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Discovery of peptides that bind to Bacillus anthracis spores.

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DISCOVERY OF PEPTIDES THAT BIND TO *BACILLUS ANTHRACIS* SPORES

by

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ABSTRACT OF DISSERTATION
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Events in the fall of 2001 elevated anthrax research to a matter of national importance and highlighted detection of *Bacillus anthracis* spores as a research focus that would benefit from both technical and intellectual advances. Current handheld *B. anthracis* spore detectors have been criticized for both their low sensitivity and low specificity. These devices use anti-spore antibodies to directly detect *B. anthracis* spores. Our research showed that one such antibody, used in a commercial spore detector, fails to recognize a *B. anthracis* spore surface component. Instead, it recognizes a common contaminant of spore preparations: the *B. anthracis* vegetative cell protein EA1. Because contaminating EA1 can be removed from spore preparations, however, it is unwise to use an anti-EA1 antibody for spore detection purposes.

We discovered a new class of molecules that can be used to detect *B. anthracis* spores: short peptides that bind to the *B. anthracis* spore surface. The TYP peptides are a family of similar peptide sequences identified after biopanning a phage display library against *B. anthracis* spores. After studying the binding of a TYP peptide to a panel of spores, we found that this peptide binds strongly to spores of both *B. anthracis* strains tested and cross-reacted with spores of only 2 out of 17 non-*anthracis* strains tested. The ATYP peptides, created by addition of an alanine to the N-terminus of TYP peptides, bind even more selectively to *B. anthracis* spores. The SLLPGL peptides, also identified

using phage display, bind only to spores of the two strains that cross-reacted with the TYP peptides. Using the ATYP and SLLPGL peptides in tandem, we devised a new assay that specifically detected *B. anthracis* spores with a greatly reduced chance of false positive results.

We used a label transfer reagent to identify two molecules on the *B. anthracis* spore surface, CotZ-1 and the product of the BA4266 locus, that likely mediate the binding of the ATYP peptides. Both of these proteins are probably components of the exosporium basal layer of *B. anthracis* spores.

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LIST OF ABBREVIATIONS

1D	one dimensional
2D	two dimensional
$ATYP_3$	Met-Ala-Thr-Tyr-Pro-Leu-Pro-Ile-Arg-Ala-Thr-Tyr-Pro-Leu-Pro-Ile-Arg-Ala-Thr-Tyr-Pro-Leu-Pro-Ile-Arg-Ala
ATYPLPIR	Ala-Thr-Tyr-Pro-Leu-Pro-Ile-Arg
ATYPLPIRGGGC-PE	Ala-Thr-Tyr-Pro-Leu-Pro-Ile-Arg-Gly-Gly-Gly-Cys-phycoerythrin
DTT	dithiothreitol
EGFP	enhanced green fluorescent protein
FACS	fluorescence-activated cell sorting
h	hour(s)
His_6	His-His-His-His-His-His
HRP	horseradish peroxidase
IEF	isoelectric focusing
IgG	immunoglobulin G
IPG	immobilized pH gradient
LCMSMS	liquid chromatography mass spectrometry mass spectrometry
mAb	monoclonal antibody
MALDI-ToF MS	matrix-assisted laser deionization time of flight mass spectrometry

min	minute(s)
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
NHS	N-hydroxysuccinimide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PE	R-phycoerythrin
pI	isoelectric point
S-layer	surface-layer
SLLPGL	Ser-Leu-Leu-Pro-Gly-Leu
SLLPGLPGGGC-PE	Ser-Leu-Leu-Pro-Gly-Leu-Gly-Gly-Gly-Cys-phycoerythrin
SDS	sodium dodecylsulfate
sSBED	sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido)hexanoamido]ethyl-1,3'-dithiopropionate
TBS	tris-buffered saline
TYP	Thr-Tyr-Pro
TYPLPIR	Thr-Tyr-Pro-Leu-Pro-Ile-Arg
TYPLPIRGGGC	Thr-Tyr-Pro-Leu-Pro-Ile-Arg-Gly-Gly-Gly-Cys
TYPXPXR	Thr-Tyr-Pro-Xxx-Pro-Xxx-Arg
UAB	The University of Alabama at Birmingham
vol/vol	volume/volume

INTRODUCTION

Anthrax bioterrorism. During the past decades, terrorist groups and unfriendly governments have developed anthrax, historically a sporadic disease primarily affecting herbivores, into a feared biological weapon. The deadly consequences of weaponized anthrax were made clear to the American public in late 2001, after intentional dissemination of spores through the U.S. mail system killed five citizens (14). These events have elevated research on *Bacillus anthracis*, the bacterium that causes anthrax, from a niche of veterinary microbiology to a focus of national security. In addition to renewing interest in the basic biology of *B. anthracis*, including its life cycle, molecular pathogenesis, and host interactions, the events of 2001 spurred applied research into topics related to preventing and responding to anthrax bioterrorism, including novel spore detection methods, decontamination strategies, and anthrax disease treatments. Currently, research and development of spore detection strategies lag behind national needs. The introduction of faster, easier, and more sensitive methods for *B. anthracis* spore detection would reduce the threat posed by this bacterium as a biological weapon.

Sporulation and spore structure. Bacteria in the genus *Bacillus* are Gram-positive, aerobic rods that form spores. The spore is a dormant, resistant form of the bacterium, which allows it to persist for a long time in adverse environments. The physical characteristics of spores are what make *B. anthracis* so well-suited as a

biological weapon: a spore can resist dessication, heat, mild alkalis and acids, organic solvents, degrading enzymes, radiation, and detergents (24). Nevertheless, the spore is not inert; it constantly senses its environment and will germinate when favorable conditions return (25).

The response of a growing bacillus to the limitation of an essential nutrient in its environment is to sporulate. Sporulation is a tightly regulated, complex developmental process which results in the creation of a single dormant spore from each vegetative cell. It requires extensive protein synthesis and is regulated by cascades of transcription factors and RNA polymerase sigma factors (26, 30). Sporulation has not been systematically studied in *B. anthracis* but is believed to be very similar to the process in *Bacillus subtilis* (described below). It is divided into eight morphological stages, each taking approximately 1 h at 37°C (22). Stage 0, at the end of exponential growth, is defined as the initiation of sporulation. Once a cell commits itself to sporulate, the first signs of its developmental switch are the condensation of its newly replicated DNA into an axial filament (Stage I) and the formation of a new septum toward one pole of the cell (Stage II). This asymmetric cell division creates a larger mother cell and smaller daughter cell, or prespore. The mother cell's septum continues to grow around the prespore and eventually engulfs it (Stage III). The daughter cell, now wholly enclosed by a double membrane within the mother cell, is then called a forespore. Next, in Stage IV, peptidoglycan is synthesized by both the mother cell and forespore. It is deposited between the two membranes of the forespore, creating the cortex of the future spore. Proteins are then synthesized and deposited onto the forespore surface (Stage V) to form the future spore coat. Specific proteolysis and cross-linking of these layers during the

spore's maturation (Stage VI) enhances its resistant character. At the end of sporulation (Stage VII), the mother cell lyses, and the mature spore is released (22).

The *B. anthracis* spore is a multilayered structure about 1 μm in diameter. The spore's dehydrated core (CR in Fig. 1) contains the 5.2 megabase-pair chromosome and a collection of inactive macromolecules (DNA and RNA polymerases, ribosomes, tRNA synthetases). Small acid-soluble proteins in the core, which account for up to 20% of total spore proteins, bind to the spore's DNA and protect it from environmental insults (7). Surrounding the core is an electron-bright peptidoglycan wall composed of two layers, the germ cell wall and cortex (CX in Fig. 1). The highly cross-linked cortex is required for maintenance of the core's dehydrated state and is the primary structure conferring heat resistance to the spore (20). The proteinaceous spore coat (SC in Fig. 1) surrounds the cortex and protects it from lytic enzymes such as lysozyme (6). For most *Bacillus* species the coat is the outermost layer of the spore, but for members of the Cereus group, including *Bacillus cereus, Bacillus thuringiensis*, *Bacillus mycoides*, and *B. anthracis*, there is an additional layer, the exosporium, which faces the environment (EX in Fig. 1). The exosporium is composed of a paracrystalline basal membrane with regularly spaced pores, plus a hair-like outer nap (9). The functions of the *B. anthracis* exosporium are not yet defined, but it is the outermost layer of the spore, and thus a role in immune recognition and/or escape is suspected. The glycoprotein nap and small pores of the exosporium may act as a molecular sieve to help shield the spore from antibody recognition.

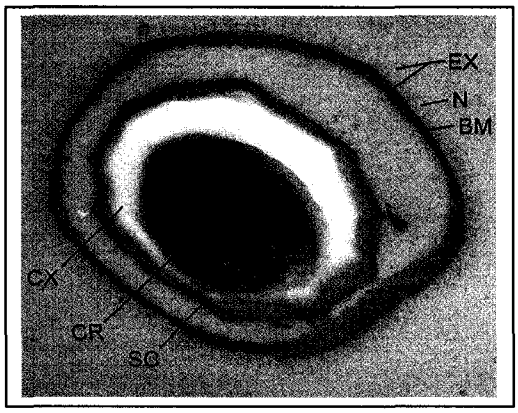

FIG. 1. An electron micrograph of a *B. anthracis* spore. CR = core, CX = cortex, SC = spore coat, EX = exosporium, N = nap, BM = basal membrane.

Understanding the composition and function of the *B. anthracis* exosporium is the primary focus of research in the Turnbough lab. Recent work by both this lab and Dr. Mock's lab at the Pasteur Institute has demonstrated that the nap of *B. anthracis* exosporium is composed of filaments of the BclA glycoprotein (29, 32). The glycoprotein's length varies between strains (29, 33) due to variation in the length of its central domain, which is composed of Gly-Xxx-Yyy repeat units, where Xxx and Yyy are typically Pro and Thr, respectively. The repeating glycines allow BclA to form helical homotrimers, with a structure similar to that of collagen. Recent unpublished work in this lab has shown that the carboxy terminus of BclA is located distally, is highly immunogenic, and probably has a globular structure. The amino terminus of the protein likely interacts with the basal layer of the exosporium to anchor the protein. Much work in this lab has also been devoted to elucidating the structure of the carbohydrate component of the BclA glycoprotein. The BclA polypeptide is conjugated to multiple

copies of a novel glycan that contains rhamnose and modified rhamnose moieties. Deletion analysis has shown that the carbohydrate component of BclA is not required for formation of a morphologically normal nap; however, the role of the glycan as a virulence factor during infection of a mammalian host has not yet been ascertained.

Anthrax pathology. Anthrax is a noncontagious, lethal mammalian disease caused by infection with *B. anthracis* spores (5, 21). Spores enter the body through three routes, that is cutaneous, gastrointestinal, or pulmonary, and the ensuing disease manifests itself differently depending on the site of infection. Cutaneous anthrax, caused by entry of spores into abrasions in the skin, is the most common naturally occurring form of the disease in humans. It is characterized by localized edema and a painless black eschar at the site of infection. The black eschar is responsible for the name of the disease, from the Greek word for coal, *anthrakis*. Natural forms of the bacterium are sensitive to common antibiotics (31). Even without antibiotic treatment, 80-90% of cutaneous anthrax lesions remain localized to the infection site, are self-limited in extent and duration, and heal without scarring in 1 to 2 weeks (5, 14). However, about 10% of untreated cutaneous anthrax cases progress from a localized lesion to a lethal systemic disease (5). Inhalational and gastrointestinal anthrax are not localized like cutaneous anthrax but are instead systemic diseases that are invariably fatal unless antibiotic treatment is begun rapidly. Inhalational anthrax, although not common in natural anthrax outbreaks, is the form of the disease likely to follow intentional dissemination of spores by a biological weapon or bioterrorism. This form of the disease, also known as pulmonary anthrax or "woolsorter's disease," has been described as a 2-stage illness. It

presents initially with nonspecific flu-like symptoms. After a few days (sometimes as soon as a few hours), during which time the patient may appear to improve, the disease rapidly progresses to high fever, bacteremia, shock, and death (13, 31).

Regardless of the route of infection, the cellular pathophysiology of systemic *B. anthracis* infections is similar. Spores are endocytosed by macrophages at the site of infection, germinate in the phagocytic vesicles of the macrophage, and escape to its cytoplasm (4, 11). The infected macrophage is carried in the lymph to regional nodes, where the growing bacteria escape from the macrophage 4 to 6 h after infection (4). The bacilli infect other immune cells in the lymph node and eventually proliferate to high titer in the bloodstream. The virulence factors of *B. anthracis*, including a poly-D-glutamic acid capsule and a tripartite toxin, are carried on two large plasmids. The tripartite toxin consists of two toxin components, edema factor and lethal factor, which associate with heptamers of the third molecule, protective antigen, in order to enter target cells (23). Edema factor, an adenylate cyclase, dramatically raises target cells' intracellular cyclic adenosine 5'-monophosphate levels (19), which causes tissue edema and impairs the effector functions of macrophages and neutrophils (18). Lethal factor is a zinc-dependent metalloprotease that cleaves six mitogen-activated protein kinase kinases to interrupt target cells' signal transduction cascade (8, 35). This severely impairs the antigen-presenting ability of dendritic cells (1). Thus both toxins disarm the adaptive immune response of the host.

***B. anthracis* spore detection.** Effective spore detection methods are essential to countering the anthrax bioterrorism threat, because prompt and accurate detection of *B.*

anthracis spores in the environment allows more focused and effective decontamination efforts and medical interventions. Rapid detection of *B. anthracis* spores is critical due to the natural course of anthrax in humans, especially the more virulent inhalational form likely to follow a bioterrorist attack. After infection by spores, exposed individuals have a short treatment window during which initiation of antibiotic therapy will successfully clear the nascent infection and avert disease. If antibiotic treatment is not begun within days, the disease reaches its fulminant second stage as the bacteria overcome the immune system and replicate in the blood. During the infection's second stage, toxin production crosses a critical threshold, after which the patient will perish even if the blood is sterilized by treatment with antibiotics (13). This highlights the importance of prompt spore detection, disease identification, and initiation of antimicrobial therapy.

Current anthrax detection strategies are generally of three types: culture of the microorganism and demonstration of Gram-positive rods, amplification of *B. anthracis*-specific DNA sequences by polymerase chain reaction (PCR), or direct detection of the spore itself using a specific ligand. The first two strategies, while sensitive and specific (2, 27), require trained lab personnel, laboratory equipment such as incubators and temperature cyclers, and time (~12 h) to culture the environmental samples prior to PCR (12). The last strategy, direct detection of spores, has many potential advantages over cell culture-based or PCR-based strategies. It is the most rapid of the three strategies, with assay time (measured in minutes) limited by the speed of ligand binding and washing. Ligand-binding assays are more easily automated into a handheld device than are PCR-based assays. Handheld spore detection devices based on ligand binding also require less training to be used correctly at the site of a suspected spore release.

Unfortunately, the use of current handheld spore detectors has been limited by the low specificity and sensitivity of the detection assays they employ. A memorandum released July 21, 2002, from the White House Office of Science and Technology Policy has recommended against using any of the currently available commercial handheld spore detectors because of their high rates of false positive results and their inability to detect low-level spore contamination, as described at http://www.gsa.gov/mailpolicy. Independent tests conducted by the Florida Department of Health Laboratory showed that the three commercially available handheld *B. anthracis* spore assays were unable to detect fewer than 10^5 spores, whereas first responders desire a test that can detect <100 spores (15).

Monoclonal antibodies used for spore detection. To date, handheld spore detectors have utilized monoclonal antibodies (mAbs) as the specific recognition molecule in immunochromatographic assays (15). The primary advantages of using mAbs for detection include the well-established protocols for the generation of mAbs (17) and the availability of sensitive binding assays employing mAbs that are well-suited for detection.

Hybridoma technology, which is the immortalization of antigen-specific B cells optimized for binding *in vivo* by the immune system, allows the isolation of high-affinity mAbs against most immunogens. Of course, the composition and purity of the immunogen determines the quality and specificity of the mAbs that are generated. Immunization with a contaminated, impure immunogen may lead to the production of antibodies against the immunogen, the contaminant, or both.

The first preprint in this dissertation describes research that demonstrates this point. This research shows that a mAb reported to bind *B. anthracis* spores and also used in a popular commercial handheld spore detector does not in fact recognize *B. anthracis* spores but instead binds a common contaminant of spore preparations. This contaminant, EA1, is an abundant protein found on the surface of vegetative *B. anthracis* cells. EA1 can be removed from spore preparations by density gradient centrifugation, which makes the purified spores nonreactive against anti-EA1 mAbs. This research shows that an anti-EA1 mAb is not suitable for spore detection purposes.

Phage display and the discovery of peptides that bind to spores. Phage display is the expression of novel peptides or proteins on the surface of filamentous phage particles. The phage particle provides a compact physical linkage between the phenotype (structure) of the displayed protein and the genotype (DNA) that encodes it. This technology was first used to display small peptides (28) and has since been used to display larger proteins such as antibody fragments (3) or DNA-binding domains (10). Phage display offers an alternative to hybridoma technology to generate specific recognition molecules. Phage libraries can be made which contain billions of individual phage, each expressing different random peptides or proteins as fusions to the phage minor coat protein pIII. As opposed to the *in vivo* selection of B cells in hybridoma technology, phage display technology relies on an *in vitro* selection of phage with a desired phenotype. Thus, phage particles within the diverse library that have a desired characteristic (e.g., ability to bind to a spore) can be purified from the rest of the library.

Recently, phage display has been used to identify short peptides that bind to *B. subtilis* and other spores (16, 34).

The second preprint in this dissertation describes a phage display selection that identified a family of short peptides that binds to *B. anthracis* spores. Peptides in this family have the consensus sequence Thr-Tyr-Pro-Xxx-Pro-Xxx-Arg (TYPXPXR); they are also called TYP (Thr-Tyr-Pro) peptides. One member of this family (Thr-Tyr-Pro-Leu-Pro-Ile-Arg or TYPLPIR) was synthesized with a Gly-Gly-Gly-Cys linker and attached to the fluorochrome R-phycoerythrin. The binding of Thr-Tyr-Pro-Leu-Pro-Ile-Arg-Gly-Gly-Gly-Cys (TYPLPIRGGGC) to *B. anthracis* spores, as well as to spores of other *Bacillus* species, was characterized using fluorescence-activated cell sorting (FACS) analysis and fluorescence microscopy. This research also showed that addition of an N-terminal alanine created an improved family of ligands, the ATYP peptides (with sequence Ala-Thr-Tyr-Pro-Leu-Pro-Ile-Arg or ATYPLPIR) that bind to *B. anthracis* spores better than the TYP peptides. We described a third peptide family, the SLLPGL peptides (with consensus sequence Ser-Leu-Leu-Pro-Gly-Leu), that binds to cross-reacting strains of spores. We demonstrated that using the ATYP and SLLPGL peptides in tandem eliminated potential false positive results from the detection assay and provided specific detection of *B. anthracis* spores.

The identification of the ATYP peptide binding targets. After the discovery of the TYP and ATYP peptides, an important unanswered question was the identity of their binding target on the *B. anthracis* spore surface. The third preprint describes research that tentatively identified the *B. anthracis* spore surface molecules that bind to the ATYP-

peptides. This work employed a cross-linking strategy to identify the candidate binding targets. The "label transfer" reagent sSBED (sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido) hexanoamido]ethyl-1,3'-dithiopropionate) covalently transferred a biotin label from an ATYP peptide to its binding target on the spore surface. Two spore proteins were biotinylated by this reagent and represent possible ATYP binding targets. They were identified, by two dimensional gel electrophoresis and mass spectrometry analysis, as CotZ-1 and the protein product of the BA4266 locus. The accessibility of these proteins to the cross-linking reagent indicates that they are likely on the *B. anthracis* spore surface, perhaps as components of the basal layer of the exosporium.

SURFACE LAYER PROTEIN EA1 IS NOT A COMPONENT OF *BACILLUS ANTHRACIS* SPORES BUT IS A PERSISTENT CONTAMINANT IN SPORE PREPARATIONS

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ABSTRACT

EA1 is an abundant, highly antigenic, surface layer protein of *Bacillus anthracis* vegetative cells. Recent reports indicate that EA1 is also a component of *B. anthracis* spores and a potential marker for spore detection. We show here that EA1 is not a spore component but a persistent contaminant in spore preparations.

Surface layers, or S-layers, are two-dimensional paracrystalline arrays that cover vegetative cells of many prokaryotic organisms (9). Typically, S-layers form by non-covalent, entropy-driven self-assembly of identical protein or glycoprotein subunits. For some species, alternative S-layers and S-layer proteins are present at the cell surface. In the case of *Bacillus anthracis*, the causative agent of anthrax, the S-layer is composed of either EA1 or Sap, which are encoded by the chromosomal genes *eag* and *sap*, respectively (3). The main S-layer protein produced in *B. anthracis* strains carrying plasmid pXO1, which contains genes necessary to produce anthrax toxins and other virulence-related proteins, is EA1 (4). In fact, EA1 is the most abundant protein and the major cell-associated antigen in these strains (3).

Several recent reports indicate that EA1 is also a component of the *B. anthracis* spore surface and could be used as a species-specific molecular marker for detection of spores (2). Here we investigate these possibilities and discuss the adverse consequences of using EA1 as a marker for *B. anthracis* spores.

Evidence indicating that EA1 is a spore component. Three reports and observations suggested that EA1 was present on spores of *B. anthracis*. First, in a

proteomic analysis of spore surface proteins of *B. anthracis*, Lai et al. reported the presence on highly washed spores of a 91,362.5-Da (isoelectric point = 5.70) cell surface antigen containing S-layer homology domains (2). They also identified this protein by mass spectrometry as protein NP_654830 in the National Center for Biotechnology Information database. Although not stated in their report, the sequence of this 862-amino acid protein is identical to EA1.

Second, we observed that from a small number of our *B. anthracis* spore preparations we were able to extract variable amounts of an approximately 100-kDa protein under conditions that solubilized proteins on the spore surface. Our spores were prepared from the avirulent Sterne strain ($pXO1^+$ $pXO2^-$ unable to produce vegetative cell capsule) grown in Difco Sporulation Medium at 37°C with shaking for 36 h (5). Under these culture conditions greater than 95% of the cells sporulate. The spores were washed extensively with water and pelleted through 50% Renografin to remove vegetative cell debris (12). Spore extracts, which contained primarily proteins of the outermost spore layer (i.e., the exosporium), were prepared by boiling 3×10^8 spores from each preparation for 8 min in 20 μl of sample buffer containing 125 mM Tris-HCl (pH 6.8), 4% sodium dodecylsulfate (SDS), 10% (vol/vol) 2-mercaptoethanol, 1 mM dithiothreitol, 0.05% bromophenol blue, and 10% (vol/vol) glycerol (10). The samples were then spun at 10,600 x *g* for 3 min, and the proteins in the supernatants were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4-15% polyacrylamide gradient gel (Ready Gels, Bio-Rad Laboratories). The variable presence of the ~100-kDa protein band was evident in the Coomassie-stained gel of proteins extracted from three spore preparations (Fig. 1a).

To identify the ~100-kDa protein, we excised the band from the gel and determined its amino-terminal amino acid sequence by automated Edman degradation. The resulting sequence was AGKSFPDVPA, which was used to search the sequence of the *B. anthracis* genome and unambiguously identified the protein as EA1 (7). The sequence AGKSFPDVPA was present in the EA1 sequence from residues 30-39, which was predicted to be the amino terminus after removal of a 29-amino acid signal peptide (3). In addition, we demonstrated that extracts of vegetative cells of *B. anthracis* (Sterne) contained a highly abundant protein that comigrated with the ~100-kDa protein variably extracted from spores (Fig. 1a). This vegetative cell protein was previously identified as EA1 (3, 4).

Third, commercial mouse monoclonal antibodies (mAbs) advertised as recognizing 92- to 94-kDa *B. anthracis* spore proteins were recently made available. The mAbs were designated SA26 (or ab2281) and M2-V129 and were sold by Novus Biologicals, Inc. (supplied by Abcam) and OEM Concepts, respectively. Reportedly, these mAbs did not cross-react with *B. anthracis* vegetative cells or with spores of *Bacillus globigii*, *Bacillus subtilis*, or *Bacillus cereus* (the latter being the most phylogenetically similar species to *B. anthracis* (6)). The size of the spore antigen(s) recognized by mAbs SA26 and M2-V129 and the highly antigenic character of EA1 prompted us to investigate the possibility that these mAbs were reacting with EA1 present in spore extracts. Therefore, we analyzed the separated proteins of the spore and vegetative cell extracts by Western blotting (Fig. 1a). After SDS-PAGE, the proteins were transferred to duplicate nitrocellulose membranes and treated as described in the manual for the Bio-Rad ImmunBlot assay kit. The membranes were probed separately

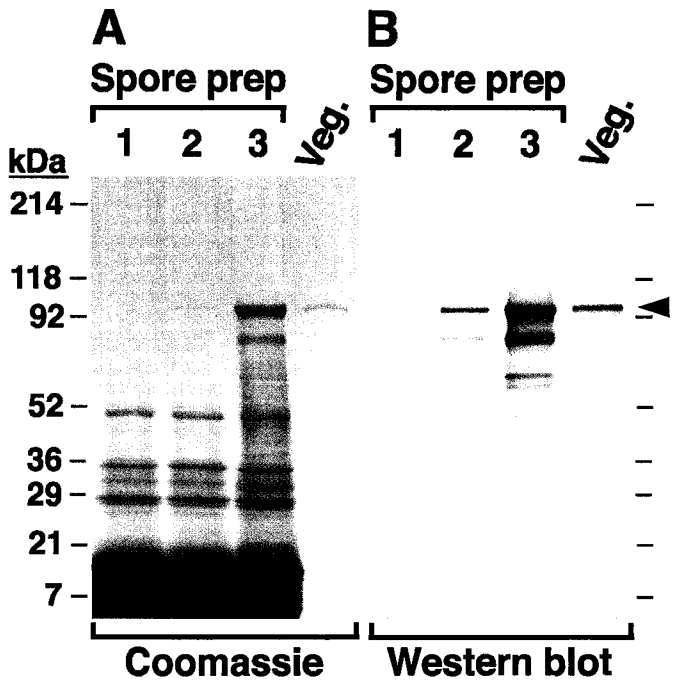

FIG. 1. Variable levels of EA1 extracted from selected preparations of *B. anthracis* spores. A typical spore preparation (prep 1) and two preparations with low (prep 2) and unusually high (prep 3) amounts of EA1 were analyzed. As a control for EA1, we also analyzed an extract of *B. anthracis* vegetative cells (Veg.). (a) Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. EA1 migrated as an ~100-kDa protein (filled arrowhead), which was a major protein in spore prep 3 and one of only two proteins observed in the vegetative cell extract (with 4 μg of protein loaded in the lane). (b) The electrophoretically separated spore and vegetative cell proteins described in panel a were analyzed by Western blotting. Separate nitrocellulose blots were probed with either SA26 or M2-V129 (5 μg/ml), and the bound mAb was detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (2 μg/ml). The results were virtually identical with either mAb, and only those obtained with SA26 are shown.

with SA26 and M2-V129, and bound mAb was detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Bio-Rad Laboratories). The results clearly show that both mAbs react with EA1 in extracts prepared from spores and vegetative cells (Fig. 1b). Cross-reactive bands that migrated faster than 100 kDa in the gel presumably were proteolytic fragments of EA1. In addition, we demonstrated that SA26 and M2-V129 reacted with intact vegetative cells of the Sterne strain of *B. anthracis* (data not shown), which is contrary to the claims of the commercial suppliers of the mAbs.

Evidence that EA1 is not a spore component but a contaminant in spore preparations. To determine if EA1 was in fact a component of the spore surface, we used fluorescence microscopy to examine directly the binding of (anti-EA1 mAb) SA26 to *B. anthracis* spores. Using all three spore preparations analyzed in Fig. 1, we dried 10^6 spores (in 10 μl) of each onto separate poly-L-lysine coated glass microscope slides (Sigma). The immobilized spores were then treated with 1% bovine serum albumin to block nonspecific binding sites and washed three times with 1 ml of cold (4°C) phosphate buffered saline (PBS) (8) containing 0.5% Tween 20 (Sigma). A 30-μl drop of SA26 (5 μg/ml in PBS) was placed on each spore sample, and the slides were incubated for 1 h at 4°C in a humid chamber. The spores were washed as above. We then placed on each spore sample a 30-μl drop containing (in PBS) Alexa 488-labeled goat anti-mouse IgG (2 μg/ml) (Molecular Probes) and 400 nM of a peptide (ATYPLPIR)-phycoerythrin (PE) conjugate that binds specifically to *B. anthracis* spores (13). (The peptide ligand was

included only to help visualize spores--see below.) The samples were incubated and washed as above and examined by phase contrast and fluorescent microscopy (Fig. 2).

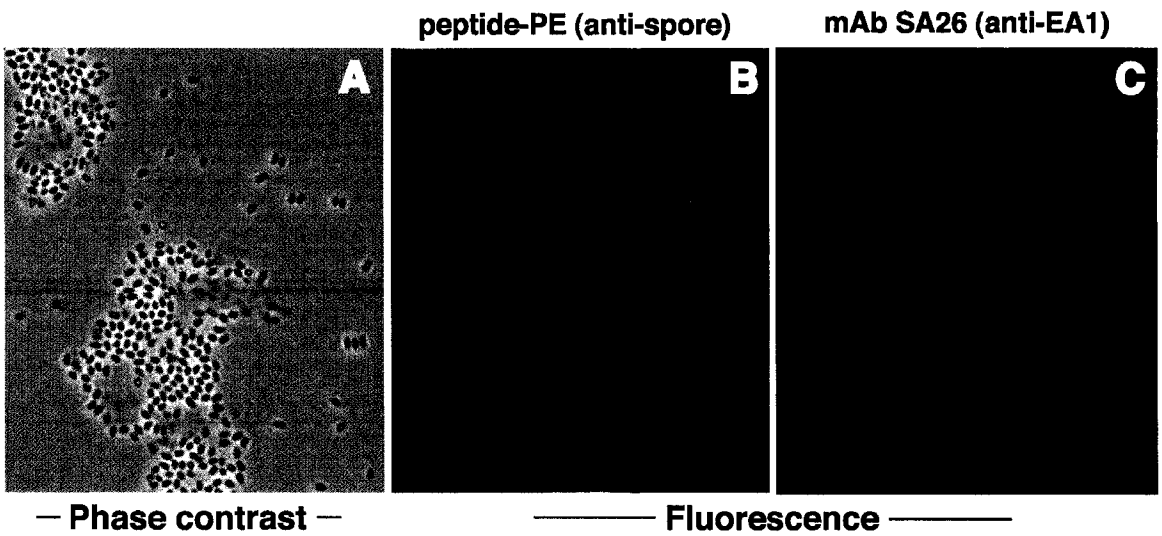

FIG. 2. Binding of an anti-EA1 mAb (SA26) to contaminating material in spore preparations but not to spores. The figure shows spores and contaminating material in spore prep 3, which contains high levels of EA1. The sample was examined by (a) phase contrast microscopy and by fluorescence microscopy under conditions that detect the fluorescence of either (b) phycoerythrin (PE) or (c) Alexa 488 (12, 13).

Examination of spores in preparation 3, which contained high levels of EA1, was most revealing (Fig. 2). Many bright spores were observed by phase-contrast microscopy (Fig. 2a), and all spores were fluorescently (red) labeled with the peptide-PE ligand (Fig. 2b). In contrast, no spores were fluorescently (green) labeled with SA26 (as detected with Alexa-labeled anti-mouse IgG) (Fig. 2c). (Note that we confirmed that the presence of peptide-PE conjugate had no effect on the reactivity of SA26/anti-mouse IgG or its detection.) Instead, SA26 bound to irregularly shaped particulate material typically much larger than a spore. This material was present in large amounts in spore preparation 3 and in low amounts in spore preparation 2 and was undetectable in spore preparation 1 (data

not shown). These results and the observed reactivity with SA26 indicated that the particulate material contained large aggregates of EA1, presumably derived from vegetative cells. The particulate material could also be observed in liquid suspension by phase contrast microscopy, although not as well as when dried. In suspension, the particulate material formed smaller clusters and was essentially devoid of vegetative cells (data not shown).

Determining the source of EA1 in spore preparations. Our results clearly demonstrated that EA1 was present in spore preparations as a contaminant. To understand how this contamination occurred and could be avoided, we monitored EA1 levels at four stages of the standard procedure for the preparation of highly purified spores (1). EA1 levels were measured after spores were harvested from 30 ml of culture medium, after the spores had been washed with 30 ml of cold (4°C) water each day for 3 days, after the spores were sedimented through 50% Renografin, and after the Renografin-purified spores had been washed three times with 10 ml of cold water. A sample containing 3×10^8 spores was removed from each fraction and treated as if to extract spore surface proteins as described above. Proteins in each sample were separated by SDS-PAGE on a 4-15% polyacrylamide gradient gel and visualized by staining with Coomassie brilliant blue (Fig. 3). The results show that a large amount of EA1, apparently contained in vegetative cell debris, was collected with the spores upon initial harvesting by centrifugation (lane 1). Extensive washing of the spores removed a negligible amount of EA1 (lane 2). However, sedimentation through 50% Renografin removed all but trace amounts of EA1 (lane 3). This small amount of EA1 was removed

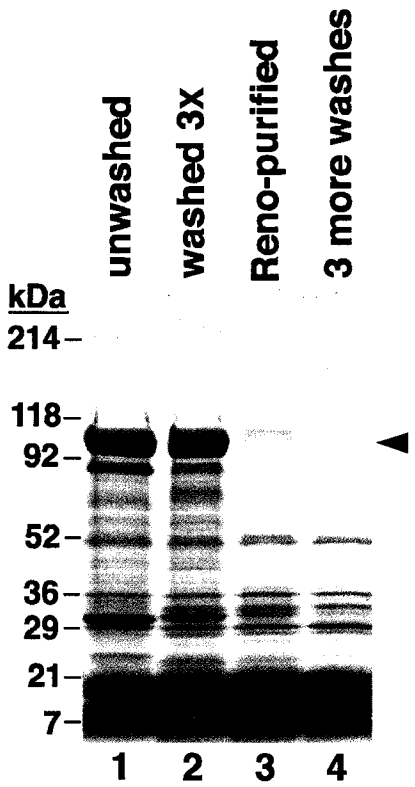

FIG. 3. Levels of contaminating EA1 during the purification of *B. anthracis* spores. For each stage, a sample of spores was treated to extract spore surface proteins, and these proteins were separated by SDS-PAGE and visualized by Coomassie staining. EA1 levels were measured after spores were harvested (lane 1, unwashed), after spores were washed three times (lane 2, washed 3X), after spores were sedimented through 50% Renografin (lane 3, Reno-purified), and after spores were washed three more times (lane 4, 3 more washes). The position of EA1 in the gel is marked with a filled arrowhead.

by additional washing (lane 4). These results indicate that contamination of spore preparations with EA1 occurs (occasionally) because of the large amount of this protein that is collected with the spores and the persistence of this protein throughout most steps of the purification protocol. In addition, the results suggest that a high level of EA1 contamination is most likely due to a problem with the Renografin purification step.

Concluding remarks. Our results demonstrate that EA1 is not present on the surface of *B. anthracis* spores. The presence of this protein in spore preparations is due to contamination. This result is consistent with the observation that inactivation of the *eag* gene has no apparent effect on sporulation or spore structure (11). However, the presence of contaminating EA1 in spore preparations is a potentially important problem for the detection of pathogenic spores of *B. anthracis*. Many detectors of *B. anthracis* spores rely on anti-spore antibodies, especially mAbs, as sensors. These antibodies are raised against spores that are typically not highly purified. Thus, these preparations are likely to be contaminated with highly antigenic EA1, and antibodies raised against these preparations are likely to react (primarily) with EA1. The incorporation of anti-EA1 antibodies into spore detectors would lead to the detection of a nonpathogenic contaminant and not spores. This situation could result in the failure to detect highly purified spores or to accurately estimate spore number. Both failures could have catastrophic consequences.

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SPECIES-SPECIFIC PEPTIDE LIGANDS FOR THE DETECTION OF *BACILLUS ANTHRACIS* SPORES

by

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ABSTRACT

Currently, available detectors for spores of *Bacillus anthracis*, the causative agent of anthrax, are inadequate for frontline use and general monitoring. There is a critical need for simple, rugged, and inexpensive detectors capable of accurate and direct identification of *B. anthracis* spores. Necessary components in such detectors are stable ligands that bind tightly and specifically to target spores. By screening a phage display peptide library, we identified a family of peptides, with the consensus sequence TYPXPXR, that binds selectively to *B. anthracis* spores. We extended this work by identifying a peptide variant, ATYPLPIR, with enhanced ability to bind to *B. anthracis* spores and an additional peptide, SLLPGLP, that preferentially binds to spores of species phylogenetically similar to, but distinct from, *B. anthracis*. These two peptides were used in tandem in simple assays to rapidly and unambiguously identify *B. anthracis* spores. We envision that these peptides can be used as sensors in economical and portable *B. anthracis* spore detectors that are essentially free of false positive signals due to other environmental *Bacillus* spores.

INTRODUCTION

The Gram-positive soil bacterium *Bacillus anthracis*, the causative agent of anthrax, has been developed into a weapon of mass destruction by numerous foreign governments and terrorist groups (8). The use of *B. anthracis* as a biological weapon, with severe consequences, was demonstrated in the fall of 2001 in the United States. The threat persists that other releases will occur with even more devastating results. *B. anthracis* is an effective agent for biological warfare and terrorism primarily because it

forms spores. Spores are resistant to extreme temperatures, noxious chemicals, desiccation, and physical damage, which makes them suitable for incorporation into explosive weapons and for concealment in terrorist devices (17). Spores enter the body through skin abrasions or by ingestion or inhalation. Once exposed to internal tissues, the spores germinate and vegetative cell growth ensues, often resulting in the death of the host within several days (9, 24). Natural strains of *B. anthracis* are sensitive to common antibiotics that can be used to treat anthrax. However, to ensure a successful outcome, treatment must begin within a day or two after exposure to spores (11). Thus, rapid detection of *B. anthracis* spores is critical in responding to the anthrax threat.

Several detection systems are currently used to identify *B. anthracis*. The most accurate systems employ either PCR-based assays or traditional phenotyping of cultured bacteria (2, 7, 24). However, these methods are complex, expensive, cumbersome, and slow, typically requiring spore germination and outgrowth of vegetative cells. Other systems, less complex and more portable, are based on antibody binding to spore surface antigens. These systems are relatively fast because they detect spores directly. However, current antibody-based detectors suffer from a lack of accuracy and limited sensitivity, which result in an unacceptably high level of both false-positive and false-negative responses, according to federal government trials at http://www.gsa/gov/mailpolicy and other independent tests (D. King, V. Luna, A. Cannons, J. Cattani, and P. Amuso, Letter, J. Clin. Microbiol. **41**:3454-3455, 2003). The lack of accuracy with these systems is compounded by the normal presence in the environment of *Bacillus* spores that resemble (and share surface antigens with) *B. anthracis* spores. Particularly problematic are spores of the opportunistic human pathogen *Bacillus cereus* and the insect pathogen *Bacillus*

thuringiensis, species which, based on genome sequence comparisons, are the most similar to *B. anthracis* (22). These three species, along with *Bacillus mycoides*, comprise the phylogenetically similar *B. cereus* group (1, 20). Therefore, due to the aforementioned limitations and deficiencies, all currently available systems for detecting *B. anthracis* are inadequate for frontline use by emergency workers and soldiers on the battlefield and for routine monitoring of public areas. Clearly, there is an urgent need for a better detector that can be used where the threat of *B. anthracis* spore exposure is the greatest.

The desired detector will, in all probability, require simple and hardy ligands capable of tight and specific binding to *B. anthracis* spores. In this report, we describe the discovery and initial characterization of such ligands: short peptides that bind selectively to spores of *B. anthracis*. We also identify an unrelated peptide that selectively binds to spores of the *Bacillus* species most closely related to *B. anthracis*. Using simple fluorescence-based assays, we demonstrate that these two classes of peptides permit unambiguous identification of *B. anthracis* spores. We envision that these peptides can be incorporated into an assortment of platforms to provide simple, rugged, and inexpensive detectors capable of accurate and direct identification of *B. anthracis* spores.

MATERIALS AND METHODS

Bacterial strains and spores. The *Bacillus* strains used in this study and their sources were as follows: the Sterne and ΔAmes strains of *B. anthracis*, *B. cereus* T, *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* B8, and *Bacillus globigii* (also called

Bacillus atrophaeus and *Bacillus subtilis* variety niger) were from John Ezzell, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md.; *B. thuringiensis* Al Hakum, *B. thuringiensis* USDA HD-571, *B. cereus* 3A (also FRI-41), *B. cereus* F1-15 (also FRI-43), *B. cereus* D17 (also FRI-13), and *B. cereus* S2-8 (also FRI-42) were from Paul Jackson, Los Alamos National Laboratory, Los Alamos, N. Mex.; *B. subtilis* (*trpC2*) 1A700 (originally designated 168), *Bacillus amyloliquefaciens* 10A1 (originally H), *Bacillus licheniformis* 5A36 (originally ATCC 14580), and *Bacillus pumilus* 8A3 (originally ATCC 7061) were from the *Bacillus* Genetic Stock Center, Ohio State University, Columbus; and *B. cereus* ATCC 4342, *B. mycoides* ATCC 10206, and *Bacillus megaterium* ATCC 14581 were from the American Type Culture Collection, Manassas, Va. Spores were produced by cells grown in liquid Difco Sporulation Medium (18) at 37°C for 48 to 72 h with shaking (except for *B. pumilis*, which was grown on solid medium at 30°C). Spores were purified by sedimentation through a Renografin step gradient as previously described (5) and were quantitated microscopically using a Petroff-Hausser counting chamber. In the biopanning experiment with *B. anthracis* ΔAmes spores, the spores were killed by gamma irradiation before use (an initial precautionary measure). In all other biopanning and spore-binding experiments (including those with ΔAmes spores), unirradiated spores were used. Gamma irradiation of spores did not appear to affect peptide binding.

Screening the phage display peptide library. The New England Biolabs (NEB) Ph.D.-7 Phage Display Peptide Library was biopanned for spore-binding phages, and these phages were analyzed as described previously (26).

Peptide synthesis and fluorochrome conjugation. Peptides were chemically synthesized and purified by high performance liquid chromatography (University of Alabama at Birmingham Peptide Synthesis Core Facility). Peptide molecules were attached to R-phycoerythrin (PE; Prozyme) by using the heterobifunctional cross-linker sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Pierce) (6).

Fluorescence Activated Cell Sorting (FACS) analysis. Spores (10^7) were mixed with a peptide-PE conjugate (at a concentration indicated in the text) in 20 µl of phosphate buffered saline (PBS) (23) and incubated at room temperature for 60 min to ensure complete binding. Unbound conjugate molecules were removed by washing spores three times in 200-µl volumes of PBS-0.5% Tween-20; after each wash, spores were collected by centrifugation at 820 x *g* and 4°C for 5 min. Spore-conjugate complexes were resuspended in 200 µl PBS, and fluorescence was measured by FACS analysis with a FACSCalibur instrument and analyzed with CellQuest Pro software (Becton Dickinson Biosciences). Spore structure was unaffected by this assay as judged by microscopic examination.

Fluorescence microscopy. Spores (10^8) were mixed with a peptide-PE conjugate, incubated, and washed essentially as described for the FACS assay. The spore pellet was resuspended in a drop of Fluoromount-G (Electron Microscopy Sciences, Fort Washington, Pa.), and a sample was examined under a Nikon Eclipse E600 microscope with a Y-FL epifluorescence attachment. Fluorescence micrographs were taken with a

Spot charge-coupled device camera (Diagnostic Instruments Inc., Sterling Heights, Mich.), using a 5-s exposure time and a gain of 8.

RESULTS

Biopanning a phage display library for peptides that bind *B. anthracis* spores. To identify peptides that bind to spores of *B. anthracis*, we screened (or "biopanned") the NEB Ph.D.-7 Phage Display Peptide Library for spore-binding phages. In the Ph.D.-7 library, random 7-mer peptides were displayed on the surface of the filamentous coliphage M13 as fusions to the surface-exposed amino terminus of the minor coat protein pIII. Each phage contained five copies of the same peptide-pIII fusion, which was encoded by the phage gene III containing a random 21-base insert. The phage display library contained 2×10^9 independent clones. In two separate experiments, we biopanned against purified spores produced by either the ΔAmes or Sterne strain of *B. anthracis*. The ΔAmes ($pXO1^-$) and Sterne ($pXO2^-$) strains are avirulent due to the absence of a plasmid necessary to produce anthrax toxins or the capsule of the vegetative cell, respectively (16). For each biopanning experiment, spores and phages were mixed to allow binding, spore-phage complexes were collected by centrifugation and washed 10 times, phages were eluted from the complexes, and the eluted phages were amplified by infecting *Escherichia coli* cells (under conditions similar to those recommended by NEB). The amplified phages were used for a second round of biopanning; in total, four rounds of biopanning were performed, after which the eluted phages (each displaying a putative spore-binding peptide) were plated to obtain single plaques. These plaques (27 and 35 with ΔAmes and Sterne spores, respectively) were

used to prepare phage stocks, from which genomic DNA was purified, and the 21-base insert in gene III of each phage was sequenced. The amino acid sequences encoded by the inserts revealed putative spore-binding 7-mer peptides.

Based on related sequences, the peptides were grouped into several families, each defined by a unique consensus sequence. However, only one peptide family was found in both biopanning experiments (i.e., with ΔAmes and Sterne spores). This family, with the consensus sequence TYPXPXR (hereafter referred to as TYP), was the largest in terms of number of phages (19 of 62) and unique peptide sequences (5) (Table 1). Often, more than one phage clone displayed a particular TYP peptide, and this peptide was encoded by the same nucleotide sequence. In one case, the peptide sequence (i.e., TYPLPIR) was encoded by two different nucleotide sequences, as permitted by the degeneracy of the genetic code. Although the TYP consensus sequence was variable at positions 4 and 6, the residues at these positions were typically similar. For example, Leu, Ile, or Val occupied position 4 in all but one unique peptide sequence.

TABLE 1. Phage display peptides selected for binding to spores of the ΔAmes or Sterne strain of *B. anthracis*

Amino acid sequence	Nucleotide sequence	No. clones/ total phages	Target spore
T Y P I P I R	ACT TAT CCT ATT CCG ATT CGT	3/27	ΔAmes
T Y P I P F R	ACT TAT CCT ATT CCG TTT CGT	3/27	ΔAmes
T Y P V P H R	ACT TAT CCG GTG CCG CAT CGG	1/27	ΔAmes
T Y P L P I R	ACG TAT CCG CTT CCG ATT CGG	8/35	Sterne
T Y P L P I R	ACG TAT CCG CTG CCT ATT AGG	3/35	Sterne
T Y P P P T R	ACT TAT CCG CCG CCG ACT CGG	1/35	Sterne

Analysis of spore binding by TYP peptides. To confirm and analyze the binding of TYP peptides to spores, we employed a FACS assay. This assay required the attachment of a fluorochrome to a test peptide prior to spore binding and analysis of spore-peptide complexes. To this end, we chemically synthesized a representative TYP peptide with the sequence TYPLPIRGGGC; the Gly-Gly-Gly-Cys extension was included as a carboxy-terminal linker for fluorochrome attachment. Approximately 10 peptide molecules were then attached (using a cross-linker) through their terminal cysteine residues to the ε-amino groups of dispersed lysine residues on one molecule of PE, a 240-kDa highly fluorescent protein. Peptide binding to *B. anthracis* (Sterne and ΔAmes) spores was then measured by incubating spores with from 4 to 4,000 nM peptide-PE conjugate, removing unbound conjugate by washing, and analyzing spore-peptide complexes by FACS. The results showed essentially identical, concentration-dependent binding of the peptide-PE conjugate to spores of the Sterne and ΔAmes strains (Fig. 1).

To examine the specificity of peptide binding, we measured (as described above) the binding of the TYPLPIRGGGC-PE conjugate to spores of 17 other *Bacillus* strains, including 6 strains of *B. cereus* (T, ATCC 4342, D17/FRI-13, 3A/FRI-41, S2-8/FRI-42, and F1-15/FRI-43), 4 strains of *B. thuringiensis* (subsp. *kurstaki*, B8, Al Hakum, and USDA HD-571), and 1 strain each of *B. mycoides*, *B. pumilus*, *B. globigii*, *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, and *B. megaterium*. These strains were all members of *Bacillus* Group 1 (of 5), within which *B. anthracis*, *B. cereus*, *B. thuringiensis*, and *B. mycoides* comprise the closely related *B. cereus* group. Seven of these strains--*B. thuringiensis* strains Al Hakum and USDA HD-57 and all *B. cereus*

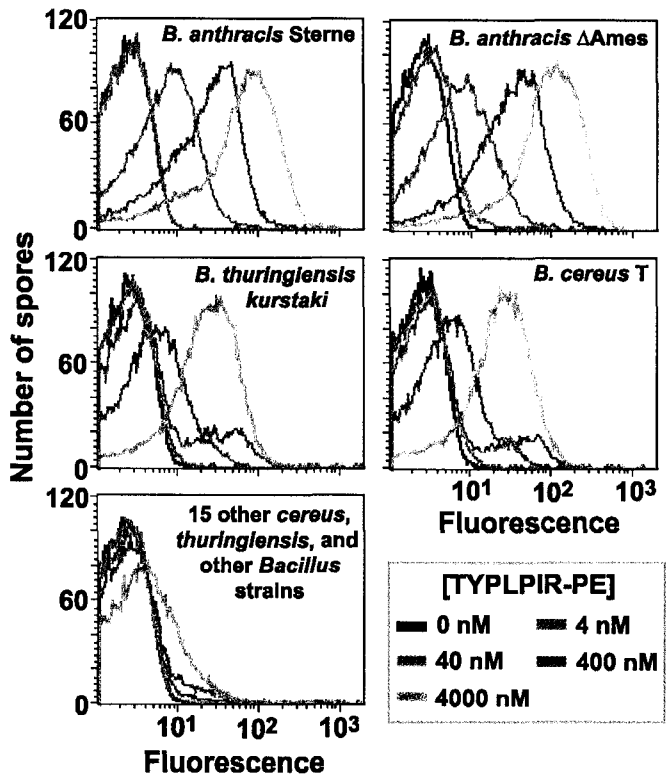

FIG. 1. FACS analysis showing the binding of the TYPLPIRGGGC-PE conjugate to selected *Bacillus* spores. The concentrations of the peptide-PE conjugate (TYP peptide-PE) are indicated. The spore species is indicated in each panel, where possible. The data shown in the bottom panel are for spores of *B. cereus* ATCC 4342 (representative of the other 15 strains), and the minimal binding at a conjugate concentration of 4,000 nM was not peptide specific. Other details are provided in Materials and Methods.

strains except T--are human pathogens and nearest neighbors to *B. anthracis* as determined by amplified fragment length polymorphism analysis (21). The binding assays showed that the peptide-PE conjugate did not bind to 15 of the other *Bacillus* strains (Fig. 1) (minimal binding at a conjugate concentration of 4,000 nM was due to nonspecific entrapment). Peptide binding was detected for spores of *B. cereus* T and *B. thuringiensis* subsp. *kurstaki*, but this binding was weaker (or less extensive) than that observed with *B. anthracis* Sterne and ΔAmes spores. These results indicated a high degree of specificity in TYPLPIR binding to spores but revealed that binding was not absolutely restricted to *B. anthracis* spores.

To control for nonspecific binding in each experiment shown in Fig. 1, several dissimilar 11-mer peptides (for example, HWHHHGHGGGC and ILPRPYTGGGC, the latter being a scrambled version of a TYP peptide) were attached to PE as described above. These conjugates were tested for spore binding. No significant binding was detected. In a related control experiment, we showed that binding of the TYPLPIRGGGC-PE conjugate to *B. anthracis* spores was not inhibited by inclusion of bovine serum albumin at 10 mg/ml in the binding and wash buffers. Furthermore, we demonstrated that the TYPLPIRGGGC-PE conjugate did not bind to vegetative cells of the Sterne and ΔAmes strains (data not shown).

Enhanced spore binding by ATYPLPIR. In a separate study, we identified a family of 7-mer and 12-mer peptides that selectively bound *B. subtilis* spores (12). This family contained a five-residue consensus sequence that permitted spore binding only when present at the amino terminus of a peptide or protein. To determine if TYP also

required a free amino terminus for spore binding, we synthesized a peptide with the sequence ATYPLPIRGGGC and attached it to PE as described above. The spore-binding ability of this conjugate was compared to that of TYPLPIRGGGC-PE, with both conjugates used at a concentration of 40 nM (Fig. 2). The results showed clearly that the TYPLPIR sequence did not require a free amino terminus to bind spores. In fact, the addition of an Ala residue permitted nearly 10-fold-enhanced binding to both Sterne and ΔAmes spores. Binding to *B. thuringiensis* subsp. *kurstaki* and *B. cereus* T spores was only slightly enhanced by the Ala addition, whereas this modification still did not permit detectable binding to spores of all other species examined. These experiments provided us with an improved ligand for *B. anthracis* spores, namely ATYPLPIR, and suggested that even better peptide ligands can be produced by additional modifications. Presumably, ATYP (i.e., ATYPXPXR) and TYP peptides bind to the same spore receptor, although this remains to be confirmed.

To demonstrate directly that the ATYPLPIR peptide was binding to the spore surface, we examined by fluorescence microscopy the binding of the ATYPLPIRGGGC-PE conjugate to *B. anthracis* spores. The results showed that, when binding occurred at a conjugate concentration of 400 nM and unbound conjugate was removed by washing, every Sterne or ΔAmes spore was completely encircled by fluorescent ligand (data not shown). At this concentration of conjugate, low-level but detectable binding to spores of *B. thuringiensis* subsp. *kurstaki* and *B. cereus* T (but not other strains) was observed. We then attempted to identify conditions under which only spores of *B. anthracis* would bind enough conjugate to be detectable by fluorescence microscopy. We found that, at a conjugate concentration of 40 nM, Sterne and ΔAmes spores were readily detectable,

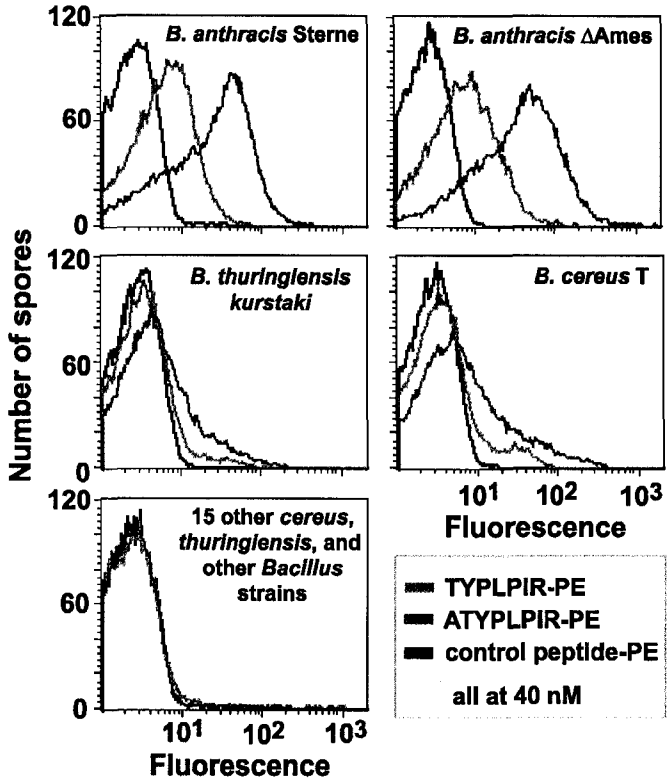

FIG. 2. FACS analysis comparing the abilities of the TYPLPIRGGGC-PE (TYP peptide-PE) and ATYPLPIRGGGC-PE (ATYP peptide-PE) conjugates to bind to selected *Bacillus* spores. Also shown are the binding results for a control peptide (HWHHHGHGGGC)-PE conjugate. Spores were mixed with a 40 nM concentration of each peptide-PE conjugate.

although with somewhat uneven fluorescence, whereas spores of the other 17 *Bacillus* strains examined in this study were essentially nonfluorescent (Fig. 3). In addition, we used several control peptide-PE conjugates to confirm that fluorescent labeling of spores required the ATYPLPIR sequence (Fig. 3 and data not shown). The reason for the uneven fluorescence observed with *B. anthracis* spores at 40 nM ATYPLPIRGGGC-PE is not known, but it appears to be unrelated to spore damage (e.g., loss of the outer spore layer).

Use of two peptides for unambiguous spore identification. In yet another biopanning experiment, we screened the Ph.D.-7 Phage Display Peptide Library for peptides that bind to spores of an uncharacterized *Bacillus* strain (probably a strain of *B. cereus* or *B. thuringiensis*) that we had isolated from an environmental sample (data not shown). These peptides revealed a single consensus sequence, SLLPGL, which was subsequently shown to bind to spores (but not to vegetative cells) of only two strains in our collection, *B. thuringiensis* subsp. *kurstaki* and *B. cereus* T. The fact that the SLLPGL peptides bind well to spores of *B. thuringiensis* subsp. *kurstaki* and *B. cereus* T, which were the only non-*B. anthracis* spores that were found to bind TYP peptides, suggested that SLLPGL peptides could be used in tandem with TYP or ATYP peptides to unambiguously identify *B. anthracis* spores. To demonstrate this application, we synthesized an SLLPGLPGGGC-PE conjugate that was equivalent to the previously described ATYPLPIRGGGC-PE conjugate. We then used a FACS assay to compare the abilities of the SLLPGL and ATYP conjugates (at 400 nM concentrations) to bind to spores of the 19 *Bacillus* strains used in this study (Fig. 4). The binding pattern for

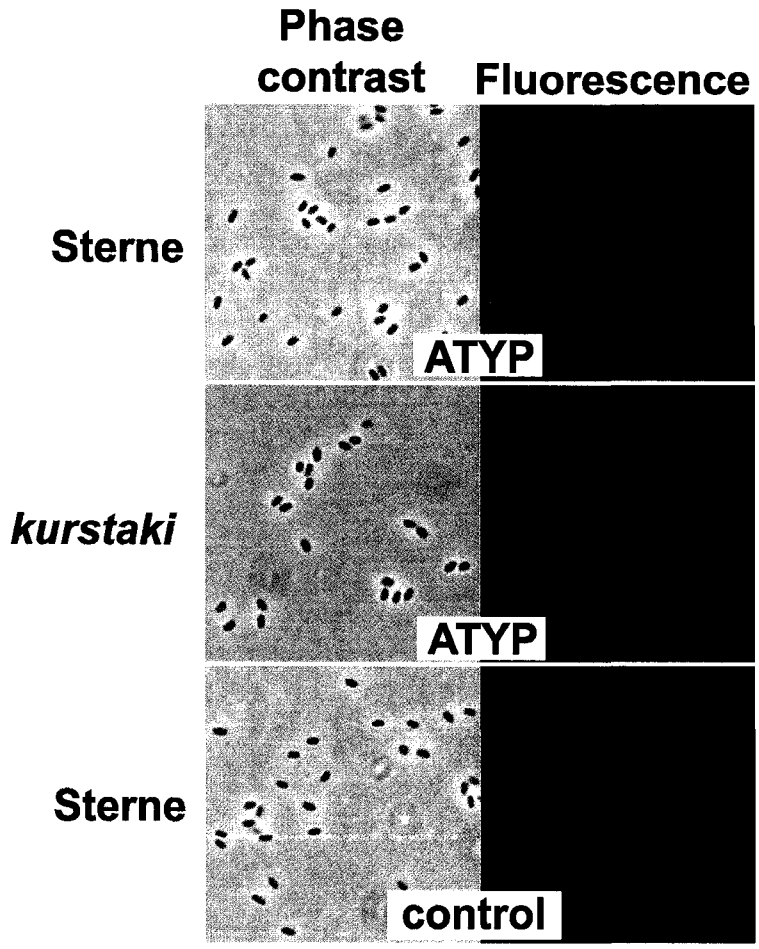

FIG. 3. Fluorescence microscopy showing selective binding of ATYPLPIRGGGC-PE to *B. anthracis* spores. The indicated spores (*B. anthracis* Sterne or *B. thuringiensis* subsp. *kurstaki*) were mixed with a 40 nM concentration of either ATYPLPIRGGGC-PE (ATYP) or a control peptide (ILPRPYTGGGC)-PE conjugate and examined by either phase-contrast or fluorescence microscopy.

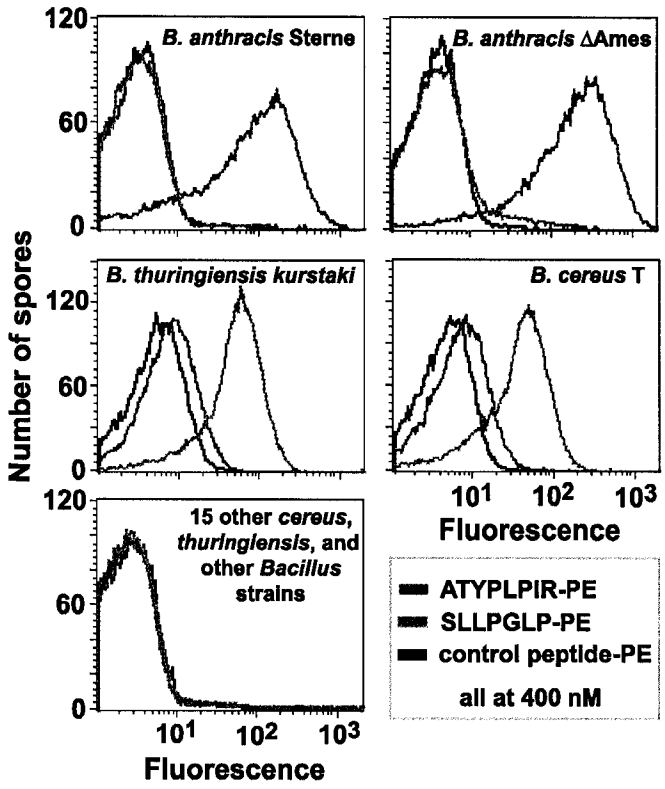

FIG. 4. FACS analysis contrasting the binding of the ATYPLPIRGGGC-PE (ATYP peptide-PE) and SLLPGLPGGGC-PE (SLLPGL peptide-PE) conjugates to selected *Bacillus* spores. Also shown are the binding results for a control peptide (HWHHHGHGGGC)-PE conjugate. Spores were mixed with a 400 nM concentration of each peptide-PE conjugate.

spores of both strains of *B. anthracis* was unique, with extensive binding by the ATYP conjugate and no binding by the SLLPGL conjugate. In clear contrast, the binding pattern for spores of *B. thuringiensis* subsp. *kurstaki* and *B. cereus* T was essentially reversed, with extensive binding by the SLLPGL conjugate and limited binding by the ATYP conjugate. No peptide-conjugate binding was observed with the other 15 spore types. Thus, the SLLPGL and ATYP conjugates can be used to clearly distinguish between spores of *B. anthracis* and the other *Bacillus* species examined.

DISCUSSION

In this study, we identified short peptides (i.e., ATYPLPIR and SLLPGLP) with differential spore-binding abilities that can be used to discriminate between spores of *B. anthracis* and those of other *Bacillus* species. For example, the ATYPLPIR peptide binds well to *B. anthracis* spores but not to other spores, with the exception of weaker binding to spores of an apparently small subset of *B. cereus* and *B. thuringiensis* strains. In contrast, the SLLPGLP peptide does not bind *B. anthracis* (or most other *Bacillus*) spores but binds well to the subset of *B. cereus* and *B. thuringiensis* spores that bind ATYPLPIR. Thus, by comparing the spore-binding abilities of the two peptides, it is possible to unambiguously identify *B. anthracis* spores. This discrimination is remarkable because it distinguishes spores of *B. anthracis* from spores of other members of the closely related *B. cereus* group. Apparently, spores of this group contain species-specific surface features (e.g., peptide receptors), which may reflect the different ecological niches and/or hosts of these species (20).

The obvious next question is whether the peptides will discriminate between *B. anthracis* and non-*B. anthracis* spores when more strains are examined. Answering this question will require the testing of a larger panel of *Bacillus* (and even non-*Bacillus*) spores, which is a study that we will undertake soon. Our first goal will be to examine a large number of virulent *B. anthracis* strains. The present study included only the avirulent Sterne and ΔAmes strains; however, these strains are likely to adequately represent the species for several reasons. The Sterne (pXO2$^-$) and ΔAmes (pXO1$^-$) strains differ from virulent strains only in the absence of one of two plasmids, neither of which is likely to alter the spore surface (22). In addition, spores produced by the ΔAmes and Sterne strains appear to be essentially identical to spores of virulent strains except for superficial differences in the length of the hair-like nap on the spore surface (13, 15, 25). Finally, *B. anthracis* strains are highly monomorphic, with genes from different isolates typically having greater than 99% nucleotide sequence identity (19).

If the peptides identified in this study are indeed generally useful in identifying *B. anthracis* spores, they offer several advantages in detector design. They bind directly to the spore, eliminating the need for extracting spore components or for growing vegetative cells. They can be easily incorporated, covalently if necessary, into detectors presently employing antibodies or into detection platforms that cannot accommodate antibodies because of size limitations or denaturing conditions. They can be easily and differentially labeled with assayable tags such as luminescent quantum dots that provide a signal sufficient to detect a single spore (3). They can be produced rapidly and inexpensively. Finally, the use of two peptides should eliminate or greatly reduce the incidence of false-positive signals. We expect that the peptides described in this paper can be used as the

probe for *B. anthracis* spores in simple, inexpensive, and portable detectors based on an assortment of analytical platforms. In our study, we employed assays based on increased fluorescence, but binding of peptides to spores can be detected by many other analytical techniques (10, 14).

We have not yet identified the sites on the *B. anthracis* spore surface to which ATYP and TYP peptides bind. However, the most likely binding sites are components of the exosporium, which is a prominent, loose-fitting, balloon-like layer that encloses the spores of *B. cereus* group strains. The exosporium, which is composed of a basal layer and an external hair-like nap, serves as a primary permeability barrier that would exclude M13 phage and PE (4). Preliminary experiments performed in our laboratory indicate that the binding sites for ATYP and TYP peptides are on the basal layer of the *B. anthracis* exosporium. In addition, we have not yet determined whether SLLPGL peptides fail to bind spores of *B. anthracis* because of the absence of receptors or because access to these receptors is blocked. Further characterization of peptide receptors and factors that influence peptide-receptor interactions is in progress.

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TWO PREDICTED EXOSPORIUM PROTEINS, COTZ-1 AND THE BA4266 GENE PRODUCT, MAY MEDIATE THE BINDING OF ATYP PEPTIDES TO *BACILLUS ANTHRACIS* SPORES

by

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ABSTRACT

Recently, short peptides with the consensus sequence ATYPXPXR (ATYP peptides) were shown to bind selectively, but not exclusively, to *Bacillus anthracis* spores. This binding ability permits the ATYP peptides to be used as a versatile sensor in a variety of *B. anthracis* spore detection assays. In this report, we describe our efforts to identify the target on the spore surface to which the ATYP peptides bind. We linked a fusion protein containing three ATYP peptide repeats to the label transfer reagent sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido)hexanoamido]ethyl-1,3'-dithiopropionate (sSBED). This cross-linking reagent, which bound to intact *B. anthracis* spores via its ATYP peptides, was photoactivated to transfer biotin tags to nearby molecules, including the binding targets of the ATYP peptide. Two proteins were thus biotinylated and had the binding characteristics one would expect of ATYP peptide targets. The first of these, CotZ-1, had been previously identified in *B. anthracis* spore extracts. The second possible target, the protein product of the BA4266 gene, was a predicted protein whose existence is confirmed here for the first time. The fact that the cross-linking reagent bound to these proteins suggests that both are located on the surface of the spore and perhaps are integral components of the exosporium. In addition to the two possible peptide-binding targets, we identified other proteins in *B. anthracis* spore extracts by mass spectrometry analysis. Further research is needed to understand the role of these proteins in the formation and structure of the outer surface of the *B. anthracis* spore.

INTRODUCTION

The recent use of *Bacillus anthracis* spores as a biological weapon and the fear of its future use has made detection of *B. anthracis* spores a national priority. Recently, a novel class of molecules useful for the direct detection of *B. anthracis* spores was described (19). A family of short peptides with the consensus sequence TYPXPXR (hereafter called TYP peptides) was identified after biopanning a phage display library against spores of two *B. anthracis* strains. Due to the large size of the phage to which these peptides were attached, the binding targets of these peptides are likely constituents of the *B. anthracis* spore surface, not its underlying layers (2). Addition of an alanine residue to the amino terminus of a TYP peptide yields an ATYP peptide (consensus sequence ATYPXPXR). The ATYP peptides are improved ligands that bind better to *B. anthracis* spores than the TYP peptides. Attaching ATYP peptides to the fluorochrome R-phycoerythrin creates a simple fluorescent sensor for *B. anthracis* spores, which can be incorporated into various spore detection technologies.

The TYP and ATYP peptides show similar binding profiles to spores of different species. Both peptides bind well to spores of *B. anthracis* strains (the ATYP peptides bind better), bind poorly to spores of two closely related strains (*Bacillus thuringiensis* Kurstaki and *Bacillus cereus* T), and do not bind at all to spores of other strains tested, including the nearest-neighbor strains of *B. anthracis* (19). Neither peptide binds to vegetative *B. anthracis* cells. Their similar binding profiles, as well as their similar sequences, suggest that the TYP and ATYP peptides recognize the same target on the spore surface. The target of the ATYP peptides could be a physiological receptor, suited to recognize a short peptide, or it could be a "fortuitous receptor" without physiological

function. Other peptide receptors have been described from spores, including the aromatic amino acid germinant receptor GerS of *B. anthracis* spores (7) and the peptide protease InhA from *B. cereus* exosporia (1). It is likely that the ATYP peptides bind to a component of the exosporium, which is the outermost layer of the spores of some species, including *B. anthracis*. The exosporium is composed of a basal layer, whose composition is presently unknown, and a hairlike nap composed of the glycoprotein BclA (13, 14). The exosporium loosely surrounds the rest of the spore and is impermeable to large molecules (2).

The experiments described here were performed to identify the binding target(s) of the TYP and ATYP peptides. To this end, a label transfer reagent was used to cross-link an ATYP peptide to its spore target and to transfer a biotin tag to the latter to allow its identification. These experiments showed that two *B. anthracis* proteins, CotZ-1 and the gene product of the BA4266 locus, probably mediate the binding of the ATYP peptides to the spore surface.

MATERIALS AND METHODS

Strains used and spore preparation. The CLT274 strain of *B. anthracis* was used throughout the experiments described here. The CLT274 strain, which has the genomic *rmlD* gene replaced with a spectinomycin resistance cassette, cannot synthesize the sugar L-rhamnose. Rhamnose is an important sugar in the polysaccharide component of the glycoprotein BclA, which makes up the hair-like nap of the exosporium and covers the spore surface (14). The CLT274 strain was used because the lack of the poly-saccharide component of BclA makes the spore surface more accessible to binding by

ATYPLPIR peptide, as demonstrated by fluorescence-activated cell sorting (FACS) (data not shown). Spores were prepared from cell cultures and then purified by Reno density centrifugation as previously described (13).

Construction and expression of the targeting fusion protein. Two complementary oligonucleotides (prJB7 = 5' CATGGCTACGTATCCGCTTCCGATT CGGGCTACGTATCCGCTTCCGATTCGGGCTACGTATCCGCTTCCGATTCGGGC and prJB8 = 5' CATGGCCCGAATCGGAAGCGGATACGTAGCCCGAATCGGAAG CGGATACGTAGCCCGAATCGGAAGCGGATACGTAGC) were annealed to form double-stranded DNA with sticky *NcoI* ends that encodes the amino acid sequence Met-Ala-Thr-Tyr-Pro-Leu-Pro-Ile-Arg-Ala-Thr-Tyr-Pro-Leu-Pro-Ile-Arg-Ala-Thr-Tyr-Pro-Leu-Pro-Ile-Arg-Ala ($ATYP_3$). This polypeptide consists of 3 serial copies of the ATYPLPIR peptide shown previously to bind the *B. anthracis* spore surface (19). The annealed oligos were ligated into the *NcoI* site of a pET21d-based expression plasmid (Novagen) which contained the coding sequence of the F64L/S65T variant of enhanced green fluorescent protein (EGFP) (3), followed by His-His-His-His-His-His (His_6). Thus created, the plasmid pCLT1135 encodes a fusion protein, called $ATYP_3$-EGFP-His_6, comprising three copies of ATYPLPIR followed by the EGFP protein and a His_6 tag. pCLT1135 was transformed into *Escherichia coli* BL21(DE3) [F- *ompT hsdS*$_B$ (r_B- m_B-) *gal dcm* (DE3)] to express the cloned gene according to the pET system manual (Novagen). Briefly, a liter culture of mid-log cells was induced with 1 mM isopropyl β-D-thio-galactopyranoside, shaken for 5 h at 37°C, and pelleted 20 min at 4,000 x *g* and 4°C. Cells were resuspended in 16 ml binding buffer (5 mM imidazole, 0.4 M NaCl, 16 mM Tris pH 7.9, 1 μg/ml DNase A) and disrupted by sonication, and cell debris was

pelleted for 20 min at 10,000 x *g* and 4°C. The supernatant containing soluble $ATYP_3$-$EGFP-His_6$ protein was mixed with 4 ml Ni-nitrilotriacetic acid agarose (Qiagen), added to a chromatography column, and washed twice with 10 ml wash buffer (20 mM imidazole, 0.4 M NaCl, 16 mM Tris pH 7.9). The intensely green-colored $ATYP_3$-$EGFP-His_6$ protein was eluted from the column in its native state with 1 ml fractions of elution buffer (250 mM imidazole, 0.4 M NaCl, 16 mM Tris pH 7.9) and then was dialyzed twice against 500 ml phosphate buffered saline (PBS).

Attachment of sSBED to $ATYP_3$-$EGFP-His_6$. The trifunctional cross-linker (or "label transfer reagent") sSBED (Pierce), as well as its conjugation products, was kept protected from light at all times due to their photoactive aryl azide moiety. Because EGFP has 20 lysine residues in addition to its amino terminus, all of which are reactive toward the N-hydroxysuccinimide (NHS) ester of sSBED, a 17-fold molar excess of sSBED was reacted with $ATYP_3$-$EGFP-His_6$. One mg (1.14 μmol) of sSBED was dissolved into 30 μl of dimethylsulfoxide and then immediately added to 2 mg (67 nmol) of purified, dialyzed $ATYP_3$-$EGFP-His_6$ protein in 0.2 ml PBS. The reaction was incubated 30 min at 25°C in the dark and then dialyzed (again, in the dark) against PBS to remove unreacted or hydrolyzed sSBED. The reaction product, $ATYP_3$-EGFP-SBED, had an apparent mass that ranged from ~35 to ~40 kDa by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), suggesting that most molecules had been decorated with 3 to 12 copies of sSBED. The concentration of $ATYP_3$-EGFP-SBED measured by Lowry assay (using bovine serum albumin as a standard) agreed with its

concentration estimated by its absorbance at A_{485} (using the EGFP molar extinction coefficient of 3.92×10^4 M^{-1} cm^{-1}).

Cross-linking $ATYP_3$-EGFP-SBED to spores for one dimensional (1D) SDS-PAGE. In the binding reactions, the spore concentration was 6.67×10^9 spores/ml, and the $ATYP_3$-EGFP-SBED concentration was 11 μM. Four microtubes were set up for each combination of washed/unwashed and light-exposed/dark samples. In each, 2.0×10^8 CLT274 (ΔrmlD) *B. anthracis* spores (enough for loading two lanes) were pelleted (3 min at 10,000 x *g* at 4°C) and then resuspended in 32 μl $ATYP_3$-EGFP-SBED diluted to 11 μM in PBS. Spores were allowed to bind to $ATYP_3$-EGFP-SBED for 60 min at 25°C shaking in the dark. For washed samples, the binding reaction was centrifuged (3 min at 10,000 x *g* at 4°C), and the supernatant was discarded. The pellet was washed twice with 1 ml PBS + 0.5% Tween-20 (resuspending the pellet and centrifuging each wash) and then finally resuspended in 32 μl PBS. To photoactivate the aryl azide of sSBED, the spore suspension was transferred to a quartz cuvette placed on ice just below a handheld ultraviolet lamp (Model UVGL-25 Mineralight Lamp, Ultra-Violet Products, Inc.) and exposed to 366 nm wavelength light for 45 min. The cuvette was agitated every 10 min to mix the spores and expose them all to the light. Eight microliters of reducing sample buffer (125 mM Tris pH 6.8, 1 mM dithiothreitol (DTT), 10% (vol/vol) mercaptoethanol, 4% SDS, 0.05% bromophenol blue, 10% glycerol) (6) was added to each tube. Samples were boiled 8 min and then centrifuged (as above) to pellet spores. For 1D PAGE, extracts from 1.0×10^8 spores were loaded onto each lane of the gel. Twenty microliters of the supernatant containing extracted proteins was loaded in duplicate on two lanes of a

4-15% polyacrylamide gradient gel (Ready Gel #161-1104, BioRad) and run out at 33 mA until the bromophenol blue dye reached the bottom. Half of the gel was stained 1 h in 0.1% Coomassie R-250 (BioRad) and then destained overnight. The other half was transferred to nitrocellulose using a TransBlot semidry electroblotting apparatus (BioRad), blocked 60 min at 25°C in tris-buffered saline + 3% gelatin and then incubated overnight at 4°C in streptavidin-horseradish peroxidase (HRP) conjugate (Amersham Biosciences) diluted 1:1,000 in tris-buffered saline + 0.05% Tween + 1% gelatin. Color was developed on the membrane using 4-chloro-1-naphthol following the manufacturer's protocol (Horseradish Peroxidase Conjugate Substrate Kit, BioRad).

Cross-linking $ATYP_3$-EGFP-SBED to spores for two dimensional (2D) electrophoresis. Protocols for 2D gel electrophoresis were adapted from the 1D SDS-PAGE protocols described above. Three times more spores were extracted, focused, and run on each 2D gel than were run on each 1D lane (3.0×10^8 spores vs. 1.0×10^8 spores). The spore concentration in the binding reactions was increased to 2.0×10^{10} spores/ml, and the $ATYP_3$-EGFP-SBED concentration was 10 μM. Due to buffer restrictions in the isoelectric focusing step (i.e., no mercaptoethanol, no salt, and only low levels of SDS were possible for good focusing), the sample extraction solution consisted of 50 mM DTT + 0.5% SDS. Also due to salt limitations, the PBS and any soluble proteins after the binding reaction were not analyzed--only the spores and proteins bound to them were boiled in sample extraction solution.

Briefly, 2.4×10^9 CLT274 *B. anthracis* spores (enough for 8 2D gels) were suspended in 120 μl PBS + 10 μM $ATYP_3$-EGFP-SBED and allowed to bind for 60 min

at 25°C shaking in the dark. Samples were washed (as required) as described above, except that spores were finally resuspended in 120 µl PBS. The aryl azide group was photoactivated as described above. After light activation, spore suspensions were centrifuged (3 min at 10,000 x *g* at 4°C) to pellet the spores and any molecules bound or cross-linked to them, and the PBS supernatant was discarded. The spore pellets were resuspended in 80 µl sample extraction solution (50 mM DTT + 0.5% SDS) and boiled 8 min. Spores were pelleted again (as above), and the supernatants containing extracted proteins were saved at –20°C.

2D electrophoresis was performed using the ZOOM IPGRunner System (Invitrogen) and followed the manufacturer's protocol with some changes. Briefly, for each 2D gel, 10 µl of the spore extract above was mixed with 150 µl of Sample Rehydration Buffer (8 M urea, 2% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.5% ZOOM Carrier Ampholytes, 0.002% bromophenol blue, and 20 mM DTT) and then loaded into the ZOOM cassette loading well. An immobilized pH gradient (IPG) strip (ZOOM strips, pH range 3-10) was inserted into the cassette and allowed to rehydrate overnight at 25°C. The ZOOM cassette was assembled into the mini-cell apparatus as described by the manufacturer. The proteins were focused using the following voltage steps and times: 100 V for 15 min, 200 V for 15 min, 450 V for 15 min, 750 V for 15 min, and 2,000 V for 75 min. After focusing, the apparatus was disassembled, and the ZOOM strip was reduced for 15 min in NuPAGE LDS Sample Buffer + NuPAGE Sample Reducing Agent and then alkylated for 15 min in NuPAGE LDS Sample Buffer + 125 mM iodoacetamide. The IPG strip was laid atop a 10-20% polyacrylamide gradient gel (Ready Gel #161-1395, BioRad), with the IPG strip's

cathodic (acidic) end next to the standards lane and then covered with 0.5 ml melted 0.5% agarose (in running buffer). The gel was run out at 33 mA for 2 h (15 min after the bromophenol blue dye ran off the bottom of the gel). 2D gels were stained with Coomassie or transferred to nitrocellulose and incubated with streptavidin-HRP in the same manner as described for 1D SDS-PAGE above.

TYPLPIR competition. A peptide with the sequence TYPLPIRGGC was chemically synthesized and purified by high performance liquid chromatography (University of Alabama at Birmingham [UAB] Peptide Synthesis Core Facility). This peptide was used (in 1,000-fold molar excess) to compete against $ATYP_3$-EGFP-SBED for binding to CLT274 *B. anthracis* spores, in a procedure similar to those described above. CLT274 spores (3.3×10^8 spores per lane) were preincubated for 30 min at 25°C with 665 μM TYPLPIRGGC peptide in PBS. Next $ATYP_3$-EGFP-SBED was added to 665 nM final concentration, and the binding reactions were incubated 60 min at 25°C in the dark. Spore suspensions were transferred to quartz cuvettes and exposed to UV light, as described above, and then centrifuged (3 min at 10,000 x *g* and 4°C) to pellet spores and any bound or cross-linked molecules. Supernatants were discarded and spores were resuspended in sample extraction solution and boiled, as described above. Extracts were run out on polyacrylamide gradient gels and either stained with Coomassie or transferred to nitrocellulose and probed with streptavidin-HRP conjugate, as described above.

Mass Spectrometry. Protein spots on Coomassie-stained 2D gels were identified by the Mass Spectrometry Shared Facility at UAB using matrix-assisted laser deionization time of flight mass spectrometry (MALDI-ToF MS) and liquid

chromatography mass spectrometry mass spectrometry (LCMSMS). After samples had undergone a 16-h tryptic digest at 37°C, MALDI-ToF MS was performed using a Voyager DE-Pro mass spectrometer (Perspective Biosystems, Foster City, CA). Tryptic peak profiles were matched by the National Center for Biotechnology Information's Mascot search engine to predicted tryptic profiles of all unique proteins in the database. Tryptic fragment identification by LCMSMS was done using a Q-Tof2 mass spectrometer (Micromass, Manchester, UK). Liquid chromatography was performed using an LC Packings Ultimate LC (LC Packings, San Francisco, CA). Samples were concentrated on a 300 μm inner diameter C18 precolumn and then flushed onto a 75 μm inner diameter C18 analytical column with a 30-min gradient of 5-100% acetonitrile. The Q-Tof2 was operated in automatic switching mode whereby multiply-charged ions were subjected to mass spectrometry-mass spectrometry if their intensities rose above 6 counts.

RESULTS

Binding $ATYP_3$-EGFP-His_6 to blotted *B. anthracis* spore extracts. Initial attempts to identify the binding target of the ATYPLPIR peptide on the *B. anthracis* spore surface used a strategy similar to a Western blot. These experiments utilized a fusion protein, $ATYP_3$-EGFP-His_6, which contained three copies of the ATYPLPIR peptide joined to EGFP, followed by a 6-histidine tag at its carboxy terminus. This fluorescent fusion protein bound well to *B. anthracis* Sterne spores, as determined by FACS analysis (data not shown). To identify the peptide binding target, $ATYP_3$-EGFP-His_6 was incubated with a blot of denatured proteins extracted from spores, and the His_6

tag was detected using an anti-His antibody. Unfortunately, no reactive bands were detected (data not shown). It is likely that ATYPLPIR, unlike an antibody used in a typical Western blot, is not complex enough to assume its own fold that is capable of binding its target. Rather, the binding of ATYPLPIR probably relies on the proper folding of a spore surface protein, which acts as either a physiological receptor or a fortuitous receptor. Due to the denaturing conditions used to extract spore proteins prior to blotting, this receptor was probably not in a binding-competent fold. Another experiment was attempted, this time extracting and running out spore proteins under native conditions; however, this also did not work because spore proteins were not extracted or solubilized under native conditions. This approach, using a Western blot to identify the binding target of ATYPLPIR, was ultimately unsuccessful.

Cross-linking $ATYP_3$-EGFP-SBED to *B. anthracis* spores. The above results suggested to us that native binding conditions, rather than denaturing conditions, would be required for the identification of the binding partner of the ATYPLPIR peptide. We decided to try using the trifunctional reagent sSBED to cross-link the ATYP peptide to its spore target under native conditions. sSBED has three flexible linker arms containing a biotin moiety, an amine-reactive sulfonated NHS ester group, and a photoactivatable aryl azide group (Fig. 1). It is used for label transfer experiments, so named because a biotin label is transferred between two interacting species. We reacted sSBED with $ATYP_3$-EGFP-His_6 (i.e., the amine-reactive NHS ester of the former reacted with the N-terminal amino group and dispersed lysine residues of the latter). The product of this reaction, $ATYP_3$-EGFP-SBED, was decorated with 3-12 SBED molecules, based on its increase in

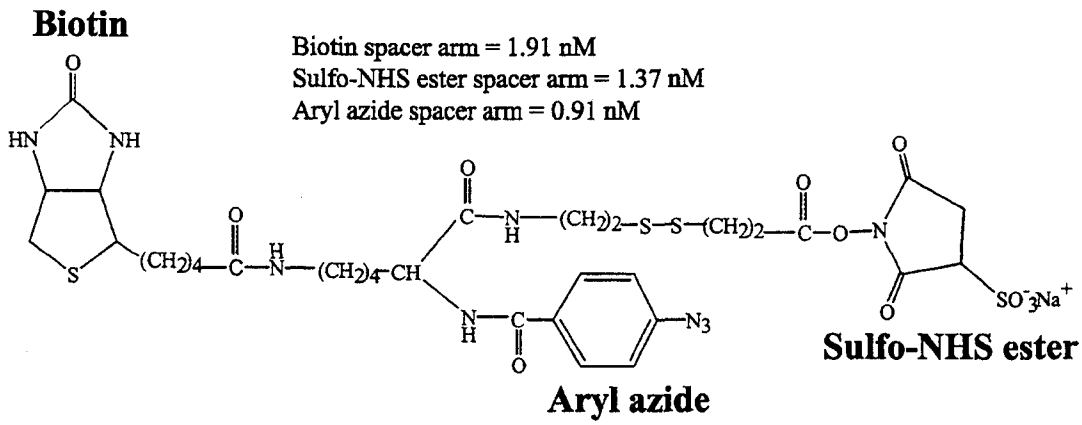

Fig. 1. Molecular structure of sSBED. The trifunctional molecule has a structure consisting of biotin, sulfo-NHS ester, and aryl azide groups linked via flexible spacer arms. The spacer arm of the NHS ester group contains a disulfide bond that allows cleavage under reducing conditions.

mass. $ATYP_3$-EGFP-SBED was then bound to *B. anthracis* spores under native conditions, under which free ATYP peptide was shown to bind well. Next, the spore-$ATYP_3$-EGFP-SBED complexes were exposed to UV light, which caused the aryl azide moiety of SBED to react with any nucleophiles in the close vicinity of the bound molecule, hopefully including the peptide receptor. Once cross-linked, the spore-$ATYP_3$-EGFP-SBED complexes were boiled in denaturing sample buffer to extract spore proteins, without concern that this would disrupt the now-covalent bond to the spore receptor. The sample buffer also provided a reducing environment, to cleave the disulfide bond in the NHS ester arm. As a result, the biotin tag was transferred from $ATYP_3$-EGFP-His_6 to the ATYP binding target.

$ATYP_3$-EGFP-SBED is a large molecule (~34 kDa; large arrow in Fig. 2) and was decorated with multiple copies of SBED. Because of this, we expected that the

cross-linking step would be relatively nonspecific, because any nearby molecules on the spore surface would be cross-linked upon photoactivation of the aryl azide group. This was in fact observed (Fig. 2b). Comparing the first two sample lanes in Fig. 2b, it can be seen that photoactivation of unwashed binding reactions labeled many spore proteins, from <6 kDa to >200 kDa, with biotin. $ATYP_3$-EGFP-SBED was itself biotinylated when the aryl azide reacted with nucleophiles on the EGFP surface. The biotinylated $ATYP_3$-EGFP-SBED band in the dark reaction was due to incidental light exposure during the course of the experiment.

From previous studies of the interaction of ATYPLPIR with spores, we knew that its binding is resistant to vigorous washing with buffer. We therefore reasoned that the blotted protein band representing the peptide binding target could be distinguished from other bands representing nonspecific interactions by its resistance to washing. To test this idea, we allowed the $ATYP_3$-EGFP-SBED conjugate to bind to spores, and then washed the complexes twice before photoactivating the cross-linker (Fig. 2, lanes 3 and 4). The results showed that washing the binding reactions prior to photoactivation prevented biotinylation of all spore proteins except for two bands, a doublet at 18 and 19 kDa (Fig. 2b, small arrows). This doublet represented a candidate spore binding target(s).

Competition for Binding with TYPLPIRGGGC. The spore protein that specifically binds the TYP peptides, as opposed to other spore proteins that may interact with low affinity, should also bind to free TYPLPIR peptide. We predicted that free TYPLPIR peptide could compete away the binding of $ATYP_3$-EGFP-SBED to the spore

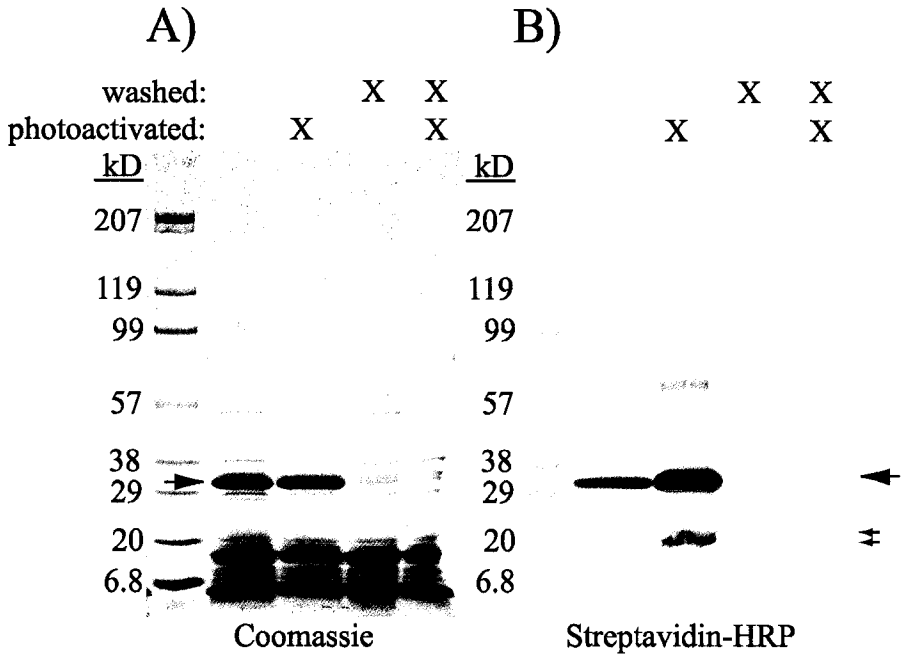

FIG. 2. The cross-linker ATYP $_3$-EGFP-SBED transfers a biotin label to multiple *B. anthracis* spore proteins, but only two are resistant to washing. A Coomassie-stained gel (a) shows the proteins extracted from spores. The cross-linker itself is also visible (large arrow) in unwashed samples. A blot of the spore extracts (b) was incubated with streptavidin-HRP to show biotinylated proteins. When unwashed spore-ATYP$_3$-EGFP-SBED complexes are cross-linked by photoactivation, many spore proteins are biotinylated. When the complexes are washed prior to photoactivation, only a doublet at 18-19 kDa (small double arrows) is biotinylated. The biotinylated band at ~35 kDa (large arrow) represents ATYP$_3$-EGFP-SBED which has cross-linked itself.

receptor, and this competition could identify the true spore receptor. Preliminary competition binding studies, analyzed by FACS, showed that a 1,000-fold excess of free TYPLPIRGGGC peptide was required to reduce the binding of fluorescent ATYPLPIRGGGC peptide to spores (data not shown). Therefore, competition experiments were initiated by incubating spores with a 1,000-fold excess of free TYPLPIRGGGC peptide. Next the $ATYP_3$-EGFP-SBED complex was added (at a 1,000-fold lower concentration) and the binding reaction was incubated to equilibrate the competition for the spore receptor. After discarding nonbinding proteins in the supernatant, the spore-$ATYP_3$-EGFP-SBED complexes were photoactivated. Analysis of the biotinylation of proteins extracted from spores showed that the 18-19 kDa doublet protein(s) bound less $ATYP_3$-EGFP-SBED when the competitor peptide was present than when it was not (Fig. 3b, lanes 2 and 3). The reduction in biotinylation was modest (about 3-fold). However, this reduction was greater than that seen with the $ATYP_3$-EGFP-SBED band (Fig. 3, large arrow), which provided an internal control for the efficiency of the competition (its cross-linking/biotinylation also decreased as it was competed away from spores and was lost into the supernatant).

Identification of the 18-19 kDa doublet. The candidate binding target(s) of $ATYP_3$-EGFP-SBED, which ran as a doublet at 18-19 kDa, shared a mass close to that of many other proteins extracted from spores. In order to identify the binding target(s) on a Coomassie-stained gel while avoiding other proteins of similar size, we decided to try separating the spore extracts by both size and isoelectric point on 2D gels. Because salt, detergents, and other chemicals are not compatible with the isoelectric focusing (IEF)

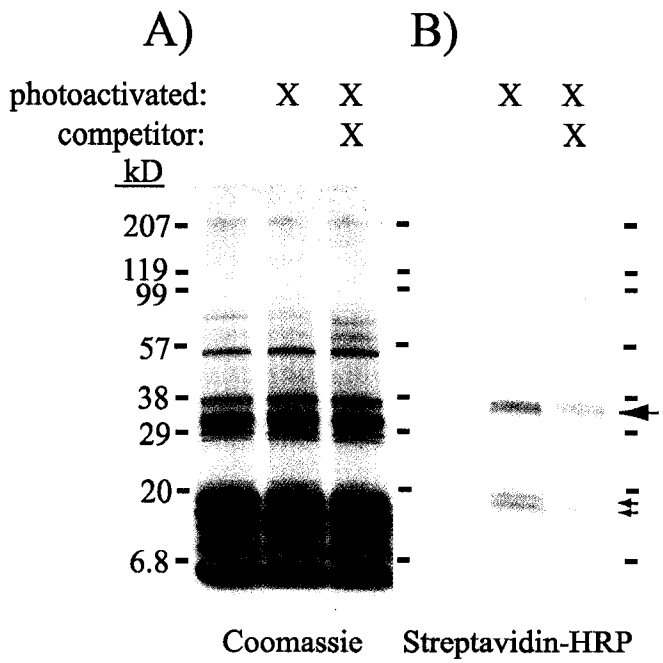

FIG. 3. Free TYPLPIRGGGC peptide is able to reduce binding of ATYP $_3$-EGFP-SBED cross-linker to the 18-19 kDa doublet band of spore extracts. The panels show both (a) Coomassie-stained gel and (b) streptavidin-HRP blot of the same. The free peptide reduces biotinylation of the 18-19 kDa doublet (small double arrows) more than it reduces nonspecific biotinylation of the cross-linker (large arrow).

step, we first had to identify IEF-compatible extraction conditions. We found that spores could be extracted in 50 mM DTT + 0.5% SDS, and this extraction buffer both allowed IEF and provided Coomassie-stained 1D protein profiles that were nearly identical to our usual extraction conditions (data not shown). After spore extraction, we focused the proteins in the supernatants using ZOOM immobilized pH gradient strips (Invitrogen) and then separated them by mass using acrylamide gels. Coomassie-stained 2D gels of spore extracts showed at least three dozen distinct protein spots (Fig. 4a). Silver-stained gels showed yet more protein spots (data not shown). More than two dozen spots were analyzed by mass spectrometry, and from this we collected data describing the identity or possible identity of 16 of the spots (Fig. 4 and Table 1, see below).

Spore proteins that had been cross-linked by $ATYP_3$-EGFP-SBED were separated by 2D electrophoresis and blotted to nitrocellulose, and their biotinylation was detected using streptavidin-HRP (Fig. 4b). When 10 μM $ATYP_3$-EGFP-SBED was cross-linked to spores, five distinct protein spots (or protein chains with varying isoelectric points) were biotinylated. Two of these, chains a and b in Fig. 4b, were also observed when only 1 μM $ATYP_3$-EGFP-SBED was cross-linked to spores (data not shown). The ~35 kDa protein chain a, which has a broad isoelectric point (pI) range from 5-7, was the most intensely biotinylated protein and likely corresponded to $ATYP_3$-EGFP-SBED that had cross-linked (biotinylated) itself. The diffuse pI range of chain a may be due to the variable number of $ATYP_3$-EGFP-His_6 molecules that were added to each molecule of EGFP. Chain b was biotinylated nearly as heavily as chain a and appeared as three distinct spots at ~19 kDa with slightly different pI's, centered around pI = 7. The cause of the three distinct pI values of chain b is unknown but could be due to variable phos-

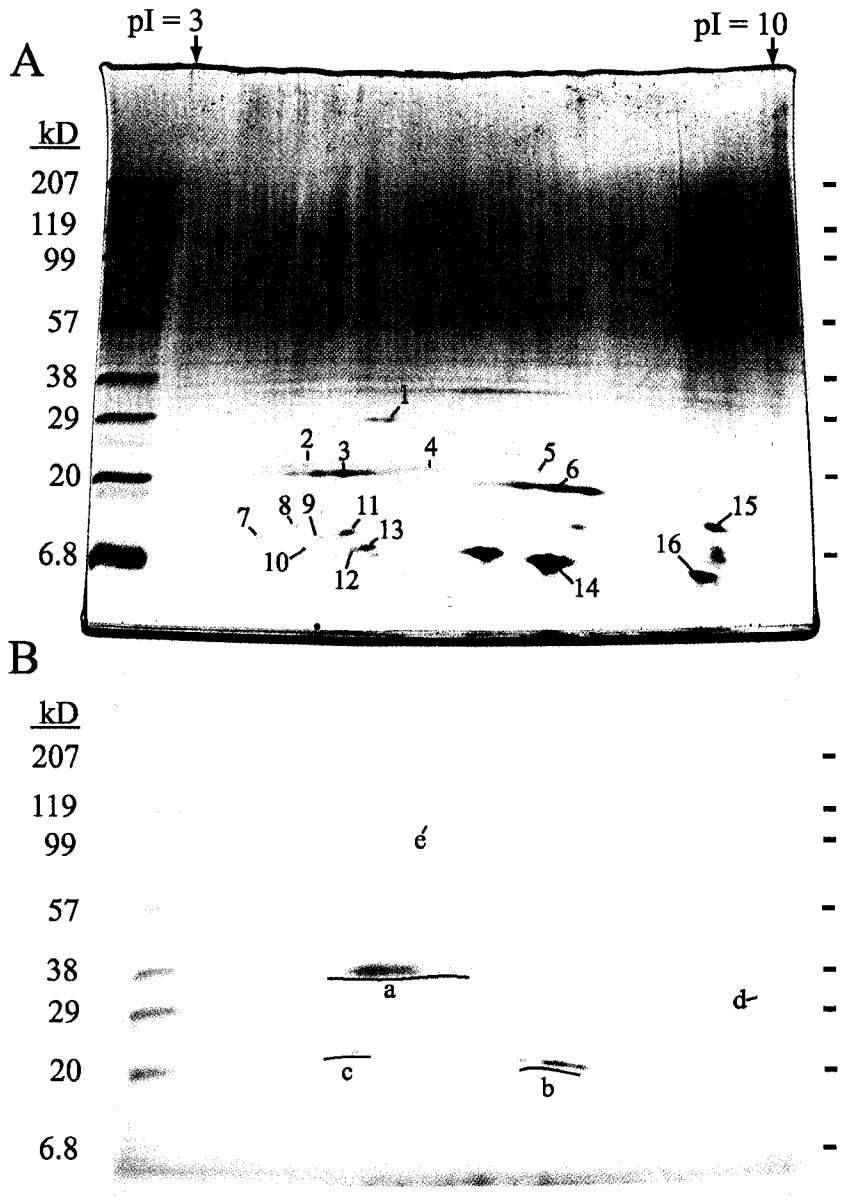

FIG. 4. 2D gel electrophoresis was used to isolate the protein bands that were biotinylated by ATYP$_3$-EGFP-SBED. (a) Extracts of *B. anthracis* spores were separated by both isoelectric point (pH gradient from 3 to 10) and mass, and then stained with Coomassie. The spots numbered from 1 to 16 were analyzed by mass spectrometry and are described in Table 1. (b) An equivalent 2D gel was blotted to nitrocellulose, and biotinylated bands were detected with streptavidin-HRP. At least 5 biotinylated spots or chains of spots were detected (indicated by lowercase letters; chains corresponding to one protein are underlined). Note that the 6.8 kDa marker band did not transfer well.

phorylation or other modifications. The pattern and position of the protein spots in chain b suggested that it corresponds to the #6 three-spot chain on the Coomassie-stained gel in Fig. 4a. Likewise, the 20 kDa biotinylated doublet labeled "c," which had a pI = 5, corresponds to the #3 doublet in Fig. 4a. Chain c was not biotinylated as heavily as chain b. Biotinylated protein chains b and c in Fig. 4b (Coomassie-stained protein chains 6 and 3 in Fig. 4a) probably correspond to the 18-19 kDa doublet which was identified on 1D streptavidin-HRP blots as the likely receptors for the ATYP peptides.

Protein identification by mass spectrometry. To identify the biotinylated proteins of bands b and c (Fig. 4b), we removed protein spots from the Coomassie-stained gel and analyzed their composition by mass spectrometry. A sample was taken from every spot visible on the gel in Fig. 4a, digested with trypsin and analyzed by MALDI-ToF MS. The Mascot program compared the tryptic digest profile of each spot to the calculated tryptic profiles of all proteins in the databases, to tentatively identify each of the 16 numbered spots in Fig. 4a. Some spots were further analyzed by LCMSMS, which provided actual sequence data to confirm the protein's identity. These data are summarized in Table 1.

Spot 3, one the two proteins which may bind to the ATYP peptides, was unambiguously identified as *B. anthracis* CotZ-1 after 77% of its entire 152 amino acid length was sequenced by LCMSMS. This protein (found under accession numbers gi:30261329, gi:21399144 (NP_655129), and gi:30255183) was previously identified in a *B. anthracis* spore extract (9). *B. anthracis* contains two *cotZ* homologues, *cotZ-1* and *cotZ-2*, located a few kilobases apart in a region of the genome with a concentration of

TABLE 1. Identification of proteins from *B. anthracis* spore extracts using 2D electrophoresis and mass spectrometry analysis

Spot ID	Size (kD)[a]	pI[a]	MS method	Protein assignments[b] Accession number	score[c]	Description
1	30	5	LCMSMS	gi:30260944	10^{-4}	CotJC, spore coat protein J
2	22	4	MALDI	gi:15896249	49	*Clostridium acetobutylicum* uracil-DNA glycosylase
3	20	5	LCMSMS	gi:30261329	10^{-58}	CotZ-1, spore coat protein Z
4	21	6	MALDI	gi:21397872	55	hypothetical protein of BA_0497 locus
5	21	7	MALDI	gi:30260172	68	hypothetical protein of BA5725 locus
				gi:30262343	56	PrsAC, protein export protein
6	19	7	LCMSMS	gi:30258768	10^{-4}	hypothetical protein of BA4266 locus
7	10	4	MALDI	gi:127525	86	*Serratia marcescens* major outer membrane lipoprotein precursor
8	12	4	MALDI	gi:21398757	63	SH3b, bacterial SH3 domain homologue
9	10	4	MALDI	gi:127525	82	*Serratia marcescens* major outer membrane lipoprotein precursor
10	8	4	MALDI	gi:21401004	53	SASP, small acid-soluble spore protein (α/β-type)
				gi:21401766	52	hypothetical protein of BA_4391 locus
11	11	5	MALDI	gi:127525	54	*Serratia marcescens* major outer membrane lipoprotein precursor
12	8	5	MALDI	gi:21399202	68	SBP_bacterial_1, bacterial extracellular solute-binding protein
				gi:21398813	58	SASP, small acid-soluble spore protein (α/β-type)
				gi:21401004	55	SASP, small acid-soluble spore protein (α/β-type)
13	8	5	MALDI	gi:21401004	85	SASP, small acid-soluble spore protein (α/β-type)
14	8	7	MALDI	gi:21402693	66	SASP, small acid-soluble spore protein (α/β-type)
15	15	9	MALDI	gi:30254051	74	SASP, small acid-soluble spore protein (γ-type)
16	7	9	MALDI	gi:21399863	88	SASP, small acid-soluble spore protein (α/β-type)

[a] Estimated from the 2D gel.

TABLE 1. (Continued)

[b] All assignments are *B. anthracis* proteins unless otherwise indicated. If the tryptic fragment profile maps to multiple *B. anthracis* proteins, all are shown.

[c] For proteins sequenced by LCMSMS, the score is an Expect (E) value, generated by NCBI's tblastn program after comparison to Genbank. For analysis after MALDI, the score shown is a Mowse score, generated by NCBI's Mascot search engine after analysis of tryptic profiles. Mowse scores are considered significant when greater than 70 ($p < 0.05$).

genes involved in formation of the exosporium, including *bclA, bxpB,* and the *rml* operon (13, 15). The CotZ-1 protein sequenced from spot 3 is similar to other CotY and CotZ proteins in closely related *Bacillus* species. It is 98% identical to the product of the *B. cereus exsY* gene (accession numbers gi:30019371 and gi:30974304). ExsY was described as being necessary for exosporium assembly in *B. cereus* (15).

Spot 6, the other protein of the doublet which binds ATYP peptides, was shown to be the gene product of the BA4266 locus. LCMSMS sequenced the first 12 (MFGSFGCCDNFR) and the last 11 (VCPTDIVAIAI) amino acids of this protein from tryptic fragments of the spot. This is the first report of expression of this protein in *B. anthracis*, in spores or otherwise. Until now, the BA4266 gene product had only been a hypothetical protein predicted from the sequence of *B. anthracis.* The BA4266 gene product contains 120 amino acids with a predicted mass of 13.4 kDa, which is smaller than its apparent 19-kDa mass seen on Coomassie-stained gels (Fig. 4). The reason for this discrepancy is unclear. During LCMSMS analysis, the mass of the carboxy terminal tryptic fragment VCPTDIVAIAI was 18 Da lower than expected, as if the peptide had cyclized and lost a water equivalent. LCMSMS analysis of the adjacent spots in the b chain (with lower and higher pIs, to the left and right of spot 6 in Fig. 4a), confirmed that these are also BA4266 gene products.

Spot 1 was analyzed by LCMSMS, and 36 of its amino acids were sequenced, which identified it as the coat protein CotJC. This protein was first identified in *Bacillus subtilis* spores, where it was shown to be a component of the undercoat (5, 12). CotJC was also recently identified in a *B. anthracis* spore extract (9).

MALDI-Tof MS analysis tentatively identified many of the protein spots in Fig. 4a, although the Mowse scores of many are less than 70 and thus are not considered to be significant. Many of the smallest proteins on the gel, in the 7-8-kDa size range, were shown to be α/β-type small acid-soluble proteins. A γ-type small acid-soluble protein was also identified (spot 15). These proteins are abundantly synthesized in the spore during late sporulation and comprise 8-15% of the total spore protein (11). They are nonspecific DNA-binding proteins, located in the spore's core, that protect the spore DNA against many types of DNA damage (10). Their presence in our spore extracts indicates either that small amounts are extractable from the core under the conditions we used (boiling 8 min in 50 mM DTT + 0.5% SDS) or, alternatively, that a small percentage of spores were completely lysed during the extraction procedure.

Mascot matched the tryptic digest profile of spot 5 (22 kDa) to two *B. anthracis* proteins (Table 1). Because the first of these proteins is predicted to be only 7 kDa in mass, the profile likely represents the second, PrsA. This lipoprotein was shown to be essential and was required for efficient export of proteins in *B. anthracis* and other Gram-positive bacteria (8, 16, 20). PrsA is attached via diacylglycerol to the *trans* side of the cytoplasmic membrane, where it functions as a posttranslational chaperone or folding factor in the space between the membrane and the cell wall (17). *B. anthracis* has three PrsA homologues, of which the PrsAC protein is represented by the tryptic profile of spot 5. Its presence in spores has not been previously reported.

The 12 kDa spot 8 was tentatively identified as SH3b, which is one of the few bacterial proteins observed to have an SH3-like domain. Interestingly, most known bacterial SH3 proteins are found in intracellular pathogens that infect eukaryotic cells,

suggesting a role in cell invasion (18). The sequence of this protein is also striking, because it is distinguished by a large central repeat region rich in glutamine, glutamic acid, lysine, and alanine residues, reminiscent of the exosporium protein BxpA (13).

The tryptic digest profiles of three nearby spots (spots 7, 9, and 11) were similar and probably represent electrophoretic variants of the same protein. Mascot matched the tryptic profiles to the major outer membrane lipoprotein from *Serratia marcescens* but was unable to match them to a predicted tryptic profile of a *B. anthracis* protein. The major outer membrane lipoprotein, found on the Gram-negative cell surface, is one of the most abundantly expressed proteins in bacteria. Because of the high Mowse scores of these matches, we considered the possibility that it indicated a contamination in our spore stock. Although microscopic inspection of spores did not reveal any evidence of this, streaking the spore stock onto a blood agar plate did reveal a very small number of β-hemolytic colonies on the background of non-β-hemolytic *B. anthacis* cells. Although the identity of these contaminants has not yet been determined, it is likely that they are indeed *S. marcescens*.

DISCUSSION

In this report, we have shown, using the label transfer reagent sSBED, that a 18-19-kDa doublet seems to be responsible for the ability of the ATYP peptides to bind to *B. anthracis* spores. This protein doublet has properties that one would expect of a TYP/ATYP peptide binding partner: it continues to bind ATYPLPIR even after washing, and free TYPLPIR peptide is able to compete off this binding somewhat. We used 2D gel electrophoresis and mass spectrometry analysis to identify the two proteins in the

doublet. The larger of the pair was determined to be CotZ-1, which is a protein previously reported in *B. anthracis* spore extracts (9), whereas the smaller protein was identified as the protein product of the BA4266 locus.

The identification of CotZ-1 is interesting for a number of reasons. The protein is nearly identical to the ExsY protein of *B. cereus*, which has been described as being required for exosporium assembly in that species (15). Although a gene deletion of *cotZ-1* in *B. anthracis* has not yet been described, the close evolutionary relationship of the two *Bacillus* species, which are both members of the Cereus group, makes it likely that this protein plays a similar role in the exosporium of *B. anthracis*. A gene deletion of *cotZ-1* in *B. anthracis* may prevent exosporium assembly in this species, too. Both *B. anthracis* and *B. cereus* chromosomes have a conserved "exosporium island" that contains, in addition to *cotZ-1* and *exsY*, other genes shown to be involved in exosporium formation (13). A nearby gene, identified in *B. anthracis* as *cotZ-2* and in *B. cereus* as *cotY*, is 86% identical to *cotZ-1/exsY*.

Less is known about the BA4266 locus and its gene product. The amino acid sequence of the BA4266 gene product is almost unique in Genbank, because the only other protein matched by a tBlastn search is its *B. cereus* homologue (gi:29897691), which shares a 92% identical amino acid sequence. The BA4266 protein is distinguished by two charged patches, 20 and 22 amino acids in length. The first patch contains seven positively charged amino acids (35%), seven negatively charged amino acids (35%), and four histidine residues (20%). The second charged patch contains six positively charged amino acids (27%), five negatively charged amino acids (23%), and nine asparagine residues (41%). The BA4266 locus likely constitutes a 1-gene operon. Intrinsic

terminators are found both 159 bp before the AUG start codon and 86 bp after the ochre stop codon (measured from the start of the run of uracil residues following the stem-loop structure of each terminator). A nearly canonical σ^K promoter ($ACn_{15}CATAnnnT$) and a good Shine-Delgarno sequence (ATGAGG) are found 27 bp and 3 bp upstream of the start codon, respectively (4).

Although suggestive, the results described here do not prove that the *cotZ-1* and BA4266 gene products bind to the ATYP peptides. Confirmation of binding will require either showing that deletion of *cotZ-1* and/or BA4266 from the *B. anthracis* chromosome abrogates ATYP peptide binding to spores or that ATYP peptides are able to bind to purified CotZ-1 and/or BA4266 expressed in *E. coli*. Both strategies have their pitfalls. As discussed above, it is possible that a *B. anthracis* strain with *cotZ-1* deleted will not form an exosporium at all; therefore, a loss of peptide binding would not be informative. Similarly, either protein may not fold correctly when expressed ectopically in *E. coli*; therefore, again a negative result would not be conclusive. These caveats aside, deleting *cotZ-1* and/or the BA4266 locus from *B. anthracis* spores is the most logical next experiment.

The results of these experiments indicated that both the *cotZ-1* and BA4266 gene products may bind to the ATYP peptides. In addition to verifying this binding as described above, we would like to discover the nature of the interactions between CotZ-1, BA4266, and the ATYP peptides. It is likely that both CotZ-1 and BA4266 are located on the spore surface and may even be components of the exosporium itself, because both are labeled by the 35-kDa $ATYP_3$-EGFP-His_6 fusion protein, whose size would exclude it from underlying spore components (2). Do CotZ-1 and BA4266 interact with each other

in the exosporium? If so, does the TYP peptide react only with the complexed proteins, or does it interact with each separately? We reported in the Results that the BA4266 gene product was biotinylated at a lower concentration of $ATYP_3$-EGFP-His_6 than was required to label CotZ-1. This observation could indicate that the ATYP peptides interact primarily with the BA4266 gene product and that CotZ-1 is occasionally labeled because of its proximity to (and perhaps interaction with) the BA4266 gene product.

The fact that the ExsY protein of *B. cereus* is 98% identical to CotZ-1 of *B. anthracis* raises questions about the source of the binding selectivity of the ATYP peptides. Why do ATYP peptides bind to *B. anthracis* spores nearly 10 times better than to *B. cereus* T spores (19)? Moreover, why do ATYP peptides bind (weakly) to one *B. cereus* strain (*B. cereus* T) but do not bind to the other strains tested (*B. cereus* ATCC4342, D17/FRI-13, 3A/FRI-41, S2-8/FRI-42, and F1-15/FRI-43)? Further research is needed to understand the presentation and local milieu of the *cotZ-1* and BA4266 gene products on the surface of spores of *B. anthracis* and *B. cereus* strains. Questions that need to be addressed include the proteins' orientation and interactions with each other and with neighboring (glyco)proteins. This understanding may clarify the factors that affect ATYP peptide binding to the spore surface.

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SUMMARY DISCUSSION

Overview. Briefly, the research described in the first preprint investigated the binding specificity of a commercial mAb, advertised as an "anti-spore" antibody capable of detecting *B. anthracis* spores. Instead, the antibody was found to recognize the EA1 protein, which is a vegetative cell protein that often contaminates spore preparations. This study highlighted the need for improved spore detectors--molecules that recognize unique *B. anthracis* spore surface components. The second preprint described the discovery of a novel class of detectors: short peptides that bind to *B. anthracis* spores. Examples of these were the TYP and ATYP peptides, which were shown to bind to *B. anthracis* spores. A second class of peptides, having consensus SLLPGL, were used in tandem with ATYP peptides to decrease false positive results and increase spore detection specificity. The third preprint in this dissertation described efforts to identify the spore surface component to which the ATYP peptides bind. This research used a trifunctional cross-linker to biotinylate the spore proteins that interacted with an ATYP peptide. The biotinylated molecules were extracted from spores and identified by gel electrophoresis and mass spectrometry. Two proteins, CotZ-1 and the protein product of the BA4266 locus, were thus tentatively identified as the spore-surface binding targets of the ATYP peptides.

Future directions. As is often the case with research findings, it seems that the more we learn, the less we know! The results of the experiments described in this dissertation provide many leads for new avenues of research, some of which I will outline briefly.

The serendipitous discovery of the ATYP variants of the TYP peptides and the improved spore-binding ability they possess suggest that additional variants can be found with even better binding properties. An initial search for better binders might entail simply substituting the other 19 amino acids, in place of alanine, at the first position of the peptide. These peptides could be directly synthesized, labeled with a fluorochrome, and assayed for their spore-binding ability.

An alternative approach to discover improved peptide ligands would be to use phage display again. First, a new library of phage could be made that has five additional amino acids of random and variable sequence preceding or, alternatively, following TYPLPIR and fused to the pIII gene as before. The new phage library could be biopanned against *B. anthracis* spores, using the same procedure described in the second preprint, with one modification: because nearly all the phage in this library would be expected to bind to spores (they all express TYP peptides, after all), it would be necessary to add an additional competition step after the usual wash steps, in which incubation with an excess of free TYP (or ATYP) peptide would compete away phage that bound with equal or lesser affinity than the TYP peptide. The phage eluted after this competition would express improved binding sequences.

Additional studies to better define the binding ability of the TYP peptides can be contemplated. These include, first, measuring the binding constants of the peptides

already discovered. Binding constants could be measured directly with a Biacore instrument or could be measured qualitatively using FACS. Second, as discussed in the second preprint, it would be useful to test the ability of TYP peptides to bind to spores of virulent *B. anthracis* strains. Last, a very useful series of experiments could investigate how different fluorochromes and different mechanisms for their conjugation to the peptides affect the ability of the attached peptide to bind its spore target and also the fluorescent signal generated after binding. It is likely that the size of the attached fluorochrome greatly influences the ability of each peptide to access its target and hence to bind. It is possible that simply changing the fluorochrome used to tag the peptides could enhance the signal generated by this class of detectors.

The results of the third preprint, describing the discovery of the spore proteins that bind to the ATYP peptides, suggest numerous additional studies. CotZ-1 and the protein product of the BA4266 locus were both identified as possible ATYP peptide receptors, but this result could be strengthened. The competition experiment described in the third preprint was admittedly weak. A 1,000-fold excess of TYPLPIRGGGC peptide was only able to partially compete off the binding of $ATYP_3$-enhanced green fluorescent protein (EGFP)-SBED from spores. This experiment could be revisited, hopefully with improved results, by using non-biotinylated $ATYP_3$-EGFP-His-His-His-His-His-His (His_6) as the competitor peptide (instead of TYPLPIRGGGC), which might allow more effective competition at a lower molar excess. The experiment also needs some negative controls, such as trying to compete the binding with an unrelated peptide (NHFLPKVGGGC) or with EGFP-His_6. A robust, unambiguous result in an improved competition experiment would improve the entire work tremendously.

Another goal of future studies is to formally prove if CotZ-1, BA4266, or both proteins are in fact the binding target of the ATYP peptides. As discussed in the third preprint, this could be done either by deleting the genes in *B. anthracis* and looking for loss of peptide binding to spores or by expressing the proteins in *Escherichia coli,* and demonstrating peptide binding to the purified proteins. In both experiments, the interpretation of the results may not be as straightforward as hoped: deletion of either gene may abolish the exosporium, or the expressed proteins may not fold correctly in *E. coli*. Another possibility is that CotZ-1 and BA4266 may interact in the exosporium, and ATYP peptides may bind to a shared site created by this interaction. It would be possible to genetically dissect an interaction of this type. A plasmid library containing point mutations in one of the genes could be expressed in its corresponding knock-out strain of *B. anthracis*, and transformed colonies could be allowed to sporulate. The spores could then be simultaneously screened by FACS, for both the presence of an intact exosporium and the loss of ATYP peptide binding, using appropriate antibodies and peptides.

It is also important to understand the cause of the binding specificity of the peptides, both the ATYP and SLLPGL families, to spores of different species and strains. Certainly, identifying the peptide receptors is the first step in this direction. That said, another important goal is to identify the binding target of the SLLPGL peptide family. The Methods used in the third paper could be applied directly to this problem. Unpublished results have shown that the SLLPGL peptide does bind to *B. anthracis* spores that have deleted either the *bclA* or *rmlD* genes. Thus the SLLPGL receptor is likely expressed in wild-type *B. anthracis* spores but is kept in a conformation that prevents peptide binding. It would be useful to identify the binding target of the

SLLPGL peptide and to understand why it is normally occluded in wild-type *B. anthracis*.

Summary. The experiments presented here provide new tools for the detection of *B. anthracis* spores, namely the TYP, ATYP, and SLLPGL peptides. This work has also created new opportunities and avenues for additional research toward understanding the composition and function of the *B. anthracis* exosporium.

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