Clinical Chemistry 50:10
1899–1906 (2004)

Oak Ridge Conference

Diagnostic Probes for *Bacillus anthracis* Spores Selected from a Landscape Phage Library

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Background: **Recent use of *Bacillus anthracis* spores as a bioweapon has highlighted the need for a continuous monitoring system. Current monitoring systems rely on antibody-derived probes, which are not hardy enough to withstand long-term use under extreme conditions. We describe new, phage-derived probes that can be used as robust substitutes for antibodies.**

Methods: **From a landscape phage library with random octapeptides displayed on all copies of the major phage coat protein of the phage fd-tet, we selected clones that bound to spores of *B. anthracis* (Sterne strain). ELISA, micropanning, and coprecipitation assays were used to evaluate the specificity and selectivity with which these phage bound to *B. anthracis* spores.**

Results: **Peptides on the selected clones directed binding of the phage to *B. anthracis* spores. Most clones exhibited little or no binding to spores of distantly related *Bacillus* species, but some binding was observed with spores of closely related species. Our most specific spore-binding phage displayed a peptide EPRLSPHS (several thousand peptides per phage) and bound 3.5- to 70-fold better to spores of *B. anthracis* Sterne than to spores of other *Bacillus* species.**

Conclusions: **The selected phage probes bound preferentially to *B. anthracis* Sterne spores compared with other *Bacillus* species. These phage could possibly be further developed into highly specific and robust probes suitable for long-term use in continuous monitoring devices and biosorbents.**

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

B. anthracis spores are a challenge to detect because several closely related *Bacillus* species are ubiquitous in the environment. *B. cereus*, an opportunistic human pathogen, and *B. thuringiensis*, an insecticide, are both genetically very similar to *B. anthracis (1)*. To avoid costly false alarms, a detection system must be sensitive enough to detect low concentrations of *B. anthracis* spores but selective enough to differentiate between *B. anthracis* and other closely related species.

There are a variety of assays available for the detection of *B. anthracis* spores [reviewed in Refs. *(2, 3)*], but to date none has been adapted for real-time continuous monitoring of the environment *(4)*. Immunoassay- and biosensor-based *(5)* detection systems are the best prospects for continuous monitoring systems, but they require specific, selective, and stable diagnostic probes with which the pathogen can be detected. Antibodies and peptides can be used for this purpose, as demonstrated by numerous recent reports *(6–11)*. Here we describe the selection of alternative probes from a landscape phage library, in which foreign peptides form dense organic landscapes on the surface of the phage.

Phage-display libraries are constructed by the genetic modification of bacterial viruses (phages) such as M13, f1, and fd. The outer coats of these filamentous phages are composed of thousands of α-helical subunits of the major

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Received June 2, 2004; accepted July 26, 2004.
Previously published online at DOI: 10.1373/clinchem.2004.038018

coat protein pVIII, which form a tube encasing the viral DNA. At the tips of the phage are several copies of each of the minor coat proteins, pIII, pVI, pVII, and pIX [reviewed in Ref. *(12)*]. To create a phage-display library, degenerate synthetic oligonucleotides are spliced in-frame into one of the phage coat protein genes, so that the "guest" peptides encoded by the degenerate oligonucleotides are fused to the coat protein and thereby displayed on the exposed surface of the virions [reviewed in Ref. *(13)*]. Each phage particle displays multiple copies of one particular peptide.

In landscape phages, as in traditional phage-display constructs, foreign peptides or proteins are fused to coat proteins on the surface of the virus particle. Unlike conventional constructs, however, landscape phages display thousands of copies of the peptide in a repeating pattern, comprising a major fraction of the viral surface. The phage body serves as an interacting scaffold to constrain the peptide into a particular conformation, creating a defined organic surface structure (landscape) that varies from one phage clone to the next. A landscape library is a huge population of such phages, encompassing billions of clones with different surface structures and biophysical properties. The landscape phage library used in this work contained random 8-amino-acid peptides fused to all 4000 copies of the major coat protein of fd-tet *(14)*.

Landscape phages have been shown to serve as substitutes for antibodies against various antigens and receptors *(15, 16)*, including live bacterial cells *(17)*. These phage probes have been used in ELISA and thickness shear mode quartz sensors to detect antigens *(18)*. Use of landscape phages as substitutes for antibodies has several advantages: they produce up to 4000 copies of the binding peptide on their surfaces, allowing multivalent interactions with the target antigen; phages can be produced rapidly and inexpensively in large quantities; they are resistant to heat *(19)*, organic solvents *(20)*, and many other stresses; and they can be stored indefinitely at moderate temperatures without loss of activity, or at 37 °C with only minimal loss of activity after 7 months (J. Brigati and V.A. Petrenko, unpublished observations).

Recently, a pIII phage-display library was used to identify peptides specific and selective for spores of various *Bacillus* species, including *B. anthracis (21–23)*. We were interested in evaluating the prospect of using not only individual peptides, but phages themselves as "building blocks" for the development of robust and inexpensive diagnostic probes and biosorbents *(17)*. As a first step toward this goal, we identified landscape phage clones from a phage-display library that bind to *B. anthracis* Sterne spores. We then studied the specificity and selectivity of their interaction with the target selector spores in comparison with other *Bacillus* species. The results of this study indicate that the landscape phage is a prospective bioselective material that can be used as an antibody substitute in monitoring devices.

Materials and Methods

STRAINS AND SPORE PREPARATION

The Sterne strain of *B. anthracis* (an avirulent veterinary vaccine strain), *B. cereus* T, and *B. thuringiensis* subsp. *kurstaki* were obtained from the US Army Medical Research Institute of Infectious Diseases (Fort Detrick, MD). *B. subtilis* (*trpC2*) 1A700 (originally designated 168) and *B. licheniformis* 5A36 (originally ATCC 14580) were provided by the *Bacillus* Genetic Stock Center, Ohio State University (Columbus, OH). *B. megaterium* ATCC 14581 was purchased from the American Type Culture Collection. Spores were produced by cells grown in liquid Difco sporulation medium at 37 °C for 48–72 h with shaking *(24)*. Remaining vegetative cells and cell debris were removed with a renografin step gradient as described previously *(25)*. Spores were stored in sterile distilled water at 4 °C.

PHAGE LIBRARY

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

PHAGE GROWTH, PURIFICATION, AND TITERING

The general procedures used for recombinant phage production and analysis, including media and buffers, are detailed in *Phage Display: A Laboratory Manual (26)*. Briefly, phage were propagated by infection of *Escherichia coli* K91 BlueKan cells *(27)*, followed by growth of the infected cells for 16 h in NZY medium containing 20 mg/L tetracycline *(28)*. Phage were purified by double polyethylene glycol precipitation as described previously *(28)*. The total number of viral particles present in phage preparations was determined spectrophotometrically by use of the formula *(26)*:

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

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

$$\text{Absorbance unit}_{269} = 6.5 \times 10^{12} \text{ virions/mL}$$

was used to determine the concentration of phage particles in a solution (physical titer). The concentration of infective phage particles (biological titer) of a phage solution was determined by infection of starved K91 BlueKan cells with the phage, followed by their spreading on a tetracycline-containing agar plate. The recombinant phage carry the gene necessary for tetracycline resistance, allowing only those cells infected by phage to form colonies on the plate *(26)*. The biological titer of these recombinant phage, expressed as colony-forming units

(CFU)[4] is typically 20-fold lower than the physical titer (virions/mL).

SELECTION OF SPORE-BINDING PHAGE CLONES

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

Wells containing *B. anthracis* Sterne spores were blocked with 10 g/L bovine serum albumin (BSA) for 1 h at 37 °C. The wells were then washed three times with 0.2 mL of Tris-buffered saline (TBS) containing 5 mL/L Tween 20 to remove unbound spores. The f8/8 phage library (1.25×10^{10} virions in 60 μL of TBS containing 5 mL/L Tween 20 and 0.1 g/L BSA) was added to each well and incubated 1 h at room temperature on an orbital shaker. Nonbound phage particles were then removed, and the wells were washed with 10 times with 0.2 mL of TBS containing 10 g/L BSA. Elution buffer (100 μL; 0.2 mol/L glycine-HCl, pH 2.2, containing 1 g/L BSA) was then added to each well and incubated for 5 min at room temperature. The eluates from all eight wells were transferred to a single microcentrifuge tube that was centrifuged for 3 min at 12 000*g* and 4 °C to pellet any spores. The eluate was then neutralized by the addition of 140 μL of 1 mol/L Tris-HCl, pH 9.1, and concentrated by use of a Centricon 100 filter to a final volume of ~100 μL. These concentrated phage clones were then propagated and purified for use in the next round of selection.

In the second round of selection, the phage clones that were selected and amplified in the first round were added to the spore-coated wells (rather than the primary phage library), but all other procedures remained the same. Likewise, in each subsequent round the phages selected and amplified in the previous round were added to the spore-coated wells. After the fourth round of selection, individual phage clones were amplified and sequenced *(29)* to determine the amino acid sequences of the displayed peptides.

PHAGE-CAPTURE ASSAY

B. anthracis spores (2×10^7 in sterile distilled water) were added to each well of a 96-well flat-bottomed microtiter plate. The plate was centrifuged for 2 min at 550*g* and then incubated at 37 °C overnight to dryness. BSA (10 g/L) was added to the wells containing spores, and the plate was incubated for 1 h at 37 °C. The wells were then gently washed with TBS containing 5 mL/L Tween 20. Candidate or control phages (~10^6 CFU in 50 μL of TBS) were added to separate spore-containing wells. After incubation for 1 h at room temperature, the plate was gently washed with TBS containing 5 mL/L Tween 20. Elution buffer (100 μL) was added to wells containing phages bound to immobilized spores and incubated for 5 min at room temperature. The eluates from each of these wells were transferred to sterile tubes and neutralized with 20 μL of 1 mol/L Tris-HCl, pH 9.1. Phage input and eluate were titered as described previously.

BIOTINYLATION OF *B. anthracis* STERNE SPORES

To biotinylate spores, we mixed 160.7 μL of 1.49 mmol/L Sulfo-NHS-LC-LC-Biotin (cat. no. 21338; Pierce) in 2 mmol/L sodium acetate with 3.954×10^9 spores in 1 mL of phosphate-buffered saline and incubated the mixture for 2 h at room temperature. Tris-HCl (300 μL; 1 mol/L, pH 9) was added, and the solution was incubated for 1 h to inactivate the remaining biotinylating agent. Spores were then centrifuged for 10 min at 9000*g* and washed with water.

ELISA WITH BIOTINYLATED SPORES

Phage preparations (3×10^{10} virions in 60 μL) were loaded in a flat-bottomed 96-well microtiter plate and incubated at 4 °C for 12 h. The plate was washed with TBS containing 5 mL/L Tween 20 in a BIO-TEK EL_x405 auto plate washer. Biotinylated *B. anthracis* spores (5×10^7 in 50 μL of TBS containing 5 mL/L Tween 20) were applied to the phage-coated wells and incubated for 2 h at room temperature on a rocker. The plate was then washed as before. Alkaline phosphatase conjugated to streptavidin (1 mg/L; Pierce) was added, and the plate was incubated for 1 h at room temperature on a rocker. After a final washing step, alkaline phosphatase substrate, *p*-nitrophenylphosphate, was added to the wells, and the absorbance at 405 nm (reference wavelength, 490 nm) was monitored for 1 h by an EL808 Ultra Microplate Reader (BIO-TEK Instruments, Inc.).

ELISA WITH NONBIOTINYLATED SPORES

The phage preparations (2.75×10^{10} virions in 55 μL of TBS) were loaded into each well of a flat-bottomed 96-well microtiter plate, and the plate was incubated overnight at 4 °C. The plate was then washed as described above. *B anthracis* spores (2.5×10^8 spores in 50 μL of TBS containing 5 mL/L Tween) were added to each well, and the plate was incubated for 2 h at room temperature with rocking. The plate was washed as before; 45 μL of the anti-*B. anthracis* spore monoclonal antibody BD8 (2.2 mg/L) was added; and the plate was incubated for 1 h. The plate was washed again, and then 40 μL of goat anti-mouse IgG–alkaline phosphatase conjugate (22.9 μg/L; cat. no. S3721; Promega) was used for detection. The substrate *p*-nitrophenylphosphate was then added, and the reaction was monitored as described above.

COPRECIPITATION ASSAY

Candidate phages (200 μL; 10^6 CFU/mL) were mixed with 2×10^7 *B. anthracis* spores and/or other *Bacillus*

[4] Nonstandard abbreviations: CFU, colony-forming unit(s); BSA, bovine serum albumin; and TBS, Tris-buffered saline.

species spores in a microcentrifuge tube and incubated for 1 h at room temperature on a rotator. Spore–phage complexes were pelleted by centrifugation for 10 min at 3000*g*. The pellets were gently washed five times with 200 μL of TBS containing 5 mL/L Tween 20, and then suspended in 200 μL of elution buffer and incubated for 10 min at room temperature with occasional vortex-mixing. The spores were pelleted by centrifugation for 10 min at 9000*g*, and the supernatant containing eluted phages was transferred to a fresh sterile tube and neutralized with 38 μL of 1 mol/L Tris-HCl, pH 9.1. Phage input and recovery were determined by biological titering as described previously.

Results

SELECTION OF PHAGE BINDING TO *B. anthracis* SPORES

The landscape library f8/8 used in this work was constructed by splicing degenerate oligonucleotides into gene gpVIII so that a foreign random octapeptide was displayed as the N-terminal portion of the major coat protein pVIII *(14)*. From this library, phage clones that bound to *B. anthracis* Sterne spores were selected through a panning procedure in which the phage library was incubated with immobilized *B. anthracis* spores, nonbound phages were washed away, and bound phages were eluted with mild acid. Phages that bound to spores in the initial selection procedure (a sublibrary) 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 after each round of selection (Fig. 1) indicated an increase in the representation of phage clones in the sublibrary that were capable of binding to *B. anthracis* spores. After four rounds of selection, 16 randomly picked clones were isolated, and a segment of genomic DNA encoding the displayed octapeptide was sequenced. Eleven unique peptide sequences were found that formed three related families, each with a particular motif or consensus sequence (Table 1). Family 1, with six members, was characterized by the presence of a negatively charged amino acid (E or D) at the first position, usually a proline residue at the second position, and a positively charged amino acid (R, K, or H) at the third position. Another interesting feature of this family was the frequent presence of a "migrating" dipeptide PH, which was replaced by PK in peptide 4. Family 2, with three members, contained the consensus sequence (D/E)(R/K)TXATXT. Family 3, with two members, contained the consensus sequence V(S/T)XXXSXS.

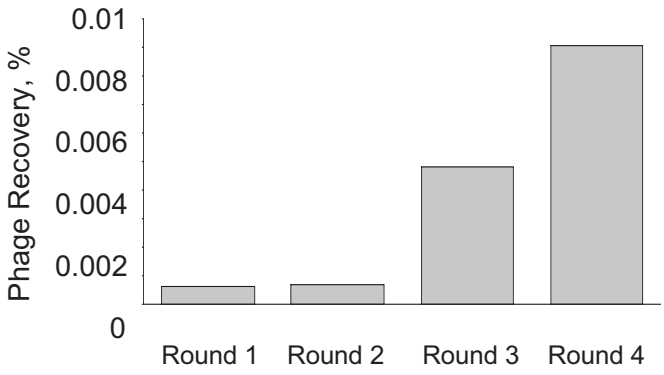

Fig. 1. Phage recovery during selection.

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

Table 1. Amino acid sequences of peptides carried by selected phage.[a]

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		

[a] The bold italics indicate common motifs.

SPECIFICITY OF PHAGE BINDING TO *B. anthracis*

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

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

In the ELISA, wells of a microtiter plate were coated with phage and then incubated with biotinylated *B. anthracis* spores. Alkaline phosphatase conjugated to streptavidin was then added to bind to the biotinylated spores, and *p*-nitrophenylphosphate was used to detect this binding. As shown in Fig. 3, many of the isolated phage clones bound to *B. anthracis* spores at a higher percentage than wild-type phage. Some clones bound strongly to *B. anthracis* Sterne in both assays, whereas other clones gave inconsistent results between the two assays. This is not completely unexpected because in the

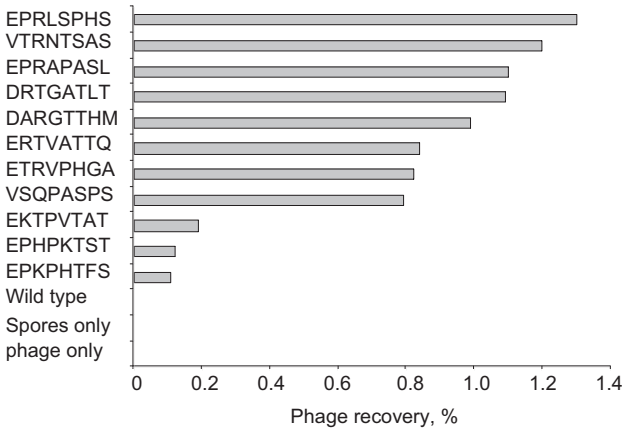

Fig. 2. Binding of selected phage to *B. anthracis* Sterne immobilized on a microtiter plate.

y axis, amino acid sequences of peptides carried by selected phage clones; *x axis*, percentage of phage recovered during the micropanning assay [recovery (%) = (eluted phage/phage input) × 100].

ELISA phage were fixed to the plate and spores were captured from solution, whereas in the phage-capture assay spores were fixed to the plate and phage were bound from solution. Thus, in these tests, phage could adopt different conformations, allowing monovalent or multivalent interactions with spore receptors, as was demonstrated in binding experiments in which β-galactosidase from *E. coli* served as a model multivalent analyte *(18)*.

To confirm that the ELISA results were not attributable to biotinylation of contaminants of the spore preparation, an ELISA was done in another format, with antibodies specific for *B. anthracis* spores *(30)*. Fig. 4 exemplifies specific binding of phages carrying the peptide VTRNTSAS to spores, revealed with monoclonal antibody BD8. It is clear from this experiment that phages were capturing

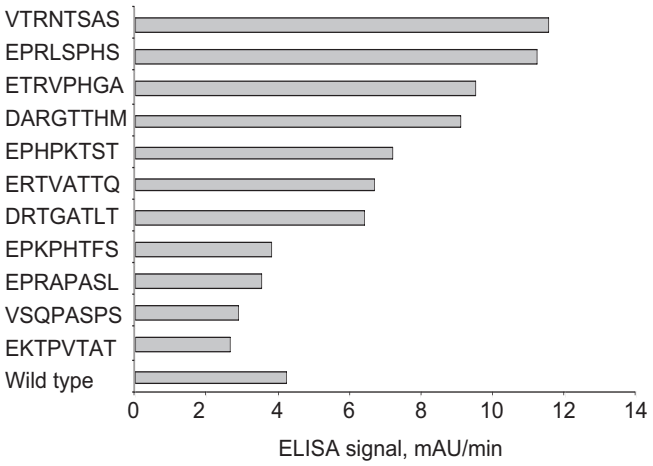

Fig. 3. Binding of biotinylated *B. anthracis* Sterne spores to selected and control phage immobilized on a microtiter plate.

y axis, amino acid sequences of peptides carried by selected phage clones; *x axis*, ELISA signal in milliabsorbance units (*mAU*)/min.

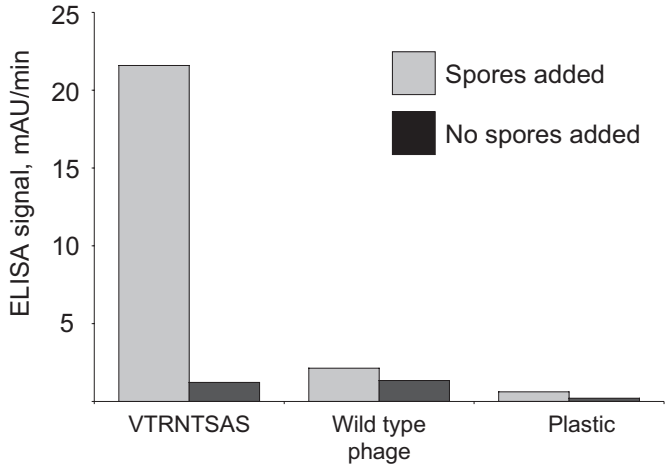

Fig. 4. Binding of nonbiotinylated *B. anthracis* Sterne spores to selected and control phage immobilized on a microtiter plate.

y axis, ELISA signal in milliabsorbance units (*mAU*)/min; *x axis*, amino acid sequences of peptides carried by selected phage clones or controls (wild-type phage and plastic)

spores and not some other contaminants of the spore preparation.

SELECTIVE BINDING OF PHAGE TO SPORES OF *B. anthracis* STERNE

We defined selectivity as the ability of a recombinant phage clone to preferentially interact with the selector in comparison with other potential targets. To determine the selectivity of phage probes for the selector *B. anthracis* spores vs spores of other *Bacillus* species, we used a coprecipitation assay. 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 were tested because of the high binding they demonstrated in the ELISA. In the coprecipitation assay, these phages were mixed individually with spores of various *Bacillus* species. After incubating, spores were collected by 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 1) exhibited very low binding to these distant relatives, whereas the clones from other families bound them nearly as well as *B. anthracis*. We found that the phage carrying the peptide DARGTTHM bound to *B. anthracis* Sterne 75-fold better than to *B. megaterium*, 25-fold better than to *B. subtilis*, and 50-fold better than to *B. licheniformis.* Phage carrying the peptide EPRLSPHS bound to *B. anthracis* Sterne 43-fold better than to *B. megaterium*, 39-fold better than to *B. subtilis*, and 70-fold

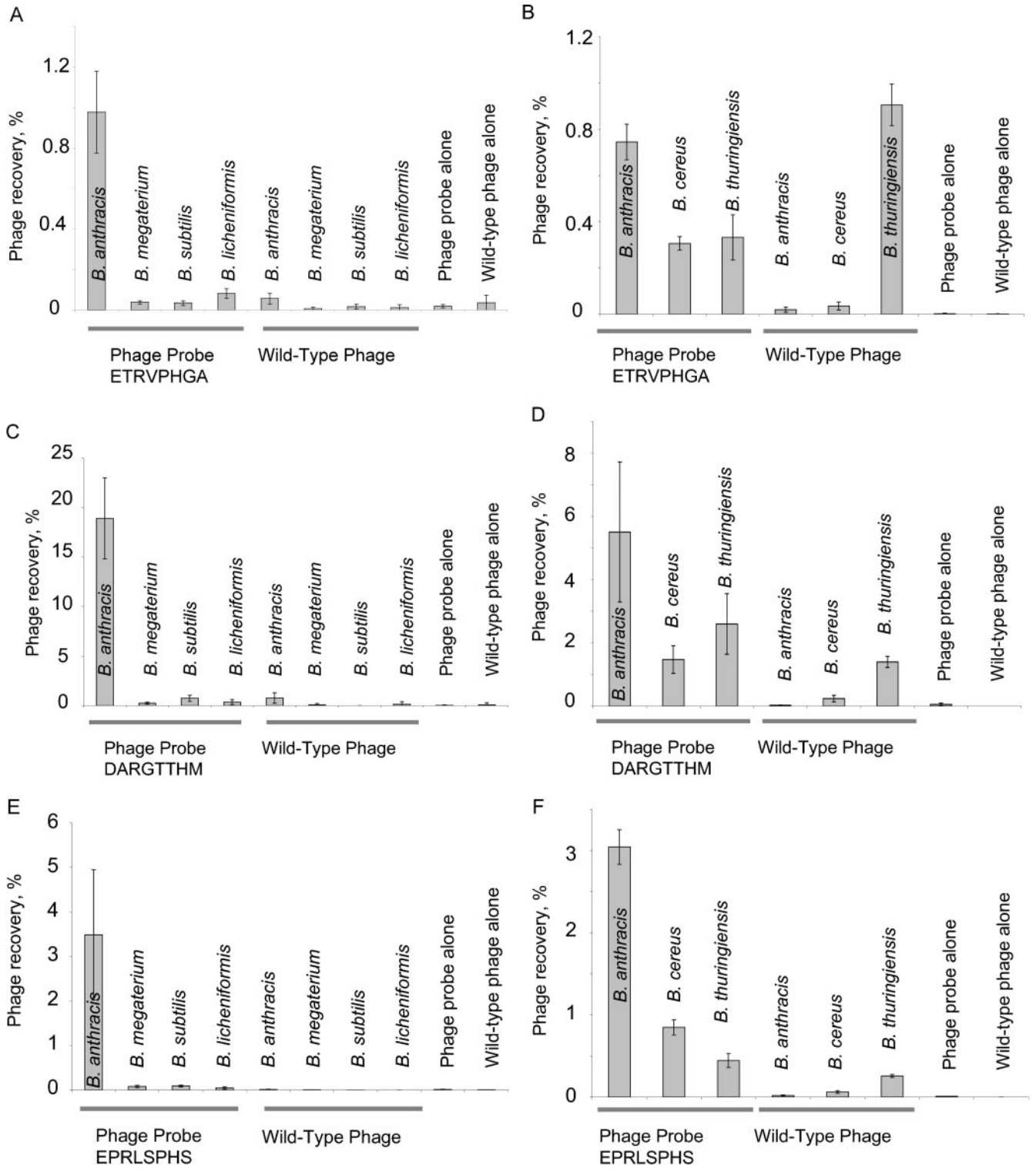

Fig. 5. Binding of selected phage clones to *Bacillus* spores in a coprecipitation assay.

y axis, species of spores that were mixed with selected phage; *x axis*, percentage of phage recovered by coprecipitation with spores [recovery (%) = (eluted phage/phage input) × 100]. *Panels A*, *C*, and *E* depict binding of phage carrying the peptides ETRVPHGA, DARGTTHM, and EPRLSPHS, respectively, to *B. anthracis*, *B. megaterium*, *B. subtilis*, and *B. licheniformis*. *Panels B*, *D*, and *F* depict binding of phage carrying the peptides ETRVPHGA, DARGTTHM, and EPRLSPHS, respectively, to *B. anthracis*, *B. cereus*, and *B. thuringiensis*.

better than to *B. licheniformis.* Phage carrying the peptide ETRVPHGA bound to *B. anthracis* Sterne 24-fold better than to *B. megaterium*, 24-fold better than to *B. subtilis*, and 12-fold better than to *B. licheniformis* (Fig. 5, A, C, and E). Phage clones from families 2 and 3 exhibited much lower selectivity and were not examined further. The three abovementioned phage probes that did not cross-react strongly with distant relatives of *B. anthracis* were examined further for binding to spores of close relatives of *B. anthracis*, namely, *B. cereus* and *B. thuringiensis*. All three phages demonstrated preferential binding to *B. anthracis* but considerable binding to the close relatives: the phage bearing the peptide DARGTTHM bound to *B. anthracis* 3.7-fold better than to *B. cereus* and 2.1-fold better than to *B. thuringiensis*; the phage bearing the peptide EPRLSPHS bound to *B. anthracis* 3.5-fold better than to *B. cereus* and 6.9-fold better than to *B. thuringiensis*; the phage bearing the peptide ETRVPHGA bound to *B. anthracis* 2.4-fold better than to *B. cereus* and 2.2-fold better than to *B. thuringiensis* (Fig. 5, B, D, and F).

Discussion

Monitoring of the environment for biological threats, such as spores of *B. anthracis*, requires probes that bind to biological agents and ensure their separation, purification, and detection *(17)*. Combinatorial probe technology is based on the principle that biological agents have unique surface markers that can bind organic molecules (probes) recruited from diverse combinatorial libraries through screening or selection procedures *(18)*. The effectiveness of this new technology has been illustrated by development of diagnostic probes for various bacterial and viral agents *(17)*, including spores of *B. anthracis (22)*. Although the peptides and antibodies identified through phage display in these examples are useful in diagnostic assays, landscape phage probes may be better suited for the exacting requirements of environmental monitoring, in which robust, selective, strong, and inexpensive binders capable of operating in severe environmental conditions are needed. Our studies of landscape phages, recombinant filamentous phages displaying 4000 foreign random peptides on their surface, showed that these phages are well suited for obtaining durable, specific probes and biosorbents *(13–15, 17, 18)*. Filamentous phages are probably the most stable natural nucleoproteins capable of withstanding high temperatures (up to 80 °C), denaturing agents (6–8 mol/L urea), organic solvents [e.g., 50% (by volume) alcohol or acetonitrile], mild acids (pH 2), and alkaline solutions. Phage-derived probes inherit the extreme robustness of the wild-type phage and allow fabrication of bioselective materials by self-assemblage of phages or their composites on metal, mineral, or plastic surfaces *(18)*.

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

We recognize that these probes are not completely ideal for identification of *B. anthracis* spores because they cross-react with spores of *B. cereus* and *B. thuringiensis*. In future experiments, cross-reacting clones such as these may be removed from the library in a biased selection procedure by depletion against competing strains, as was demonstrated previously *(17, 31–33)*. The depleted library could serve as a reservoir of probes for unique *B. anthracis* spore markers. If necessary, affinity of probes toward the target agent may be adjusted by mutagenesis of phage and selection of the spore-binders in more stringent conditions [reviewed by Ref. *(17)*]. We believe that this phage evolution technique could be used to gradually enhance the performance of the selected phage-derived probes, allowing them to serve as robust substitutes for antibodies in concentration and detection systems for biological threat agents.

This work was supported by Army Research Office/ Defense Advanced Research Projects Agency Grant DAAD 19-01-10454 (to V.A.P.), NIH Grant NIH-1 R21 AI055645 (to V.A.P.), Auburn University Animal Health & Disease Research Funds Grant 354 (to V.A.P.), DARPA MDA972-01-1-0030 (to C.L.T.), and ARO DAAD19-00-1-0032 (to C.L.T.). We are grateful to Professor Bryan A. Chin, Director of Auburn University Detection and Food Safety Center, for supporting of this project and for fruitful discussions, and Professor John F. Kearney (University of Alabama at Birmingham) for the kind gift of monoclonal antibodies against *B. anthracis* spores.

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