Review article

Discovery of phage display peptide ligands for species-specific detection of Bacillus spores

Charles L. Turnbough Jr.*

Department of Microbiology, University of Alabama at Birmingham, 409 Bevill Biomedical Research Building, 845 19th Street South, Birmingham, AL 35294, USA

Abstract

Short peptides are capable of tight and specific binding to physiological or fortuitous receptors on the surface of cells. These peptides can be used to tag or capture target cells in an assortment of detector platforms. As part of an effort to identify small-molecule ligands for advanced detectors for spores of Bacillus anthracis, the causative agent of anthrax, we are screening (or biopanning) commercial phage display peptide libraries for peptides that bind tightly and selectively to spores of several Bacillus species. In addition to B. anthracis, these species include B. cereus, B. subtilis, and B. globigii. This review summarizes the methods used in our studies, the results from the biopanning experiments, and the characterization of the spore-binding peptides identified to date. Briefly, several unique families of peptides, with consensus sequences ≤seven-amino-acids long, were identified that exhibit preferential binding to spores (but not vegetative cells) of either one or only a few Bacillus species. At least one peptide family binds well to spores of multiple strains of B. anthracis, while binding poorly or not at all to spores of phylogenetically similar species. This review also discusses other points of interest regarding the use of peptide ligands for spore detection and for the detection of other types of cells.

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1. Introduction

As a mechanism of long-term survival, certain species of bacteria form endospores (or spores) upon nutrient deprivation (Stragier and Losick, 1996). The spore is dormant and highly resistant to extreme temperatures, radiation, desiccation, harsh chemicals, and physical damage. These properties allow spores to survive adverse environments, for many years in some cases, until encountering conditions that trigger germination and vegetative cell outgrowth (Nicholson et al., 2000). Spore-forming bacteria are primarily members of the low G+C subdivision of Gram-positive eubacteria. The best studied spore-formers are the rod-shaped soil bacteria belonging to the genera Bacillus and Clostridium, which are characterized by aerobic and anaerobic growth, respectively. These bacteria have a long history of exploitation by man. Selected species are used for fermentation processes, as sources of products of industrial importance, and scientifically as a simple model for the study of cellular differentiation. In addition, several species are medically important because they cause diseases such as anthrax (B. anthracis), food poisoning (B. cereus and C.

* Tel.: +1-205-934-6289; fax: +1-205-975-5479.
E-mail address: ChuckT@uab.edu (C.L. Turnbough).

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In recent decades, the world has witnessed a most unfortunate use of a spore-forming bacterium, namely, the development by numerous countries and terrorist groups of *B. anthracis* spores as a weapon of mass destruction (Inglesby et al., 1999). The terrible consequences of using *B. anthracis* spores as a weapon, even on a small scale, were vividly demonstrated in the United States in the fall of 2001. For large-scale use, weaponized spores would most likely be dispersed in aerosol form to maximize exposure and contamination. Spores can enter the body through skin abrasions or by ingestion or inhalation. Once exposed to internal tissues, spores can germinate and grow vegetatively, which in many cases will result in the death of the human (or animal host) within several days. However, anthrax caused by natural strains of *B. anthracis* can be successfully treated with common antibiotics, provided treatment begins within a day or two of exposure to spores (Jernigan et al., 2001). Therefore, rapid detection of *B. anthracis* spores is critical in responding to the anthrax threat.

Unfortunately, the current detectors capable of reliable identification of *B. anthracis* spores are large, complicated, expensive, and slow—typically requiring spore germination and outgrowth (Bell et al., 2002; Swartz, 2001). These drawbacks limit the use of current detectors in frontline situations and for general monitoring. Clearly, there is a need for simpler but more advanced detectors that directly identify *B. anthracis* spores. The most important components of such advanced detectors will be simple and hardy ligands that bind tightly and specifically to *B. anthracis* spores.

For several years, my lab and the labs of several collaborators have engaged in the discovery and development of small-molecule ligands capable of species-specific binding to *Bacillus* spores, with the ultimate goal of producing a simple, inexpensive, rugged, and accurate detector of *B. anthracis* spores. The primary focus of my lab is the identification of short peptides capable of species-specific spore binding. In addition to *B. anthracis* spore ligands, we are...
also interested in identifying peptide ligands for spores of *B. cereus*, *B. thuringiensis*, *B. subtilis*, and *B. globigii*. *B. cereus* and *B. thuringiensis* are phylogenetically the most similar species to *B. anthracis* (Fig. 1). These three species plus *B. mycoides* comprise the so-called *B. cereus* group. Spores of this group are widely disseminated in nature and are structurally similar, possessing a well-developed exosporium (Gerhardt, 1967; Gerhardt and Ribi, 1964; Gerhardt et al., 1976) (Fig. 2A). Peptide ligands capable of binding selectively to spores of each member of the *B. cereus* group would be valuable tools for discriminating between spores of *B. anthracis* and its less dangerous close relatives. Our interest in ligands for spores of *B. subtilis* and *B. globigii* is because these spores are used as nonpathogenic simulants for *B. anthracis* spores during detector development, including laboratory and field testing. *B. subtilis* and *B. globigii* spores share many of the properties of *B. anthracis* spores, although they do not possess a readily detectable exosporium (Henriques and Moran, 2000) (Fig. 2B).

2. Biopanning of phage display peptide libraries for *Bacillus* spore-binding ligands

To identify the desired spore-binding peptides, we chose to screen (or biopan) the Ph.D.-7 and Ph.D.-12 Phage Display Peptide Libraries available from New England Biolabs (NEB). These libraries display random 7- and 12-mer peptides, respectively, on the surface of the filamentous coliphage M13. These peptides are fused to the surface-exposed amino terminus of the minor capsid protein pIII, which is present in five copies at one end of the phage particle (Webster, 1996). Thus, each phage displays five copies of one particular peptide. The Ph.D. libraries each contain approximately $2 \times 10^9$ independent phage clones. Each clone produces a protein pIII–peptide fusion that was created by the insertion of a random 21- or 36-base DNA fragment into a cloning site at the start of gene III of the phage. Consequently, the sequence of any peptide that binds a target spore can be readily determined by cloning the phage displaying this peptide and sequencing the peptide-encoding region of the phage genome. Spores were produced by cells grown in liquid Difco sporulation medium at 37 °C for 48–72 h with shaking (Nicholson and Setlow, 1990). Spores were purified by sedimentation through a Renografin step gradient as previously described (Henriques et al., 1995).

For a typical biopanning experiment, $10^9$ spores and $10^{11}$ phage are mixed in 1 ml of sterile TBST [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% Tween-20] for approximately 20 min at room temperature with gentle shaking (Fig. 3). Spores and bound phage are collected by centrifugation (12,000 × g at 4 °C for 5 min), and unbound phage in the supernatant are discarded. The pelleted spore–phage complexes are washed 10 times with 1 ml (each) of cold (4 °C) TBST to remove unbound phage. The pelleted spore–phage complexes are resuspended in 1 ml of elution buffer [0.2 mM glycine–HCl (pH 2.2), 1 mg/ml bovine serum albumin] and gently mixed for 5 min at room temperature. This sample is centrifuged as above for 5 min, and the supernatant, which contains eluted phage, is quickly removed and neutralized by 0.1 M Tris–HCl (pH 8.0) to increase the pH to 7.5. The supernatant is then neutralized by 0.5 M Tris–HCl (pH 7.5) to increase the pH to 7.5. The eluted phage are then amplified by infection of fresh cells, and the biopanning process is repeated for a total of 4 rounds. After the 4th round, individual clones are isolated and genomic DNAs are sequenced.

Fig. 3. Biopanning a phage display peptide library for phage peptides that bind spores.
the addition of 150 μl of 1 M Tris–HCl (pH 9.1) to prevent phage killing. The eluted phage are then amplified by infecting a phage-sensitive strain of *Escherichia coli* (e.g., strain ER2738 from NEB). The resulting phage stock is used for a second round of biopanning, which is performed exactly as described above. A total of four rounds of biopanning are performed, after which the final eluted phage are plated to obtain single plaques. These plaques (typically around 30) are used to prepare phage stocks, from which genomic DNAs are extracted by using Qiagen Spin M13 columns. The sequence of the region of each genomic DNA encoding a putative spore-binding peptide is determined. Additional details can be found in the instructions for the NEB Phage Display Peptide Library Kit.

During each round of biopanning, a small sample of input phage, supernatants from the initial collection of spore–phage complexes and selected washes, and eluted phage are saved for titering. The number of phage found in these samples provides an excellent indicator for the expected enrichment of phage populations. Titering data for a typical Ph.D.-7 biopanning experiment with *B. subtilis* spores is shown in Table 1. Compared to the titers in Round 1, the titers for the late washes (5 and 10) and the eluted phage in Rounds 2–4 were \(10^7–10^9\) higher, indicating that a large enrichment for spore-binding phage had occurred.

### Table 1

<table>
<thead>
<tr>
<th>Phage sample</th>
<th>Number of phage(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Round 1</td>
</tr>
<tr>
<td>Input(^b)</td>
<td>(5.4 \times 10^{10})</td>
</tr>
<tr>
<td>Supernatant(^c)</td>
<td>(5.7 \times 10^{10})</td>
</tr>
<tr>
<td>Wash 1(^d)</td>
<td>(3.0 \times 10^9)</td>
</tr>
<tr>
<td>Wash 5(^d)</td>
<td>(4.8 \times 10^7)</td>
</tr>
<tr>
<td>Wash 10(^d)</td>
<td>(1.5 \times 10^6)</td>
</tr>
<tr>
<td>Eluant(^e)</td>
<td>(5.0 \times 10^5)</td>
</tr>
</tbody>
</table>

\(^a\) Error in titering phage (plaque forming units) was \(± 20\%\).
\(^b\) Phage initially mixed with spores in each round.
\(^c\) Supernatant from initial centrifugation of spore–phage complexes.
\(^d\) Supernatant from indicated wash.
\(^e\) Phage eluted from spore–phage complexes.

## 3. Results of biopanning experiments

We performed separate biopanning experiments against spores of the following *Bacillus* strains: *B. anthracis* (Sterne), *B. anthracis* (ΔAmes), *B. cereus* T, *B. subtilis* 168, and *B. globigii*. (Biopanning against *B. thuringiensis kurstaki* spores is in progress.) Sequencing the appropriate regions of the genomes of phage recovered after the fourth round of biopanning revealed the identity of putative spore-binding peptides, which were inspected for consensus sequences. In every experiment, we obtained at least one large family of similar peptides from which we could derive an apparent consensus sequence for spore binding. These consensus sequences were typically different for each *Bacillus* species. These consensus sequences are included in two recently submitted papers, and therefore they cannot be included in this review. These peptides will be described only in general terms and identified by the first two amino acid residues in the consensus sequence.

Biopanning was performed with the Ph.D.-7 library against spores of two strains of *B. anthracis*, namely Sterne and ΔAmes. The ΔAmes and Sterne strains are avirulent because they lack a plasmid necessary to produce either the anthrax toxins (i.e., pXO1) or the capsule of the vegetative cell (i.e., pXO2), respectively (Mock and Fouet, 2001). The absence of these plasmids has no known effect on spore structure. The ΔAmes spores had been killed by gamma-irradiation (40 Gy) before use. In the experiment with ΔAmes spores, in which 27 phage were examined, we found a single family of similar peptides (the TY-peptide family). This family consisted of three unique peptides, which were displayed by seven phage (i.e., multiple phage displayed the same peptide). The TY-peptides contain a seven-amino-acid consensus sequence, with residues 1, 2, 3, 5, and 7 being invariant. With the Sterne spores, 35 phage were examined and 12 displayed two new TY-peptides. Two other phage displayed similar peptides (the DM-peptide family), with invariant residues again at positions 1, 2, 3, 5, and 7. Using assays described below, we have shown that the TY- and DM-peptides bind to both ΔAmes and Sterne spores (without detectable bias), and that the TY-peptides bind these spores more extensively than the DM peptides. We also showed that the TY-peptides exhibit
clear preferential binding to *B. anthracis* spores. These peptides bind only weakly to spores of *B. cereus* and *B. thuringiensis* (several strains for each species were examined) and not at all to spores produced by *Bacillus* species outside the *B. cereus* group. Furthermore, all the spore-binding peptides fail to bind vegetative cells of the ΔAmes and Sterne strains.

Biopanning was performed with both the Ph.D.-7 and Ph.D.-12 libraries against spores of *B. cereus* T. In both cases, nearly every phage examined displayed a peptide containing a five-amino-acid consensus sequence (the SP-peptide family). Although only residues at positions 1 and 2 appear invariant in this consensus sequence, residues at the other positions are highly conserved. The species-specificity for spore binding of these peptides is presently being determined, but we have shown that they do not bind vegetative cells of *B. cereus*.

Biopanning was also performed with both the Ph.D.-7 and Ph.D.-12 libraries against spores of *B. subtilis* 168. Approximately 50 phage were examined in the two experiments and every phage displayed a peptide containing a five-amino-acid consensus sequence (the NH-peptide family). In this consensus sequence, the first four residues are invariant, and they are always found at positions 1–4 in the displayed peptide. We have determined that this location (at the amino terminus of the peptide) is essential for spore binding. Using only the Ph.D.-7 library, biopanning was performed against spores of *B. globigii*. As with the *B. subtilis* spores, every phage recovered from the *B. globigii* spores displayed an NH-peptide. Further characterization revealed that the NH-peptides apparently bind exclusively to spores of *B. subtilis*, *B. globigii*, and three other species present on a single branch of the *Bacillus* phylogenetic tree (Fig. 1). The NH-peptides do not bind to vegetative cells of any of these species.

### 4. Confirmation of peptide binding to spores

In addition to spore binding, Ph.D. phage can be enriched during biopanning by preferential amplification. We have found that the most reliable method for excluding this possibility (and confirming spore binding) is to measure phage enrichment without amplification. The procedure is to first prepare (and confirm) a phage mixture containing 99.9% phage from the Ph.D. library and 0.1% phage displaying the putative spore-binding peptide. (For weakly binding phage, a 99:1 mixture is more appropriate.) A sample of this mixture containing $10^{10}$ total phage is then mixed with $10^9$ target spores and a single round of biopanning is performed. The eluted phage (without amplification) are plaque purified and 10 plaques are used to determine the sequences of the peptide-encoding regions of the genomic DNAs. If the examined peptide indeed binds the target spores, then many (and perhaps all) of the eluted phage will display this peptide. When a phage displaying an NH-peptide was examined for binding to *B. subtilis* spores by this procedure, 7/10 eluted phage displayed the NH-peptide- a 700-fold enrichment. It is advisable to perform this assay before proceeding with further characterization and development of a putative spore-binding peptide.

### 5. Assays for peptide binding to spores

We routinely assay peptide binding to spores by using fluorescence-activated cell sorting (FACS) and fluorescence microscopy. Both methods require the attachment of a fluorochrome to the peptide of interest, usually prior to spore binding and analysis of the spore–peptide complex. A generally useful procedure is to chemically synthesize the peptide, including a GGGC carboxy-terminal extension, and attach it to the large (240 kDa), highly fluorescent protein R-phycoerythrin (Prozyme). Approximately 10 peptide molecules are attached through their carboxy-terminal cysteine residues to the ε-amino groups of dispersed lysine residues on one molecule of phycoerythrin, using the heterobifunctional cross-linker sulfo succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Pierce) (Hermanson, 1995). For the assay, spores are mixed with a peptide-phycoerythrin conjugate in TBST at room temperature for 10–60 min (optimum binding times need to be determined empirically), unbound conjugate molecules are removed by one to three washings with TBST, and the spores are analyzed by FACS (BD FACSCalibur) or fluorescence microscopy. An example of a FACS assay
showing the binding of an NH-peptide-phycoerythrin conjugate to spores of *B. subtilis* is shown in Fig. 4.

Another convenient assay procedure that works well with spores lacking an exosporium is to fluorescently label the M13 phage displaying the peptide of interest and use the phage as the ligand in a spore-binding assay essentially as described above. We use two methods to label the phage. In the first method, phage are labeled directly with Alexa Fluor 488 by using the Alexa Fluor 488 Protein Labeling Kit from Molecular Probes. The Alexa dye contains a succinimidyl ester that reacts with a subset of the exposed primary amines on the surface of the phage capsid (primarily ε-amino groups of lysine residues in the major capsid protein pVIII). We have varied the density of Alexa labeling from 50 to 500 dye molecules per phage, without inhibiting spore binding. A fluorescent micrograph showing the binding of an Alexa-labeled phage displaying an NH-peptide to spores of *B. subtilis* is shown in Fig. 5. The second method is to label phage indirectly with an Alexa-labeled monoclonal antibody (mAb) that binds to capsid protein pVIII. In this case, the mAb is mixed with preformed spore–phage complexes. The anti-pVIII mAb (Amersham) is Alexa-labeled (four to nine Alexa molecules per mAb) as described above. The use of phage as a spore-binding ligand generally is not recommended for spores with an exosporium because the hair-like nap (Fig. 2A) apparently prevents efficient binding of the filamentous (approximately 1 μm long) phage.
6. Technical problems, limitations, deficiencies, and other points of interest

Several technical problems were encountered in our studies. Our first biopanning experiments were done with *B. subtilis* spores, which were washed with PBS or TBS to remove unbound phage. However, after 10 washings, we had lost essentially all of the spores because of inefficient pelleting during centrifugation. This problem was solved by including Tween-20 in the wash buffers, which allowed nearly quantitative spore recovery. The *B. cereus* group spores pellet well even in the absence of Tween-20. A much bigger problem was encountered when we first identified the TY-peptides after biopanning *B. anthracis* (D Ames) spores. We immediately Alexa-labeled phage displaying a TY-peptide and attempted to show spore binding. No evidence of binding was seen by FACS or fluorescence microscopy. The problem was that exosporium-containing spores bind the phage very inefficiently, with less than one phage bound per many thousands of spores. While this inefficient binding is sufficient to retrieve spore-binding phage by biopanning, which is an extremely powerful enrichment technique, it is grossly inadequate for general labeling of spores. Demonstration of TY-peptide binding to spores required the use of other assays described in Sections 4 and 5. Finally, in biopanning experiments with *B. anthracis* spores, we found that a large percentage of the eluted phage displayed the same peptide, encoded by an identical phage DNA sequence. When examined directly, neither these phage nor the displayed peptide actually bound to the target spores. Apparently, the enrichment of these phage occurred by a mechanism other than preferential spore binding.

Other experimental limitations also are worthy of mention. For example, some peptides (e.g., NH-peptides) require a free amino terminus for spore binding. Thus, attachment of a fluorochrome or other adduct to this end of the peptide will prevent spore binding. Another possible limitation is that the elution buffer used to recover spore-bound phage in our biopanning experiments is not sufficient to dislodge the tightest binding phage/peptides. Consequently, we developed another method for recovering spore-bound phage. Instead of eluting phage from spore–phage complexes to an early log-phase culture of phage-sensitive, tetracycline-resistant *E. coli* cells (e.g., strain ER2738), which were growing in LB medium containing 12.5 μg/ml tetracycline. In this medium, spores germinate and release phage that will infect the *E. coli* cells, while outgrowth of *Bacillus* cells is prevented by tetracycline. We used this alternative phage recovery method during biopanning with spores of the Sterne strain of *B. anthracis*. Finally, our approach of reiteratively biopanning a phage display peptide library usually yielded a single consensus sequence for the target spore species, presumably the best binding peptide under the conditions employed. Possibly, a different peptide could be recovered by employing different biopanning conditions (e.g., buffer, number of washes, etc.), or by using a library displaying peptides in a different context (e.g., on the M13 capsid protein pVIII (Smith and Petrenko, 1997)).

One current deficiency in our research on the peptide ligands is the lack of quantitative spore-binding information. To obtain this information, small monovalent peptide ligands are presently being developed. One obstacle to this development has been a high level of nonspecific binding with small ligands. Preliminary studies indicate that this problem can be overcome and that peptide binding is not dependent on a polyvalent ligand. Another deficiency in our knowledge is the identification of the receptors to which the peptides bind. These receptors may be physiological or fortuitous. In at least one case, that of *B. subtilis*, growing evidence indicates that the NH-peptides bind to a physiological receptor. The NH-peptide consensus sequence is present in proteins (some enzymes) that are targeted to the spore surface during spore formation and maturation. A likely scenario is that the NH-peptide sequence in these proteins is the targeting signal that binds to a spore-surface receptor, thus concentrating the proteins at a site where their activities are required.

Finally, an obvious concern with respect to using peptide ligands to detect spores is possible interference from extraneous material in environmental samples. Important considerations in evaluating this potential problem are the type of detector platform in which the peptides are incorporated and the nature of the sample analyzed. Research in these areas has only begun, but it seems likely that even currently
available technologies for sampling air, water, and soil are sufficient to provide spore samples that will not interfere with detection devices that are presently being designed. With respect to detector design, it is noteworthy that the NH-peptides have been covalently attached to the surface of a glass slide and used to efficiently capture spores of *B. subtilis* from an aqueous suspension. As yet, the other peptides have not been tested for the ability to capture their target spores in the same manner. The ability to capture spores is a critical ligand property in several detector platforms presently under development or in use with anti-spore antibodies (Luppa et al., 2001).

### 7. Concluding remarks

By screening phage display libraries, we have successfully identified at least one family of peptide ligands that binds spores of every *Bacillus* species examined. The peptides appear to bind spores in a highly selective manner, binding only to the target spore species and spores of phylogenetically similar species. In the most interesting case, we have identified at least one family of peptides that binds much better to spores of *B. anthracis* than to spores of the closely related species *B. cereus* and *B. thuringiensis*. Work is in progress to improve both the binding strength and specificity of these peptides. Potentially, these peptides or improved versions can be used in a detector to specifically identify spores of *B. anthracis*. Our studies also suggest that phage display libraries could be successfully screened for peptides that bind to any cell of interest, including other species of spores, pathogenic microorganisms, or isolated eukaryotic cells. These peptides could be used for detection and identification, as described above, or an assortment of other therapeutic and commercial uses.

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