DEVELOPMENT AND STUDY OF PHAGE-DERIVED DETECTION PROBES

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DEVELOPMENT AND STUDY OF PHAGE-DERIVED DETECTION PROBES

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VITA

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DISSERTATION ABSTRACT

DEVELOPMENT AND STUDY OF PHAGE-DERIVED DETECTION PROBES

Jennifer Brigati

Doctor of Philosophy, May 13, 2005
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Directed by Valery A. Petrenko

The threat of bioterrorism has created a demand for continuous monitoring of the environment for threat agents. Existing monitoring systems use antibody-derived detection probes which are not hardy enough to withstand long-term use in harsh environments. Alternative phage-derived probes have been shown to function as robust and stable substitutes for antibodies in a variety of platforms. I describe here the study and development of phage-derived probes for potential use in detection of the threat agent *Bacillus anthracis*.

Naturally occurring phages are resistant to heat, organic solvents, and proteases. We hypothesized that landscape phage probes inherit the thermostability of their parental phage. The stability of a phage probe and a monoclonal antibody that bind to β-

galactosidase was examined at 25 °C to 76 °C. The phage probe was found to be more stable than the antibody at 37 °C and above, and had a half life of ~2.5 years at 37 °C. The activation energy (the amount of energy required to ensure that the reaction occurs) of phage degradation was found to be 31,987 cal/mol.

Feasibility of identifying landscape phage probes that could be used to detect threat agents was demonstrated using *B. anthracis* Sterne spores as a model. It was hypothesized that landscape phage clones that bind to *B. anthracis* spores could be selected from a landscape phage display library in biased and non-biased selection procedures. Several phage clones that bound to *B. anthracis* spores in a highly specific and moderately selective manner were identified in the non-biased procedure; the most selective spore-binding phage displayed several thousand copies of the peptide EPRLSPHS, and bound from 3.5- to 70- fold better to *B. anthracis* than to spores of other *Bacillus* species. The use of biased selection procedures to identify new probes, or the modification of the surface of existing probes may allow the development of highly specific and selective robust probes suitable for long term use in continuous monitoring devices and biosorbents.

The evolution of the landscape phage display library f8/8 was examined during biased selection procedures. It was hypothesized that examining the evolution of the library during selection might reveal predictors of successful isolation of target-binding clones, and offer clues about which clones are the strongest target binders. No reliable predictor of target binding ability was found, but characteristics of a successful selection procedure were identified.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1. Introduction

Recent incidents of bioterrorism have highlighted the need for durable, rapid, accurate, and inexpensive methods for monitoring the environment for the presence of biological threat agents, such as spores of *Bacillus anthracis,* which have been a threat agent of special concern since their distribution through the US postal service in 2001. At the time of the attack no monitoring systems were in place, so the presence of *B. anthracis* spores was only suspected after the initial victims were diagnosed with anthrax.

In the years since 2001, numerous assays have been developed to detect *B. anthracis* spores. Many of these tests are immunoassay-based and rely on antibodies for recognition of spores. While monoclonal antibodies are able to accurately differentiate between *B. anthracis* spores and spores of other bacillus species, they are not completely ideal probes for use in environmental monitoring. Monoclonal antibodies are expensive to produce and can be unstable in environmental conditions. They are also limited in that they can only be produced against antigens to which animals have a strong immune response.

Recently, an alternative type of probe has been proposed for use as a substitute for antibodies in immunoassays: landscape phage. Landscape phages are filamentous bacteriophages that express foreign peptides fused to their major coat protein. An individual phage expresses thousands of copies of the foreign peptide in a constrained conformation on its surface, creating a "landscape" on the surface of the phage. These phage can be used in their entirety as probes in platforms where antibodies have traditionally been used.

To justify replacement of antibodies with phage probes in detection platforms, it was necessary to demonstrate that phage probes can bind specifically to *B. anthracis* and are more stable than antibodies under environmental conditions. We hypothesized that landscape phage are resistant to degradation at high temperatures. We also hypothesized that *B. anthracis*-specific probes could be identified through standard and biased selection procedures.

The first chapter of this dissertation will review current knowledge about *B. anthracis* spores, current methods for detection and identification of *B. anthracis* spores, and ways in which current detection methods could be improved. Also in the first chapter will be a brief description of phage biology and phage display, and a more in-depth look at how phage may help to solve some of the problems with current threat agent detection methods.

The second chapter of this dissertation describes experiments that were performed to determine the resistance of phage to degradation when stored at high temperatures, and to compare the stability of phage probes to the stability of antibodies. Specific aims included:

I. Determine if phage probes resist degradation at high temperatures in comparison to antibodies

II. Calculate the degradation constants of a recombinant phage probe at temperatures ranging from 25°C to 76°C and create an Arrhenius plot for this phage

The third chapter of this dissertation describes the selection of landscape phage clones that bind to *B. anthracis* Sterne spores in a non-biased selection procedure. Specific aims included:

I. Identify phage clones from a landscape phage display library that bind specifically to *B. anthracis* Sterne spores.

II. Determine which of the identified clones cross-reacts the least with spores of other *Bacillus* species.

The fourth chapter of this dissertation describes the selection of landscape phage clones that bind to *B. anthracis* Sterne spores through biased selection procedures in which the library was depleted of *B. cereus*, *B. subtilis* and *B. thuringiensis* binders prior to selection. This chapter includes an in-depth analysis of the changes which occurred in the phage display library during selection. Specific aims included:

I. Identify new phage clones from a landscape phage display library that bind specifically to *B. anthracis* Sterne spores.

II. Study the selection process in depth, monitoring the diversity of the library following each round of selection.

2. B. anthracis

2.1. General description of bacteria & disease

B. anthracis, the causative agent of anthrax, is an aerobic, gram-positive, spore forming bacillus. Vegetative *B. anthracis* cells are 1-1.5 μm x 3-10 μm in size, may occur singly or in chains, and are typically nonmotile (Sneath, 1984). These cells are encapsulated by a poly- γ- D – glutamyl capsule (Sneath, 1984). Vegetative cells cannot survive outside a host for extended periods of time (>24 hours) and are not capable of establishing an infection in a new host (Inglesby et al., 2002). *B. anthracis* spores are formed when anthrax-infected body fluids are exposed to air, and local nutrient resources are depleted (Inglesby et al., 2002). Endospores form in a central position within the mother cell, and are elliptical in shape (Sneath, 1984). These spores are extremely hardy and can survive for decades despite harsh environmental conditions.

Virtually all mammals, including humans, are susceptible to anthrax. Vegetative *B. anthracis* cells cannot initiate disease, and no examples of live animal to live animal transmission have been reported (Hanna and Ireland, 1999). Infection is initiated by the entry of *B. anthracis* spores into the host through a skin abrasion, an insect bite, consumption of contaminated food or inhalation of airborne particles. Regardless of the route of infection, spores are taken up by macrophages where they germinate and are transported to regional lymph nodes (Mock and Fouet, 2001). The course of the disease is dictated by the route of infection, and may present as one of three forms; cutaneous anthrax, gastrointestinal anthrax, or inhalational anthrax.

Cutaneous anthrax occurs when spores enter the body through an abrasion, cut or insect bite. A small pimple will usually appear on the skin within 3 days, and a black eschar will form a few days later. If bacilli remain localized, the eschar will resolve irrespective of treatment within 6 weeks, but in ~20% of patients septicemia and death will occur (Spencer, 2003).

Gastrointestinal anthrax occurs when *B. anthracis* spores are ingested. Eschars form along the GI tract after a short 2-5 day incubation period, and frequently septicemia and death occur before a diagnosis can be made (Erickson and Kornacki, 2003).

Inhalational anthrax develops when aerosolized *B. anthracis* spores enter the lungs of a host. Symptoms develop 2 days to 6 weeks after inhalation of spores. The illness begins with a mild flu-like phase, which then rapidly progresses to an acute illness and without treatment typically results in death (Spencer, 2003).

2.2. Significance as a biological weapon

Biological warfare techniques have been used since before the true mechanisms of disease spread were fully understood. In the 14th century, armies catapulted cadavers of persons who died from plague into an enemy city, initiating a plague epidemic and causing opposing forces to retreat (Christopher et al., 1997). When specific microorganisms were identified as the causative agents of infectious diseases, more advanced biological warfare techniques were rapidly developed.

B. anthracis spores have long been recognized as a biological warfare agent because of their hardiness and ability to cause mortality in humans and animals. Germany used *B. anthracis* to fight the allied forces during World War I; they infected

sheep and other livestock with *B. anthracis* for export to allied forces. The Japanese conducted research on the use of *B. anthracis* as a weapon from 1932-1945, performing experiments on prisoners, and attacking Chinese cities by contaminating food and water. Allied forces also experimented with weaponized spores of *B. anthracis*, studying dispersion of spores delivered by bombs. The United States also studied *B. anthracis* in its offensive biological program, producing 5000 bombs filled with spores during World War II (reviewed in Christopher et al., 1997).

Modern biological threats seem less likely to come in a bomb, and more likely to be silently released. In this manner, the agent can be spread and infect hundreds, or even hundreds of thousands of people before it is detected. *B. anthracis* is ideal for this because the hardy spores can be lyophilized and spread in a powder, or aerosolized and spread through a ventilation system or by a crop duster. An accidental release of aerosolized *B. anthracis* spores in Sverdlovsk, USSR in 1979, resulted in 96 cases of human anthrax and numerous cases of anthrax in livestock in a narrow geographical region downwind from the source of the spores (Meselson et al., 1994). It has been estimated that the release of 50–100 Kg of *B. anthracis* spores over an urban population would result in 250,000 to 3 million casualties, many of whom would die without treatment (reviewed in Inglesby et al., 1999).

B. anthracis spores were used for bioterrorism against the United States in October of 2001. The spores were delivered to victims in a powder enclosed in letters sent through the US postal system. Most of those infected were postal workers who were presumably exposed by handling the letters or by breathing in aerosols created by mail-sorting equipment. Other victims worked in the offices where the letters were delivered

or had an unknown route of exposure (Jernigan et al., 2001). All together, twenty-two confirmed or suspected cases of anthrax occurred as a result of the attack (Inglesby et al., 2002).

2.3. Genetics and comparison to other Bacillus sp.

B. anthracis is part of the *B. cereus* group of bacilli, which also contains *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, and *B. medusa* (Lechner et al., 1998; Nakamura, 1998; Turnbull, 1999). *B. anthracis* is physiologically and genetically very similar to other group members *B. cereus* and *B. thuringiensis*. It has even been argued that these three species of bacteria should really be considered one species (Helgason et al., 2000). *B. anthracis* is believed to have evolved from *B. cereus* by the acquisition of virulence plasmids pXO1 and pXO2, and multiple chromosomal changes (reviewed in Jensen et al., 2003).

Plasmid pXO1 carries the genes *pag*, *lef*, and *cya*, which encode the toxin proteins protective antigen (PA), lethal factor (LF) and edema factor (EF). This plasmid also encodes trans-acting regulator genes *atxA* and *atxR* that regulate the transcription of the toxin protein genes, as well as a number of other unrelated genes. Plasmid pXO2 carries the genes required for synthesis of the poly-γ-D-glutamic acid capsule of vegetative cells, *capA*, *capB*, *capC*, *dep*, and *acpA,* as well as a number of other genes. The presence of both plasmids is necessary for virulence of *B. anthracis* in most hosts, although some mice are sensitive to $pXO1^{-}/pXO2^{+}$ and $pXO1^{+}/pXO2^{-}$ strains (reviewed in Little and Ivins, 1999).

While these plasmid-encoded genes are unique to *B. anthracis*, they are not considered ideal for detection purposes because *B. anthracis* strains exist which lack both of these plasmids. While none of the known $pXO1^-/pXO2^-$ strains are virulent, there is concern that a virulent strain could be engineered. Ideally, both chromosomal and plasmid sequences unique to *B. anthracis* should be identified for use in detection of spores.

The similarities between *B. anthracis* and its close relatives are troublesome when developing assays for detection of *B. anthracis* because both *B. thuringiensis* and *B. cereus* are commonly found in the environment. *B. thuringiensis* has been isolated worldwide from soil, insects, stored-product dust, and deciduous and coniferous leaves (reviewed by (Schnepf et al., 1998). *B. cereus* is ubiquitous in soil and has been isolated as a contaminant of various food products (reviewed by (Drobniewski, 1993).

Differentiation of *B. anthracis* from its close relatives is extremely important during a bioterrorist attack to prevent inappropriate antibiotic use and avoid costly evacuation of buildings that have not actually been contaminated. During the attack in 2001, two unclassified *Bacillus* species that met initial microbiological criteria for *B. anthracis* were identified (Bottone, 2003). These bacteria were encapsulated and susceptible to penicillin, and formed nonhemolytic colonies. They were only determined not to be *B. anthracis* strains because they were resistant to lysis by γ-phage. Unfortunately, the phage-lysis assay is typically only performed at state laboratories, leaving a gap of several days between initial identification and confirmation of identification. Clearly, a better system is needed to make a rapid and definitive identification of unknown Bacilli.

2.4. Spore structure

Bacillus species form spores when exponentially growing vegetative cells exhaust nutrients such as carbon, nitrogen, or phosphorus (Nicholson, 1990). While spores are generally dormant, existing as a vehicle to preserve DNA, they can undergo some small morphological changes when exposed to different environmental conditions (Driks, 2003). Spores are extremely resistant to wet and dry heat, dessication, UV radiation, and a wide variety of toxic chemicals (reviewed in Nicholson et al., 2000). The structure of *Bacillus* spores has been extensively studied in recent years because the spore is the infectious form of *B. anthracis*.

Spores of *Bacillus* species have three to four major layers; the core, which houses the DNA complexed with small acid soluble proteins; the cortex, which contains peptidoglycan structures; the coat, which contains highly crosslinked proteins; and in some species the exosporium, which contains polysaccharides, lipids and proteins (reviewed in Jedrzejas, 2002; Driks, 2003). The exosporium is a multilayered shell that surrounds the spore coat, but is not directly connected to it. It may have filamentous, hair-like or pilus-like structures associated with it (reviewed in Charlton et al., 1999; Driks, 1999). In species that have an exosporium, it is the outer-most layer of the spore and therefore the layer most likely to interact with a host or be recognized by a detection device.

B. anthracis and its close relatives *B. cereus* and *B. thuringiensis* all have exosporia, but there are differences in the carbohydrate and glycoprotein composition of their spores. Rhamnose, 3-*O*-methyl rhamnose and galactosamine have been found in the

spores of all three, but fucose and 2-*O*-methyl rhamnose were found only in *B. cereus and B. thuringiensis* spores (reviewed in Fox et al., 2003). A novel tetrasaccharide named anthrose is found in the exosporia of *B. anthracis* but not *B. cereus* or *B. thurinigiensis* (Daubenspeck et al., 2004).

Exosporia of *B. anthracis*, *B. cereus* and *B. thuringiensis* have all been found to contain glycoproteins. The major glycoprotein present in the *B. anthracis* exosporium is a 382 amino acid protein named BclA (Sylvestre et al., 2002). BclA was found to be present in the hair-like projections (filaments) of the exosporium (Sylvestre et al., 2002). BclA has a repetitive collagen-like region that can vary in length between strains of *B. anthracis*; the length of this region is directly proportional to the length of the filaments seen on the surface of spores (Sylvestre et al., 2003). The hair-like projections, or filaments, of the exosporium are the outermost structure of *B. anthracis* spores, making them a potential target for identification and even possibly differentiation of strains. While the major glycoproteins of *B. thuringiensis* and *B. cereus* have been identified and are different from BclA (Garcia-Patrone and Tandecarz, 1995; Todd et al., 2003), there is some evidence that BclA or a BclA homologue is present in the *B. cereus* exosporium (Todd et al., 2003). BclA was found to be the immunodominant moiety of the *B. anthracis* exosporium, as the majority of monoclonal antibodies raised against *B. anthracis* bound to BclA (Steichen et al., 2003). Monoclonal antibodies against spores of *B. cereus* and *B. thuringiensis* do not appear to react with BclA, indicating that if BclA is present in these species, it is not a major constituent of the exosporium (Steichen et al., 2003).

In addition to BclA, 136 proteins have been identified as associated with the *B. anthracis* exosporium (Liu et al., 2004). While many of these proteins have not yet been fully described, a number of them have been studied and their presence in the exosporium confirmed. Proteins known to be associated with the exosporium of *B. anthracis* include BxpA, BxpB (ExsF), CotY, ExsY, CotB, ExsK, alanine racemase, inosine hydrolase, and superoxide dismutase (Steichen et al., 2003; Redmond et al., 2004). It is important to note, however, that most of these characterized proteins have homologues in the *B. cereus* exosporium, so they may not be good targets for unambiguous identification of *B. anthracis*.

The coat proteins of *B. anthracis* spores, while generally hidden under the exosporium, may also be important in detection of the organism. In an electron microscopy study of *B. anthracis* spore structure, spores were found to exist in two different morphological classes; spores with intact exosporia, and spores that appeared to have lost their exosporia during culturing or preparation for microscopy (Chada et al., 2003). *B. anthracis* spores without exosporia are just as infectious as those with exosporia, so their detection is also important. The *B. anthracis* coat has been found to contain many proteins with high homology to proteins of *B. subtilis*, a distant relative (Lai et al., 2003). However, it is expected that each of these species also has unique spore proteins (Lai et al., 2003) that could be used as targets for detection. *B. anthracis* spores without exosporia were found to contain 744 distinct proteins from a wide variety of functional categories, indicating great potential for identification of targets unique to this species (Liu et al., 2004). One *B. anthracis* spore coat protein, designated Cotά, was found to be shared only with close relatives *B. cereus* and *B. thuringiensis* (Kim et al.,

2004). While this protein is not a suitable target for detection, the lack of a homologue in more distant relatives gives hope that some *B. anthracis*–specific coat proteins may be identified.

While rapid progress is being made in the identification and characterization of proteins in the *B. anthracis* exosporium and spore coat, complete analysis will be very time consuming. While the identification of unique, species-specific proteins exposed on the surface of the *B. anthracis* spore is anxiously anticipated, assays for the detection and identification of *B. anthracis* must be developed now without such information.

3. Detection of *B. anthracis*

3.1. Introduction

Prior to the attacks in 2001, little effort was put forth to develop rapid, specific and sensitive assays for the detection and identification of *B. anthracis* spores. In 2001, no monitoring systems were in place to detect *B. anthracis* spores in the environment, so the first sign of the attack was when a victim arrived at a hospital with anthrax. Traditional microbiological methods were used to diagnose the initial victims, with more advanced tests being used only for confirmation and study of the spores. In the time that has elapsed since the 2001 attacks, a large number of new methods for the detection of *B. anthracis* spores have been developed, including rapid immunoassay and nucleic acid-based detection systems. Prototype continuous monitoring systems have been developed using these new technologies and are under trial use in a few selected government buildings (Larkin, 2003; Meehan et al., 2004). However, since use of monitoring systems

is still limited, if another attack were to occur today detection and identification would still most likely occur only upon the appearance of an infected person at a hospital, and would still rely on traditional microbiological methods.

3.2. Traditional identification/detection methods

At this time, there are no systems in widespread use to continuously monitor the environment for the presence of threat agents, so the first sign of an attack is the presentation of an infected patient. The time between exposure to *B. anthracis* spores and development of anthrax may vary between 2 and 43 days (Meselson et al., 1994). The currently accepted method for identification of *B. anthracis* as published by the Laboratory Response Network is based entirely on traditional microbiological methods (http://www.bt.cdc.gov/agent/anthrax/index.asp). A sample is presumed to contain *B. anthracis* if the following criteria are met: direct smears from clinical samples reveal encapsulated gram-positive rods, bacteria isolated from growth on sheep blood agar (SBA) are large gram positive rods, colonies grow rapidly, aerobically, and cling tenaciously on SBA, bacteria are catalase positive, bacteria are non-motile, and bacteria grown on SBA are nonhemolytic and form colonies with a ground-glass appearance. These procedures take a minimum of 24-48 h to complete, making the total time between spore release and detection of the attack at least three days. These procedures can also lead to false positive or negative identifications when dealing with atypical *B. anthracis* strains or strains of other *Bacillus* species (Papaparaskevas et al., 2004).

A phage sensitivity test is commonly used for confirmation of *B. anthracis* identification, but is currently only performed at a limited number of laboratories in the

United States. In this assay, a nutrient agar plate is spread with bacteria from an overnight broth culture, then a loopful of the *B. anthracis*–specific γ-phage is applied to the center of the plate. If the phage application results in an area devoid of bacterial growth, then the bacteria are confirmed to be *B. anthracis* (Logan et al., 1985). However, even this test can yield false positive results because some rare forms of *B. cereus* are susceptible to lysis by γ-phage (Turnbull, 1999).

To reduce the risk of death from anthrax following exposure to *B. anthracis* spores, early detection is critical. Screening of a portion of blood from blood drives for detection of bioterror agents has been proposed, but after careful evaluation was found to be too expensive to be practical and too slow to prevent deaths (Kaplan, 2003). Clearly, environmental monitoring for threat agents in high risk areas is essential to reduce the susceptibility of the US population to bioterrorism.

3.3 Rapid detection methods

3.3.1 Mass spectrometry methods

Mass spectrometry is based on the principle that all proteins can be broken down into component amino acids that yield a complex mass spectrum that contains species-specific patterns. Several groups have recently reported the use of mass spectroscopy for identification of *B. anthracis* spores and differentiation of these spores from those of close relatives *B. cereus* and *B. thuringiensis*. Some assays have focused on detection of dipicolinic acid (DPA), a compound found in spore coats of multiple species (Beverly et al., 1996; White et al., 2002). While these are useful for rapid detection of spores, they

are not specific for pathogenic species and could cause costly false alarms. Other assays have focused on the identification of species- and strain-specific biomarkers of *B. anthracis* spores (Elhanany et al., 2001) and vegetative cells (Krishnamurthy et al., 1996), or spores of *B. anthracis* simulates such as *B. atrophaeus* (Fergenson et al., 2004).

While species- and strain-specific biomarkers have been identified in several of these assays, mass spectroscopy is still far from ready for use in environmental monitoring. A variety of technical difficulties, including difficulties reproducing data, interference caused by contaminating atmospheric particles, and differences in results depending on the growth conditions of the target organisms, have thus far limited its application (Fergenson et al., 2004). While mass spectroscopy methods show great potential for rapid identification of biological threats, it will likely be many years before they can achieve the sensitivity and specificity needed for monitoring. Immunoassays and nucleic acid-based detection techniques will have to be relied upon for the first generation of environmental monitoring devices.

3.3.2. Immunoassay detection methods

Immunoassays are a group of antigen recognition methods that rely on the detection of the specific binding of a probe to an antigen. They have developed rapidly since the first radioimmunoassay for detection of insulin was developed in 1959 (reviewed in Andreotti et al., 2003). Immunoassays typically rely on poly- or monoclonal antibodies as probes, but have also been developed using peptides and aptamers (reviewed in Andreotti et al., 2003). Immunoassays adapted for detection of

biological threat agents have included direct fluorescent antibody assays (De et al., 2002), biosensor-based assays (Branch and Brozik, 2004), immunochromatographic lateral flow assays, enzyme-linked immunofluorescent assays (ELISA), time resolved fluorescence assays (TRF), immunomagnetic separation-electrochemiluminescent assays (reviewed in Peruski and Peruski, 2003), and flow cytometric assays (Alvarez-Barrientos et al., 2000; Stopa, 2000)

Direct fluorescent assays are the simplest immunoassays, in which a fluor-labeled antibody is incubated with target antigen, excess antibody is washed away, and antigen-antibody complexes are visualized by fluorescent microscopy. A two component direct fluorescent-antibody assay was developed for the identification of *B. anthracis* vegetative cells in 2002 (De et al., 2002). This assay could detect more than 99% of *B. anthracis* isolates tested as vegetative cells by labeling with antibodies targeting two different vegetative cell surface antigens. While the assay took only 3-6 hours, the bacteria had to be grown as vegetative cells prior to evaluation, greatly increasing the total assay time.

A biosensor-based immunoassay was recently developed for the detection of *Bacillus* spores (Branch and Brozik, 2004). The biosensor used in this assay was a love wave (surface acoustic wave) sensor that detects changes in mass on its sensing surface. The sensor was coated with an antibody with a high affinity for *B. anthracis* spores, and sensitivity was demonstrated using *B. anthracis* simulate *B. thuringiensis* B8.

A number of immunochromatographic lateral flow assays for the detection of *B. anthracis* spores are commercially available. Lateral flow assays consist of a labeled antibody on a pad at the tip of a wicking membrane, and a capture antibody in a line further down the membrane. Samples are applied to the pad containing the labeled

antibody, then antibody-antigen complexes are wicked down the membrane until they bind to the capture antibody, where they form a visible line. An independent laboratory tested the sensitivity and accuracy of three of the commercially available assays and found that the Biowarfare Agent Detection Devices (Osborne Scientific, Lakeside, AZ), and Anthrax SMART II (New Horizons Diagnostics, Columbia, Md) kits were able to consistently detect 10^5 *B. anthracis* spores within 5 minutes (King et al., 2003). A third kit, Anthrax Bio-Threat Alert (Tetracore, Gaithersburg, MD) could only consistently detect 10^6 spores. None of the kits mistakenly identified *B. cereus* as *B. anthracis*, but SMART II did give a positive result when tested with *B. thuringiensis*.

Traditional sandwich ELISAs are performed by adsorbing a target-specific antibody to a surface, allowing the target antigen to bind, and detecting the bound antigen with a second enzyme-linked detector. Traditional ELISAs are not very useful for detection of threat agents because they require cumbersome plate washers and readers. One ELISA developed for detection of *Bacillus* spores uses a miniaturized biochip detection system that makes the assay portable for field use (Stratis-Cullum et al., 2003). The potential of this assay for field use was proven by its ability to detect as few as 100 *B. globigii* spores collected through a portable bioaerosol sampler. Unfortunately, this assay still requires several long incubation steps, making it less than ideal for monitoring uses. A rapid ELISA for detection of antibodies against the anthrax toxin component PA was recently approved by the FDA for use in confirmation of anthrax infection in patients. The test can be completed in less than 1 hour and has a less than 1% chance of giving a false positive reading (Stephenson, 2004). This test however is only useful for detection of antibodies produced by infected patients, not for detection of spores.

ELISAs are not the only sandwich-style immunoassays used for detection of threat agents. A microarray assay was created, in which a flow module was attached to the surface of a slide covered with capture antibodies. Samples containing spores (or other agents) were forced through the flow module, followed by wash buffer and then fluorescent-labeled detection antibodies. This format permitted detection of *B. globigii* spores at a concentration of 10^4 cfu/ml in just 15 minutes (Delehanty and Ligler, 2002).

TRF assays are also similar to ELISAs in their set-up, but they use lanthanide chelate-labeled detector antibodies with very long fluorescent decay times for detection of bound antibody. In a comparison of TRF assays with traditional ELISAs for detection of biological threat agents, it was found that TRF assays were significantly more sensitive, with improvements in detection limits ranging from 3-fold to 2000-fold (Peruski et al., 2002).

Several immunomagnetic electrochemiluminescence (ECL) – based threat agent detection assays have been developed in recent years (reviewed in Yu et al., 2000). In these assays, paramagnetic beads coated with spore-specific antibodies are used to capture and concentrate spores. Captured spores are then detected with a ruthenium (II) trisbipyridal chelate–labeled antibody. An assay based on concentration of *B. anthracis* spores by incubation with paramagnetic beads coated with *B. anthracis*-specific antibodies was first demonstrated in 1995 (Gatto-Menking et al., 1995). In this assay, as few as 100 spores could be detected in 50 µl of PBS. The assay did not translate well to environmental samples however, and the detection limit of spores in a soil suspension was ~10^5 spores in a 65 µl sample (Bruno, 1996). A slightly different version of this assay was developed in 1999, in which short DNA sequences (aptamers) capable of

binding to *B. anthracis* spores replaced the antibodies on the magnetic beads (Bruno and Kiel, 1999). Detection was achieved by binding captured spores with biotinylated aptamers, and then adding streptavidin–linked $Ru(bpy)^{2}{}_{3}{}^{+}$. This assay was able to detect as few as 10 spores in 250 µl of buffer. An ECL assay was also incorporated into the Automated Biological Agent Testing System: a laboratory based system developed to analyze up to 300 environmental samples per day for the presence of threat agents (Byrne et al., 2003).

Flow cytometry is a method of counting, sorting, and characterizing particles (cells, bacteria, etc.) labeled with fluorescent molecules. A flow cytometer can differentiate particles based on their size, granularity, and fluorescence. Typical flow cytometric assays involve several binding and washing steps, requiring several hours for sample preparation. Rapid protocols have been developed, however, that eliminate the need for these washes. An assay was developed for the detection of *B. anthracis* that could detect as few as 10^3 spores in less than 10 minutes (Stopa, 2000). However, use of this particular assay for detection was limited because of inability to differentiate between *B. anthracis* and close relatives *B.cereus* and *B. thuringiensis*. Fortunately, further research allowed the development of a fast and specific flow cytometric assay for the detection of *B. anthracis* spores.

The first autonomous continuous monitoring device created for the detection of *B. anthracis* and other threat agents, reported in 2003, relied on flow cytometry for the detection and identification of target bacteria and toxins (McBride, 2003). The monitoring device mixes particles collected by an aerosol collector with antibody-coated fluorescent microbeads; detector antibodies labeled with phycoerythrin are then added,

and the complexes are analyzed with a Luminex Lx100 flow cytometer. The system could detect a number of different threat agents at once, because microbeads with different fluorescent properties were coated with different antibodies, allowing simultaneous classification of each bead's target (indicated by bead fluorescence) and detection of bound target (indicated by phycoerythrin). The next version of this device was improved by adding washing steps and using a two-step detection procedure (biotinylated detector antibody followed by streptavidin-phycoerythrin) to reduce background fluorescence and increase specific antigen binding (Hindson et al., 2004). Washing was accomplished without compromising the speed and autonomy of the assay by using a membrane to contain the beads while allowing buffers to pass through. The limit of detection of this system is between 10^5 and 10^6 spores/mL.

Another approach used to reduce the cross reactivity seen in flow cytometric assays without adding lengthy washing steps exploits frequency resonance energy transfer (FRET). In FRET, antibodies to the same target organism are labeled with two different fluorophores, one which has an emission wavelength close to the excitation wavelength of the other. In FRET, when antibodies carrying the two different fluorophores bind close to one another, and the donor fluorophore is excited, the donor fluorophore is quenched and the emission fluorescence of the acceptor fluorophore is detected. In a FRET assay developed with polyclonal antibodies specific for *B. anthracis*, selectivity was improved 10-fold with respect to *B. thuringiensis* and 100-fold with respect to *B. subtilis* when compared to a single fluorophore assay (Zahavy et al., 2003).

In 2003, a very unique immunoassay was developed in which B lymphocytes carrying membrane-bound antibodies specific for a target pathogen were engineered to express a bioluminescent protein upon binding of their target (Rider et al., 2003). B lymphocytes specific for agents such as *B. anthracis* were engineered to express a calcium-sensitive luminescent protein. Crosslinking of membrane-bound antibodies leads to a rise in intracellular calcium levels, so the B lymphocytes would become luminescent when they came in contact with target antigen. Using these cells, it was possible to detect 1000 spores in a 1 mL sample in about 5 minutes.

Improvements in traditional immunoassays and the development of new cutting edge technologies have greatly enhanced the prospect of using immunoassays for the detection of *B. anthracis* spores. While many of these immunoassays show great promise and have been incorporated into commercially available test kits and prototype continuous monitoring systems, they are not without difficulties. The use of antibodies as probes in the majority of these assays is problematic because antibodies are not very stable in environmental conditions, making the shelf life of these assays short. Natural antibodies are also problematic because they typically only recognize one or a few immunodominant proteins on the surface of the spore, making it possible for a minor modification of a spore surface protein to make spores undetectable. New types of probes that are stable and bind to a variety of spore surface antigens are needed to circumvent these problems and improve current immunoassays. Ideally, improved immunoassays will be used in combination with nucleic acid-based detection methods to increase sensitivity and decrease the possibility of misidentification of non-pathogenic spores.

3.3.3. Nucleic acid detection methods

Recent advances in polymerase chain reaction (PCR) and microarray technology, as well as microelectromechanical systems and microfluidics, have driven the development of systems designed to detect threat agents based on their nucleic acid sequences (Ivnitski, 2003). The field has also been advanced by the availability of complete genome sequences for most of the bacterial threat agents identified by the Centers for Disease Control and Prevention (Fraser, 2004). Nucleic acid based detection methods attempt to identify *B. anthracis,* and differentiate between this pathogen and its close relatives, by targeting unique areas of the *B. anthracis* genome. While *B. anthracis*, *B. cereus*, and *B. thuringiensis* are genetically similar, differences do exist that can be used to reliably distinguish the species. In one study, 93 DNA sequences were found to be present in *B. anthracis* but absent from other *Bacillus* species (Radnedge et al., 2003).

Many of the assays developed for *B. anthracis* detection rely on amplification of species-specific gene sequences through PCR. The high throughput Automated Biological Agent Testing System, mentioned previously, uses real time PCR in addition to an immunoassay for identification of threat agents (Byrne et al., 2003). A portable real time PCR assay suitable for use in the field has been developed using the handheld advanced nucleic acid analyzer (HANAA)(Higgins et al., 2003). Real time PCR confirmation of *B. anthracis* detection was also incorporated into the first autonomous pathogen detection system developed in 2003 (McBride, 2003).

A number of different chromosomal and extrachromosomal regions of the *B. anthracis* genome have been used as targets for PCR amplification. The 16s rRNA gene

was one of the first targets identified as a possible region of variability between *B. anthracis* and other closely related *Bacillus* species. In 2002, the 16S rRNA genes of 86 *B. anthracis* isolates, as well as 10 *B. cereus* and 11 *B. thuringiensis* strains were sequenced (Sacchi et al., 2002). All of the *B. anthracis* strains tested had identical 16S rRNA gene sequences, which differed by at least 1 base pair from the sequences of the *B. cereus* and *B. thuringiensis* strains examined. This established the 16s rRNA gene as a target for *B. anthracis* identification, in spite of the knowledge that some *B. anthracis* 16S rRNA gene sequences found on Genbank were identical to those in *B. cereus*, and not to the gene sequence identified in the 86 *B. anthracis* strains examined by this group. A fluorescent heteroduplex assay using fluorescent DNA probes derived from the *B. anthracis* 16s rRNA gene was developed (Merrill et al., 2003). This assay could separate bacteria from the *B. cereus* group into two subgroups, but could not unambiguously differentiate between *B. anthracis* and its close relatives. This work indicated that the 16S rRNA gene is not an ideal target for detection or identification of *B. anthracis*. This was later confirmed in a study that found 100% homology between *B. anthracis* and *B.cereus* across the entire 16s rRNA gene (Blackwood et al., 2004).

Other target genes have been identified that allow unambiguous differentiation of *B. anthracis* from its close relatives (Ramisse et al., 1996; Fasanella et al., 2001; Qi et al., 2001). One of these targets is the *rpoB* gene, which encodes the beta subunit of RNA polymerase. A real-time PCR assay for identification of *B. anthracis* spores was developed using a primer-probe set based on the *rpoB* gene and the *lef* gene, which is the lethal factor gene carried on pXO1 (Oggioni et al., 2002). This assay could differentiate between *B. anthracis* Sterne and wildtype *B. anthracis* isolates, as well as between *B.*

anthracis and *B. cereus*. It was also found that primer-probe sets against *rpoB* alone could be used to identify *B. anthracis*, without risk of cross reactivity with *B. subtilis* or *B. cereus* (Drago et al., 2002), or *B. thuringiensis* (Ko et al., 2003).

Other targets of PCR-based assays have included; the *cap* genes, which are needed for encapsulation, and the *pag* gene, which encodes a component of the protective antigen (Cheun et al., 2003; Makino and Cheun, 2003); the gene *acpA,* which encodes the trans-activator of encapsulation, and the chromosomal region Ba813 (Levi et al., 2003); a 279bp fragment of *tlf*, the toxin lethal factor (Breadmore et al., 2003); the gene *vrrA*, which is presumed to encode a 30kDa protein (Andersen et al., 1996); and the *capA* gene, which encodes a capsular protein (Higgins et al., 2003). Some of these targets, such as Ba813, have since been found in other *Bacillus* species, but others are believed to be specific for *B. anthracis* (Papaparaskevas et al., 2004). Several real-time PCR assays have been developed in which genes on pXO1, pXO2, and the bacterial chromosome are amplified to allow differentiation between pathogenic and non-pathogenic strains of *B. anthracis* (Bell et al., 2002; Ellerbrok et al., 2002; Patra et al., 2002).

Recently, a multiprobe microarray hybridization assay was developed which could identify multiple *B. anthracis* strains (with and without pXO1 and pXO2) and differentiate them from closely related species *B. cereus* and *B. thuringiensis* (Volokhov et al., 2004). This assay was time consuming, requiring growth of the bacteria as vegetative cells, isolation of DNA, two PCR reactions, and a hybridization step, but it was very specific. The assay detected the presence of genes *cyaA*, *pagA* and *lef* on pXO1, genes *capA*, *capB*, and *capC* on pXO2, and chromosomal region BA-5449 (a region which includes BA813, but contains sequences unique to *B. anthracis*). By

looking at these seven sequences, all six *B. anthracis* strains examined were correctly identified and none of the 56 other *Bacillus* species gave a false positive result.

While amplification and detection of species-specific DNA sequences through PCR is a popular method for identification of *B. anthracis*, it is not the only nucleic acid-based method that has been developed. A biosensor that can detect *atxA* (anthrax toxin activator) RNA with an oligonucleotide sandwich hybridization assay has also been reported (Hartley and Baeumner, 2003). The assay itself, in which the target sequence binds to an immobilized oligonucleotide probe and is then detected by the binding of a second oligonucleotide probe coupled to dye-encapsulating liposomes, takes only 15 minutes. However, to detect spores a total of 12 hours are required to grow the bacteria as vegetative cells, extract RNA, and amplify the target RNA using nucleic acid sequence-based amplification. The advantage of this protocol over many of the others is that it has a very low limit of detection, and was able to identify a single *B. anthracis* spore. The same group also developed a "universal biosensor" that can be adapted to detect any specific nucleic acid sequence in about 15 minutes (Baeumner et al., 2004). In this biosensor, streptavidin is immobilized to the surface of the detection zone of the membrane, so biotinylated capture probes (oligonucleotides complementary to a segment of the target sequence) can be easily attached. Reporter oligonucleotide probes are designed to have their 3' end complementary to a generic liposomal oligonucleotide, while their 5' end is complementary to the target sequence. Sensitivity of this "universal" assay adapted for detection of *AtxA* was virtually identical to that of the specific assay developed earlier.

Another research group developed a similar sandwich-style assay for electrochemical detection of a *B. cereus* target DNA on a biochip (Gabig-Ciminska et al., 2004). In this assay, which could easily be adapted for detection of *B. anthracis*, a target DNA sequence (amplified through PCR or in a crude spore lysate) is bound by a complementary capture oligonucleotide conjugated to a magnetic bead. Biotinylated detection oligonucleotides are added to the reaction mixture at the same time, and the complexes are removed from solution with a magnet. An extravidin-alkaline phosphatase complex is added that binds to the biotinylated probes, and p-aminophenyl phosphate is added as substrate. The product of the reaction is pumped over the surface of an electric chip where it is redox-recycled and produces an electric current that is related to the number of target DNA molecules present in the sample. Using this method, 1 pM target PCR product could be detected, and the target sequence could be detected in 10^7 disrupted spores. While more complex and time consuming, this assay has an advantage over the other sandwich methods in that it does not require nucleic acid purification and amplification prior to detection.

Ribotyping was also explored as a possible method for identification of *B. anthracis*. Ribotyping is a method in which DNA extracted from bacteria is digested, then probed with a region of the rRNA operon to reveal the pattern of rRNA genes. A Riboprinter® (Qualicon Europe Ltd.) was used in an attempt to identify several pathogens including *B. anthracis* (Grif et al., 2003). Unfortunately, the system's EcoRI-based database did not include data for *B. anthracis*, so when only EcoRI was used for analysis the bacteria was misidentified as *B. cereus*. However, when other restriction enzymes such as PvuII were used, *B. anthracis* could be distinguished from other

Bacillus species. This demonstrated that while ribotyping using multiple restriction enzymes could be useful as an identification method for pathogens, the databases of commercially available systems do not currently contain sufficient data about these bacteria for simple use.

Nucleic acid amplification and detection methods show great promise for use in detection of *B. anthracis* because multiple unique genomic regions can be detected to allow unambiguous identification of spores. Testing for the presence of multiple *B. anthracis*-specific targets reduces the possibility of a modified spore escaping detection. While many nucleic acid-based spore detection methods have been developed in recent years, few have actually been used in clinical or environmental testing.

3.3.4. Nucleic acid-based detection in clinical/environmental samples

The nucleic acid-based detection methods described above were developed in laboratory settings, working with purified spores, vegetative cells and/or nucleic acids extracted from bacteria. To determine the feasibility of using some of these assays to detect *B. anthracis* in clinical and environmental samples, several groups have studied their detection limits and reliability when mock clinical or field samples were analyzed.

One limitation of many of the aforementioned assays is that they have a high limit of detection. In the real time PCR assay in which the *rpoB* and *lef* genes were targeted, the detection limit of non-lysed spores eluted from nasal swabs was found to be ~2000 cells/1 µl sample (Oggioni et al., 2002). This is a much higher concentration than one would expect to find even in a heavily contaminated nasal swab or environmental sample. Sensitivity of the assay was significantly increased by growth of the bacteria in culture,

or isolation of genomic DNA, but these procedures extend the assay time considerably. Another group examined the ability of assays using primers targeting Ba813, *rpoB*, *lef,* and *pag* to amplify DNA templates extracted from spores on nasal swabs using four different extraction methods (Rantakokko-Jalava and Viljanen, 2003). Even the best conditions (High Pure or glassmilk DNA isolation) yielded a limit of detection of 2000 spores – the same limit seen when non-lysed spores were added directly to the PCR reaction. A high limit of detection is also problematic in food safety applications. The limit of the Ruggedized Advanced Pathogen Identification Device (RAPID, a portable real time PCR device) for detection of *B. anthracis* spores in milk was found to be 2500 spores/ml (Perdue et al., 2003). This detection limit was achieved only after a DNA purification step, which lengthens the procedure considerably.

The ability of traditional PCR and real time PCR to detect *B. anthracis* vegetative cells in simulated clinical blood samples was also evaluated (Rantakokko-Jalava and Viljanen, 2003). The detection limits of assays using primers targeting Ba813, *rpoB*, *lef,* and *pag* were evaluated using DNA templates extracted from vegetative cells in blood samples using four different extraction methods. A real time PCR assay using primers targeting *rpoB* could detect DNA extracted from vegetative cells at a concentration of 400 cfu/ml. While higher than ideal, an assay with this detection limit might be sufficient for identification of *B. anthracis* in the blood of patients with septic anthrax.

The detection of *B. anthracis* spores in soil samples has also proved to be challenging. A real time PCR assay that was shown to be capable of detecting a single *B. anthracis* spore trapped on the filter of an air monitoring device, was unable to detect less than 1000 spores extracted from a soil sample (Cheun et al., 2003). A single spore could

be detected from the soil matrix only if two rounds of enrichment were performed by cultivating the bacteria in trypticase soy broth prior to isolation of DNA.

To make an RT-PCR assay more portable for use in the field, the handheld advanced nucleic acid analyzer (HANAA) instrument was used to detect *B. anthracis* spores with primer-probe sets for the genes *vrrA* and *capA* (Higgins et al., 2003). While the real-time PCR reaction gave a positive signal very rapidly (32 – 22 min) in this study, the assay was performed with DNA isolated from vegetative cells, and therefore the total assay time was more than 24 hours. Clearly, rapid detection of *B. anthracis* spores in environmental samples is a challenge.

Clinical and environmental samples also present a challenge for nucleic-acid based detection systems because these samples can contain PCR inhibitors. Amplification of target DNA in soil samples can be inhibited by humic compounds, phenolic compounds, heavy metals, and high concentrations of non-target bacterial DNA (reviewed in Wilson, 1997). Amplification of target DNA in blood samples can be inhibited by hemoglobin, heparin, DNA binding proteins, or other unknown inhibitors (reviewed in Wilson, 1997).

The need for removal of PCR inhibitors, and growth of bacteria and isolation of DNA from spores extracted from clinical and environmental samples, greatly increases the total assay time required to detect the presence of *B. anthracis* spores. In incidences of bioterrorism, a rapid response is essential to control the spread of spores and treat those exposed. To reduce the assay time and limit of detection of nucleic-acid based assays, methods for rapid purification and concentration of *B. anthracis* spores must be developed.

3.3.5. Methods of purification and concentration of spores and spore DNA

In assays run on purified *B. anthracis* spores, simply heating the spores at 95°C to 100°C for 15 to 30 minutes is sufficient to prepare them for use as a PCR template (Drago et al., 2002; Makino and Cheun, 2003). Even autoclaved (30 min, 98°C) spores can be used as a PCR template (Fasanella et al., 2003). However, clinical and environmental samples may contain PCR inhibitors making such simple preparation methods insufficient for detection of small numbers of spores.

The detection of spores from clinical and environmental samples can be improved by purification of spore DNA prior to PCR amplification. A microchip-based procedure was developed in which PCR amplifiable DNA could be isolated from spores in under 30 minutes (Breadmore et al., 2003). In this procedure, spores are passed through a device and allowed to bind to silica beads, then PCR inhibiting materials are washed away, and purified DNA is eluted. FTA filters (Whatman Bioscience) were also shown to be useful for the preparation of DNA templates from *Bacillus* spores. The detection limit for *B. subtilis* spores lysed on an FTA filter and then detected by PCR amplification of the 16s rRNA gene is 53 spores (Lampel et al., 2004).

Not all DNA purification methods have been successful for use with *B. anthracis* spores. The FastDNA SPIN Kit for Soil (Bio101, Vista, CA), a bead beating extraction kit, was not able to extract PCR-amplifiable DNA from spores (Levi et al., 2003). Other bead beating methods were used successfully though, and in combination with a paramagnetic bead DNA extraction procedure, this method has been put to use in the Automated Biological Agent Testing System (Byrne et al., 2003).

While DNA purification methods are useful, they do not separate the DNA of the target organism from the DNA of all the other organisms in the sample, and this can be troublesome in later PCR assays. A variety of methods have been developed for the separation and concentration of bacterial cells from various sample matrices (reviewed in Benoit and Donahue, 2003; Stevens and Jaykus, 2004). Many of these methods are non-selective; they concentrate and separate all bacteria in the sample, not just the organism of interest. Non-selective concentration methods include adsorption of bacteria onto solid surfaces of food components, ion exchange resins, and lectins (Stevens and Jaykus, 2004). Dielectrophoresis, aqueous two-phase partitioning, high speed centrifugation, differential centrifugation, density gradient centrifugation, coagulation and flocculation, and filtration can also be used to separate bacteria non-selectively from their matrix (Stevens and Jaykus, 2004). While these methods are useful, a high concentration of non-target DNA (from other spores/bacteria harvested by these methods) can still inhibit subsequent PCR reactions.

To selectively concentrate the target bacteria or spores, a target-specific probe must be used in an immunoaffinity procedure. In most immunoaffinity procedures developed to date, antibodies are attached to the surface of magnetic beads that can be removed from the sample matrix (taking the target spores/bacteria with it) using a strong magnet.

Immunomagnetic concentration of *B. anthracis* spores has been demonstrated using biotinylated polyclonal antibodies linked to streptavidin-coated paramagnetic beads (Gatto-Menking et al., 1995; Bruno, 1996). Concentration of spores in PBS was accomplished by first reacting the spores with biotinylated monoclonal antibodies, then

adding the streptavidin-coated beads and pulling the complexes out of solution (Gatto-Menking et al., 1995). Separation of spores from soil was accomplished by incubating prepared paramagnetic bead-antibody complexes with spores in a soil matrix (Bruno, 1996). While separation of spores from the soil matrix was possible, the recovery of spores was much lower from soil than from PBS.

Immunomagnetic separation of *Bacillus* spores from matrices including clay, pepper, milk, and sandy soil has been demonstrated (Blake and Weimer, 1997). To achieve separation, polyclonal antibodies against *B. stearothermophilus* were fixed to paramagnetic beads, and the beads were mixed with *B. stearothermophilus* alone or in a mixture with *B. subtilis* spores in each of the aforementioned matrices. The beads were shown to bind and concentrate only the *B. stearothermophilus* spores, allowing them to be separated from the sample matrix and contaminating *B. subtilis* spores.

Immunomagnetic separation, while popular, is not the only selective method to isolate bacteria from sample matrices. Capture of *B. globigii* spores on large monoclonal antibody-coated 3mm glass beads by the ImmunoFlow method has also been demonstrated (Weimer et al., 2001). In this method, the spore or bacteria-containing fluid is forced through a cartridge that contains large antibody coated-beads. This method was able to capture a higher percentage of spores spiked into river water than in PBS. However, this method was developed for use in an ELISA style assay, in which the spores remained bound to the beads in the cartridge, so modifications would be necessary to use it for concentration prior to a nucleic-acid based assay. The antibody-coated beads used in the assay were also found to decrease in activity significantly during the first two

weeks of incubation in either sodium phosphate buffer or river water at ~4°C, making them less than ideal for commercial use.

While use of antibodies in immunomagnetic and flow-through style separation systems has been somewhat successful, there are still challenges remaining in this area. Monoclonal and polyclonal antibodies have limited potential for use with environmental and food samples because they are often susceptible to degradation when exposed to organic solvents, detergents and proteases (Shone et al., 1985; Pancrazio et al., 1999). Use of antibody fragments engineered for high stability may be one way around this problem (Molloy et al., 1995). Naturally occurring and engineered bacteriophages may also potentially be used as probes for affinity separation.

Bacteriophages are viruses that infect bacteria. While there are no published examples of the use of recombinant bacteriophages as probes for the separation and concentration of bacteria or spores, a naturally occurring bacteriophage has been used for this purpose. The 'Sapphire' lytic T4 phage was immobilized to the wells of a microtiter dish and used to capture *Salmonella typhimurium* from PBS (Bennett et al., 1997). While capture of *Salmonella* with phage in this format was not very efficient, it was selective and prevented inhibition of PCR by competitor microorganisms such as *E. coli*. This phage also showed potential for separation of *Salmonella typhimurium* when attached to magnetic particles.

Recombinant bacteriophages engineered and/or selected for binding to biothreat agents may be advantageous over naturally occurring bacteriophages for separation uses for several reasons. Recombinant bacteriophages can be selected for binding to virtually any target, including spores and toxins, as opposed to natural bacteriophages that only

interact with bacterial cells. Recombinant bacteriophages will not harm bacterial targets, as lytic phages can. Finally, the stringency of separation can be controlled with recombinant bacteriophages by selecting a phage that binds to a target unique to an individual bacterium or spore, or a phage that binds to a target found on numerous bacteria and spores.

4. Phage and Phage display

4.1. Phage Biology

Bacteriophages, viruses that infect bacterial cells, belong to a diverse group of organisms. Phage may have single- or double-stranded DNA or RNA genomes, and may be filamentous or have icosahedral capsids. The phages that this work will focus on are filamentous phages with single-stranded DNA genomes from the family Inoviridae.

The Ff class of filamentous phages, which includes M13, f1, and fd, have been used extensively in the development of phage display vectors. These three phages are approximately 7 nm wide and 900 nm long. Their coat consists of 5 proteins; one major capsid protein, pVIII, present in 2700 or more copies per phage; and four minor capsid proteins, pIII, pVI, pVII, and pIX, present in a few copies per phage (reviewed in Webster, 1996; Webster, 2001).

Phages M13, f1, and fd are known as Ff phage because they infect *Escherichia coli* by interaction with the F-pilus. Upon binding of minor coat protein pIII to the F-pilus, the pilus retracts, allowing the phage to interact with other receptors on the bacterial cell surface. Through unclear mechanisms, the phage coat disassembles into the

bacterial cytoplasmic membrane and phage DNA is translocated to the cytoplasm. Here, DNA complementary to the viral strand is synthesized to create the replicative form, which is then replicated by the rolling circle mechanism (reviewed in Webster, 1996; Webster, 2001).

Phage capsid proteins are produced by the host cell machinery and integrated into the bacterial cell envelope. These capsid proteins assemble around the phage DNA while it is extruded through the envelope. Phage progeny are secreted continuously and their production does not kill the host cell. Bacterial cells tolerate well the infection by Ff phage, having a generation time only ~50% longer than uninfected cells (reviewed in Webster, 1996; Webster, 2001).

Ff phage are frequently used as cloning vehicles because their replication and assembly are not inhibited by changes in genome size. When a length of foreign DNA is inserted into a nonessential region of the genome, the phage particle is simply made larger. The addition of foreign DNA encoding a peptide or protein into one of the coat protein genes can result in the display of a foreign peptide or protein on the phage surface. This concept of displaying a foreign peptide or protein on the surface of a phage is known as phage display (reviewed in Smith and Petrenko, 1997).

4.2. Phage display

Phage display is the use of phage as a vector to display foreign peptides or proteins on its surface. Phage display libraries are mixtures of phage clones each carrying a different foreign DNA insert in one of its coat protein genes and therefore expressing a different peptide or protein on its surface. Phage display libraries can be

created with phage having icosahedral heads, but this work will focus on libraries created from filamentous phages.

As described earlier, the outer coats of filamentous phages are composed of thousands of alpha-helical subunits of the major coat protein pVIII, and several copies of each of the minor coat proteins, pIII, pVI, pVII, and pIX. Foreign peptides and/or proteins have been fused to all of these proteins (reviewed in Webster, 2001) . Foreign peptides or proteins can be expressed at the N-terminus of pVII, pVIII, and pIX, the C-terminus of pVI and either the N or C terminus of pIII. Libraries can be created such that foreign peptides or proteins are expressed on all copies of the selected coat protein, or just a portion of the copies. Genetically, the expression of a foreign peptide or protein on just a portion of the copies of a coat protein is accomplished by splicing a second wild-type coat protein gene into the phage genome, or by placing the modified gene into a phagemid that is accompanied by a wild-type helper phage. A phagemid is a specialized plasmid that carries both a plasmid replication origin and a phage origin, as well as an antibiotic resistance gene.

Phage display libraries have been created to display fragments of antigens, proteins and protein domains, mutagenized proteins, antibodies and antibody fragments, cDNA encoded proteins, and random peptides (reviewed in Kay, 1996). This work will focus on phage display libraries in which random peptides are displayed on the phage surface. To create a random peptide phage display library, degenerate synthetic oligonucleotides are spliced in-frame into one of the phage coat protein genes, so that the "guest" peptides encoded by the degenerate oligonucleotides are fused to the coat protein and thereby displayed on the exposed surface of the virions. Each phage particle displays

multiple copies of one particular peptide, but a library contains billions of different phage clones carrying billions of different peptides. The length of the random peptides expressed on the phage surface have ranged from 5 amino acids to 40 amino acids (reviewed in Smith and Petrenko, 1997).

Phage display libraries have been used for many applications, including epitope mapping of antibodies, generation of immunogens, cDNA expression screening, and isolation of high affinity antibodies and peptides (reviewed in Kay, 1996). The vast majority of phage display methods involve the isolation of phage carrying peptides or proteins with the ability to bind to a target. Typically, a target antigen is fixed to a solid support, and then the phage display library is added in solution to allow phage carrying target-specific peptides or proteins to bind. Phage that do not bind to the target are washed away, and then bound phage are eluted. The phage population eluted from the target, which is enriched for phage carrying peptides or proteins that bind to the target, is then amplified in bacterial cells. This collection of amplified phage clones is then applied to an identical preparation of target antigen for a second round of affinity selection. After several rounds of selection, individual phage clones can be isolated and the peptide or protein responsible for their affinity to the target can be divulged by sequencing the corresponding coding sequence in the phage's DNA.

Different types of phage display libraries are advantageous for different applications. Large proteins and peptides are best displayed on pIII or on a portion of the pVIII proteins to avoid interfering with phage viability and infectivity, while short peptides can be displayed on some or all copies of any of the coat proteins. During selection, a phage library displaying one copy of a peptide will likely yield few binders,

but those binders will be expected to have a high affinity for the target. A large number of binders will typically be isolated from a library in which phage clones display many copies of a peptide on their surface, because the avidity of the many peptides will be stronger than the affinity of an individual peptide (Smith and Petrenko, 1997). While this can be a disadvantage if one is seeking individual peptides with a high affinity for a target, it can be a great advantage if the phage clone carrying the peptides can itself be used as a probe. Libraries in which peptides are expressed on all copies of the major coat protein, known as landscape libraries, were created specifically for the isolation of these phage probes.

4.3. Landscape phage display

In landscape phage display, a foreign peptide or protein is fused to all copies of the major coat protein on a phage particle. The major coat protein, pVIII, is initially synthesized with an N-terminal signal peptide, which is cleaved as the protein is inserted into the bacterial cell envelope. The processed protein spans the cytoplasmic membrane, so the amino terminus is exposed to the periplasm, while the carboxy terminus of pVIII interacts with the sugar phosphate backbone of the phage DNA in the cytoplasm. When phage particles are extruded through pIV channels, the amino terminus of pVIII becomes exposed to the environment. Each of the 2700^{+} pVIII proteins contributes to the formation of a right-handed helical phage coat, with the individual monomers tilted at an ~20 degree angle to the long axis of the phage particle (reviewed in Webster, 1996; Smith and Petrenko, 1997; Webster, 2001).

In landscape phage, as in traditional phage-display constructs, foreign peptides or proteins are fused to coat proteins on the surface of the virus particle. Unlike conventional constructs, however, landscape phage display thousands of copies of the peptide in a repeating pattern, comprising a major fraction of the viral surface. The phage body serves as an interacting scaffold to constrain the peptide into a particular conformation, creating a defined organic surface structure (landscape) that varies from one phage clone to the next. Genetically, DNA encoding a foreign peptide is inserted into the pVIII gene between the N-terminal domain and the signal peptide, so that the foreign peptide is expressed on the outer surface of the phage particle (Smith and Petrenko, 1997). While the pVIII gene can accommodate large inserts, and pVIII proteins carrying large foreign peptides or proteins can be produced, phage production is hindered by foreign peptide additions of more that 10 amino acids. To accommodate peptides or proteins larger than this, wild type pVIII molecules must supplement the modified proteins to create a mosaic phage particle. Only phage composed solely of modified pVIII proteins are considered landscape phage, so landscape phage can carry a maximum of 9 - 10 foreign amino acids (Smith and Petrenko, 1997).

A landscape library is a huge population of landscape phages, encompassing billions of clones with different surface structures and biophysical properties. The landscape phage library used in this work contains random 8 amino acid peptides fused to all 4000 copies of the major coat protein of an fd-tet based vector (Petrenko et al., 1996). The random 8 amino acid peptides carried on the surface of these phage clones are encoded by the degenerate oligonucleotide Gnk $(nnk)_6$ nnG, where positions designated n have an equal mixture of all four bases, and positions designated k have an equal mixture

of G and T. The library contains 1.5 x 10^9 clones, which have been amplified so that multiple copies of each clone are present.

4.4. Phage stability

It has long been known that naturally occurring and recombinant bacteriophages are hardy and resistant to degradation. Filamentous phage and phage-derived vectors have been found to retain infectivity after exposure to 20% 1-propanol (Olofsson et al., 1998; Olofsson et al., 2001), 20–99% acetonitrile (Olofsson et al., 1998; Olofsson et al., 2001), 30–55% ethanol (Berglund, 1998; Olofsson et al., 1998; Olofsson et al., 2001), 50% diethyl ether (Amako and Yasunaka, 1977), 50% acetone (Amako and Yasunaka, 1977), 50-65% dimethylformamide (Olofsson et al., 1998; Olofsson et al., 2001), and 50–80% methanol (Amako and Yasunaka, 1977; Olofsson et al., 1998; Olofsson et al., 2001).

Phage have also been found to be resistant to proteolytic digestion. Filamentous phage fd was found to be resistant to trypsin, chymotrypsin, and endoproteinase Glu-C. The only enzyme the phage was found to be vulnerable to was subtilisin, which attacked the N-terminal portion of pVIII (Schwind et al., 1992).

A limited number of thermostability studies of naturally occurring and recombinant phage have been previously documented. Early studies of filamentous phage fd demonstrated that virions are not disrupted by temperatures below 70°C (Wiseman, 1972), and that infectivity of virions is not reduced after heating to 80°C for 10 minutes (Hoffmann-Berling et al., 1963). Other filamentous phages such as If1 and Ec9 have also demonstrated thermostability of virions at 70°C to 80°C (Meynell, 1968). In a more focused study, the short term thermostability of pIII on phage fd was examined

with an infection assay, and an ELISA that detected binding of the phage to bacterial TolA receptor (Holliger et al., 1999). This study revealed that pilus-independent infection of *E. coli* and TolA binding increased as temperatures rose from 37°C to 60°C, then decreased to non-detectable levels between 60°C and 85°C, while infection of *E.coli* in a pilus-dependent manner was not affected by temperatures up to 80°C. Filamentous bacteriophages from extreme thermophiles such as *Thermus themophilus* have been shown to be stable at even higher temperatures. These bacteriophages were shown to be structurally stable at temperatures of 80°C to 90°C, and preliminary results indicated stability even at 130°C (Sakaki and Oshima, 1975; Pederson et al., 2001). Another highly thermostable phage, HR, was actually isolated by heating sewage to 90°C for 10 minutes to kill other phages (Hsu, 1968).

While there is little information available about the stability of filamentous phages, the data is even sparser for lytic phages. A study of soil bacteriophages that infect and lyse *B. anthracis* found that these phages retained infectivity after 12 h incubations at temperatures ranging from -20°C to 37°C, but were damaged by temperatures of 55°C or higher (Walter, 2003). A naturally occurring lytic bacteriophage active against *Lactococcus lactis* was shown to be stable in M17 broth for at least 14 weeks at 4°C (Parada and de Fabrizio, 2001).

The stability of phage under a variety of harsh conditions lead to the idea of using recombinant phage as probes for environmental monitoring. Recombinant target-specific phage have been shown to function as substitutes for antibodies in a variety of platforms.

4.5. Use of phage as substitutes for antibodies

Detection and identification of bacteria by use of specific phage is not a new idea. One of the traditional microbiological assays used to identify *B. anthracis* is a phage susceptibility test. Phage have already been shown to function as recognition elements in bacterial detection assays for *S. typhimurium* and *E. coli* (Goodridge et al., 1999; Mosier-Boss et al., 2003). Naturally occurring phages can only interact with vegetative cells however, limiting their use with spores. To use phage for detection of bacterial spores, recombinant phage selected for spore-specific binding can be identified.

Landscape phages have been shown to serve as substitutes for antibodies against various antigens and receptors (Petrenko and Smith, 2000; Romanov et al., 2001), including live bacterial cells (Petrenko and Sorokulova, 2004). These phage probes have been used in ELISA and thickness shear mode quartz sensors to detect antigens (Petrenko and Vodyanoy, 2003). Use of landscape phage as substitutes for antibodies has several advantages: phage express up to 4000 copies of the binding peptide on their surface, allowing multivalent interactions with the target antigen; phage can be produced rapidly and inexpensively in large quantities; they are resistant to heat (Holliger et al., 1999), organic solvents (Olofsson et al., 1998), and many other stresses; and they can be stored indefinitely at moderate temperatures without loss of activity, or at 37ºC with only minimal loss of activity after 7 months (Brigati, 2004).

4.6. Selection of spore-specific probes from phage display libraries

Recently, a pIII phage display library was used to identify peptides that are specific and selective for spores of various *Bacillus* species, including *B. anthracis* (Knurr, 2003; Turnbough, 2003; Williams, 2003). Human scFv capable of binding to various *Bacillus* species have also been selected through phage display (Zhou et al., 2002). We were interested in evaluating the prospect of using not individual peptides or antibody fragments, but phages themselves as "building blocks" for the development of robust and inexpensive diagnostic probes and biosorbents (Petrenko and Sorokulova, 2004).

Earlier in this review the similarities between *B. anthracis* and its close relatives were described extensively. The many common antigens shared by these bacteria make the random selection of a selective probe unlikely. Subtractive phage display procedures can be used to reduce the likelihood of isolating cross-reactive phage clones. In subtractive phage display, phage clones that bind to common antigens are removed by pre-incubation of the library with non-target antigens before panning on the target, or co-incubation of the library with target and non-target antigens. Through subtractive procedures, a phage-displayed peptide that binds to ICAM-1 expressing epithelial cells, but not to normal epithelial cells was selected (Belizaire et al., 2003). Subtractive procedures were also used to identify phage-displayed peptides that bind to malignant glioma cells, but not to normal brain cells (Samoylova et al., 2003). Human scFv fragments specific for keratinocytes yet not cross-reactive with melanoma cells (Stausbol-Gron et al., 2001), and specific for thymic stromal cells yet not cross-reactive

with lymphoid cells (Van Ewijk et al., 1997) were also selected through subtractive phage display. In a more relevant example, human scFv specific for a pathogenic *Streptococcus suis* serotype 2 strain were selected from an antibody phage display library through a subtractive procedure that eliminated antibodies that cross-reacted with a non-pathogenic *S. suis* serotype 2 strain (de Greeff et al., 2000). In another relevant example, the identification of human scFv that bind to *B. subtilis* but not to *B. licheniformis* was achieved by subtractive phage display (Zhou et al., 2002).

If subtractive selection techniques do not allow the identification of target-selective phage clones, mutation of cross-reactive clones, and subsequent selection may allow the identification of better candidates. Phage clones identified in initial selection procedures can be modified in areas of pVIII surrounding the peptide insert to change their affinity. With these procedures, it should be possible to create phage probes selective for virtually any target.

While subtractive procedures and molecular evolution can be time consuming, initial selection of target binding clones can be accomplished in just a few weeks. The ability to rapidly select phage probes for engineered biological warfare agents makes them ideal for use in response to new threats. It is anticipated that advances in genetic research will allow the development of advanced biological warfare agents engineered to have novel effects and to avoid detection by systems designed for currently known threat agents (Petro et al., 2003). A combinatorial approach such as phage display could allow development of probes for detection of a new threat agent before virtually any characterization of the agent is completed.

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CHAPTER II

THERMOSTABILITY OF LANDSCAPE PHAGE PROBES

1. Introduction

Immunoassays are relied upon for detection and identification of a wide variety of infectious and hazardous agents. Traditionally, these assays have relied on the use of monoclonal or polyclonal antibodies as probes. While antibody-based probes work fairly well in a controlled laboratory setting, their sensitivity to environmental stresses makes them less than ideal for use in field testing and continuous monitoring.

An ideal probe for use in field testing and continuous monitoring would be stable for shipping, storage, and operation at ambient temperatures. Probes are typically kept at moderate temperatures (4 °C–25 °C), but could be exposed to temperatures as high as 45 °C in desert locations, and possibly higher temperatures during shipping to these locations. The stability of monoclonal antibodies can vary depending on the unique structure of each antibody, but they are generally susceptible to degradation at high temperatures. The binding activity of some mouse monoclonal antibodies was found to begin to decrease after a mere 2 h at 37 °C. At 70 °C–80 °C the binding activity of these antibodies was completely eliminated after 2 h (van der Linden et al., 1999). Another study found that a monoclonal antibody lost ~10% of its activity after 15 days at

37 °C in a buffer with pH 10, and ~5% of its activity after 15 days at 37 °C in a buffer with pH 4 (Usami et al., 1996).

Antibody fragments consisting of only the variable region of an antibody (scFv) have also been found to be unstable, even when stored at cool temperatures. In one study, an scFv stored in solution at 4 °C was found to degrade significantly within 6 months (Kramer et al., 2002). Another study found a 45% decrease in scFv binding to a target antigen after storing the antibody fragment at 4 °C for 7 weeks (Brichta, 2003). At higher temperatures, scFv was even more unstable, losing activity after a mere 4 h at 37 °C in PBS (Reiter et al., 1994). Some stable scFv frameworks have been found, and stable antibody fragments with different specificities have been created by grafting different antigen-binding residues to these frameworks. In one example, the antigen binding residues of an unstable scFv (degraded after 30 min at 37 °C) were transferred to the framework of a stable scFv to create an scFv stable for 20 h at 37 °C (Willuda et al., 1999). An antibody designed with a different stable framework was found to be stable for 6 months when dried to a chip and stored at 4 °C (Steinhauer et al., 2002). Another stabilized scFv retained 50% of its activity after a 3 h incubation at 41 °C (in comparison to an unstabilized scFv that lost 50% of its activity after 3 h at 34 °C) (Dooley et al., 1998). Another type of antibody fragment, which contains constant regions in addition to the variable regions found in scFv, may be more stable than scFv. These antibody fragments, known as F_{ab}, last more than a year at 4 °C without degradation (Kramer et al., 2002). Their stability at elevated temperatures was not evaluated.

Recently, the possibility of using landscape phage as detection probes in place of antibodies has been presented (Petrenko and Smith, 2000; Petrenko and Vodyanoy, 2003;

Brigati et al., 2004; Petrenko and Sorokulova, 2004). Phage probes are selected from landscape phage display libraries that are constructed by the genetic modification of filamentous bacteriophage fd-tet. The outer coats of the phage are composed of thousands of subunits of the major coat protein pVIII, which form a tube encasing the viral DNA. At the tips of the phage are several copies of each of the minor coat proteins, pIII, pVI, pVII, and pIX (reviewed in (Webster, 2001)). To create a landscape phage display library, degenerate synthetic oligonucleotides are spliced in-frame into the pVIII coat protein gene, so that the "alien" peptides encoded by the degenerate oligonucleotides are fused to the major coat protein pVIII and thereby displayed as a landscape of 4,000 copies on the exposed surface of the virions (reviewed in (Smith and Petrenko, 1997)). These alien peptides are arranged in a repeating pattern, comprising a major fraction of the viral surface. The phage body thus may serve as an interacting scaffold to constrain the peptide into a particular conformation, creating a defined organic surface structure (landscape) that varies from one phage clone to the next. A landscape library is a huge population of such phages, encompassing billions of clones with different surface structures and biophysical properties. The landscape phage library f8/8 from which the phage clone used in this work was selected contains random 8 amino acid peptides fused to all 4000 copies of the major coat protein of fd-tet (Petrenko et al., 1996).

The landscape phage clone used in this work was obtained by panning of a landscape phage display library against β-galactosidase (from *E.coli*) fixed to a polystyrene Petri dish (Petrenko and Smith, 2000). The amino acid sequence of the peptide expressed at the N-terminus of all 4000 copies of the major coat protein of this phage is (Ala)-Asp-Thr-Phe-Ala-Lys-Ser-Met-Gln, where the bracketed alanine belong to

the native pVIII protein and other amino acids replace its Glu-Gly-Asp peptide. This phage clone was shown to serve as a substitute for antibodies in the detection of β-galactosidase from *E.coli* in an ELISA (Petrenko and Smith, 2000) and a thickness shear mode quartz sensor (Petrenko and Vodyanoy, 2003).

Use of landscape phages as substitutes for antibodies has several advantages: phage probes express 4000 copies of an antigen-binding peptide on their surface, allowing strong multivalent interactions with a target; phages can be produced rapidly and inexpensively in large quantities; and phages are very stable and resistant to degradation by organic solvents, proteases and heat. Filamentous phage and phage-derived vectors have been found to retain infectivity after exposure to 20% 1-propanol (Olofsson et al., 1998; Olofsson et al., 2001), 20–99% acetonitrile (Olofsson et al., 1998; Olofsson et al., 2001), 30–55% ethanol (Berglund, 1998; Olofsson et al., 1998; Olofsson et al., 2001), 50% diethyl ether (Amako and Yasunaka, 1977), 50% acetone (Amako and Yasunaka, 1977), 50-65% dimethylformamide (Olofsson et al., 1998; Olofsson et al., 2001), and 50–80% methanol (Amako and Yasunaka, 1977; Olofsson et al., 1998; Olofsson et al., 2001). Elevated temperatures can decrease the stability of phage in some solvents. For example, exposure of phage to 50% ethanol at elevated temperatures (30°C or higher) significantly reduced infectivity (Olofsson et al., 2001).

Phage have also been found to be resistant to proteolytic digestion. Filamentous phage fd was found to be resistant to trypsin, chymotrypsin, and endoproteinase Glu-C. The only enzyme fd was vulnerable to was subtilisin, which attacked the N-terminal portion of the major coat protein pVIII (Schwind et al., 1992).

A limited number of thermostability studies of naturally occurring and recombinant phage have been previously documented. Early studies of filamentous phage fd demonstrated that virions are not disrupted by temperatures below 70 °C (Wiseman, 1972), and that infectivity of virions is not reduced after heating to 80 °C for 10 minutes (Hoffmann-Berling et al., 1963). Other filamentous phages such as Ifl and Ec9 also have demonstrated thermostability of virions at 70 °C to 80 °C (Meynell, 1968). In a more focused study, the short-term thermostability of pIII on phage fd was examined with an infection assay, and an ELISA that detected binding of the phage to bacterial TolA receptor (Holliger et al., 1999). This study revealed that pilus-independent infection of *E. coli* and TolA binding increased as temperatures rose from 37 °C to 60 °C, then decreased to non-detectable levels between 60 °C and 85 °C, while infection of *E.coli* in a pilus-dependent manner was not affected by temperatures up to 80 °C. Filamentous bacteriophages from extreme thermophiles such as *Thermus themophilus* have been shown to be stable at even higher temperatures. These bacteriophages were shown to be structurally stable at temperatures of 80 °C to 90 °C, and preliminary results indicated stability even at 130°C (Sakaki and Oshima, 1975; Pederson et al., 2001),. Another highly thermostable phage, HR, was actually isolated by heating sewage to 90 °C for 10 minutes to kill other phages (Hsu, 1968).

While there is little information available about the stability of filamentous phages, the data are even sparser for lytic phages. A study of soil bacteriophages that infect and lyse *B. anthracis* found that these phages retained infectivity after 12 h incubations at temperatures ranging from -20 °C to 37 °C, but were damaged by temperatures of 55 °C or higher (Walter, 2003). A naturally occurring lytic bacteriophage

active against *Lactococcus lactis* was shown to be stable in M17 broth for at least 14 weeks at 4 °C (Parada and de Fabrizio, 2001). While these studies all demonstrate the thermal stability of phages, none examine the stability of recombinant phages used as probes.

In this study, the stability of a recombinant phage at temperatures ranging from 25 °C to 76 °C was examined and compared to the stability of a commercially available monoclonal antibody that recognized the same target. Stability was measured by monitoring the ability of the phage and monoclonal antibodies to interact with their target antigen following incubation at each temperature for a period of time ranging from one day to 7 months.

2. Materials and methods

2.1 Materials

The β-galactosidase-binding phage clone used in this work, which carries thousands of copies of a peptide with the amino acid sequence DTFAKSMQ, was described by Petrenko & Smith (Petrenko and Smith, 2000) and is designated 1G-40. Phage 1G-40 was propagated in *Escherichia coli* K91 BluKan cells and purified by PEG precipitation as previously described (Yu and Smith, 1996). The total number of viral particles present in phage preparations was determined spectrophotometrically using the formula (Barbas and Carlos, 2001):

$$\text{Virions/ml} = (A_{269} \times 6 \times 10^{16}) \text{ /number of nucleotides in the phage genome}$$

For the recombinant phage used in this work (9198 nucleotides), the formula:

$$\text{absorbance unit } (AU)_{269} = 6.5 \times 10^{12} \text{ virions/mL}$$

was used to determine the concentration of phage particles in a solution.

Monoclonal mouse anti- β-galactosidase antibody was obtained from Promega (Z3783) as a 2.3 mg/mL solution. Lyophilized β-galactosidase was obtained from Sigma (G-5635) and a 3 μM suspension was prepared in 50% glycerol. *o*-Nitrophenyl β-D-Galactopyranoside (ONPG) was obtained from Sigma (N-1127), and stored as an 8 mg/mL solution in 0.1 M sodium phosphate. Z-buffer (0.06 M $Na_2HPO_4 \cdot 7H_20$, 0.04 M $NaH_2PO_4 \cdot H_2O$, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 7H_2O$, 0.05 M β-mercaptoethanol, pH 7.0) was filter sterilized and stored at 4 °C. High binding 96-well EIA/RIA plates were obtained from Costar (3369), and plate sealing tape was obtained from Nalgene (232701).

2.2 Preparation of enzyme immunoassay (EIA) plates

Phage 1G-40 was diluted to a concentration of 5 x 10^{11} virions/ml in tris-buffered saline (TBS) containing 50 mM Tris-HCl pH 7.5, 0.15 M NaCl. The monoclonal mouse anti- β-galactosidase antibody was diluted to 10 μg/ml in TBS. The phage and antibody solutions were loaded into separate columns of wells (60 μl/well) of EIA/RIA plates. The plates were incubated overnight at 4 °C to allow adsorption of the phage and antibodies to the wells. The loaded wells only were then washed with TBS containing 0.5% Tween 20 with an ELx50 plate washer. The loaded wells were then filled with TBS and the

plates were sealed with plate sealing tape. As a day 0 control, the wells of an unwashed column in one plate were immediately loaded with phage 1G-40 (60 µl/well), and the plate was incubated overnight at 4 °C to allow adsorption of the phage. The remaining plates were placed in humidified boxes at 25 °C, 37 °C, 50 °C, 63 °C and 76 °C. After 4 h (76 °C), 8 h (76 °C), 1 day (50 °C, 63 °C, 76 °C), or one week (25 °C and 37 °C) and every 8-16 h (76 °C), 7 days (50 °C and 63 °C) or 21 days (25 °C and 37 °C) thereafter, a plate was removed from incubation at each temperature, allowed to cool to room temperature, and then phage was added to an unwashed column of wells as previously described. This phage, added after the incubation at the indicated temperature, will be referred to as "fresh phage."

2.3 ELISA

All wells of the prepared plate were washed with TBS containing 0.5% Tween 20 five times with shaking by an ELx405 plate washer. β-Galactosidase was gradually diluted in Z-buffer to concentrations of 200 nM, 67 nM, 22 nM, 7.4 nM, 2.5 nM, 823 pM, 274 pM, and 91 pM, loaded (50 µl) into different wells containing phage, antibody, fresh phage, and no probe (plastic control), and the plate was incubated for 1 h at room temperature with gentle rocking. After this incubation, all wells of the plate were washed again as previously described. ONPG was diluted to 4 mg/ml with Z-buffer, and 90 µl of this solution was added to each well of the plate. The absorbance at 405 nm (reference

wavelength 490 nm) was monitored for 1 h using an EL808 Ultra Microplate Reader (BIO-TEK Instruments, Inc.).

3. Results and Discussion

In this study, the stability of a phage probe and a monoclonal antibody specific for β-galactosidase were monitored with a simple ELISA. Phage probe and monoclonal antibody were fixed to the wells of 96-well plates, incubated at various temperatures, and then tested for the ability to bind β-galactosidase. β-galactosidase is an enzyme capable of cleaving a readily available substrate, ONPG, and therefore its binding was visualized without use of an enzyme-linked antibody. A microplate reader was used to quantify the kinetics of the reaction and determine the relative β-galactosidase binding.

Initially, stability of phage and monoclonal antibodies at 25 °C and 37 °C was examined. Consistent β-galactosidase binding throughout the experiment demonstrated that both phage and monoclonal antibodies were stable at 25 °C for more than 6 months when fixed to an EIA/RIA plate. Fig. 1 and Fig. 2 show the ELISA signals obtained at each time point for phage and monoclonal antibodies. The signals are expressed as a ratio: signal of experimental well / signal of well coated with fresh phage. This was done to prevent possible slight variations in β-galactosidase concentration, ONPG concentration, or room temperature from affecting the results. The thermostability of the

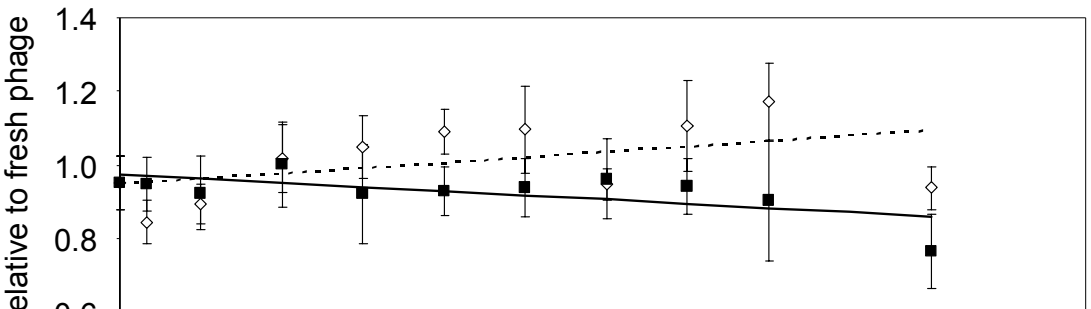

Fig.1. Phage binding of β-galactosidase after incubation at 25 °C and 37 °C. Phage 1G40 and antibody were fixed to the wells of EIA/RIA plates and incubated at 25 °C (◊) and 37 °C (■). At various intervals, plates were removed from the incubator, fresh phage was added to an unused set of wells, and β-galactosidase was allowed to bind to the phage. β-galactosidase binding was visualized by the addition of substrate ONPG. ELISA signal values (mOD/min) are relative to the signals in the wells containing fresh phage. Error bars are indicative of standard deviation of values obtained at 3 β-galactosidase concentrations.

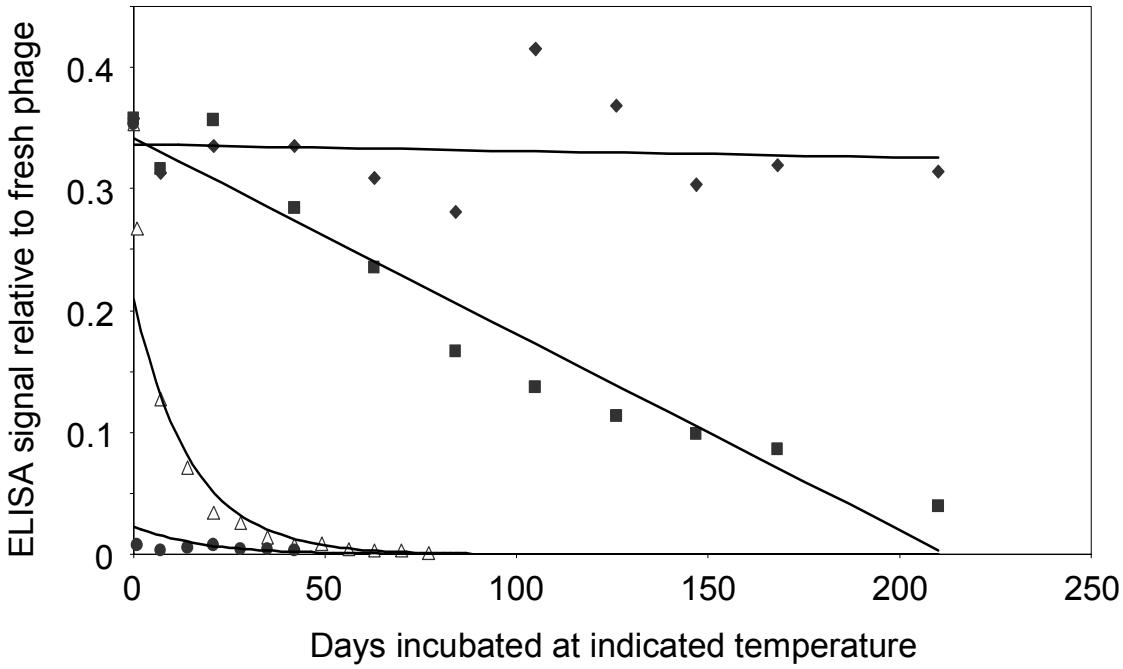

Fig.2. Antibody binding of β-galactosidase after incubation at 25 °C, 37 °C, 50 °C, and 63 °C. Monoclonal antibody specific for β-galactosidase was fixed to the wells of EIA/RIA plates and incubated at 25 °C (♦), 37 °C (■), 50 °C (Δ), and 63 °C (●). At various intervals, plates were removed from the incubators, fresh phage was added to an unused set of wells, and β-galactosidase was allowed to bind to the phage and antibodies. β-galactosidase binding was visualized by the addition of substrate ONPG. ELISA signal values (mOD/min) shown in this example are relative to the signals in the wells containing fresh phage at a 200 nM concentration of β-galactosidase.

phage and the antibody probes was characterized by the degradation constants (k) equal to the change in binding ability over the change in time, which is equal to the slope of the lines in figure 1. The degradation constants were not statistically different from zero for both phage and monoclonal antibodies at 25 °C, but differed dramatically at higher temperatures. At 37 °C, phage degraded only slightly, while monoclonal antibodies lost virtually all of their activity by the end of the 30 week study. The degradation constant (k) of phage at 37 °C was calculated to be 0.0005/day, and the half life of phage as a probe at this temperature was calculated to be 950 days. The degradation constant of the monoclonal antibody at 37 °C was 0.001/day.

The stability of phage and antibodies at moderate temperatures prompted an examination of these probes at more extreme temperatures. Fig. 2 and Fig. 3 show the ELISA signals obtained at each time point for phage and monoclonal antibodies at 50 °C and 63 °C. At 50 °C, both phage and monoclonal antibodies progressively degraded, but monoclonal antibody activity was undetectable after just 5 weeks, while phage still retained more than 50% of its activity at the same time point. At 63 °C, monoclonal antibodies were found to be completely inactivated after just 24 h. The phage probe was significantly more stable at this temperature, maintaining detectable activity for 6 weeks. The degradation constant (k) of phage at 50 °C was calculated to be 0.0066/ day and at 63 °C was calculated to be 0.019/ day. The half life of phage probe 1G40 at 50 °C and 63 °C was calculated to be 83 days and 22 days, respectively.

To further test the thermostability of phage 1G40, its ability to bind β-galactosidase was then examined after incubation at 76 °C. The results, shown in Fig. 4,

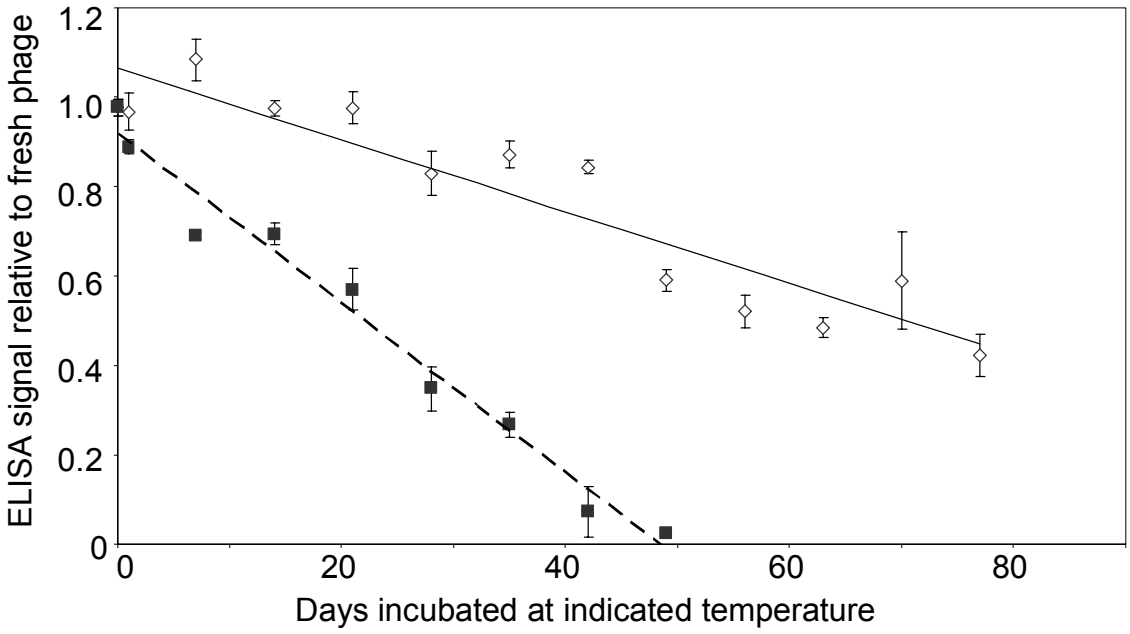

Fig.3. Phage binding of β-galactosidase after incubation at 50°C and 63°C. Phage 1G40 and antibody were fixed to the wells of EIA/RIA plates and incubated at 50°C (◊)and 63°C (■). At various intervals, plates were removed from the incubator, fresh phage was added to an unused set of wells, and β-galactosidase was allowed to bind to the phage. β-galactosidase binding was visualized by the addition of substrate ONPG. ELISA signal values (mOD/min) are relative to the signals in the wells containing fresh phage at a 200nM concentration of β-galactosidase. Error bars are indicative of standard deviations of triplicate wells.

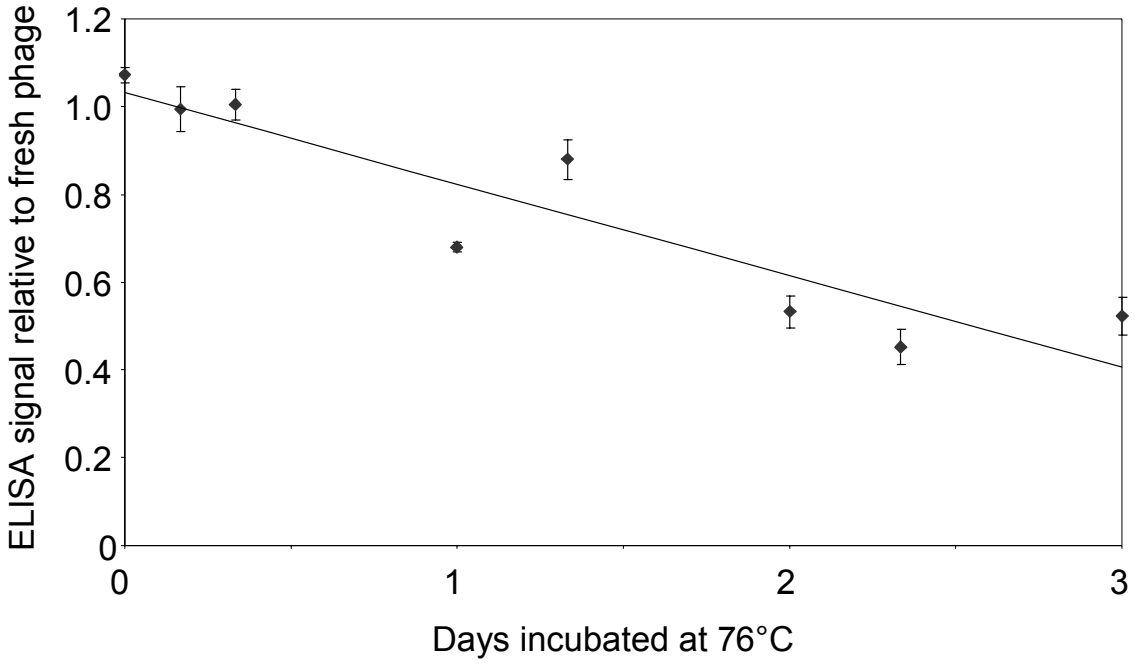

Fig.4. Phage binding of β-galactosidase after incubation at 76 °C. Phage 1G40 was fixed to the wells of EIA/RIA plates and incubated at 76 °C. At various intervals, plates were removed from the incubator, fresh phage was added to an unused set of wells, and β-galactosidase was allowed to bind to the phage. β-galactosidase binding was visualized by the addition of substrate ONPG. ELISA signal values (mOD/min) are relative to the signals in the wells containing fresh phage at a 200 nM concentration of β-galactosidase. Error bars are indicative of standard deviations of triplicate wells.

demonstrated that phage retained binding activity after short incubations at very high temperatures. While a small amount of degradation was detectable after only 4 h, phage retained detectable binding ability even after 72 h at 76 °C. The degradation constant (k) of phage at 76 °C was calculated to be 0.21/ day.

To derive the activation energy of phage probe 1G40, and develop an equation to predict its stability at temperatures other than those examined in this experiment, an Arrhenius plot was created using the data generated. The Arrhenius plot is shown in Fig. 5, with k values shown in logarithmic scale on the x-axis and 1000/absolute temperature shown on the y-axis. To calculate the activation energy from this plot, the following formula was used (Segal, 1976):

$$k = Ae^{(-Ea/RT)}$$

where k is the degradation constant, A is a constant for the particular reaction, E_a is the activation energy, R is the universal gas constant, and T is the absolute temperature. From this equation, a more useful equation was derived:

$$\text{Slope} = -Ea/2.3R$$

where slope refers to the slope of the line in the Arrhenius plot (log k vs 1/T).
Using this equation, the activation energy of phage 1G40 was determined to be 31, 987 cal/mol - a value corresponding to the disruption of peptide bonds. Thus, the stability of the landscape phage probes is comparable to the stability of isolated peptides. For example, the activation energy of the 37 amino acid peptide pramlintide, as derived from the Arrhenius equation is even lower than determined for phage - 21,900 cal/mol (Kenley

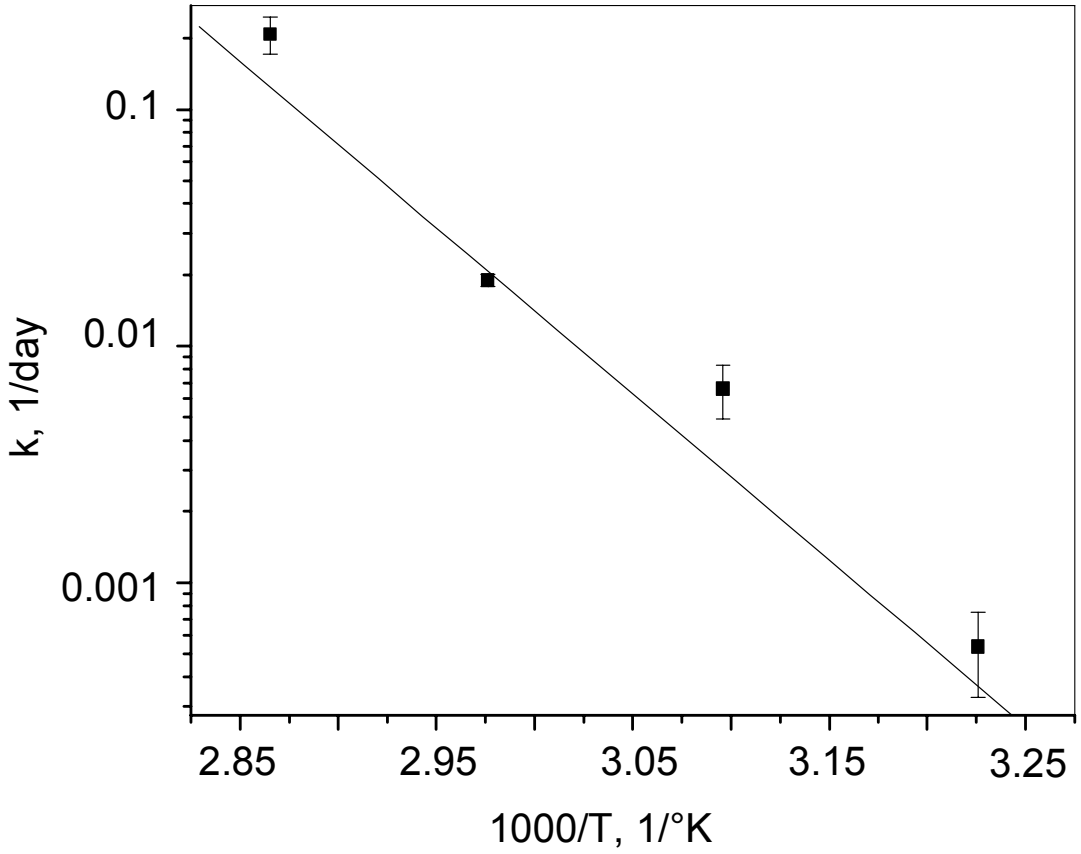

Fig.5. Arrhenius plot for phage 1G40 bound to an EIA/RIA plate and stored in TBS. Squares represent experimental values of the rate constant of thermal degradation (k) at various temperatures (plotted as the reciprocal of the absolute temperature). The line derived from this plot is described by the equation $y = -7.022x + 19.215$ ($P = 0.041$).

et al., 2000). Using the Arrhenius plot, it was also possible to estimate the half life of phage at 25 °C as ~29 years.

The instability of the monoclonal antibody examined in this work is in agreement with previously published data on the thermostability of monoclonal antibodies. The purpose of examining a monoclonal antibody in this study was to provide a direct comparison of its stability versus stability of a phage probe in identical conditions. The monoclonal antibody examined here appeared to be more stable than others which have been previously examined. This was most likely due to the conditions used, in which antibody was bound to a plate rather than stored in solution. In spite of the increased stability of fixed antibody over free antibody at high temperatures, it was still not as stable as the phage probe. While long-term studies have not been done regarding the stability of phage probes in solution, phage are known to withstand 10 minute incubations at 70 °C without any loss of infectivity. Phage 1G40 in solution has also been shown to retain full β-galactosidase binding ability after 3 years at 4 °C (Petrenko, unpublished data).

4. Conclusions

In this study, the thermostability of phage probes when fixed to plastic has been shown to be superior to that of monoclonal antibodies and approximately equal to that of peptides. The landscape phage probes are as stable at ambient and elevated temperatures as parental wild-type phage. This was demonstrated by phage 1G40, but it is expected that other phage probes have similar thermostability. The thermostability of phage

probes makes them suitable for uses in which probes need to function at ambient temperatures or withstand shipment and storage without refrigeration. While this study examined the probes in an ELISA format, they may be useful in other immunoassay and biosorbent applications as well, for detection of a wide range of antigens.

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CHAPTER III

DIAGNOSTIC PROBES FOR *BACILLUS ANTHRACIS* SPORES SELECTED FROM A LANDSCAPE PHAGE LIBRARY

1. Introduction

Spores of *Bacillus anthracis*, the causative agent of anthrax, were recently used in successful bioterrorism attacks in the United States. In instances of bioterrorism, rapid recognition of exposure is essential to allow early initiation of antibiotic treatment, which can greatly reduce mortality. Detection of *B. anthracis* spores used in a bioterrorism attack before the onset of symptoms in victims requires the development of a system to continually monitor the air for spores.

B. anthracis spores are a challenge to detect because several closely related *Bacillus* species are ubiquitous in the environment. *B. cereus*, an opportunistic human pathogen, and *B. thuringiensis*, an insecticide, are both genetically very similar to *B. anthracis* (Radnedge et al., 2003). To avoid costly false alarms, a detection system must be sensitive enough to detect low concentrations of *B. anthracis* spores, but selective enough to differentiate between *B. anthracis* and other closely related species.

There are a variety of assays available for the detection of *B. anthracis* spores (reviewed in (Ivnitski, 2003; Peruski and Peruski, 2003)) but to date none has been

adapted for real-time continuous monitoring of the environment (King et al., 2003). Immunoassay- and biosensor-based (Cunningham, 1998) detection systems are the best prospects for continuous monitoring systems, but they require specific, selective and stable diagnostic probes with which the pathogen can be detected. Antibodies and peptides can be used for this purpose, as demonstrated by numerous recent reports (Gatto-Menking et al., 1995; Bruno, 1996; Yu et al., 2000; De et al., 2002; McBride, 2003; Zahavy et al., 2003). Here we describe the selection of alternative probes from a landscape phage library, in which foreign peptides form dense organic landscapes on the surface of the phage.

Phage display libraries are constructed by the genetic modification of bacterial viruses (phages) such as M13, f1 and fd. The outer coats of these filamentous phages are composed of thousands of alpha-helical subunits of the major coat protein pVIII, which form a tube encasing the viral DNA. At the tips of the phage are several copies of each of the minor coat proteins, pIII, pVI, pVII, and pIX (reviewed in (Webster, 2001)). To create a phage display library, degenerate synthetic oligonucleotides are spliced in-frame into one of the phage coat protein genes, so that the "guest" peptides encoded by the degenerate oligonucleotides are fused to the coat protein and thereby displayed on the exposed surface of the virions (reviewed in (Smith and Petrenko, 1997)). Each phage particle displays multiple copies of one particular peptide.

In landscape phages, as in traditional phage-display constructs, foreign peptides or proteins are fused to coat proteins on the surface of the virus particle. Unlike conventional constructs, however, landscape phages display thousands of copies of the peptide in a repeating pattern, comprising a major fraction of the viral surface. The

phage body serves as an interacting scaffold to constrain the peptide into a particular conformation, creating a defined organic surface structure (landscape) that varies from one phage clone to the next. A landscape library is a huge population of such phages, encompassing billions of clones with different surface structures and biophysical properties. The landscape phage library used in this work contained random 8 amino acid peptides fused to all 4000 copies of the major coat protein of fd-tet (Petrenko et al., 1996).

Landscape phages have been shown to serve as substitutes for antibodies against various antigens and receptors (Petrenko and Smith, 2000; Romanov et al., 2001), including live bacterial cells (Petrenko and Sorokulova, 2004). These phage probes have been used in ELISA and thickness shear mode quartz sensors to detect antigens (Petrenko and Vodyanoy, 2003). Use of landscape phage as substitutes for antibodies has several advantages: they express up to 4000 copies of the binding peptide on their surface, allowing multivalent interactions with the target antigen; phage can be produced rapidly and inexpensively in large quantities; they are resistant to heat (Holliger et al., 1999), organic solvents (Olofsson et al., 1998), and many other stresses; and they can be stored indefinitely at moderate temperatures without loss of activity, or at 37ºC with only minimal loss of activity after 7 months (Brigati & Petrenko, unpublished).

Recently, a pIII phage display library was used to identify peptides which are specific and selective for spores of various *Bacillus* species, including *B. anthracis* (Knurr, 2003; Turnbough, 2003; Williams, 2003). We were interested in evaluating the prospect of using not only individual peptides, but phages themselves as "building blocks" for the development of robust and inexpensive diagnostic probes and biosorbents

(Petrenko and Sorokulova, 2004). As a first step towards this goal, we identified landscape phage clones from a phage display library that bind to *B. anthracis* Sterne spores. We then studied the specificity and selectivity of their interaction with the target selector spores in comparison with other *Bacillus* species. The results of this study indicate that the landscape phage is a prospective bioselective material that can be used as an antibody substitute in monitoring devices.

2. Materials and Methods

2.1 Strains and spore preparation

The Sterne strain of *B. anthracis* (an avirulent veterinary vaccine strain), *B. cereus* T, *B. thuringiensis* subsp. *kurstaki* were obtained from the U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD. *B. subtilis* (*trpC2*) 1A700 (originally designated 168) and *B. licheniformis* 5A36 (originally ATCC 14580) were provided by the *Bacillus* Genetic Stock Center, Ohio State University, Columbus, OH. *B. megaterium* ATCC 14581 was purchased from the American Type Culture Collection, Manassas, VA. Spores were produced by cells grown in liquid Difco sporulation medium at 37°C for 48-72 h with shaking (Nicholson, 1990). Remaining vegetative cells and cell debris were removed with a renografin step gradient as previously described (Henriques et al., 1995). Spores were stored in sterile distilled water at 4 ºC.

2.2 Phage library

The f8/8 landscape phage library, containing ~2 x 10^9 different clones, was previously described (Petrenko et al., 1996). The library was constructed by replacing amino acids E2, G3 and D4 on every copy of the pVIII coat protein of vector f8-1 (fd-tet derivative) with eight random amino acids.

2.3 Phage growth, purification, and titering

The general procedures used for recombinant phage production and analysis, including media and buffers, are detailed in *Phage Display, A Laboratory Manual* (Barbas and III Carlos F., 2001). Briefly, phage were propagated by infection of *Escherichia coli* K91 BlueKan cells (Yu and Smith, 1996), followed by growth of the infected cells for 16 h in NZY medium containing 20 mg/L tetracycline (Smith and Scott, 1993). Phage were purified by double Polyethylene glycol precipitation as previously described (Smith and Scott, 1993). The total number of viral particles present in phage preparations was determined spectrophotometrically by use of the formula (Barbas and Carlos, 2001):

$$\text{Virions/ml} = (A_{269} \times 6 \times 10^{16}) \text{ /number of nucleotides in the phage genome}$$

For the recombinant phage used in this work (9198 nucleotides), the formula:

$$\text{absorbance unit } (AU)_{269} = 6.5 \times 10^{12} \text{ virions/ml}$$

was used to determine the concentration of phage particles in a solution (physical titer). The concentration of infective phage particles (biological titer) of a phage solution was

determined by infection of starved K91BlueKan cells with the phage, followed by their spreading on a tetracycline-containing agar plate. The recombinant phage carry the gene necessary for tetracycline resistance, allowing only those cells infected by phage to form colonies on the plate (Barbas and III Carlos F., 2001). The biological titer of these recombinant phage, expressed as colony forming units (CFU) is typically 20-fold lower then the physical titer (virions/ml).

2.4 Selection of spore binding phage clones

B. anthracis Sterne spores (10^7 in 25 µl of sterile distilled water) were applied to 8 wells of a Costar flat-bottom EIA/RIA 96-well plate. The plate was centrifuged for 2 min at 550*g* to obtain an even coating of the wells with spores. The plate was incubated at 37 ºC overnight to dryness.

Wells containing *B. anthracis* Sterne spores were blocked with 1% bovine serum albumin (BSA) for 1 h at 37 ºC. The wells were then washed three times with 0.2 ml of Tris-buffered saline (TBS) containing 0.5% Tween 20 to remove unbound spores. The f8/8 phage library (1.25×10^{10} virions in 60 µl of TBS containing 0.5% Tween 20 and 0.01% BSA) was added to each well and incubated 1 h at room temperature on an orbital shaker. Non-bound phage particles were then removed, and the wells were washed 10 times with 0.2 ml of TBS containing 1% BSA. Elution buffer (100 µl, 0.2 M glycine-HCl, pH 2.2, containing 0.1% BSA) was then added to each well and incubated for 5 min at room temperature. The eluates from all 8 wells were transferred to a single microcentrifuge tube that was centrifuged for 3 min at 12,000*g* and 4 ºC to pellet any

spores. The eluate was then neutralized by the addition of 140 µl 1 M Tris-HCl, pH 9.1 and concentrated using a Centricon 100 filter to a final volume of ~100 µl. These concentrated phage clones were then propagated and purified for use in the next round of selection.

In the second round of selection, the phage clones that were selected and amplified in the first round were added to the spore-coated wells (rather than the primary phage library), but all other procedures remained the same. Likewise, in each subsequent round the phages selected and amplified in the previous round were added to the spore-coated wells. After the fourth round of selection, individual phage clones were amplified and sequenced (Haas and Smith, 1993) to determine the amino acid sequences of the displayed peptides.

2.5 Micropanning assay

B. anthracis spores (2×10^7 in sterile distilled water) were added to each well of a 96-well flat bottom microtiter plate. The plate was centrifuged for 2 min at 550*g*, and then incubated at 37 ºC overnight to dryness. BSA (1%) was added to the wells containing spores, and the plate was incubated for 1 h at 37 ºC. The wells were then gently washed with TBS containing 0.5% Tween 20. Candidate or control phage (~10^6 CFU in 50 µl of TBS) were added to separate spore-containing wells. After incubation for 1 h at room temperature, the plate was gently washed with TBS containing 0.5% Tween 20. Elution buffer (100 µl) was added to wells containing phage bound to immobilized spores, and incubated for 5 min at room temperature. The eluates from each

of these wells were transferred to sterile tubes and neutralized with 20 µl of 1 M Tris-HCl, pH 9.1. Phage input and eluate were titered as previously described.

2.6 Biotinylation of B. anthracis Sterne spores

To biotinylate spores, we mixed 160.7 µl of 1.49 mM Sulfo-NHS-LC-LC-Biotin (Pierce, cat#21338) in 2 mM sodium acetate with 3.954×10^9 spores in 1 ml of phosphate-buffered saline and incubated for 2 h at room temperature. Tris-HCl (300 µl, 1 M, pH 9) was added and the solution was incubated for 1 h to inactivate the remaining biotinylating agent. Spores were then centrifuged for 10 min at 9,000*g* and washed with water.

2.7 ELISA with biotinylated spores

Phage preparations (3×10^{10} virions in 60 µl TBS) were loaded onto a flat bottom 96-well microtiter plate and incubated at 4 ºC for 12 h. The plate was washed with TBS containing 0.5% Tween 20 using a BIO-TEK EL_x405 auto plate washer. Biotinylated *B. anthracis* spores (5×10^7 in 50µl of TBS containing 0.5% Tween 20) were applied to the phage coated wells and incubated for 2 h at room temperature on a rocker. The plate was then washed as before. Alkaline phosphatase conjugated to streptavidin (1 mg/L) was added, and the plate was incubated for 1 h at room temperature on a rocker. After a final washing step, alkaline phosphatase substrate, *p*-nitrophenylphosphate, was added to the

wells and the absorbance at 405 nm (reference wavelength 490 nm) was monitored for 1 h by an EL808 Ultra Microplate Reader (BIO-TEK Instruments, Inc.).

2.8 ELISA with non-biotinylated spores

Phage preparations (2.75 x 10^{10} virions in 55 µl of TBS) were loaded into each well of a flat bottom 96-well microtiter plate, and the plate was incubated overnight at 4 ºC. The plate was then washed as described above. *B. anthracis* spores (2.5 x 10^{8} spores in 50 µl of TBS containing 0.5% Tween 20) were added to each well and the plate was incubated for 2 h at room temperature with rocking. The plate was washed as before, and then the anti-spore monoclonal antibody BD8 (45 µl, 2.2 µg/ml) was added and the plate was incubated for 1 h. The plate was washed again, and then goat anti-mouse IgG, alkaline phosphatase conjugate (Promega, cat# S3721) (40 µl, 22.9 ng/ml) was used for detection. The substrate *p*-nitrophenylphosphate was then added, and the reaction was monitored as described above.

2.9 Coprecipitation assay

Candidate phages (200 µl, 10^{6} CFU/ml) were mixed with 2 x 10^{7} *B. anthracis* spores and/or other *Bacillus* species spores in a microcentrifuge tube and incubated for 1 h at room temperature on a rotator. Spore-phage complexes were pelleted by

centrifugation for 10 min at 3,000*g*. The pellets were gently washed with TBS containing 0.5% Tween 20, and then suspended in elution buffer and incubated for 10 min at room temperature with occasional vortexing. The spores were pelleted by centrifugation for 10 min at 9,000*g*, and the supernatant was transferred to a fresh sterile tube. The supernatant (containing phage) was then neutralized with 1 M Tris-HCl, pH 9.1. Phage input and recovery were determined by biological titering as previously described.

3. Results and Discussion

3.1 Selection of phage that bind to B. anthracis spores

The landscape library f8/8 used in this work was constructed by splicing degenerate oligonucleotides into gene gpVIII so that a foreign random octapeptide was displayed as the N-terminal portion of the major coat protein pVIII (Petrenko et al., 1996). From this library, phage clones that bind to *B. anthracis* Sterne spores were selected through a panning procedure in which the phage library was incubated with immobilized *B. anthracis* spores, non-bound phages were washed away and bound phages were eluted with mild acid. Phages that bound to spores in the initial selection procedure (a sub-library) were amplified and used as the input (instead of the primary library) in the next round of selection. This procedure was repeated for four rounds of selection. The numbers of infective phage particles present in the input, washes, and eluate of each round of selection were determined by titering. The increase in phage recovery following

each round of selection (Fig. 1) indicated an increase in the representation of phage clones in the sub-library that were capable of binding to *B. anthracis* spores. After four rounds of selection, 16 randomly picked clones were isolated, and a segment of genomic DNA encoding the displayed octapeptide was sequenced. Eleven unique peptide sequences were found that formed three related families, each with a particular motif or consensus sequence (Table 1). Family 1, with six members, was characterized by the presence of a negatively charged amino acid (E or D) at the first position, usually a proline residue at the second position, and a positively charged amino acid (R, K, or H) at the third position. Another interesting feature of this family was the frequent presence of a "migrating" dipeptide PH, which is replaced by PK in peptide 4. Family 2, with three members, contained the consensus sequence (D/E)(R/K)TXATXT. Family 3, with two members, contained the consensus sequence V(S/T)XXXSXS.

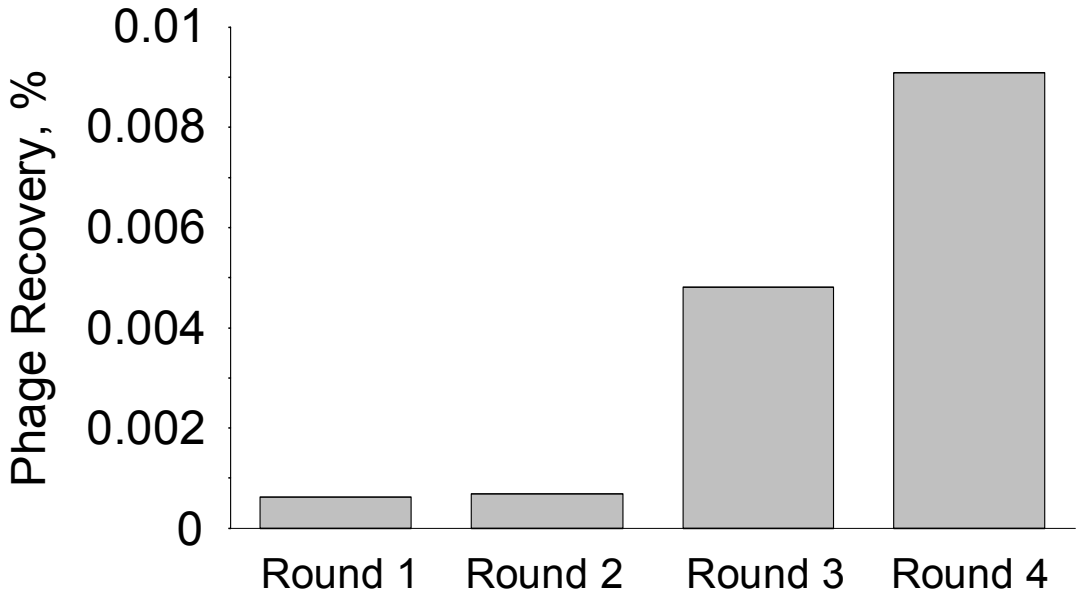

Fig.1. Phage recovery during selection. Phage input and recovery was monitored during each round of selection (x axis), and the percent recovery (y axis) was calculated [recovery, % = (phage input/eluted phage) x 100].

Table 1

Amino acid sequences of peptides carried by selected phage. Bold italics indicate common motifs.

Family 1	Family 2	Family 3
EPHPKTST	***DRT***GA***T***LT	***VS***QPA***S***P***S***
EPKPHTFS	***EKT***PV***T***AT	***VT***RNT***S***A***S***
EPRAPASL	***ERT***VA***T***TQ	
EPRLSPHS		
ET***R***VPHGA		
DA***R***GTTHM		

3.2 Specificity of phage binding to B. anthracis

We define specificity as the ability of the recombinant phage to interact with spores as a result of the presence of a specific peptide sequence displayed on the surface of the phage. To determine the specificity, we compared the binding of the selected phage clones with that of wild-type phage (f8-1, see Methods) and non-related recombinant phage from the f8-1/8mer library.

The relative binding of the isolated phage clones to *B. anthracis* spores was measured by a phage-capture assay and an ELISA. In the phage-capture assay, a procedure very similar to the selection procedure was used to determine relative binding of phage clones to immobilized *B. anthracis* spores. Briefly, selected phage clones were added to the wells of a microtiter plate that were coated with *B. anthracis* spores. After an incubation to allow binding, non-bound phage were washed away and bound phages were eluted and titered. The percent recovery was determined as a ratio of the eluted phages to input phages. As shown in Fig. 2, selected clones bind at a much higher percentage than the wild-type phage to *B. anthracis* spores in this assay.

In the ELISA, wells of a microtiter plate were coated with phage and then incubated with biotinylated *B. anthracis* spores. Alkaline phosphatase conjugated to streptavidin was then added to bind to the biotinylated spores, and *p*-nitrophenylphosphate was used to detect this binding. As shown in Fig. 3, many of the isolated phage clones bound to *B. anthracis* spores at a higher level than wild-type phage. Some clones bound strongly to *B. anthracis* Sterne in both assays, while other clones

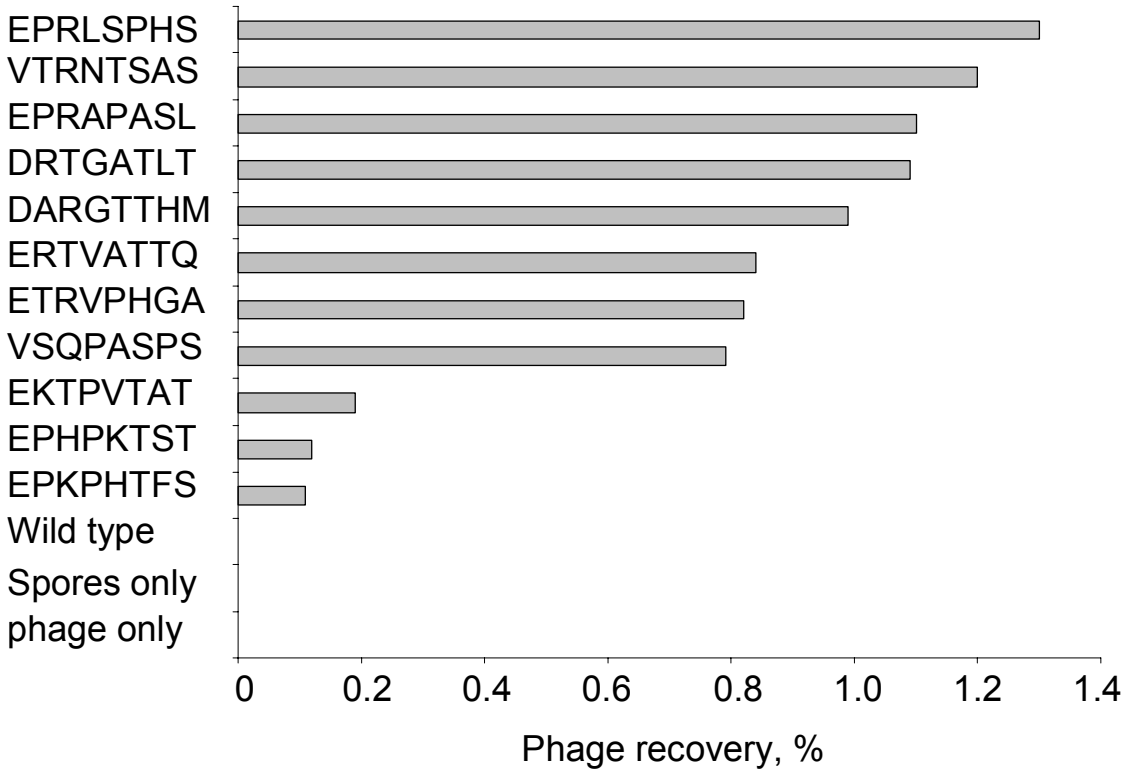

Fig.2. Binding of selected phages to *B. anthracis* Sterne immobilized on a microtiter plate. y-axis, amino acid sequences of peptides carried by selected phage clones; x-axis, percent of phage recovered during the micropanning assay [recovery, % = (phage input/eluted phage) x 100].

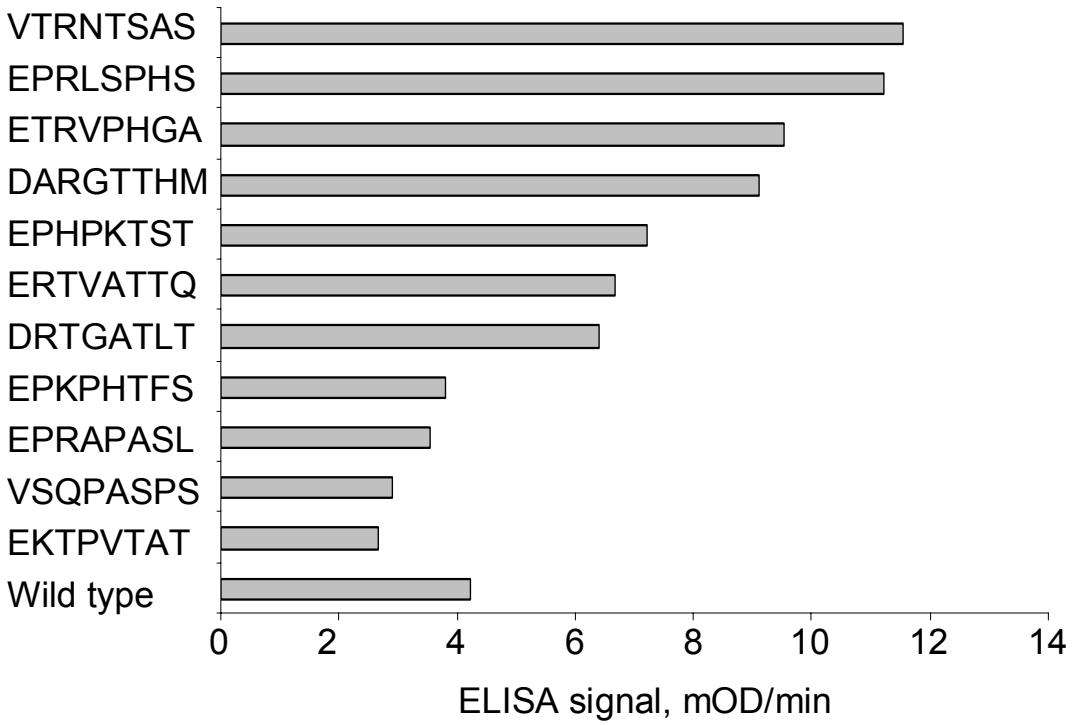

Fig. 3. Binding of biotinylated *B. anthracis* Sterne spores to selected and control phages immobilized on a microtiter plate. y-axis, amino acid sequences of peptides carried by selected phage clones; x-axis, ELISA signal in mOD/min.

gave inconsistent results between the two assays. This is not completely unexpected because in the ELISA phage are fixed to the plate and spores are captured from solution, while in the phage-capture assay spores are fixed to the plate and phage are bound from solution. Thus, in these tests phage could adopt different conformations allowing monovalent or multivalent interactions with spore receptors, as was demonstrated in binding experiments in which β-galactosidase from *E.coli* served as a model multivalent analyte (Petrenko and Vodyanoy, 2003).

To confirm that the ELISA results were not attributable to biotinylation of contaminants of the spore preparation, an ELISA was done in another format, with antibodies specific for *B. anthracis* spores (Williams and Turnbough, 2004). Fig. 4 exemplifies specific binding of phages carrying the peptide VTRNTSAS to spores, revealed with monoclonal antibody BD8. It is clear from this experiment that phages were capturing spores and not some other contaminants of the spore preparation.

3.3 Selective binding of phage to spores of B. anthracis Sterne

We defined selectivity as the ability of a recombinant phage clone to preferentially interact with the selector in comparison with other potential targets. To determine the selectivity of phage probes for the selector *B. anthracis* spores versus spores of other *Bacillus* species, a coprecipitation assay was used. Phage displaying the peptides DARGTTHM, EPRLSPHS, and VTRNTSAS were initially examined because of their high binding in both the ELISA and micropanning assays. Phage displaying the peptides DRTGATLT and EPRAPASL were tested because of the high binding they

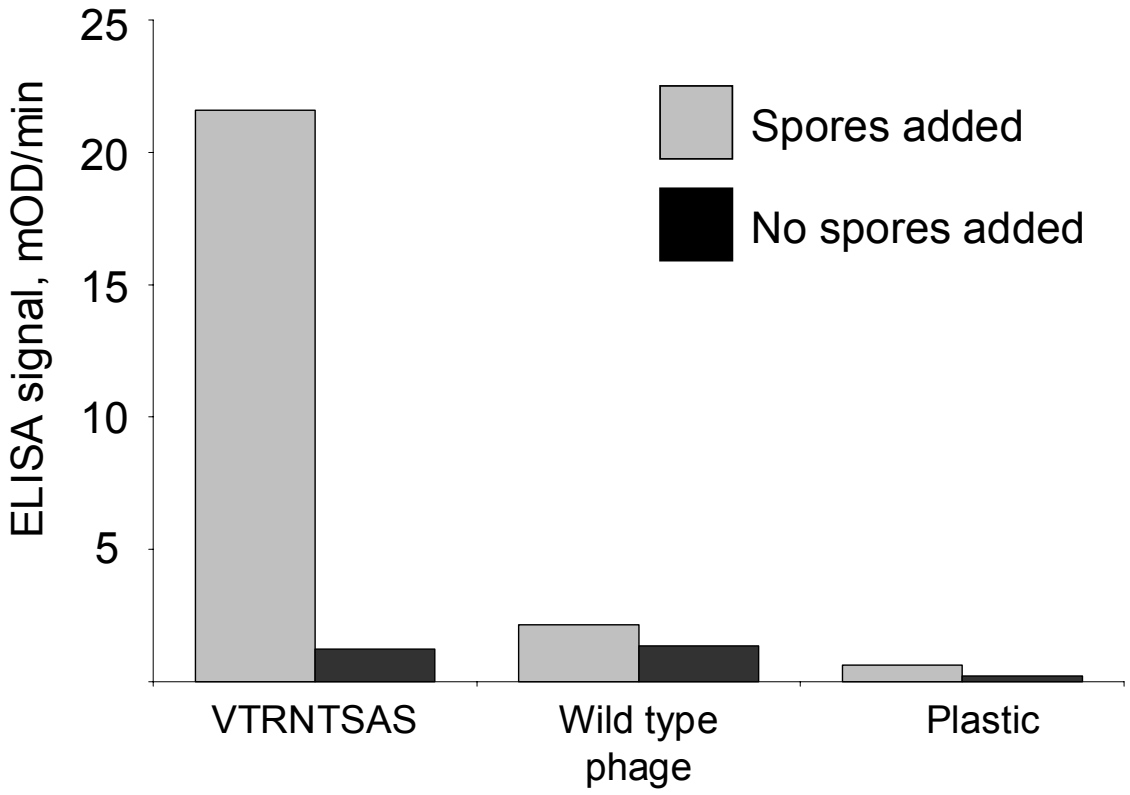

Fig.4. Binding of non-biotinylated *B. anthracis* Sterne spores to selected and control phage immobilized on a microtiter plate. y-axis, ELISA signal in mOD/min; x-axis, amino acid sequences of peptides carried by selected phage clones, or controls (wild-type phage, plastic).

demonstrated in the phage-capture assay, and phage carrying the peptide ETRVPHGA were tested because of the high binding they demonstrated in the ELISA. In the coprecipitation assay, these phages were mixed individually with spores of various *Bacillus* species. After incubating, spores were collected by a low speed centrifugation, so that only phage bound to spores would be found in the pellet. Phages without spores were used as a control to ensure that the phage were not aggregating and precipitating on their own. Initial tests were done with distant relatives of *B. anthracis*; *B. megaterium*, *B. subtilis*, and *B. licheniformis*. Some clones exhibited very low binding to these distant relatives, while others bound them nearly as well as *B. anthracis* We found that the phage carrying the peptide DARGTTHM bound to *B. anthracis* Sterne 75-fold better than to *B. megaterium*, 25-fold better than to *B. subtilis*, and 50-fold better than to *B. licheniformis.* Phage carrying the peptide EPRLSPHS bound to *B. anthracis* Sterne 43-fold better than to *B. megaterium*, 39-fold better than to *B. subtilis*, and 70-fold better than to *B. licheniformis.* Phage carrying the peptide ETRVPHGA bound to *B. anthracis* Sterne 24-fold better than to *B. megaterium*, 24-fold better than to *B. subtilis*, and 12-fold better than to *B. licheniformis* (Fig. 5, A,C,E). Phage clones from families 2 and 3 exhibited much lower selectivity and were not examined further. The three above mentioned phage probes that did not cross react strongly with distant relatives of *B. anthracis* were examined further for binding to spores of close relatives of *B. anthracis*, namely *B. cereus* and *B. thuringiensis*. All three phages demonstrated preferential binding to *B.anthracis*, but considerable binding to the close relatives: the phage bearing the peptide DARGTTHM bound to *B. anthracis* 3.7-fold better than to *B. cereus* and 2.1-fold better than to *B. thuringiensis*; the phage bearing the peptide EPRLSPHS bound to

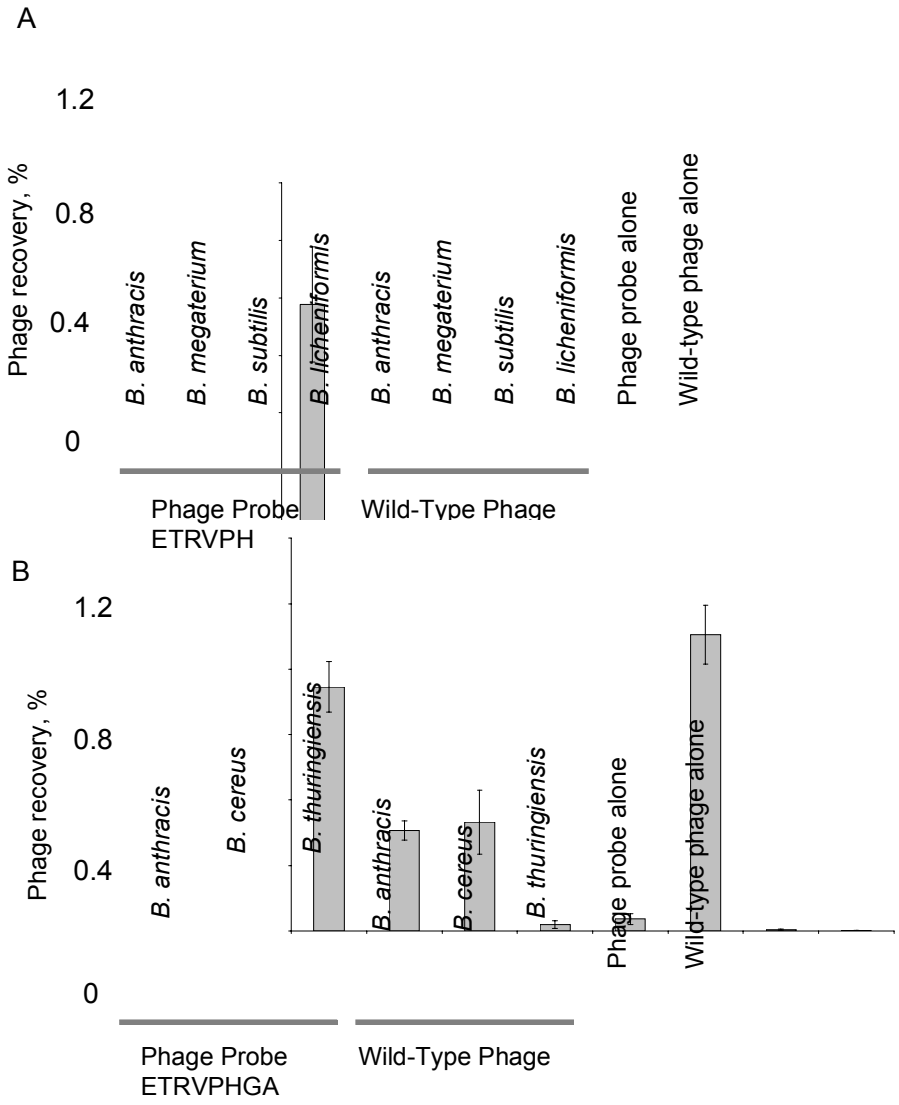

Fig. 5. Binding of selected phage clones to *Bacillus* spores in a coprecipitation assay. y-axis, species of spores that were mixed with selected phage; x-axis, percent of phage recovered by coprecipitation with spores [recovery, % = (phage input/eluted phage) x 100]. (a), (c), and (e) depict binding of phage carrying the peptides ETRVPHGA, DARGTTHM, and EPRLSPHS, respectively to *B. anthracis*, *B. megaterium*, *B. subtilis*, and *B. licheniformis*. Parts (b), (d), and (f) depict binding of phage carrying the peptides ETRVPHGA, DARGTTHM, and EPRLSPHS, respectively to *B. anthracis*, *B. cereus*, and *B.thuringiensis*.

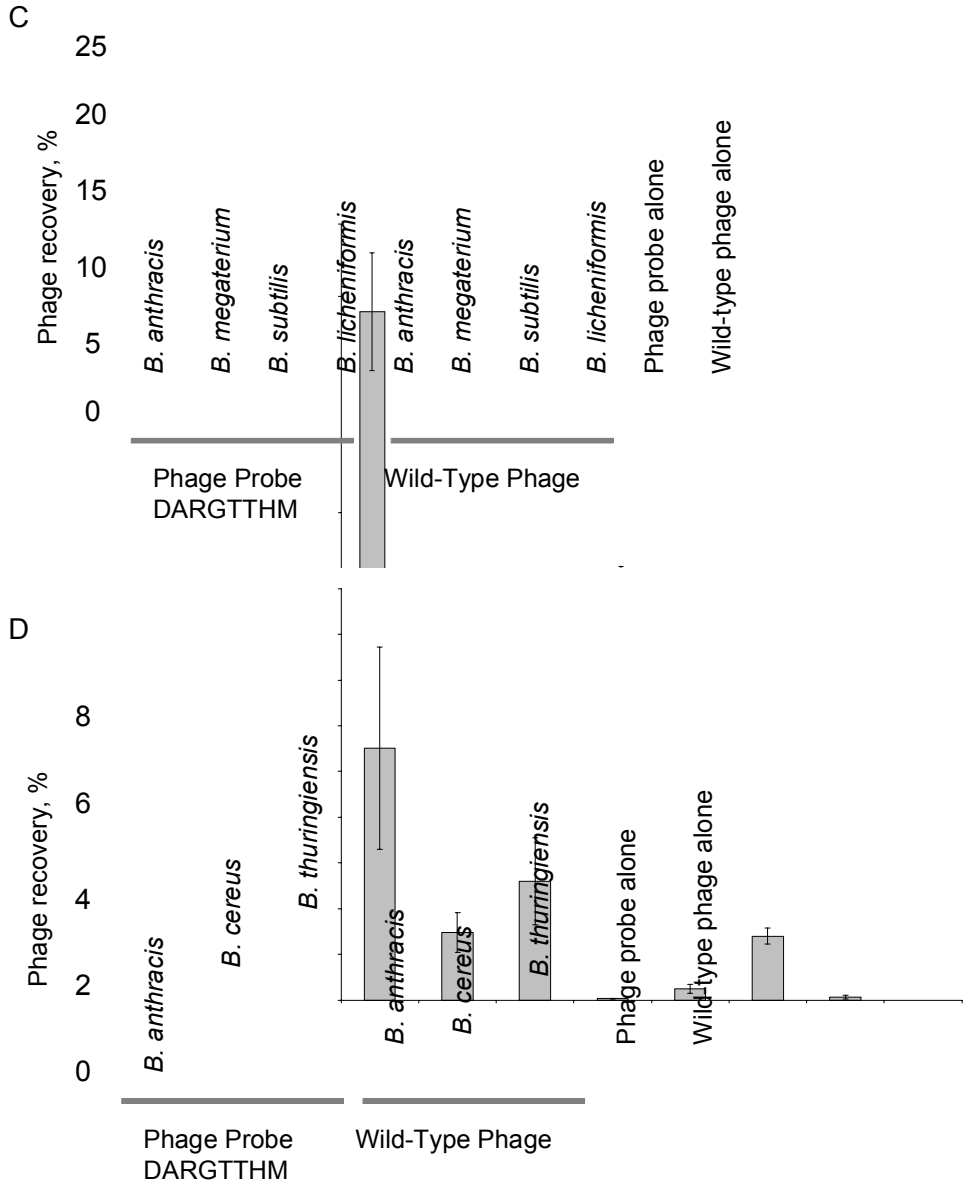

Fig. 5. continued

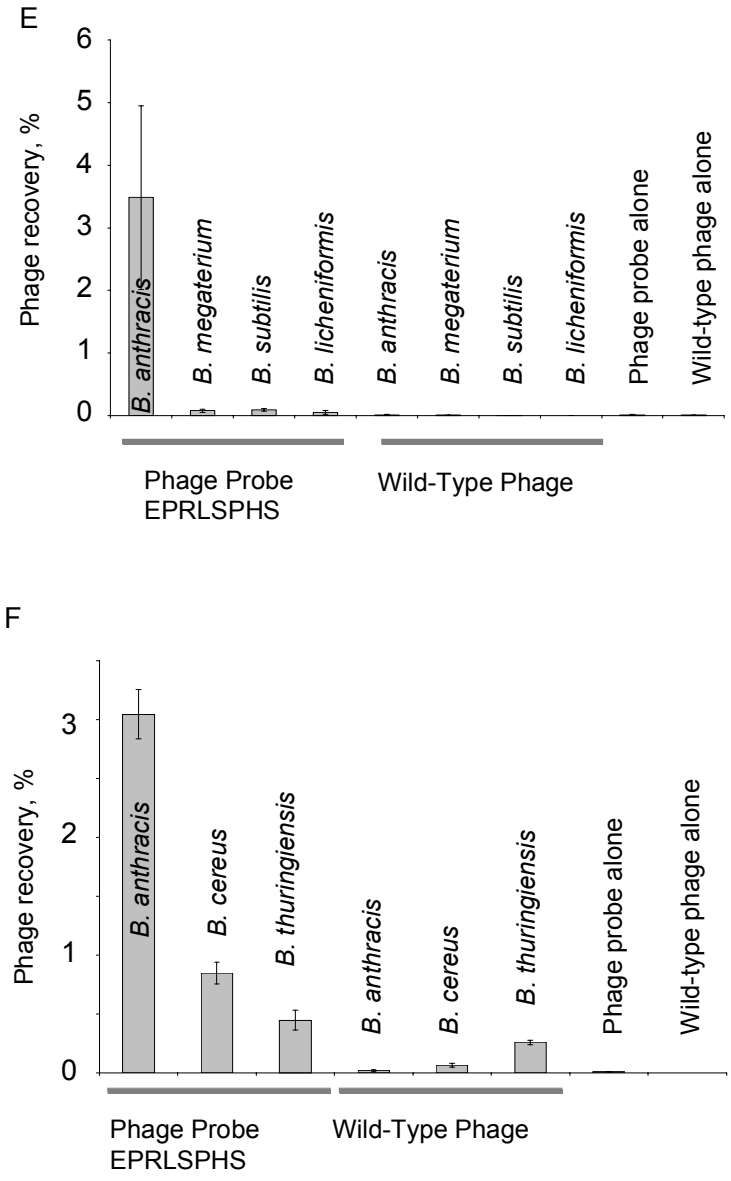

Fig. 5. continued

B. anthracis 3.5-fold better than to *B. cereus* and 6.9-fold better than to *B. thuringiensis*; the phage bearing the peptide ETRVPHGA bound to *B. anthracis* 2.4-fold better than to *B. cereus* and 2.2-fold better than to *B. thuringiensis* (Fig. 5, b,d,f).

4. Conclusions

Monitoring of the environment for biological threats, such as spores of *B. anthracis*, requires probes that bind to biological agents and ensure their separation, purification and detection (Petrenko and Sorokulova, 2004). Combinatorial probe technology is based on the principle that biological agents have unique surface markers that can bind organic molecules (probes) recruited from diverse combinatorial libraries through screening or selection procedures (Petrenko and Vodyanoy, 2003). The effectiveness of this new technology has been illustrated by development of diagnostic probes for various bacterial and viral agents (Petrenko and Sorokulova, 2004), including spores of *B. anthracis* (Williams, 2003). Although the peptides and antibodies identified through phage display in these examples are useful in diagnostic assays, landscape phage probes may be better suited for the exacting requirements of environmental monitoring, in which robust, selective, strong, and inexpensive binders capable of operating in severe environmental conditions are needed. Our studies of landscape phages, recombinant filamentous phages displaying 4,000 foreign random peptides on their surface, showed that these phages are well suited for obtaining durable, specific probes and biosorbents (Petrenko et al., 1996; Smith and Petrenko, 1997; Petrenko and Smith, 2000; Petrenko and Vodyanoy, 2003; Petrenko and Sorokulova, 2004). Filamentous phages are probably

the most stable natural nucleoproteins capable of withstanding high temperatures (up to 80^{o}C), denaturing agents (6-8 mol/L urea), organic solvents (50% alcohol, acetonitril, etc.), mild acids (pH 2), and alkaline solutions. Phage-derived probes inherit the extreme robustness of the wild-type phage and allow fabrication of bioselective materials by self-assemblage of phages or their composites on metal, mineral or plastic surfaces (Petrenko and Vodyanoy, 2003).

In this work we demonstrated that the landscape phage library contains many potential probes for surface markers of *B. anthracis* spores. Phage probes were isolated in a nonbiased multistage selection procedure using immobilized spores as a selector. We have characterized three landscape phage clones that bound to *B. anthracis* spores and did so at a higher level than to other species of *Bacillus* spores. We expect that these phage could serve as biosorbents and diagnostic probes for monitoring of *B. anthracis* spores by various platforms in which antibodies or peptides have previously been used. Since the isolated phage bind strongly to *B.anthracis* spores, they may be used for separation and purification of spores before their identification by PCR, immunoassays, flow cytometry, or other methods.

We recognize that these probes are not completely ideal for identification of *B. anthracis* spores because they cross-react with spores of *B. cereus* and *B. thuringiensis*. In future experiments, cross-reacting clones such as these may be removed from the library in a biased selection procedure by depletion against competing strains, as was previously demonstrated (de Kruif et al., 1995; Boel et al., 1998; de Greeff et al., 2000). The depleted library could serve as a reservoir of probes for unique *B. anthracis* spore markers. If necessary, affinity of probes towards the target agent may be adjusted by

mutagenesis of phage and selection of the spore-binders in more stringent conditions (reviewed by (Petrenko and Sorokulova, 2004)). We believe that this phage evolution technique could be used to gradually enhance the performance of the selected phage-derived probes, allowing them to serve as robust substitutes for antibodies in concentration and detection systems for biological threat agents.

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CHAPTER IV

EVOLUTION OF A LANDSCAPE PHAGE DISPLAY LIBRARY DURING SELECTION OF TARGET-BINDING CLONES

1. Introduction

Phage display libraries have been used to identify peptides and phage landscapes which bind specifically to a variety of targets (reviewed by (Kay, 1996; Smith and Petrenko, 1997; Szardenings, 2003; Petrenko and Sorokulova, 2004). Hundreds of publications have appeared in the last ten years describing the selection of target-specific peptides from phage display libraries, but very few have done any in-depth analysis of the selection procedure itself. In this paper we describe the evolution of a phage display library during several different selection procedures.

To identify target-specific peptides from the billions of peptides represented in a library, a target antigen is typically fixed to a solid support, and then the phage display library is added in solution to allow phage carrying target-specific peptides to bind. Phage that do not bind to the target are washed away, and then bound phage are eluted, amplified, and applied to an identical preparation of target antigen for a second round of affinity selection. After several rounds of selection, individual phage clones are isolated and the peptide responsible for their affinity to the target is determined by sequencing the corresponding coding sequence in the phage's DNA. Once the peptide sequences are

determined, the search for dominant motifs and highly fit clones begins. Traditionally, dominant motifs have been identified by looking at a limited number of sequences and lining them up manually. The presence of dominant motifs within the selected phage borne peptides has been assumed to indicate that these motifs mediate binding, and that clones carrying these motifs are the strongest target binders in the set of selected clones. However, no studies have been done to prove that the peptides containing a dominant motif are actually the strongest binders.

The recent development of programs designed for analysis of groups of peptides encouraged us to investigate the changes occurring in a phage display library during a selection procedure, and also to look for ways to predict the best target binders from the group of phage clones identified through selection. The programs we used in this research are part of the Receptor Ligands Contacts (RELIC) bioinformatics server for combinatorial peptide analysis (Mandava et al., 2004). These programs are designed to search for common motifs within a set of sequences, estimate the diversity of groups of peptides (overall and at each position of the peptide), calculate the prevalence of specific amino acids in a group of peptides, and calculate the information content of individual peptides within a group.

We studied the selection of target-specific landscape phage clones in depth, examining changes in the library occurring during each round of selection following different types of library depletion and target-bound phage elution. By sequencing a portion of the library after each round of selection, we were able to monitor changes in diversity and prevalence of amino acids, occurrence of rare peptides in the library, and development of common motifs throughout the selection process. We also examined the

influence of propagation on the appearance of dominant motifs in a selection procedure. Our goal was to determine if monitoring of the library could allow prediction of the success of a selection procedure as a whole, and also of the performance of individual phage clones.

In this work, the process of selection of target-binding phage clones was examined using a landscape phage display library. In a landscape phage display library, degenerate synthetic oligonucleotides are spliced in-frame into the pVIII coat protein gene, so that the foreign peptides encoded by the degenerate oligonucleotides are fused to the major coat protein pVIII and thereby displayed as a landscape of 4,000 copies on the exposed surface of the virions (reviewed in (Smith and Petrenko, 1997)). These alien peptides are arranged in a repeating pattern, comprising a major fraction of the viral surface. The phage body thus may serve as an interacting scaffold to constrain the peptide into a particular conformation, creating a defined organic surface structure (landscape) that varies from one phage clone to the next. A landscape library is a huge population of such phages, encompassing billions of clones with different surface structures and biophysical properties.

The landscape phage library f8/8 which was used in this work contains random 8 amino acid peptides fused to all 4000 copies of the major coat protein of fd-tet phage (Petrenko et al., 1996). The random peptides carried by the phage in this library are encoded by Gnk $(nnk)_6$ nnG, where n = G, A, T or C and k = G or T. The diversity of this library was previously estimated to be 0.0750 (where a library with diversity of 1.0 contains all possible amino acids in equal proportions at all positions), which is only slightly lower than the diversity of pIII based libraries (Makowski and Soares, 2003).

The diversity of all peptide phage display libraries is significantly below 1.0 because of uneven numbers of codons encoding each amino acid, and biological censorship (Rodi et al., 2002).

The target used in this study was spores of *B. anthracis* Sterne. The need to identify robust probes which bind specifically to *B. anthracis* spores has lead to numerous investigations in which ligands have been selected from phage display libraries (Zhou et al., 2002; Knurr, 2003; Turnbough, 2003; Williams, 2003). Recently, we selected landscape phage probes that bind specifically to *B. anthracis* Sterne (Brigati et al., 2004). We performed this study of the evolution of a landscape phage display library during our effort to select a new set of *B. anthracis* binding clones that do not cross-react with other *Bacillus* species.

The many common antigens shared by *Bacillus* species make the random selection of a probe which binds to *B. anthracis* but not to close relatives *B. cereus* and *B. thuringiensis* unlikely. Our initial studies yielded probes that cross-reacted with spores of these closely related species. In an effort to eliminate this cross-reactivity, new phage probes were selected by a subtractive phage display procedure. In subtractive phage display, phage clones that bind to common antigens are removed by pre-incubation of the library with non-target antigens before panning on the target, or co-incubation of the library with target and non-target antigens. Subtractive procedures have been used to identify phage-borne probes which can be used to differentiate between different types of cells (Van Ewijk et al., 1997; Stausbol-Gron et al., 2001; Belizaire et al., 2003; Samoylova et al., 2003) and bacteria (de Greeff et al., 2000; Zhou et al., 2002). It was during our efforts to use subtractive phage display to identify new sets of *B. anthracis*

binding landscape phages that we monitored the evolution of the landscape phage display library f8/8.

As expected, different methods of depletion and the use of different elution buffers resulted in the selection of unique sets of clones, all of which were different from those clones selected in the original non-biased procedure. The diversity of the library was found to decrease with each round of selection, while the number of isolated clones with high information content (low probability of being selected at random) tended to increase following each round of selection. These two factors, along with elimination of vector phage, may be useful in determining the success of a selection procedure as a whole with this type of phage display library. However, neither the information content of its displayed peptide, nor its prevalence in the library, could predict the target binding strength of a phage clone. Unfortunately, this means that the only way to identify the best target binder in a group of selected clones is to perform target binding assays with each clone.

2. Materials and methods

2.1 Bacterial strains

The Sterne strain of *B. anthracis* (an avirulent veterinary vaccine strain), *B. cereus* T, and *B. thuringiensis* subsp. *kurstaki* were obtained from the U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD. *B. subtilis* (*trpC2*) 1A700 (originally designated 168) and *B. licheniformis* 5A36 (originally ATCC 14580) were provided by the *Bacillus* Genetic Stock Center, The Ohio State University,

Columbus, OH. *B. megaterium* ATCC 14581 was purchased from the American Type Culture Collection, Manassas, VA. Spores were produced by cells grown in liquid Difco sporulation medium at 37°C for 48-72 h with shaking (24) or by cells grown on solid Difco sporulation media (liquid media with agar added). Remaining vegetative cells and cell debris were removed with a renografin step gradient as previously described (25). Spores were stored in sterile distilled water at 4 ºC.

The f8/8 landscape phage library, containing ~2×10^9 different clones, was previously described by Petrenko, et. al. (14). The library was constructed by replacing amino acids E2, G3 and D4 on every copy of the pVIII coat protein of vector f8-1 (fd-tet derivative) with eight random amino acids.

2.2 Phage library and phage manipulations

The general procedures used for recombinant phage production and analysis, including media and buffers are detailed in "Phage Display, A Laboratory Manual" (Barbas and III Carlos F., 2001). Briefly, phage were propagated by infection of *E. coli* K91 BlueKan cells (Yu and Smith, 1996), followed by growth of the infected cells for 16 h in NZY medium containing 20 mg/L tetracycline (Smith and Scott, 1993). Phage were purified by double PEG precipitation as previously described (Smith and Scott, 1993). The total number of viral particles present in phage preparations was determined spectrophotometrically using the formula (Barbas and III Carlos F., 2001):

virions (vir)/ml = ($A_{269} \times 6 \times 10^{16}$) /number of nucleotides in the phage genome

For the recombinant phage used in this work (9198 nucleotides), the formula:

$$\text{absorbance unit } (AU)_{269} = 6.5 \times 10^{12} \text{ vir/mL}$$

was used to determine the concentration of phage particles in a solution (physical titer). The concentration of infective phage particles (biological titer) of a phage solution was determined by infection of starved K91BlueKan cells with the phage, followed by their spreading on a tetracycline-containing agar plate. The recombinant phage carry the gene necessary for tetracycline resistance, allowing only those cells infected by phage to form colonies on the plate (Barbas and III Carlos F., 2001). The biological titer of these recombinant phage (expressed as colony forming units or CFU) is typically 20-fold lower then the physical titer (vir/mL).

2.3 Selection of spore-binding phage probes

B. anthracis Sterne spores (2×10^7 in 50 μL of sterile distilled water) were applied to 8 wells of a Costar flat-bottom EIA/RIA 96-well plate. The plate was incubated at 37ºC overnight to dryness.

Meanwhile, the f8/8 phage library (2.5×10^{11} vir in 400 μL Tris –buffered saline (TBS)/0.5% Tween 20/0.01% BSA) was depleted of phage which bind to common *Bacillus* antigens. The library was mixed with 5×10^8 *B. cereus* spores and incubated for 30 minutes on a rotator. The spore/phage solution was then centrifuged for 10 minutes at 3,000 *g* to pellet the spores and any spore-bound phage. The supernatant ("depleted library") was transferred to a new tube and stored at room temperature, or depleted further against *B. thuringiensis* and *B. subtilis* before use.

Wells containing *B. anthracis* Sterne spores were blocked with 200 µL 1% bovine serum albumin (BSA) for 1 h at 37 ºC. The wells were then washed 3 times with 0.2 mL TBS/ 0.5% Tween 20 to remove unbound spores. The depleted f8/8 phage library (50 µL) was added to each well and incubated 1 h at room temperature with gentle rocking. Non-bound phage particles were then removed, and the wells were washed 10 times with 0.2 mL TBS/ 0.01% BSA/ 0.5% Tween 20. Acidic elution buffer (100 µL, 0.2 M glycine-HCl, pH 2.2/0.1% BSA) was then added to each well and incubated for 5 min at room temperature. The eluate from all 8 wells was transferred to a single microcentrifuge tube. The wells were washed once with 100 µL TBS and this wash was added to the eluate. The tube containing the acidic eluate and wash was centrifuged for 3 min at 12,000 *g* to pellet any spores. The eluate was then neutralized by the addition of 150 µL 1 M Tris-HCl, pH 9.1 and concentrated using a Centricon 100 filter to a final volume of ~100 µL.

Meanwhile, 40 µL deoxycholate elution buffer (2 % sodium deoxycholate, 10mM Tris, 2mM EDTA, pH adjusted to 8.0 with HCl) was added to each of the 8 spore-containing wells. After 30-minute incubation at room temperature with gentle rocking, the deoxycholate eluate was transferred to a microcentrifuge tube and stored at 4ºC. The phage contained in each of the eluates (acidic and deoxycholate) were then propagated and purified for use in the next round of selection. In the second round of selection, the phage clones that were selected and amplified in the first round were added to separate sets of spore-coated wells (phage from the acidic eluate were kept separate from phage from the deoxycholate eluate). From the wells into which amplified phage from the acidic eluate were added, only phage eluted by acid were retained and amplified. From

the wells into which amplified phage from the deoxycholate eluate were added, only phage eluted by deoxycholate were retained. Likewise, in each subsequent round the phages selected and amplified in the previous round were added to spore-coated wells, keeping the phage eluted with acidic buffer separate from those eluted with deoxycholate buffer. Following each round of selection, the DNA encoding the peptide inserts of ~100 individual phage clones was sequenced to determine the amino acid sequences of the displayed peptides (Sorokulova et al, submitted).

2.4 Identification of fast-growing phage clones

Separate aliquots of starved K91 Blue Kan *E. coli* cells (10 μl each) were inoculated with unique phage clones from round 2 of selection with acidic elution buffer after depletion with three *Bacillus* species (from colonies of infected *E. coli* cells) by transfer with a sterile toothpick. Cells were incubated for 10 min at room temperature to allow infection, then 180 μl NZY, 0.2 μg/ml tetracycline was added to each tube and tetracycline resistance was allowed to develop for 45 minutes at 37ºC. The contents of all tubes were then added to one flask of NZY containing 20 μg/ml tetracycline, and grown overnight with shaking at 200 rpm at 37ºC. Phage were purified and titered as is usual in propagation. Phage clones were randomly chosen for sequencing and analysis.

2.5 Analysis of selected phage probes

The peptide sequence analysis programs included in RELIC (Receptor Ligand Contacts) (Rodi et al., 2002; Makowski and Soares, 2003; Rodi et al., 2004) were used to evaluate the original landscape phage display library (used in the first round of selection)

and the phage clones sequenced following each round of selection. DIVAA was used to calculate the amino acid diversity at each position of the random peptides in the library and following each round of selection. AAFREQ was used to calculate the frequency of occurrence of each amino acid in the library and following each round of selection. INFO was used to calculate the information content of each peptide found on the surface of the landscape phage in the library and following each round of selection.

2.6 Phage capture ELISA

B. anthracis Sterne spores (2×10^7 in 50 μL of sterile distilled water) were applied to half of the wells of a Costar flat-bottom EIA/RIA 96-well plate. The plate was incubated at 37 ºC overnight to dryness. All wells of the plate were blocked with 100 μL 0.1% bovine serum albumin (BSA) for 1 h at room temperature. The wells were then washed 3 times with 200 μL TBS/ 0.5% Tween 20. Each purified phage clone (50 μL, 1×10^{12} vir/ml) was added to one well coated with spores and one empty well of the plate, and allowed to bind for 1 h at room temperature with gentle rocking. All wells of the plate were then washed 5 times with 200 μL TBS/ 0.5% Tween 20. Biotinylated anti-fd IgG (0.12 μg/mL, 45 μL/well) was then added to each well and allowed to bind for 1 h at room temperature with gentle rocking. After 5 washes, 40 μL dilute alkaline phosphatase streptavidin conjugate (APSA) (1.25 μg/ml APSA in 0.05 M Tris-HCl, 0.15 M NaCl, 0.1% Tween 20, 0.1% BSA) was added to each well and allowed to incubate 1.5 h at room temperature with gentle rocking. After a final washing step, alkaline phosphatase substrate, *p*-nitrophenylphosphate, was added to the wells and the absorbance at 405 nm

(reference wavelength 490 nm) was monitored for 1 h using an EL808 Ultra Microplate Reader (BIO-TEK Instruments, Inc.).

2.7 Phage capture assay

A phage-capture assay was performed as previously described (Brigati et al., 2004), to examine the binding ability of a portion of the selected phage clones. Briefly, *B. anthracis* spores (2×10^7 in sterile distilled water) were added to each well of a 96-well flat bottom microtiter plate, which was incubated at 37 ºC overnight to dryness. BSA (1%) was added to the wells containing spores, and the plate was incubated for 1 h at 37 ºC. The wells were then gently washed with TBS/0.5% Tween 20. Candidate or control phages (~10^6 CFU in 50 μL TBS) were added to separate spore-containing wells. After incubation for 1 h at RT, the plate was gently washed with TBS/0.5% Tween 20. Elution buffer (100 μL) was added to wells containing phages bound to immobilized spores, and incubated for 5 min at RT. The eluates from each of these wells were transferred to sterile tubes and neutralized with 20 μL 1 mol/L Tris-HCl, pH 9.1. Phage input and eluate were titered as previously described.

3. Results and discussion

Phage clones that bind to *B. anthracis* Sterne spores were selected from the f8/8 landscape phage display library through a biased panning procedure. In this procedure, the phage library was depleted of phages that bound strongly to spores of different *Bacillus* species by pre-incubation with *B. cereus* only, or by sequential incubation with *B. subtilis*, *B. thuringiensis* and *B. cereus*. Phages bound to the spores were removed,

and the depleted library was then incubated with *B. anthracis* Sterne spores absorbed to the wells of a microtiter plate. Non-bound phages were washed away and bound phage were eluted, first with an acidic buffer, and then with a deoxycholate buffer. The two elution buffers were used to break up hydrophilic and hydrophobic interactions, respectively. Phage which bound to spores in the initial selection procedure (a sub-library) were amplified and used as the input (instead of the primary library) in the next round of selection. This procedure was repeated for four rounds of selection. Following each round of selection, the DNA encoding the foreign peptides carried by a random subset of phage clones was sequenced.

Table 1 summarizes the phage clones analyzed during the selection procedure. In early rounds of selection, there were typically a large number of vector phage and phage carrying short (<8 amino acids) peptides. The presence of vector and phages carrying short peptides is expected to decrease with each round of selection if target-specific phages are being selected. Vector phages and phages with short inserts grow more rapidly than phages carrying foreign 8mer peptides and therefore will increase in prevalence in the library if there is not a strong selection for target binders between amplification steps. As seen in the table, vector presence generally decreased, except in the deoxycholate eluate selection after depletion with *B. cereus.* This particular selection procedure was not successful in isolating strong binding, target-specific clones (data not shown).

Table 1
Results of each round of selection for each biased selection protocol.

Depletion: *B. cereus*, Selection: Acidic Elution Buffer				
Round	Number of clones	Number of clones carrying 8mer peptides	Number of unique 8mer peptides	Number of vector / short insert clones
1	104	91	91	13
2	100	92	80	8
3	94	90	54	4
4	89	86	25	3
Depletion: *B. cereus*, Selection with Deoxycholate Elution Buffer				
1	91	87	87	4
2	83	64	47	19
3	79	54	33	25
4	96	59	19	37
Depletion: *B. subtilis, B. thuringiensis,* & *B. cereus*, Selection: Acidic Elution Buffer				
1	88	71	53	17
2	100	92	70	8
3	99	96	40	3
4	96	87	26	9
Depletion: *B. subtilis, B. thuringiensis,* & *B. cereus*, Selection: Deoxycholate Elution Buffer				
1	70	46	34	24
2	93	76	29	17
3	46	40	14	6
4	46	46	11	0

In early rounds of selection, there are very few phage clones with such a high prevalence in the library that they will be found more than once in the 40 – 100 clones sequenced. The number of unique clones found in the sequenced sample of the library typically decreases with each round, as a dominant peptide motif becomes apparent. It is clear in table 1 that this occurred in each selection procedure.

The phage clones isolated in this set of experiments were all different from the phage clones previously isolated in a non-biased selection procedure with the same target. Table 2 shows the amino acid sequences of the peptides carried by phage isolated in the non-biased procedure (Brigati et al., 2004), along with those carried by phage isolated in each of the biased procedures after four rounds of selection. In each selection procedure, a unique dominant motif became apparent after 4 rounds of selection. In the *B. cereus* depletion / acidic elution procedure, the motif VDRXSXXX was found on nearly 40% of phages after four rounds of selection. In the *B. cereus* depletion / deoxycholate elution procedure, the peptide EFTARPSS was found on 38.5% of phages after four rounds of selection. In the *B. subtilis*, *B. cereus*, and *B. thuringiensis* depletion / acidic elution procedure, the peptide AGRAGGGV was found on 35% of phages after four rounds of selection, and a similar peptide (50% identity) was found on 7% of phages. In the *B. subtilis*, *B. cereus*, and *B. thuringiensis* depletion / deoxycholate elution procedure, the peptide EDGRGGTA was found on 32% of phages, the peptide EDRVFPST was found on 24% of phages after four rounds of selection. In addition to the phage clones carrying the dominant motif, each selection procedure allowed the identification of a supplemental set of unique spore-binding clones. While none of these

Table 2

Amino acid sequences of peptides carried by selected phage clones.

A	B	C	D	E
DARGTTHM	ASRSSGAL	AARSVTDS	AARQPAGM	ARSSPSLA
DRTGATLT	ATRTAPTS	ATRTTSPM	AARQPMAS	DDAGRGTP
EKTPVTAT	DPRAAVTA	DKLSSSGT	ADRVPYPS	DLGDRSQG
EPHPKTST	PARDPVNM	DPSAYSRA	**AGRAGGGV**	DTRETGSS
EPKPHTFS	VARSTGDS	DSRALIAP	**AGR**GPGLP	**EDGRGGTA**
EPRAPASL	**VDRGSATS**	DYDAVMRT	AHREMPQG	**EDRVFPST**
EPRLSPHS	**VDR**S**S**TTT	DYPTKTNS	ANRVPPTS	EERQNPSG
ERTVATTQ	**VDR**T**S**SPA	**EFTARPSS**	ARPSDGLS	ENSRSTQM
ETRVPHGA	VGREGGAV	EPKMQAAQ	ARSAGPLP	EPRLAGDQ
VSQPASPS	VPRPDATS	EPSRGPGS	ARSALPSS	GPESQTAS
*VTRN*TSAS	VPTRTPQG	ERAATDQT	ARSNPALT	VSDRVSPA
	VPTSRADQ	ESRGVGTE	ARSQPALS	
	VPTTRETS	EYERASQG	ASRDGAVM	
	VQPTAAPP	VPPPSSST	ASRTSGLP	
	VRPTPTDT	VSRTQEHV	ATRPASSM	
	VSDRGTAT	VTDARTPA	AVRDQPNL	
	VSPTQQQT	*VTRN*PSDA	EPMRDMAS	
	VSREAAAS	VTSASSSQ	GLRTTPNT	
	VSRMESTP	VVRGSDGA	GQRTPPTT	
	VSTRPTET		VDRGTTLS	
	VTPRADST		VDRTPPSQ	
	VTRGSMNT		VGRANPSS	
	*VTRN*PAAS		VNQTAQPA	
	VVREPTHS		VSRIPSET	
			VTNANSPS	
			VTRDLSSS	

Phage clones were isolated after four rounds of selection under the following conditions; non-biased selection with acidic elution buffer (A), depletion against *B. cereus* and acidic elution buffer (B), depletion against *B. cereus* and deoxycholate elution buffer (C), depletion against *B. subtilis*, *B. thuringiensis*, and *B. cereus* and acidic elution buffer (D), and depletion against *B. subtilis*, *B. thuringiensis*, and *B. cereus* and deoxycholate elution buffer (E). Peptides in bold contain the dominant motif isolated in each selection procedure. Peptides in italics contain motifs isolated with more than one selection procedure.

clones appeared following more than one selection procedure, a few clones from different procedures did carry common motifs. For example, VTRNTSAS appeared in the non-biased selection procedure, VTRNPAAS appeared following four rounds with acidic elution following depletion with *B. cereus*, and VTRNPSDA appeared following four rounds with deoxycholate elution following depletion with *B. cereus*.

To gain insight into the role propagation plays in influencing the outcome of a selection procedure, the growth of a set of clones from round two of the *B. subtilis*, *B. cereus*, and *B. thuringiensis* depletion / acidic elution selection procedure was examined. Separate aliquots of starved K91 Blue Kan *E. coli* cells were infected with 69 unique clones, then mixed together and grown in a liquid culture overnight just like phage that are propagated between rounds of selection. The amplified phage were purified, 100 randomly picked clones were sequenced, and the results of this sequencing are shown in table 3. Clearly, certain clones became more prevalent during growth, including those carrying the peptides ETRADTMP, AAPRDMPA, and AGRGPGLP. Two of these clones, carrying ETRADTMP and AAPRDMPA were apparently eliminated from the library due to poor binding in round 3. The phage clone displaying AGRGPGLP, however, became a dominant clone in rounds 3 and 4. This dominance was most likely due at least in part to its favorable growth properties, because it was not found to be one of the strongest target-binders identified in this selection procedure. The phage clone which increased in prevalence the most between rounds 2 and 3 of this selection procedure, and was also the dominant clone in round 4, carried the peptide AGRAGGGV. This did not seem to be a fast growing clone, as only one out of the 100

Table 3

Changes in the phage population during propagation

Insert	N	Insert	N	Insert	N
ETRADTMP	14	ANGRQPPT	1	DDSRTALT	0
AAPRDMPA	9	ARPSDTML	1	DERTSVMG	0
AGRGPGLP	9	ARSAGPLP	1	DGRANTLT	0
DIREPQGV	6	ASRPLPSQ	1	DGRGPGLP	0
ARPNSSIA	4	AVRPPSST	1	DLERPSGM	0
EERMTPLP	4	DDRPGSTA	1	DLGSGAAP	0
AMRPGADT	3	DGRPEMVP	1	DPTAQAIS	0
APPRDMNP	3	DGTERSSP	1	DVSRGPEL	0
ARPSDGLS	3	DTGGPPGP	1	EAGRDQVQ	0
DSTPASYT	3	EDRTQPGP	1	EATPQHLS	0
ESAGNATS	3	GAPPQAVE	1	EEMRNPLP	0
EVRDATTA	3	VDRDTAMV	1	EGRDPPIM	0
ADGQTQGA	2	VPIRTPES	1	ENREAISA	0
AHREMPQG	2	VQSAETPE	1	EPRDPSYL	0
ARGPTPIP	2	VSPQAGTA	1	EQREGSPP	0
DERPVPTS	2	AARQPAGM	0	ESSPESTT	0
DVTSPTTA	2	AARPQPSV	0	ETTPPAVP	0
EERLPQGS	2	ADRVPYPS	0	GELQQPSG	0
EHTRTEYQ	2	AMRPTSPM	0	GHTTSPVQ	0
EPRELGAL	2	APSYSSIA	0	GQAVPAIE	0
AERDGDAV	1	ASRPGALA	0	VDDRSLIA	0
AHARPELT	1	DDPRAPST	0	VGDRPVHS	0
AGRAGGGV	1	DDPRLSSV	0		

N = the number of times a phage clone carrying the given sequence was found out of 100 randomly chosen phage clones in the amplified population.

clones sequenced after propagation carried this sequence. Although this clone was also not one of the strongest target-binders identified, it was a stronger binder than the clone carrying AGRGPGLP. This data indicates that dominant phage clones are selected based on a combination of their binding and growth properties, meaning that fast-growing mediocre binders compete with slower growing strong binders for dominance.

The diversity of amino acids at each position in the landscape phage display library and the sublibraries created after each round of selection was evaluated using the RELIC program DIVAA. Diversity is a statistical measure of the proportion of the 20 possible amino acids that are observed at any given position (Rodi et al., 2004). If a position in the peptide is populated by equal proportions of all 20 amino acids, then the diversity is 20/20 or 1.0. If a position in the peptide is populated by only one amino acid in all sequences, then the diversity is 1/20 or 0.05. In Fig. 1, the diversity of the library and sublibraries at each of the eight positions of the foreign peptide are shown for two of the selection procedures. The diversity in positions one and eight is significantly lower than in the other positions because the random peptide is encoded by Gnk $(nnk)_6$ nnG, where n = G, A, T or C and k = G or T. In all selection procedures, the diversity in all positions was generally found to decrease with each round of selection, although some positions retained more diversity than others. Positions 4 and 5 in particular seemed to maintain more diversity than other positions. In rounds where one or two dominant phage clones made up a large percentage of the library in later rounds, the DIVAA program could not accurately calculate the diversity of amino acids because there were not enough unique peptide sequences. Intuitively, it is clear that if one or two peptides

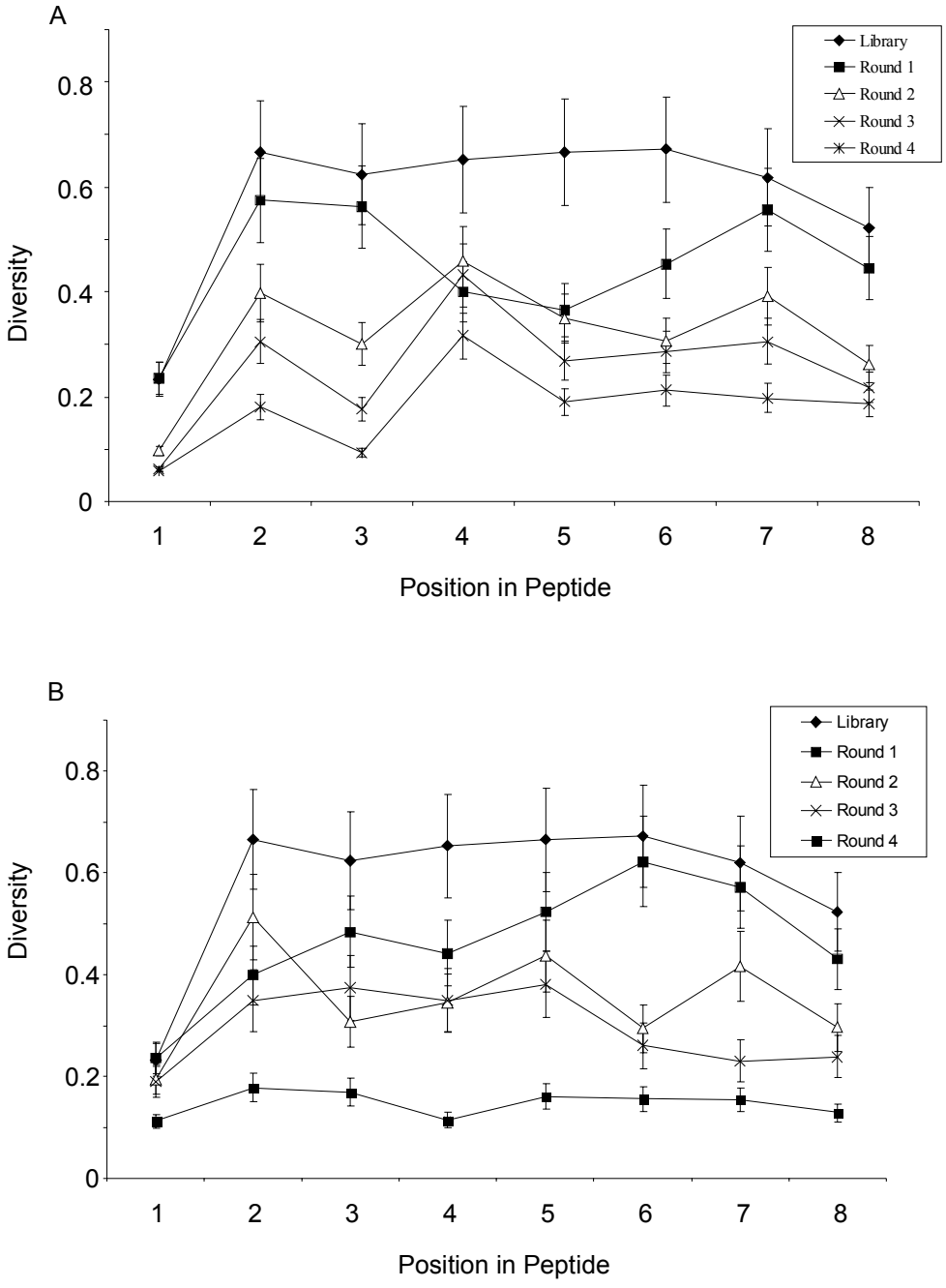

Fig.1. Diversity of amino acids in the primary phage display library and after each round of selection with (A) acidic elution buffer or (B) deoxycholate elution buffer. Data shown here are from the procedure in which *B. cereus* was used for depletion.

dominate the library, the diversity of amino acids at each position in the library will likely be lower than if many unique sequences are present.

The frequency of amino acid occurrence in the random peptides carried by the isolate phage clones was also evaluated, using the RELIC program AAFREQ. This program determines the frequency of an amino acid in the population of peptides as a whole, and also lists the number of occurrences of a given amino acid at each position in the peptide (Mandava et al., 2004). The program throws out duplicate sequences, so where a dominant peptide is present the results are skewed, but still useful for general evaluations. The amino acid frequency in the landscape phage library prior to selection, as well as following each round of selection, is shown in Fig. 2. It is immediately noticeable that cysteine is absent from this library. All other amino acids are represented in the library, although some are virtually eliminated during the selection procedure. In both biased selection procedures in which phage were eluted from *B. anthracis* spores with an acidic elution buffer, there was a decrease in leucine, lysine, phenylalanine, and histidine. In both procedures, there was an increase in arginine, but threonine and valine only increased in one of the procedures. Phage eluted with deoxycholate carried peptides with less leucine and histidine, but more arginine, serine, and tyrosine than the library. Glycine also increased during one of the deoxycholate elution procedures.

To further characterize the phage clones isolated after four rounds of selection with the four different biased selection procedures, the information values of the peptides carried by these clones were determined. The information values of the peptides were calculated using the RELIC program INFO. The information value of a given peptide is equal to the negative logarithm of the probability of its natural occurrence. The lower the

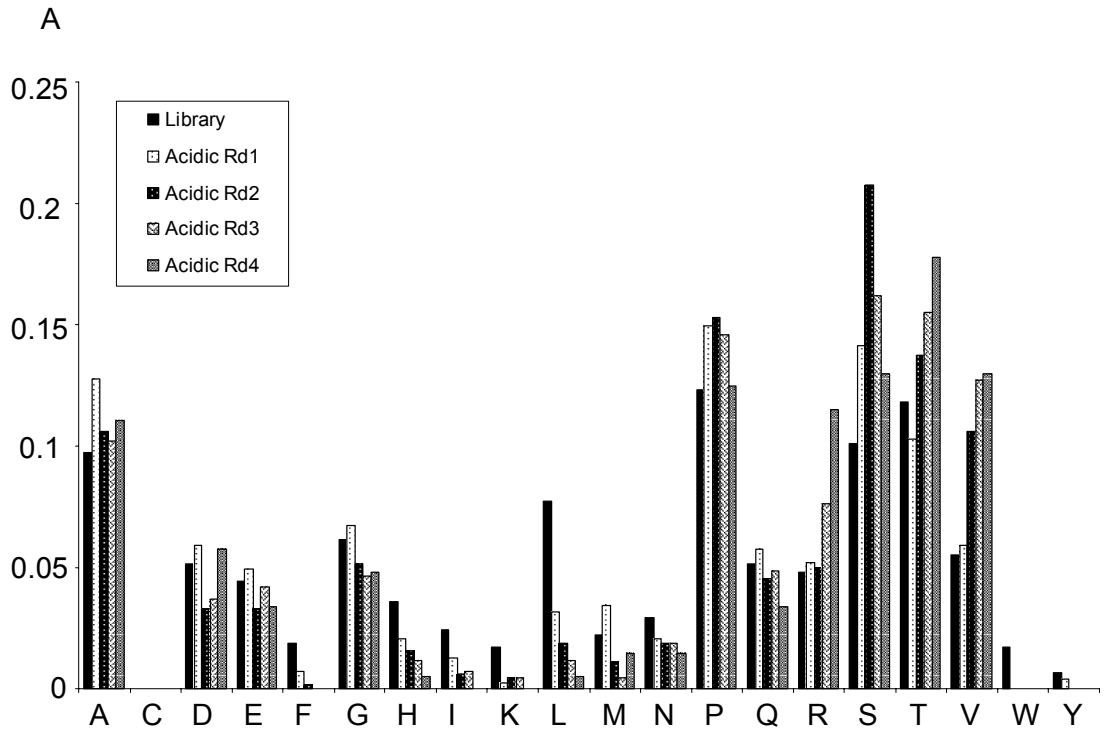

Fig. 2. Frequency of occurrence of amino acids in the primary phage display library and after each round of selection. (A) Selection using acidic elution buffer following depletion of the library against *B. cereus* (B) Selection using deoxycholate elution buffer following depletion of the library against *B. cereus* (C) Selection using acidic elution buffer following depletion of the library against *B. subtilis, B. thuringiensis* and *B. cereus* (D) Selection using deoxycholate elution buffer following depletion of the library against *B. subtilis, B. thuringiensis* and *B. cereus.*

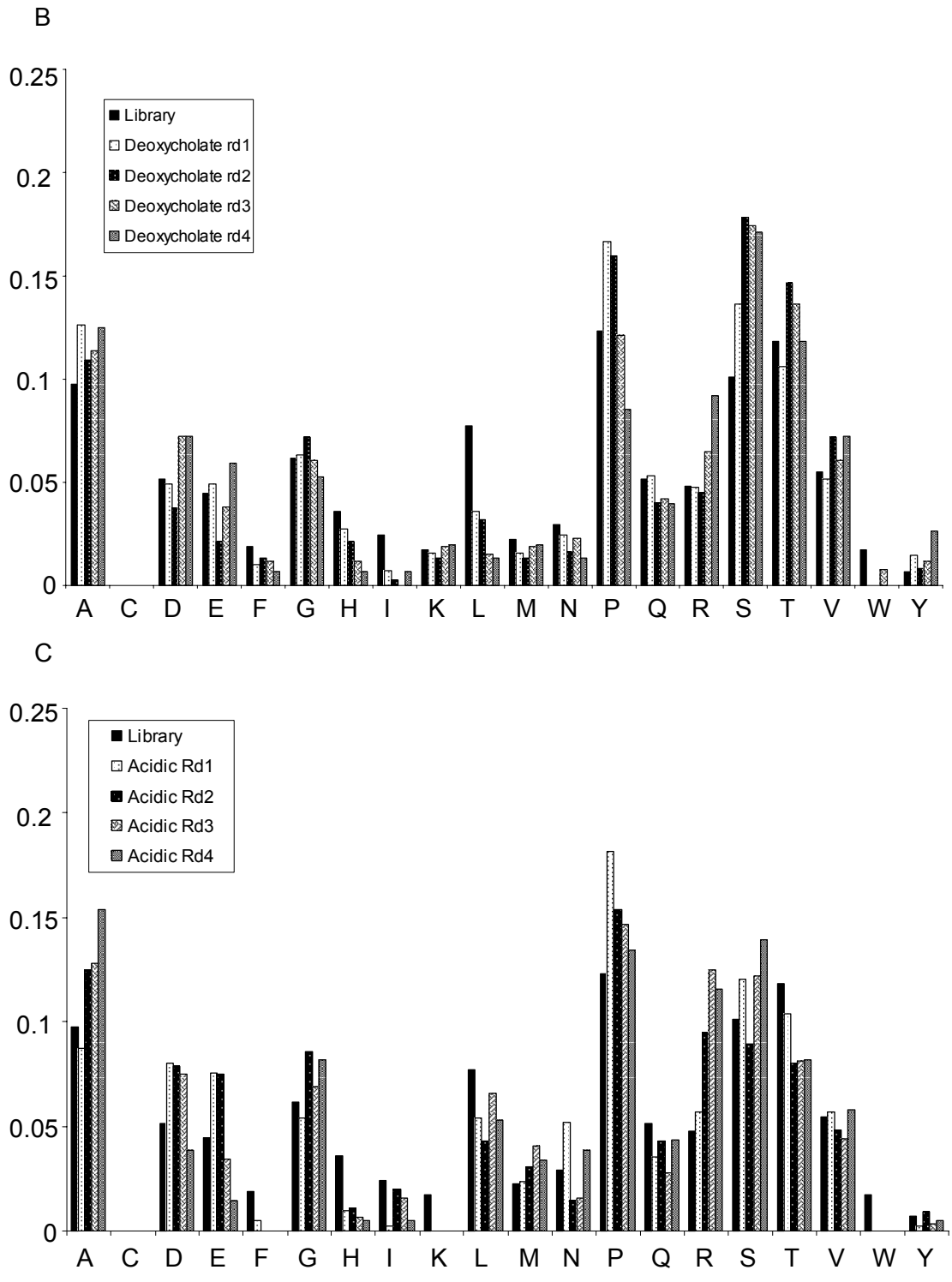

Fig. 2. Continued

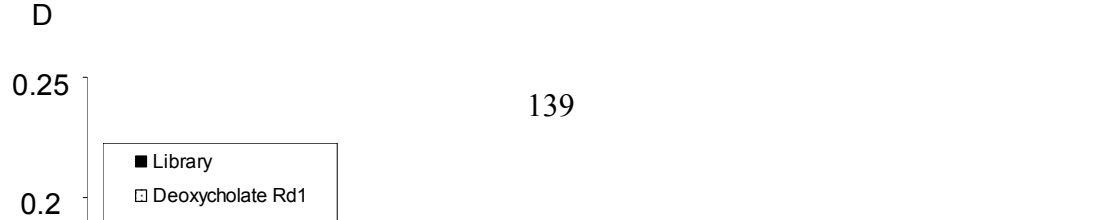

Fig.2. continued

probability of occurrence is, the higher the information value will be, and the higher the probability of occurrence is, the lower the information value will be. Peptides with high information values are rare in the parent library, and therefore it is expected that their presence after selection is due to affinity to the target. The INFO program calculates the probability of a peptide's random occurrence by multiplying the probability of each amino acid occurring at each position (based on the position specific frequencies of amino acids in the parent library) within the peptide (Mandava et al., 2004).

Histograms representing the information content of the peptides present after four rounds of selection versus the information content of the initial library are shown in Fig. 3. It is clear from these histograms that in each selection procedure a subset of high information peptides was selected. The actual information value of these peptides, and the relative occurrence of high information peptides in the library varied with the selection procedure. Histograms created based on the peptides sequenced after each round of selection showed that there was a change in the information content of the library after each round of selection, and that higher information peptides tended to occur more frequently with each round of selection (data not shown).

The information content and prevalence [(number of copies of clone carrying a given peptide)/(number of clones analyzed) × 100] of the peptides isolated after the fourth round of selection with acidic elution buffer are shown in Tables 4 and 5. One of the goals of this work was to determine if information or prevalence could be used to predict the binding ability of selected phage clones. If one of these factors correlated well with binding ability, it would greatly reduce the amount of labor needed to analyze the results of a selection procedure. To determine if there was any correlation between

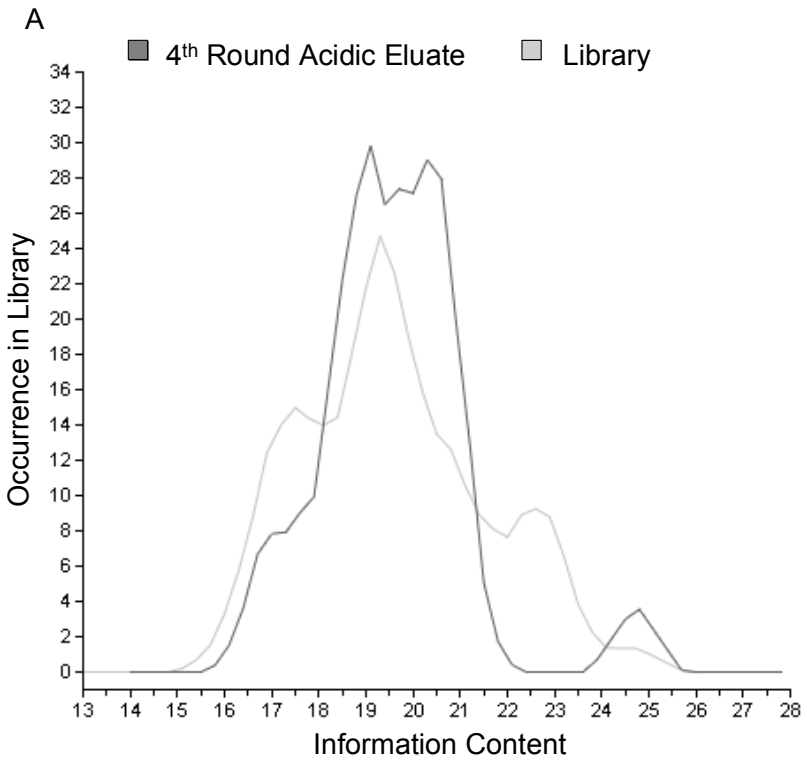

Fig.3. Histograms demonstrate the information values of peptides contained in the library before selection and after various selection schemes. (A) Selection using acidic elution buffer following depletion of the library against *B. cereus* (B) Selection using deoxycholate elution buffer following depletion of the library against *B. cereus* (C) Selection using acidic elution buffer following depletion of the library against *B. subtilis, B. thuringiensis* and *B. cereus* (D) Selection using deoxycholate elution buffer following depletion of the library against *B. subtilis, B. thuringiensis* and *B. cereus.*

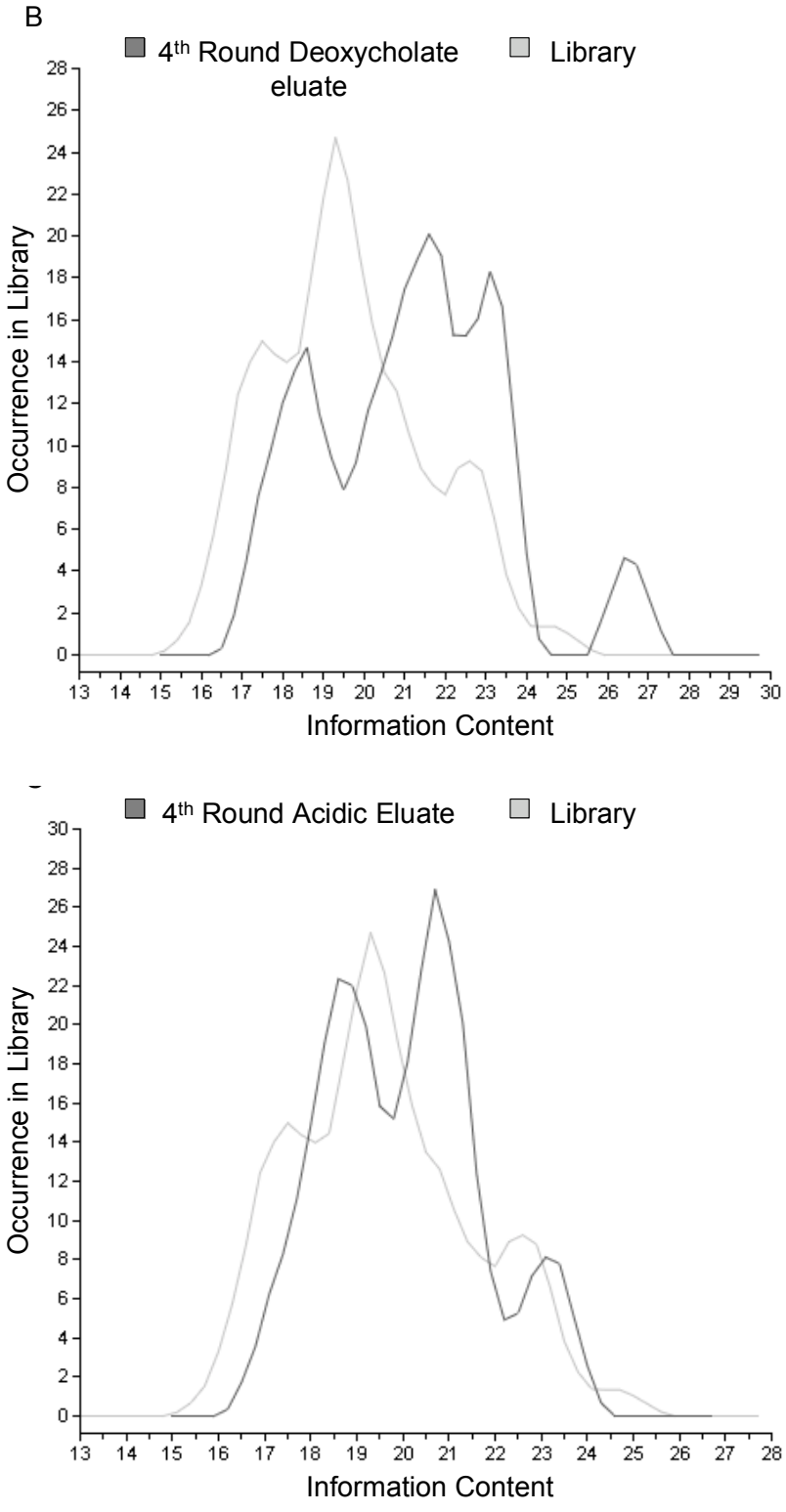

Fig.3. continued

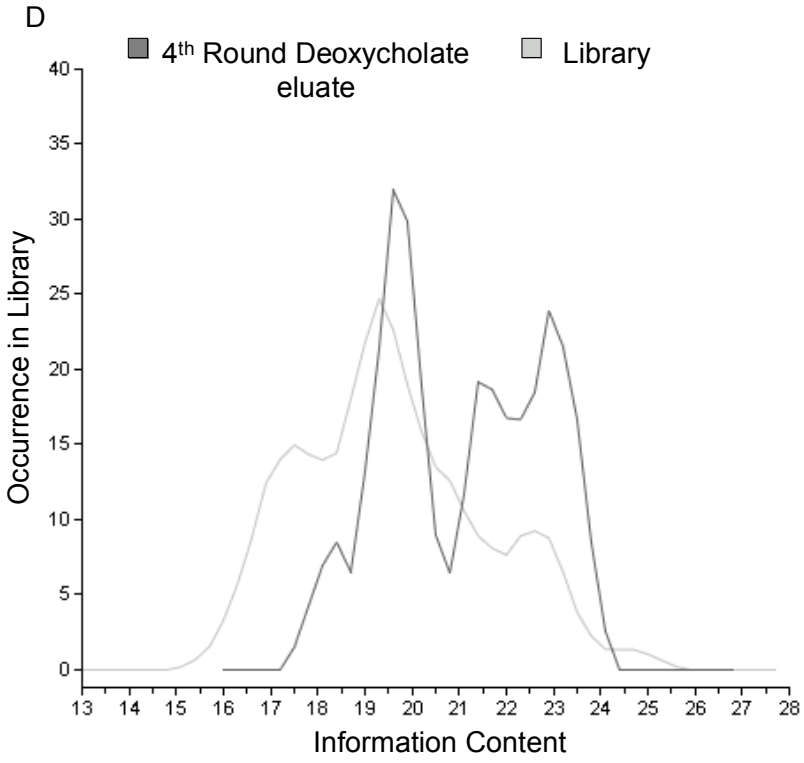

Fig.3. continued
Table 4

Information and prevalence of peptides present after four rounds of selection with acidic elution buffer (depletion against *B. cereus*).

Peptide	Information	Prevalence after 4 Rounds (% library)
VGREGGAV	24.722	2.2
VSDRGTAT	21.203	14.6
VDRTSSPA	20.847	11.2
VRPTPTDT	20.569	1.1
VVREPTHS	20.533	2.2
VSREAAAS	20.474	6.7
VDRGSATS	20.405	16.9
VARSTGDS	20.292	1.1
DPRAAVTA	19.941	1.1
VSRMESTP	19.827	2.2
VQPTAAPP	19.786	1.1
VTRNPAAS	19.750	1.1
VSPTQQQT	19.347	1.1
VPRPDATS	19.019	1.1
VTPRADST	19.006	2.2
VSTRPTET	18.960	2.2
VPTSRADQ	18.901	1.1
VTRGSMNT	18.859	1.1
VPTTRETS	18.431	1.1
VDRSSTTT	18.313	11.2
ASRSSGAL	18.277	1.1
VPTRTPQG	17.515	1.1
ATRTAPTS	17.150	2.2
PARDPVNM	16.701	3.4

Table 5
Information and prevalence of peptides present after four rounds of selection with acidic elution buffer (depletion against *B. subtilis, B. thuringiensis* and *B.cereus*).

Peptide	Information	Prevalence after 4 Rounds (% library)
AGRAGGGV	25.282	35.4
ASRDGAVM	23.385	2.1
EPMRDMAS	22.377	1
VGRANPSS	22.083	2.1
AARQPAGM	21.680	6.2
VTNANSPS	21.609	1
VSRIPSET	21.380	2.1
AARQPMAS	20.784	1
VTRDLSSS	20.356	2.1
AGRGPGLP	19.914	7.3
ARPSDGLS	19.886	2.1
VNQTAQPA	19.853	1
ARSAGPLP	19.827	4.2
VDRGTTLS	19.635	2.1
ARSQPALS	19.616	1
ARSALPSS	19.375	1
ARSNPALT	19.365	1
ATRPASSM	19.347	1
ASRTSGLP	18.910	6.2
GQRTPPTT	18.808	1
VDRTPPSQ	18.623	1
AHREMPQG	18.235	1
AVRDQPNL	18.101	5.2
ANRVPPTS	17.237	2.1
ADRVPYPS	16.712	1
GLRTTPNT	15.529	1

information content and binding ability of selected phage clones, a portion of the phages were amplified, purified, and evaluated with binding assays.

The specificity of binding of phage probes to *B. anthracis* Sterne was first determined with an ELISA. In this assay, *B. anthracis* Sterne spores were adsorbed to the wells of an EIA/RIA plate. Individual phage clones were allowed to bind and were detected with biotinylated anti-fd antibodies followed by APSA and PNPP. Phage binding to plastic was also evaluated. As shown in Fig. 4, the majority of isolated phage probes bound to *B. anthracis* more strongly than a control (unrelated) phage probe. None of the phage probes reacted significantly with plastic (ELISA signal <2 mOD/min). The best binding phage clones identified in the acidic elution procedure after depletion against *B. cereus* carried the peptides VTRNPAAS and VTRGSMNT. Not surprisingly, these two clones carry a similar motif. It is also worthy of note that they are similar to the peptide VTRNTSAS isolated in the non-biased selection procedure. Since this phage clone cross-reacted with spores of numerous *Bacillus* species, this may be an indication that a single depletion with one type of spore may not be sufficient to remove all binding phage. It also indicates that the motif VTR N/G interacts strongly with a spore surface antigen. The best binding phage clones identified in the acidic elution procedure after depletion against *B. subtilis, B. thuringiensis and B. cereus* carried the peptide VGRANPSS. This is again somewhat similar to VTRNTSAS isolated in the non-biased procedure, although it is different enough that it may not cross-react with other *Bacillus* species. Other high binders in this selection procedure included phage probes carrying the related peptides ARQPAGM, ARSQPALS, AARQPMAS, and AVRDQPNL. The

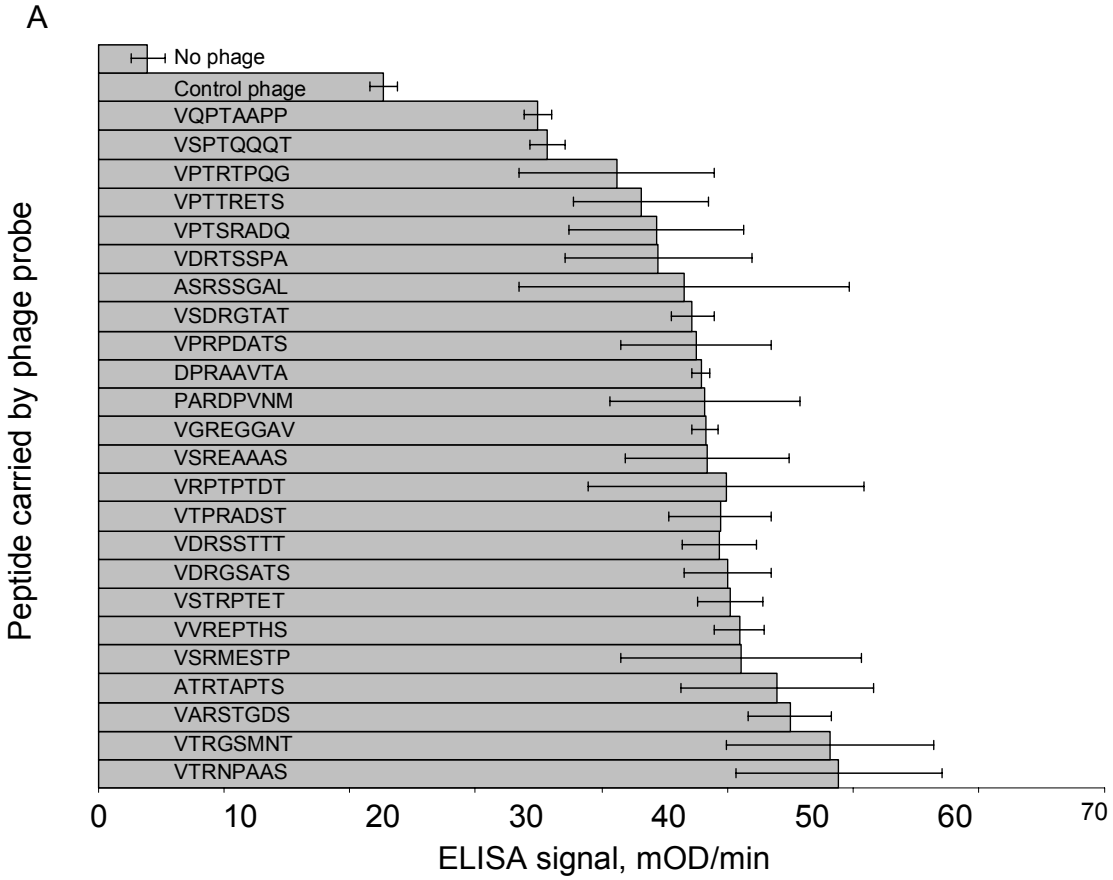

Fig. 4. Binding of selected phage clones to immobilized *B. anthracis* Sterne spores as detected with an ELISA. Individual phages were allowed to bind to immobilized spores, and then were detected with biotinylated anti-fd antibodies followed by APSA and PNPP. A phage clone carrying an unrelated peptide sequence was used as the control. None of the phage clones interacted with plastic. Error bars are indicative of the standard deviation of triplicate wells. (A) Phage probes isolated through selection using acidic elution buffer following depletion of the library against *B. cereus* (B) Phage probes isolated through selection using acidic elution buffer following depletion of the library against *B. subtilis, B. thuringiensis* and *B. cereus.*

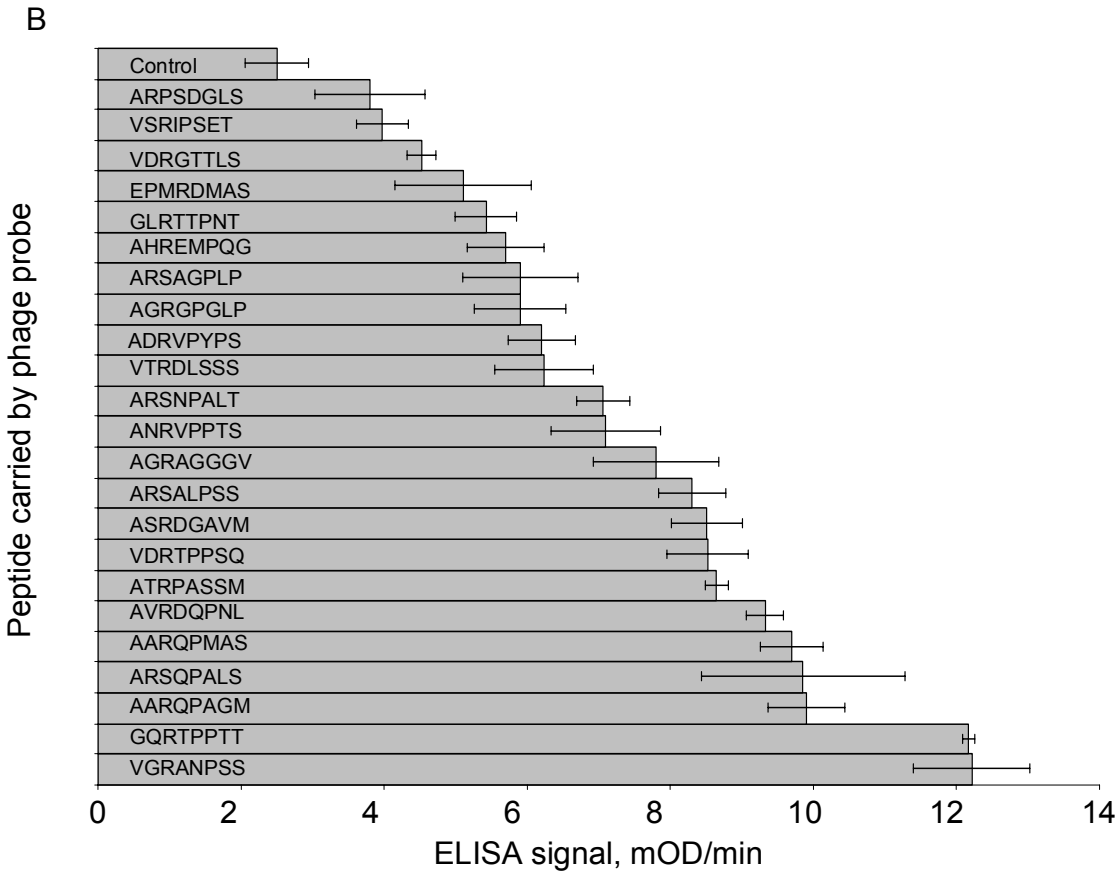

Fig. 4. continued

phages from the *B. cereus* depletion / deoxycholate selection procedure which were examined demonstrated poor binding (data not shown). This was not unexpected: the selection of vector phage and short insert clones during this selection procedure indicated that fast growing clones were being selected rather than target-specific clones.

The ELISA, while useful for quick screening processes, can underestimate the binding ability of some phage clones because it relies on polyclonal antibodies which bind to the pVIII coat protein in the region which is modified in our phage clones (Kneissel et al., 1999). To further characterize the selected phage clones, we used a phage-capture assay which provides a true quantitative measure of phage binding to spores. The phage-capture assay virtually replicates the selection procedure, and binding of phages is detected by titering the number of phages which are eluted from the target. The results of the phage-capture assay can be seen in fig. 5. The best binding phage clones identified in the acidic elution procedure after depletion against *B. cereus* carried the peptide VTRGSMNT. The best binding phage clones identified in the acidic elution procedure after depletion against *B.subtilis, B. thuringiensis and B. cereus* carried the peptide AARQPAGM.

To determine whether or not the information content of a peptide is a good predictor of the ability of a phage clone carrying that peptide to give a high binding signal, the percent recovery of each phage clone in the phage-capture assay was plotted against the information content of the peptide it carries. As seen in Fig. 6, no correlation was found between the information content and binding signal of the phage clones isolated in these selection procedures ($R^2 = 0.0082$). Evaluation of other phage clones selected against other complex targets in this manner also failed to reveal a correlation

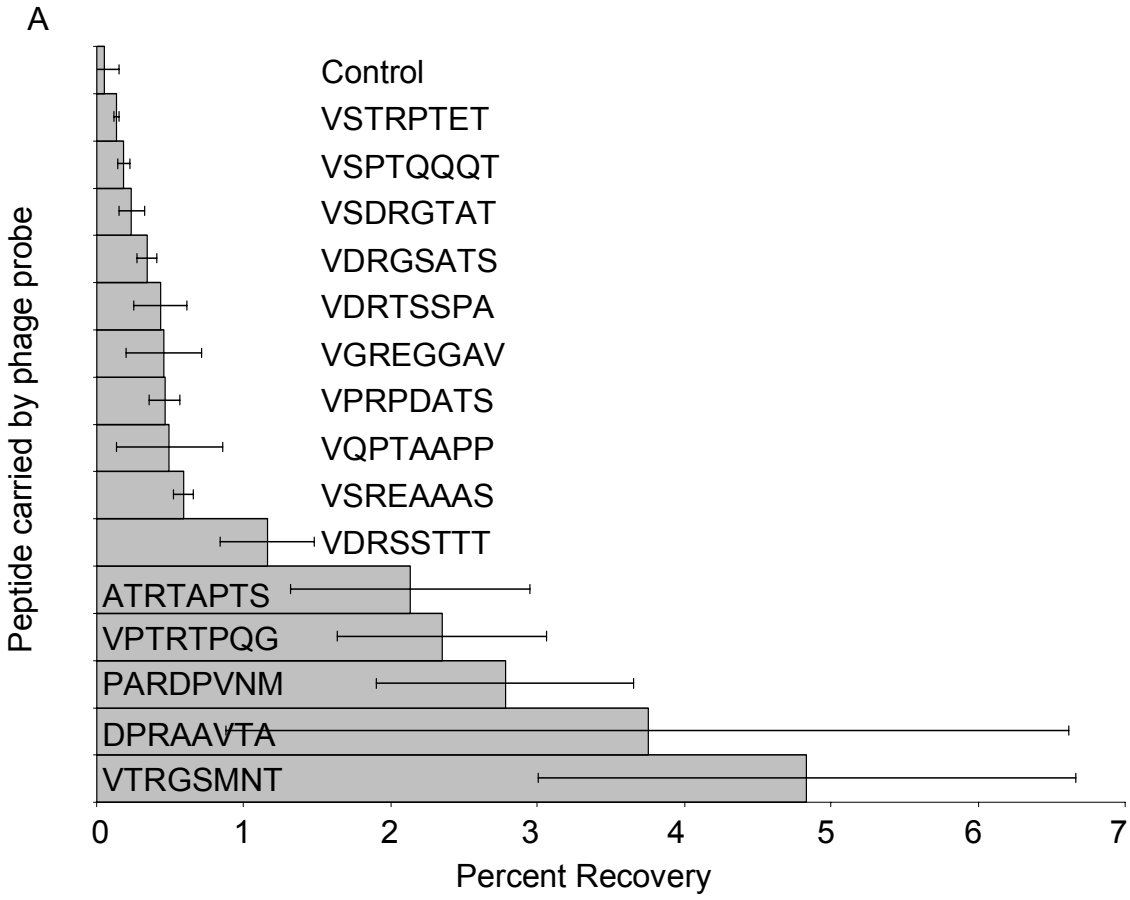

Fig. 5. Binding of selected phage clones to immobilized *B. anthracis* Sterne spores as detected in a phage-capture assay. Individual phages were allowed to bind to immobilized spores, then non-bound phages were washed away and bound phages were eluted and titered. The percent recovery of each phage clone was calculated by dividing the number of phage eluted by the phage input, and multiplying by 100. Error bars are indicative of the standard deviation of triplicate platings. (A) Phage probes isolated through selection using acidic elution buffer following depletion of the library against *B. cereus* (B) Phage probes isolated through selection using acidic elution buffer following depletion of the library against *B. subtilis, B. thuringiensis* and *B. cereus.*

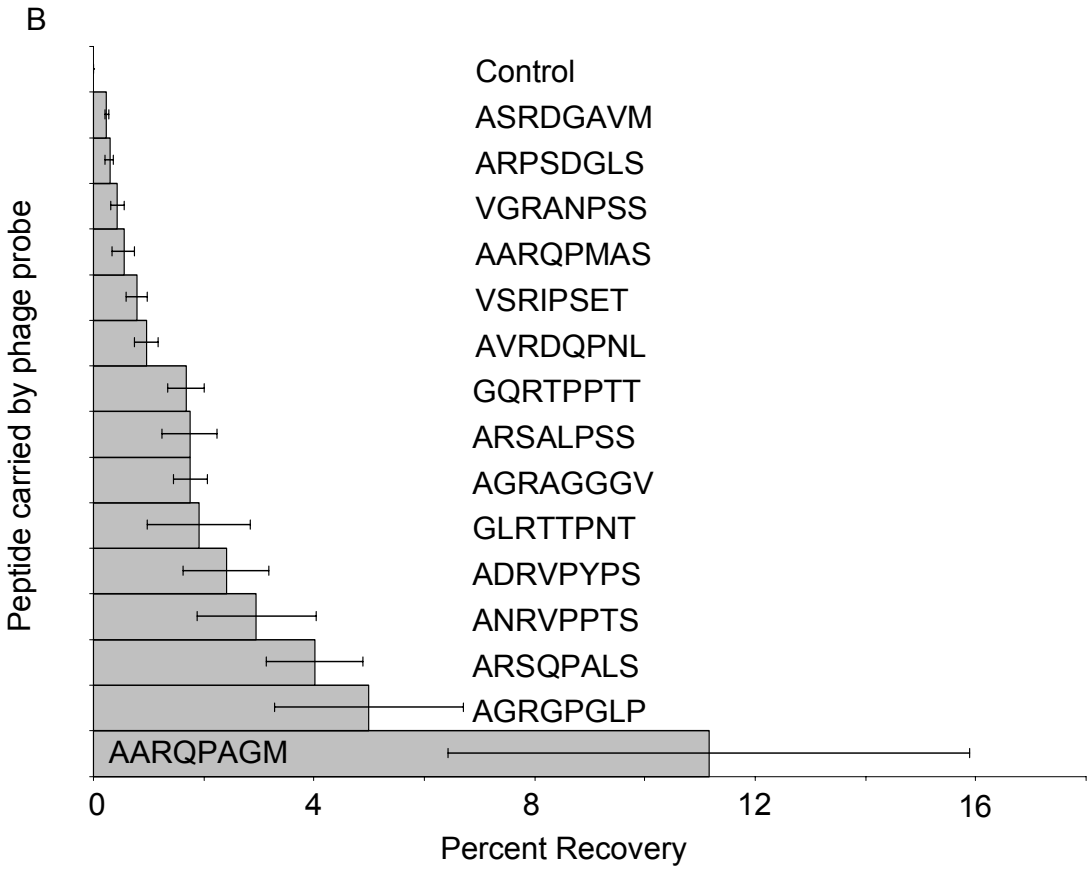

Fig. 5. continued

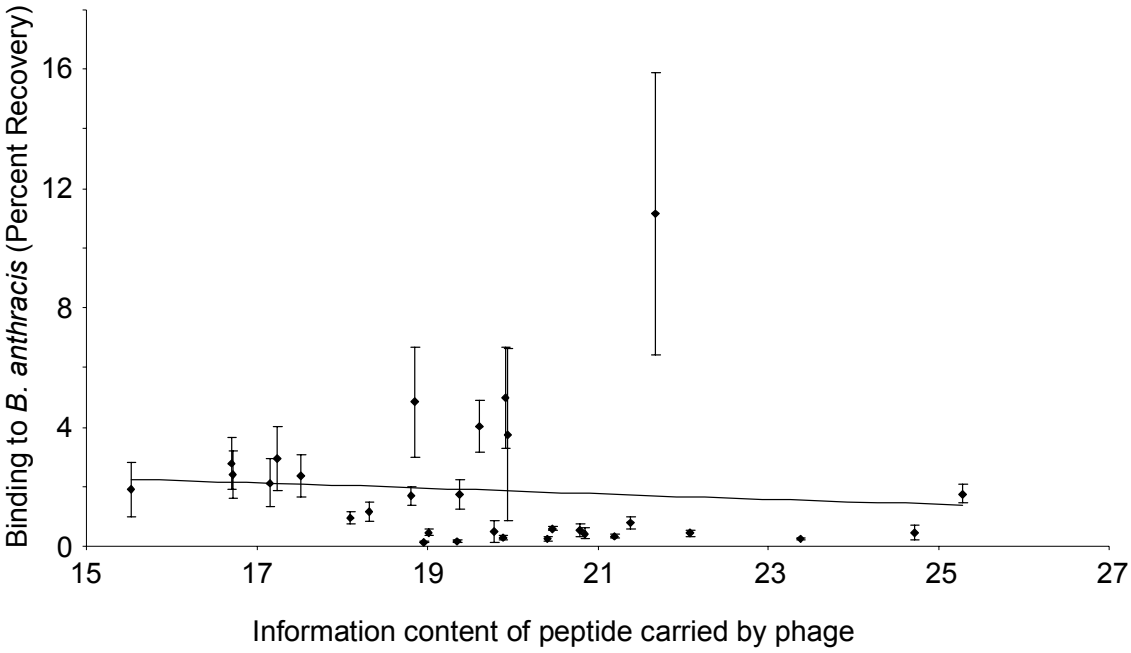

Fig. 6. Relationship between information and binding ability of peptides carried by selected phage clones. The ability of phage clones carrying the selected peptide to bind to a target was determined with a phage-capture assay. Error bars represent the standard deviation of triplicate platings.

between information content and binding signal (unpublished data). This does not exclude the possibility that there could be a relationship between information content of a peptide and affinity of a phage clone carrying that peptide. Binding signal is dependent on a combination of the affinity of a phage probe for its target, and the abundance of binding sites on the target. This merely suggests that for complex systems involving multivalent interactions information content cannot be used to predict binding signal.

To determine if prevalence of a clone in the pool of selected phages is an indication of binding ability, the prevalence of each phage probe was plotted against its percent recovery in the phage-capture assay. As seen in fig. 7, there was no correlation between the prevalence of a phage clone carrying a certain peptide or motif and its binding signal ($R^2 = 0.0023$). There are a number of possible reasons for the high prevalence of phage clones which do not yield a high binding signal, but the most likely is that these clones are fast growers and gain an advantage during the amplification steps between each round of selection.

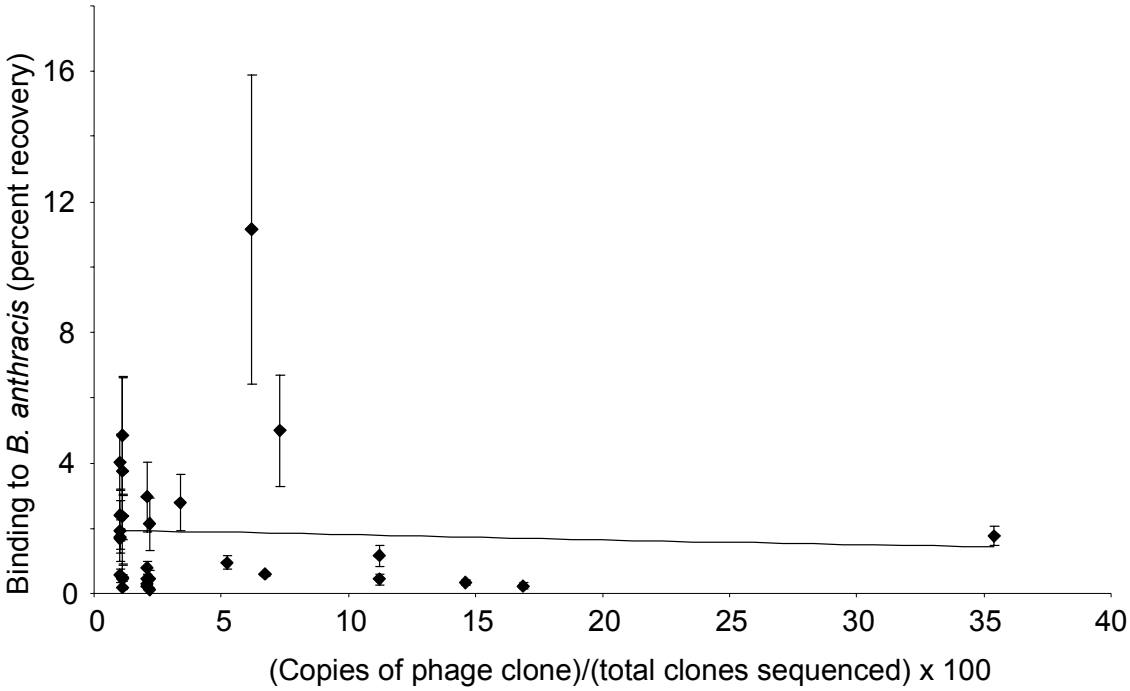

Fig. 7. Relationship between prevalence of a phage clone in the library after four rounds of selection and the ability of that phage clone to bind to its target. The ability of phage clones to bind to their target was determined with a phage-capture assay. Error bars represent the standard deviation of triplicate platings.

4. Conclusions

The use of depletion techniques allows the identification of different target-specific phage clones than those isolated in non-biased selection procedures. In a successful selection procedure, the percent of vector phage remaining in the library after each round of selection will decrease, in spite of the growth advantage vector phage has over phages carrying foreign inserts. As expected, the diversity of amino acids present in any position of a foreign peptide carried on a phage vector tends to decrease following each round of selection, although not all positions lose diversity at the same rate. In the landscape library examined, as in all random peptide libraries, some amino acids were more prevalent than others. During selection, the overall prevalence of amino acids in the library changed, and this change appeared to be influenced by the type of elution buffer used. A subset of high information peptides was identified in each selection procedure, but there was no clear correlation between information content and target binding signal as measured by a phage-capture assay. Prevalence after multiple rounds of selection was also not found to have any correlation with binding signal. Binding signal as measured by the assay used in this study is dependent on both affinity of the phage probe for its target and the abundance of target molecules on *B. anthracis* spores, so this conclusion does not exclude the possibility of a relationship between information content and affinity. These results indicate that while monitoring the selection procedure closely and using the RELIC programs can help to predict the overall success of a selection procedure, they cannot be relied upon to predict which individual phage clones will give high detection signals when binding to complex targets.

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CHAPTER V

CONCLUSIONS

The threat of bioterrorism has driven a need to develop rugged and portable devices for continuous monitoring of both indoor and outdoor environments for threat agents. Many assays for detection of threat agents rely on antibodies for recognition or concentration of the agent. While monoclonal antibodies can often be used to detect target agents without fear of false positive results, they are not very stable in environmental conditions and their use can inhibit long term field deployment of monitoring systems. The recent development of phage probes which can be used as substitutes for antibodies in a variety of detection platforms lead to a question of whether these probes might be suitable for use in detection of biothreats.

In one portion of this work, the thermostability of a phage probe when fixed to plastic was demonstrated. The phage probe examined was found to have a half life of ~2.5 years at 37°C, and to withstand temperatures of 76°C for three days without losing all of its activity. The phage probe performed far better than a monoclonal antibody specific for the same target after incubation at high temperatures. While this work examined only one phage probe, which binds to β-galactosidase, work by others has demonstrated similar thermostability of phage probes with specificity for *B. anthracis* (Bryan Chin, personal communication).

The remainder of the work was performed to determine the feasibility of developing phage probes for detection of threat agents, and was focused on selection of phage probes specific for *B. anthracis* spores. A number of different selection procedures were used to isolate these spore-specific probes, including a non-biased procedure, and several biased procedures in which phage that bound to spores of other *Bacillus* species were removed from the phage library prior to selection. Through these procedures, a large number of *B. anthracis* binding phage clones carrying different peptides were isolated for further study. All of the examined clones were found to bind to *B. anthracis* with higher affinity than the vector phage, although some clones cross-reacted significantly with other *Bacillus* species in co-precipitation assays. The phage clone with the least cross-reactive properties, which carries thousands of copies of the peptide EPRLSPHS, bound to *B. anthracis* 3.5 to 70 fold better than to spores of other *Bacillus* species in a co-precipitation assay. Further work with this clone has revealed that cross-reactivity can be reduced by examining binding of spores to phage fixed to a solid surface (Bryan Chin, personal communication). This spore-capture assay was closer to an actual application of phage-probe use to detect *B. anthracis* spores, so the increased selectivity of probe EPRLSPHS in this format is encouraging. Work is underway to develop a high throughput system to test the selectivity of all of the identified *B. anthracis* spore binding phages using a spore-capture assay.

During the selection of phage probes that bind to *B. anthracis* spores, the molecular evolution of the phage display library was examined in detail. This examination was performed for three reasons; to learn basic information about what happens to a landscape phage library during selection; to determine if certain changes in

the library might indicate a successful selection procedure; and to determine if any qualities of the selected phage clones might be able to help predict their target-binding ability. It was found that during a successful selection procedure the peptides in the library tend to decrease in amino acid diversity, but increase in information content. It was also found that a combination of target binding ability and fast growth properties determine the prevalence of a phage clone following each round of selection. The influence of growth properties on the prevalence of phage clones in the library is probably the reason that the phage clone which becomes dominant after several rounds of selection is not always the best target binder. No correlation was found between the prevalence of a phage clone after four rounds of selection and its target-binding ability. It was hypothesized that there might be a positive correlation between the information content of a peptide and the target-binding ability of a phage clone carrying that peptide, since random isolation of a clone carrying a high information peptide is rare. However, no correlation was found between the information content of a peptide and its target binding ability. These results confirm the need to develop rapid systems for screening of phage clones to determine both the specificity and selectivity of their binding.

Phage-derived probes have great potential for use in monitoring devices for the detection of threat agents in the environment. Their ability to withstand extreme environmental conditions, including extended exposure to high temperatures, gives them a distinct advantage over antibodies. Phage-probes specific for a biological threat agent can be selected from a landscape library in a relatively short amount of time, and small changes in the selection procedure can allow the identification of a variety of different probes with different affinities for the target. While probes identified through non-biased

selection procedures may cross-react with similar targets, the use of biased selection procedures to identify new probes, or the modification of the surface of existing probes may allow the development of highly selective and robust probes suitable for long term use in continuous monitoring devices and biosorbents.