on immunotubes in either coating buffer or PBS. The phage obtained after each round of panning and amplification were tested by a polyclonal phage ELISA using whole cells or LPS as antigen. Wells coated with coating buffer alone were used as negative antigen controls. The proportion of phage showing nonspecific binding or phage binding to plastic increased with each round. There was no increase in specific activity with each round. This occurred consistently, irrespective of the antigen (whole cells or LPS) or the type of panning method (panning in suspension or panning on immunotubes). We tried panning on both of the antigens on immunotubes without amplifying the eluted phage in between each panning round. However, when the eluted phage at the end of third round were tested in a monoclonal phage ELISA, there was no useful signal either with whole cells or LPS. We also tried coating the bacteria in the presence and absence of glutaraldehyde in the antigen suspension. We used 3% (w/v) BSA instead of casein to suspend the phage particles in panning to rule out the potential detrimental effects of Tween on the binding of LPS. However, we did not obtain any useful phage antibodies. In all, 10 attempts were made using the phage from Griffin.1 library.

We then obtained the next generation of phage – the Tomlinson I and J libraries. These phage have several advantages over the Griffin.1 library detailed in the Introduction and Materials and Methods. Phage from the Tomlinson I library were panned on whole S. Typhimurium cells, LPS, or flagellar extracts. When the library was panned on whole cells either in suspension or on immunotubes, with amplification in between rounds, the number of eluted phage increased with each round, suggesting

enrichment of specific phage. The phage obtained after each round were tested by a polyclonal phage ELISA using whole cells and LPS as antigens, and wells coated with Vibrio cholerae cells or with coating buffer were used as negative-antigen controls. The background activity in the wells coated only with coating buffer did not increase with each round. The reactivity against purified LPS was very low and was only slightly higher than the wells not coated with any antigen (Figure 3-2). There was a gradual increase in reactivity against *S.* Typhimurium with each round, but there was higher activity against *V. cholerae* cells. Assuming cross reactivity of phage antibodies with the surface antigens of *V. cholerae*, we incubated the cross-reactive phage with excess of *V. cholerae* cells to absorb the cross-reactive phage antibodies. Potentially cross-reactive phage should have been removed by pelleting *V. cholerae* cells by centrifugation.

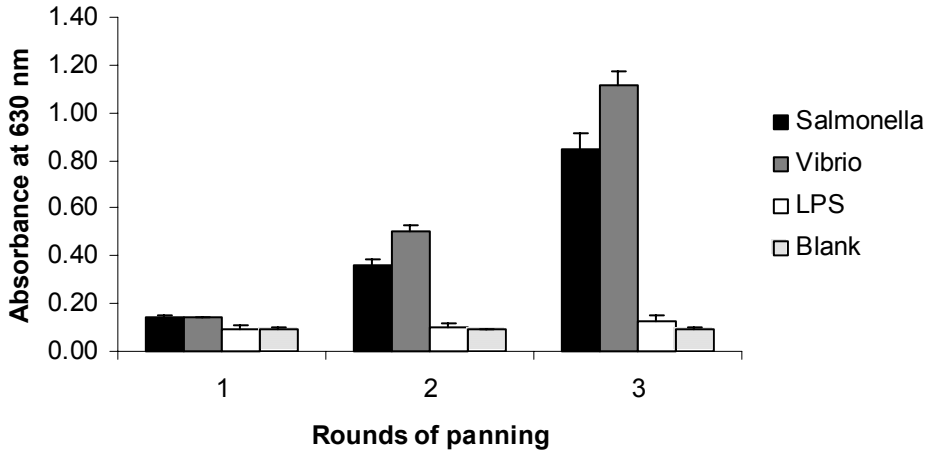

Figure 3-2: Activity of phage obtained after each round of panning on S. Typhimurium cells in suspension. Phage from the Tomlinson I library were used for panning on whole S. Typhimurium cells in suspension. Three rounds of panning were done with amplification of eluted phage in between rounds. Phage from rounds 1, 2, and 3 were tested for their activity against *S.* Typhimurium (Salmonella) cells, *V. cholerae* (Vibrio) cells, purified LPS of *S.* Typhimurium (LPS), and no-antigen (Blank) control wells in a polyclonal phage ELISA. The data presented are the means and standard deviations of the absorbance values at 630 nm 40 min after addition of the substrate.

Unexpectedly, there was no increased activity against *S.* Typhimurium or a decrease in binding to *V. cholerae* when the phage remaining in the supernatant were tested by an ELISA. To isolate clones specific to *S.* Typhimurium from the phage pool showing reactivity towards both *S.* Typhimurium and *V. cholerae*, phage were absorbed on *S.* Typhimurium, and the specific phage antibodies were eluted with trypsin. The eluted phage were used to infect *E. coli* TG1 and plated on 2xTY AG plates. Each colony was screened individually by whole-cell ELISA to see if the same colony had activity against *V. cholerae* and *S.* Typhimurium or if there were two populations of phage with separate activities. However, the activity against *V. cholerae* remained higher than for *S.* Typhimurium for all of the clones screened. None of the phage obtained by panning on *S.* Typhimurium reacted with purified LPS, although LPS is the predominant antigen on the cell surface.

As an alternative to panning on whole cells, purified LPS of *S.* Typhimurium suspended in either PBS or carbonate buffer was used as an antigen for coating on immunotubes. Successive rounds of panning were done either with or without amplification in between the panning rounds. When panning was done in succession without amplification, LPS was coated at progressively decreasing concentrations for each round, starting from 10 μg/mL to 0.1 μg/mL, to isolate phage with higher affinities (67).

Phage obtained after three rounds of panning with amplification in between rounds of panning were tested in polyclonal phage ELISA. The phage pool did not show any specific activity against either purified LPS or whole bacterial cells. The phage obtained after three rounds of panning without amplification were screened in a monoclonal phage

ELISA. Of the 120 clones examined, some clones showed moderately high activity against purified LPS giving a signal of 1.1 units, but with whole cells the highest signal obtained was in the range of 0.4 units. Some clones showed equally high reactivity giving signal in the range of 0.5 units with both *S.* Typhimurium and *V. cholerae*, which was used as a negative-antigen control. The phage obtained from the clones showing reactivity with both *S.* Typhimurium and *V. cholerae* were used as primary antibody in a Western blot using *S.* Typhimurium and *V. cholerae* whole cells as antigens. The phage did not react with proteins or LPS of either *S.* Typhimurium or *V. cholerae*. Therefore, we were unable to determine the specificity and nature of these dual-reactive phage. Study of these clones was not pursued further.

As an alternative to LPS, flagellar extract was used as the antigen for panning. Isolating phage antibodies to proteins is relatively easier than for carbohydrate antigens, because the affinity of the anti-carbohydrate antibodies is generally a 1000-fold lower than the affinities of the antibodies that can be isolated by panning (68,69). The flagella of *S*. Typhimurium were extracted (62) and analyzed by SDS-PAGE. A single protein band of approximately 58 kD was observed, which corresponded to the molecular weight of flagellin (34,70). The flagellar extract was tested by ELISA with Accurate YVS6301 anti-flagellar and USB10 anti-LPS antibodies. Both of the antibodies gave a signal of approximately 1.2, indicating the presence of LPS also. Flagellar extract was coated on immunotubes at a concentration of 10 μg/mL in PBS. Three pannings were done with phage from the Tomlinson I library without amplification in between rounds of panning. As explained in the Materials and Methods, when trypsin was used for elution, the eluted phage could not be used for panning without amplification, as the scFv antibody

sequence is lost. Therefore, when panning without amplification between rounds of panning was done with phage from the Tomlinson library, elution was done with 0.1 M glycine, pH 2.8 in the first two rounds, and in the third round 1 μg/mL trypsin was used for elution. Fifty-nine colonies were obtained after three rounds of panning. Phage were produced from each colony separately and were screened by monoclonal phage ELISA using flagellar extracts and whole bacterial cells as antigens. Thirteen clones showed very good reactivity giving a signal higher than 1.6 units with flagellar extract and poor activity against whole cells (Table 3-7). As detailed below, it turned out that 11 of the 13 were multiple isolates of the same clone and the remaining two were isolates of another clone.

To determine how many of the thirteen clones were clonal representing multiple copies of the same phage, restriction digestion of the plasmids extracted from *E. coli* infected with each of these phages was done. The four base pair recognizing restriction enzymes *Bst*N1, *Hae*III, *Alu*I, or *Sau*3A were used to cut the plasmids to produce a restriction pattern for each separate clone. The restriction patterns were not conclusive because of the small size of the insert compared to the whole plasmid and the relatively low probability of a novel restriction site being present in the variable portion of the scFv-encoding sequences. All of the thirteen clones and even the anti-BSA clone gave an apparently similar pattern. These plasmids were sequenced with primers Fdseq1, hybridizing upstream of the scFv gene, and Lmb3, binding to DNA in the 5' region of gIII. Analysis of the DNA sequences with GCG software package showed that there were only two different clones among the thirteen. The distinct phage clones were named SF1 and SF2. As expected, the anti-BSA phage had a unique DNA sequence.

Table 3-7: Activity of phage obtained after panning on flagellar extracts

Clone number	Salmonella	Flagella
2	0.38 ± 0.02	1.98 ± 0.09
4	0.45 ± 0.03	1.98 ± 0.07
6	0.39 ± 0.04	1.98 ± 0.03
9	0.56 ± 0.03	1.96 ± 0.03
11	0.30 ± 0.08	1.94 ± 0.05
12	0.30 ± 0.04	1.95 ± 0.03
17	0.35 ± 0.02	1.97 ± 0.06
29	0.4 ± 0.03	1.7 ± 0.04
44	0.27 ± 0.01	1.83 ± 0.08
45	0.39 ± 0.02	1.79 ± 0.10
48	0.29 ± 0.03	1.83 ± 0.05
54	0.28 ± 0.04	2.04 ± 0.09
55	0.31 ± 0.10	2.01 ± 0.11

59 colonies obtained after three rounds of panning on flagellar extracts of *S.* Typhimurium were screened by a monoclonal phage ELISA using either 10 μg/mL flagellar extract (Flagella) or whole *S.* Typhimurium cells (Salmonella) at 10^7 CFU/mL as antigen. The data presented are the mean absorbance and standard deviation values of the positive clones.

To examine the specificity of SF1 and SF2 anti-flagellar antibodies, *S*. Typhimurium cells and flagellar extracts were resolved by SDS-PAGE, and the antigens were transferred onto a nitrocellulose membrane. The blot was probed with SF1 or SF2 phage antibodies at concentration of 10^8 phage/mL or with Accurate YVS6301 anti-flagellar monoclonal antibody at a concentration of 10 μg/mL. HRP conjugated anti-M13 or anti-mouse IgG antibodies at a dilution of 1:2000 were used as secondary

antibody, and 4-CN was used as the substrate. The monoclonal antibody failed to recognize the flagellar protein, while both of the phage antibodies recognized the band in both whole cell sample and flagellar extract (Figure 3-3).

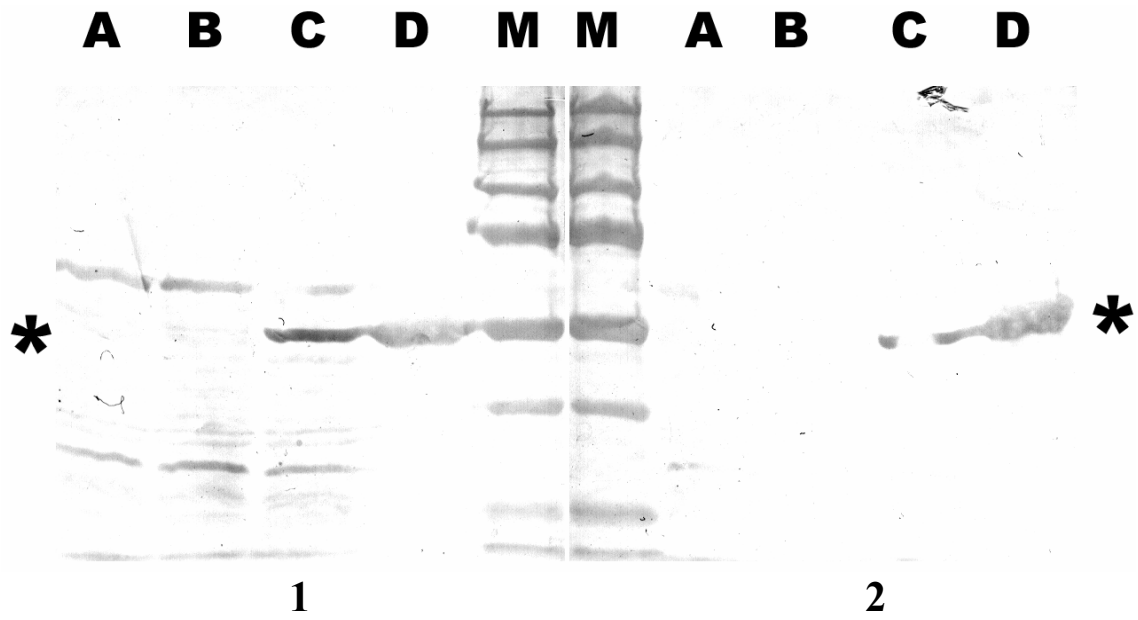

Figure 3-3: Western blot to determine the specificity of SF1 and SF2 anti-flagella phage antibodies. Whole cells of two non-flagellated strains of Salmonella, *S.* Typhimurium (*fliC*) (A) and *S.* Typhimurium (*flhD*) (B), *S.* Typhimurium (wild-type) (C), and flagellar extract of *S.* Typhimurium (D) were resolved on SDS-PAGE and a Western blotting was done using SF1 (1) or SF2 (2) anti-flagella phage as primary antibody. Molecular weight marker (M). The position of the flagellar protein is shown by asterisk (*).

The specificities of SF1 and SF2 anti-flagellar phage antibodies were tested using two non-flagellated strains of *S.* Typhimurium. Approximately 10^8 cells each of *S.* Typhimurium χ3000 (wild-type), *S.* Typhimurium (*fliC*::Tn10), *S.* Typhimurium (*flhD*), and 5 µg/mL of purified flagellar protein were resolved by SDS-PAGE, and the proteins were transferred onto a nitrocellulose membrane. The blot was probed with SF1 or SF2 phage antibodies at concentration of 10^8 phage/mL or with Accurate YVS6301 anti-flagellar monoclonal antibody at a concentration of 10 µg/mL as primary antibody. HRP conjugated anti-M13 or anti-mouse IgG antibodies at a dilution

of 1:2000 were used as secondary antibody, and 4-CN was used as the substrate. The monoclonal antibody failed to recognize the flagellar protein, while both of the phage antibodies recognized the band in both whole cell sample and flagellar extract (Figure 3-3).

Determining the Activity of scFv Antibodies vs. Phage Antibodies

scFv antibodies can be obtained as soluble antibody molecules instead of scFv-pIII fusion proteins expressed in the context of intact phage. The genes for scFv and gIII are separated by a TAG amber stop codon. When the phagemid is present in a non-suppressor strain such as *E. coli* χ1918, synthesis of the peptide chain is terminated at the amber stop codon, and a soluble antibody molecule is released. The scFv gene has a *pelB* leader sequence at the 5' end of the gene enabling the secretion of scFv antibody molecules into periplasm and culture supernatant. scFv antibodies can be harvested directly from the culture supernatant or from periplasmic by osmotic shock (62). scFv antibody molecules produced from phage obtained from the Tomlinson library have protein A and protein L sites in addition to the hexahistidine tag and c-myc epitope present in scFv antibodies obtained from the Griffin.1 library. It should therefore be possible to determine the activity of scFv antibodies by ELISA or Western blotting using protein A conjugates or anti-c-myc conjugates.

scFv antibodies were produced by infecting *E. coli* χ1918 with phage that gave a good signal by monoclonal phage ELISA. To understand the properties of scFv antibodies in general, anti-BSA scFv antibody, which was obtained as a positive control with the Tomlinson library, was used. One hundred microliters of BSA (10 μg /mL) in PBS was used as antigen. Anti-BSA scFv antibody produced in *E. coli* χ1918 and harvested from the culture supernatant was diluted 1:2 in casein blocking buffer and used

as primary antibody. Protein A-HRP conjugate was used as secondary antibody at a dilution of 1:2000. The signal obtained with the anti-BSA scFv antibody was 1.2 units, comparable to the signal obtained with anti-BSA phage antibody. Anti-BSA scFv antibodies recognized the band corresponding to BSA when used as primary antibody in a Western blot with BSA as antigen (data not shown).

scFv antibodies were produced from SF1 and SF2 phage clones. The culture supernatant containing the scFv antibodies were diluted 1:2 in casein blocking buffer and used for immunological analysis. The activities of the scFv antibodies were tested by an ELISA and a Western blot with whole bacterial cells and flagellar extracts as antigens. Protein A-HRP conjugate was used at a dilution of 1:2000 as secondary antibody. There was no signal with any of the anti-flagellar scFv antibodies against flagellar extract or against whole cells in an ELISA. scFv antibodies also failed to recognize the flagellar protein in a Western blot.

To determine if scFv antibodies were being produced and secreted into the culture medium, ELISA was done using the culture supernatants having SF1, SF2, and anti-BSA scFv antibodies as antigens for coating the wells. Protein A-HRP conjugate was used at a dilution of 1:2000 as primary antibody. There was no signal with any of the scFv antibodies. The same samples of culture supernatant were resolved by SDS-PAGE, and the antigens were blotted onto a nitrocellulose membrane and probed with protein A-HRP conjugate. There were no bands corresponding to any of the scFv antibodies. Fifteen milliliters of culture supernatant containing anti-BSA scFv antibody was concentrated by affinity chromatography with a protein A-Sepharose column and eluting with 0.1 M citric acid, pH 3 (61). The eluted fraction was resolved by SDS-PAGE, and the gel was stained

with Coomassie Blue. Alternatively, the antigens in the gel were transferred onto a nitrocellulose membrane, and the membrane was probed with protein A-HRP. No bands were observed on either gel. The scFv antibodies might be present in concentrations below the limits of detection by a Coomassie Blue staining or Western blotting.

Genetically Fusing scFv Antibodies to Biotin

Biotinylation of antibody molecules and detection with streptavidin conjugates is a common strategy to amplify the signal obtained in immunological analysis (71-73). Proteins such as scFv or monoclonal antibodies are most often chemically derivitaized with biotin; however, chemical biotinylation has disadvantages of variability and possibly inactivating the antibody. Therefore, we chose to take advantage of the ability of genetically modifying scFv antibodies to enable enzymatically mediated and site-directed biotinylation.

Avitag is a 15-peptide sequence that is specifically recognized and biotinylated by BirA enzyme of *E. coli* (Avidity). *birA* is stably integrated into the chromosome of *E. coli* AVB100. Avitag vectors pAC4, pAC5, and pAC6 have the sequence coding for Avitag peptide at the 3' end of a multiple cloning site. The genes for SF1, SF2, and anti-BSA scFv were subcloned as *Hin*dIII/*Not*I fragment into the pAC5 vector, and electroporated into *E. coli* AVB100 cells. Production of scFv was induced with 1 mM IPTG. *birA* was induced with 0.4% (w/v) L-arabinose after adding d-biotin to the culture medium to a final concentration of 50 μM. To confirm the synthesis of the biotinylated antibodies, approximately 10^8 cells of *E. coli* AVB100 bearing the pAC5 plasmid with the anti-BSA scFv, SF1, or SF2 genes were boiled and resolved by SDS-PAGE. The antigens were transferred to a nitrocellulose membrane and probed with streptavidin-HRP

conjugate. All of the clones showed a band of 28 kD corresponding to biotinylated scFv (Figure 3-4).

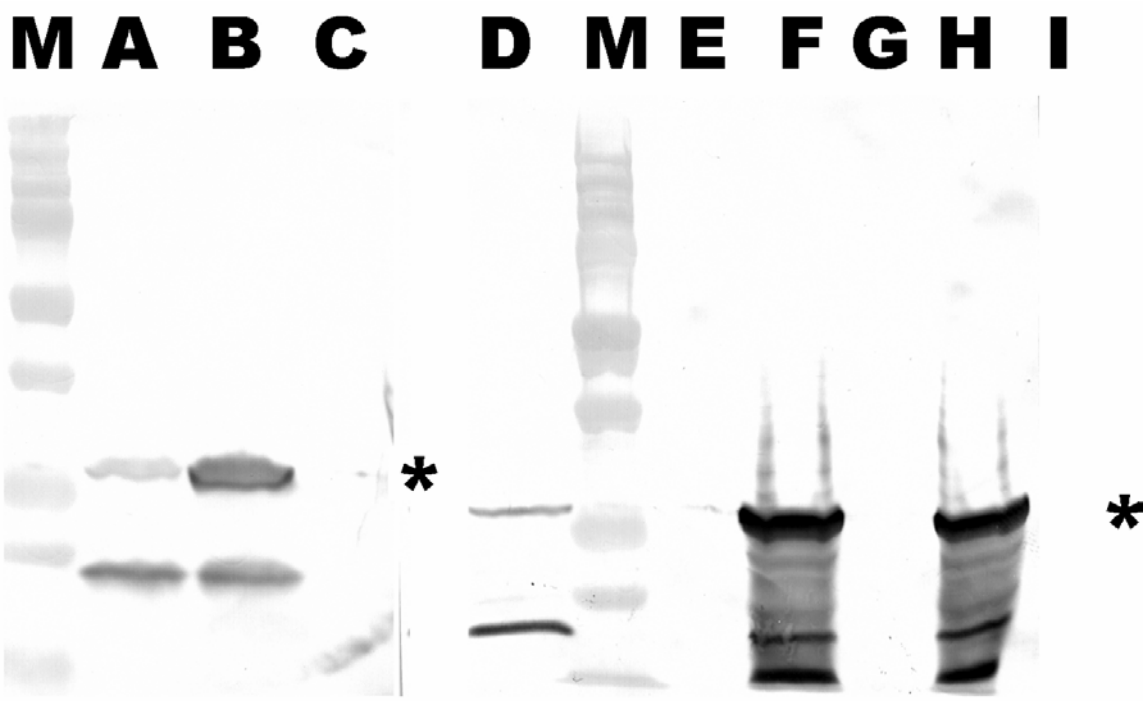

Figure 3-4: Confirmation of synthesis of biotinylated scFv antibodies. *Escherichia coli* AVB100 cells containing the SF1, SF2, or anti-BSA scFv genes cloned into pAC5 Avitag vectors or the culture supernatants were resolved on SDS-PAGE and the antigens were transferred onto a nitrocellulose membrane. Streptavidin-HRP conjugate was used for probing. Molecular weight marker (M), Biotinylated anti-BSA scFv positive control (A,D), SF1-Avitag in whole cells (B) or culture supernatant (C), SF2-Avitag in whole cells (F) or in culture supernatant (E), anti-BSA scFv-Avitag in whole cells (H) or culture supernatant (G)

scFv antibodies were harvested from either culture supernatant or from periplasm. scFv antibodies in the periplasmic space were obtained by cold osmotic shock method (62). The culture supernatant and periplasmic extract were diluted 1:2 in casein blocking buffer and used as primary antibody in an ELISA using BSA or flagella as antigens as appropriate. Protein A-HRP conjugate was used as secondary antibody for unmodified scFv antibodies, while streptavidin-HRP conjugate was used as secondary antibody for biotinylated antibodies. With anti-BSA scFv antibodies in the culture supernatant, the

signal obtained with biotinylated molecules was lower than with unmodified antibodies, the absorbance values being 1.42 and 1.08, respectively (Table 3-8). The periplasmic extracts showed a slightly higher activity than the culture supernatant (Table 3-8). There was no activity with the SF1 and SF2 scFv antibodies either in the unmodified or biotinylated forms in either the culture supernatant or periplasmic extract. Biotinylation of SF1, SF2 , and anti-BSA scFv antibodies did not have any benefit over the unmodified antibodies. However, as seen with anti-BSA scFv, the genetic fusion of scFv to a biotinylation cassette was successful.

Table 3-8: Activities of unmodified and biotinylated scFv antibodies

	Anti-BSA scFv	SF1 scFv	SF2 scFv
Culture supernatant			
Unmodified scFv	1.42 ± 0.03	0.16 ± 0.02	0.10 ± 0.02
Biotinylated scFv	1.08 ± 0.06	0.18 ± 0.02	0.07 ± 0.02
Periplasmic extract			
Unmodified scFv	1.67 ± 0.05	0.11 ± 0.04	0.09 ± 0.01
Biotinylated scFv	1.31 ± 0.03	0.12 ± 0.01	0.09 ± 0.01

The activities of unmodified and biotinylated anti-BSA, SF1, and SF2 scFv antibodies harvested from either culture supernatant or periplasm were tested by ELISA using 10 µg/mL BSA or 10 µg/mL flagellar extract as antigen, as appropriate. Protein A-HRP conjugate or streptavidin-HRP conjugate at a dilution of 1:2000 was used as secondary antibody for unmodified and biotinylated scFv antibodies, respectively. The data presented are the mean and standard deviation of the absorbance values after 40 min after the addition of the substrate. However, the amounts of protein in each sample were not quantified.

CHAPTER 4
DISCUSSION

S. Typhimurium accounts for over a million cases of gastroenteritis in the United States every year caused by eating contaminated food products (29). Salmonella infections also cause huge losses in the poultry and diary industries (74). *S.* Typhimurium can also be used as an agent of bioterrorism (3,5). The first step in managing the above-mentioned problems would be accurate and timely detection of contamination. A biosensor system with capture antibodies attached to an optic fiber and detection antibodies labeled with fluorescent dyes has been shown to be able to detect bacteria sensitively and rapidly (26). Such systems would be of great practical and economic utility. Efforts are underway at the University of South Florida to develop such a system for detecting *S.* Typhimurium. As a part of that project, we screened several murine monoclonal antibodies recognizing surface epitopes of *S.* Typhimurium by ELISA for their potential use in the biosensor system.

Recombinant phage display antibodies recognizing an antigen can be isolated from a phage display library by panning the library on the desired antigen and isolating the phage that bound specifically to the antigen. In this procedure antibodies can be isolated very rapidly, when compared with producing a monoclonal antibody using hybridoma technology. Griffin.1 and Tomlinson I human synthetic scFv phagemid libraries were used for panning on whole *S.* Typhimurium cells or cell extracts to isolate antibodies recognizing surface epitopes of *S.* Typhimurium.

This thesis describes the efforts done to develop a protocol for ELISAs using bacterial cells as antigens, to characterize several commercially available monoclonal antibodies for their use in the biosensor system, and to isolate recombinant phage display antibodies recognizing *S.* Typhimurium and test their potential usefulness in the biosensor system.

Specific Aim 1: Standardization of a Protocol for Whole Bacterial Cell ELISAs

ELISA is a very simple, economical, and rapid method for immunological analysis of antibodies (64). There are many different variations of the basic protocol, each suitable for detecting antigens or antibodies in a complex mixture or for characterizing antibodies. The basic procedure has been adapted by us to suit the requirements for using whole bacterial cells as antigens. We examined the following parameters in optimizing our procedure: antigen concentration, coating conditions such as buffer for suspending the antigen, blocking buffer, and reaction development time. In standardizing these conditions, we used goat anti-*S.* Typhimurium serum as the primary antibody and donkey anti-goat IgG-alkaline phosphatase conjugate as the secondary antibody. All of the incubations were done at 4 ºC. To enhance the binding of the antibodies to antigens, incubations were done for 2 h rather than 30 min to 1 h as used in ELISAs using proteins as antigens (64). Glutaraldehyde stabilizes the cell membrane by cross-linking the amine groups of the surface proteins (57). Therefore, glutaraldehyde was added to the antigen suspension to a final concentration of 0.05% (w/v) to stabilize the cell membranes. (It does not have a role in increasing adhesion as we were thinking all these days). Each condition was tested in triplicate, and the mean absorbance value was calculated. The signal to noise ratio was calculated by dividing the mean value of the antigen-positive wells with mean value of antigen-negative wells.

We chose to use enzyme-linked secondary antibodies, as opposed to fluorescently labeled antibodies or fluorogenic substrates, mainly for economy. The EL_x 800UV microtiter plate reader was used to read the absorbance values from product development in ELISAs. Rather than stopping the enzyme reactions at an arbitrary time, as is commonly done, we allowed color development to continue during reading so that we could use data at the optimum, rather than arbitrary, time of reaction. However, the EL_x 800UV plate reader takes approximately 1 min for reading all of the 96 wells of a microtiter plate. Absorbance values in wells coated with the same concentrations of bacteria and treated in identical conditions differed by a significant amount depending on the location of wells on the plate when the plate was read after only a short time, e.g., 5 to 10 min after the addition of substrate (i.e., the plate reading time contributed significantly to the development time of the different wells). However, there was no difference in absorbance values if the plate was read after approximately 30 min, since the one minute required to read the plate constituted an insignificant part of the total development time. Therefore, the development reaction was allowed to proceed for 40 min to prevent discrepancies introduced by the slowness of the plate reader. The components of the ELISA were therefore adjusted for optimum development over such an extended period.

Experiments were done to optimize the concentration of bacteria that needed to be coated for obtaining a good signal without increasing the background activity. When the concentration of bacteria is high, the coating of the cells might not be even, leading to formation of clumps and thereby inhibiting optimal binding of antibody due to steric hindrances (63). If the concentration of the antigen is low, the signal obtained might be too low for interpreting the results properly. Initial experiments were done with bacteria

at an arbitrarily chosen concentration of 1.0 x 10^9 CFU/mL. When the wells were coated with bacteria at serial five-fold dilutions and the change in the intensity of signal was observed, a concentration of 8.0 x 10^6 CFU/mL gave signal of 1.8 units, which was similar to the signal obtained with higher concentrations (Figure 3-1). In addition, at a concentration of 8.0 x 10^6 CFU/mL the rate of development of reaction was linear with respect to time and reached peak intensity towards the end of the monitoring period of 40 min. The signal to noise ratios were similar for all the concentrations of bacteria above 8.0 x 10^6 CFU/mL, because wells coated with only coating buffer were used as antigen-negative control wells. Further lowering of the concentration of bacteria affected the peak signal obtained (Figure 3-1). Therefore, a concentration of 1.0 x 10^7 CFU/mL was determined to be appropriate for the whole-cell ELISAs.

Nonspecific binding of the antibodies to the matrix on which the antigen is deposited, such as the wells of a microtiter plate or a nitrocellulose membrane, is a common problem in immunological analysis (63). Several different types of agents are used for preventing this nonspecific binding, the common ones being casein, BSA, and dextran sulfate (75,76). These blocking agents bind to the sites where the antigen is not bound and prevent nonspecific binding of primary and secondary antibodies to the matrix. The primary and secondary antibodies are diluted in blocking buffer for the same purpose. Some blocking buffers have detergents such as Tween which might affect the binding of molecules such as LPS to the matrix. Two common agents used for blocking, casein and BSA, suspended in PBS were tested for their blocking efficiencies in whole-cell ELISAs. Both of the blocking buffers had 0.1% (w/v) Tween 20. One percent (w/v) BSA, 1% (w/v) casein, or a mixture of 0.5% (w/v) casein and 0.5% (w/v)

BSA was used as blocking buffer in an ELISA with *S.* Typhimurium cells as antigen. Casein performed better than either BSA alone or the mixture of BSA and casein. The signal obtained in each case was in the range of 3.35 to 3.55 absorbance units. The background activity was 0.13 units with casein, while with BSA the value was 0.34 units. The signal to noise ratio was therefore doubled with casein, and casein was used as the blocking buffer for subsequent experiments.

S. Typhimurium was suspended in either the carbonate buffer or PBS to study the effect of coating buffer on the signal obtained. The antigen was at a concentration of 10^7 CFU/mL, and the ELISA was done using USB10 as primary antibody. There was no significant difference in the signal obtained, with the values being 1.11 and 1.14 for carbonate buffer and PBS, respectively.

Based on the above results, we determined that for characterizing monoclonal antibodies *S.* Typhimurium would be used at a concentration of 10^7 CFU/mL suspended in carbonate buffer with glutaraldehyde at final concentration of 0.05% (w/v). PBS-T (0.1% (w/v)) containing 1% (w/v) casein would be used as the blocking buffer. The reaction would be allowed to develop for 40 min.

Specific Aim 2: Characterization of Commercially Available Monoclonal Antibodies to *S.* Typhimurium

Several monoclonal antibodies to various antigens of *S.* Typhimurium are available commercially. We analyzed monoclonal antibodies recognizing surface antigens for their suitability in the biosensor system. Since the final conditions of the biosensor probe closely resemble the conditions of an indirect sandwich ELISA, rather than other immunological methods such as immunoblotting, all the antibodies were characterized by indirect ELISA. We considered that an indirect ELISA would be more appropriate than

an indirect sandwich ELISA, as we were primarily interested in the reactivities of the antibodies rather than their ability to stick to the microtiter plate and serve as capture reagents.

LPS was chosen as the principal antigen for study because the purpose of the biosensor is to detect intact cells rather than cell extracts. LPS is abundant on the cell surface enabling the binding of many antibody molecules on the surface rather than other antigens such as outer membrane proteins, which might be present only in few copies (33). Antibodies recognizing LPS have the added advantage of being able to be used both as capture antibody and detection antibody, as LPS is present in many copies on the bacterium. The selected antibodies recognized different epitopes such as O4 and O12 (Table 2-2).

Another antigen of interest was the flagellar protein. Flagella are made up of repeating units of monomeric flagellin, a 60-kD protein. Antibodies recognizing flagella offer all of the advantages of those recognizing LPS. The disadvantages include their inability to recognize non-flagellated strains. *S*. Typhimurium also exhibits phase variation of their flagella (34,70). The genes coding for the two forms of flagella are highly homologous, but are not identical, making the two forms of flagella antigenically distinct (34). The antibodies recognizing one form of flagella might not recognize the other, giving false-negative results.

Using the standardized protocol, antibodies were tested at various concentrations, and their activities were noted. In the initial experiments *E. coli* LE392 was used as a negative-antigen control. With the monoclonal antibodies the signal obtained with *E. coli* coated wells was equal to wells coated with coating buffer only. For subsequent

experiments wells coated with coating buffer alone were used as negative-antigen controls. The antibodies were ranked on both the peak signal obtained and the signal to noise ratio. A signal greater than one absorbance unit was considered satisfactory.

Antibodies recognizing LPS and other undetermined cell surface antigens were characterized by ELISA using whole bacterial cells and purified LPS as antigens. USB10 anti-*S*. Typhimurium LPS monoclonal antibody recognizing the O4 epitope performed superiorly to all of the antibodies screened (Table 3-2). This antibody gave a signal higher than 1.3 in whole-cell ELISAs. It gave a similar signal when purified LPS at a concentration of 5 μg/mL was used as antigen. This antibody was tested at various concentrations, and it gave a signal of 0.3 absorbance units at concentrations as low as 0.1 μg/mL (Table 3-3). Biosp53 anti-*S*. Typhimurium LPS antibody also performed similarly at concentrations as low as 0.1 μg/mL. RDI and Virostat 6341 monoclonal antibodies specific to groups A, B, and D Salmonella performed moderately well at concentrations of 10 μg/mL giving a signal of approximately 0.6 absorbance units. The other antibodies recognizing LPS or uncharacterized whole cell epitopes did not give any satisfactory signal in whole-cell ELISAs.

It is interesting to note that there is a hundred-fold difference in the potencies of the antibodies allegedly recognizing the same epitopes. For example, USB10 recognizing O4 epitope gave a good signal at an antibody concentration of 0.1 μg/mL, while USB15 recognizing the same epitope was not satisfactory even at concentrations as high as 10 μg/mL. The difference in the activity could be due to difference in affinity of the antibodies produced from different clones, even though they recognize the same epitope.

Antibodies recognizing flagella were characterized by ELISA and Western blotting using whole bacterial cells and flagellar extracts as antigens. Of the anti-flagellar monoclonal antibodies Accurate YVS 6301 performed well at concentrations of 10 μg/mL giving a signal of 1.03 absorbance units when flagellar extract at a concentration of 10 μg/mL in PBS was used as the antigen (Table 3-4). However, the signal obtained in a whole-cell ELISA was less than 0.2 absorbance units. The other anti-flagellar monoclonal antibody, Virostat 6301, did not give any signal higher than 0.2 units either with flagellar extracts or whole cells. The lack of activity with Virostat 6301 antibody may be explained by the fact that it recognizes a different phase of flagellum than present on the bacterium used in the experiment. Neither of the antibodies recognized the flagellar protein band on a Western blot. This could be explained by the possibility that the antibodies were recognizing conformational epitopes, which could have been destroyed by denaturation in the SDS-PAGE. As described in the Introduction section, anti-flagellar antibodies have most of the advantages of anti-LPS antibodies in recognizing whole cells, though detecting non-flagellated strains would be difficult.

Whole-cell ELISAs were done using *S.* Enteritidis of different serogroups to determine the specificity of USB10 and Biosp53 monoclonal antibodies. Both of them showed neglible activity with non-group B *Salmonella*, except that they gave a moderate signal of 0.4 units with *S.* Worthington, belonging to group G (Table 3-5). However, the authenticity of the strain cannot be verified, and the strain may in fact be a group B salmonella. Additionally, there could be some partial cross-reactivity between groups B and G.

USB10 and Biosp53 were determined to be useful for further study in the biosensor system because of their potency and specificity.

Specific Aim 3: Isolation of Recombinant Phage Display Antibodies to *S.* Typhimurium and Genetically Fusing scFv Antibodies with Ligands or Reporter Molecules

Phage display of antibody fragments offers a powerful, rapid, and economical approach to screen libraries of large complexities ranging from 10^7 to 10^9 variants (66,67). Griffin.1 and Tomlinson I + J Human Synthetic $V_H + V_L$ scFv libraries are scFv phagemid libraries made from synthetic V-gene segments by cloning the heavy and light chain variable regions of human immunoglobulin genes into phagemid vectors (43). The genes coding for the antibody fragments are cloned in frame with gene gIII coding for the coat protein pIII. Genes coding for a hexahistidine tag and a c-myc tag are cloned at the carboxyl terminus of the antibody gene for the purposes of purification of scFv from culture supernatant. Sequences coding for protein A and protein L binding sites also are present in the constructs of Tomlinson library. A TAG amber stop codon is engineered at the junction of the scFv gene and gIII (42).

Isolating antibodies from phage display libraries by panning the phage libraries on antigen of choice is an efficient, rapid, and economical method of obtaining antibodies of desired specificity. We used Griffin.1 and Tomlinson I libraries for panning on whole bacteria, purified LPS, or flagellar extracts for isolating antibodies. Phage obtained from Griffin.1 library were used for panning on whole *S.* Typhimurium cells and purified LPS. Phage obtained from Tomlinson I library were used for panning on flagellar extracts of *S.* Typhimurium, in addition to whole cells and purified LPS.

Overcoming the Problem of Insert-less Phage in the Phage Pool

Generally, three rounds of panning were done with each antigen. The eluted phage after each round were used for infecting *E. coli* TG1 and amplification of the phage bearing antibody sequences of desired specificity, or the phage were used in the next round of panning. Phage particles were produced by superinfecting *E. coli* TG1 with helper phage. This amplified phage pool was used for panning in the subsequent round. However, in each amplification step a significant fraction of the output phage do not display the scFv-pIII fusion protein. This might be because of the relative growth advantage of the phagemids not bearing the scFv gene (67). To overcome the problem of insert-less phage dominating the culture, panning was also done without the amplification step in between the rounds of panning. The eluted phage were suspended in blocking buffer and used for panning immediately. The number of phage particles input in each subsequent round of panning would be exponentially decreasing if the eluted phage are not amplified, thereby greatly decreasing the non-specific phage obtained after the third round. The number of phage particles obtained at the end of the third round were typically around 100, enabling screening of each clone by a monoclonal phage ELISA. When a phage pool gave a good signal by polyclonal phage ELISA, the *E. coli* TG1 bearing the phagemids were serially diluted and plated to obtain single colonies, phage produced from which were screened by a monoclonal phage ELISA. By eliminating the amplification step in between, the need for isolating single clones from the pool was obviated. This method has added advantages of being less time-consuming and being cost-effective than the traditional method of panning. However, a disadvantage is that specific phage might be lost and not recovered because they are not amplified.

Reactivity of Phage Antibodies

Phage from Griffin.1 library were used for panning on either whole cells or purified LPS. The phage obtained after three rounds of panning both in suspension and on immunotubes did not show any significant specific activity against either LPS or whole cells. The phage pool showed nonspecific binding or binding to the plastic surface of the microtiter plate. This result was the same with either of the antigens and was irrespective of the method of antigen coating. Panning was done without amplifying the eluted phage in between rounds of panning, and the output phage of the third round were screened in a monoclonal phage ELISA. Most of the phage clones showed no activity, and the remaining phage showed nonspecific activity. Many clones gave a very good signal irrespective of the presence or absence of any antigen. These apparently "plastic binding" phage were considered useful, because these phage could be used in the biosensor system for attaching the capture antibodies to the probe by constructing a hybrid scFv antibody with the plastic-specific scFv coupled to the desired antigen-specific scFv. Since, the genes coding for the antibodies are readily available, they can be genetically manipulated to create an antibody showing specificity towards the desired antigen and towards plastic. These antibodies can be used to coat the plastic surface of the optic fiber, such that the antibody recognizing the microbes is exposed into the solution. This would be better than attaching the antibodies to the probe by chemical means, as the process might destroy the structure and hence the function of the antibody. However, such experiments were not performed in these studies.

When panning was done with phage from Tomlinson I library on whole bacteria with amplification in between rounds, there was a gradual increase in the activity of the phage pools against whole bacteria (Figure 3-2). There was no increase in the

background activity with each round, as was observed with phage from Griffin.1 library. This could be the result of better elimination of nonspecific phage in the eluted fraction by trypsin treatment. However, the activity against antigen-negative control *V. cholerae* cells was higher than with *Salmonella*. We considered the possibility of the two gram-negative bacteria sharing some common epitopes, and so we tried to remove the cross-reactive phage from the pool. The phage pool was incubated with excess of *V. cholerae* cells, and the cells with the dual-reactive phage bound to them were removed by centrifugation. The supernatant was tested for activity by ELISA. However, there was no change in the specificity of the phage pool. As another approach to isolate the phage antibodies specific to *S.* Typhimurium from the pool containing cross-reactive phage, the phage pool was incubated with *S.* Typhimurium cells and the phage bound to the antigens were eluted with trypsin. Instead of amplifying the eluted phage, they were used to infect *E. coli* TG1, and the infected cells were plated on 2xTY AG plates. Phage were produced from each of the clones, and the phage were tested by a monoclonal phage ELISA. However, there was no increased specificity with any of the clones.
S. Typhimurium and *V. cholerae* cells were run on a SDS-PAGE, and the antigens were transferred onto a nitrocellulose membrane. Phage from a few representative clones showing dual reactivity were used as primary antibody. None of the phage recognized any antigen of either bacterium. The study of these clones was not pursued further.

The difficulty in isolating a phage specific to LPS or whole cell epitopes might be explained by the fact that the affinities of anti-carbohydrate antibodies are in low micromolar range (68). The panning procedure involves many washing steps, and it is possible that the phage bearing antibody sequences against carbohydrates were being lost

in the washing process. With the phage display technique the lowest affinity of the antibodies isolated is generally in the nanomolar range (68). This could explain the fact that there are very few instances in literature of isolation anti-carbohydrate antibodies, while antibodies to proteins seem to be relatively easy to isolate. Therefore, flagella were chosen as an alternative antigen for panning.

Flagella were extracted from *S.* Typhimurium and used as antigen for coating immunotubes. Three successive rounds of panning were done without amplification steps between rounds of panning. Fifty-nine colonies were obtained when the eluate of the third round was used to infect *E. coli* and plated on 2xTY AG plates. When these colonies were screened by monoclonal phage ELISA using flagellar extracts and *S.* Typhimurium cells as antigens, thirteen clones showed good reactivity with flagella giving a signal of 1 absorbance unit but showed poor reactivity with whole cells (Table 3-8). To investigate the reason for lower reactivity of the phage with whole cells when compared to flagellar extracts, bacteria were coated at hundred-fold higher concentration, and the ELISA was repeated, but there was no increased signal. This result is similar to the one obtained with anti-LPS monoclonals, in which bacteria coated at 10^7 or 10^9 CFU/mL gave a similar absorbance value.

To determine if each of the thirteen clones was different or if they represented repeated isolations of the same clone, restriction digestion of the plasmid DNA of each clone was done. Plasmid DNA was extracted from *E. coli* infected with each of these phage, and restriction digestion was done with either *Bst*NI, *Sau*3A, *Alu*I, or *Hha*I, all being four-base pair recognizing enzymes, to generate a restriction pattern. The restriction patterns were inconclusive. The size of the vector pIT2 is 4.2 kb, while the

size of the scFv insert is approximately 900 bp. The relatively smaller size of the insert when compared with the vector made analyzing the restriction pattern difficult. Additionally, the variable portion of the scFv-encoding sequences is even a smaller target for potentially distinctive restriction enzymes. All of the thirteen clones and the anti-BSA clone gave apparently an identical restriction pattern. Therefore, the plasmid DNAs from the thirteen anti-flagellar clones were sequenced, and the sequences were aligned using GCG software package. Eleven of the thirteen clones turned out to be siblings. Approximately 10^{12} phage particles were used in the first round of panning, and the size of the library is approximately 1.2 x 10^{9} variants. Therefore, statistically a thousand copies of each antibody were present in the initial suspension. It was not unlikely that multiple copies of the same antibody were isolated, even though the eluted phage were not amplified in between rounds of panning. The diversity in the sequence was observed in the V_H region. The two anti-flagellar clones were named SF1 and SF2.

The specificities of SF1 and SF2 were tested in a Western blot using wild type *S.* Typhimurium and two non-flagellated mutants of *S.* Typhimurium cells and flagellar extract of *S.* Typhimurium as antigens. Both of the anti-flagellar phage antibodies recognized the flagella in the whole cell sample and purified flagellar extract. No protein band corresponding to flagella was seen in the lanes with either of the two non-flagellated strains (Figure 3-3).

Reactivity of scFv Antibodies vs. Phage Antibodies

Antibody molecules can also be obtained as soluble scFv molecules instead of fusion proteins displayed on the pIII protein of the phage particle by expressing the phagemids in a non-suppressor strain of *E. coli*.

The anti-BSA scFv showed a moderate activity giving a signal of 0.6 units when tested by an ELISA using 10 μg/mL BSA as antigen and anti-c-myc-HRP conjugate as secondary antibody. However, there was no activity with either of the anti-flagellar antibodies with either whole cells or flagellar extracts. To determine if the protein was being made, the culture supernatant was used in an ELISA as the antigen and protein A-HRP conjugate was used for detection. There was no signal either with anti-BSA scFv or with the anti-flagellar scFvs. The same supernatants were resolved on a SDS-PAGE, and the antigens were transferred onto a nitrocellulose membrane. When probed with protein A-HRP, no bands appeared corresponding to any of the scFvs. The scFv antibodies were concentrated by passing the culture supernatant on a protein A-sepharose column and eluting with 0.1 M citric acid, pH 3 (61). The eluted fractions were tested for their activity by ELISA and Western blot. None of the fractions showed any activity by either an ELISA or a Western blot.

The instability of the scFv molecule might explain the activity of these antibodies as scFv-pIII fusion proteins and not as soluble molecules. The factors on which the stability of the scFv molecules depend include intradomain disulfide bonds and the stability of V_H-V_L interface (77). Alternatively, there could be a problem in the secretion of different scFvs. The amount of scFv antibodies secreted into the culture supernatant might be very low for use as detection reagents.

Two strategies were used to obtain increased yields of scFv antibodies, using anti-BSA antibody as a model. Periplasmic extracts were analyzed for the presence of scFv protein and its activity to rule out the possibility that most of the protein was present in the periplasm and was not being released into the supernatant. As an alternative

approach, sucrose was added to a final concentration of 0.4 M while the culture was in mid-exponential phase to increase the yield of scFv antibodies in the supernatant as shown by Kipriyanov et al (78). The culture supernatant and the periplasmic extract were tested for the presence of scFv antibodies by a Western blot using protein A-HRP conjugate as detection reagent. No band was observed corresponding to the antibody in either periplasmic extract and culture supernatant. The activity of the antibodies in the periplasmic extract and culture supernatant was measured by a Western blot and an ELISA using BSA as antigen. There was no useful signal in either of the assays.

Biotinylation of antibodies is a common technique used to amplify the signal in immunological analysis by taking advantage of the high affinity of biotin for the multivalent binding sites of streptavidin and the wide variety of streptavidin conjugates available for detection (72,73). SF1, SF2, and anti-BSA scFv antibody molecules were biotinylated *in vivo* by cloning the respective genes into pAC5 Avitag vector and expressed in *E. coli* AVB100. scFvs were harvested from periplasm and culture supernatant and were tested for their activity by ELISA. There was no activity with the biotinylated or unmodified SF1 and SF2 harvested from either the periplasm or culture supernatant (Table 3-8). The biotinylated anti-BSA scFv gave a lower signal than unmodified scFv, the absorbance values being 1.08 and 1.42, respectively with the antibodies harvested from culture supernatant, 1.31 and 1.67, respectively with the antibodies from periplasmic extract. However, the amount of protein in each sample was not quantified, making the comparison of activities inaccurate. These experiments showed that scFv antibodies can be biotinylated easily without the loss of activity.

Future Directions

Studies are being done in our lab to isolate phage antibodies to whole cell epitopes of *S.* Typhimurium by panning on whole cells or purified LPS. Two strategies are being used to isolate antibodies to LPS. One of the strategies being followed is to conjugate the O antigen portion of the LPS molecule to a carrier molecule such as BSA for use as antigen in panning. The rationale behind this approach is that purified LPS being a hydrophobic molecule might not be sticking in enough amounts to immunotubes for serving as the antigen for panning. By conjugating the O antigen to a carrier protein such as BSA, it is possible that the antigen could be coated on immunotubes in adequate amounts. Another strategy is to obtain a new version of Tomlinson library that is obtained by superinfecting the original bacterial library with Hyperphage instead of KM13 helper phage. When phage were obtained from the original library for the first time, KM13 helper phage was used for superinfection. The antibodies thus obtained might be displaying wild type pIII and scFv-pIII molecules, instead of only scFv-pIII molecules (65). When such phage were used for panning, the phage specifically recognizing LPS might be washed away in the first round of panning because of the low affinity of anti-carbohydrate antibodies. However, in theory if the phage were obtained with Hyperphage, all copies of pIII would be scFv-pIII. Avidity effects could play a role in increasing the binding of the antibodies to the antigen and hence an improved chance of isolating anti-carbohydrate antibodies, as shown by MacKenzie et al (68).

Incubating biotinylated scFv antibodies with streptavidin could yield a tetrameric form of the antibody, as each streptavidin can bind to four biotin molecules (71). Multivalency of the antibody could be useful with anti-carbohydrate antibodies with intrinsic low affinities (65). When streptavidin conjugates are used for forming such a

tetrameric complex, the same complex can be used both as primary and secondary antibody.

A biosensor system that can detect microbial contamination in food and water and on surfaces is of practical and economic importance. This thesis describes the efforts done towards developing such a system for detecting *S.* Typhimurium. Commercially available murine monoclonal antibodies were screened for their potential usefulness in the biosensor system. Recombinant phage display antibodies that recognize flagellar protein of *S.* Typhimurium were isolated. Although the phage display antibodies we isolated were not very useful in recognizing whole cells, the general methods developed for panning and isolating antibodies could be used to isolate antibodies with greater affinities and higher potencies. Alternatively, the isolated anti-flagellar antibodies could be modified genetically to increase their affinity.

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BIOGRAPHICAL SKETCH

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