Biochemical and Biophysical Research Communications 395 (2010) 93–98

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Peptides panned from a phage-displayed random peptide library are useful for the detection of *Bacillus anthracis* surrogates *B. cereus* 4342 and *B. anthracis* Sterne ☆

Shilpakala Sainath Rao [a], Ketha V.K. Mohan [a,*], Nga Nguyen [b], Bindu Abraham [c], Galina Abdouleva [b], Pei Zhang [d], Chintamani D. Atreya [a]

[a] Section of Cell Biology, Laboratory of Cellular Hematology, Center for Biologics Evaluation and Research, FDA, Bethesda, MD 20892, USA
[b] Facility for Biotechnology Resources, Center for Biologics Evaluation and Research, FDA, Bethesda, MD 20892, USA
[c] Laboratory of Biochemistry and Vascular Biology, Center for Biologics Evaluation and Research, FDA, Bethesda, MD 20892, USA
[d] Laboratory of Plasma Derivatives, Center for Biologics Evaluation and Research, FDA, Bethesda, MD 20892, USA

ARTICLE INFO

Article history:
Received 18 March 2010
Available online 27 March 2010

Keywords:
Bacillus anthracis
Counter-terrorism
Peptide ligands
Phage-display
Plasma
Quantum dots

ABSTRACT

Recent use of *Bacillus anthracis* as a bioweapon has highlighted the need for a sensitive monitoring system. Current bacterial detection tests use antibodies as bio-molecular recognition elements which have limitations with regard to time, specificity and sensitivity, creating the need for new and improved cost-effective high-affinity detection probes. In this study, we screened a commercially available bacteriophage-displayed random peptide library using *Bacillus cereus* 4342 cells as bait to identify peptides that could be used for detection of *Bacillus*. The method enabled us to identify two 12-amino acid consensus peptide sequences that specifically bind to *B. cereus* 4342 and *B. anthracis* Sterne, the nonpathogenic surrogates of *B. anthracis* strain. The two *Bacillus*-binding peptides (named BBP-1 and BBP-2) were synthesized with biotin tag to confirm their binding by four independent detection assays. Dot-blot analysis revealed that the peptides bind specifically to *B. cereus* 4342 and *B. anthracis* Sterne. Quantitative analysis of this interaction by ELISA and fluorometry demonstrated a detection sensitivity of 10^2 colony forming U/ml (CFU/ml) by both assays. When the peptides were used in combination with Qdots, the sensitivity was enhanced further by enabling detection of even a single bacterium by fluorescence microscopy. Immunoblot analysis and protein sequencing showed that BBP-1 and BBP-2 bound to the S-layer protein of *B. anthracis* Sterne. Overall, our findings validate the usefulness of synthetic versions of phage-derived peptides in combination with Qdot-liquid nanocrystals as high sensitivity bioprobes for various microbial detection platforms.

Published by Elsevier Inc.

1. Introduction

Bacillus anthracis is a causal agent of anthrax, a serious and often fatal infection of livestock and humans [1]. Because of its highly pathogenic nature and spore-forming capability, it is considered one of the most effective biological weapons of mass destruction [2]. The bacterium can infect humans by cutaneous, gastrointestinal, or respiratory routes. Anthrax bacilli are often distinguished on the basis of time-consuming morphological or phenotypic characteristics, such as Gram-positive staining, spore-forming capability, and nonhemolytic reaction on sheep blood agar, sensitivity to penicillin, nonmotile nature, and inability to ferment salicin [3]. *B. anthracis* is distinguished from other members of the closely related *Bacillus cereus* group of bacteria by the presence of its toxin-encoding pXO1 and capsule-encoding pXO2 plasmids. Both plasmids are required for the bacterial virulence and absence of either plasmid in the bacterial cell results in attenuation [4].

Majority of the bioprobes used in rapid detection methods described in the literature are antibodies. However, this procedure has some disadvantages, such as cross-reaction and tedious procedures. Monoclonal antibodies could minimize these problems, but hybridoma techniques are expensive and time-consuming. An effective alternative to antibodies are affinity-selected short peptides derived from phage-displayed random peptide libraries for specific biological targets [5,6]. Phage-display is being routinely used for isolation of peptide ligands to a wide variety of targets including small molecules [7], complex targets such as prostate cancer cells [8,9], serum antibodies [10], identification of several

☆ *Disclaimer:* The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination policy.

* Corresponding author. Address: Bldg. 29A, Room 2C-15, HFM-335, CBER/FDA, NIH Campus, 8800 Rockville Pike, Bethesda, MD 20892, USA. Fax: +1 301 827 1825.
E-mail address: krishna.ketha@fda.hhs.gov (K.V.K. Mohan).

0006-291X/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.bbrc.2010.03.145

microorganisms such as *B. anthracis* spores [11] *Salmonella typhimurium* [5], and *Heamophilus influenzae* [12].

Detection strategy for *B. anthracis* lethal agent during the development stages requires specialized and expensive facilities such as bio-safety level-3^{+}. This can be avoided by using its nonpathogenic or non-virulent strains such as *B. anthracis* Sterne and *B. cereus* 4342 as surrogates. *B. cereus* 4342 is closely related in morphological, physiological and genetic properties to *B. anthracis* [13–15]. The cell wall structural features of peptidoglycan identified for *B. cereus* 4342 primarily represent the same characteristics of the cell wall peptidoglycan of the highly monomorphic isolates of *B. anthracis* [16,17]. In this study, utilizing the above two surrogate strains in a step-wise fashion, we first employed a whole-cell phage-display approach to isolate peptides that specifically bind to the cell surface of *B. cereus* 4342 and subsequently analyzed the ability of these peptides to bind to *B. anthracis* Sterne as well. The bacterial detection sensitivity of these peptides was further enhanced by combining with high fluorescence nanomaterials such as quantum dots.

2. Materials and methods

2.1. Bacterial strains, bacteriophage, and reagents

Bacterial cultures of *B. cereus* (RSVF1 strain 4342), *B. cereus* 11778, *B. cereus* 10876, and *Bacillus thuringiensis* 10792 were obtained from ATCC (Manassas, VA). Animal vaccine strain of *B. anthracis* (Sterne 34F2) originally obtained from Colorado Serum Co. (Denver, CO) was a generous gift from Dr. Duncan, CBER, FDA. This strain was cultured in brain–heart infusion (BHI) broth (Becton–Dickinson, Sparks, MD). Stock cultures of all bacteria were stored in ATCC media #20-2200 at −70 °C.

2.2. Biopanning of phage-displayed peptides

Selection of peptides from a random peptide phage-display library (Ph.D-12; New England Biolabs, Ipswich, MA) for a given target called biopanning was as described [18]. Briefly, approximately 10^{10} phages were incubated with *B. cereus* 4342 cells for 20 min at room temperature. After eight repeat washings with 10 mM Tris–HCl buffer (pH 7.5) containing 0.02% Tween 20, the phage particles were eluted from the complex with 0.1 M HCl for 8 min at room temperature. The eluted phage was amplified in *Escherichia coli* strain ER2738 by infection method. The amplified stock was used for a second round of biopanning as described above. After six rounds of biopanning the eluted phage was used to prepare phage stocks to isolate phage genomic DNA for nucleotide sequencing. The DNA sequences were translated into amino acids by using 'Gene Runner' software (www.generunner.net).

2.3. Peptide synthesis

Peptide sequences identified from the biopanning experiment were compared to arrive at two consensus sequences termed *Bacillus*-Binding Peptide-1 (BBP-1) and BBP-2 for this study (Table 1). The peptides were synthesized at our Core Facility in CBER, FDA, biotinylated with a C6-linker and purified by high pressure liquid chromatography (HPLC). Peptides were reconstituted in 100 mM $NaPO_4$ and 150 mM NaCl, pH 7.2, buffer at room temperature to a final concentration of 1 M (stock solution). Prior to the binding assays, these peptides were tested to ensure that the peptides have the biotin linkers and are recognized by streptavidin-HRP conjugate.

2.4. Dot-blot assay

Five milliliters early-log-phase cultures of *B. cereus* 4342, *B. cereus* 11778, *B. thuringiensis,* and *B. cereus* 10876 were grown in LB broth and *B. anthracis* (Sterne) was grown in BHI broth for 3 h followed by centrifugation at 3000*g*. The cell pellet was resuspended and 10-fold serially diluted bacterial suspension was spotted directly onto a nitrocellulose membrane essentially as described [19]. Peptides alone were spotted to serve as positive controls for detection. Membranes were blocked with 5% bovine serum albumin (BSA) for 2 h and incubated individually with BBP-1 and BBP-2 peptides (1.5 mM) for 1 h at 4 °C. Subsequently the membranes were washed with TBST buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20), incubated with streptavidin-HRP conjugate (Upstate, Temecula, CA) for 60 min at RT, washed with TBST buffer and developed using a DAB (3,3′-diaminobenzidine) substrate kit (Invitrogen, Carlsbad, CA). Colored spots on the membrane in locations where the bacteria were spotted was inferred as positive for peptide binding.

Table 1
B. cereus 4342-binding peptide sequences encoded by phage clones.

Sequences	Number of clones with identical sequence
AETVESCLAKSH	9
ALTLHPQPLDHP	4
QNMMSPIEGVRI	1
APRYTQTPQALA	1
FMGPQESTLQRL	1
TALATSSTYDPH	1
KSWLPLSQEVRF	1

2.5. Enzyme-linked immunosorbent assay (ELISA)

The assay was carried out as described previously [19]. Ten-fold serial dilutions of log-phase bacterial cultures were made in 1× PBS as described above and the bacterial suspensions were then added to wells of a 96-well microplate and incubated overnight at RT. Subsequently, cells were fixed with ethanol and blocked with 5% BSA for 60 min at RT. Peptides (10 mM) were then added to all wells and incubated for 15 min. Following incubation, wells were washed with PBST buffer (PBS, pH 7.4, 0.01% Tween 20) and incubated with streptavidin-HRP conjugate (Upstate, Temecula, CA) for 15 min. Tetramethylbenzidine (TMB) peroxidase substrate (Zymed Laboratories, Carlsbad, CA) was used to detect peptide binding and the color development was recorded using a microplate reader (Synergy 4™, BioTek Instruments, Winooski, VT) at 450 nm wavelength.

2.6. Qdot-based fluorescence assay

Log-phase cultures of *B. cereus* 4342, *B. anthracis* Sterne, *B. cereus* 11778, *B. cereus* 10876, and *B. thuringiensis* were centrifuged and the pellets were resuspended in 10-fold serial dilutions made in 1 ml of human plasma. 10^3 CFU/ml was selected as the spiking titer based on the rationale that current limit of detection of bacteria in blood and blood components using rapid bacterial detection system is at or above 10^{3-5} CFU/ml (www.veraxbiomedical.com). Peptide binding was performed in a final volume of 100 μl in Eppendorf tubes kept at RT for 90 min. Subsequently, tubes were centrifuged for 5 min at 3000*g* and the pellets were washed with PBS and resuspended in 100 μl of diluted 30 nM streptavidin-conjugated Qdots (QD 605) solution (Invitrogen, Oregon). After incubating for 90 min at RT, the tubes were centrifuged again as above and the pellets were resuspended in 150 μl of PBS. The samples were placed on a glass slide or a microplate and analyzed either under a fluorescence microscope using Nikon Eclipse TE2000-U (Nikon Instruments, Melville, NY) or by fluorometry using a micro plate reader (BioTek Instruments, Winooski, VT), respectively [19].

2.7. Bacterial cell lysate preparation

Five milliliters of early-log-phase bacterial cultures of *B. cereus* 4342, *B. anthracis* Sterne, and *B. cereus* 11778 were subjected to centrifugation at 3000*g*. The cell pellet was resuspended in 500 µl of immunoprecipitation buffer (Thermo Scientific, Rockford, IL) and disrupted by 3 s × 30 s bursts of sonication at 70% pulse, with ice bath cooling, using a model Virtis Sonicator (VirTis Co., Gardiner, NY, USA) and the samples were stored at −20 °C, until use.

2.8. Coomassie staining and peptide-binding assay

To identify which protein of the *Bacillus* bacterium does the BBP-1 and BBP-2 bind lysates were prepared from *B. cereus* 4342, *B. anthracis* Sterne, and *B. cereus* 11778 and ran in duplicate for separation on a 4–20% Tris–glycine gel (Invitrogen, Carlsbad, CA). Following electrophoresis, one set of electrophoresed proteins on the gel was stained with simply blue safe stain (Invitrogen, Gaithersburg, MD) while the other set of electrophoresed proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked at room temperature for 1 h in 5% bovine serum albumin and then incubated with BBP-1 and BBP-2 peptides for 2 h at 37 °C. The membrane was then processed as per the dot-blot assay protocol and peptide binding was detected by DAB. The most prominent peptide-binding band was compared with the Coomassie-stained gel and the corresponding band in the stained gel was excised for identification by protein sequencing.

2.9. Protein identification by LC–MS/MS

The most prominent band showing binding to the BBP-1 and BBP-2 peptides was found to be between 90 and 100 kDa. The protein band was excised from the SDS–PAGE gel and the protein was identified by LC–MS/MS. In-gel protein digestion and extraction of peptides from gel slices were performed as previously described, with minor modifications [20]. The LC–MS/MS data was interpreted using Sequest and MASCOT (Matrix Science) [21].

2.10. Statistical analyses

All assays were performed in triplicates and their mean values were estimated. Mean value ± SD (standard deviation) of triplicates was calculated using Microsoft Excel®. Statistical analyses were performed using Student's *t*-test and values were considered significant when $P < 0.05$.

3. Results

3.1. Biopanning of a phage-display library against B. cereus 4342 resulted in identifying two consensus peptides BBP-1 and BBP-2

To identify peptides that bind to *B. cereus* 4342, we screened (or "biopanned") the NEB Ph.D-12 Phage Display Peptides Library for *B. cereus* 4342-binding phages. After six rounds of biopanning the eluted phages were plated to obtain single plaques from which individual phage clones were amplified and genomic DNA was extracted. The amino acid sequences encoded by the inserts revealed two putative *Bacillus*-binding peptides (BBP) that were the result of consensus sequences represented by nine and four clones for BBP-1 (AETVESCLAKSH) and BBP-2 (ALTLHPQPLDHP), respectively (Table 1).

3.2. Specificity and selectivity of peptides binding to B. anthracis

To determine the ability of the phage-displaying peptides to interact with the target bacterium, we synthesized the two peptides BBP-1 and BBP-2 with a C6-linker for biotin tag. The binding capacity of these peptides to *B. cereus* 4342, *B. anthracis* Sterne, *B. cereus* 11778, *B. thuringiensis*, and *B. cereus* 10876 was analyzed by four independent methods: (1) dot-blot assay, (2) ELISA, (3) fluorometry, and (4) fluorescence microscopy.

3.3. BBP-1 and BBP-2 are highly specific to B. cereus 4342 and B. anthracis Sterne

We tested BBP-1 and BBP-2 peptides for their ability to bind to *B. cereus* 4342, *B. anthracis* Sterne, *B. cereus* 11778, *B. thuringiensis*, and *B. cereus* 10876. Results from the dot-blot assay showed that both these peptides bound strongly to *B. cereus* 4342 and *B. anthracis* Sterne at a titer of 10^3 CFU/ml (Fig. 1). The BBP-1 and BBP-2 peptides were highly specific and did not bind to *B. cereus* 11778, *B. thuringiensis*, and *B. cereus* 10876 (Fig. 1).

3.4. BBP-1 and BBP-2 detect up to 10^2 CFU/ml of Bacillus by ELISA

To confirm the binding and to analyze the detection limit of the BBP-1 and BBP-2 to *B. cereus* 4342 and *B. anthracis* Sterne we analyzed the binding of the peptides by ELISA. As shown in Fig. 2, both BBP-1 and BBP-2 showed significant binding to *B. cereus* 4342 and *B. anthracis* Sterne when the bacteria were at 10^2 CFU/ml ($P < 0.05$). Both these peptides did not show binding to *B. cereus* 11778, *B. thuringiensis*, and *B. cereus* 10876 as observed with the dot-blot method.

3.5. Fluorometry reveals a sensitivity of 10^2 CFU/ml

BBP-1 and BBP-2 peptides were incubated individually with *B. cereus* 4342, *B. anthracis* Sterne, *B. cereus* 11778, *B. thuringiensis*, and *B. cereus* 10876. Binding of each peptide to the bacteria was detected using Qdot-nanocrystal cores conjugated with streptavidin. Fluorometric analysis revealed that in this experimental setting BBP-1 and BBP-2 were able to bind to *B. cereus* 4342 and *B. anthracis* Sterne as indicated by the significantly higher levels of fluorometric counts when the bacteria were at 10^2 CFU/ml ($P < 0.05$) (Fig. 3). Binding of BBP-1 and BBP-2 to *B. cereus* 4342

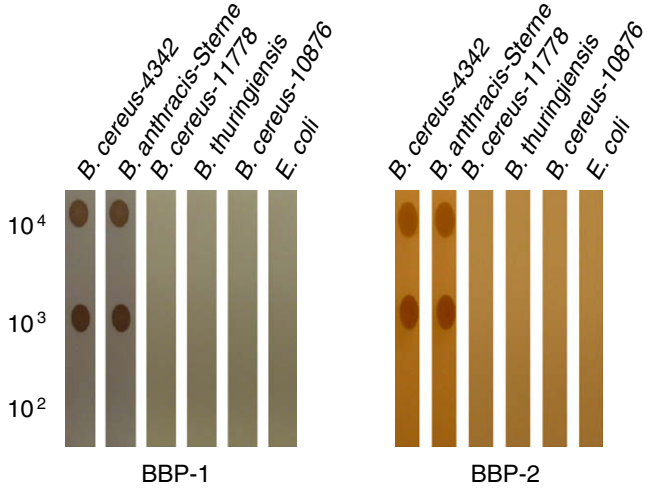

Fig. 1. Demonstration of binding of BBP-1 and BBP-2 peptides to the bacteria by dot-blot analysis. Ten-fold dilutions of bacteria (*B. cereus* 4342, *B. anthracis* Sterne, *B. cereus* 11778, *B. thuringiensis*, and *B. cereus* 10876) were made with PBS and blotted onto a nitrocellulose membrane and incubated individually with BBP-1 and BBP-2. Positive binding was confirmed by the appearance of colored spots when developed with DAB. (For interpretation of references to colour in this figure legend, the reader is referred to see the web version of this article.)

and *B. anthracis* Sterne was highly specific as they did not cross-react with other *Bacillus* strains.

3.6. Fluorescence microscopy enables detection of single bacterium

To demonstrate directly that the BBP-1 and BBP-2 peptides were binding to *B. anthracis* Sterne and *B. cereus* 4342 we examined the binding of the peptides by fluorescence microscopy. Briefly, human plasma samples spiked with *Bacillus* cultures were incubated with peptides BBP-1 and BBP-2 individually and the bacteria–peptide complex was detected using streptavidin-conjugated Qdots under a fluorescence microscope. The analysis clearly identified that the peptides specifically bind to the outer surface of *B. cereus* 4342 and *B. anthracis* Sterne bacterium, providing visual evidence that BBP-1 and BBP-2 in combination with the Qdot labeling method enhance the detection sensitivity up to a single bacterium in the spiked plasma (Fig. 4).

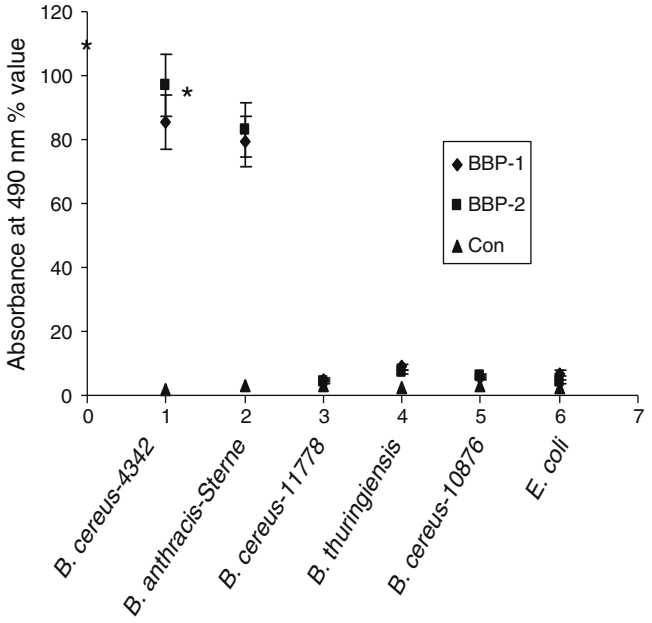

Fig. 2. ELISA-based analysis of the binding of BBP-1 and BBP-2 to the bacteria tested. Results are shown as an average of three individual experiments. Ninety-six-well microplates were coated with bacterial suspensions and incubated with biotinylated BBP-1 and BBP-2 prior to incubation with streptavidin-HRP. TMB substrate was used to detect the positive binding of peptides to bacteria. Error bars indicate standard deviations. Student's *t*-test indicates significant reactivity ($P < 0.05$) as indicated by an (∗).

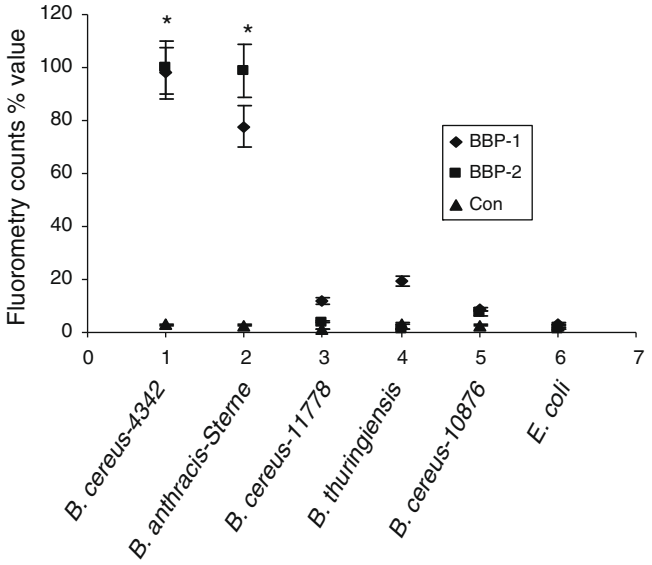

Fig. 3. Fluorometric results of BBP-1 and BBP-2 binding to the bacteria. Human plasma spiked with bacteria (*B. cereus* 4342, *B. anthracis* Sterne, *B. cereus* 11778, *B. thuringiensis*, and *B. cereus* 10876) was incubated individually with BBP-1 and BBP-2. Streptavidin-Qdot conjugate was added and the positive binding was analyzed by a fluorescence plate reader. Results are shown as an average of three independent experiments. Error bars indicate standard deviations. (∗) indicates statistically significant difference ($P < 0.05$).

3.7. BBP-1 and BBP-2 peptides bind to bacterial cell wall S-layer protein EA1

The peptide-binding protein from the Coomassie-stained gel as shown in Fig. 5 was subjected to protein sequencing. Results from Mascot and Sequest searches were submitted to a program called Scaffold to generate a comprehensive listing of all the proteins found in each sample, with information on subcellular localization and cellular function. Scaffold results identified S-layer protein EA1 as the most likely protein in the sample. The results were in concordance with those generated by Sequest and Mascot.

4. Discussion

Our goal was to identify peptides from a phage-displayed random peptide library that selectively bind to *B. anthracis* surrogates strains *B. anthracis* Sterne and *B. cereus* 4342 in spiked plasma as a model for *B. anthracis* detection under bio-safety level 2 (BSL2) conditions. The advantage of using the Sterne vaccine strain is that it differs from the virulent strain only in the absence of one of two plasmids [4,22]. *B. cereus* 4342 may be considered a *B. anthracis* cured of its virulence plasmids [23]. We identified two groups of peptides, panned from the screening whose consensus was represented in two short synthetic peptides BBP-1 and BBP-2 that bind strongly to the cells of *B. anthracis* Sterne and *B. cereus* 4342. These peptides did not bind to the other *Bacillus* species tested (*B. cereus* 11778, *B. thuringiensis*, and *B. cereus* 10876). The results thus demonstrated the binding specificity of these peptides only to the two bacterial species. Our approach of reiterative biopanning of a phage-display library followed by arriving at consensus peptides by comparing clones of phage-derived peptide sequences that bound to the bacteria yielded best binding synthetic peptides under the conditions employed. Our results clearly show that the BBP-1 and BBP-2 peptides consistently demonstrated selective binding to *B. cereus* 4342 and *B. anthracis* Sterne in the membrane based dot-blot method. Though the peptides were able to show reactivity to the bacteria, the membrane assay does not reveal the quantitative nature of this binding. Therefore, quantitative estimates were achieved by ELISA and fluorometry. These assays further confirmed that the peptides showed significant binding ($P < 0.05$) only to *B. cereus* 4342 and *B. anthracis* Sterne. When combined with Qdots, the detection sensitivity of BBP-1 and BBP-2 was enhanced to a single bacterium in the spiked plasma by fluorescence microscopy. Based on the published literature so far with extensive comparisons to other reported peptide sequences we found that both BBP-1 and BBP-2 are novel peptide sequences with potential application for use in the detection of *B. anthracis* [19,24,11,25–29]. Our analysis also confirms the *Bacillus*-binding potency of BBP-1 and BBP-2 by four independent assays. Identification of these novel peptide sequences is only the first step towards designing a superior detection system for *Bacillus* in terms of specificity, sensitivity, time taken and cost involved [19,24,25,27–29]. Future work on these two promising peptides would involve further characterization of the binding interaction and developing of

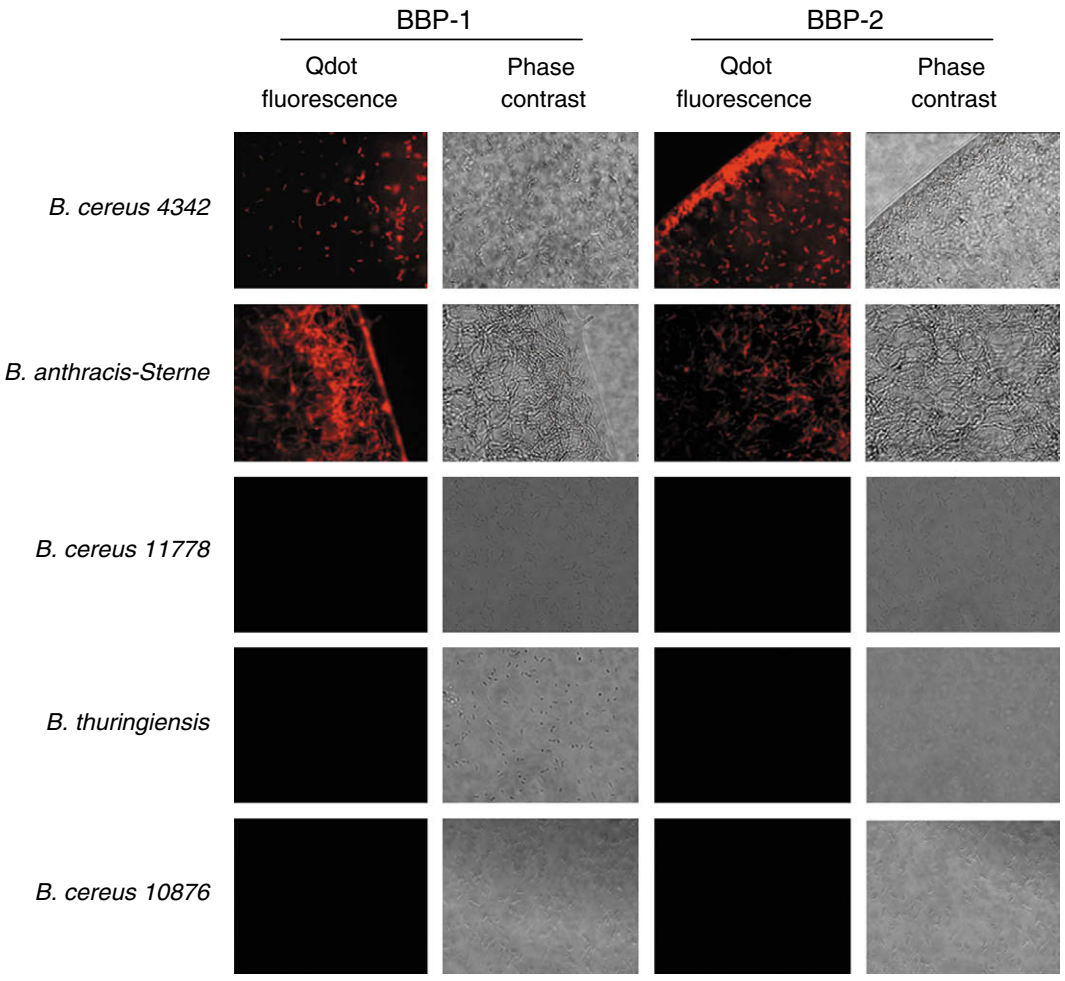

Fig. 4. BBP-1 and BBP-2 binding to the bacteria by fluorescence microscopy. Human plasma spiked with bacteria (*B. cereus* 4342, *B. anthracis* Sterne, *B. cereus* 11778, *B. thuringiensis*, and *B. cereus* 10876) was incubated with peptides and the complex was detected by incubating with streptavidin-conjugated quantum dots. Bacterial cells were visualized by fluorescence microcopy and phase contrast microscopy.

a diagnostic assay that could meet all the requirements of a rapid, sensitive and economical test system.

In addition to confirming these *Bacillus*-binding properties of the peptides we further identified the S-layer protein EA1 on the cell wall of *B. anthracis* Sterne as the binding target for BBP-1 and BBP-2 peptides, which in the near future should aid in designing refined bioprobes for detection of *Bacillus* bacteria. It is interesting to note though that the EA1 protein has been reported to be a contaminant in *B. anthracis* spore preparations and not a component of the spores themselves suggesting that these peptides would be very useful for the detection of vegetative forms [30,31].

5. Conclusion

In conclusion, we report here two novel peptides that bind specifically to *B. cereus* 4342 and *B. anthracis* Sterne, the surrogates of *B. anthracis*. Overall, the results reported here validate the usefulness of application of synthetic versions of affinity-selected recombinant filamentous phage-derived peptides in combination with Qdot-liquid nanocrystals as high sensitivity detection probes for any desired bacteria and suitable for various detection platforms.

Acknowledgments

This work was partially supported by Biomedical Advanced Research Development Authority (BARDA) funds to C.A. S.S. is a recipient of a postdoctoral fellowship at the Center for Biologics Evaluation and Research (CBER) administered by the Oak Ridge Institute for Science and Education (ORISE) through an intra agency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration. Authors thank Drs. Xuan Chi and Mikhail Ovanesov, OBRR, CBER for their review of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.03.145.

References

[1] W. Beyer, P.C. Turnbull, Anthrax in animals, Mol. Aspects Med. 30 (2009) 481–489.
[2] D.G. Bouzianas, Medical countermeasures to protect humans from anthrax bioterrorism, Trends Microbiol. 17 (2009) 522–528.
[3] J.A. Higgins, M. Cooper, L. Schroeder-Tucker, S. Black, D. Miller, J.S. Karns, E. Manthey, R. Breeze, M.L. Perdue, A field investigation of *Bacillus anthracis* contamination of U.S. Department of Agriculture and other Washington, D.C., buildings during the anthrax attack of October 2001, Appl. Environ. Microbiol. 69 (2003) 593–599.
[4] K.A. Edwards, H.A. Clancy, A.J. Baeumner, *Bacillus anthracis*: toxicology, epidemiology and current rapid-detection methods, Anal. Bioanal. Chem. 384 (2006) 73–84.
[5] I.B. Sorokulova, E.V. Olsen, I.H. Chen, B. Fiebor, J.M. Barbaree, V.J. Vodyanoy, B.A. Chin, V.A. Petrenko, Landscape phage probes for *Salmonella typhimurium*, J. Microbiol. Methods 63 (2005) 55–72.

[6] V.A. Petrenko, G.P. Smith, Phages from landscape libraries as substitute antibodies, Protein Eng. 13 (2000) 589–592.
[7] I. Saggio, R. Laufer, Biotin binders selected from a random peptide library expressed on phage, Biochem. J. 293 (Pt. 3) (1993) 613–616.
[8] V.I. Romanov, D.B. Durand, V.A. Petrenko, Phage display selection of peptides that affect prostate carcinoma cells attachment and invasion, Prostate 47 (2001) 239–251.
[9] V.I. Romanov, T. Whyard, H.L. Adler, W.C. Waltzer, S. Zucker, Prostate cancer cell adhesion to bone marrow endothelium: the role of prostate-specific antigen, Cancer Res. 64 (2004) 2083–2089.
[10] G.A. Kouzmitcheva, V.A. Petrenko, G.P. Smith, Identifying diagnostic peptides for lyme disease through epitope discovery, Clin. Diagn. Lab. Immunol. 8 (2001) 150–160.
[11] J. Brigati, D.D. Williams, I.B. Sorokulova, V. Nanduri, I.H. Chen, C.L. Turnbough Jr., V.A. Petrenko, Diagnostic probes for *Bacillus anthracis* spores selected from a landscape phage library, Clin. Chem. 50 (2004) 1899–1906.
[12] S.L. Bishop-Hurley, F.J. Schmidt, A.L. Erwin, A.L. Smith, Peptides selected for binding to a virulent strain of *Haemophilus influenzae* by phage display are bactericidal, Antimicrob. Agents Chemother. 49 (2005) 2972–2978.
[13] E. Helgason, D.A. Caugant, I. Olsen, A.B. Kolsto, Genetic structure of population of *Bacillus cereus* and *B. thuringiensis* isolates associated with periodontitis and other human infections, J. Clin. Microbiol. 38 (2000) 1615–1622.
[14] E. Helgason, O.A. Okstad, D.A. Caugant, H.A. Johansen, A. Fouet, M. Mock, I. Hegna, A.B. Kolsto, *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* – one species on the basis of genetic evidence, Appl. Environ. Microbiol. 66 (2000) 2627–2630.
[15] P.C. Turnbull, Definitive identification of *Bacillus anthracis* – a review, J. Appl. Microbiol. 87 (1999) 237–240.
[16] P. Keim, L.B. Price, A.M. Klevytska, K.L. Smith, J.M. Schupp, R. Okinaka, P.J. Jackson, M.E. Hugh-Jones, Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*, J. Bacteriol. 182 (2000) 2928–2936.
[17] T.D. Read, S.L. Salzberg, M. Pop, M. Shumway, L. Umayam, L. Jiang, E. Holtzapple, J.D. Busch, K.L. Smith, J.M. Schupp, D. Solomon, P. Keim, C.M. Fraser, Comparative genome sequencing for discovery of novel polymorphisms in *Bacillus anthracis*, Science 296 (2002) 2028–2033.
[18] P. Zhang, M.Y. Yu, R. Venable, H.J. Alter, J.W. Shih, Neutralization epitope responsible for the hepatitis B virus subtype-specific protection in chimpanzees, Proc. Natl. Acad. Sci. USA 103 (2006) 9214–9219.
[19] S. Sainathrao, K.V. Mohan, C. Atreya, Gamma-phage lysin PlyG sequence-based synthetic peptides coupled with Qdot-nanocrystals are useful for developing detection methods for *Bacillus anthracis* by using its surrogates, *B. anthracis*-Sterne and *B. cereus*-4342, BMC Biotechnol. 9 (2009) 67.
[20] A. Vassilev, K.J. Kaneko, H. Shu, Y. Zhao, M.L. DePamphilis, TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm, Genes Dev. 15 (2001) 1229–1241.
[21] J.R. Yates III, J.K. Eng, A.L. McCormack, D. Schieltz, Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database, Anal. Chem. 67 (1995) 1426–1436.
[22] A. Fasanella, S. Losito, T. Trotta, R. Adone, S. Massa, F. Ciuchini, D. Chiocco, Detection of anthrax vaccine virulence factors by polymerase chain reaction, Vaccine 19 (2001) 4214–4218.
[23] R. Schuch, D. Nelson, V.A. Fischetti, A bacteriolytic agent that detects and kills *Bacillus anthracis*, Nature 418 (2002) 884–889.
[24] G. Acharya, D.D. Doorneweerd, C.L. Chang, W.A. Henne, P.S. Low, C.A. Savran, Label-free optical detection of anthrax-causing spores, J. Am. Chem. Soc. 129 (2007) 732–733.
[25] B. Dhayal, W.A. Henne, D.D. Doorneweerd, R.G. Reifenberger, P.S. Low, Detection of *Bacillus subtilis* spores using peptide-functionalized cantilever arrays, J. Am. Chem. Soc. 128 (2006) 3716–3721.
[26] Y. Guo, X. Liang, Y. Zhou, Z. Zhang, H. Wei, D. Men, M. Luo, X.E. Zhang, Construction of bifunctional phage display for biological analysis and immunoassay, Anal. Biochem. 396 (2010) 155–157.
[27] S. Pai, A.D. Ellington, M. Levy, Proximity ligation assays with peptide conjugate 'burrs' for the sensitive detection of spores, Nucleic Acids Res. 33 (2005) e162.
[28] C.L. Turnbough Jr., Discovery of phage display peptide ligands for species-specific detection of *Bacillus* spores, J. Microbiol. Methods 53 (2003) 263–271.
[29] D.D. Williams, O. Benedek, C.L. Turnbough Jr., Species-specific peptide ligands for the detection of *Bacillus anthracis* spores, Appl. Environ. Microbiol. 69 (2003) 6288–6293.
[30] D.D. Williams, C.L. Turnbough Jr., Surface layer protein EA1 is not a component of *Bacillus anthracis* spores but is a persistent contaminant in spore preparations, J. Bacteriol. 186 (2004) 566–569.
[31] D.B. Wang, R. Yang, Z.P. Zhang, L.J. Bi, X.Y. You, H.P. Wei, Y.F. Zhou, Z. Yu, X.E. Zhang, Detection of *B. anthracis* spores and vegetative cells with the same monoclonal antibodies, PLoS One 4 (2009) e7810.