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13. ABSTRACT (Maximum 200 words)

We introduced new formats of phage display—landscape phage and mosaic landscape phage, in which all 4,000 copies of the major coat protein in the phage bear one or two foreign peptides. Libraries of landscape and mosaic landscape phages contain billions of clones with different peptides. This work demonstrated a great potential of the landscape phage probes as elements of biosensors and threat agent detectors. We learned that landscape phages affinity-selected against various agents (bacteria, spores, proteins) demonstrate high specificity, selectivity and extreme robustness that commend them well as candidate probes for separation, concentration and continuous monitoring of biological threats. We showed that selected bacterium- and virus-binding phages may be used as probes for identification of surface components of these pathogens—potential targets for vaccines and drugs development. It is significant, too, that this work represented the first test of a phage evolution strategy that may have broad applicability in any system where directed enhancement of the probe's performance is required. In particular, it may improve operational affectivity of already existing detection and monitoring devices. It suggests also a new strategy of genetically directed nano-manipulation, which may be beneficial in creating new materials for nano-electronics, medicine and fundamental research.

14. SUBJECT TERMS
Phage display, landscape phage, mosaic phage, detection, biological threats, environmental monitoring, probes, Bacillus anthracis, Salmonella typhimurium.

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c. Final Progress Report

(1) Foreword

The probe technique originated from early attempts of Anton van Leeuwenhoek to contrast microorganisms under the microscope using plant juices, successful staining of tubercle bacilli with synthetic dyes by Paul Ehrlich, and discovery of a stain for differentiation of gram-positive and gram-negative bacteria by Hans Christian Gram (Reviewed by Petrenko and Sorokulova, 2004). The technique relies on the principle that pathogens have unique structural features, which can be recognized by specifically labeled organic molecules. A hundred years of extensive screening efforts led to discovery of a limited assortment of organic probes that are used for identification and differentiation of bacteria. A new challenge--continuous monitoring of biological threats—requires long lasting molecular probes capable of tight specific binding of pathogens in unfavorable conditions. To respond to the challenge, probe technology is being revolutionized by utilizing methods of combinatorial chemistry, phage display and directed molecular evolution (Reviewed by Petrenko and Vodyanoy, 2003) . This report describes how phage evolution methods developed in PI's laboratory under support of the ARO are applied for development of phage probes against biological threat agents, such as spores *Bacillus anthracis* and bacteria *Salmonella typhimurium*. The performance of the probes in detection of these threats is illustrated by a precipitation test, enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), magnetostrictive sensors, and fluorescent, optical and electron microscopy.

(2) Table of Contents

(3) List of Illustrations and Tables

(4) Statement of the problem studied

There are a variety of assays available for the detection of *B. anthracis* spores and other threats, but thus far none have been adapted for real-time continuous monitoring of the environment. Immunoassay and biosensor-based detection systems are the best prospects for continuous monitoring systems, but they require specific, selective and robust diagnostic probes with which the pathogen can be detected. In this report we describe the development of a new classes of phage-derived probes--landscape and mosaic phages--which fit well to the strong requirements of environmental monitoring.

(5) Summary of the most important results

We have shown that filamentous phage can form on its surface an indefinite number of potential antigen-binding sites by displaying random peptides fused to the major coat protein pVIII. We constructed billion-clone libraries with random peptides fused to all 4,000 copies of pVIII in various formats (landscape libraries), and selected phages that act as substitute antibodies specific for a panel of test antigens and threat agents. We demonstrated that phage-derived probes bind biological agents and, as a part of analytical platforms, generate detectable signals. Phages are prospective probes in a new generation of sensors for food safety control and environmental in-a-real-time monitoring. As elements of field-use detectors, they are superior to polyclonal and monoclonal antibodies, since they are inexpensive, highly specific, selective and strong binders, extremely robust and resistant to unfavorable environmental conditions.

To accomplish the overall goals of the project, the following specific aims have been achieved:

1. Construct filamentous phage landscape libraries displaying randomized peptides in various formats on every copy of the major coat protein.

2. Create libraries of mosaic phage on whose surface displays a mixture of two different peptides.
3. Select from the landscape and mosaic libraries phages that bind model antigens with high affinity. Characterize the specific binding between selected phages and antigen.
4. Use "test" pathogens to select binding phage.
5. Identify target receptors on the bacterial and viral particles.

Specific Aim 1. Construct of filamentous phage landscape libraries displaying randomized peptides in various formats on every copy of the major coat protein

New Libraries. Because our first 8-mer landscape library was contaminated with ~8% of the vector phage, which could hinder the selection procedure, for construction of new libraries we designed a new vector f8-6 which:

•Has four unique sites of restriction in p8-coding area
•….PstI….BamHI…NheI…MluI…
and two TAG-stop codons at the beginning of the gp8 ….TAG-TAG….
•Grows in amber suppressor E, in which pVIII gains Gln in the N-terminus AEQQDPAKAA…
•Does not grow in sup (-) strains
•Provides selection of recombinant phages with fused random foreign peptides, which grow in sup (-) strain
•Shows very low reversion to WT type in sup (-) strain $<<10^{-7}$%.

Using this vector, we constructed a new landscape library f8/9 and studied its diversity, evolution and censoring. This large (10^9-clone) landscape library was constructed by splicing the underlined degenerate coding sequence below into the beginning of the pVIII coat-protein gene, replacing wild-type codons 2–5:

```
DNA    GCAGNKNNKNNKNNKNNKNNKNNKNNKCCCGCAAAAGCGGCCTTTGAC…
pVIII   A  X  X  X  X  X  X  X  X  X  P  A  K  A  A  F  D…
        1  a  b  c  d  e  f  g  h  i  6  7  8  9  10 11 12…
```

where each N represents an equal mixture of all four nucleotides (A, G, C and T) and K-mixture of G and T. As a result of this modification, every pVIII subunit in a phage is five amino acids longer than wild-type, and displays a "random" sequence of nine amino acids: X_9 above. In any single clone, the random nona-mer is the same in every particle, but every clone displays an unique random nonamer. The nonamers are arranged regularly around the outside of the virion, occupying a substantial fraction of the surface. In another libraries, amino acids in positions 12-19 of pVIII are also deleted and substituted with random peptides (Petrenko et al. 2002).

Censoring and evolution of the library. Randomization of significant part of the major coat protein may result in appearance of clones with defects in phage morphogenesis and/or toxicity towards the host *E.coli* cells. These clones should be eliminated during phage propagation (censored). To study the censoring and evolution process, we sequences 50 primary clones which produced phage particles, nine clones which did not produced phage virions, and 64 phage clones from over-night grown library (amplified library). All nine clones which supported

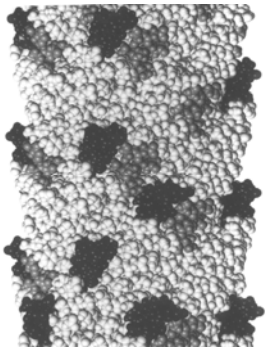
Table 1. Censoring in primary (un-shaded) and amplified (shaded) clones in the 9-mer library. .

Amino Acid	Occurrence/expectation of amino acids in position																	
	a		b		c		d		e		f		g		h		i	
Charged (RKHYCDE)	1.8	2.4	1.9	2.0	1.6	2.0	1.0	1.3	1.2	1.6	1.4	1.5	1.1	1.6	1.2	1.8	1.8	3.8
Acidic (DE)	1.8	2.4	1.7	1.4	1.7	1.9	1.6	1.1	0.8	1.7	1.4	1.9	1.1	2.0	0.9	2.0	2.2	5.7
Basic (KR)	0/0	0/0	0.4	0.2	0.6	0	0	0.6	0.6	0.4	0	0.2	0.6	0	1.0	0.4	0.2	0
Polar (NCQSTY)	0/0	0/0	1.7	3.7	2.9	3.4	2.2	3.3	3.0	3.6	2.2	2.9	3.6	3.7	2.0	3.4	1.7	3.1
Hydrophobic (AILFWV)	0.6	0.7	1.2	1.7	1.0	1.0	1.3	1.0	1.1	1.2	1.2	2.0	0.8	1.3	1.5	1.2	1.2	0.2
A Ala	0.5	0.7	0.6	2.5	0.3	2.2	0.9	0.9	0.9	0	0.6	2.5	0.9	1.9	1.9	0.9	0.6	0.3
C Cys	0/0	0/0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D Asp	2.1	**3.2**	**5.6**	**3.1**	**5.6**	2.5	**3.1**	2.5	1.9	**3.7**	1.2	**5.0**	0.6	**3.7**	2.5	**5.0**	**3.7**	**16.7**
E Glu	1.6	1.6	1.2	2.5	1.2	**5.0**	3.1	1.9	1.2	**3.1**	**4.3**	2.5	**3.7**	**4.3**	1.2	**3.1**	**5.0**	**6.2**
F Phe	0/0	0/0	0.6	2.5	1.2	0	1.2	1.9	0	1.2	1.9	0	0	1.2	1.2	0.6	0	0
G Gly	0.9	1.4	2.2	1.6	0.9	3.1	2.8	2.8	1.2	2.8	2.2	1.2	1.6	2.8	1.6	1.9	1.2	1.2
H His	0/0	0/0	0.6	1.9	0	1.9	0	1.2	1.2	0	1.2	0	0	1.2	0.6	0.6	0.6	0
I Ile	0/0	0/0	0.6	1.2	0	1.9	1.9	2.5	0	1.2	0.6	1.2	0	1.2	0	0.6	0.6	0
K Lys	0/0	0/0	0.6	0	0.6	0	0	0	0.6	0	0	0	0.6	0	0.6	0.6	0	0
L Leu	0/0	0/0	0.4	0.2	1.2	0	1.0	0	1.0	0.6	0.8	1.4	0.6	0.4	1.0	0.2	1.2	0.2
M Met	0/0	0/0	1.9	0.6	0	0	1.2	1.2	1.2	2.5	1.2	1.9	0	0.6	0.6	2.5	1.2	0
N Asn	0/0	0/0	0.6	2.5	3.1	1.9	1.9	3.1	0.6	2.5	2.5	3.1	2.5	2.5	1.9	1.2	0.6	3.7
P Pro	0/0	0/0	0.3	0.6	0.9	1.9	0	2.8	0.9	0.6	0.9	0.6	0.9	0	0.6	0.6	0.9	0.3
Q Gln	0/0	0/0	0	0	1.9	1.9	0.6	2.5	1.9	3.7	1.9	0.6	1.2	5.6	1.2	0.6	1.9	0.6
R Arg	0/0	0/0	0.2	0.2	0.4	0	0	0.6	0.4	0.4	0	0.2	0.4	0	0.8	0.2	0.2	0
S Ser	0/0	0/0	0.8	2.3	1.7	1.2	1.2	1.6	1.2	1.2	0.4	1.9	2.7	1.5	1.4	2.3	0.8	2.1
T Thr	0/0	0/0	0.6	0.9	0.3	1.9	1.2	0.9	2.2	1.6	0.6	0.9	0.9	0.9	0.3	2.2	0.6	0.9
V Val	0.8	0.6	2.2	1.2	0.3	0.3	0.6	0.3	1.2	1.9	0.9	1.6	0.9	0.6	0.6	2.2	1.2	0
W Trp	0/0	0/0	0.6	0	0.6	0	0	0	0	0	0	0.6	0	0.6	1.2	0	0.6	0
Y Tyr	0/0	0/0	2.5	3.7	1.2	2.5	0	0.6	1.2	1.2	1.9	0.6	0.6	0.6	0	0.6	0.6	0

Notes: The numbers in the columns a-i are ratios of observed appearance of the amino acid in the position a-i of the insert peptide to the expectation of the amino acid in this position, which depends on the number of codons used for coding of this amino acid in the degenerate segment of DNA; O/O means that the amino acid in this position was not coded by the degenerate insert (using GNK codons) and was not observed; Ratios of occurance to expectation higher that three are marked as bold.

transformation of bacterial cells but didn't produce secreting phage virions had TAG-stop codons in the random DNA inserts leading to abortion of translation of the major coat protein in the nonsuppressive strain K91BK and inability of phage to assemble. The high portion of the clones with *amber*-stop codons (39%) in the nine-mer encoding DNA segment was expected, since their portion in the total number of degenerate codons NNK was 1/32.

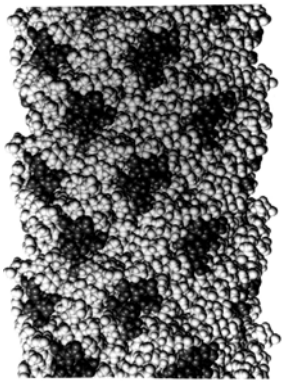
Primary clones obtained after transformation of bacterial cells with the degenerate DNA demonstrated a high diversity of inserted peptides as shown in the Table 1 (un-shaded columns).



The negatively charged amino acids Asp and Glu are over-displayed in all positions of the inserts, probably compensating the loss of Asp in positions 4 and 5 and Glu in position 2 of the major coat protein. Other amino acids are presented more randomly (exceptions: Cys, which is banned in all positions, as was observed in other landscape libraries (Petrenko et al., 1996, 2002). After propagation of the library, the composition of the amino acids in the inserts changed in favor of amino acids Asp and Glu, as shown in the Table 1 (shaded columns). The most significant censoring effect of propagation was observed in the position i of the insert corresponding to the position 5 of the major coat protein.

These data show that replacement of amino acid Asp in position 5 of the major coat protein leads to a strong censoring of the library and dominance of these amino acids in all positions of the "random" inserts. Thus, in the libraries of next generations we plan to retain this amino acid and increase diversity of the libraries by the increase of the length of the inserted peptides.

Specific Aim 2. Create libraries of mosaic phage on whose surface displays a mixture of two different peptides.



Mosaic phage. We designed a library (alpha-f8/6) with six degenerate codons in gene VIII, specifying amino acids 12, 13, 15–17 and 19 of the major coat protein pVIII. This library showed great diversity of amino acids at the randomized positions (shown as dark area in the model on Figure 1 to the right), and diversity did not diminish noticeably during repeated subculture. One of the motivations in constructing of this library was its potential use in mosaic libraries. Initially we planed to construct a mosaic landscape phage library by co-infecting a bacterial host with two separate "parent" landscape libraries displaying their foreign peptides as N-terminal and central part of major coat protein. The doubly-infected cells were expected to secrete "mosaic" virions coated with a mixture of two types of coat proteins, as shown on Figure 2 to the right. Although a mosaic virion's coat has subunits contributed by both parents, inside it has only one type of DNA, and therefore only one type of gpVIII gene. Thus, genotypically it is "homozygous." We expected that close interaction between neighboring random peptides, like CDR fragments in antibodies, may create new high-affinity binding sites

A billion clone mosaic landscape phage library was constructed by co-infecting a bacterial host with two separate "parent" libraries: alpha library f8/6 and landscape library c8/8 which displays the foreign random peptides in the same format as previously reported f8/8 library (Petrenko, 1996) but harbors chloramphenicol resistance gene (CAT) instead of tetracycline resistance gene. Co-infected cells were selected over singly-infected cells in the culture medium with both antibiotics. Unexpectedly, we found that one cell can accept more than two different phage genomes expressing many different variants of major coat proteins fused with different random peptides. Since all these proteins can participate in phage assembly, the infected cell secretes mosaic virions coated with a mixture of several different coat proteins. A composition of mutant major coat proteins in one randomly chosen mosaic clone is shown

below, where four peptides come from c8/8 library (left) and one peptide comes from f8/6 library (right).

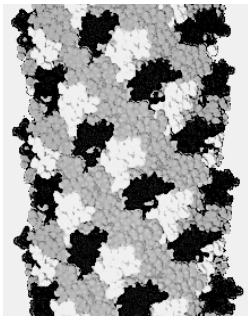
A**AASGGTDT**DPAKAAFDSLQASATEY…
A**GYDGSGSS**DPAKAAFDSLQASATEY…
A**GS**DPAKAAFDSLQASATEY…
A**ES**DPAKAAFDSLQASATEY

AEGEDPAKAAF**IE**LQ**TQ**A**Q**EY…

Individual mosaic clones were characterizes by electrophoresis in comparison with their parental phage. In contrast to parental landscape phages, demonstrating compact bands in agarose gel, the mosaic phages appear as smears comprising a mixture of many different structures (data not shown).

We used the mosaic library for selection of clones binding β-galactosidase – a model target protein. Mosaic clones that bound immobilized β-galactosidase were propagated separately in a media with tetracycline and a media with chloramphenicol to isolate parent clones contributing to formation of β-galactosidase-binding mosaic phage. After propagation and coinfection of fresh *E.coli* cells, these two groups of phage formed a new mosaic library, which was used at the second round of selection. After three round of selection, mosaic clones were propagated in two media with different antibiotic, as described above, and DNA of parent phage was sequenced. We found that mosaic clones were composed of phage bearing N-terminal peptides with structures which were previously determined in selection experiments with non-mosaic individual landscape libraries. A partner phage demonstrated usually wild type genotype. Thus, the "mechanical" combination of two landscape libraries did not allow us to generate principally new binding entities. Furthermore, the unexpected formation of multiply infected cell clones raised serious doubts in possibility of controlled and reproducible generation of mosaics of this type. The serious pitfalls in attempts to realize the "shuffling" scheme of the mosaics formation encouraged us to focus on another combinatorial scheme outlined in our proposal—Directed Phage Evolution.

Directed Phage Evolution



We formulated and justified a new conception of genetically stable mosaic phage clones, which are produced stepwise by Directed Phage Evolution (DPE). We proposed that performance of phage-derived probes may be enhanced by inducing mutations in the areas neighboring phage-borne binding peptides that may increase affinity, selectivity and stability of phage-derived probes.. The principle of DPE is illustrated by the Figure 3 to the right, in which black areas show primary N-terminal binding peptides, selected in the first round of affinity selection, and white areas show amino acids which are mutated to form a new sublibrary, which is used for selection of improved phage probes. These areas correspond to the segments of pVIII shown in the structure below.

Black **Grey** **White** **Grey** **----------*Buried*----------**

AXXXXXXXXDPAKAAFXXXXXXXXEYIGY*AWAMVVVIVGATIGIKLFKKFTSKAS*

DPE strategy was developed using the model phage 1G40 binding β-Galactosidase (β-Gal) selected from landscape library f8/8 (Petrenko, 1996). The phage displays peptide DTFAKSMQ that lies in the area shown black in the Figure 3 above. To introduce random mutations in the neighboring area (white in the Figure 3) we use random oligonucleotide-directed mutagenesis of

U-containing phage DNA. Degenerate oligonucleotide forms with phage DNA a mixture of heteroduplexes inducing random mutations of underlined amino acids

```
6   7   8   9  10  11  12  13  14  15  16  17  18  19  20  21  22  23  24  25
P   A   K   A   A   F   D   S   L   Q   A   S   A   T   E   Y   I   G   Y   A
CCC GCA AAA GCG GCC TTT GAC TCC CTG CAA GCC TCA GCG ACC GAA TAT ATC GGT TAT GCG T
  3′CGT TTT CGC CGG AAA nnm nnm GAC nnm nnm nnm CGC nnm CTT ATA TAG CCA ATA5′
```

where n = G, A, T or C; and m = G or C.

For mutagenesis, single stranded phage DNA (ssDNA) containing uracil instead of thymine was prepared by standard procedures from phage 1G40 grown on uridine-containing media in *ung*$^-$ host bacteria. Ss DNA and random oligos were annealed under gradually decreased temperature and incubated with components of polymerase reaction. The formation of DNA heteroduplexes was controlled by electrophoresis in agarose gel. Heteroduplex DNAs were electroporated into *E.coli* cells MC106, which were plated onto tetracycline-containing agar plates. The resulting colonies of transformed bacterial were used for PCR and DNA sequencing. To optimize the mutagenesis procedure, we varied parameters of polymerase reaction, such as: chemical or enzymatic oligo phosphorylations, ratio of the oligos and the DNA templates and regime of their annealing, concentration of polymerase and DNA ligase in solution, origin of DNA polymerases (T4-, T7- (sequenase), Klenow fragment). Following varying mutagenesis protocols, we prepared nine analytical random libraries for sequencing of representative clones and evaluation of mutagenesis efficiency. In the best experiment, 50 % (6 out of 12) of analyzed colonies were mutants: 2 mutants out of these 6 had stop codons, 4 mutants had the randomized areas as were designed in oligo (Table 2). These data demonstrated, in accordance with our assumption, that phage bearing 8-mer peptide at the N-terminus of the major coat protein pVIII can be additionally mutagenized at the central part of pVIII to produce a mosaic sub-library for selection of improved diagnostic phage probes with higher performance. However, efficiency of Kunkel's mutagenesis appeared to be not sufficient for obtaining a highly diverse library (data not shown).

Table 2. Mutant phage clones

Mutants	Comments
DSL**QAS**A**T**	**Wild-type**
CLL**PDL**A**P**	**mutants**
PNL**TLI**A**S**	
APL**QRD**A**S**	
QPL**TFP**A**S**	
TVL**EVP**A**I**	
TNL**TEP**A**V**	
YEL**FPC**A**H**	
TQL**TPYs**A**T**	

A diverse mosaic landscape library was constructed by cloning of a synthetic oligonucleotide duplex encoding the β-galactosidase-binding peptide DTFAKSMQ into existent f8/6 α-library with random amino acids in positions 12, 13, 15-17 and 19 (Petrenko et al., 2002) using summary replicative form (RF) DNA of the library's 10^8 clones as cloning vectors. Briefly, *E. coli* K91 BlueKan cells were infected with 1.5×10^9 virions from the f8/6 α-library (~10^9 clones) and double-stranded RF DNAs were prepared from the culture. The RF DNAs were cleaved by *Pst*I and *Bam*HI endonuclease and the synthetic duplex bearing appropriate sticky ends was ligated into the vectors yielding 30-50 % of ligated DNA, as evidenced by electrophoresis in agarose gel. However, the yields of primary clones of a new mosaic library was unexpectedly low – 1.2×10^6 clones, even when the highly competent cells (3.3×10^8 TU per 1 μg of RF DNA) and 4 μg of ligated RF DNA were used in this experiment. The library was grown and purified yielding 8.4×10^{13} virions. The library, named G-α-library, was characterized by sequencing of 74 primary clones that were obtained after electroporation of bacterial cells and

plating them on the agar medium. Out of these 74 clones 44 (59 % of the population) revealed frame shifts and stop codons in pVIII gene, so these clones were not able to produce phage particles and were not present in the propagated phage library. The remaining 30 clones comprised 7 clones (23 %) bearing ß-Gal binding peptide along with mutated amino acid 12-19 of the pVIII protein, 19 clones had no inserts and belonged to f8/6-library, one clone was f8-5 vector bearing ß-Gal binding epitope and 3 clones turned out to be a parental f8-5 vector. All 7 mosaic clones were able to produce phage virions when were grown individually. Thus, an estimated diversity of mosaic clones in the library was 2.8×10^5 variants. This library was used to study whether the mutations surrounding the binding peptides on the phage landscape can affect their binding efficiency.

Search for better binders: affinity selection and ELISA

To find out whether a better ß-Gal binders can be derived from the 1G40 phage, which bears ß-Gal binding peptide DTFAKSMQ, we performed three rounds of affinity selection of G-α-library against immobilized ß-Gal. First round was performed under mild conditions favoring binding of any clones having ß-Gal binding peptide. Second and third round of selection have been carried out under more stringent conditions. The yield of phages for the first round of selection was 0.3%, for the second - 0.8% and for the third one- 2.4%. Sequence analysis of randomly picked up clones from each round revealed increasing number of the clones bearing ß-Gal binding peptide: estimated number in original G-α-library was 23% of phage population, after the first round of selection its fraction increased to 58%, after the second – to 82 % and after the third one - up to 93.5%. Altogether these data suggested selection of the clones that specifically bind ß-Gal. Individual phage clones bearing ß-Gal binding peptide and mutated 2-19 amino acids of pVIII protein were tested by ELISA, in which immobilized phages interacted with ß-Gal (Table 3 below). Although the majority of the clones showed ELISA signals lower then the signal produced by the parent 1G40 phage (data not shown), some of them demonstrated increased signal (157 % vs.100% for the phage 1G40).

Table 3. Structure of phage clones and their activity in ELISA

Sequence of amino acid 12-19	Direct ELISA, %	Competitive ELISA, %
DSLHGQAM	157	1.3
DQLNATAL	113	1.0
DSLQASAT (WT) Parent phage with β-Gal-binding peptide	100	6.9
SNLEMMAT	100	1.4
ADLTVQAN	97	1.6
DSLHGQAM		1.3
AELTTRAE	94	2.5
DQLTVSAQ	84	17.5
DSLTLQAQ	79	-
EDLTQRAL	66	2.8
DTLTHEAT	63	2.4
ESLNHQAE	12	20.9
NDLVGQAH	39	21.4
EELTVHAT	64	69.4
DELTVAAN	0	100
DSLQASAT (WT) Control phage without β-Gal-binding peptide	0	100

Note: All clones except a last control one bear β-Gal-binding peptide at N-terminus of pVIII

To exclude a possibility that ELISA signals of mosaic phages vary because of their different affinity to plastic, all promising clones and several control clones showing very low signal were tested by competitive ELISA. In this assay wells of a microtiter dish coated by phage 1G40 were treated with different phages pre-incubated with ß-Gal (Table 3). ELISA signal of f8-5, vector, which doesn't bind ß-Gal specifically were considered as 100%. The results of the competitive ELISA were in

agreement with phage capture ELISA: the clones which revealed strong binding with ß-Gal in phage capture ELISA showed strong binding with enzyme in solution; phages that did not bind ß-Gal when they were immobilized on plastic surface, also did not bind it in solution. All promising clones from both tests were undergone further ELISA to obtain the binding curves of phages with ß-Gal (Table 3.). This test revealed that mutants AELTTRAE and DSLTLQAQ were able to bind ß-Gal with the same ability as original 1G40 (DSLQASAT) and two clones with mutated areas DQLNATAL and DSLHGQAM were able to bind ß-Gal even better then 1G40. Three clones (ADLTVQAN, DQLTVSAQ, SNLEMMAT) bound ß-Gal less effectively then the parent phage with the wild-type sequence 12-19. These data demonstrate a dramatic effect of phage amino acids neighboring a foreign peptide on the binding properties of the peptide and, probably, on its specific conformation controlled by an integral phage organic landscape. The results of this study strongly confirm our conception of phage surface landscapes as materials with emergent properties (Petrenko et al., 1996) and suggest a way how a performance of phage-derived probes can be enhanced by the phage evolution.

Comparative analysis of parented f8/6 α-library and G-α-library; diversity and censoring

As was shown in the previous subsection, amino acids neighboring the N-terminus binding peptide on the phage landscape can control its binding efficiency. This conclusion was further confirmed by comparison of diversities of β-Gal binding mosaic clones versus original f8/6 α-library. Fig. 4 shows diversities of amino acids in positions 12,13,15-17 and 19 in 62 random clones picked from the primary 8/6 α-library (upper panel), 108 clones from amplified f8/6 α-library (middle panel) and 77 clones from enriched G-α-library (bottom panel), calculated using a package of statistical programs RELIC. It is not surprising that a strict feature of the parental 8/6 α-library—absence of cysteine, proline and tryptophan--is inherited by both amplified and G-α-libraries. Original f8α-library have wide dispersion of amino acids in all 6 randomized positions, which does not change dramatically after 3 cycles of propagation (in amplified library). In opposite to the amplified library,

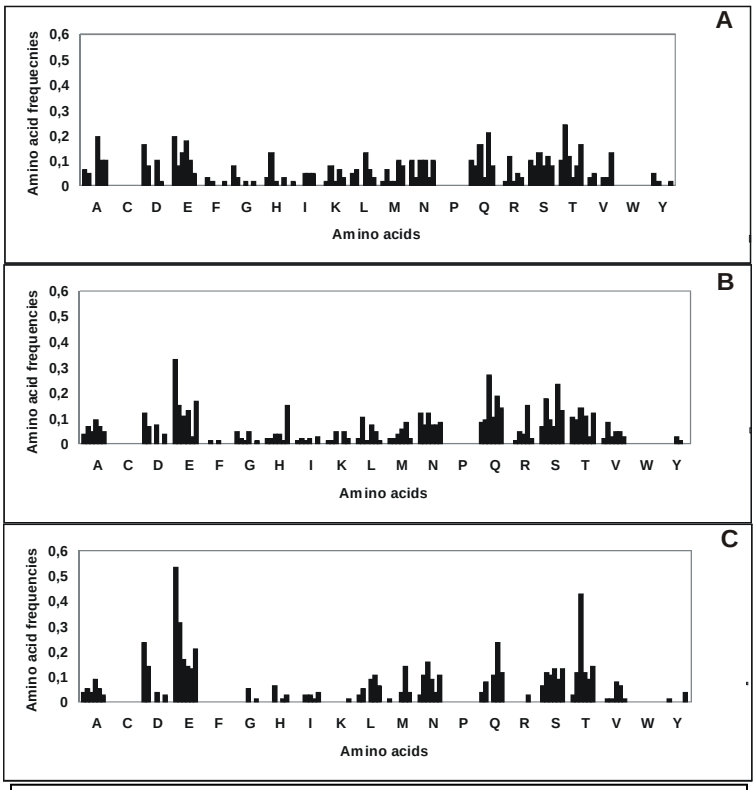


Figure 4. Dispersion profile of amino acid residues in landscape libraries: A – original f8/6 α-library; B – amplified f8/6 a-library; and C – G-a-library after the first round of selection

the G-α-library demonstrates dispersion of the amino acids quite different from the parental f8/6 α-library, with more prevailing glutamic acid and threonine and poorly presented F, G, H, K, R and Y.

These results clearly demonstrated that amino acids that belong to the N-terminal and the central segments of pVIII can impel each other and constitute a common specific organic landscape. Diversity of mutation in the region of 12-19 amino acid of pVIII gene in G-α-library is sufficiently lower then the diversity of the parented f8/6 α-library which showed the influence of N terminal sequence on the mutated area, and vise versa--mutation in the area of 12-19 amino acid able to dramatically change the landscape of the phage, so the binding activity of it against molecular target could be increased or completely destroyed. This observation allowed us to propose a new type of phage landscape mosaic library with two mutated area: at the N-terminal part of pVIII and at 12-19 region of it, in which different mutations in 12-19 area increase conformational diversity of foreign peptides inserted at the N-terminus of pVIII protein. This highly diverse hypothetical library may be a rich source of landscape phage probes, in which an extended area of phage surface may favor a specific and strong binding to complementary surface landscapes of the targeted threat agents.

Specific Aim 3. Use various model antigens to select phage that bind antigens with high affinity. Characterize the specific binding between selected phage and antigen.

We have shown that different targets select phage families with different lead motifs of fused peptides, as exemplified in the Table 4 to the right. The high selectivity of the selection procedure may be illustrated by results of selection of landscape phages against two proteins with identical function – binding of biotin, but different mode of this binding. Reflecting the similarity and the difference of these protein, the phage clones are also similar, but have a strong selectivity to the target proteins (data not shown).

Table 4. Phage borne peptides in phages selected against different targets

***Bacillus anthrasis* spores**	**β-Galactosidase**	**Streptavidin from *Streptomyces avidinii***
EPRA**PR**SL	**DTFAK**MA**Q**	**VPEGAF**GS
EPK*PH*TFS	**DTFAK**SM**Q**	**VPEGAF**SS
E*PH*PKTST	**ETFAK**MS**Q**	**VPEGAF**SQ
ESR**V*PH***GA	**ETFAK**MT**Q**	**VPDGAF**SQ
DA**R**GTTHM	V**TF**AAANT	**VPEGAF**ST
DRTGLTL		**VPESAF**AQ
Avidin from egg white	**Human prostate cells**	**Dioxin**
VPEYSRPS	**DPR**SATMT	**EPFP**NQQSDP
VPEYKGAS	**DPR**TAAMA	**EPFP**RDRPDP
VPEYTGRP	**DPR**IATMS	**EPFP**SFAEDP
VPEYVNTK	**DPR**ATTST	**EPFP**AHGPDP
VPEYTNRP	**DPR**GAAIS	G**EPFP**MMQDP
VPEYTRT	**DPR**SSAMT	**E**FT**PFP**SGDP

Specific aim 4. Use "test" pathogens to select binding landscape phage.

We selected phages that act as diagnostic probes against spores *Bacillus anthracis* and food-poisoning bacteria *Salmonella typhimurium*. The list of test pathogens was corrected according to the current bio-security demands and DARE Program (Darpa, Dr. Millie Donlon).

Bacillus anthracis. Phage clones that bind to *B. anthracis* Sterne spores were selected from the landscape library f8/8 through a panning procedure in which the phage library was incubated with immobilized *B. anthracis* spores, non-bound phages were washed away and bound phages

were eluted with mild acid. Phages that bound to spores in the initial selection procedure (a sub-library) were amplified and used as the input (instead of the primary library) in the next round of selection. This procedure was repeated for four rounds of selection. The numbers of infective phage particles present in the input, washes, and eluate of each round of selection were determined by titering. The increase in phage recovery following each round of selection (data not shown) indicates an increase in the representation of phage clones in the sub-library that were capable of binding to *B. anthracis* spores. After four rounds of selection, 16 randomly picked clones were isolated and a segment of genomic DNA encoding the displayed octapeptide was sequenced. Eleven unique peptide sequences were found that formed three related families, each with a particular motif or consensus sequence (Table 5). Family 1, with six members, is characterized by the presence of a negatively charged amino acid (E or D) at the first position, usually a proline residue at the second position, and a positively charged amino acids (R, K, or H) at the third position. Another interesting feature of this family is the frequent presence of a "migrating" dipeptide PH, which is replaced by PK in peptide 4. Family 2. with three members, contains the consensus sequence (D/E)(R/K)TXATXT. Family 3, with two members, contains the consensus sequence V(S/T)XXXSXS.

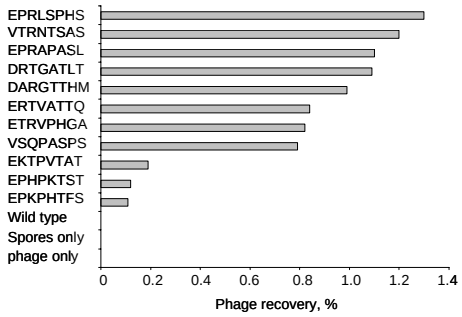
Table 5. Amino acid sequences of peptides carried by selected phage

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

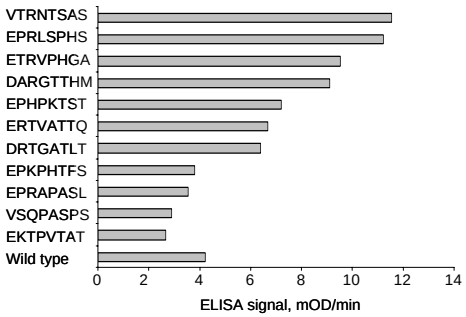
Specificity of phage binding to *B. anthracis*

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

The relative binding of the isolated phage clones to *B. anthracis* spores was measured by a phage-capture assay and an ELISA. The phage-capture assay, a procedure very similar to the selection procedure, was used to determine relative binding of phage clones to immobilized *B. anthracis* spores. Briefly, selected phage clones were added to the wells of a microtiter plate that were coated with *B. anthracis* spores. After an incubation to allow binding, non-bound phage were washed away and bound phage were eluted and titered. The percent recovery was determined as a ratio of the eluted phages to input phages. As shown in the Figure 5 to the right, selected clones bind at a much higher level than the wild-type phage to *B. anthracis* spores in this assay.



In the ELISA, wells of a microtiter plate were coated with phage, then incubated with biotinylated *B. anthracis* spores. Alkaline phosphatase conjugated to streptavidin was then added to bind to the biotinylated spores, and *p*-nitrophenylphosphate was used to detect this binding. As shown in the Figure 6 to the left, many of the isolated phage clones bound to *B. anthracis* spores at a higher level than wild-type phage. Some clones bound strongly to *B. anthracis* Sterne in both assays, while other clones gave inconsistent results between the two



assays. This is not completely unexpected because in the ELISA phage are fixed to the plate and spores are captured from solution, while in the phage-capture assay spores are fixed to the plate and phage are bound from solution. Thus, in these tests phage can adopt different conformations allowing monovalent or multivalent interactions with spore receptors, as was demonstrated in binding experiments in which β-galactosidase from *E.coli* served as a model multivalent analyte (Petrenko and Vodyanoy, 2003). To confirm that the ELISA results were not due to biotinylation of contaminants of the spore preparation, an ELISA was done in another format, with antibodies specific for *B. anthracis* spores (data not shown)

Selective binding of phage to spores of *B. anthracis* Sterne:

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

In the coprecipitation assay, these phages were mixed individually with spores of various *Bacillus* species. After incubating, spores were collected by a low speed centrifugation, so that only phages bound to spores would be found in the pellet. Phages without spores were used as a control to ensure that the phage virions were not aggregating and precipitating on their own. Initial tests were done with distant relatives of *B. anthracis*: *B. megaterium*, *B. subtilis*, and *B. licheniformis*. Clones from family 1 (Table 5) exhibited very low binding to these distant relatives, while the clones from other families bound them nearly as well as *B. anthracis* It was found that the phage carrying the peptide DARGTTHM bound to *B. anthracis* Sterne 75-fold better than to *B. megaterium*, 25-fold better than to *B. subtilis*, and 50-fold better than to *B. licheniformis.* Phage carrying the peptide EPRLSPHS bound to *B. anthracis* Sterne 43-fold better than to *B. megaterium*, 39-fold better than to *B. subtilis*, and 70-fold better than to *B. licheniformis.* Phage carrying the peptide ETRVPHGA bound to *B. anthracis* Sterne 24-fold better than to *B. megaterium*, 24-fold better than to *B. subtilis*, and 12-fold better than to *B. licheniformis*. Phage clones from families 2 and 3 exhibited much lower selectivity and were not examined further. The three aforementioned phage probes which did not cross react strongly with distant relatives of *B. anthracis* were examined further for binding to spores of close relatives of *B. anthracis*, namely *B. cereus* and *B. thuringiensis*. All three phages demonstrated preferential binding to *B.anthracis*, but considerable binding to the close relatives: the phage bearing the peptide DARGTTHM bound to *B. anthracis* 3.7-fold better than to *B. cereus* and 2.1-fold better than to *B. thuringiensis*; the phage bearing the peptide EPRLSPHS bound to *B. anthracis* 3.5-fold better than to *B. cereus* and 6.9-fold better than to *B. thuringiensis*; the phage bearing the peptide ETRVPHGA bound to *B. anthracis* 2.4-fold better than to *B. cereus* and 2.2-fold better than to *B. thuringiensis.*

In this work we have identified three landscape phage clones which bind to *B. anthracis* Sterne and do so at a higher level than other *Bacillus* species. These phage probes can be used for detecting *B. anthracis* spores in any platform in which antibodies or peptides have previously been used. We recognize that these probes may be not completely ideal for identification of *B.anthracis* because they cross-react with *B. cereus* and *B. thuringiensis*. In future work, library depletion and molecular evolution will be used to eliminate such cross-reactive phage.

Selection of phage probes for *Salmonella typhimurium*

Landscape phage library f8/8 was used for selection of diagnostic probes against *Salmonella typhimurium*. We studied three different schemes of selection, as summarized in the Table 6 below.

Table 6. Selection of *Salmonella typhimurium* binding phage

	Separation of bacteria/phage complexes	Blocking	Library Depletion	Washing	Elution	Rounds
1	Immobilization	0.1% BSA	NO	0.1% Tween, TBS	a) Acid, pH 2.2 b) 2% DOC	5
2	Immobilization	0.1% BSA	Plastic, BSA-coated plastic	0.5% Tween, TBS.	a) Acid, pH 2.2 b) 2% DOC	4
3	Precipitation	NO	Centrifugation	0.5% Tween, TBS	a) Acid, pH 2.2 b) 2% DOC	3

Selection 1. Phage virions bound to *Salmonella* cells were recovered consecutively with low pH elution buffer and with 2% deoxycholate (DOC) lysis buffer. Since DOC is known to solubilize bacterial lipopolysacharides, it was exploited in the selection procedure to bias it towards isolation of membrane-bound phage. Eluted phages were amplified and used as input in the next round of selection. The yield of *Salmonella*-associated phage, determined as a ratio of output/input phage, increased from one round to another, indicated the successful selection of specific phage clones (Table 7). This increase was distinctly higher when the phage was eluted with acid. After the fifth round of selection, individual phages were propagated, phage DNA's were isolated and sequenced for analysis of the peptide-encoding regions. Phage clones isolated by acid demonstrated four different types of peptide sequences with the main motif V $^{S}/_{T}$ P $^{P}/_{Q}$ (Table 8). In phages recovered by DOC several different types of sequences were found. Three of them had the similar amino acid motif as those from elution fraction. Eight clones (53%) had proline (P) in the third and fourth position

Selection 2. To exclude selection of unrelated clones, we depleted the library against plastic and BSA and used a higher concentration of Tween-20 for washing. In contract to Selection 1, the phage yield in the acid fraction sharply increased at the 3rd round of selection, while no enrichment of DOC fraction occurred during 4 rounds (Table 7). After the forth round of selection, acid and DOC-eluted phages were cloned and sequenced. Ten from eighteen analyzed phage clones isolated from the acid fraction encoded peptide VPPP (Table 4), similar to the motif V(T/S)PP present in the phages isolated by acid elution in Selection 1. Four phage clones

displayed the sequence VTPP and VTP. One phage isolated from DOC fraction had the same peptide VTPP, while majority of phages displayed motif (D/E)P(K/R/H).

Selection 3. Since bacterial cells can display different receptors under different conditions, we modified selection procedure to allow phages to bind *Salmonella typhimurium* in suspension. In this selection protocol, phage library was incubated at 70^0 C to decrease aggregation of phage and depleted from residual aggregates by centrifugation at 13,000 rpm. The library was incubated with a suspension of *Salmonella* cells for 1 hour. *Salmonella* cells with bound phages were precipitated at 3,500 rpm and washed with 0.5% Tween/TBS. Bound phage virions were eluted from *Salmonella* cells with acid and DOC. The highest recovery of the phage was observed after the 3rd round of selection in elution fraction (Table 3). In contrast to Selection 1 and 2, we could not recover any phage clones with DOC. Twenty three representative clones eluted in the 3rd round of selection were propagated and sequenced. Eighteen of 23 phage clones had identical peptide sequence **VS**SNE**APP** (Table 4). This peptide is similar to peptide **VS**PQS**APP** isolated from the acid fraction in the Selection 1. Four identical phage clones with peptide DRSPSSPT have clear similarities to some phages isolated with DOC in Selection 1 and 2.

Table 7. Recovery of phage during selection against Salmonella typhimurium

	Yield, % × 10^2 / round of selection				
	1	2	3	4	5
Selection 1					
Acid fraction	1.1	0.24	1.1	3.8	16
DOC fraction	0.91	0.88	0.86	2.3	4.7
Selection 2					
Acid fraction	0.86	1	15	13	
DOC fraction	0.63	0.0022	0.0031	0.013	
Selection 3					
Acid fraction	0.024	0.31	3		
DOC fraction	0	0	0		

Table 8. *Salmonella*-binding phage

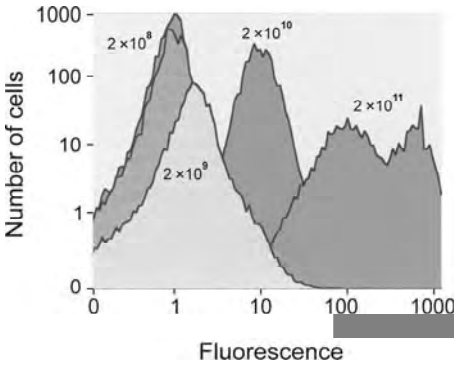
	Phage-borne peptides	
	Acid fraction	**DOC fraction**
1	**VTPPSQHA**(10)	**ERPP**NPSS(8)
	VTPPTQHQ(4)	**ER**SSQANM
	VSPPPQHS	**ER**TTSAHT
	VSPQSAPP	**DR**TSNQAT
		DLTSNQAT
		VTPPSQHA
		VSPPSNPS(2)
2	**VPPPSP**QS(2)	**DPRSPASL**(2)
	VPPPSPHS(3)	**DPKSPLHT**
	VPPPSASS	**DPKSPQQT**
	VPPPSQSQ(2)	**DPKGPHSM**
	VPPPSNPS	**DPR**PAQHT
	VPPPGQHQ	**DPH**LAGGL
	VPPSSSSP	**DPSKRTQP**
	VPQQNKAQ	**EPR**LAHGA
	VTPPQSSS	**EPHR**AASV
	VTPPTSPQ	**EP**NKHSQS
	VTPSSPHS	**DRPSP**NTV
	VTPQGSHP	**D**NKMTSQS
	VSTQSTHP	**VTPP**QQGS
	TPGQDKAQ	
3	**VS**SNQAPP(18)	
	VPIPYNGE(1)	
	DRSPSSPT (4)	

Striking similarities of phage-borne peptides in the groups isolated from acid eluates in all three selections and their clear distinction from peptides of DOC fractions can indicate that these peptides may target different surface receptors of *Salmonella*. One of the intriguing features of isolated peptides is unusually high concentration of proline. We noted that some of the selected proline-rich phage can inhibit growth of the host *E.coli* cells, that is consistent with known antibacterial activity of some proline-rich peptides. Representative phage clones were assessed for their ability to bind *Salmonella* in phage capture and *Salmonella* capture ELISAs. Clones demonstrating higher signals than control wild-type phage were characterized in precipitation test to confirm their specificity to *Salmonella* cells. This test, used by other authors for analysis of

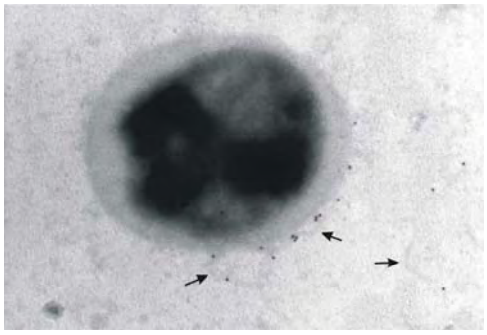
phage binding to zoospores and bacterial spores, was optimized in this work to avoid aggregation and self-precipitation of phage.

Phage (~ 10^9 cfu/ml in TBS buffer) was heated 10 min at 70^0 C and centrifuged at 13,000 rpm to precipitate aggregated phages. Phage from supernatant was incubated with *Salmonella* cells 1 h, centrifuged at 3500 rpm, washed and titered in parallel with the input phage. Phage without *Salmonella* cells subjected to the same procedures served as a control. In the precipitation test, yields of *Salmonella*-bound phage were 12,000-22,000 times higher than the yield of the control wild-type phage, indicating very high specificity of the selection procedure.

Binding of phage probes to *Salmonella* was also assayed by fluorescence-activated cell sorting (FACS), fluorescent microscopy and transmition electron microscopy (TEM). The phage was fluorescently labeled by Alexa Fluor 488 (Molecular Probes) with a density of 300 dye molecules per phage. For the FACS assay, fluorescently labeled phage (10^9 cfu) was incubated with *Salmonella* cells at room temperature for 1h and centrifuged at 3,500 rpm, as in the precipitation test. The pellet was washed and analyzed by FACS and fluorescence microscopy. Figure 6 to the left exemplifies results of FACS assay showing binding of selected phage to *Salmonella* cells. The complex of phage with bacteria was visualized directly by fluorescence microscopy (not shown) and transmition electron microscopy (TEM) (Figure 7 below to the right), demonstrating a multivalent character of the phage-bacteria binding.



We also observed binding of *Salmonella* to immobilized phage directly, by high power optical microscope (Signaton, Inc.). In this test, one gold plate was coated with selected phage and another—with unrelated phage using chemisorptions and/or hydrophobic absorption. The plates were soaked in a suspension of *Salmonella* cells, washed and analyzed microscopically. Using this test we observed specific binding of bacterial cells to immobilized phage in real time and in the liquid flow.



Selectivity of the best phage candidate for *Salmonella typhimurium* was studied in co-precipitation test in comparison with nine other gram-negative bacteria, predominately *Enterobacteriaceae*. A small amount of cross reactivity of this phage was noted with *Yersinia enterocolitica* and *Citrobacter freundii* (data not shown).

Phage as bioselective element of biosensor

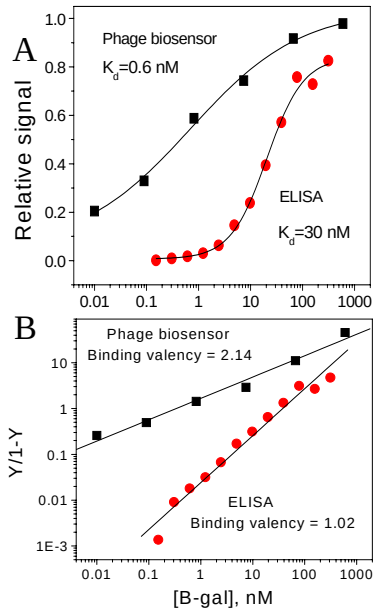
A critical step in the development of the sensor system is the immobilization of the capture probes to the platform area on the chip where the analytes (bacteria, bacteria components, toxins, or complementary DNA, etc.) will bind in a positive response. We have developed three methods of immobilization of phage-derived probes onto the sensor surfaces:

- Self-assembling of a phage layer on the preformed Langmuir-Blodgett (LB) film by biotin/streptavidin coupling;

- Direct physical adsorption of phage to the sensor surface, and
- Phage skinning method allowing coating of the sensor with self-assembling phage-derived peptide probes.

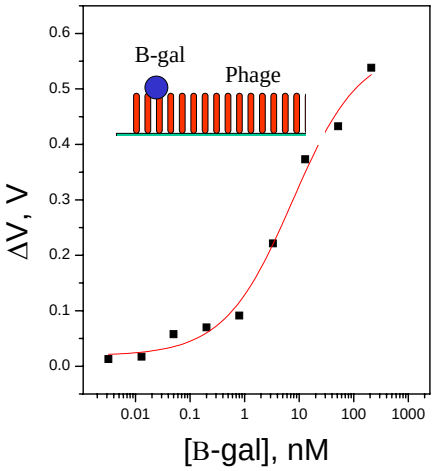
Self-assembling of a phage layer on the preformed Langmuir-Blodgett (LB) film.

Monolayers containing biotinylated phospholipids were transferred onto the gold surface of the acoustic wave sensor using the Langmuir-Blodgett technique and treated consequently with streptavidin and biotinylated phage. Experiments were carried using a TM-400 Maxtek thickness monitor with a frequency resolution of 0.05 Hz at 5 MHz. Figure 7A on the left demonstrates specific dose-dependent binding of β-galactosidase to the phage immobilized to the acoustic wave sensor in comparison with phage immobilized onto ELISA plate. It was observed that affinity of the complex depends on the mode of phage immobilization and type of analytical platform: 0.6 nM in TSM quartz sensor *versus* 30 nM in ELISA. The difference in affinities can be attributed to the monovalent (in ELISA) and divalent (in sensor) interaction of the phage with β-galactosidase, as is indicated by Hill-presentation of binding curves (Fig. 7B). One or another mode of interaction probably depends on the conformational freedom of the phage immobilized to the solid surface. Binding of the phage is quite specific because the response is reduced by 85% if β-galactosidase is preincubated with 4 nM phage. Binding of the phage to β-galactosidase is very selective: presence of 1000-fold excess of bovine serum albumin in mixture with β-galactosidase does not considerably change the ELISA signal and reduces the biosensor signal only by 4%.

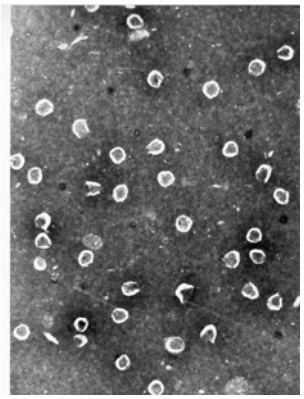


Direct physical adsorption of phage to the sensor.

Proteins adsorb strongly and irreversibly onto the gold surfaces due to hydrophobic interactions—effect that was previously employed for immobilization of a wide range of molecules including human serum albumin, antibodies, and viral proteins. In our experiments, the quartz TSM sensor with cleaned gold surface was exposed to a phage suspension containing 2.3×10^{11} virions/mL for 1 hour. After incubation, the sensor was rinsed in water and placed in wet chamber at 4 °C for 24 hours before tests with β-galactosidase began. The typical sensor response to the β-galactosidase in solution is shown on the Figure 8 to the left. The sensor shows the value of EC_{50} of about 5 nM, what is comparable with the results obtained by the described above self-assembling LB method.



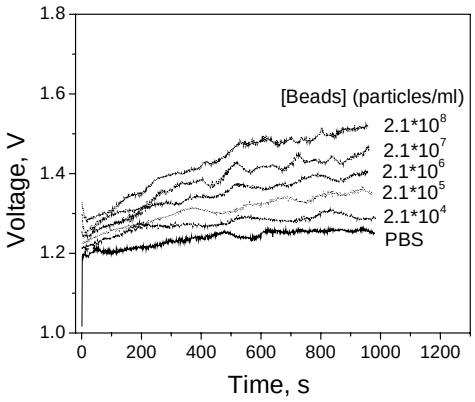
Fabrication of monolaers of phage-derived a target-binding coat proteins

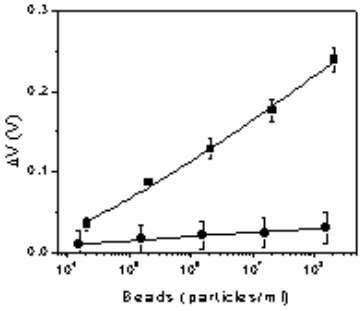


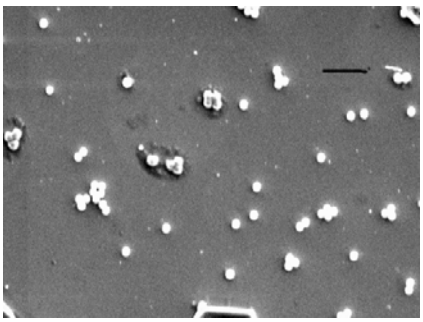
Phage-skinning method, invented in this project, is based on three successive steps. Phages are first converted into spheroids, which are then transformed into monolayers of the major coat protein pVIII. The layers are finally deposited onto the sensor surface by LB method. Spheroids were prepared by treatment of phage with chloroform and were characterized by electron microscopy (Figure 9 to the right). The conversion of phage to spheroids was monitored by gel electrophoresis of whole phage and spheroid particles (Petrenko et al. 1996).

Monolayers of phage coat proteins were made by allowing the spheroid suspension to run down a vertical glass rod that was partially submersed into the subphase. The formed monolayer was compressed to rupture the spheroids and create a new monolayer of phage coat proteins. The compressed monolayer was then transferred onto sensor surface by LB method.

We have shown that monolayers prepared by the phage skinning method produce a functional coating of high quality providing high sensitivity, specificity and selectivity of detection. For example, Figure 10 below on the left demonstrates signals generated by quartz crystals covered with the monolayer formed from phage specific for streptavidin in reaction with streptavidin-coated ~1 micron beads. For each bead concentration (10^4-10^8 particles/ml) the sensor signal approached a steady-state value within 500 s. In Figure 10 in the center, the mean values of the steady state output sensor voltages are plotted as a function of bead concentration (upper curve, squares),. The interaction of the beads with the peptide is specific since the signal is significantly lower for beads coated with BSA (lower line, circles). Binding of the beads to the sensor was confirmed by scanning electron microscopy (Figure 10 below on the right, the black bar is 10 μm long).



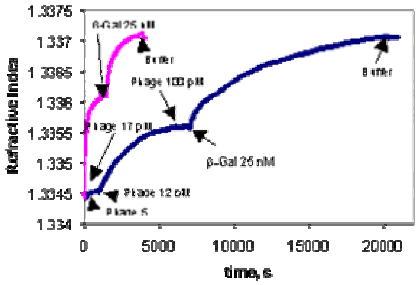




Surface plasmon resonance (SPR)

SPR, another sensor platform that we exploited in this project, is a well-established noninvasive technique, which gained application as a powerful tool for biorecognition characterization. In our preliminary experiments (with Dr. A. Simonyan, Auburn University) we have used the Spreeta

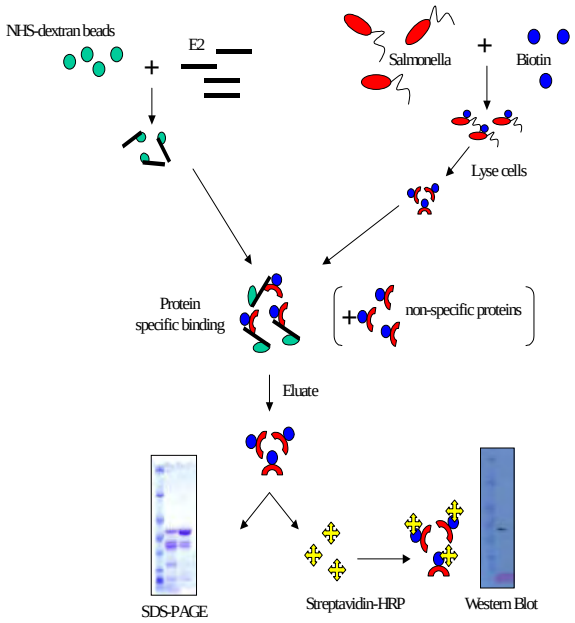
sensor--a miniature (approximately 7 g), fully integrated surface plasmon resonance device produced by Texas Instruments. 1401). Using this miniature device, we have shown that the phage selected against β-galactosidase immobilized to the SPR device by physical absorption shows a strong binding to nanomolar concentrations of β-galactosidase, as shown on the Figure 11 (50 nm on the upper curve and 25 nM on the lower curve).

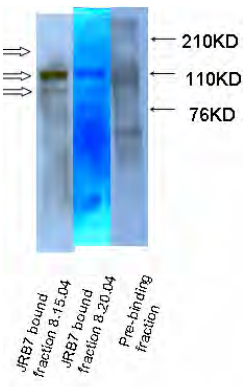


The data obtained in this project show that landscape phage and phage-derived peptide probes demonstrate many features (high affinity, specificity, selectivity field stability and availability) which commend them very well as substitute antibodies in biological detectors and prospective probes for detection of *B.anthracis* spores and other biological threats.

Specific aim 5. Identify target receptors on the bacterial and viral particles.

We developed a major scheme of identification of phage-binding receptors on the targeted organisms that is illustrated by a Figure 12 below to the right. Briefly, a selected phage that binds a target agents (bacterium or spores) was crosslinked with NHS-dextran (Smith, Petrenko et al., 1998) and mixed with a biotinylated lysate of the agent (Samoylova et all., 2004). Phage-bound receptors were separated by low-speed centrifugation and analyzed by gel-electrophoresis and Western blot. Using this technique we identified phage-binding receptors on *Salmonella typohimurium* cells (Figure 12 to the right) and *Bacillus anthracis* spores (Figure 13 below to the left) with molecular masses 60 and 110 KD correspondingly.The later protein have been revealed in exosporium with rabbit anti-*Bacillus anthracis* spore protein polyclonal antibodies and with mouse anti-*Bacillus anthracis* spore protein monoclonal antibodies which appear to bind the ~ 110 KD band to which the selected phage most strongly binds. To identify the "p60" and "p110" proteins, we plan to use Nano-LC/MS/MS technique, which become the standard method for protein identification when sensitivity and accuracy of the analysis are needed, especially when many proteins are present in a sample (Midwest Bio Services, LLC, Hemlock St, KS). The processing of the sample in this method starts with proteolytic digestion, typically with trypsin. The resulting peptide mixture is concentrated on a peptide trap column and washed to get rid of salts and other impurities. Then the peptides are separated on a microcapillary C18 reverse-phase chromatography column, such as PicoFrit columns that enable direct spray





of the eluting peptides from the tip of the column into the mass spectrometer, eliminating post-column losses. Full MS and MS/MS spectra will be acquired by the LCQ Deca XP Plus ion trap mass spectrometer (ThermoFinnigan). MS/MS spectra will be obtained after precursor peptide ions are isolated and fragmented inside the ion trap of the mass spectrometer and the resulting series of daughter ions are detected in a tandem mass spectrometry experiment. The sequences of the parent peptides will be inferred by matching the MS/MS spectra to protein sequence databases using the TURBOSEQUEST software.

Summary

We introduced new formats of phage display—landscape phage and mosaic landscape phage, in which all 4,000 copies of the major coat protein in the phage bear one or two foreign peptides. Libraries of landscape and mosaic landscape phages contain billions of clones with different peptides. This work demonstrated a great potential of the landscape phage probes as elements of biosensors and threat agent detectors. We learned that landscape phages affinity selected against various agents (bacteria, spores, proteins) demonstrate high specificity, selectivity and extreme robustness that commend them well as candidate probes for separation, concentration and continuous monitoring of biological threats. We showed that selected bacterium- and virus-binding phages may be used as probes for identification of surface components of these pathogens—potential targets for vaccines and drugs development. It is significant, too, that this work represented the first test of a phage evolution strategy that may have broad applicability in any system where directed enhancement of the probe's performance is required. In particular, it may improve operational affectivity of already existing detection and monitoring devices. It suggests also a new strategy of genetically directed nano-manipulation, which may be beneficial in creating new materials for nano-electronics, medicine and fundamental research.

(6) Publications and technical reports

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1. Brigati J., D.D. Williams, I.B. Sorokulova, V. Nanduri, I-H. Chen, C.L. Turnbough, Jr. and V.A. Petrenko (2004). Diagnostic probes for *Bacillus anthracis* spores selected from a landscape phage library. *Clinical Chemistry* V.50, No.11, p.1899-1906.
2. Samoylova T.I. , N.R. Cox, N.E. Morrison, L.P. Globa, V. Romanov, H.J. Baker, and V.A. Petrenko (2004). Phage matrix for isolation of glioma cell-membrane proteins. *BioTechniques.* V.37, No. 2, p.254-260
3. Petrenko V.A. and I.B. Sorokulova (2004) Detection of Biological Threat Agents. A challenge for Combinatorial Biochemistry. The Journal of Microbiological Methods Vol 58/2 pp 147-168.
4. Petrenko V.A. and V.J. Vodyanoy (2003) Phage display for detection of biological threat agents. The Journal of Microbiological Methods, 53/2 pp. 243-252.
5. Petrenko V.A., Smith G.P., Mazooji M.M. and Quinn T. (2002) Alfa-helically constrained phage display library. Protein Engineering, V.15, No.11, pp.943-950.

6. Kouzmitcheva G.A., Petrenko V.A. and Smith G.P. (2001) Diagnostic Peptides for Lyme Disease Through Epitope Discovery. Clinical and Diagnostic Laboratory Immunology, V8, p.150-160.

7. Petrenko V.A. and Smith G.P. (2000) Phage from landscape libraries as substitute antibodies. Protein Engineering, V.13, N8, p.101-104.

Papers published in non-peer-reviewed journals or in conference proceedings

1. S.B.Shankar Ganesh, Valery A. Petrenko, Aleksandr L.Simonian. Prevention of Non-specific binding as a Way to Increase Sensitivity of SPR-based Biosensors. The Electrochemical Society, Inc. 2004 Joint International Meeting. October 3-October 8, 2004. Honolulu, Hawaii, Hilton Hawaiian Village.

2. Viswaprakash Nanduri, Alexandre M. Samoylov, Valery A. Petrenko, Vitaly Vodyanoy, and Aleksandr L. Simonian. Comparison of Optical and Acoustic Wave Phage Biosensors. The Electrochemical Society, Inc. 2004 Joint International Meeting. October 3-October 8, 2004. Honolulu, Hawaii, Hilton Hawaiian Village.

3. Petrenko V.A. Robust Bioselective Materials for Concentration, Storage, Transportation and Analysis of Biological Threat Agents. Proc. of First International Conference on Fate of Biological Agents. 7-10 June 2004. Williamsburg Hospitality House, Williamsburg, Virginia.

4. Petrenko V.A., Sorokulova, I.B., Brigatti J., Olsen E., Nanduri V., Chen I., Barbaree J., Symonyan A., Vodyanoy V., Chin B. Substitution of Antibodies by Phage-Borne Probes in Detection Devices. Proc. of 36th Annual Oak Ridge Conference "Tomorrow's Technology Today. Pushing the Technology Envelope. An Exploration of the Future of Clinical Laboratory Testing." April 29-30, 2004, The Fairmont San Jose, California.

5. Jennifer R Brigati, David Williams, Iryna Sorokulova, Viswaprakash Nanduri, Charles Turnbough, Jr., and Valery Petrenko. Diagnostic probes against *Bacillus anthracis* selected from a landscape phage library. Proc. of GSC Research Forum. Auburn University Graduate Student Council, Auburn University Hotel and Dixon Conference Center, March 3-4, 2004.

6. Valery A. Petrenko, Iryna B. Sorokulova, Jennifer Brigatti, Eric Olsen, I-Hsuan Chen, Jim Barbaree, Aleksander Symonyan, Vitaly J. Vodyanoy and Brian A. Chin. Rigid Phage-Derived Probes for Continuous Monitoring of Biological Threats. Proceedings of 2nd Joint Conference On Point Detection, 1-5 March 2004, Williamsburg Hospitality House Williamsburg, Virginia.

7. Petrenko V.A. “Landscapes against Landscapes. Targeting Complex Biological Systems: Bacteria, Spores and Tumor Cells, with Multivalent Phage Probes”. Understanding Phage Display 2003, January 17-20, 2003, Morris L. Wosk Centre for Dialogue, Simon Fraser University, Vancouver.

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10. Petrenko V.A. Display at different positions of pVIII and the design of mosaic phage. Understanding Phage Display—Structure, Biology, and Applications. September 21-24, 2000, Simon Frazer University, Vancouver, Canada.
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Manuscripts submitted, but not published

1. Sorokulova I.B., E.V. Olsen, I-H. Chen, B. Fiebor, J.M. Barbaree, V.J. Vodyanoy, B.A. Chin and V.A. Petrenko (2004). Landscape Phage Probes for *Salmonella typhimurium.* The Journal of Microbiological Methods (In Press).

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(7) Participating scientific personnel and their contribution

2. Valery A. Petrenko – Professor, PI, planning and supervising of all stages of the project;
3. Iryna B. Sorokulova – Ph.D., Dr.Sc., Visiting Professor, selection of *Salmonella typhimurium*-binding phage;
4. Galina A. Kouzmitcheva – Ph.D., Postdoctoral Fellow, construction of landscape and mosaic phage libraries, development of phage evolution methods;
5. Jane Mount – Ph.D., Postdoctoral Fellow, identification of receptors on *Bacillus anthracis* spores targeted by selected phages;
6. I-Hsuan Chen – M.S., Research Associate, participation in constructing of phage libraries and selection experiments, analysis of phage clones by ELISA;
7. Jennifer R. Brigati – Ph.D. Student, selection of *Bacillus anthracis* spore-binding phages;
8. Eric Olsen – M.S., Ph.D. Student, characterization of *Salmonella typhimurium*-binding phages, identification of phage-binding receptors of *Salmonella;*
9. Viswaprakash Nanduri – Ph.D. Student, ELISA of *Bacillus anthracis* spore-binding phages.

(8) Report of Inventions

1. U.S. Utility Patent Application. Biospecific Probes Against Salmonella. Valery A. Petrenko, Iryna B. Sorokulova, Bryan A. Chin, James M. Barbaree, Vitaly J. Vodyanoy, I-Hsuan Chen, Alexander M. Samoylov. Appl..#10/835,405. Filed April 29, 2004.
2. International Patent Application corresponding to U.S. Provisional Application No. 60/451,918. Methods of Forming Monolaers of Phage-Derived Products and Uses Thereof. Valery A. Petrenko, Vitaly J. Vodyanoy, Jennifer Cannon Sykora. Filed March 3, 2004.
3. U.S. Utility Application. Methods of Forming Monolaers of Phage-Derived Products and Uses Thereof. Appl. No. 10/792,187; Valery A. Petrenko, Vitaly J. Vodyanoy, Jennifer Cannon Sykora. Filed March 3, 2004.
4. U.S. Provisional Patent Application. Biospecific Probes Against Salmonella. Appl. No. Not yet assigned. Valery Petrenko, Iryna Sorokulova, James Barbaree, Bryan Chin, Vitaly Vodyanoy. Filed April 29, 2003.
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6. International Patent Application No PCT/US02/35758, corresponding to U.S. Provisional Application #60/340,017 filed November 7, 2001 and 60/415,037 filed October 1, 2002. Phaqe Ligand Sensor Devices and Uses Thereof. Our Ref: 035721/256633. Valery A. Petrenko, Vitaly J. Vodyanoy, Alexandre M. Samoylov, Iryna Sorokulova, Viswaprakash Nanduri, Bryan A. Chin, James A. Barbaree, W. Charles Neely. Filed: November 7, 2002
7. U.S. Patent Application. Phage Ligand Sensor Devices and Uses Thereof. Appl. #10/289,725; Our Ref. No.035721/253299. Valery A. Petrenko, Vitaly J. Vodyanoy, Alexandre M. Samoylov, Iryna Sorokulova, Viswaprakash Nanduri, Bryan A. Chin, James A. Barbaree, W. Charles Neely. Filed: November 7, 2002. Pub. No: US 2004/0005540 A1, Jan, 8, 2004.
8. U.S. Provisional Patent #60/415.037. “Phage binding for continuous spore detection”, Valery A. Petrenko, Vitaly J. Vodyanoy, Alexandre M. Samoylov, W. Charles Neely, Bryan A. Chin, Iryna Sorokulova, Viswaprakash Nanduri. Filed: October 1, 2002.
9. U.S. Provisional Patent Application. “RGD phage as affinity matrix for isolation and purification of integrins and other RGD binding proteins.” Appl. # not yet assigned. Auburn University Technology Disclosure (AU# 02-027). Tatiana I. Samoylova, Valery A. Petrenko, Nancy R. Cox, Nancy E. Morrison, Henry J. Baker, Ludmila P. Globa. Filed July 3, 2002
10. US Provisional Patent Appl #60/340,017; "Phage as Bio-selective Elements in Biosensors", Valery A. Petrenko, Vitaly J. Vodyanoy, Rachana Agrawal, Alexandre M. Samoylov, Charles Neely, James A. Barbaree, Bryan A. Chin. Filed: November 7, 2001.

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