DEVELOPMENT AND STUDY OF PHAGE-BASED MICROARRAY AND

DOT-BLOT

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DEVELOPMENT AND STUDY OF PHAGE- BASED MICROARRAY AND

DOT-BLOT

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DEVELOPMENT AND STUDY OF PHAGE - BASED MICROARRAY AND

DOT-BLOT

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THESIS ABSTRACT

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DOT-BLOT

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The sequencing of cell genomes along with the development of DNA microarrays established a foundation for the discovery of new genes and enabled the exploration of the whole transcriptome in a single assay [1]. However, there is no absolute correlation between the mRNA transcription levels and the corresponding protein expression levels [2, 3, 4]. Hence, protein-specific high-throughput technologies are urgently needed. We developed a novel variant of a protein microarray, based on landscape bacteriophages where 4000 copies of specific peptides were displayed on the phage surface as an N-terminal part fused to the pVIII protein. In our model system, we used phages binding streptavidin and displaying the VPVGAYSDT or VPEGAFSS peptides. The control

phage displayed the non - related EPRLSPHS peptide. All phages were immobilized on an epoxy - coated glass slide and the binding was monitored with fluorescently labeled streptavidin. We demonstrated that the sensitivity of our phage array is very high: VPVGAYSDT - phage specifically recognizes the target at concentration ~ 1.0 nM (60 fmoles in quantity). The system we described shows several advantages: 1) the robustness of the recombinant phage inherited from its natural predecessor (phage fd), which allows its use in harsh environments, 2)   high density of the binding component to the total protein mass compared to antibodies and other proteins and 3)   high sensitivity.

The developed phage microarray was also adapted as a prospective high-throughput method for screening of phage clones specific for *B. anthracis* spores. An array variant fluorescent dot-blot was also designed for rapid examination of the discriminative selectivity of selected phage probes when exposed to mixed populations of spores of the *Bacillus* family.

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PREFACE

The first chapter of this thesis contains a review of current knowledge on microarray technology, in particular DNA and protein microarrays. Advantages, applications, and limitations of these methods are also described. It also includes a brief description of phage biology and phage display methods, and how these methods can help to solve current immunoassay problems. The main focus of the first chapter is the description of the experiments that were performed to immobilize landscape phage on a microarray platform and to justify its use as a screening and diagnostic method with high sensitivity, specificity and selectivity. Specific aims included:

I.   Immobilize landscape phage probes on commercially available glass slides.

II.   Determine the sensitivity of phage based microarrray with Texas Red Streptavidin as a model target.

III.   Determine the phage microarray specificity.

IV.   Determine the selectivity of the phage microarray by competition assays with related and non-related targets.

The second chapter of this thesis contains a review on *B. anthracis* spores and valid methods for their detection and identification. It also describes experiments

designed to justify the direct application of phage microarray and fluorescent dot-blot for binding of *B. anthracis* spores and its suitability as a screening tool for candidate clones from landscape phage libraries. Specific aims included:

I. Demonstrate binding of *B. anthracis* spores by phage microarray with appropriate phage clones.

II. Determine the specificity of phage clones by fluorescent dot-blot assay with mixed populations of fluorescently labeled *B. anthracis* and *B. subtilis* spores.

CHAPTER 1

PHAGE MICROARRAY

I. LITERATURE REVIEW

Completion of the human genome project allowed scientist to obtain huge amount of important information for understanding the control of complex cellular events. DNA microarrays were established as high-throughput hybridization systems, enabling the exploration of the genetic information in a single assay [1]. Many lines of research demonstrated a lack of correspondence between mRNA transcription levels and the protein expression levels. Moreover, it was impossible to deduce the functional state of proteins purely from their expression and it was impossible to determine their controlling role in disease and non-disease phenotypes [2, 3, 4]. Therefore, additional high-throughput technologies were required to facilitate the analysis of the interactions within the proteome. A variety of immunoassays and protein microarray methods were developed as diagnostic approaches, which complement DNA based techniques and target the key molecules with high accuracy, providing an integrated view of disease mechanisms and cellular processes at the protein level [5, 6]. In these clinical studies, monoclonal antibodies (mAb) are recognized as unique and indispensable tools. Nevertheless, traditional methods for preparing mAbs are expensive and labor intensive

[7]. These hurdles limit antibodies to be used as capture agents in association with high-throughput technology. More stable, reliable and economic ligands for protein microarrays are needed. Currently, a large variety of new methods are being developed to fulfill the demand of diagnostic probes for immunoassays. Landscape phage display method represents a new, powerful technique for development of substitutes for antibodies in immunoassays [8]. Landscape phages are filamentous bacteriophages that express foreign peptides fused to their major coat protein. An individual virion expresses thousands of copies of a foreign peptide in a constrained conformation on its surface, creating a landscape with binding properties [8]. Over the last decade, these molecules showed an ability to be employed in their entirety as probes in platforms where antibodies have traditionally been used. Historically, the investigation of viruses has been centered on their function as infectious agents and as tools to elucidate cell biology. Viruses, however, are now finding a new expanded role as nanoplatforms with direct applications in materials sciences and medicine [9]. As a low cost and innovative approach we developed a variant of protein microarray, based on landscape bacteriophages immobilized through covalent attachment on commercially available epoxy glass slides. To justify replacement of antibodies in microarray platforms, it was necessary to demonstrate that phage probes can bind a model molecule, such as streptavidin conjugated with Texas Red fluorescent label with high sensitivity, specificity and selectivity.

In our work we hypothesized that landscape phage can be immobilized on epoxy glass slides and the developed phage microarray would identify its target with high sensitivity, specificity and selectivity, and by these means would present a reliable alternative of current protein microarray approaches based on antibodies. The described method was also adapted as an array approach for screening of phage clones binding *B. anthracis* spores.

1. Microarray technology

The concept of microarray technology was first introduced as an ambient analyte model, relying on immobilization of interacting elements on a few square microns. This technology is a logic and multiplex assay of biological molecules laid on a solid planar platform, generally a glass slide. The resulting presentation is referred to as an array and sometimes called chip. In principle, the model is capable of detecting analytes with higher sensitivity than conventional macroscopic immune assays [10]. Also, it allows researchers to collect evidence for each molecule independently but simultaneously, conserving agents, samples and increasing speed for analysis. Biomolecules commonly immobilized on microarrays include, PCR products, oligonucleotides, proteins, lipids, peptides and carbohydrates. [11]

2. Array formats

Arrays are commonly printed on glass slides and are referred to as planar or two-dimensional arrays (2-D). Three-dimensional arrays refer to glass slides modified to contain hydrogel (protein arrays) or polyacrylamide (gene arrays) layers into which probes are introduced. Bead-based microarrays are an alternative format of planar arrays [12]. Protein microarrays were urged as a technique to exploit the worthy knowledge of nucleic acids array methods. Hence, a brief review of DNA microarray technology is provided in this chapter.

3. DNA Microarrays

DNA microarray technology is based on methods which exploit the remarkable and crucial discovery of the DNA structure [13]. This finding, suggested that the two strands could be separated by heat or alkali treatment and the reverse process, commonly known as DNA renaturation or molecular hybridization, can occur because of complementarity of DNA strands in the double helix [14]. Consequently, new experimental procedures were established to measure the rate of interaction of RNA molecule and the DNA from which it was transcribed and enabled ways to determine numbers of repeated sequences such as the ribosomal genes using labeled rRNA as a probe [15].

After the development of recombinant DNA technology, it was concluded that specific sequences in recombinant clones can be screened, applying molecular hybridization directly to bacterial colonies lysed and fixed to a membrane. The same technique was devised as a related method for phage plaques [16, 17].

A step forward was the demonstration of the possibility to synthesize complex nucleic acids, and polynucleotides of any sequence up to 200 nucleotide residues [18]. These findings had a great impact and were followed by the establishment of large scale analysis of bacteria and yeast cells carrying recombinant DNAs. Novel approaches enabled analysis of multiple hybridization targets in parallel by applying them to a filter known as dot-blot. In the dot-blot format, multiple targets are arrayed on a support. The probe, normally, a single sequence, is labeled and applied under hybridization conditions [19]. Current microarray formats are based on a variant called reverse dot-blot. This method employs multiple probes, attached as an array, and the target to be analyzed is labeled [20]. The first arrays were prepared on impervious supports [21]. Ultimately, to increase the density of spots, the manual procedures used to pick and spot clones onto filters were replaced by robotics. Automation increased the speed of the operation, removed human errors and improved the accuracy of placing samples [22]. Routinely, DNA microarrays are fabricated by in situ synthesis, inkjet printing or microspotting to create custom microarrays by direct deposition of biomolecules of interest on microscope glass slides, membranes or other surfaces [23, 24]. At the present, the technology is an

essential tool for tumor classification, risk assessment, prognostics, expression, mutations and polymorphism analysis.

4. Protein microarrays

DNA microarrays became an indispensable approach for the interpretation of sequence information obtained from the genome of multicellular organisms. However, they provide limited data for the process of actual protein expression and even less insight on protein–protein communication or the proteins' biochemical activity. These processes cannot be understood fully at the level of nucleic acids, since they occur by post-transcriptional control of protein translation, post-translational modifications or by protein proteolysis [25, 26, 27]. Furthermore, prediction of protein function is complicated by the fact that no role is known for a large amount of the proposed proteins. Also, the dynamic fluctuation of protein expression ranges greatly [28]. For the purpose of comprehending cellular functioning at the protein level, there is a great demand for large-scale methods in the field of proteomics.

4.1 Types of protein microarrays

Three major types of protein microarrays are employed to study the biochemical activities of proteins: analytical microarrays, functional microarrays, and reverse phase microarrays [29].

Analytical microarrays are used as profile complex mixtures of proteins in order to measure binding affinities, specificities, and protein expression levels. This implies the use of libraries of antibodies, aptamers, or affibodies, arrayed on a glass microscope slide. The array is then targeted with an analyte in solution. These types of microarrays can be used to monitor gene expression profiles at the translational levels and for clinical diagnostic tests [29]. Examples include profiling responses to environmental stress and healthy versus diseased tissues [30].

Functional protein microarrays are commonly composed of arrays containing full-length functional proteins or protein domains. These types of arrays are employed as tools to study protein: protein, protein: DNA, protein: RNA, protein: phospholipid, and protein: small molecule interactions [31, 32].

Functional protein microarrays are related to analytical microarrays. They are known as reverse phase protein microarrays (RPA). In RPA, cells are isolated from various tissues of interest and lysed. The lysate is arrayed onto a nitrocellulose slide using a contact pin microarrayer. The slides are then probed with antibodies against the target protein of interest, and the antibodies are typically detected with chemiluminescent,

fluorescent, or colorimetric assays. Typically, posttranslational modifications of expressed proteins, which are altered as a result of disease, can be detected using this method. Once the cell's dysfunctional protein pathway is determined, a specific therapy can be applied to correct the protein pathway, and thus, treat the disease [33].

4.2 Applications of protein chips

Protein arrays allow analysis of thousands of analytes in a parallel format. In addition to characterizing the functions of previously unknown proteins, protein arrays have also been used to discover new functionalities for previously characterized proteins. Furthermore, protein arrays have been reported to elucidate protein–protein interactions [32], protein–DNA interactions [31], protein–lipid interactions [32], protein–drug interactions [26], protein–receptor interactions [34], and antigen–antibody interactions [35].

There have been several reports on yeast protein kinase studies using protein chips. Among them, a recent study, using a silicone elastomer nanowell sheets placed onto glass slides, examined the activity of 119 yeast kinases and discovered new properties of known proteins. Using 117 different substrates, 27 yeast kinases were found to act *in vitro* as tyrosine kinases. This was roughly triple the number of tyrosine kinases originally thought to exist in yeast [36].

Protein phosphorylation in yeast using proteome chips and radiolabeled ATP was also monitored. An investigation reported 4200 phosphorylation events affecting 1325 different proteins. A global kinase–substrate map for yeast was assembled [37].

Proteome chips have also been used successfully to screen patient's sera for the presence of autoantibodies [38, 39]. Recently, a diagnostic array was described, based on spotted corona virus proteins. The array unambiguously detected Cy-3 labeled antihuman IgG and IgM in affected patients [40].

Another important application of protein arrays is drug discovery. Entire proteomes printed on an array platform can be screened simultaneously and their interactions with drugs of interest examined. An analysis of the small molecule inhibitor of rapamycin (SMIR) was probed to find protein targets that may be involved in the target - ofrapamycin (TOR) dependent nutrient response network. Through this approach it was discovered a protein of previously unknown function to be a target of the SMIR [34].

Protein recruitment to receptors can also be monitored in a high-throughput fashion. Using protein-based-arrays, specific binding domains that interact with different epidermal growth factor receptors (EGFR), involved in a variety of cellular responses, were recently reported. Using the data obtained from the experiment, it was possible to calculate the dissociation constants of the protein–receptor binding [41]. Other prominent examples of protein array advantages include serum profiling [40] and evaluation of calmodulin binding proteins and their role in regulation of yeast genome expression [32].

Protein arrays constitute an excellent high-throughput method for the analysis of proteins and their specific function and biochemistry. This new technology provides a practical way to discover novel multifunctional proteins and reveals more about unsuspected roles of well-studied proteins. However, preparation of protein arrays is a difficult task. Proteins have a complex nature which requires suitable immobilization platforms allowing them to remain in their active functional state.

4.3 Solid supports for protein microarrays

The first report on protein microarray production, which used standard arraying equipment and slide scanners available from DNA microarray studies, generated perspectives in functional and comparative proteomics [42]. However, the chemical aspects of DNA microarray surfaces could not be adopted easily, since there are fundamental biophysical and biochemical differences between the two classes of molecules. Proteins, in general, being polymers of 20 amino acids and displaying immense chemical, physical and structural diversity, present additional problems when immobilized on a microarray.

In contrast to DNA, denaturation, dehydration or oxidations of proteins are often observed. They cause loss of structure and biochemical activity. Furthermore, the detection of proteins by antibody–antigen interactions is characterized by a wide range of specificity and affinity. Additionally, binding affinities of antibodies were found to be

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reduced upon immobilization [43]. Consequently, there is a need for more sophisticated immobilization chemistries to perform global analysis of the proteome of thousands of proteins simultaneously. For comparative proteome screening of healthy and disease tissues, microarrays will require the use of a probe, a sensor molecule on the chip surface, which can recognize native and postranslationally modified proteins. Commonly, the multiplexed high-throughput protein microarrays are based on recombinant antibodies or other non-antibody high-affinity ligands [44, 45]. Unlike the negatively charged nucleic acids, proteins are amphiphatic molecules, which as a result show considerable surface activity. The high degree of protein adsorption is caused by hydrophobic, electrostatic, van der Waals and Lewis acid–base force interactions, as well as conformational changes and restricted lateral diffusion in the vicinity of a surface [46]. The mode of interaction with a surface differs widely between proteins. Therefore, achieving a low degree of unspecific binding is complicated if a complex protein sample containing thousands of molecules is to be analyzed. For most biological assays to be successfully carried out in a microarray, it is crucial that immobilized proteins and peptides are oriented in an active state and with a high density [47].

Originally protein microarrays were developed in the format of enzyme immunosorbent assays in microtiter plates of 96 wells. ELISAs became the gold standard and were optimized furthermore by adaptation to 384 - well format for high-throughoutput and lower consumption of materials [48]. However, the binding mechanism on classical support media used for ELISA, such as polystyrene, is based on a

partial denaturation of proteins on the hydrophobic surface, which may cause an important decrease in immunoreactivity. Proteins adsorbed to hydrophobic surfaces tend to denature [49]. Apart from the large volume required, the main disadvantage of the microtitre plate format is the inherent limitation with regard to further assay miniaturization and thus volume reduction. Important advantages, however, are the capability of stirring the incubation solution, avoiding depletion effects—and the ability to process in parallel, various protein samples [50].

Absorption of proteins on hydrophilic provide new analytical opportunities. Physical absorption and binding through amino groups of lysines and arginines are common to all protein classes and portrays the simplest process of protein binding, although it is rather uncontrollable. Close vicinity with the absorptive surface and the reactive protein's site influences the affinity for an analyte target unfavorably. Surfaces may also be susceptible to exchanging absorbed protein with proteins in the surrounding solution. Stringent washing can destabilize protein attachment [51].

Compared to the conventional and well known immune assays such as ELISA, relying on partial denaturation, covalent binding of proteins to a surface represents a more rational and robust approach and can be subdivided at random and oriented attachment. Glass slides have the ideal surface for microarray applications because they are inexpensive and with low intrinsic fluorescence. At the same time they also possess a relatively homogeneous chemical surface which when used with appropriate bioconjugate chemistry, are capable of immobilizing biomolecules at very high densities.

This translates into higher sensitivity in detection of proteins/ peptides in most microarray assays. The surface of the glass slide is usually derivatized with chemicals to generate different types of molecular layers. Biomolecules such as proteins or DNA molecules are usually immobilized on amine- or aldehyde-derivatized surfaces by covalent attachment between the amino groups (lysine residues on proteins), amino-modified oligonucleotides, or PCR products by the Schiff base reaction. Similarly, epoxy-derivatized surfaces can be used for immobilizing proteins, amino-modified oligonucleotides or PCR products. The amino groups of biomolecules react with the epoxide group on the surface, forming a covalent bond [47, 51]. Thus, for stable immobilization of proteins/peptides, arraying takes advantage of covalent linkages or non-covalent absorption in an oriented and non-oriented way. Evaluation, with regard to performance and functionality of commercial and self-made supports, was recently reviewed [6, 47].

4.4 Antibodies as capture molecules for protein arrays

Antibody/antigen microarrays represent an approach compatible with DNA microarrays, since it aims simultaneous analysis of several thousand proteins of biological samples. Antibodies are the capture agents of choice used in bio - detecting assays. At present, immunoassays based on monoclonal antibodies are still the most important diagnostic methods widely used in clinical and research areas [52]. Several

tens of thousands of monoclonal antibodies are currently commercially available. Nevertheless this number is insufficient for large-scale protein profiling since it is based on hybridoma technology which is time consuming and limited in economical sense [7]. Fab fragments can be proteolitically obtained from antibodies. However, this way of production is even more laborious. The absence of C_H domain of antibodies lowers their stability, solubility and affinity. Both antibodies and Fab fragments have immobilization problems on solid surfaces [53]. On the other hand, polyclonal antibodies are not the best choice for microarray purposes, since they are not highly selective and thus unsuitable for large-scale methods. Cross reactivity is a major drawback in for targeting proteins with and without sequence homology. Moreover, the probability of detecting unspecific recognition can increase with the analyte and array complexity [54, 55]. Furthermore antibody mini-arrays and antigen microarrays were successful in demonstrating the usefulness of the technology in principle, but with moderate quality and control [56, 57]. Moreover, antibody microarray systems are incapable of producing data from very low concentrated samples, which are frequent in a clinical environment [58]. Also, only analyses of samples of inferior complexity were really successful [59]. The theoretical detection limit of the first microspot array with antibody monolayers was predicted to be a few femtograms or less [60]. Nevertheless it has been difficult, until now, to produce a detectable signal in the picogram range even with artificial one - antibody one - antigen test systems, which avoid the background problems [61].

These hurdles limit antibodies for greater commercial growth in association with high throughput technology. More stable, reliable and economic ligands for protein microarrays are needed. To meet the requirements, new strategies such as ribosomal display, lipocalin, fibronectin and aptamers are being developed [61, 62, 63, 64]. A promising and remarkable technique, capable of fulfilling the demand of reliable, robust screening and diagnostic probe, is the phage display method.

5. Phage biology

Bacteriophages are viruses that infect bacterial cells, generally showing specificity and a predisposition for a weakness of the host, in order to transfect their nucleic acid. Phages may have single- or double-stranded DNA or RNA genomes, protected by filamentous or icosahedral capsids. The phages used in this study are filamentous, single-stranded DNA bacterial viruses, from the family Inoviridae. The Ff class of the filamentous bacteriophages (f1, f8, fd and M13) have been extensively studied. The DNA sequences of these phages show 98% similarity. The Ff phage particle is approximately 7 nm wide and 900 nm long. The genome (encoding eleven genes) is a single stranded, covalently closed DNA molecule of about 6400 nucleotides that is encased in a flexible protein cylinder. The coat consists of 5 proteins; one major capside protein, pVIII, is a 50-amino-acid protein, present in 2700 or more copies; and four minor capsid proteins, pIII, pVI, pVII and pIX, present in five copies per phage [65, 66].

Phages of the Ff class are neither lytic nor lysogenic vectors for *E. coli*. They infect bacteria via the plasmid-encoded F-pilus. Upon binding of minor coat protein pIII to the F-pilus of *Escherichia coli*, 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 the phage DNA is translocated into the cytoplasm. Subsequently, DNA complementary to the viral strand is synthesized to produce the replicative form, which is then replicated by the rolling circle mechanism. The capsid proteins are produced by the host and merged into the bacterial cell membrane. During the DNA extrusion through the host membrane, the capsid proteins assemble around the phage DNA. The progeny particles are secreted continuously without lysing the host cell. Bacterial cells tolerate well the infection by Ff phage, since their generation time is only ~ 50% longer than uninfected cells [65, 66, 67].

5.1 Phage display

Ff phages are commonly used as cloning vectors, since their replication and assembly are not inhibited by changes in genome size. Foreign DNA addition in a nonessential region of the genome does not have a lethal effect on the phage particle; virions are made larger [68]. As a result of foreign DNA insertion, encoding peptide or protein, virion particles can display them on their surface. This concept is commonly known as phage display [69].

Phage display is a method taking advantage of phage as a cloning vector to display foreign peptides or proteins on its surface. Mixtures of phage clones, created through this technique, can generate libraries of virions, each expressing different peptide or protein on its surface, as a result of foreign DNA insertion [69]. Phage display libraries can be made with bacteriophages having either icosahedral or filamentous capsid. However, this work focuses essentially on an application of libraries created using filamentous phages.

As mentioned earlier, the outer coats of filamentous phages consist mainly of the major coat protein pVIII, assembled in thousands of alpha-helical subunits, tightly packed around the viral genome [65, 66, 67]. Before being assembled into phage particles, the hydrophobic segment of pVIII spans the inner membrane of E. coli, with the negatively charged N-terminal segment outside in the periplasm and the positively charged hydrophilic C-terminal segment inside in the cytoplasm. In the native virion, the carboxy-terminal 10-13 residues of pVIII line the inner surface of the sheath, where they neutralize the negative charge of the DNA core [70] The amino-terminal portion of pVIII is present on the outside of the particle. When phage particles are extruded through pIV channels, the amino terminus of pVIII becomes exposed to the environment. Each of the 2700 copies of pVIII protein, contributes to the formation of a right - handed helical coat, with the individual monomers tilted at a ~ 20 degree angle to the long axis of the phage particle [65, 66, 71]. Also, there are five copies of each of the minor coat proteins, pIII, pVI, pVII and pIX, on both tips of the particle. Foreign peptides and/or proteins have

been fused to all of these proteins. These 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 [71].

Generation of libraries implies expression of foreign peptides or proteins on all copies of the coat protein of choice or just on a portion of the copies. Genetically, this is accomplished by splicing a second wild type coat protein sequence into the phage genome, or by introducing the adjusted gene into a phagemid that is assisted by wild-type phage as a helper. Phage display libraries have been generated to display fragments of antigens, proteins and protein domains, mutagenized proteins, antibodies and antibody fragments, cDNA encoded proteins, and random peptides [72, 73].

A random peptide library is generated by splicing in-frame synthetic degenerate oligonucleotides into the gene of one of the coat protein of the phage. This approach causes each phage particle to display multiple copies of one particular peptide. As a consequence, a library contains billions of different phage clones, carrying billions of different peptides. Random peptide length, expressed on the phage filamentous capsid, ranges from a few amino acids to up to 40 amino acids [74, 75]. This work focuses on a novel application of phage display libraries in which random peptides are displayed on the phage surface and the whole body of the phage is subsequently exploited as a ligand on a microarray platform.

The majority of phage display methods suggest selection and isolation of phages carrying particular peptide sequence or protein with the ability to bind a target. Usually, a target antigen of interest is immobilized on a solid support followed by the addition of a

solution carrying the phage display library for screening of specific transformants. Phages that failed to bind to the target are washed away, whereas bound, target-specific phages are eluted. Afterwards, the eluted phage population exhibiting binding is amplified in bacterial cells and further enriched through affinity selection. After several rounds of selection, individual phage clones can be isolated and characterized through DNA and amino acid sequencing and the best binders can be used in binding assays.

In most situations, the phage display systems of choice are pIII and pVIII. Depending on the application, different display libraries are designed. To avoid lower phage viability and infectivity, large proteins and peptides are displayed on pIII or on a portion of pVIII coat proteins. Smaller peptides can be displayed on some or all copies of any of the coat proteins. Libraries containing transformants displaying one or a few copies of a particular peptide will more likely yield few binders of high affinity for a given target during the selection procedures. Whereas, 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. The display of many copies of a particular peptide increases the avidity for a target and could be stronger than the overall affinity showed by displaying an individual peptide [74]. Despite the fact that this multiple display hinders the isolation of individual peptides with high affinity, it shows great potential for selecting of effective phage clone carrying numerous copies of one peptide. As a consequence, the whole phage particle can be used as a probe for multivalent interaction [75, 76].

5.2 Landscape phage display

Phage libraries in which peptides are expressed on all copies of the major coat protein, pVIII, are commonly known as landscape libraries. In landscape phage display, a foreign peptide is fused to all copies of the major coat protein on a phage particle. Unlike conventional constructs, landscape display allows thousands of copies of one peptide to be displayed in a repeating pattern on a major part of the viral surface. Furthermore, the whole phage body can perform as an interacting scaffold to constrain the peptide into a particular conformation, generating a defined organic landscape, which varies from one phage clone to the next [75]. Genetically, this means that a foreign DNA sequence, encoding 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 after the pVIII protein has processed into mature form. Theoretically, the pVIII gene can adapt large inserts. However, phage production is hindered by addition of foreign peptides that are longer than 10 amino acids. To achieve fusion of peptides larger than this, wild type pVIII molecules must complement the modified proteins to create a mosaic particle. However, only phage composed exclusively of modified pVIII proteins are considered landscape phage. In this situation, landscape phage can carry a maximum of 9-10 foreign amino acids [74, 75]. A landscape phage library is a huge collection of landscape phages, comprising billions of clones with different peptides displayed on their surface [75].

Remarkable progress has been achieved in adapting phage-display selections to robotics. Novel methods for automatation analysis by phage ELISA, clone picking and sequencing are routinely employed. As a theoretical consequence, specific clones can be selected,virtually for any organic or inorganic target [76]. Phage displays, as it is practiced today, mirrors natural immune systems, since these organic collections are dynamic and can be refined to have immense range of specificities.

5.3 Landscape Phage display applications

Landscape phages have been shown to serve as substitute antibodies against various antigens and receptors [8]. A key advantage of phages over antibodies is their stability, durability, reusability and low cost production [76]. Prominent applications include detection of bacteria and *B. anthracis* spores [77, 78]. Phages were already described as specific probes for Lyme disease sera [79], and prostate cancer cells [80]. In conjunction with an acoustic wave device, phages were used for the detection of bacteria [81]. These studies substantiated with evidence the possibility to develop an array chip as a high - throughput method based on landscape phages.

5.4 Immobilization of phage on solid supports

Not long after the description of phage display technique, methods to immobilize phages on surfaces were developed for the purpose of affinity measurement or biosensor measurement. Physical absorption for ELISA, covalent binding and molecular recognition were proposed for a variety of applications. Phages have been immobilized by peptide bond between amino and carboxyl terminal groups on solid platforms. Other proposed approaches for immobilization of phages are through disulfide bonds and thiol groups, or by specific recognition between hexahistidine tags on the phage and nickel coated surface [82-88].

5.6 Phage microarrays

Phage microarrays were used recently to monitor sera from breast cancer patients on amine slides [89]. Another study proposed phage arrays to track immune responses in HIV patients by mimetic peptides. Arrays were prepared on a nitrocellulose membrane [90]. In both cases the display system of choice was pIII. The use of individual display peptides in these reports has been successful. However, an inborn advantage of landscape phage over other display systems is the use of the entire body of the phage as a block for multivalent interaction. As a novel model for a phage microarray chip, we took advantage of the pVIII landscape display system. This approach exploits all 4000 copies of the

major coat protein. It generates a defined landscape of N-terminal foreign peptide inserts,

available to interact in a multivalent way with increased avidity with a given target [8, 75,

76]

II. Introduction

The successful completion of genome sequencing projects created the need of methods to interrogate individual molecules in a high throughput scale in a sensitive, selective and specific way. While these major requirements were satisfactorily attained with regard to DNA information, a universal reliable chip for analysis of complex protein samples is still unavailable. Given the central role that proteins play in establishing phenotypes of living organisms in normal and disease state, there is an urgent need to study their abundance and activity. Protein-based microarrarrays offer one such approach [91].

Microspot assay for the detection of proteins in minute sample volumes has already been described and at the present is a valuable tool for proteomic research [92]. A great challenge for protein microarrays remains the generation of capture agents with high affinity, specificity and selectivity that can bind their targets in very low concentrated samples [58]. Until the present time, antibodies are the most important capture agents used in microarrays. Nevertheless, antibodies are ligands with inborn drawbacks. Lack of specificity, loss of activity when immobilized on microarray platforms, high cost, cumbersome production, and limited target spectrum are some of the hurdles which prevent them for greater commercial deployment together with high throughput systems [35, 47, 93].

A promising and robust alternative to antibodies are filamentous phages, affinity-selected from a landscape library for specific and selective binding to target molecules. A key feature of the Ff class of bacteriophage is its flexibility in tolerating the incorporation of foreign DNA, thus, enabling expression of alien peptides on its surface for presentation to a complementary target. In this way, landscape phages can act as substitute antibodies, possessing undeniable advantages over antibodies, including durability, stability, standardization and low cost production, while being sensitive and selective [8].

Historically, phage libraries screens for immunoreactivity have been performed through evaluation of recombinant phages by ELISA, Western blots, membrane plaque lift procedure or microarrays on filters [94]. These methods were efficient for variety of applications but also exhibited some limitations. Foremost, they need large sample volumes, for both printing of antigens and for samples. Furthermore, these assays are time-consuming and laborious. Hence, a complementary technology is required for analysis of complex samples with high sensitivity, selectivity and specificity. In the present study these parameters were analyzed in a high throughput scale for three affinity selected landscape phages immobilized on commercially available epoxy slides.

1. Materials

1.1 Reagents

1.1.1 Texas red streptavidin (TRS)

Streptavidin is a 60,000 dalton protein. It can be isolated from the microorganism *Streptomyces avidinii*. It is composed of four identical subunits, each of which has a binding site for biotin. The label is Texas Red® Sulfonyl Chloride with approximate molecular weight MW 625 Da. This red fluorescent product excites at about 595nm and has an emission maximum at about 615 nm. TRS is commercially available (Vector laboratories, USA), (1 mg at 1 mg/ml stored at 4º C).

1.1.2 Bovine serum

BSA, biotin free (Powder / 96 %), purchased from Sigma-Aldrich, USA, (catalog number A7030).

1.1.3 Streptavidin (ST)

ImmunoPure Streptavidin (Powder/ 98%), provided by Pierce Biotechnology, USA)

1.2 Glass slides

Amine and epoxy slides were manufactured by TeleChem International, Inc. ArrayIt® Division, USA.

1.3 Phage clones

We used phages selected from landscape libraries as streptavidin binders. Each clone displayed 4000 copies of the specific peptide on the surface as an N-terminal part of the fused pVIII major coat protein. Phage SAE10 was selected as a streptavidin binder

from 9-mer library and displayed VPVGAYSDT peptide (selection procedure described by Kouzmitcheva et al., unpublished data), phage 7B1 – selected from 8-mer library as streptavidin binder and displayed VPEGAFSS peptide, selection procedure reviewed in [75]. Phage JRB7 displayed EPRLSPHS peptide, found in 8-mer library as *B. anthracis* (Sterne) spores binder (selection procedure reviewed in [78]), was used as a negative control in this study.

2. Methods

To fabricate phage microarrays, we used two arraying machines. The potential of our method was demonstrated by printing and sucssesful immobilization of phages on epoxy slides. Nevertheless, the robot machine OmniGrid® 100 (Genomic Solutions®; Department of Biological Sciences, Auburn University) performed poorly with regard to spot uniformity. The lack of precise control for the spot volume and constant distance between each dot was expressed in varying fluorescent intensities, and made data analysis difficult (Image 1, p. 52). In order to solve this difficulty, we employed a high-precision prototype robot to enable spotting of phage samples in nanoliter volumes, resulting in spot sizes of 100-110μm. (Ultimate Microarray Printer constructed by the bioinstrumentation group at the Lawrence Berkeley National Laboratory, Berkeley, CA, USA; Department of Genetics, University of Alabama at Birmingham) [95]. As a result, each of the microspots in the arrays displayed a relatively constant amount of specific

peptides/epitopes. It was important to print phage arrays with spot uniformity to determine with accuracy the performance of each phage clone. A large number of microarray printers have been built and many are commercially available. However, all these printers have significant limitations and do not allow the manufacture of arrays that take full advantage of the potential of the technique. Commercial printers typically use metal pins with small slots to print the arrays. The pins are inefficient in their use of printing solutions, and the arrays that can be printed are limited in the density of the spots on the array [95]. The prototype machine we employed, equipped with a channeled quartz pin, eliminated the possibility to cross contaminate our arrays with samples of the control clone. With regard of the speed of the experiments and the image quality (Ultimate Microarray Printer, Lawrence Berkeley National Laboratory, Berkeley, CA, USA; Department of Genetics, University of Alabama at Birmingham), also performed much better than OmniGrid® 100 (Genomic Solutions®; Department of Biological Sciences, Auburn University)

2.1 Phage immobilization

Phage clone 7B1 was labeled with fluorescent dyes Alexa 488 (Alexa Fluor ® 555 Microscale Protein Labeling Kit (A30007)) according to the protocol suggested by Molecular Probes, Invitrogen Corporation. Briefly: to a volume of 0.5ml of phage 7B1 (stock solution 1.4×10^{13} cfu/ml in PBS) 50µl of 1 M sodium bicarbonate was added to

raise the pH of the reaction mixture, because succinimidyl ester moieties (of Alexa Fluor 488) react efficiently at pH 7.5-8.5. The phage solution was transferred to the reactive dye vial and left for one hour at room temperature. Afterwards, the labeled phages were centrifuged for 10min / 12000 rpm, and the dyes were removed delicately. Phages were re-suspended in PBS and kept in the dark at 4º C. Before the experiment, phage stock was adjusted to a final concentration of 6.0×10^{12} cfu/ml in PBS and printed on one amine and one epoxy slide (TeleChem International, Inc. ArrayIt® Division, USA) in a 10×10 array format (OmniGrid® 100, Genomic Solutions®; Department of Biological Sciences, Auburn University) . The same printing was performed on amine and epoxy slides with the same phage clone but without the fluorescent label.

2.2 Optimization of the phage concentration for printing phage array

To determine the concentration of phage necessary for phage microarray construction, two-fold dilutions of phage clone SAE10 were prepared as follows: 6.0×10^{12} cfu/ml, 3.0×10^{12} cfu/ml, 1.5×10^{12} cfu/ml, 7.5×10^{11} cfu/ml and suspended in PBS. Subsequently, each dilution was printed in a 5×20 array format and hybridization with decreasing concentrations 5000, 1000, 500 and 50ng/ml of Texas Red Streptavidin was performed. Evaluation of array quality and signal intensities was monitored after scanning.

2.3 Construction of phage array to target Texas Red Streptavidin

All three phage clones were adjusted to an equimolar concentration of 6.0×10^{12} cfu/ml in phosphate-buffered saline (PBS, 0.15 M NaCI, 5 mM NaH_2PO_4, pH 7.0) and printed with an approximate spot volume of 0.1nl, spot-to-spot distance of 200μm, in 5 × 20 format (Ultimate Microarray Printer constructed by the bioinstrumentation group at the Lawrence Berkeley National Laboratory, Berkeley, CA, USA) on three epoxy slides (TeleChem International, Inc. ArrayIt® Division,USA). The humidity (45-50%) and temperature (21º C) were maintained during all printing operations. Twenty-four identical arrays were printed in groups of three for each phage clone on each slide. The printed slides were incubated for 4h in a humid chamber (85-90%).

2.4   Hybridization and data acquisition

Hybridizations were performed in Microplate Microarray Hardware (TeleChem International, Inc. ArrayIt® Division, USA) providing 96 separate wells with the capability to harbor 4 slides at a time. The design of the printing enabled each phage clone array (3 × 100 spots) to fit in one well. All hybridization steps were performed at 25º C for 1 hour with mild agitation. Slides were blocked with 2% BSA, suspended in PBS / 0.05% Tween-20. Subsequently, the blocking solution was removed from the chambers and the arrays inside were rinsed one time with PBS 0.05% v/v Tween-20,

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followed by rinsing three times with water for 1min. Sets of six wells of the first slide were incubated with decreasing concentrations 5000, 1000, 500 and 50ng/ml of Texas Red Streptavidin to determine the sensitivity and specificity of the phage microarray. The two other slides were utilized for competition assays with a related and non-related competitor, to obtain data for the selectivity of the phage array. Sets of two wells were incubated with constant concentration of 250ng/ml of Texas Red Streptavidin and increasing (two-fold) concentration of the related competitor Streptavidin, finishing with 14 times excess (3500ng/ml). The first two wells were used as a control with no competitor. Sets of three wells in the last slide were incubated with constant concentration of 250ng/ml Texas Red Streptavidin, but in a mixture of increasing concentration of BSA, finishing with 4000 times excess. Hybridization reagents were added in all wells with 75μl volume. The first three wells were used as control, no competitor. Hybridization agents in all wells of the three slides were subsequently removed and the slides were washed one time with 0.05% Tween-20 and three times with water. Thereafter, all three slides were dried in a dark place. After drying all slides were scanned with GenePix 4000B (Molecular Devices Corp., USA). The same laser power setting was used for all slides, according to the excitation and emission wavelengths of Texas Red Streptavidin. The data were obtained and corrected by subtracting local background using GenePix Pro 4.1 software algorithm (Molecular Devices Corp., USA). In addition spots that had obvious defect were flagged and then removed from further analysis.

3. Results and Discussion

The aim of the present study was to develop a microarray method based on landscape phage as a binding probe and to evaluate its sensitivity, specificity and selectivity. To accomplish this, it was necessary to find conditions for effective immobilization of phage on commercially available amine and epoxy slides.

The trials for immobilization of Alexa-labeled phage on amine slides were not successful. The possible reason for this is the lack of negatively charged amino acids, since the f 8/8 landscape phage library was constructed by replacing amino acids E2, G3, D4, as reviewed in [75], which lowers the presence of negatively charged groups and hinders the formation of ionic bonds with the positively charged amine groups on the slide surface. In contrast, Alexa-labeled phage was readily immobilized on the epoxy slides because covalent binding is possible between N-terminal groups of the pVIII major coat protein and the epoxy groups of the slide surface. No signal was observed after scanning of the arrays prepared with control of non-labeled phage; hence the signal is not due to intrinsic fluorescence of phage (Image 2 and 3, p. 52). The optimal concentration of phage for array construction was established to be 6.0×10^{12} cfu/ml, since the arrays with less concentrated phage gave a low intensity signal inappropriate for data analysis with the scanning software (Image 4, p. 53).

Subsequently, it was essential to monitor performance of the three clones-based array targeting low sample volumes of Texas Red Streptavidin (TRS), in absence and

presence of related and non-related competitors. The hybridizations with decreasing concentration of TRS clearly established the presence of immobilized phage particles on the phage microarrays at expected locations. The fluorescent signal observed substantiates with evidence that the foreign peptides fused to the N-terminal part of all 4000 copies of pVIII major coat protein are the component binding TRS. Along with binding TRS, these foreign peptide inserts allow immobilization of the whole phage particle on the glass slide. Furthermore, a relationship between the analyte concentration and the fluorescence intensities was determined. The signal decreased as the concentration of hybridized TRS was lowered (Image 5., Fig. 1., p. 54).

Having demonstrated that landscape phage particles can be printed and detected on the arrays, we investigated the selectivity of the displayed peptides by a pair of competition assays with related and non-related targets. The hybridizations with a constant concentration of TRS and increasing excess of related competitor, in this assay unlabeled streptavidin, verified that the fluorescence signal on the arrays is dependent on the concentration of the target analyte, and is not due to nonspecific recognition between the printed phage clones and the fluorescent moiety Texas Red. No binding was found for the negative control, phage JRB7 displaying peptide EPRLSPHS, selected as *B. anthracis* spores binder. Whereas, phage clone 7B1, with N-terminal insert VPEGAFSS, also selected as a streptavidin, showed a weak signal, inadequate for data analysis (Image 6., Fig. 2, p. 55). The competition assay, with non-related target, such as BSA, strengthens our hypothesis that the developed phage microarray is a specific and selective model for

more complex samples. No significant decrease in signal was detected between control wells and those hybridized in conjunction of competitor excess. In addition, fluorescent intensities, present from wells with BSA, showed uniform signal, which substantiates the selectivity of the landscape phage probes for streptavidin, but not for unrelated analyte (Image 7., Fig. 3, p. 56). Moreover, both competition assays performed specifically. No detectable signal was obtained from arrays of the control phage JRB7 with N-terminal insert VPEGAFSS, selected as *B. anthracis* spores binder.

As a novel and advantageous method in this work, we described a sensitive, specific and selective method based on landscape phages immobilized on commercially available epoxy slides, using the common principle of microarray technology. Currently, immunoassays based on monoclonal antibodies are still the most important diagnostic methods widely used in analytical assays and clinical research areas [52]. Several tens of thousands monoclonal antibodies are currently commercially available. Nevertheless this number is insufficient for large-scale protein profiling since it is based on hybridoma technology which is time consuming and limited in economical sense [7]. On the other hand, polyclonal antibodies are not the best choice for microarray purposes, since they are not highly selective and thus unsuitable for large-scale methods. Cross reactivity is a major drawback in targeting proteins with and without sequence homology [55]. These hurdles limit antibodies for greater commercial growth in association with high through output technology. More stable, reliable and economic ligands for protein microarrays are needed. To meet the requirements, new strategies such as ribosomal display, affibodies,

lipocalin, fibronectin and aptamers are being developed [61-64]. A promising and remarkable technique, accustomed to fulfill the demand of reliable, robust screening and diagnostic probe, is the phage display method. Phage display is a method taking advantage of phage as a cloning vector to display foreign peptides or proteins on its surface [74]. In most situations, the phage display systems of choice are pIII and pVIII coat proteins. Depending on the application, different display libraries are accustomed. Phage display microarrays were already reported as successful probes to monitor sera from breast cancer patients and to track immune responses in HIV patients by mimetic peptides, exploiting the pIII display system [89, 90].

To develop the phage microarray for our experiments, we took advantage of the pVIII landscape display system. This approach exploits all 4000 copies of the major coat protein displaying a uniform and defined landscape of N-terminal foreign peptide inserts. These surface peptides are available to interact in a multivalent way with increased avidity with a target [8] in contrast to the commonly used pIII display system relying on just five copies of the minor coat protein pIII [71, 73]. Moreover, landscape phages have been shown to serve as substitute antibodies against various antigens and receptors [8]. A key advantage of phages over antibodies is their stability, durability, reusability and low cost production [76]. Prominent applications have been demonstrated including detection of bacteria and spores of the *B. anthracis* [77, 78]. Phage probes were already described as specific for Lyme disease sera [79], prostate cancer cells [80] and furthermore, these were used in conjunction with acoustic biodetector devices [81]. These studies proved

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possible an application of phage array as a chip in the diagnostics of cancer and other diseases. On the other hand, immobilization of phages was stable, and signal intensities were not hindered by the washing procedures. Straight covalent immobilization of protein arrays has followed a similar immobilization strategy applied for nucleic acids. Amino acids (such as lysine, glutamate, aspartate, histidine, tyrosine, cystein or any N- or C-terminus acids) provide an appropriate functional capacity for covalent immobilization [6, 47, 93]. In the developed assay, plenty of surface amine groups of the phage particle were enabled to interact covalently with the epoxy groups of the glass surface, allowing stable immobilization of the phage particle. Furthermore, landscape phage has the undeniable innate utility as a microarray probe, since it tolerates harsher environments compared to antibodies [96, 97] which tend to denature and loose affinity upon immobilization [47]. The performance of our model method supports the idea that microarrays based on landscape phage probes can overcome common drawbacks associated with antibodies, such as cross reactivity, lack of stability, and limited target spectrum [35, 47, 93]. At the same time it demonstrates the sensitivity envisioned in earlier studies of protein array technology [98].

4. Conclusion

In the present work, we have tested whether protein-engineered landscape phages selected for a target of interest, can serve as molecular recognition probes in microarray

platforms. We conclude that the proposed approach represents a valid strategy for high-throughput profiling of complex protein samples, with high sensitivity, specificity and selectivity achieved through landscape phage. Given the multivalent display of the foreign peptide insert, stable covalent immobilization on a solid platform can be achieved. Moreover, phages interact with their analyte with increased avidity, in contrast to phage based microarrays where pIII, minor coat protein, is proposed as a display system . Furthermore, the model described is highly practical, since selection of clones for a particular target is a low cost process. In numerous studies, phages proved to be robust alternative to antibodies, because they inherit a distinct stability from their natural predecessor (phage fd). Hence, we believe that landscape phage display combined with microarray technology would allow in near future to target essential components and pathways within many different diseases, including cancer, AIDS, cardiovascular disease, autoimmune disorders and bioterrorism threat agents.

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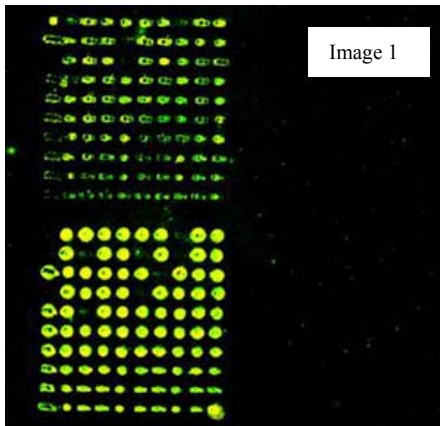
Image 1. Phage arrays printed with OmniGrid® 100 (Genomic Solutions®)

Array pattern of the phage clones 7B1, upper array, and SAE10, lower array, characterized by the lack of spot uniformity and the uneven fluorescence intensities.
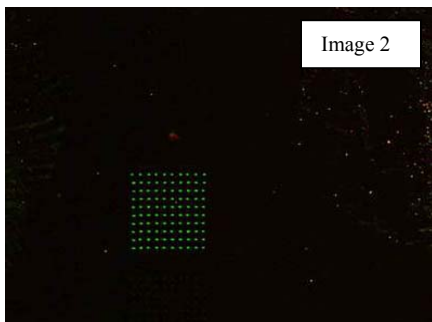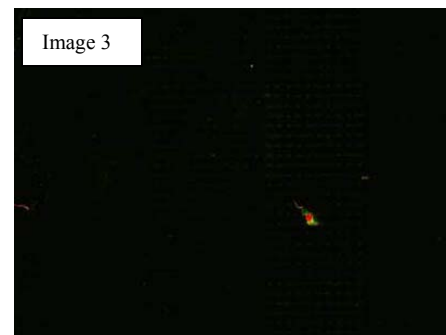

Images 2 and 3.

2) Array pattern of Alexa-labeled phage clone 7B1, printed in a 10 x 10 array format.

3) Non – labeled phage clone 7B1, printed in a 10 x 10 array format. No signal observed.
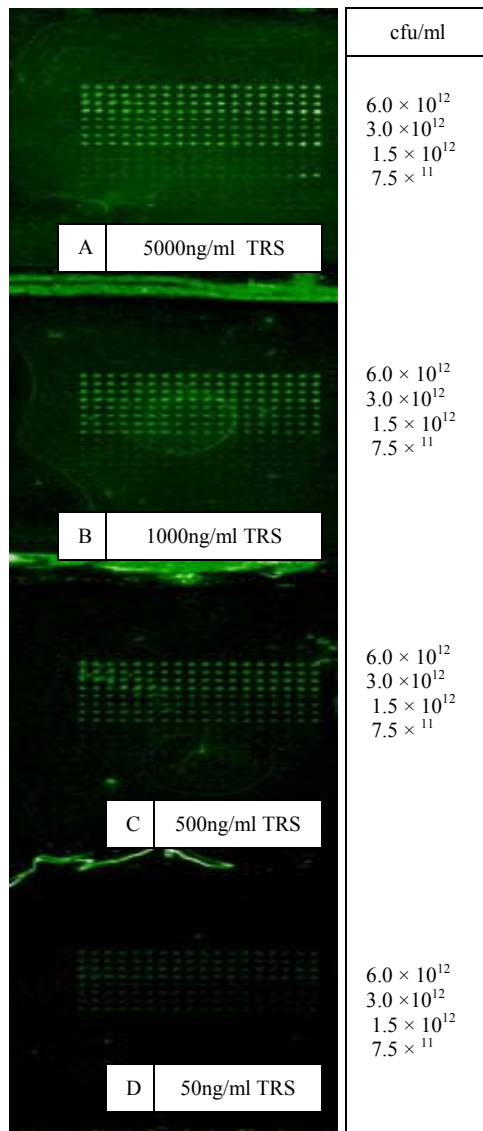
Image 4. From top to bottom, arrays of 5 x 20 spots (6.0×10^{12}, 3.0×10^{12}, 1.5×10^{12}, 7.5×10^{11} cfu/ml) of the phage clone SAE10, were incubated with the decreasing concentrations of 5000, 1000, 500 and 50ng/ml of TRS (A, B, C, D). The signal had adequate intensities from spots 6.0×10^{12} and 3.0×10^{12} cfu/ml.
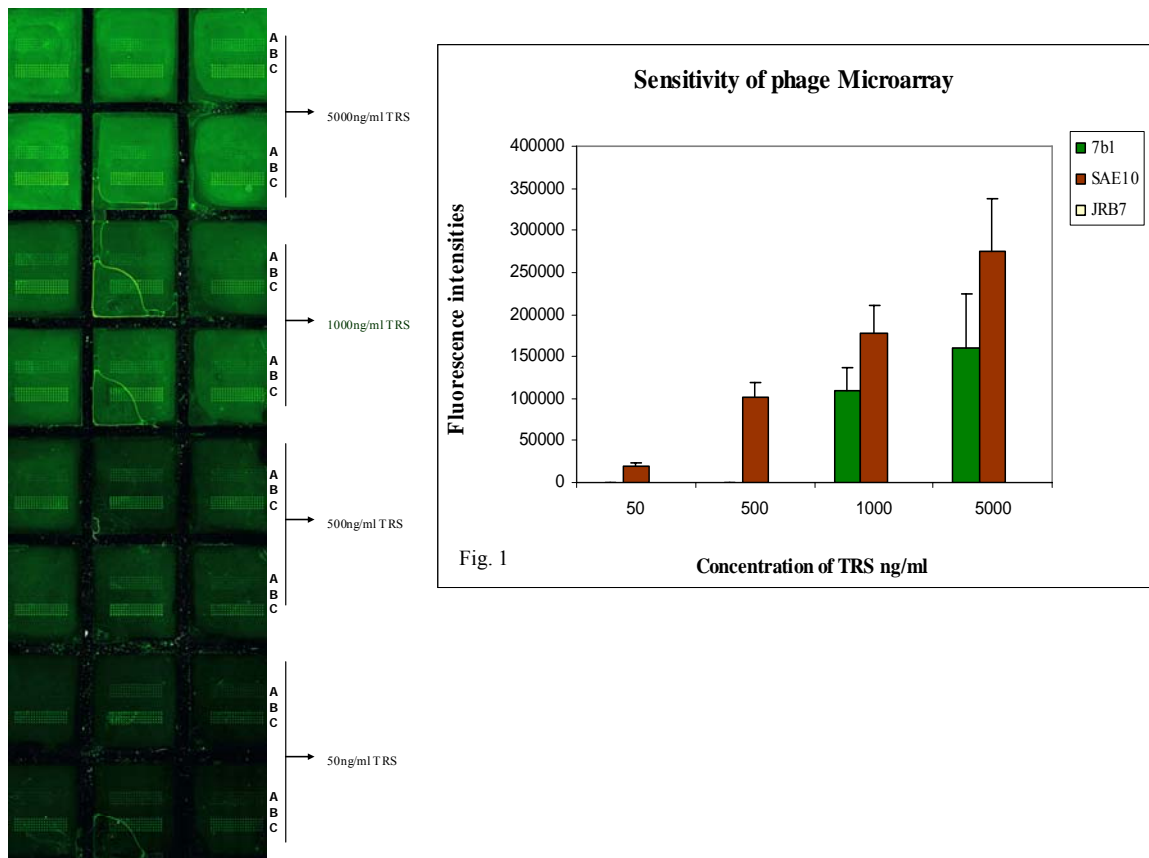
Image 5. From top to bottom, clusters of six wells, each well containing three phage clone arrays were incubated with 5000, 1000, 500 and 50ng/ml of Texas Red Streptavidin. The first array, in each well, is a moderate binder (7B1) of streptavidin (A); the middle array is the control phage (JRB7), no signal (B). The third array of each well is a strong binder (SAE10) of streptavidin (C).

Fig. 1. Fluorescence intensities of the phage array showed the clone SAE10 to recognize the target TRS at a concentration of 50ng/ml. The phage clone 7B1, performed with 20 times lower sensitivity. All three phage clones had an equal concentration prior to spotting. Signals differed as a consequence of the specificities of the displayed peptides.

Image 6. From top to bottom, the first pair of wells were incubated with 250ng/ml TRS as a control. Subsequent wells were incubated with two-fold excess of streptavidin starting at 55 and finishing with 3500ng/ml. The first array, in each well, is the moderate binder (7B1) of streptavidin (A), not taken in account for the plot. The middle array is the control phage (JRB7), (B), no signal. The last, third array is the strong binder of streptavidin (SAE10), (C).

Fig. 2. The fluorescence signal decreased as the excess of unlabeled streptavidin (ST) was added. The fluorescence intensities were dependent on the concentration of the target (ST) and not due to recognition of the fluorescent moiety Texas Red. Phage clone 7B1 showed inadequate signal intensities for the data analysis.
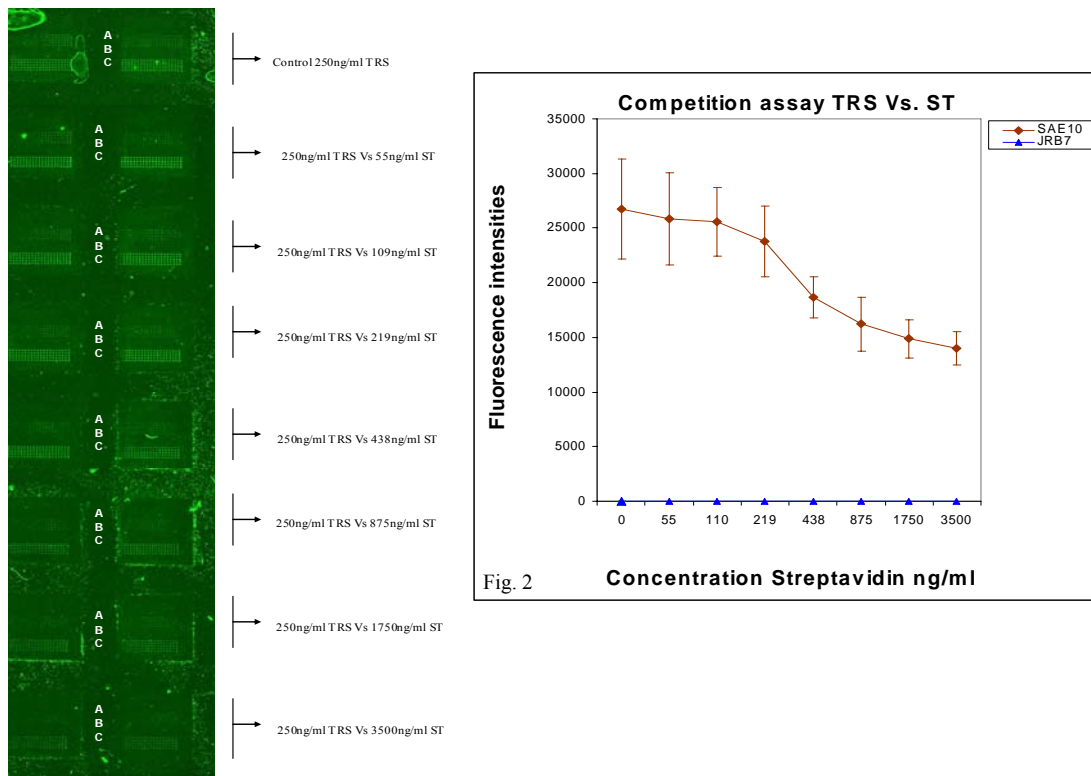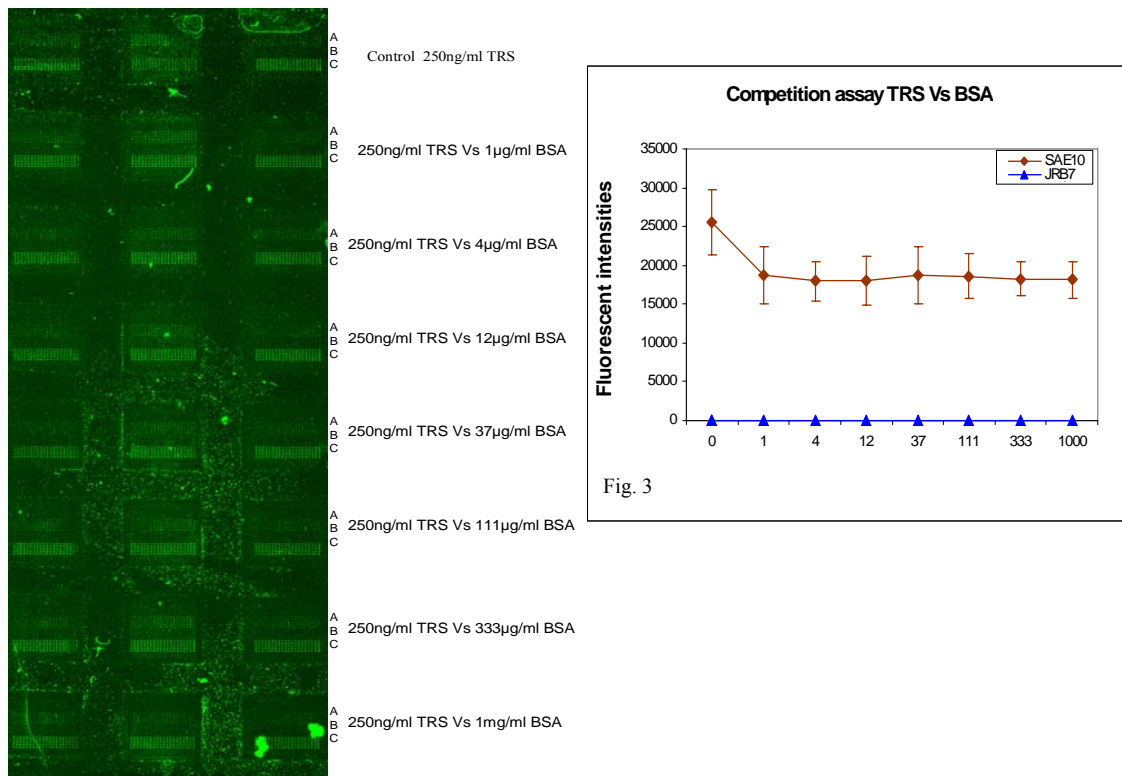
Image 7. From top to bottom, the first three wells were incubated with 250ng/ml TRS as a control. All subsequent wells were incubated with 250ng/ml TRS but in a mixture of increasing excess of BSA starting at 1µg/ml and finishing at 1mg/ml. The first array, in each well, is a moderate binder (7B1) of streptavidin (A), not taken in account for the plot. The middle array is the control phage, no signal (B). The last third array represents a strong binder of streptavidin (C).

Fig. 3. No significant decrease in the signal was detected between the control wells and those hybridized with increasing excess of the competitor BSA. Fluorescent intensities, from wells with BSA, showed a uniform signal, proving the phage clone SAE10 selective for streptavidin but not for the unrelated competitor BSA.

CHAPTER 2

PHAGE MICROARRAY AND DOT-BLOT FOR CHARACTERIZATION OF BINDERS

OF *Bacillus anthracis*

I. LITERATURE REVIEW

1. *Bacillus anthracis,* general description of bacteria and disease

B. anthracis are anaerobic, gram-positive, spore forming bacteria that cause anthrax. In their vegetative form, *B. anthracis* spores are 1-1.5μm × 3-10μm in size and are typically nonmotile, occurring either as a single or chain forming cells. *B. anthracis* are protected by a poly - γ – D - glutamic capsule [1]. Vegetative cells are incapable of surviving outside a host for extended periods of time. Thus they are not able to establish infection in a new host. As a long-term survival strategy, upon exposure to a low nutrient environment, radiation, desiccation or harsh chemicals, *B. anthracis* enters a dormant state and forms spores. These spores are extremely resistant and hardy, and can survive for decades in this state [2]. Sporulating cells carry elliptic, centrally located spores [3].

Anthrax is mainly a disease of herbivores. Nevertheless all mammals, including humans, are susceptible. The disease starts upon entry of spores into the host body such as through skin abrasions, insect bites, consumption of contaminated food or inhalation of airborne particles. There is no available evidence of live animal to live animal transmission [4]. Early diagnosis is difficult and the disease can develop into a systemic form that becomes treatment-resistant and rapidly fatal with shock-like symptoms, sepsis and respiratory failure [5]. Regardless of the route of infection, spores are taken up by macrophages where they germinate and are transported to the regional lymph nodes [3]. Nevertheless, the course of the disease is dictated by the route of infection, thus expressed as cutaneous anthrax, gastrointestinal anthrax, or inhalation anthrax [6, 7].

2. Significance as biological weapon

Biological warfare strategies were used in ancient times, long before true mechanisms of disease spread were fully understood. During the 6th century B.C., the Assyrians poisoned enemy wells with a fungus that would make the enemy delusional. In the Middle Ages, victims of the bubonic plague were used for biological attacks. Their corpses were thrown over castle walls with catapults. When the specific microorganisms were identified as the causative agents of certain diseases, more advanced biological weapons evolved [8].

For a long period of time *B. anthracis* spores were acknowledged as a biological weapon with great potential. The hardiness and robustness against harsh environments and their ability to cause mortality in humans and animals after short incubation periods made them a deadly biological weapon. During World War I, Germany contaminated livestock exported to the Allied forces with *B. anthracis* spores. In World War II, the Japanese attacked Chinese cities by contaminating food and water with the spores. The United States also studied *B. anthracis* in its offensive biological program, producing 5000 bombs filled with spores [8].

However, present biological threats are more likely to be silently released rather than delivered in a bomb. The agent can be spread and infect hundreds or even thousands of people before it is detected. *B. anthracis* is an ideal agent, since hardy spores can be spread in powder or aerosols through ventilation systems or by crop dusters. Accidental aerosolized releases have been already observed. In Sverdlovsk, USSR, in 1979, the spread of *B. anthracis* resulted in 96 cases of human anthrax and numerous cases of livestock death in a tight geographical region [9]. 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 [2].

The spores of *B. anthracis* mailed in the US aggravated the aftermath of September 11, 2001. The spores were delivered to victims in a powder enclosed in letters sent through the U.S. postal system [10]. The incident caused 23 cases of confirmed

anthrax. Perpetrators remain unidentified and the risk of future exposure through unconventional means still exists.

3. Genetics and comparison to other *Bacillus* species

B. anthracis is part of the *B. cereus* group of *Bacillus*, which also includes *B. thuringensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, and *B. medusa* [5, 11, 12]. *B. anthracis* is physiologically and genetically very similar to *B. cereus* and *B. thuringensis*. In some studies it has been argued that these three species of bacteria should be considered one [13]. *B. anthracis* is believed to have evolved from *B. cereus* by the acquisition of virulence plasmids, pXO1 and pXO2, together with multiple chromosomal changes [14].

Studies of 16S and 23S rRNA revealed that *B. anthracis* is quite similar to other *Bacillus* species [15, 16, 17]. For *B. anthracis,* a 100% 16S rRNA sequence identity with *B. cereus* was discovered. Whereas, a difference of only four to nine nucleotides from the sequences of *B. mycoides* and *B. thuringiensis* was detected [16]. Furthermore, only two-nucleotide difference in the 23S rRNA sequence was observed between *B. anthracis* and *B. cereus.* Investigations of the 16S–23S intergenic spacer region (ISR), showed only a single nucleotide deletion difference between *B. anthracis* and *B. cereus,* and a 13-nucleotide distinction versus the sequence of *B. mycoides*. The region coding for the ISR between the two subunits of DNA gyrase, *gyrA* and *gyrB*, of *B. cereus* and *B. mycoides*

differed from that of *B. anthracis* by one and two nucleotides, respectively [18]. *B. anthracis* is even more similar to itself [16]. Homology studies of different *B. anthracis* strains and their genome suggested that the organism has a similarity greater than 90%, and it may be the most molecularly homogenous bacteria known. The quiescent and stable spore-state protects the genome from injurious events such as the presence of phages and during the constant replication of DNA [13, 19, 20].

The virulent strains of *B. anthracis* carry two large plasmids, pXO1 and pXO2, which encode the primary virulence factors, toxin production and capsule formation, respectively. The complete DNA sequences of pXO1 and pXO2 are known. The GC content (33%) of these plasmids is similar to that of the *B. anthracis* chromosome. The pXO1 was isolated from the Sterne strain and is 181,654 nucleotides long with 143 open reading frames, covering about 61% of the DNA. The pXO1 carries the structural toxin genes, *pagA*, *lef*, and *cya* which encode the toxin proteins protective antigen (PA), lethal factor (LF) and edema factor (EF). Respectively, pXO1 also carries regulatory elements, such as a resolvase and a transposase; and *gerX*, in a three-gene germination operon [21, 22]. This entire sequence resides within a 44.8-kbp segment flanked by inverted IS1 627 elements. The region has been termed a pathogenicity island. A reported inversion implies it is able to transpose. The plasmid also carries DNA topoisomerase [23]. The pXO2 carries *capB*, *capC*, *capA*, and *dep*, required for the synthesis of the poly - γ – D - glutamic capsule of vegetative cells. The pXO2 was isolated from a Pasteur strain and little is known about its putative functions. The presence of both plasmids is necessary

for the virulence of *B. anthracis* [24]. Plasmid-encoded genes are not considered ideal for detection purposes, since there are *B. anthracis* strains lacking both. None of the known strains, pX01$^-$/ pX02$^-$ is virulent. However, there is concern that a virulent strain could be engineered. For a reliable detection method, both chromosomal and plasmid sequences unique to *B. anthracis* should be taken into account.

The close similarity between *B. anthracis* and its close relatives makes the development of assays for detection of *B. anthracis* very difficult, since both *B. thuringiensis* and *B. cereus* are frequently found in the environment. *B. thuringiensis* has been found in soil worldwide, as well as in insects and store product dust, whereas *B. cereus* has been isolated from soil and food products [25, 26].

4. Spore surface structure

Upon nutrient deprivation, after exhausting elements such as carbon, nitrogen or phosphorus, species of the *Bacillus* family form spores. These are extremely resistant to wet and dry heat, desiccation, UV radiation and a wide spectrum of toxic chemicals [27]. The structure of *Bacillus* spores has been thoroughly studied, since it is the infectious form of *B. anthracis*.

4.1 The Exosporium

The spore surface is the first structure for interaction with the host. The major component of the exosporium of *B. cereus* is protein, but lipid (18%) and carbohydrate (20%) are also present [28]. A spore usually contains three hexagonal lattice layers [29, 30], and its surface is decorated with filamentous appendages [31]. As with Gram-negative pili, these filaments may be of importance for spore attachment to surfaces or ligands. The function of the exosporium is unknown, but it does not appear to be important either for dormancy or germination [32]. It may perform as a barrier, preventing exoenzymic attack on the spore coat and cortex layers [33]. *B. anthracis* and its close relatives, *B. cereus* and *B. thuringiensis* all have exosporiums which contain carbohydrates and glycoproteins. However, there are some differences in the composition. Rhamnose, 3-o-methyl rhamnose and galactosamine have been found only in *B. cereus* and *B. thuringiensis* spores [34]. A novel tetrasaccharide named anthrose was found in the exosporium of *B. anthracis* but not in *B. cereus* or *B. thuringiensis* [35]. The major glycoprotein found in *B. anthracis* exosporium is a 382 amino acid peptide called BclA and was observed to be present in the hair-like projections of the exosporium. The major glycoprotein of *B. cereus* and *B. thuringiensis* has been found to be homologous to BclA, but different from the glycoprotein present in the exosporium of *B. anthracis* [36, 37]. BclA was found to be the immunodominant moiety of the *B. anthracis* exosporium, as the majority of monoclonal antibodies raised against *B.*

anthracis spores, bound to BclA. However, monoclonal antibodies generated against *B. cereus* and *B. thuringiensis* do not appear to react with BclA, suggesting that BclA is present in this species but it is not a major component of the exosporium [38].

4.2 The Capsule

Additionally, the cytoplasmic membrane and peptidoglycan layers found in all bacteria, *B. anthracis* bacilli have two other surface structures, namely a capsule and an S-layer. The *B. anthracis* parietal architecture is very complex; few bacteria possess both of these structures. The capsule contributes to pathogenicity by enabling the bacteria to evade the host-immune defenses and provoke septicemia. The *B. anthracis* capsule inhibits phagocytosis, since it is a monotonous linear polymer that is weakly immunogenic. The capsule is a polymer of poly - γ – D - glutamic. The molecular weight of the polyglutamic chains is between 20 and 55 kDa *in vitro* and estimated to be 215 kDa *in vivo*. Although peptide capsule is not common in bacteria, some species, including *B. subtilis, B. megaterium,* and *Bacillus licheniformis,* synthesize glutamic acid polymers. The glutamyl polypeptide of *B. anthracis* adheres to the host cell by an as yet unknown mechanism. *B. anthracis* capsule synthesis is encoded by the plasmid pXO2. Three pXO2 genes are sufficient for poly - γ – D - glutamic acid synthesis in *Escherichia coli* [39, 40, 41,42] . These genes, *capB, capC,* and *capA,* encode enzymes of 44.8, 16.5, and 46.4 kDa, respectively. Their sequences suggest that they are membrane associated enzymes.

No specific enzymatic function has yet been assigned to any of these *B. anthracis* or *B. subtilis* proteins. A fourth gene in the *B. anthracis cap* locus, gene *dep*, is associated with depolymerization of the capsular polymer [43]. *Dep* appears to catalyze the hydrolysis of the poly - $\gamma - D$ - glutamic acid polymer, thus controlling the size of the capsule.

4.3 S-Layer

The capsule is the outermost element of the cell wall. When *B. anthracis* does not produce its capsule, the exterior of the cell wall appears layered owing to the S-layer. However, the presence of an S-layer is not required for normal encapsulation of *B. anthracis* bacilli, but it may modify its fine structure [44]. The S-layer, overlaying the peptidoglycan, is composed of fragments displaying a highly patterned ultrastructure [31, 45]. S-layers are proteinaceous paracrystalline sheaths that completely cover the cell surface. Various functions have been proposed for S-layers, including shape maintenance and molecular sieving, and they may also be virulence factors [46]. Nevertheless, the capsule and the S-layer seem to have a cumulative effect, increasing resistance to complement pathway-mediated defenses [3].

5. Detection of *B. anthracis*

The *B. anthracis* detection is challenging because of its great genetic resemblance to *B. cereus*, *B. thuringiensis*, and *B. mycoides*, the four species that altogether compose the *B. cereus* group of bacteria [13]. The principal difference is the presence of plasmids coding for insecticidal toxins in *B. thuringiensis* and the accompanying two virulence plasmids in *B. anthracis*. The Sterne and Pasteur vaccine strains of *B. anthracis* lack either the capsule plasmid pXO2 or the toxin plasmid pXO1.Whereas the Carbosap strain includes both plasmids [47]. Such strains maintain species-dependent virulence, and upon immunization, exhibit fluctuation in effectiveness towards subsequent exposures with virulent *B. anthracis* [47, 48]. Additionally, there is significant overall similarity between *B. anthracis* and other members of the *Bacillus* genus. While there are some striking differences between *B. anthracis* and other closely related species like *B. cereus* and *B. thuringiensis*, such as that *B. anthracis* can cause disease in humans and produces a polypeptide capsule, an unambiguous identification remains a challenge [13, 18, 49].

5.1 Traditional identification methods

Identification of *B. anthracis* can be performed using standard morphological considerations. It is sensitive to penicillin, non-motile, and is not β - hemolytic on sheep- or horse-blood agar plates. Furthermore, it is susceptible to lysis by gamma phage, and

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has a distinctive appearance [5]. *B. anthracis* selectively grows on polymyxin-lysozyme EDTA-thallous acetate (PLET) agar [50], but this method requires 1–2 days and is still subject to further confirmation [51, 52]. This may be followed by McFadyean's capsule-staining test which causes rods of *B. anthracis* to become dark blue and be surrounded by a pink capsule upon staining heat-fixed smears of blood with polychrome methylene blue [53]. Identification based on colonial morphology and biochemical tests yields an initial diagnosis of *Bacillus* infection after an additional 12–24 h, but definitive identification of *B. anthracis* requires an additional 1–2 days of testing [2]. Blood cultures do provide definitive identification; however, cultures from skin or lesions cannot be used as they yield positive results in only 60–65% of the samples tested [54].

5.2 Rapid methods

5.2.1 Mass spectrometry

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. Methods based on this principle have enabled successful identification of *B. anthracis* and the discrimination of these from the closely related *B. cereus* and *B. thuringiensis*. Limitations of these methods, however, are the inferences caused by contaminating particles and the differences in results depending on the growth of the target organisms [55].

5.2.2 Immunoassay methods

Immunoassays are methods based on recognition of an antigen by a specific capture ligand. They have developed since the first immunoassay for detection of insulin. Generally these methods rely on poly- or monoclonal antibodies as probes [56]. Antibodies to *B. anthracis* spores, vegetative cells, and toxin proteins have been utilized in a variety of immunoassays for *B. anthracis* detection [57]. Immunoassays based on surface antigens can provide a rapid detection method for spores. However, specific serological detection is difficult, due to cross-reactivity of both polyclonal and monoclonal antibodies, and between antigens of spores of *B. anthracis* and closely related species [58-60].

A successful immunofluorescence system to detect spore surface antigens unique to *B. anthracis*, as determined by the lack of cross-reactivity with the closely related *B. cereus* species, has been reported [61, 62, 63]. This method involved immobilizing of 10^4 spores on a slide and then viewing the spores one at a time. Spores also have been detected by ELISAs as well as immunoradiometric assays (IRMA) [63].

A method based on magnetic beads labeled with streptavidin attaches spores's specific antibodies labeled with biotin and with an N-hydroxysuccinimide ester derivative of ruthenium (II) trisbipyridine [Ru $(bpy)_3^{2+}$]. Hence, a sandwich complex between the beads and the spores is formed. The method was able to detect spores rapidly in soil but has detection limits ranging from less than 100 spores for the Sterne strain to 10^4–10^5

spores for the Vollum strain. The analysis of the Ames strain yielded intermediate sensitivity [64].

Another method of detection used monoclonal antibodies against vegetative cell surface antigens. However, the antibodies were only effective with non-encapsulated vegetative cells and suffered from cross-reaction with cells of *B. cereus* [65].

Flow cytometry has also been used to detect fluorescein-labeled antibodies bound to *B. anthracis* spores. However, the main drawback of this rapid technique is the high limit of detection (10^3 spores) [66].

The detection of capsular and cell wall antigens has been accomplished through direct fluorescent antibody (DFA) assays. A fluorescently labeled antibody binds specifically to its target antigen and provides fluorescence when viewed under a fluorescence microscope. Unbound antibodies are eliminated by centrifugation of the antibody–antigen complexes. This direct method, with only one antibody showed some cross-reactivity. No cross-reactivity was observed when assays were performed using two antibodies when 56 other Bacillus strains were tested. Using this method, a detection limit of 10^4 cells/ml was reported [67].

In addition to the detection of the entire organism, enzyme immunoassays are available to detect all three of the toxin proteins in the blood, but these must be done when the disease is already at an advanced stage [68]. Many diagnostic techniques associated with anthrax are designed to occur after infection is suspected, such as the detection of PA using a dot-blot ELISA [69], detection of antibodies to PA by indirect

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hemagglutination assay (IHA) [70], detection of serum antibodies to PA and LF by electrophoretic-immunotransblots (EITB) [71], and detection of antibodies to PA [72], EF, LF [73] or poly-D-glutamic acid capsule by ELISAs [71]. Immunoassays directed towards the protective antigen or capsular components have been found to be the most reliable, versus those directed towards lethal or edema factors for the detection of a prior infection or immunization [73]. In a study of human serum samples following an outbreak of cutaneous anthrax, 50 and 91.7% of known positive cases were confirmed by EITB when screened for antibodies against LF and PA, respectively [71].

Several varieties of ELISAs for anti-PA IgG have been reported, including a direct assay with purified PA, allowing anti-PA antibodies in serum to bind [74]; an antigen-capture ELISA where a monoclonal anti-PA antibody was immobilized, and recombinant PA was added, followed by the introduction of serum samples [75]; and a competitive ELISA [76, 77]. Detection was accomplished using an enzyme-linked secondary antibody and appropriate substrate. These assays had varying sensitivity and specificity, with sensitivity ranging from 71 to 91.4% [78] frequency for detection in confirmed clinical cases. False positives have also been reported with rates depending on the ELISA format. The production of the required purified PA from *B. anthracis* for ELISAs is not a trivial task, but has been overcome through the use of a recombinant form of PA [74, 75]. Although, ELISAs have proven to be reproducible and sensitive assays, they are intended for the measurement of one analyte at a time. For the analysis of

potential biothreat agents, a multiplexed assay, such as the one made possible by the Luminex system, is desirable.

The Luminex system relies on the unique light transmission from beads containing red and infrared dye which are coupled to various recognition elements. Multiplex detection is made possible using a flow cytometry format to detect the distinct signature of each of the beads combined with a labeled secondary recognition element upon forming a sandwich with the antigen [79, 80]. This approach was used to yield an MDC of 0.01 and 0.001μg/ml for anti-PA IgG and anti-LF IgG, respectively, when detected in a multiplex format. When used as a monoplex assay, the Luminex-based assay yielded an MDC of 0.006μg/ml for anti-PA IgG [80, 81].

While these detection methods are of considerable interest in medical diagnostics and epidemiology, they are not relevant to rapid pathogen detection geared towards preventing exposure, since they are only applicable after exposure to the organism. The drawback, of these otherwise very effective immunoassays, is that death normally results in patients prior to sufficient antibody levels being produced, or before a blood culture of *B. anthracis* can be grown for the detection of antibodies through agar diffusion methods [82]. In addition, anti-PA antibodies may already be present in some susceptible species, where the disease is geographically endemic [78].

5.2.3 Nucleic acid methods of identification

A broad set of reliable nucleic acid amplification-based techniques, including PCR [48], real-time PCR [83, 84, 85], and multiplex PCR [87, 88], are available for the detection of *B. anthracis*. These techniques depend on the availability of nucleic acid probes that are unique for *B. anthracis* and do not cross-react with other species. Primers are often chosen to amplify targets such as *lef*, *pag*, or *cap*, found on either, or both, of the virulence plasmids, pXO1 and pXO2 [88, 89]. PCR has been reported to detect the presence of both pXO1 and pXO2 plasmids and can be used to characterize *B. anthracis* vaccine strains [48] and to detect spores from soil samples. Nevertheless, some strains lacking these plasmid sequences might remain occult. To overcome such problems, some studies have focused on chromosomal sequences as trustworthy markers.

A sequence known as BA-5449 has been reported to be specific for *B. anthracis* amongst 62 Bacillus strains tested in a microarray assay [90]. Another reliable sequence is the 175bp *rpoB*. It has been shown to be highly selective for *B. anthracis*. Only one strain cross-reacted, out of 175 bacilli strains tested in a real-time PCR assay [48]. This sequence discriminates between *B. anthracis* and other Bacillus strains by a four-nucleotide difference. Using this target, other real-time PCR assays have been reported [91, 92]. However some have found false - positive signals for *rpoB* with strains of *B. cereus*, apparently due to slight mispriming of a single nucleotide difference at the 3 ′ end [89].

The DNA replication gene *gyrA* has also been used successfully as a target for *B. anthracis* identification [93]. This assay yielded 100% sensitivity amongst the 171 organisms from 29 genera tested. The 832bp sequence SG-850 has been identified as species specific, belonging to the *B. cereus* group. Restriction and digestion, after amplification of this fragment, by the endonuclease AluI and subsequent gel analysis showed two fragments containing approximately 90 and 660bp that were unique to *B. anthracis* strains [93]. Other potential markers include AC-390 [94], 16S rRNA [96, 97], and 16S–23S internal transcribed spacers [98].

Chromosomal sequences exhibit great potential as applicable markers for *B. anthracis* detection. Still, they are not ideal, since PCR identification based on *gyrA* and *rpoB* rely on the detection of single-nucleotide differences between *B. anthracis* and other Bacillus species and are based on stringent assay conditions [99]. Whereas, minimal differences exist in the 16S and 16S–23S sequences [100], and the analysis of some targets, like SG-850, requires additional time-consuming and labor-intensive steps. While these targets are not 100% specific to *B. anthracis*, they provide sequences to screen for avirulent or plasmid-cured strains that may be reason for false alarms [101]. As a consequence, amplification methods usually take advantage of both chromosomal and plasmid markers and become reliable assays, altogether with phenotypical analysis [5, 88, 89, 102, 103].

Real-time PCR utilizes fluorescent-label incorporation and detection of amplified DNA through fluorescence resonance energy transfer (FRET). The advantage of this

method is the possibility to amplify and at the same time detect particular sequences, reducing cross-contamination. A study, using this technique with primers for *pagA* and *capB,* reported detection within 1h [100]. However, the method required a growth period of 18–24 h and extraction for 0.5–1h prior to amplification. During the 2001 U.S. anthrax outbreak, to test 542 isolates from this outbreak and previous outbreaks, Real-time PCR using primers and probes for regions on pXO1, pXO2, and a segment of the *B. anthracis* chromosome reported to detect 1pg DNA (which corresponded to ~167 cells or 5–10 spores) [104].

Multiplex PCR has also been used to detect DNA from *B. anthracis* in several reports. This approach depends on simultaneous amplification of unique regions of DNA applying several sets of primers in one reaction chamber. Obvious benefit is the elimination of false positive and false negative results, since it amplifies and detects simultaneously multiple targets [87, 105].

5.2.4 Prototype systems for detection of *B. anthracis*

The U.S. Postal Service has installed real-time PCR units for anthrax screening units at several sorting facilities [106]. These systems are fully automated and are able to detect suspect samples within 30min. The commercial technology employed by Cepheid takes advantage of ultrasonic energy to lyse samples, and microfluidic chambers to purify

and concentrate the resulting nucleic acids prior to PCR amplification of *pag* and *capB* along with internal controls [107].

Other commercially available systems for detection of pathogens include the Liat Analyzer and the Luminex system. The Liat Analyzer utilizes segregated tubes which contain all components necessary for real-time PCR. Samples are added to each tube, which are then selectively mixed by the instrument prior to the analysis. Four optical channels are available that permit multiplex detection [108]. The Luminex system is used for automated analysis of aerosolized *B. anthracis* and *Y. pestis* [79]. This system also serves as a component of the autonomous pathogen detection system (APDS). The APDS is an instrument combining aerosol sampling, sample preparation, multiplex immunoassay detection and confirmatory real-time PCR that is intended for continuous monitoring of pathogens [109, 110].

A device known as the Handheld Advanced Nucleic Acid Analyzer (HANAA) during emergency situations can simultaneously detect up to four pathogens and output results within 30min. It has been used for the detection of *B. anthracis* through amplification of *capA* and *vrrA* genes, as well as for other pathogens including *Erwinia herbicola* and *Escherichia coli* [7].

Each of these techniques has its own inborn advantages, but they are not ideal for the detection of low levels of specific bacteria in environmental samples. Furthermore, DNA and antibody-based techniques do not indicate whether the pathogens are still viable [111]. Since this discrimination has been traditionally done by culturing samples

into vegetative bacteria or by inoculating animals with suspect samples to test for lethality [5, 51], mRNA, or rRNA, detection methods are a better indicator of viability than DNA. In addition, mRNA has a shorter half life, which is another advantage for screening through the RT-PCR-based technique. This technique, while promising for RNA amplification, requires DNAse step to remove DNA so that it is not co-amplified along with RNA [112]. Nevertheless, the DNAse treatment has shown to be not very effective and additional complex steps are required [113]. An assay called nucleic acid sequence-based amplification (NASBA) combined with a lateral flow assay has been successfully demonstrated and is an excellent alternative for the rapid and sensitive detection of viable *B. anthracis*. These assays enable extremely low detection limits of 1.5 fmol per assay. The biosensor assay itself is completed in 15min and can be quantified visually or using a handheld reflectometer. Two mRNA sequences, *atxA* and *pag*, were chosen, providing high specificity for viable *B. anthracis*. In the case of *atxA* detection, one spore could be detected using 8h of sporulation and enrichment culture with subsequent NASBA amplification and biosensor detection. In the case of *pag* as target analyte, only 30min of sporulation in a growth media was required (instead of 8h). Due to higher expression levels and more optimal NASBA amplification primers, this method shortens the overall detection time for 10 spores to 4h total. In both cases, no cross-reactivity with any other *Bacillus* species was determined [114, 115].

II. Introduction

The causative agent of anthrax, spores of *Bacillus* anthracis, were recently reported as a successful biological weapon to perpetrate terrorist attacks in the United States. Rapid detection system of spores is required before the onset of symptoms in victims. *B. anthracis* spores are hardy and robust against harsh environments. They cause mortality in humans after short incubation period which makes them a deadly biological weapon [2, 27]**.** Although specific and sensitive, nucleic acid based methods for detection of *Bacillus* species are very complex, expensive, and cumbersome and usually require spore germination and outgrowth of vegetative cells. Immunoassays and biosensor based detection systems are so far the best alternative for continuous monitoring [116]. Antibodies and peptides can be employed for this purpose as demonstrated by several recent reports. These systems are relatively fast because they detect spores directly. However, current antibody-based detectors suffer from lack of accuracy and limited sensitivity, which results in an unacceptably high level of both false-positive and false-negative responses, according to federal government trials [117] and other independent tests [118]. Additionally, *B. anthracis* has several related *Bacillus* species, such as *B. cereus* and *B. thuringiensis,* and the development of specific and sensitive probes to avoid false alarms, has been a great challenge [13]. Hence, new types of robust and specific probes are required. We envision that in near future, landscape phage probes can be used as alternative capture agents on commercially available array platforms designed

as *B. anthracis* spores detectors. Recently, the possibility of using landscape phage probes in place of antibodies has been presented [119, 125, 126, 127]. Nevertheless, procedures for selection and binding performances of designed clones, specific for a target of choice, are time consuming. Hence, we developed two systems for rapid high-throughput characterization of phage clones selected as *B. anthracis* binders.

1. Materials

1.1 *Bacillus anthracis* spores:

The *Bacillus* strains used in this study were as follows: the Sterne strain of *B. anthracis* (an avirulent veterinary vaccine strain) and *B. subtilis* (trpC2) 1A700 (originally designated 168). Spores were kept in equal concentrations of 10^9 spores/ ml in 40% ethanol at 4º C.

Spores were labeled with fluorescent dyes Alexa 488 (Alexa Fluor ® 555 Microscale Protein Labeling Kit (A30007)) and Texas (Texas Red®-X Protein Labeling Kit (T-10244)) according to the protocol suggested by Molecular Probes (Invitrogen Corporation). Briefly: to a volume of 0.5ml of *B. anthracis* and *B. subtilis* spores (stock solution 10^9 spores/ml in water) 50µl of 1 M of sodium bicarbonate was added to raise the pH of the reaction mixture, since succinimidyl ester moieties (of both, Alexa Fluor 488 and Texas red) react efficiently at pH 7.5-8.5. The spore solutions were transferred to the respective reactive dye vial and left for one hour at room temperature. Afterwards,

the labeled spores were centrifuged for 10 min/10,000 rpm, and the dyes were removed carefully. Spores were re-suspended in 40% v/v alcohol and kept in the dark at 4º C. Before each experiment, the concentrations of both *B. anthracis* and *B. subtilis* stocks were adjusted to 10^8 spores/ml in 4% v/v alcohol using Neubauer counting chamber.

1.2 Phage clones:

We employed phages selected from landscape libraries which were identified using ELISA as *B. anthracis* binders. Each clone displayed 4000 copies of specific peptide insert on as an N-terminal part of the fused pVIII major coat protein. Phage JRB7 displayed the EPRLSPHS peptide, selected from an 8-mer library (selection procedure reviewed in [119]). Phage 1E24 displayed the ASRPMPVS peptide, selected from an 8-mer library (selection procedure described by Kouzmitcheva et al., unpublished data), both used as *B. anthracis* (Sterne) spores binders. Phage 7B1 – from an 8-mer library - displayed the VPEGAFSS peptide and was selected as streptavidin binder (selection procedure reviewed in [120]) and was used as a negative control.

2. Methods:

2.1 Phage microarray trials

Phage clones JRB7 and 7B1 were adjusted to an equimolar concentration of 6.0 × 10^{12} cfu/ml in phosphate-buffered saline (PBS, 0.15 M NaCI, 5 mM NaH_2PO_4, pH 7.0)**.** Afterwards phages were printed, 0.5-1.0nl of each phage suspension, by a robot machine (Omni Grid^{TM} Microarrayer / Gene Machines USA, equipped with one steel pin) in a 10 × 10 array format (150-200μm diameter and 400μm space between spots) on one Epoxy glass slide (Corning, USA). Phage particles were incorporated on the slides at 25ºC and 80-90% humidity. After printing, all slides were immediately placed in a humid box for 4h, to allow the attachment of the virion suspensions through their amino surface groups to the epoxy coating of the glass slide. Subsequently, the arrays on the glass slides were sealed in RPI secure hybridization chambers. Each chamber has 200μl volume (Research Products International Corp.). Three chambers were placed on the glass platform. Each chamber was blocked with 2% BSA (Sigma-Aldrich, USA) suspended in PBS/0.05% Tween-20 for 1h. Each chamber contained three arrays, one for each phage clone. The blocking solution was removed from the chambers and the arrays inside were rinsed one time with PBS 0.05% v/v Tween-20, followed by three times rinsing with water for 1 min. Non-labeled *B. anthracis* spores were added to each chamber (200μl, 10^8 spores/ml, 4 % ethanol). After 1h of incubation, the excess of spores was removed and wells were washed one time with PBS 0.05% v/v Tween-20 and three times with water. The slide

was left to dry at room temperature. Afterwards, the slide was observed with a DIC microscope (Differential Interference Contrast; Olympus America Inc.) and images were recorded.

2.2 Fluorescent Dot-blot trials

The epoxy glass slide (Corning, USA) was placed in microarray microplate 96-wells (4 × 24 cm, ArrayIt, Microarray Technology). All three phage clones were adjusted to an equimolar concentration of 6.0×10^{12} cfu/ml in phosphate-buffered saline (PBS, 0.15 M NaCI, 5 mM NaH_2PO_4, pH 7.0). Hybridizations were performed in Microplate Microarray Hardware (TeleChem International, Inc. ArrayIt® Division,USA) providing 96 separate wells with the capability to harbor 4 slides at a time. Each phage clone was deposited in one separate well of the microplate, 75µl volume, covering the entire surface of the well. Phages were immobilized through covalent attachment to the epoxy coating of the glass slide (4h, 25º C, ~ 90% humidity). Wells were blocked in 2% BSA (Sigma-Aldrich, USA) in PBS/0.05% Tween-20. Subsequently, the blocking solution was removed from the wells and they were rinsed one time with PBS 0.05% v/v Tween-20, followed by three times washing with water for 1 min. Equal mixtures (10^8 spores/ml, 4% ethanol) of *B. anthracis* spores labeled with Alexa 488 and *B. subtilis* spores labeled with Texas Red fluorescent moiety were added to three wells incubated with a specific phage clone. At the same time, one well was incubated with the spore's mixture without prior treatment with phage, to estimate what portion of the binding is due to interaction with

phages with regard to the total input of spores. Additionally, another well was incubated with BSA solution with the goal to determine if binding is specific and is not due to surface factors or other reagents. After 1h of incubation, excess of spores was removed and wells were washed with PBS 0.05% v/v Tween-20. The slide was left to dry at room temperature, mounting media was added, and samples were sealed with cover slips and observed with a fluorescent microscope. Fluorescent images of spores were recorded and subsequently counted, and the results were presented as a percentage bound by phages.

3. Results and discussion

The aim of the present study was to develop a method that allows rapid and simultaneous characterization of several landscape phage clones, binders of *B. anthracis* spores from landscape libraries on a microarray platform. Historically, phage library screens for immunoreactivity have been performed through evaluation of recombinant phages by ELISA, Western blots, membrane plaque lift procedure or microarrays on filters [121]. These methods were efficient for variety of applications but also exhibited some limitations. Foremost, they need large sample volumes for both printing of antigens and for samples. Furthermore, these assays are time-consuming and laborious. A new rapid assay for characterization of phage libraries is needed since phages have demonstrated being excellent alternative of antibodies. Nevertheless, it is a challenging task to screen libraries for binding candidates through current methods.

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Several studies have described the pIII phage display library to identify specific peptides selective for the *Bacillus* species, including *B. anthracis* [122, 123, 124]. The use of individual display peptides in these reports has been successful. However, an inborn advantage of landscape phage over other display systems is the use of the entire body of the phage as a block for multivalent interaction with a given target. The landscape phage displays a particular peptide combination, which is fused to all 4000 copies of the major coat protein [125]. Furthermore, landscape phages have been shown to serve as substitute antibodies against various antigens and receptors [125]. A key advantage of phages over antibodies is their stability, durability, reusability and low cost production [126], allowing the use of the entire phage particle for multivalent interaction with a given target. With regard to detection of biological threat agents, prominent applications of landscape phage have been demonstrated, including the detection of bacteria and spores of the *B. anthracis* [119, 127].

While spores binding was confirmed with phage microarray for an expected specificity and array pattern(Images 8A, 8B, 8C., p.107), the spores' size was inadequate for binding to the printed array spots (Images 1A, 1B, p.107). The images suggest that some spores unbound during the washing procedures due to a rupture by share forces. An improved fluidic system is needed to solve the problem. As an alternative to overcome this hurdle, we employed a fluorescent dot-blot variant of microarray. In our study, fluorescent dot-blot technique, resulted in preferential binding of spores by clone JRB7 (displaying peptide EPRLSPHS). Moderate binding was observed when phage 1E24

(displaying peptide ASRPMPVS) was used. Both phages, however, exhibited some binding of *B. subtilis*. Unspecific binding was observed with control clone 7B1 (displayed peptide VPEGAFSS) for both *B. anthracis* and *B. subtilis*. Low binding accounted for wells incubated with BSA prior to spore incubation. The wells with no phage (total input) were references for subtraction and obtaining the percentage that accounts for the phage binding. The fluorescent images of spores were recorded (Image 8, p. 110) and subsequently counted as real numbers, and the results were presented as a percentage bound by phages (table 1, p. 108; fig. 4, p. 109) by the formula:

$$\frac{\text{Phage binding spores counts} - \text{BSA binding}}{\text{Total spores input}} \times 100 = \text{\% due to phage binding}$$

The method eliminates the need of an array spotter and at the same time offers plenty of phage particles to bind directly to the spores with no need for extracting spore components or for growing vegetative cells. In addition, this approach makes possible the multiple and simultaneous screening of available phage libraries, increasing the chance of rapid evaluation of clones carrying peptide inserts that can be used as probes for *B. anthracis* spores. The labeling with different fluorescent dyes of related *Bacillus* spores enhances the possibility to examine several phages simultaneously and examine their specificity for *B. anthracis*. The method can also to be performed as a selection high-throughput procedure for candidate binders with lower cross-reactivity. Afterwards phage

clones with higher specificity can be produced rapidly and inexpensively, and be employed in a variety of detection devices or easily labeled with fluorescent dyes or tags, such as luminescent quantum dots that provide a signal sufficient to detect a single spore [128].

Nevertheless, an outstanding question remaining is whether the clones JRB7 (displaying peptide EPRLSPHS), 1E24 (displaying peptide ASRPMPVS), and other clones from landscape libraries will discriminate between more closely related *Bacillus* members such as *B. cereus* and *B. thuringiensis*, and non - *B. anthracis* spores when more strains are examined. Answering this question will require the testing of a broader spectrum of *Bacillus* (and even non – *Bacillus*) species. Although specific clones with determined peptide inserts bound selectively to *B. anthracis* spores, *B. subtilis* binding was also observed. Another drawback of the fluorescent dot-blot developed here is the sensitivity limit of 10^8 spores/ml for binding. This is a high concentration, to use this as a detection tool.

Another goal will be to examine a larger number of landscape phage clones simultaneously, both, with virulent *B. anthracis* and non-virulent strains. The present study included only the avirulent Sterne strain. This strain adequately represents the specie for several reasons. The Sterne (pXO2⁻) strain differs from virulent strains only in the absence of one of the two plasmids, neither of which is likely to alter the spore surface [129]. In addition, Sterne strains appear to be essentially identical to spores of virulent strains excepting differences in the length of the hair-like nap on the spore

surface [130]. Finally, *B. anthracis* strains are highly monomorphic with genes from different isolates typically having greater than 99% nucleotide sequence identity [13].

4. Conclusion

The phage microarray method proved to be a prospective high-throughput screening approach for the monitoring of binding of selected phages to *B. anthracis* spores. The technique shows the theoretical possibility to screen and characterize hundreds of clones simultaneously. Nevertheless it requires an improved fluidic system for the washing procedures. To solve this hurdle, fluorescent dot-blot for evaluation of phage selectivity against mixed populations of related *Bacillus* spores was also developed. An advantage of this technique over phage microarray is the ability of the assay to discriminate relatives of spores of the *Bacillus* family. Through fluorescent dye labeling of spores the fluorescent dot-blot enables rapid and specific recognition of landscape phage clones preferentially binding *B. anthracis* spores.

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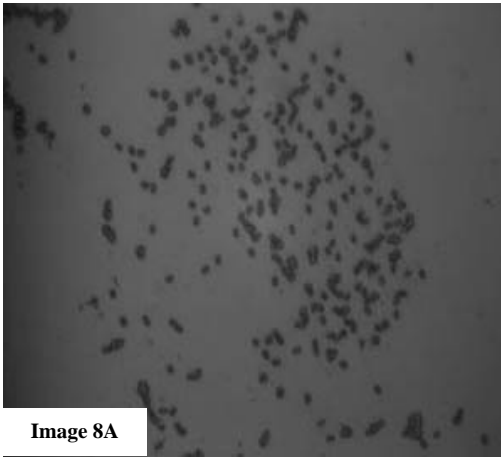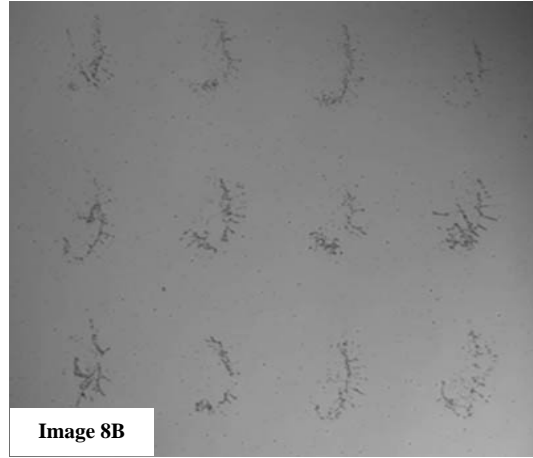
Image 8A

Image 8B
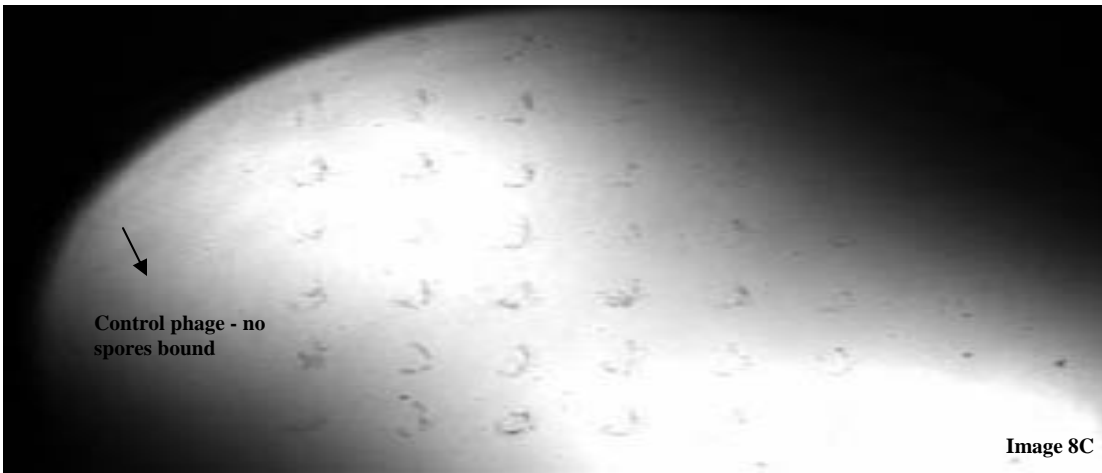

Control phage - no spores bound

Image 8C

The first image (8A) depicts one phage dot with bound spores (x100 Magnification). The second image (8B), shows the array pattern with 12 spots (x40 Magnification). The images show some spores unbound the spots where the phages were printed during the washing procedures due to the rupture by share forces. The third image (8C) shows part of the entire array pattern with bound *B. anthracis* spores (x10 Magnification). No binding was registered for the control

Table 1. Fluorescent dot-blot spore counts in percentage, subtracting unspecific binding

of spores to BSA from total input of spores (incubation of spores/ no phage)

Type of spores mixtures incubation	Spores counts (real numbers)	Percentage due to phage binding
No phage, total input spores *B. anthracis*	3640	0
No phage, total input spores *B. subtilis*	2740	0
BSA, binding *B. anthracis*	5	0
BSA, binding *B.subtilis*	43	0
JRB7, binding *B.anthracis*	139	3.7%
JRB7, binding *B.subtilis*	13	0
1E24, binding *B. anthracis*	129	2.9%
1E24, binding *B. subtilis*	45	0
7B1, binding *B. anthracis*	34	0.8%
7B1, binding *B. subtilis*	25	0.8%

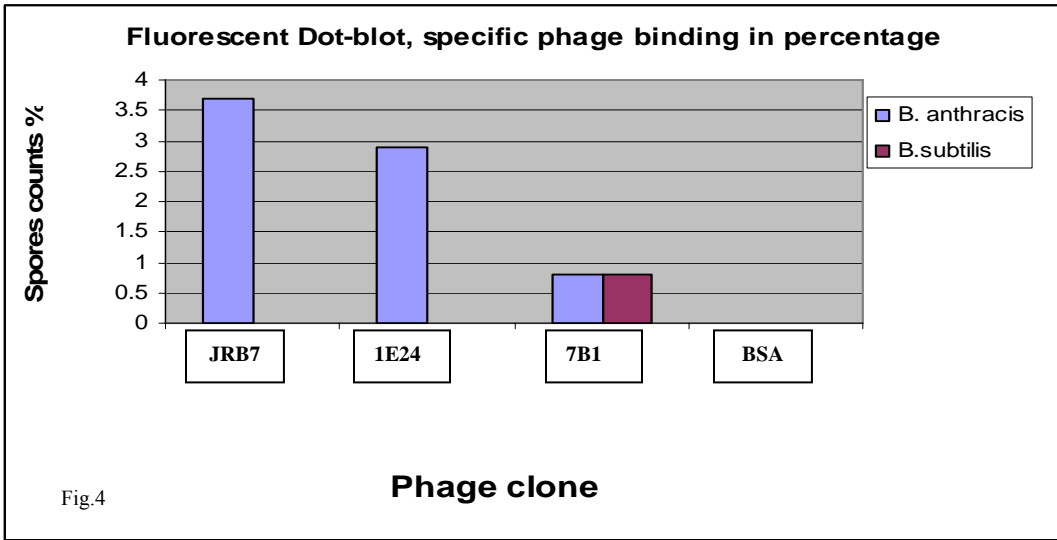**Fluorescent Dot-blot, specific phage binding in percentage**

Fig.4

Fig. 4. Spore counts due to phage binding. Real numbers were converted to percentages after subtracting the total input of spores incubated without phage. Phages JRB7 and 1E24 performed more specifically since they were selected as *B. anthracis* binders. Some unspecific binding accounts for phage 7B1 and BSA.
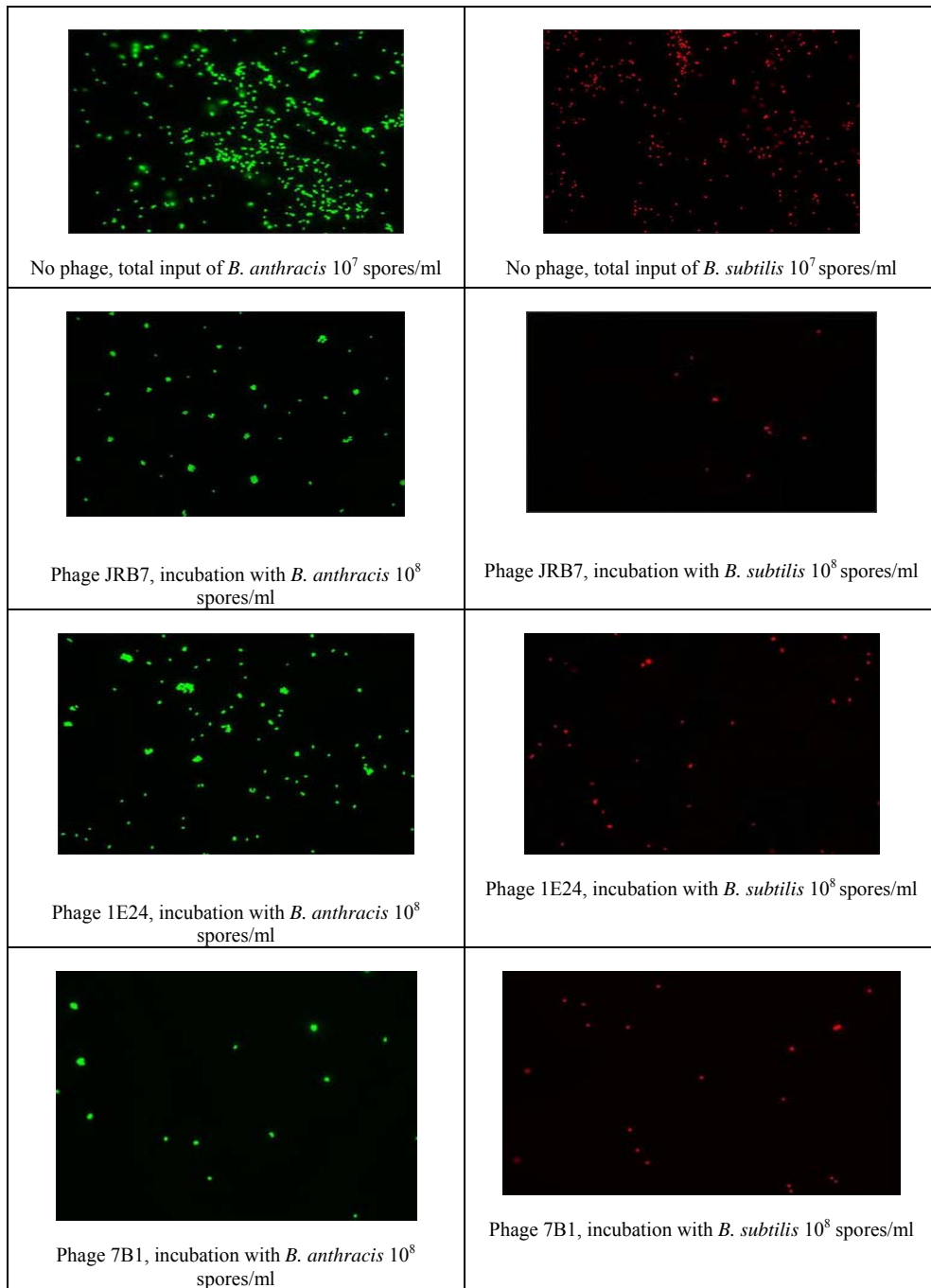
No phage, total input of *B. anthracis* 10^7 spores/ml	No phage, total input of *B. subtilis* 10^7 spores/ml
Phage JRB7, incubation with *B. anthracis* 10^8 spores/ml	Phage JRB7, incubation with *B. subtilis* 10^8 spores/ml
Phage 1E24, incubation with *B. anthracis* 10^8 spores/ml	Phage 1E24, incubation with *B. subtilis* 10^8 spores/ml
Phage 7B1, incubation with *B. anthracis* 10^8 spores/ml	Phage 7B1, incubation with *B. subtilis* 10^8 spores/ml

Image 9. continued

BSA, incubation with *B. anthracis* 10^8 spores/ml   BSA, incubation with *B. subtilis* 10^8 spores/ml
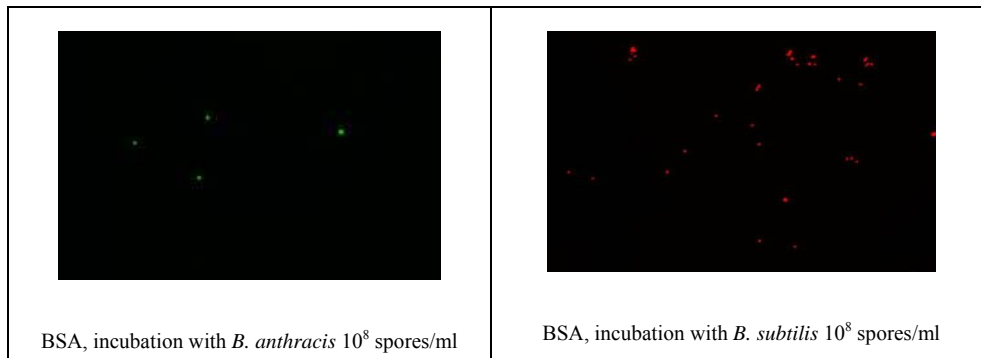
Image 9. Each image represents a well incubated with a specific phage clone or BSA as a control. The fluorescent dot-blot technique resulted in preferential binding of spores by clone JRB7 (displaying peptide EPRLSPHS). Moderate binding was observed when phage 1E24 (displaying peptide ASRPMPVS) was used. Both phages, however exhibited some binding of *B. subtilis*. Unspecific binding was observed with control clone 7B1 (displayed peptide VPEGAFSS) for both *B. anthracis* and *B. subtilis*. Low binding accounted for wells incubated with BSA prior to spore incubation. The wells with no phage (total input) were references for subtraction and obtaining the percentage due to phage binding.