Secondary cell wall polysaccharides of *Bacillus anthracis* are antigens that contain specific epitopes which cross-react with three pathogenic *Bacillus cereus* strains that caused severe disease, and other epitopes common to all the *Bacillus cereus* strains tested

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The immunoreactivities of hydrogen fluoride (HF)-released cell wall polysaccharides (HF-PSs) from selected *Bacillus anthracis* and *Bacillus cereus* strains were compared using antisera against live and killed *B. anthracis* spores. These antisera bind to the HF-PSs from *B. anthracis* and from three clinical *B. cereus* isolates (G9241, 03BB87, and 03BB102) obtained from cases of severe or fatal human pneumonia but did not bind to the HF-PSs from the closely related *B. cereus* ATCC 10987 or from *B. cereus* type strain ATCC 14579. Antiserum against a keyhole limpet hemocyanin conjugate of the *B. anthracis* HF-PS (HF-PS-KLH) also bound to HF-PSs and cell walls from *B. anthracis* and the three clinical *B. cereus* isolates, and *B. anthracis* spores. These results indicate that the *B. anthracis* HF-PS is an antigen in both *B. anthracis* cell walls and spores, and that it shares cross-reactive, and possibly pathogenicity-related epitopes with three clinical *B. cereus* isolates that caused severe disease. The anti-HF-PS KLH antiserum cross-reacted with the bovine serum albumin (BSA)-conjugates of all *B. anthracis* and all *B. cereus* HF-PSs tested, including those from nonclinical *B. cereus* ATCC 10987 and ATCC 14579 strains. Finally, the serum of vaccinated (anthrax vaccine absorbed (AVA)) Rhesus macaques that survived inhalation anthrax contained IgG antibodies that bound the *B. anthracis* HF-PS-KLH conjugate. These data indicate that HF-PSs from the cell walls of the bacilli tested here are (i) antigens that contain (ii) a potentially virulence-associated carbohydrate antigen motif, and (iii) another antigenic determinant that is common to *B. cereus* strains.

**Keywords:** antigens/*Bacillus anthracis*/ *Bacillus cereus*/ polysaccharides specificity /

Introduction

Anthrax is primarily a disease of herbivores although humans can also be infected. The etiologic agent of anthrax is *Bacillus anthracis*. Systemic anthrax, secondary to any of its associated routes of entry – cutaneous, gastrointestinal, and inhalation – is, if untreated, potentially fatal. The potential for using *B. anthracis* as a weapon has been widely reported (Hilleman 2002; Baillie 2005). Since the anthrax bioterrorism events in 2001, there has been a renewed interest in effective diagnostic tools and medical countermeasures. The carbohydrate antigens of *B. anthracis* have not been extensively investigated.

In general, Gram-positive bacteria have a cell surface comprising several classes of polysaccharides. These include teichoic acid, a polysaccharide that consists of repetitive sugar-phosphate residues that can have noncarbohydrate substituents such as D-alanine; lipoteichoic acid, a type of teichoic acid that is anchored to the membrane via a glycolipid; and teichuronic acid; a polymer similar to teichoic acid except that one or more of the saccharide moieties consist of glycuronosyl residues. Gram-positive bacteria also often contain additional neutral and acidic polysaccharides in their cell walls, including polysaccharide capsules that are not teichoic acid, lipoteichoic acid, or teichuronic acid. Many of these polysaccharides, as well as teichoic acid and teichuronic acid, are linked to the cell wall peptidoglycan (PG). In an attempt to classify the polysaccharides in Gram-positive bacteria, Schaffer and Messner (2005) termed polysaccharides of the teichoic acid and teichuronic acid type as “classical” secondary cell wall polymers (SCWPs), while the others were grouped as “nonclassical” SCWPs.

Our objective is to determine whether carbohydrates either on *B. anthracis* spores or on vegetative cells are antigenic and have structural or immunochemical properties that may make them suitable for the development of improved diagnostic methods and new or improved vaccines. Recently, two *B. anthracis* carbohydrate antigens have been identified that show this potential (Daubenspeck et al. 2004; Choudhury et al. 2006; Mehta et al. 2006). One of these carbohydrates is an oligosaccharide that is part of the collagen-like protein, BeLA, on the spore exosporium (Daubenspeck et al. 2004; Mehta et al. 2006), and the second is a nonclassical secondary cell wall polysaccharide found in the vegetative cell wall (Choudhury et al. 2006).

The research described in this report focuses on the secondary cell wall polysaccharide that is released from the *B. anthracis* cell wall by aqueous hydrogen fluoride (HF-PS). For *B. anthracis*, it was shown that the HF-PS anchors cell surface proteins, such as S-layer proteins, to the peptidoglycan (Mesnage et al. 2000). It is thought that the HF-PS is the ligand for the carbohydrate-binding SLH domain of the surface protein.
while a HF-labile phosphate bond anchors the PS to the peptidoglycan (Mesnage et al. 2000). A recent report identified 23 B. anthracis genes that encode proteins with SLH domains and, further, demonstrated that one of these genes, bslA, is present on the pXO1 pathogenicity island and that its product is necessary for adherence of B. anthracis to host cells (Kern and Schneewind 2008). We have previously shown, by examining the cell walls of B. anthracis and related Bacillus cereus strains, that B. anthracis produces a specific HF-PS structure that is identical in the investigated B. anthracis strains, i.e. Ames, Sterne, and Pasteur, but different from that of B. cereus cell walls (Choudhury et al. 2006; Leoff, Choudhury, et al. 2008, Leoff, Saile, et al. 2008). As shown in Figure 1, our structural investigations showed that the B. anthracis HF-PS comprises an amino sugar backbone of →6)-α-GlcNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc-(→ in which the α-GlcNAc residue is substituted with α-Gal and β-Gal at O3 and O4, respectively, and the β-GlcNAc substituted with α-Gal at O3 (Choudhury et al. 2006). In comparison, the HF-PS from the closely related B. cereus ATCC 10987 consists of α-GalNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc-(→ backbone in which the α-GalNAc is substituted at O4 with a β-Gal residue and the β-ManNAc is acetylated at O3 (Leoff, Choudhury, et al. 2008). To date, our structural investigations into the B. cereus HF-PSs from B. cereus ATCC 10987 and from the more distantly related B. cereus type strain ATCC 14579 revealed a common structural theme (see Figure 1) consisting of a HexNAc-ManNAc-GlcNAc backbone that is substituted with terminal galactosyl (Gal) or glucosyl (Glc) residues or noncarbohydrate substituents such as acetyl groups (Leoff, Choudhury, et al. 2008).

The presence of strain-specific structural features as well as a general common structural theme in these HF-PSs prompted us to further investigate whether these polysaccharides might be (i) antigenic and, if so, (ii) to characterize their immunochemical specificities. In this paper, we show that the HF-PS from B. anthracis is antigenic in that anti-HF-PS IgG antibodies are found in the antisera from rabbits inoculated with B. anthracis live or killed spores. In addition, we demonstrate that HF-PSs from pathogenic B. cereus clinical isolates of human patients suffering from severe or fatal pneumonia (Hoffmaster et al. 2004, 2006; Avashia et al. 2007), i.e. B. cereus strains G9241, 03BB87, and 03BB102, share carbohydrate antigen epitopes with B. anthracis and that these epitopes are not found on the nonpathogenic B. cereus ATCC 10987 or the B. cereus type strain ATCC 14579. We show, using antisera against a keyhole limpet hemocyanin (KLH) conjugate of B. anthracis HF-PS, that the five B. cereus and three B. anthracis strains tested share a common epitope in their HF-PS-BSA conjugates. Finally, using antisera from Rhesus macaques that survived inhalation anthrax, we demonstrate that the HF-PS antigen is expressed during B. anthracis infection in vivo.

Results

Reactivity of antispore antisera with HF-PS from B. anthracis and B. cereus strains

Immunoreactivity of HF-PS extracts from selected B. anthracis and B. cereus strains was evaluated by enzyme-linked immunosorbent assay (ELISA). Antiserum to both live and killed B. anthracis spores contained IgG antibodies that bound conjugates of the HF-PSs from B. anthracis and the B. cereus clinical isolates, G9241, 03BB87, and 03BB102, isolated from cases of severe or fatal pneumonia (Figure 2A and B). In contrast however, these antisera did not bind the B. cereus ATCC 14579 HF-PS-BSA conjugate. The binding of anti-B. anthracis spore antiserum to the synthetic AntRha2-BSA conjugate was also observed, as previously reported (Mehta et al. 2006). There was no detectable binding of these antisera to the negative control bovine serum albumin (BSA) or maltosepta-BSA conjugate. Furthermore, anti-B. cereus ATCC 14579 spore antiserum bound to a B. cereus ATCC 14579 HF-PS-BSA conjugate but not to the HF-PS-BSA conjugates from B. anthracis, or the B. cereus G9241, 03BB87, and 03BB102 clinical isolates (Figure 2C). These data support the conclusion that the B. anthracis HF-PS epitopes are present on B. anthracis spores and that cross-reactive epitopes exist between B. anthracis spore HF-PS antigen and the HF-PSs from the three clinical B. cereus isolates that caused severe or fatal pneumonia.

The specificity of the antibody binding to the HF-PSs from B. anthracis and B. cereus was further evaluated using inhibition ELISAs where a B. anthracis Pasteur HF-PS-BSA conjugate was used as the capture antigen and unconjugated HF-PSs from B. anthracis Pasteur, B. anthracis Ames, B. cereus ATCC 10987, B. cereus ATCC 14579, and B. cereus G9241 were used as the inhibitors (Figure 3). The data show that the HF-PSs from B. anthracis Pasteur and B. anthracis Ames were effective inhibitors and that the B. cereus G9241 HF-PS was able to inhibit binding but to a lesser extent; i.e., 50% inhibition required a 10-fold greater concentration of B. cereus G9241 HF-PS compared to B. anthracis HF-PS. In contrast to these HF-PSs, the HF-PSs from B. cereus ATCC 14579 and from B. cereus ATCC 10987 were not effective inhibitors, even when presented at 50-fold excess (wt/wt). Also, no inhibition was observed when using the chemically synthesized spore AntRha2 trisaccharide indicating that the reactivity to HF-PS is due to epitopes different from those on AntRha2.
Reactivity of anti-\textit{B. anthracis} HF-PS-KLH conjugate antiserum with HF-PS from \textit{B. anthracis} and \textit{B. cereus}

Rabbit anti-\textit{B. anthracis} HF-PS-KLH conjugate antiserum reacted to similar levels in ELISA with HF-PS-BSA conjugates of \textit{B. anthracis} and \textit{B. cereus} ATCC 14579 extracts indicating the presence of common cross-reactive epitopes in these HF-PS-protein conjugates (Figure 4). The presence of these common cross-reactive epitopes in the HF-PS-protein conjugates of all the \textit{B. anthracis} and \textit{B. cereus} strains was further examined by immuno-dot blot assays which are described below. A low level of binding to a maltoheptaose-BSA conjugate was observed but this occurred only at the highest serum concentration and was at the assay threshold of 0.5 OD units. In contrast, no binding to the AntRha3 trisaccharide or to the maltoheptaose or BSA controls (Figure 5, Panel A). This result indicates that the AntRha2 trisaccharide is a distinct antigen from the HF-PS and, also, that one or more structural motifs of HF-PS are present as antigens in spores. Panel B of Figure 5 shows that the binding of rabbit anti-\textit{B. anthracis}
HF-PS-KLH antiserum to unconjugated HF-PSs could be observed down to a threshold level of 0.1 µg for the *B. anthracis* HF-PS, 1–3 µg for the HF-PSs from the three *B. cereus* clinical isolates G9241, 03BB87, and 03BB102, and no detectable binding for up to 5 µg of the HF-PS from nonclinical *B. cereus* ATCC 14579-type strain. However, a different reactivity pattern was observed with the HF-PS-BSA conjugate antigens for which antiserum against *B. anthracis* HF-PS-KLH reacted strongly to the HF-PS-BSA conjugates from all species and strains, including that from *B. cereus* ATCC 14579 (Figure 5, Panel B). This latter result indicates that conjugation to protein produces or exposes an antigenic determinant that is common to protein conjugates of the HF-PSs from all of the *B. cereus* strains used in this study.

Panel C of Figure 5 shows that rabbit anti-*B. anthracis* HF-PS-KLH antiserum was reactive against whole cells and cell walls of all *B. anthracis* strains used in this assay, as well as whole cells and cell wall extracts from *B. cereus* clinical isolates G9241, 03BB87, and 03BB102. The detection threshold limit for binding the cell walls of the *B. anthracis* strains was 0.1 µg. In comparison, this threshold limit for binding the cell walls from the *B. cereus* clinical isolates G9241 and 03BB87 was increased to about 1.0 µg, and even greater for the clinical isolate 03BB102 requiring 10 µg. No binding of this antiserum to the cells and cell walls of *B. cereus* ATCC 14579 and *B. cereus* ATCC 10987 was observed, a result which is consistent with the data described above showing that anti-*B. anthracis* spore antiserum binds the HF-PSs from *B. anthracis* and the three *B. cereus* clinical isolates, but not the HF-PS from these latter two *B. cereus* strains. This cross-reactivity of the *B. anthracis* HF-PS with the HF-PS from *B. cereus* clinical isolates that caused fatal pneumonia is intriguing and indicative of a shared structural epitope among these pathogenic bacilli, a conclusion that is consistent with the similar glycosyl compositions of these HF-PSs.

**Reactivity of Rhesus macaque (Macacca mulata) anti-AVA and postinfection sera with *B. anthracis* HF-PS**

The presence of IgG antibodies that bind the *B. anthracis* HF-PS in animals inoculated with *B. anthracis* spores prompted an examination of available antiserum from naïve and anthrax vaccine adsorbed (AVA) vaccinated Rhesus macaques that had survived aerosol challenge with *B. anthracis*. Prechallenge and convalescent sera were obtained from five anthrax-vaccinated (RM1, RM3, RM4, RM5, RM6) and three naïve (RM8, RM9, and RM10) Rhesus macaques. Vaccinated animals had received three doses (week 0, 4, 26) of a 1:10 (RM4), 1:20 (RM1, RM3, RM6), or 1:40 (RM5) dilution of AVA and survived aerosol challenge with 20–422 LD_{50} equivalents (7 × 10^{3}–4 × 10^{6} CFUs) of *B. anthracis* Ames strain given at week 52. Sera were evaluated by ELISA using the *B. anthracis* Pasteur HF-PS-KLH conjugate as the capture antigen (Figure 6). None of the animals showed a prevaccination response (week 0). Three of the five vaccinated animals (RM3, RM5, RM6) had an above-threshold response at week 30 indicating that AVA may contain HF-PS and all of the vaccinated animals responded above the threshold on day 14 postexposure at levels much greater than those in naïve animals (Figure 6A). None of the naïve animals had a detectable preexposure response above the threshold and only one of the three unvaccinated animals (RM10) mounted an immune response above the threshold on day 14 postexposure (Figure 6B). All animals mounted an antiprotective antigen (PA) IgG response postexposure, confirming that they had been infected with *B. anthracis* (data not shown).

**Discussion**

The data presented in this report demonstrate that the major polysaccharides released from the cell walls of a selection of *B. anthracis* and *B. cereus* strains by aqueous HF are antigenic and animals exposed to spores of these strains generated antipolysaccharide IgG antibodies to *B. anthracis* and *B. cereus*, respectively. Postinfection Rhesus macaque serum also reacted to *B. anthracis* HF-PS indicating that this antigen is expressed during infection, and the presence of anti-HF-PS antibodies in the serum from vaccinated animals prior to spore exposure indicated that HF-PS is likely present in the AVA. Further, immunochemical analysis of these polysaccharide antigens showed that they contain both common and strain-specific epitopes depending on the antiserum–antigen combination used for investigation.

Common cross-reactive epitopes were demonstrated by the reaction of rabbit anti-*B. anthracis* HF-PS-KLH antiserum with
the HF-PS-BSA conjugate antigens from all *B. anthracis* and *B. cereus* strains investigated. This antiserum reacted strongly with the BSA conjugates of the HF-PSs from *B. cereus* strains ATCC 10987 and ATCC 14579 as well as with these same antigens from *B. anthracis* and the three clinical *B. cereus* isolates that caused severe or fatal pneumonia. The identity of the structural features in the HF-PSs responsible for the observed common cross-reactive epitopes is unknown, but this cross-reactivity depended on conjugation of the isolated HF-PSs to a protein. This dependence suggests that the common cross-reactive epitopes are normally cryptic and not exposed in the cells, cell walls, or unconjugated HF-PSs. One possible explanation is that the combination of releasing the HF-PS from the cell wall with conjugation to a protein exposes a common structural feature that becomes immunoreactive. Data from this laboratory suggest that the HF-PS from *B. anthracis* and all of the *B. cereus* strains examined here have a backbone repeating unit structure that is rich in aminoglycosyl residues (Figure 1), of which two residues are GlcNAc and ManNAc with another being either GlcNAc or GalNAc, and that this backbone structure is substituted with Gal or Glc residues or noncarbohydrate groups such as acetyl substituents (Leoff, Choudhury, et al. 2008). It may be that conjugation to proteins involves a ManNAc-GlcNAc- common structural motif in these HF-PSs that, when conjugated to protein, becomes a more accessible epitope for the host’s immune response and for antibody binding. A second possible explanation is that a common structural motif may be present in the form of a highly conserved linkage group between these HF-PSs and the PG, e.g., if the HF-PSs of all of these *B. anthracis* and *B. cereus* strains were attached to the PG via the same -HexNAc-P(P)-PG glycosyl-phosphate (or pyrophosphate) bridge. In the cell wall, such a common -HexNAc-P(P)-PG region in each of the polysaccharides would be in the innermost portion of the cell wall and not directly accessible to the host’s immune system while the structurally variable portion of the polysaccharide is more exposed and accessible. However, when the polysaccharides are released by HF cleavage of the phosphate bridge, the common structural region that was linked to the PG is “uncovered” and, therefore, more accessible to the host’s immune system. Conjugation of the isolated HF-PS to the protein may enhance this accessibility and result in the observed cross-reactivity between anti-*B. anthracis* HF-PS-KLH antiserum and all of the HF-PS-BSA conjugates. At this time, it is not known if all of these HF-PSs have a common structural region at their reducing ends (i.e., the end that would have been attached to the PG via a phosphate bridge). There is evidence, however, that cell wall teichoic acid polymers of certain bacilli are linked to the peptidoglycan through a common -ManNAc-GlcNAc-P(P)-PG linkage (Bhavsar et al. 2004; Freymond et al. 2006; Ginsberg et al. 2006). It has also been shown that other secondary cell wall polysaccharides from several bacilli are linked from a GlcNAc residue to the PG muramic acid residue via phosphate or pyrophosphate (Schaffer et al. 1999, 2000; Steindl et al. 2005). Investigation into the existence and structures of the PG linkage region of the *B. anthracis* and *B. cereus* HF-PSs is underway.

Specific epitopes were demonstrated by the reaction of antisera raised against live or killed *B. anthracis* spores with the isolated HF-PS or HF-PS-BSA conjugate antigens from *B. anthracis* strains. Also, these antisera reacted, at a reduced level, with HF-PS-BSA conjugate antigens from the clinical *B. cereus* isolates that caused fatal or severe pneumonia. However, no reaction was observed with the HF-PS-BSA conjugate from the *B. cereus* type strain ATCC 14579. Likewise, antiserum to the spores from *B. cereus* ATCC 14579 only reacted with the HF-PS-BSA conjugate of that strain. We also demonstrated the existence of specific epitopes in cells, cell walls, and isolated but unconjugated HF-PSs from *B. anthracis* strains and from the three clinical *B. cereus* isolates through their reactivity with an antiserum raised against the *B. anthracis* HF-PS-KLH conjugate. This antiserum did not react with the same extracts from *B. cereus* strains ATCC 14579 or ATCC 10987. It was also observed that this anti-*B. anthracis* HF-PS antiserum reacted with *B. anthracis* spores. Thus, in addition to its specificity, the reactivity of the anti-HF-PS-KLH antiserum with *B. anthracis*
spores, as well as the presence of anti-HF-PS IgG antibodies in antiserum generated against \textit{B. anthracis} killed spores, supports the conclusion that this HF-PS structure is a spore antigen or a component of these spore preparations, as well as a vegetative cell wall antigen.

As stated above, we observed that cross-reactive epitopes that bound \textit{B. anthracis} spore antiserum were present in the HF-PSs from three clinical isolates of \textit{B. cereus} that caused severe or fatal pneumonia, G9241, 03BB87, and 03BB102 (Hoffmaster et al. 2004, 2006; Avashia et al. 2007), indicating structural conservation or relatedness in the HF-PS antigens of these strains to that from \textit{B. anthracis}. The cross-reactive epitopes were not observed in the HF-PSs from the closely related \textit{B. cereus} ATCC 10987 strain or the \textit{B. cereus} ATCC 14579 type strain. The lack of cross-reactive epitopes on these latter two \textit{B. cereus} HF-PSs is likely due to the fact that the structures of these molecules differ significantly from the \textit{B. anthracis} HF-PS (Choudhury et al. 2006; Leoff, Choudhury, et al. 2008; Leoff, Saile, et al. 2008). On the other hand, the cross-reactive epitopes on the HF-PSs from the three clinical \textit{B. cereus} isolates are most likely due to the similarity in their structures to that of the \textit{B. anthracis} HF-PSs. These HF-PSs are very similar in glycosyl residue composition to the \textit{B. anthracis} HF-PSs (Leoff, Saile, et al. 2008), and recent structural analysis indicates that they all have the same aminoglycosyl trisaccharide backbone structure as the \textit{B. anthracis} HF-PS but with more extensive substitution by Gal residues (Choudhury et al., in preparation). We hypothesize, therefore, that these results indicate the existence of pathogenicity-related conserved structural elements in these cell wall antigens. If these hypothesized cross-reactive structural features in the HF-PSs are confirmed, they could be particularly useful for the development of multivalent vaccines that would be effective against both \textit{B. anthracis} as well as against \textit{B. cereus} strains that cause severe illness.

At present, we do not know the details of the relationship between pathogenicity and HF-PS structures. However, it is likely that the HF-PS has important functions for growth and/or pathogenicity, e.g., involving the carbohydrate binding domain (CBD) of cell surface proteins. It is known that surface proteins in \textit{B. anthracis}, S-layer proteins and others, have a CBD. The CBD, e.g., in the \textit{B. anthracis} S-layer proteins Sap and EA1, is a protein domain that normally comprises three short amino acid stretches with a motif known as the SLH motif (for S-layer family) (Choudhury et al. 2006; Leoff, Choudhury, et al. 2008). In the case of Sap and EA1 from \textit{B. anthracis}, their export and anchoring to the cell wall are mediated by the SLH domain to form a crystalline array in the surface of the cell (Mesnage et al. 2000). It is thought that the SLH protein domain binds to the HF-PS which, in turn, is covalently bound via a phosphatidyl residue to the PG of the cell wall (Mesnage et al. 2000). In addition to Sap and EA1, it was recently reported that another surface protein, BslA, that is encoded on the pXO1 plasmid contains a SLH domain and is responsible for adherence of \textit{B. anthracis} to host cells (Kern and Schnewind 2008). Thus, the HF-PS of pathogenic strains could be involved in exporting/anchoring proteins, such as BslA, that are necessary for virulence. However, to date no proof has emerged that shows directly this protein’s exporting/anchoring function or its involvement in host--cell interactions and other functions that may be required for growth. We have embarked on testing this hypothesis by preparing and characterizing the phenotypes (i.e., growth and virulence properties, HF-PS structure, and binding affinities to surface proteins) of \textit{B. anthracis} mutants carrying mutations in genes thought to encode enzymes required for the synthesis of the HF-PS.

Finally, we showed that sera from all vaccinated Rhesus macaques that were exposed to \textit{B. anthracis} spores contain IgG antibodies that bind the \textit{B. anthracis} HF-PS. This result supports further investigation into the potential use of the HF-PS conjugates to detect exposure of primates to \textit{B. anthracis}, and for use as an alternative antigen component for the development or improvement of anthrax vaccines. These investigations as well as investigations into the HF-PS structures from the pathogenic \textit{B. cereus} strains are in progress.

Material and methods

\textbf{Bacterial strains and culture conditions}

The strains/isolates used in this work and their phylogenetic relatedness are listed in Table I. All \textit{B. anthracis} strains were obtained from the CDC culture collection. Cells cultured overnight in the brain heart infusion medium (BHI) (BD BBL, Sparks, MD) containing 0.5% glycerol were used to inoculate four 250 mL volumes of BHI medium in 2 L Erlenmeyer flasks the next morning. Cultures were grown at 37°C (\textit{B. anthracis}) or 30°C (\textit{B. cereus}) with shaking at 200 rpm. Growth was monitored by measuring the optical density of the cultures at 600 nm. In the mid-log phase, cells were harvested by centrifugation (8000 \times g, 4°C, 15 min), washed two times in sterile saline, enumerated by dilution plating on BHI agar plates, and then autoclaved for 1 h at 121°C before further processing.

\textbf{Preparation of bacterial cell walls}

Bacterial cell walls were prepared from previously enumerated autoclaved bacterial cells (3 \times 10^8 to 3 \times 10^9 CFU/mL) that were disrupted in 40 mL sterile saline on ice by four 10 min sonication cycles. The complete or near complete disruption of cells was checked microscopically. Unbroken cells were removed by a low speed centrifugation run (8000 \times g, 4°C, 15 min). The separated pellet and supernatant fractions were stored at −70°C. The cell walls were separated from the low speed supernatants by ultracentrifugation at 100,000 \times g, 4°C for 4 h. The resulting cell wall pellets were washed by suspension in cold, deionized water followed by an additional ultracentrifugation at 100,000 \times g, 4°C for 4 h, and lyophilized.

\textbf{Release of phosphate-bound polysaccharides from the cell wall}

Phosphate-bound polysaccharides were released from the cell walls by treatment with aqueous HF according to the modification of the procedure described by Ekweni et al. (1991). Briefly, the cell walls were subjected to 47% HF under stirring at 4°C for 48 h. The reaction mixture was neutralized with NH_4OH, subjected to a 10 min low speed centrifugation, and the supernatant with the released polysaccharides lyophilized, redissolved in deionized water, and subjected to a chromatographic size separation on a BioGel P2 column (Bio-Rad). The fractions eluting from the Bio-Gel P2 column were monitored using a refractive index detector. Polysaccharide-containing fractions were pooled, lyophilized, and analyzed by gas chromatography-mass spectrometry as previously described (Choudhury et al. 2006).
**Preparation of spores**

Spores of *B. anthracis* were prepared from liquid cultures of the phage assay (PA) medium (Green et al. 1985) grown at 37°C, 200 rpm for 6 days. Spores of *B. cereus* ATCC 14579 were prepared from liquid cultures of the PA medium grown at 30°C, 200 rpm for 6 days. Spores were harvested by centrifugation and washed two times by suspension in cold (4°C) sterile deionized water followed by centrifugation at 10,000 × g. They were then purified in a 50% Reno-60 (Bracco Diagnostics Inc., Princeton, NJ) gradient (10,000 × g, 30 min, 4°C) and washed a further four times in cold sterile deionized water. After suspension in sterile deionized water, spores were quantified by surface spreading on BHI agar plates (BD BBL, Sparks, MD) and counting the colony forming units (cfu). Spore suspensions were stored in water at −80°C.

For the preparation of killed spores, 500 μL aliquots of spore suspensions in water, prepared as described above and containing approximately 3 × 10^8 CFU/mL, were irradiated in 2 mL Sarstedt freezer tubes (Sarstedt, Newton, NC) in a gamma cell irradiator with an absorbed dose of 2 million rads. Sterility after irradiation was confirmed by spread-plating 10 μL aliquots of irradiated spore suspension on BHI agar plates. The plates were incubated for 72 h at 37°C and monitored for colony growth. The absence of growth was taken as an indicator of sterility.

**Preparation of rabbit anti-spore antiserum and Rhesus macaque infection sera**

Anti-spore antiserum against spores of *B. anthracis* Sterne and *B. cereus* ATCC 14579 were prepared in female New Zealand White rabbits (2.0–3.5 kg) purchased from Myrtle’s Rabbitry (Thompson Station, TN). Each of two rabbits was inoculated intramuscularly at two sites in the dorsal hind quarters with 0.5 mL of washed live-spore or killed-spore inoculum (3 × 10^8 total spores). Rabbits were vaccinated at 0, 14, 28, and 42 days. Serum was collected prior to the first immunization (preimmune serum) and at day 7 and day 14 after each injection of antigen. Terminal bleeds were collected on day 14 after the last immunization. All animal protocols were approved by the CDC Animal Care and Use Committee (ACUC) and implemented under the direction of the CDC attending veterinarian. Rhesus macaque sera were made available from anthrax correlates of protection studies at CDC (C.P. Quinn, unpublished data).

**Conjugation of HF-PS to BSA or KLH**

Conjugation was performed by modification of a previously described method (Bystricky et al. 2000; Shafer et al. 2000). Approximately 1 mg of freeze-dried polysaccharide was dissolved in 0.15 M HEPES buffer, pH 7.4. While stirring, 4 mg of 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) in acetonitrile (90 μL) was slowly added to a solution of the polysaccharide to avoid precipitation. After activation of the polysaccharide (30 s), aqueous triethylamine (120 μL of 0.3 M triethylamine) was added and stirred for 2 min. Finally, 4 mg of BSA (Sigma, St. Louis, MO) or keyhole limpet hemocyanin (KLH; Sigma) were dissolved in 348 μL 0.01 M phosphate buffered saline (PBS), pH 7.4, and added to the reaction mixture. After stirring for 18 h at 4°C, the reaction mixture was quenched with the addition of 120 μL of 0.5 M ethanolamine in the 0.75 M HEPES buffer, pH 7.4. After 15–20 min of stirring, the unconjugated sugars in the mixture were separated from the protein–polysaccharide conjugate by centrifugation at 3200 × g using a centrifugal filter device (Centriplus YM-10, Millipore, Billerica, MA). The conjugate was lyophilized and stored at room temperature. The percentage of sugars in the conjugates was determined by the preparation and GC-MS analysis of trimethylsilyl methyl glycosides (York et al. 1985). Briefly, 200 μg of the HF-PS-KLH or -BSA conjugate was methanolyzed in methanolic 1 M HCl, derivatized into trimethylsilyl ethers, and analyzed by GC-MS. Using this procedure, the percent mass of hexose and the amount of carbohydrate in the HF-PS-protein conjugates was determined from the known hexose percent present in the unconjugated HF-PS, e.g., based on Gal for *B. anthracis* and on Glc for *B. cereus* ATCC 14579 HF-PS-protein conjugates.

**Preparation of antiserum to the *B. anthracis* HF-PS-KLH conjugates—*B. anthracis***

*B. anthracis* Pasteur HF-PS was conjugated to KLH as described above and used for the preparation of anti-HF-PS antiserum. For antiserum production each of two female (2.0–3.5 kg) New Zealand White rabbits (Myrtle’s Rabbitry, Thompson Station, TN) were inoculated intramuscularly at two sites in the dorsal hind quarters. For the primary injection, 1.0 mL of MPL + TDM + CWS Adjuvant System (Sigma) with 500 μg of the HF-PS-KLH conjugate was divided into two injections per rabbit. For the booster shots, 1.0 mL of MPL + TDM + CWS Adjuvant

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**Table I. *Bacillus anthracis* and *B. cereus* group strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MLST Clade, Lineage</th>
<th>Clinical information</th>
<th>Source/provider</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. anthracis</em> Ames</td>
<td>Clade 1 Anthracis</td>
<td>Veterinary isolate</td>
<td>Bovine anthrax isolate (1981, TX) (Van Ert et al. 2007)</td>
<td></td>
</tr>
<tr>
<td><em>B. anthracis</em> 34F2 Sterne</td>
<td>Clade 1 Anthracis</td>
<td>Veterinary vaccine strain (Italy)</td>
<td>(Sterne 1937)</td>
<td></td>
</tr>
<tr>
<td><em>B. anthracis</em> 4229 Pasteur</td>
<td>Clade 1 Anthracis</td>
<td>na</td>
<td>Unknown, 1880s (Green et al. 1985)</td>
<td></td>
</tr>
<tr>
<td><em>B. cereus</em> ATCC 10987</td>
<td>Clade 1, Cereus I</td>
<td>na</td>
<td>Dairy isolate (30s) (Smith 1952)</td>
<td></td>
</tr>
<tr>
<td><em>B. cereus</em> 03BB102</td>
<td>Clade 1, Cereus III</td>
<td>Fatal pneumonia</td>
<td>Human blood isolate (2003, TX) (Hoffmaster et al. 2006)</td>
<td></td>
</tr>
<tr>
<td><em>B. cereus</em> 69241</td>
<td>Clade 1, Cereus IV</td>
<td>Severe pneumonia</td>
<td>Human blood isolate (1994, LA) (Hoffmaster et al. 2004)</td>
<td></td>
</tr>
<tr>
<td><em>B. cereus</em> 03BB87</td>
<td>Clade 2 Tolworthii</td>
<td>Fatal pneumonia</td>
<td>Human blood isolate (2003, TX) (Hoffmaster et al. 2006)</td>
<td></td>
</tr>
<tr>
<td><em>B. cereus</em> ATCC 14579</td>
<td>Clade 2 Tolworthii</td>
<td>na</td>
<td>B. cereus type strain; possibly dairy isolate (1916) (Ford and Lawrence 1916)</td>
<td></td>
</tr>
</tbody>
</table>

*The phylogenetic relatedness of strains on the basis of multilocus sequence typing (MLST) was adopted from Priest et al. (2004) with modifications (see footnote b).

*b The classification of these strains in Cereus IV is proposed by Hoffmaster et al. (2008).

*na = not available.*
System with 250 μg of the HF-PS-KLH conjugate was used. Rabbits were immunized at 0, 14, 28, and 42 days. Serum was collected prior to the first immunization (preimmune serum) and at day 7 and day 14 after each injection of antigen. Terminal bleeds were collected 14 days after the last immunization.

**Enzyme-linked immunosorbent assay determination of IgG binding to B. anthracis and B. cereus HF-PS-protein conjugates**

The immunochemical reactivity of serum from rabbits inoculated with B. anthracis spores and that of serum from Rhesus macaques that survived inhalation anthrax were tested against protein-conjugated HF-PS extracts from B. anthracis Ames and B. cereus ATCC 14579 by ELISA. Slightly different protocols were used to examine these antisera.

The rabbit anti-B. anthracis spore antisera were assayed using the wells of a 96-well microtiter plate (Immulon II-HB, Thermo Labsystems, Franklin, MA) in which each well was coated with the 100 μL of a 5 μg/mL solution of HF-PS-BSA conjugate in 100 μL of 0.01 M PBS, pH 7.4, and incubated overnight at 4°C. The next day, the plates were washed three times with the wash buffer (0.01 M PBS, pH 7.4, 0.1% Tween-20) followed by the blocking buffer (5% nonfat dry milk in 0.01 M PBS, pH 7.4, 0.5% Tween-20) for 1 h at room temperature. The plates were then washed again, and serial dilutions (100 μL per well) of spore rabbit antisera in the blocking buffer were added and the plates incubated for 1 h at room temperature. The plates were then washed three times with the wash buffer. Horseradish peroxidase (HRPO)-labeled goat anti-rabbit IgG, 1:5000 dilution, was added (100 μL/well) and incubated for 1 h at room temperature. Plates were washed five times with the wash buffer before adding 100 μL of ABTS/H2O2 peroxidase substrate (KPL, Gaithersburg, MD) for 10 min. The color development was stopped with the addition of 100 μL of ABTS peroxidase stopping solution (KPL, Gaithersburg, MD) and the optical density of each well was read at a wavelength of 405 nm with a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA).

The Rhesus macaque sera were assayed as described above with the exception that the B. anthracis HF-PS-KLH conjugate rather than the BSA conjugate was used to coat the microtiter plates. Samples were tested three times and average OD and standard deviation were calculated. Anti-HF-PS IgG responses were expressed as a “fold response” over a reactivity threshold (RT) value. The RT was determined from the average OD value plus two standard deviations (SD) from the sera of 88 true negative Rhesus macaques tested against HF-PS-KLH by ELISA. Each sample was tested twice at a 1:100 dilution in the dilution buffer. The RT was calculated as an OD value of 0.22.

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Conflict of interest statement
None declared.

Abbreviations

Ant, anthrose (4-N-β-hydroxyisovaleryl-4,6-dideoxy-D-glucose); AVA, anthrax vaccine adsorbed; BSA, bovine serum albumin; CBD, carbohydrate binding domain; KLH, keyhole limpet hemocyanin; Rha, rhamnose.

References


