Secondary cell wall polysaccharides in *Bacillus anthracis* and *Bacillus cereus* strains

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ABBREVIATIONS

ATCC	American Type Cultre Collection
Ba	*B. anthracis*
Bc	*B. cereus*
BHI agar	Brain heart infusion agar
BSA	Bovine albumine serum
CDAP	1-cyano-4-dimethylaminopyridinium tetrafluroborate
CHO	Carbohydrates
CM	Cytoplasmic membrane
CPS	Capsular polysaccharide
Cry	Crystal protein (*B. thuringiensis*)
CWS	delipidated, deproteinized, cell wall skeleton
Cyt	Cytolytic protein
CytK	Cytotoxin K
DI water	Deionized water
DNA	Deoxyribonucleic acid
EA1	Extractable antigen 1
EdTx	Edema toxin
EF	Edema factor
FAO	Food and Agriculture Organization of the United Nations
Gal	Galactose
GalNAc	N-acetylgalactosamine
GC-MS	Gas chromatography mass spectrometry
Glc	Glucose
GlcN	Glucosamine
GlcNAc	N-acetylglucosamine
Hbl	Hemolysin BL
IgG	Immune globuline G
kb	kilo base pair

kDa	kilo Dalton
KLH	Keyhole limp hemocyanine
LeTx	Lethal toxin
LF	Lethal factor
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
ManNAc	N-acetylmannosamine
MAPKK	Mitogen activated protein kinase kinase
MPL	Monophosphoryl lipid A a derivative of diphosphoryl lipid A from *Salmonella minnesota* R595
MurN	Muramic acid
MurNAc	N-acetylmuramic acid
N-CAM	neural cell adhesion molecule
Nhe	Non hemolytic enterotoxin
OD	Optical density
OIE	World Organisation for Animal Health
OMe	O-methyl group
ORF	Open reading frame
PA broth	Phage assay broth
PG	Peptidoglycan
Pyr	Pyruvate
rRNA	Ribosomal Ribonucleic acid
Sap	Surface array protein
SCWP	Secondary cell wall polymer
SLH	Surface layer homology
TA	Teichuronic acid
TDM	trehalose dimycolate purified cord factor from *mycobacterium phlei*
TI-1	T-cell independent 1
TI-2	T-cell independent 2

WHO   World Health Organisation

WTA   Wall teichoic acid

Figures are numbered for each chapter separately. If not otherwise stated, the mentioned figure numbers refer to the figures in the same chapter.
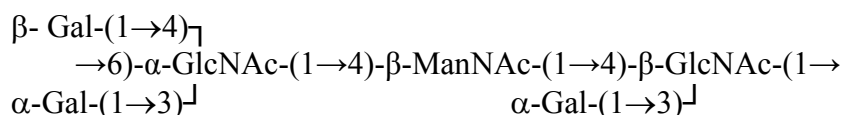
SUMMARY

The *Bacillus cereus* group of bacteria is comprised of the closely related species *B. cereus*, *B. anthracis, and B. thuringiensis*. As animal or human pathogens, all three species are of high biological relevance. A comparison of the cell wall glycosyl (CWG) composition of species from the *B. cereus* group has, to date, not been performed.

This thesis presents a systematic comparison of cell walls from closely related *B. cereus, B. thuringiensis* and *B. anthracis* strains. The strains used in this study have phylogentically been grouped into two clades and four lineages. Our findings showed that the CWG compositions varied significantly both qualitatively and quantitatively among the investigated strains. The cell walls from *B. anthracis* strains (Clade 1/Anthracis) contained glucose (Glc), galactose (Gal), *N*-acetylmannosamine (ManNAc), and *N*-acetylglucosamine (GlcNAc). Qualitatively, *B. cereus* strains belonging to Clade 1/Cereus IV (such as *B. cereus* G9241 and *B. cereus* 03BB87) had the same CWG composition as strains belonging to Clade 1/Anthracis. The cell walls from strains of Clade 1/Cereus III (e.g. *B. cereus* 03BB102) differed from those of Clade 1/Anthracis in that they contained an additional mannose (Man) residue. Strains belonging to Clade I/Cereus I (e.g. *B. cereus* ATCC 10987) differed from *B. anthracis* in that their cell walls additionally contained *N*-acetylgalactosamine (GalNAc). The cell walls from strains belonging to Clade 2 (e.g. the *B. cereus* type strain and *B. thuringiensis* strains) lacked Gal and contained GalNAc. Our findings suggest the CWG composition in the various *B. cereus* group strains display differences that correlate with their phylogenetic relatedness and that the carbohydrate profiles may have diagnostic value for strain recognition (**Chapter 2/{Leoff, 2008 #10326}**).

The treatment of the cell walls with aqueous hydrofluoric acid (HF) released a polysaccharide structure (named HF-PS). A comparison of the glycosyl compositions from the released and purified HF-PS structures of the different strains closely matched the differences that were observed for the cell wall compositions, suggesting that this cell wall fragment is likely a major or the major carbohydrate structure in these bacterial strains. Moreover, shifts in the relative amounts of the various sugars in the cell walls from *B. anthracis* strains with different plasmid content showed a possible impact of the
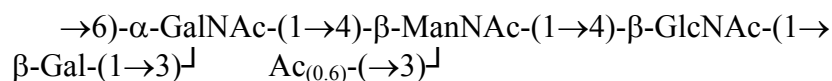
plasmids on the CWG composition. These results are, however, preliminary and need further investigations (**Chapter 2/{Leoff, 2008 #10326}**).

The structural analysis of the HF-PS isolated from *B. anthracis* cell walls showed that it is comprised of hexasaccharide repeating units with the following structure:

$$\beta\text{- Gal-}(1{\rightarrow}4){\urcorner}$$
$$\rightarrow 6)\text{-}\alpha\text{-GlcNAc-}(1{\rightarrow}4)\text{-}\beta\text{-ManNAc-}(1{\rightarrow}4)\text{-}\beta\text{-GlcNAc-}(1{\rightarrow}$$
$$\alpha\text{-Gal-}(1{\rightarrow}3){\lrcorner} \qquad\qquad \alpha\text{-Gal-}(1{\rightarrow}3){\lrcorner}$$

Microheterogeneity exists in this structure due to variation in the number of repeating units and in the level of substitution by galactosyl residues (3, 2, 1 or 0 Gal substitutions can occur). Details of this work are described in **Chapter 3 {Choudhury, 2006 #10325}.**

The structure analysis of the HF-PS from *B. cereus* ATCC 10987 showed that it is comprised of tetrasaccharide repeating units with the structure (**Chapter 4/{Leoff, 2008 #10510}**):

$$\rightarrow 6)\text{-}\alpha\text{-GalNAc-}(1{\rightarrow}4)\text{-}\beta\text{-ManNAc-}(1{\rightarrow}4)\text{-}\beta\text{-GlcNAc-}(1{\rightarrow}$$
$$\beta\text{-Gal-}(1{\rightarrow}3){\lrcorner} \qquad Ac_{(0.6)}\text{-}({\rightarrow}3){\lrcorner}$$

The HF-PS from *B. cereus* ATCC 10987 was heterogeneous due to a variation in the number of repeating units, the presence of O-acetyl groups (Ac), and the addition of ManNAc, GlcNAc, and GalGalNAc, respectively, to the di-, and, possibly, mono- and tri-repeat unit structures.

Mass spectrometric data obtained for the HF-PS from *B. cereus* type strain ATCC 14579 revealed that this HF-PS preparation consists of one, two, and three HexNAc$_3$Hex tetrasaccharide repeat units as observed for the *B. cereus* ATCC 10987 HF-PS, except that there was no evidence that the *B. cereus* ATCC 14579 HF-PS is acetylated. To date, the comparison of the different structures would suggest a conserved structural element in the sugar back bone of the HF-PSs composed of GlcNAc and ManNAc and, depending on the strain, a varying sugar of GlcNAc or GalNAc (**Chapter 4/{Leoff, 2008 #10510}**).

The analysis of structural data of HF-PSs from three pathogenic *B. cereus* strains (*B. cereus* G9241*, B. cereus* 03BB87 *and B. cereus* 03BB102) demonstrated more

similarities with the HF-PS from *B. anthracis* in that all three strains have the same amino-sugar backbone found in the *B. anthracis* HF-PS composed of [$\rightarrow$6)-α-D-GlcNAc-(1$\rightarrow$4)-β-D-ManNAc-(1$\rightarrow$4)-β-D-GlcNAc-(1$\rightarrow$]. The HF-PS of the pathogenic *B. cereus* isolates differed from the *B. anthracis* HF-PS only in that they have more galactosyl residues attached to several positions of the amino-sugar backbone (**Chapter 5**).

The suspected presence of a shared element in the HF-PS structures was supported by investigations into the immunochemical properties of the HF-PS structures. Enzyme linked immunosorbent assays (ELISA) revealed that antisera against live or killed spores from *B. anthracis* recognized not only the HF-PS from *B. anthracis* but also (albeit weaker) to the HF-PSs from the three pathogenic *B. cereus* strains G9241, 03BB87 and 03BB102. This demonstrated the antigenic nature of the HF-PS wall fragments. As these sera did not bind the HF-PSs from the closely related *B. cereus* ATCC 10987 or from *B. cereus* type strain ATCC 14579, they reacted specifically and indicated the possibility of shared epitopes in the HF-PS antigen structure from *B. anthracis* and those of the pathogenic *B. cereus* strains. These findings correlate well with the results from our CWG composition and the HF-PS structure analysis.

An antiserum against a *B. anthracis* HF-PS-keyhole limpet hemocyanine (KLH) conjugate was generated to investigate the immunogenicity of the HF-PS. This aforementioned antiserum contained IgG antibodies that bound spores, HF-PSs, cells and cell walls from *B. anthracis* strains as well as from the pathogenic *B. cereus* strains G9241, 03BB87, and 03BB102. However, the serum did not bind to HF-PS, cells and cell walls of the *B. cereus* type strain ATCC 14579 or of the closely related *B. cereus* ATCC 10987 strain. It did however bind to the HF-PS-BSA conjugates of all *B. cereus* strains tested, including *B. cereus* type strain ATCC 14579 (contrary to the anti spore sera!). This result suggests that the HF-PS-protein conjugates, but not the unconjugated HF-PSs, cells, or cell walls have common immunodominant epitopes.

In addition, we tested the sera from *Rhesus macaques* that had been exposed to an aerosol of *B. anthracis* spores and demonstrated that they contain IgG antibodies that recognize and bind to the *B. anthracis* HF-PS. The data also showed that (Anthrax Vaccine Adsorbed) AVA vaccinated animals contain low titers of HF-PS antibodies which increase to high antibody titers after the exposure to spores. These results support

the potential use of the HF-PS-conjugates to detect exposure of primates to *B. anthracis*, and for use as an alternative antigen component for the development of a new vaccine or to improve the current vaccine (**Chapter 6/{Leoff, 2008 #10546}**).

1

GENERAL INTRODUCTION

The *Bacillus cereus* group of bacteria:

The *Bacillus cereus* group of bacteria is a group of phylogenetically closely related species. It includes the species *B. anthracis, B. cereus, B. thuringiensis, and B. weihenstephanensis* (Taxonomy browser/Pubmed). The members of this group are Gram-positive, rod-shaped, aerobic or facultative anaerobic bacteria with the ability to form endospores (50). Bacilli can be observed as single cells or long chains of individual cells. The individual cell size ranges from 1 µm to 10 µm (28). The *B. cereus* group is comprised of non-pathogenic bacteria, but also of a number of important pathogenic members. The bacteria are easily isolated from almost all natural habitats and pose a risk to food safety and human health (53). There are considerable gaps in our knowledge of their molecular composition and architecture. For example, the occurrence, structure and function of carbohydrates in these bacteria is to large part unexplored despite the fact that they may have potential for medical uses such as for the developments of new vaccines, diagnostics, and therapeutics.

The taxonomy of the *B. cereus* group of bacteria:

An area currently of particular interest is the taxonomic and phylogenetic relatedness of members of the *B. cereus* group of bacteria. It turned out that the taxonomic and phylogenetic arrangement of strains within the genus *Bacillus* has been challenging and is, to a degree, still a matter of debate. Traditional taxonomy has used differential characteristics of the species within the *Bacillus cereus* group: that includes the growth at pH<6 in Voges-Proskauer broth, production of catalase, anaerobic growth and growth temperature, resistance to lysozyme, antibiotic sensitivity, metabolic carbohydrate utilization, and bacteriophage susceptibility (28), but none of these criteria

clearly distinguished between *B. cereus* and *B. thuringiensis* strains (28). Many new methods for the classification of bacteria have been developed, most of them on the basis of chemical composition and molecular architecture (66). Among the most promising methods is the hybridization of 16S and 23S rRNA to immobilized DNA probes, the comparison of 16S or 23S rRNA sequences or 16S-23S rRNA spacer regions (32, 65). However, within the members of the *B. cereus* group the sequences or spacer regions were >99% identical and therefore not useful for the differentiation between these closely related strains (15, 16, 19, 31). Overall, the phylogenetic picture that emerged from the use of these modern molecular methods for the *B. cereus* group is only partially in accordance with the schemes resulting from traditional classification (18, 24, 58, 59, 68, 72, 73).

One of the new methods that was introduced for the identification of pathogenic bacteria is called multilocus sequence typing (MLST) (85). The MLST analysis compares polymerase chain reaction (PCR) amplified and sequenced internal fragments from a number (usually 7) of different housekeeping genes. This method has been shown to be a fast, accurate, and highly discriminating way of taxonomical differentiation (85). Five separate MLST schemes have been used for the typing of strains from the *B. cereus* group (59, 73, 107, 124, 130). Despite some discrepancy within the different *B. cereus* group schemes, the results were consistent in dividing this group of related bacteria into three main phylogenetic clusters. One cluster contains the *B. anthracis* strains and a number of *B. cereus* and *B. thuringiensis* strains most of which are clinical isolates. A second cluster contains *B. cereus*, including the *B. cereus* type strain ATCC 14579 (73, 107, 124) and *B. thuringiensis* strains from various sources, and a third cluster includes the cold tolerant *B. weihenstephanensis* isolates and a few *B. cereus* strains (131). The classifications based on the MLST schemes from Ko *et al*. (73), Priest *et al*. (107), and Sorokin *et al*. (124) are especially interesting as they place the pathogenic *B. cereus* group strains phylogenetically close to *B. anthracis*.

Pathogenicity of the *B. cereus* group

□ *Insect pathogen: Bacillus thuringiensis*

B. *thuringiensis* is mainly known as an insect pathogen (27). An interesting characteristic of *B. thuringiensis* is the formation of one or more parasporal crystalline inclusion bodies during the sporulation which is frequently used as a criterion to differentiate *B. thuringiensis* from other *Bacilli* (8, 9, 27). The inclusion bodies, now known as crystal toxin or crystal protein, were first described in 1953 (56). Experiments showed that the crystal toxin caused midgut paralysis in most insects, whereas, the spores induced septicemia (10, 11). This led to its industrial use in the production of specific and environmentally safe insecticides. Today, *B. thuringiensis* is widely used in cotton production in Texas, Mississippi, and Louisiana as well as in the production of fruits and vegetables in California, Arizona, and Florida (136-138).

□ *Human pathogens: Bacillus cereus and Bacillus anthracis*

B. *cereus* is mainly known for causing two types of food poisoning (51). One type, the emeritic form, exhibits nausea, vomiting and abdominal cramps after a short incubation time of 1- 6h (129). These symptoms are caused by the emetic toxin (78, 79). The emetic toxin or cereulide is a cyclic dodecadepsipeptide that is heat and acid stable (133). Cereulide functions through the 5-HT$_3$ receptor and stimulates the *vagus afferent* (3). It was also connected to the inhibition of hepatic mitochondrial fatty acid oxidation and liver failure (2, 84).

The second type of food poisoning is also called the diarrheal form. It manifests itself in abdominal cramps and diarrhea after ingestion and 8-16h incubation time (129). Three enterotoxins are described to cause the symptoms of the diarrheal form of food poisoning: the protein complex Hbl (hemolysin BL), the protein complex Nhe (non hemolytic enterotoxin) and a single protein CytK (cytotoxin CytK) (20, 52, 82). The Hbl is a three component hemolytic enzyme that promotes cell lysis and fluid accumulation (20, 35). The Nhe complex is also a three component protein complex which induces osmotic lysis by forming pores (52). The CytK is a necrotic and hemolytic toxin (82). Its cytotoxic effects were demonstrated for intestinal epithelia which most likely contribute significantly to the diarrheal symptoms (57).

B. cereus has also been connected to a variety of non-gastrointestinal infections such as endocarditis (126), wound infections (69), osteomyelitis (114) and even infections of the root canal or periodontal pockets (86). Most concerning, however, were reports of anthrax-like infections caused by *B. cereus* that were shown to carry *B. anthracis* toxin genes (60, 61).

B. anthracis, the probably best known pathogen in the *B. cereus* group, is the etiological agent of anthrax (75). Anthrax is mainly a disease in herbivores although humans can be infected through exposure to *B. anthracis* spores. The three clinical forms of anthrax; cutaneous, gastrointestinal and pulmonary anthrax are, if untreated, potentially fatal. The cutaneous form accounts for approximately 95% of the incidents. A cut or abrasion of the skin while handling contaminated wool, hides, leather or infected animals is the point of entry for the bacterium. The hallmark symptom of cutaneous anthrax is a raised bump that develops into a black necrotic ulcer. Gastrointestinal anthrax can be conducted through the consumption of contaminated meat. Symptoms are rather general and include nausea, loss off appetite, fever followed by abdominal cramps, vomiting of blood and severe diarrhea which is fatal in $25 - 60\%$ of the anthrax cases. Pulmonary anthrax is by far the most severe form. Initial symptoms similar to a common cold rapidly progress within a few days. The disease is usually fatal due to late or incorrect diagnosis (26, 33).

Virulence factors of *B. anthracis*

The virulence factors of *B. anthracis* currently known to be responsible for the anthrax disease are encoded on two virulence plasmids pXO1 and pXO2 that carry among others the genes for toxins and the capsule formation (94, 100, 122). The plasmids are not essential for growth, and a number of *B. anthracis* strains derivatives exist that do not carry both virulence plasmids. Besides *B. anthracis* Ames strains that contain both plasmids pXO1 and pXO2, *B. anthracis* Sterne strains contain plasmid pXO1 but not pXO2, while *B. anthracis* Pasteur strains contain plasmid pXO2 but not pXO1. Strains with both plasmids are considered to be genetically complete and highly pathogenic, although strains with only one of the plasmids retain some of their

pathogenicity at least for some host organisms, e.g. the *B. anthracis* Sterne is still a potent mouse pathogen (146).

Plasmid pXO1 harbors the *B. anthracis* toxin genes *pagA, cya,* and *lef* (100). These genes encode for the proteins protective antigen (PA), edema factor (EF) and lethal factor (LF). The proteins form two toxin complexes that were the first multicomponent toxins described in the literature (122). The toxin complexes are comprised of protective antigen (PA) and one of two enzymatic parts, lethal factor (LF) or edema factor (EF) to build the lethal toxin LeTx (LeTx=PA+LF) complex or the edema toxin EdTx (EdTx=PA+EF) complex (47, 125). The LF factor is a zinc endoprotease. As LeTx complex, it inactivates members of the mitogen-activated protein kinase kinase (MAPKK) family (37, 140). The EF factor has an adenylate cyclase acitivity. As EdTx toxin complex it increases intracellular cAMP levels (81). The PA factor is a secreted protein that is activated through binding to extracellular cell surface receptors. Upon cleavage into a small and a large subunit, the larger unit heptamerizes to form a prepore complex while the smaller one diffuses away. LF or EF bind to the heptamer and are internalized via receptor mediated endocytosis (76). For details on the structure and functions of the *B. anthracis* toxins complexes see Mock *et al.*(94) and Collier *et al.*(29).

Treatment and prevention of anthrax

□ *Antibiotic treatment*

A prompt and timely antibiotic treatment is crucial for the recovery from the disease. Mild cases of cutaneous anthrax are usually treated with intramuscular procaine penicillin (600mg every 12-24h) or intramuscular benzylpenicillin (penicillin G, 250 000 units every 6h). In severe cases of gastrointestinal or pulmonary anthrax a combination treatment of penicillin G (1200mg intravenous per day with streptomycin, 1-2g intramuscular per day) is recommended. Alternative antibiotics include tetracyclines, chloramphenicol, gentamycin and erythromycin. From the tetracycline family, doxycycline is especially effective whereas quinolone and ciprofloxacin are also suitable (48).

□ Vaccines

The currently available veterinary vaccines are using toxigenic non-encapsulated pXO1$^+$/pXO2$^-$ *B. anthracis* Sterne 34F$_2$ strains for vaccination (134). Human vaccines are available in form of live spore vaccine (in China and in countries of the former USSR) and in form of cell free culture filtrates of either *B. anthracis* Sterne 34F$_2$ (in UK) or non-encapsulated, non proteolytic derivatives of bovine isolate V770-NP1-R (Serotype-2) (in US) (134). The anthrax vaccines for humans as well as for animals induce in the immune system the production of anti-protective antigen (anti-PA) antibodies. Immune responses to the other two components, lethal factor (LF) and edema factor (EF) may contribute to the protection (62, 132). Currently, a number of vaccines are being developed that make use of recombinant proteins, such as rPA. However, to date, neither a fully synthetic vaccine has been developed nor have other potential antigenic components, such as cell surface carbohydrates, been tested as vaccine components.

Scope of medically related uses of carbohydrates:

Until recently, carbohydrates were thought to have two main functions. There was extensive knowledge about (i) their role as energy source for animals, plants and microorganisms as well as (ii) their ability to give structural support in plant and bacterial cell walls. We now know that carbohydrates are ubiquitous in humans and higher developed animals where they play a role in many physiological aspects oftentimes in the form of glycoproteins. A substantial portion of today's carbohydrate research is to understand the involvement of carbohydrates in human disease and utilize them in diagnostics, treatment and prevention.

□ *Diagnostic use of carbohydrates*

Diagnostic tests based on carbohydrate recognition are widely used in microbial pathogen recognition. For example, diagnostic serotyping based on the surface expression of carbohydrates such as lipopolysaccharide O-antigen or K-antigen (capsular polysaccharide) have been developed for *E. coli, Salmonella*, *Haemophilus influenza, Neisseria meningitides* and others (5, 43, 44, 46, 49, 102, 147). An example for the use of carbohydrates in diagnostics of Gram-positive bacteria is *Streptococcus*

pneumonia, where the pneumococcal polysaccharides consist of a large variety of structures making extensive serotyping possible (6, 64, 96).

□   *Carbohydrate based vaccines*

The potential of carbohydrates as potent components in the development of vaccines has been recognized for some time already. Vaccines based on capsular polysaccharide or neoglycoconjugates are in use in the prevention of infections with *Neisseria meningitides*, *Streptococcus pneumoniae*, *Haemophilus influenza* type b (Hib) and *Salmonella typhi* (1). Even anti-cancer vaccines based on carbohydrates are in development and are currently tested for their efficacy in cancer prevention (120, 121).

□   *Carbohydrate based therapeutics*

This is currently an important and intense area of research. The usefulness of carbohydrates for novel therapeutic approaches has already been demonstrated, e.g. there use brought a breakthrough in anti-inflammatory and anti-thrombotic treatment where synthetic heparin has been used to prevent coagulation (115). Carbohydrates are also usedl in new therapeutics for the treatment of type-2 diabetes or experimentally used for HIV-1 therapeutics (17, 115).

One question that underlies the work presented in this thesis is whether bacteria belonging to the *B. cereus* group contain cell wall carbohydrates that could hold promise for the development of new and improved medical uses for the diagnosis, prevention, and treatment of anthrax and disease caused by pathogenic *B. cereus* strains. To address the objectives of this work in more detail, a short review of the carbohydrate in Gram-positive bacilli, in particular in the *B. cereus* group of bacteria is needed.

Architecture and Carbohydrates of the Gram positive bacilli cell wall:

□   *Gram positive cell envelope::*

The cell envelope is the outer part of the bacterial cell. This includes in Gram-positive bacteria the cytoplasmic membrane, a thick peptidoglycan layer with associated secondary cell wall polymers and, in some species, an additional capsule and/or S-layers.

□ *Capsules:*

A capsule is a layer of excreted polysaccharides or polypeptides that surrounds the bacterial cell. It can have multiple functions as a protective shield and/or can enable the bacteria to evade the host immune system while inhibiting the phagocytosis through macrophages (70, 87). *Bacillus anthracis* produces a poly-D-glutamic acid capsule. The genes for capsule formation are arranged on *B. anthracis*'s second virulence plasmid, pXO2, in an operon of four genes *capB, capC, capA* and *dep. The CapB, CapC, and CapA enzymes are membrane-associated with molecular masses ranging from 16.5 kDa to 46.4 kDa. The Dep protein has mass of 51.4 kDa; it is thought to control the size of the poly-glutamate capsule by catalyzing the hydrolysis of this polypeptide (87, 135). The exact biosynthetic steps of capsule formation in *B. anthracis* are still unclear. However, speculation is that its biosynthesis has some similarity to the polypeptide biosynthesis in *B. licheniformis* (76, 80). Much remains to be learned about capsule formation. The capsule was the first virulence factor described for *B. anthracis* (106). Its production enhances the virulence of pXO2 carrying *B. anthracis* strains (149) and can be induced in vitro through growth with >5% CO_2 and added bicarbonate or serum (91). There are also indications for the involvement of the global regulator *atxA*. In a genetically complete strain, containing both pXO1 and pXO2, *atxA* has been shown to be indirectly involved in the upregulation of the capsule biosynthesis operon *capBCAD* located on pXO2 (36, 139).

Although *B. thuringiensis* and *B. cereus* are typically not encapsulated (28), a non-poly-D-glutamic acid capsule production has been observed in three *B. cereus* strains associated with severe pneumonia (127). The capsule production was verified by PCR analysis. This analysis showed the presence of putative polysaccharide capsule biosynthesis genes on a previously unknown plasmid pBC218 in *B. cereus* G9241 and *B. cereus* 03BB87 (61, 127). The genes for capsule production in the third strain *B. cereus* 03BB102 have not yet been confirmed by PCR analysis. This could mean that either the primer binding regions are slightly altered in that strain and, therefore, could not be detected or the genes for the capsule production differ in this strain from the other two strains (127). The existence of capsules were confirmed, however, for all three strains at various expression levels depending on growth conditions, using Indian ink as capsule stains (127).

□ *S-layers*

When present, S-layers are the outermost layer of bacterial cells. They represent an important interface between these cells and their environment (21, 90, 119). S-layers are proteinacious surface layers composed of proteins or glycoproteins that form uniform, paracrystalline lattices of square, hexagonal or oblique appearance on the cell surface. They function as a protective coat, as molecular sieves in the ultrafiltration range, or as a molecule and ion traps. Very recently, it has been noticed that they may also be involved in peptidoglycan metabolism(4) They also represent target structures which promote cell adhesion and surface recognition (71, 118). Based on their involvement in bacteria-host interaction, S-layers have been suggested as a virulence factor (23, 67, 77, 90).

B. anthracis synthesizes two S-layer proteins, namely Sap (surface array protein) and EA1 (extractable antigen 1) (40, 41, 89, 93). Their expression requires the transcription of one of two chromosomal genes, *sap* or *eag* that lay close together as independent transcription units on the chromosome of the bacteria. They translate into two 94 kDa proteins that form a two dimensional crystalline layer of proteins on the *B. anthracis* cell surface (Figure 3) (42). It has been shown that *B. anthracis* expresses Sap during exponential growth and EA1 during the stationary phase (93), indicating that S-layers may be growth dependent dynamic structures. The global regulator *atxA* of *B. anthracis* is also indirectly involved in the transcription of the S-layer proteins. Here *atxA* is responsible for the pXO1 mediated suppression and activation of *sap* and *eag* respectively (92).

Both protein structures of Sap and EA1 start with a short signal peptide (117) that is followed by three S-layer homology motifs (SLH-motif). SLH domains are short sequence motifs that play an essential role in the targeting of surface proteins to the cell wall. In *B. anthracis* Ames genome, there are 20 chromosomal genes, two genes on plasmid pXO1 and one on pXO2 that carry a SLH-domain (94). These genes encode various proteins with a range of surface or enzymatic functions. For Sap and EA1 it has been shown that the SLH-domain of the protein non-covalently binds to secondary cell wall structure (i.e. a carbohydrate polymer) which in turn firmly anchors the S-layer proteins to the peptidoglycan (83, 101, 108, 109). Obligatory for a successful anchoring is hereby the operon *csaAB*. In *B. anthracis* the product of the gene *csaB* is reportedly

involved in the addition of a pyruvyl group to a cell wall associated polysaccharide (88). It can be expected that in *B. anthracis* other proteins with SLH-domains are anchored to the cell wall in a similar fashion to Sap and EA1, via a carbohydrate polymer. Our current picture on these cell wall associated polysaccharides is discussed in more detail below.

□ *Peptidoglycan and cytoplasmic membrane*

The structural integrity of the *Bacillus* cell is secured by a thick layer of peptidoglycan (PG) that surrounds the cytoplasmic membrane; it constitutes a rigid layer that prevents lysis of cells through osmotic pressure and confers form and strength to cells by withstanding tensile forces (74, 116). The complex PG structure is a composite of multiple linear polysaccharide strands with alternating residues of β-1,4 linked N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) residues, cross-linked by amino acid side chains. Secondary modifications to the glycan part e.g. deacetylation and variation in the amino acid combination of the peptide linker or the position of the interpeptide bridge are possible (141). The degree to which the peptide linker can vary is limited based on the occurrence of enzymes such as muramic acid ligases (MurC, MurD, MurE and MurF) or transferases that mediate in the biosynthesis and turnover of the PG (25). The three dimensional assembling of the PG is still subject of an ongoing debate where two mutually exclusive models – peptidoglycan sheet and peptidoglycan scaffolding model - are under discussion (34, 142).

The cytoplasmic membrane is a semi-permeable membrane with a complex composition. It separates the cell inside from the outside, retains the cytoplasm of the cell, and is a barrier against uncontrolled ion exchange. It consists of a phospholipid bilayer with embedded proteins with main functions in ion transport, as receptors, and enzymes (128).
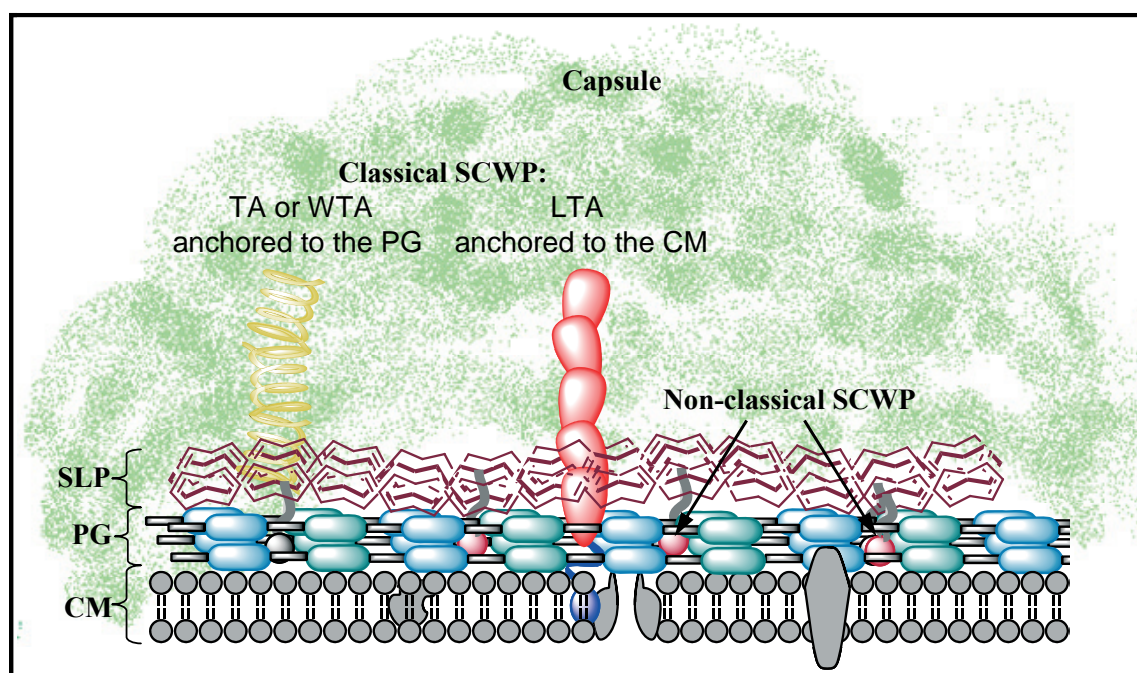
Figure 3: **Schematic representation of cell wall associated structures in Gram-positive *Bacillus* bacteria (the structures are to degree assumptions as a number of details have not yet been described):** The peptidoglycan (PG), is one of the largest components of the Gram-positive cell; it lies between the cytoplasm enclosing cytoplasmic membrane (CM) and the S-layer proteins (SLP). Secondary cell wall polymers (SCWP; in *B. anthracis* a SCWP [red ball] confers SLP attachment to PG) are anchored either to the CM (e.g. lipoteichoic acid (LTA/red) or to the PG (e.g. wall teichoic acid (WTA, yellow), teichuronic acid (TA). The whole cell can be surrounded by an additional capsule (green) (113, 145).

□ *Classical and non-classical cell wall associated carbohydrates of the* B. cereus

   group

   › Classical SCWP

Gram-positive bacteria have linked to the cytoplasmic membrane and the PG so called secondary cell wall polymers (SCWP) that are of glycosylic nature (111). The SCWP of Gram-positive bacteria can be divided into three groups based on their structural characteristics. (i) Teichoic acids or lipoteichoic acids are short phosphate containing polymer chains that can be attached to the PG over a lipid anchor (13, 14, 45). (ii) Teichuronic acids are uronic acid-containing heteropolysaccharides (55, 143). (iii) A third group includes neutral and acidic polysaccharides which cannot be placed in one

of the other groups (12, 98), and include the non-classical SCWP which are described further below.

The first two groups are given the attribute "classical" because originally they were thought to contribute to the basic cell functions (14, 105). However, much remains to be learned about SCWP cell functions. For example, only recently it was demonstrated that wall teichoic acids are not essential for viability of *B. subtilis* and *S. aureus* (30, 144). Most research on teichoic acids in the *Bacillaceae* family has been focused on *B. subtilis* (which is not a member of the *Bacillus cereus* group). In *B. subtilis*, one lipoteichoic acid (LTA), two distinct wall teichoic acids (WTA) and one teichuronic acid (TA) were reported (22). The expression of the teichoic acids and the teichuronic acid is genetically regulated and depends on phosphate concentration (39, 54, 123). Hardly any information is available about the classical SCWC of *B. cereus* group members. In *B. thuringiensis,* a few studies looked into the presence of teichoic acids and teichuronic acids. For *B. cereus;* the little research that has been done on the cell walls of these bacteria indicates that carbohydrates seemed to vary from strain to strain (63).

An indicative marker commonly used to screen in Gram-positives for wall teichoic acids is the presence of a wall teichoic acid biosynthetic pathway, especially the protein TagF. The TagF is involved in the polymerization of polyolphosphate chains (104, 112). As none of the *B. cereus* group members have the gene *tagF,* it was suggested *that* they are incapable of synthesizing teichoic acids (7). A genome comparison between members of the *B. cereus* group with *B. subtilis* gene orthologs to the *B. subtilis dltABCD* operon were found in all members of the *B. cereus* group (7). The genes in the *dltABCD* operon encode for enzymes involved in D-alanylation of teichoic and lipoteichoic acids (99, 103). As the functions of these genes have not yet been clarified, the synthesis of lipoteichoic acids in *B. cereus* group members at this point cannot be completely ruled out. In fact, there have been older reports about the isolation and biosynthesis of polyglycerolphosphates in *B. cereus* AHU1030 (97, 110) This observation is in need of clarification; it questions the reliability of a classification method based on the presence or absence of teichoic acids to differentiate into strains belonging to *B. subtilis* or strains belonging to *B. cereus* and its relatives.

For *B. anthracis* there is reported biochemical evidence that it does not form teichoic or lipoteichoic acids (76, 95). Genetic studies also showed that *B. anthracis* lacks crucial transport genes and the genes for teichoic acid permeases, such as *tagG* (www.tigr.org). Therefore, at least for the time being, it is assumed that *B. anthracis* is not capable of biosynthesizing teichoic and lipoteichoic acid. The presence or absence of teichuronic acids in *B. anthracis* has not yet been addressed and remains an open question.

› Non-classical SCWP

Non-classical SCWP are carbohydrate structures of neutral or acidic character that can not be placed into the group of teichoic-, lipoteichoic or teichuronic acids (12, 98, 111). A few of these non-classical SCWP polysaccharide structures have been determined and are shown in Figure 4.

In *B. anthracis* cells, a cell wall fragment that is falling into the class of non-classical SCWP is a carbohydrate fragment that can be released from the cell wall through treatment with aqueous hydrofluoric acid (HF). This isolated polysaccharide fragment with a mass of 12 kDa was until recently the only reported cell wall associated carbohydrate (38). The polysaccharide is composed of galactose, N-acetylglucosamine and N-acetylmannosamine (38). It is proposed to be a ligand of the SLH domain that connects the S-layer protein with the peptidoglycan via a HF-labile phosphate bond (88). It was also suggested that this anchoring mechanism is widely spread among prokaryotes and requires the pyruvylation of the polysaccharide in order to anchor or export the S-layer proteins (88). The molecular structure of this glycosyl anchor and its functional details were until recently unknown and are part of this thesis. However, related structures have been reported for a few *B. cereus* and *B. subtilis* strains (111) and are shown in Figure 4. For example, for *B. cereus* AHU 1356 a N-acetylglucosaminyl pyrophosphorylundecaprenol is reported to be a precursor in the synthesis of the non-classical SCWP of this strain (148). Nevertheless, much remains to be learned about the occurance and structural variability of the non-classical SCWP in the *B. cereus* group of bacteria. This will lay the groundwork for future investigations in their functions in cell viability and pathogenicity as well as their usefulness for applied purposes.

Group 1:
-*Paenibacillus polymyxa* AHU1335
-*P. alvei* CCM2051
-*B. spaericus* CCM2177

Pyr-(4,6)⌐
→3)-β-ManNAc-(1→4)-β-GlcNAc-(1→

-Kojima et al. 1988
-Schäffer & Messner 2005
-Schäffer & Messner 2005

Group 2:
-*Geobacillus stearothermophilus* NRS 2004/3a

→4)-α-Man-2,3-di-NAcA-(1→6)-β-Glc-(1→4)-α-Man-2,3-di-NAcA-(1→
→3)-β-GlcNAc-(1→

-Schäffer & Messner 2005

-*Geobacillus tepidamans* GS5-97

$R_{1,2,3}$⌐
→4)-α-Man-2,3-di-NAcA-(1→6)-β-Glc-(1→4)-α-Man-2,3-di-NAcA-(1
→3)-β-GlcNAc-(1→

(R_1=CONH$_2$, R_2=CONHCOCH$_3$, R_3=CON(COCH$_3$)$_2$)

-Schäffer & Messner 2005

Group 3:
-*B. subtilis* AHU1219

β-GlcNAc-(1→3)⌐
→3)-α-GalNAc-(1→4)-β-ManNAc-(1→3)-α-GlcNAc-(1→
α-Glc-(1→6)⌐ β-GlcNAc-(1→6)-β-GlcNAc-(1→6)⌐

-Iwasaki et. al. 1989

-*B. cereus* AHU1356

β-GlcNAc-(1→3)⌐
→6)-α-GalNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc-(1→
β-Glc-(1→3)⌐

-Amano et al. 1977

Figure 4: Structures of non-classical SCWP's of S-layer carrying *Bacillaceae*. Pyr - pyruvate, Man-2,3-di-NAcA – 2,3-di-N-acetylmannosaminuronic acid, GlcNAc – N-acetylglucosamine, GalNAc – N-acetylgalactosamine, Glc – glucose,

Questions and objectives addressed in this work:

As exemplified above, carbohydrates have been proven to be involved in various disorders and diseases and a more detailed knowledge about their structures and functions is indispensable in order to optimize disease management and development of new carbohydrate-based approaches in disease treatment and prevention. Our knowledge about cell wall carbohydrates in members of the *B. cereus* group is marginal and unsatisfactory and much remains to be learned about this group of bacteria.

To date, no systematic investigation into the molecular composition and structures of non-classical secondary cell wall polymers of the *B. cereus* group of bacteria has been performed. Hence, it is not known how wide spread and specific these structures in this bacterial group are and whether they have antigenic properties. Therefore, the specific questions of this thesis were:

- What is the carbohydrate composition of the cell walls and the non-classical SCWP from *B. cereus* members? Are the carbohydrate compositions from all *B. cereus* group members identical or are there species or strain specific variations?

- What is the molecular structure of the non-classical SCWP isolated from *B. anthracis*?

- What is the molecular structure of the non-classical SCWP in pathogenic and non-pathogenic *B. cereus* strains?

- How do the non-classical SCWP structures of *B. cereus* compare with the structure from *B. anthracis*?

- What are the immunochemical properties of the isolated non-classical SCWP structures, the cell walls or cells from *B. anthracis* and *B. cereus* strains?

The main objectives of this work have been to characterize the cell wall carbohydrate, in particular the non-classical SCWPs in the *B. cereus* group of bacteria with regard to occurrence and structural uniqueness. These investigations are crucial for future studies into the role of the non-classical SCWPs in cell viability and pathogenicity. This will also lay the groundwork for applied investigations into their usefulness as diagnostic tools, therapeutics and potential vaccine components.

The following chapters will describe the outcomes of this quest in form of published articles or manuscripts that are in preparation for publication. Each chapter

has its own introduction and discussion specifically tailored to elaborate the described findings and their value for this area of research.

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Cell wall carbohydrate compositions of strains from the *B. cereus* group of species correlate with phylogenetic relatedness

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Running Title: *Bacillus anthracis* cell wall carbohydrates.

Contribution to this publication:

I prepared the first draft of this manuscript and was involved in the design and configuration of tables and figures. The isolation of the cell walls and purification of the HF-PS as well as their analyses by gas chromatography mass spectrometry was performed by me. Elke Saile was responsible for growing the bacterial cultures used in the experiments and preparing them for shipment. The bacterial strains required cultivation in BSL2 and BSL3 laboratories at the Center for Disease Control (CDC).

Key findings and conclusions:

- The cell wall glycosyl (CWG) compositions of *B. cereus* group members vary in a manner that suggests possible correlations with their phylogenetic relatedness.
- The CWG compositions of *B. cereus* group members vary qualitatively between the lineages and quantitatively between strains of the same lineage.
- The CWG composition of different *B. anthracis* strains varies in a plasmid dependent manner.
- CWG composition of *B. cereus* strains that caused severe pneumonia is most similar to the one from *B. anthracis*.
- The glycosyl composition of isolated polysaccharide structures followed the strain specific trend.
- These findings could be useful in the taxonomic classification of the *B. cereus* group.
- The CWG composition could serve as criteria in the differentiation of strains from this group.

ABSTRACT

Members of the *Bacillus cereus* group contain cell wall carbohydrates that vary in their glycosyl compositions. Recent multi-locus sequence typing (MLST) refined the relatedness of *B. cereus* group members by separating them into clades and lineages. Based on MLST, we selected several *B. anthracis*, *B. cereus* and *B. thuringiensis* strains and compared their cell wall carbohydrates. The cell walls of different *B. anthracis* strains (Clade 1/Anthracis) were composed of glucose (Glc), galactose (Gal), *N*-acetyl mannosamine (ManNAc), and *N*-acetyl glucosamine (GlcNAc). In contrast, the cell walls from Clade 2 strains (*B. cereus* type strain ATCC 14579, and of *B. thuringiensis* strains) lacked Gal, and contained *N*-acetylgalactosamine (GalNAc). The *B. cereus* Clade 1 strains had cell walls that were similar in composition to *B. anthracis* in that they all contained Gal. However, the cell walls from some Clade 1 strains also contained GalNAc which was not present in *B. anthracis* cell walls. Three recently identified Clade 1 strains of *B. cereus* that caused severe pneumonia, i.e. strains 03BB102, 03BB87, and G9241, had cell wall compositions that closely resembled those of the *B. anthracis* strains. It was also observed that *B. anthracis* strains cell wall glycosyl compositions differed from one another in a plasmid-dependent manner. When plasmid pXO2 was absent, the ManNAc/Gal ratio decreased while the Glc/Gal ratio increased. Also, deletion of *atxA*, a global regulatory gene, from a pXO2-minus strain resulted in cell walls with an even greater level of Glc.

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INTRODUCTION

The *Bacillus cereus* group of bacteria contains the closely related species *B. cereus*, *B. anthracis,* and *B. thuringiensis*. *Bacillus cereus* can be a potent opportunistic pathogen, while *B. thuringiensis* is an insect pathogen, and *B. anthracis* is the causative organism of anthrax. The distribution of *B. anthracis* spores in the US mail system in 2001 demonstrated their potential as a bioterrorist weapon. Because of the risks these strains pose for public health, rapid differentiation and identification of the members, the *B. cereus* group of strains, through molecular means is an important and ongoing endeavor in many laboratories.

To date, the cell wall carbohydrates in the *Bacillus cereus* group of strains have, nonetheless, not been investigated systematically with regard to occurrence, structural peculiarities and their usefulness for taxonomic classification and strain identification. Carbohydrates are a common feature of bacterial cell walls, e.g. as capsules, as S-layer protein components, or various other cell wall glycoconjugates. In the *B. cereus* group of strains, these cell wall components can vary from strain to strain and display heterogeneity: For example, while *B. cereus* and *B. thuringiensis* are typically not encapsulated, in a number of pathogenic *B. cereus* strains the occurrence of, as yet not fully characterized capsules was indicated, which seems to be, at least in one case, carbohydrate in nature (45). In contrast, pathogenic *B. anthracis* cells are surrounded by capsules that are comprised of poly-γ-D-glutamic acid (18). In a survey on S-layer distribution in strains of the *B. cereus* group, about 40 % of all strains had S-layers. Of the strains that originated from clinical isolates, the vast majority (approximately 70 %) contained S-layers in their cell walls (35). The carbohydrate components of S-layers in *B. cereus* group of strains have so far not been characterized systematically.

In many bacterial genera the cell walls are well established as diagnostic targets (1, 34, 48, 49), carbohydrate-based vaccine antigens (30, 49), and virulence factors (36). Therefore, the characterization of cell walls of *B. anthracis* and other strains of the *B. cereus* group could be important for identifying potential vaccine antigens, diagnostics, and to elucidate the molecular basis for their virulence and pathogenicity. Infection by pathogenic strains of the *B. cereus* group likely involves multiple components of the cell wall, including the cell wall carbohydrate-containing components that interact with the

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host. During an infection, these cell wall components may function in bacterial adhesion to host cells and also as barriers to the host defense mechanism, thereby acting as virulence factors. Should the cell wall carbohydrate of *B. anthracis* prove to have such functions, as is the case with many other bacteria, they would ensure its structural conservation and, thus, make it a potentially good candidate for identification and classification of *Bacillus* species, as well as for development into a vaccine antigen.

Traditionally, *Bacillus* species have been differentiated based on their phenotypic and biochemical characteristics. Recently, molecular methods of classification have become more prevalent (2, 3, 19, 20, 23, 25, 27). These molecular classification methods have been used to re-group *Bacillus* strains. The phylogenetic picture that is emerging from these studies for strains of *B. cereus* group is only partially in accordance with the more traditional classification scheme and is, to a degree, still in flux. For example, the *B. cereus* group strains have traditionally been classified as three species; *B. cereus, B. thuringiensis,* and *B. anthracis*. In contrast, fluorescent heteroduplex analysis placed these species in only two subgroups (32). Similarly, albeit on a different set of bacterial strains, the analysis of small acid soluble proteins in the *B. cereus* group with mass spectrometry (SASPs) led also to only two subgroups (6, 7). These recent findings, as well as those based on other methods including comparative *Bacillus* species genome analyses, will alter the more traditional *Bacillus* taxonomic groupings (40, 42). In particular, among these molecular approaches, multi-locus sequence typing (MLST) analysis (2, 20, 27, 39) is widely used because of its power to resolve the relatedness of even closely related strains because its findings are unambiguous, and because the method is truly portable among laboratories (20) .

Using MLST, a study published in 2004 reported that a collection of *B. cereus* group strains representing 59 sequence types could be assigned to 3 clades and 9 lineages (39). Also using MLST, the same laboratory evaluated the phylogeny of invasive *B. cereus* isolated from clinical infections (2). Interestingly, the study showed that pathogenic strains were not restricted to a single clonal group or lineage but were genomically diverse and related to strains traditionally grouped as *B. anthracis*, *B. cereus*, or *B. thuringiensis*. These findings were particularly interesting since it showed that all *B. cereus* group strains obtained from human or animal infections, including anthrax and bacterial pneumonia, are closely related to each other (2, 39). Little is

known about the carbohydrates that comprise the cell walls of pathogenic *B. cereus* group strains. However, recent insights into the relatedness of these strains raise the intriguing question whether function or phylogenetic relatedness governs the occurrence of their cell wall carbohydrate.

Previous studies have established a precedent for distinctive glycosyl compositions of the total cell walls of representative strains from *B. anthracis*, *B. cereus*, and *B. thuringiensis*. For example, galactose (Gal) was found only in *B. anthracis* cell walls, while glucose (Glc) and *N*-acetylgalactosamine (GalNAc) were present in *B. cereus* cell walls (17, 50). These published data suggest that there could be cell wall carbohydrates that are specific to each of these three *Bacillus* species. However, a systematic comparison of the cell wall compositions/structures from members of the *B. cereus* group of bacteria as a function of the more detailed MLST phylogenetic classification has not been reported. In the present study we investigated the glycosyl compositions of the cell walls from a collection of strains of the *B. cereus* group (Table 1) with characterized phylogenetic relatedness based on MLST analysis. The strains in this report were picked on the basis of their MLST phylogenetic relatedness as put forward by Priest *et al.* (39) with a suggested modification adding the Cereus IV lineage to Clade 1 (37). In addition, since recent sequencing projects of whole genomes from *B. cereus* group strains showed that genes involved in carbohydrate biosynthesis and metabolism are localized not only on the chromosome, but can also be encoded on plasmids (41), we investigated whether cell wall composition is influenced by the virulence plasmid content in selected *B. anthracis* strains. The data demonstrate that there is variation in the glycosyl compositions of cell walls among even closely related *B. cereus* group strains and that this compositional variation correlates with differences in phylogenetic relatedness. Further, we show that at standard laboratory growth conditions the types of carbohydrates found in the cell walls of *B. anthracis* strains may depend, to some extent, on their virulence plasmid content.

MATERIAL AND METHODS

Bacterial strains and culture conditions.

Most *B. anthracis* strains were obtained from the CDC culture collection. The strains *B. anthracis* 7702 and UT60 were obtained from T. Koehler, University of Texas/Houston Health Science Center. A list of bacterial strains used in this study and their sources are given in Table 1. Cells cultured over night in 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 1-L Erlenmeyer flasks the next morning. Cultures were grown at their optimum growth temperatures: *B. anthracis* at 37 °C, and *B. cereus* and *B. thuringiensis* at 30 °C with shaking at 200 rpm. Growth was monitored by measuring the optical density of the cultures at 600 nm. In mid-log phase (i.e. at an OD_{600} of approximately 2.3-2.7 for *B. anthracis* and 4.0-4.6 for *B. cereus* and *B. thuringiensis*), cells were harvested by centrifugation (8,000 x *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.

Preparation of bacterial cell walls.

The bacterial cell walls were prepared by modification of a previously described procedure (5). The autoclaved bacterial cells (3×10^8 to 3×10^9 CFU/ml) were disrupted in 40 ml sterile saline on ice by four 10-minute sonication cycles. The complete or near complete disruption of cells was checked microscopically. Unbroken cells were removed by a low speed centrifugation run ($8,000 \times g$, 4 °C, 15 min). The separated pellet and supernatant fractions were stored at $-70^{\circ}C$. 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 $^{\circ}C$ for 4 h and lyophilized.

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 a modification of the procedure described by Ekwunife *et al*. (14). Briefly, the cell walls were subjected to 47 % hydrogen fluoride (HF) under

stirring at 4 $_o$C for 48 h. The reaction mixture was neutralized with NH$_4$OH, 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 BioGel P2 column were monitored using a refractive index detector. Polysaccharide-containing peaks were pooled, lyophilized and analyzed by gas chromatography-mass spectrometry as described below. These HF-released polysaccharides are referred to as HF-PSs.

Glycosyl composition analysis.

The carbohydrate profiles were determined by gas chromatography-mass spectrometry (GC-MS) analysis of the trimethylsilyl (TMS) methylglycosides as previously described by York *et al.* (51). The cell walls and HF-PSs were subjected to methanolysis at 80 °C for 18 h in methanolic HCl (1 M). The resulting methyl glycosides were *N*-acetylated, trimethylsilylated and then analyzed by GC-MS analysis (5890A GC-MS; Agilent Technologies, Palo Alto, CA) using a 30-m DB-1 fused silica capillary column (J&W Scientific, Folsom, Calif.). Inositol was used as an internal standard, and retention times were compared to authentic standards. Composition analysis was done on samples obtained from at least two independent cultures of each strain, and each sample was also analyzed at least twice.

RESULTS

Glycosyl composition analysis for members of the **B. cereus** *group.*

The strains investigated in this study and their classifications are given in Table 1.

Strain	MLST Clade, Lineage[1,2]	Clinical Information[3]	Source/ Provider[4]	Reference
B. anthracis Ames	Clade 1 Anthracis	Veterinary isolate	Cow (1981, Texas)	(47)
B. anthracis 4229 Pasteur		Veterinary vaccine strain (Italy)	Unknown, 1880's	(38)
B. anthracis 34F2 Sterne		Veterinary vaccine strain	Cow (1930s, South Africa)	(44)
B. anthracis 7702		n/a	Koehler, Univ. of Texas, Houston	(8)
B. anthracis UT60 (strain 7702 Δ *atxA)*		n/a	Laboratory derived deletion mutant	(10)
B. cereus F666	Clade 1, Cereus I	Gastrointestinal illness	Human stool isolate, (1981, North Carolina)	(37)
B. cereus ATCC 10987		n/a	Dairy isolate (1930)	(43)
B. cereus B5780	Clade 1, Cereus III	Unknown	Human blood isolate (1970, Texas)	(37)
B. cereus 03BB102		Fatal pneumonia	Human blood isolate (2003, Texas)	(21)
B. cereus G9241	Clade 1, Cereus IV	Severe pneumonia	Human blood isolate (1994, Louisiana)	(22)
B. cereus 03BB87		Fatal pneumonia	Human blood isolate (2003, Texas)	(21)

| *B. cereus* ATCC 14579	Clade 2 Tolworthi	n/a	*B. cereus* type strain; possibly dairy isolate (1916)	(15)
B. thuringiensis ATCC 33679	Clade 2 Kurstaki	Entomocidal	ATCC; originally isolated from diseased insect larvae	(12)
B. thuringiensis ATCC 35646	Clade 2 Sotto	Larvicidal to horn flies	CDC; originally isolated from sewage in Israel	(46)

Table 1: Strains investigated: MLST groupings, clinical manifestation, and source. 1 The phylogenetic relatedness of strains on the basis of multi locus sequence typing (MLST) was adopted from Priest *et al.* (39) with modifications (see point 2). 2 The classification of these strains in Cereus IV is proposed (37) 3 Abbreviation: n/a, not available 4 Strains *B. anthracis* 7702 and *B. anthracis* UT60 were kindly provided by Dr. Theresa Koehler, University of Texas—Houston Health Science Center, Houston

Examples of GC profiles comparing a *B. anthracis* Sterne 34F2 cell wall sample with those of *B. cereus* ATCC 10987 and with *B. cereus* ATCC 14579 (the *B. cereus* type strain) are given in Figure 1. The compiled glycosyl compositions for the cell wall samples from each strain are shown in Table 2. The glycosyl composition of the cell walls from all the *B. anthracis* strains contained Glc, Gal, ManNAc, and GlcNAc. Qualitatively, *B. cereus* strains belonging to Clade 1/Cereus IV had the same cell wall glycosyl components as strains belonging to Clade 1/Anthracis. The cell walls from strains of Clade 1/Cereus III differed from those of Clade 1/Anthracis in that they contained additional Man, while strains belonging to Clade I/Cereus I differed in that their cell walls additionally contained GalNAc, and the cell walls from all strains belonging to Clade 2 lacked Gal and contained GalNAc.

There were some notable differences with regard to the relative amounts of certain glycosyl residues even among strains belonging to the same clade and lineage. For example, strain *B. anthracis* Sterne 34F2 had cell walls with notably decreased levels of ManNAc compared to *B. anthracis* Ames and *B. anthracis* Pasteur 4229, while *B. anthracis* 7702 and its *atxA* deletion mutant UT60 showed an increase in cell wall Glc levels compared to the other *B. anthracis* strains. Quantitative differences in various

glycosyl components were also present between *B. cereus* Clade 1/Cereus I strains F666 and ATCC 10987. Relative to strain *B. cereus* ATCC 10987, strain *B. cereus* F666 contained significantly increased amounts of Glc and decreased amounts of Gal. Differences were also noticeable in strains *B. cereus* B5780 and 03BB102 cell walls, both belonging to Clade 1/Cereus III. Strain B5780 had a much higher level of Glc and lower levels of both Gal and ManNAc compared to strain 03BB102.
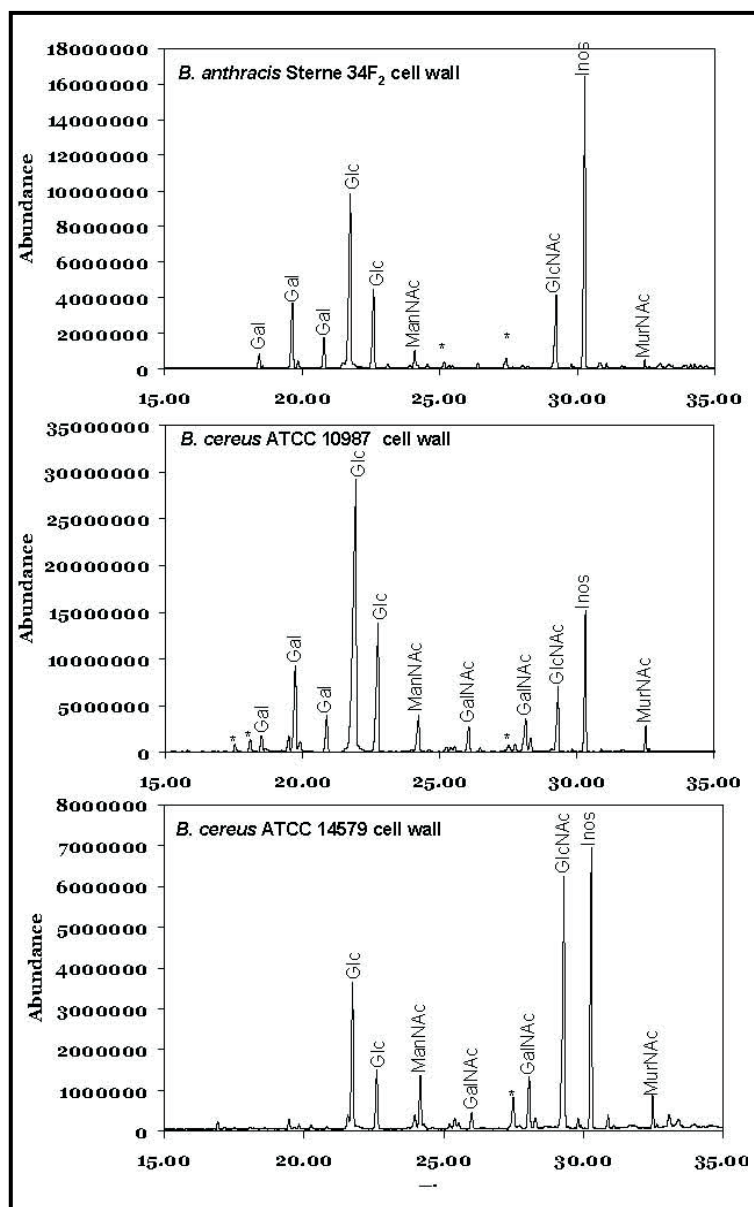
Figure 1. **Gas chromatographic-mass spectrometric (GC-MS) sugar profiles** obtained from *B. anthracis* Sterne 34F2, *B. cereus* ATCC 10987, and type strain *B. cereus* ATCC 14579 vegetative cell walls after hydrolysis of the total cell wall preparations and derivatization into trimethylsilyl (TMS) methylglycosides. The sample origin is indicated in the profiles. Gal, galactose; Glc, glucose; ManNAc, *N-*

acetylmannoseamine; *N*-acetyl-glocosamine; Inos, inositol (internal standard); MurNAc, *N*-acetylmuramic acid; *, non-carbohydrate components (not further characterized).

MLST Clade, Lineage	Strain	Sugar composition*					
		Man	Glc	Gal	ManNAc	GlcNAc	GalNAc
Clade 1 Anthracis	*B. anthracis* Ames	n.d.	6.2 ± 1.1	54.2 ± 7.4	13.2 ± 4.3	26.2 ± 4.2	n.d.
	B. anthracis Pasteur 4229	n.d.	5.6 ± 1.2	52.3 ± 7.7	13.2 ± 3.6	28.6 ± 4.9	n.d.
	B. anthracis Sterne 34F2	n.d.	8.5 ± 1.7	61.3 ± 6.7	4.7 ± 2.1	25.4 ± 5.0	n.d.
	B. anthracis 7702	n.d.	15.0 ± 1.6	54.5 ± 8.6	8.1 ± 5.8	21.3 ± 2.4	n.d.
	B. anthracis UT60 (atxA deletion mutant of 7702)	n.d.	23.0 ± 1.1	49.3 ± 3.1	7.2 ± 0.9	20.2 ± 3.0	n.d.
Clade 1 Cereus I	*B. cereus* F666	n.d.	24.5 ± 7.9	13.2 ± 5.8	12.8 ± 3.6	32.3 ± 6.5	16.8 ± 3.7
	B. cereus ATCC 10987	n.d.	2.6 ± 1.3	31.8 ± 6.2	15.4 ± 0.2	25.7 ± 3.0	24.5 ± 4.2
Clade1 CereusIII	*B. cereus* B5780	2.7 ±1.4	68.3 ± 2.7	1.2 ± 0.2	2.9 ± 1.5	24.9 ± 3.2	n.d.
	B. cereus 03BB102	0.9 ±0.5	5.1 ± 0.8	61.7 ± 5.8	9.5 ± 3.9	22.9 ± 2.7	n.d.
Clade 1 Cereus IV	*B. cereus* G9241	n.d.	5.2 ± 0.7	63.4 ± 2.1	9.5 ± 1.9	21.7 ± 1.0	n.d.
	B. cereus 03BB87	n.d.	2.5 ± 0.9	57.4 ± 9.2	11.4 ± 6.6	28.0 ± 3.2	n.d.
Clade2 Tolworthi	*B. cereus* ATCC 14579	n.d.	27.7 ± 2.2	n.d.	14.5 ± 4.5	45.2 ± 2.5	12.2 ± 3.1
Clade 2 Kurstaki	*B. thuringiensis* ATCC 33679	n.d.	55	n.d.	7.2	30	7.7

Clade 2 Sotto	*B. thuringiensis* ATCC 35646	n.d.	20	n.d.	15	49	17

Table 2: Sugar composition of cell walls from members of the *B. cereus* group. Values are given in percent (+/- one standard deviation) of total carbohydrate before HF treatment. *For the strains *B. anthracis* Sterne 34F2 and *B. cereus* ATCC 10987, high Glc content was occasionally observed in cell wall preparations. The sugar compositions given here are from cell wall preparations confirmed in independent culturing experiments. n.d. = none detected (i.e., relative percentage levels below 0.5 %); Man = mannose; Glc = glucose; Gal = galactose; ManNAc = *N*-acetylmannosamine; GlcNAc = *N*-acetylglucosamine; GalNAc = *N*-acetylgalactosamine. *N*-acetylmuramic acid was also detected in the cell wall preparations, but not quantified. For MLST classifications see Table 1.

Effects of plasmid content on the glycosyl composition in **B. anthracis** *cell walls.*

In order to determine whether the plasmid content has an effect on glycosyl composition of cell walls in the different *B. anthracis* strains, we normalized the glycosyl residue percentages shown in Table 2 to the amount of Gal for each sample. The reason for normalizing to Gal is that, as will be described below, Gal is the major glycosyl residue found in the HF-PSs for each of the *B. anthracis* strains, and the HF-PSs of these strains all have the same structure as reported by Choudhury *et al.* (9). These Gal-normalized values are given in Table 3 together with the plasmid content in the different strains. Qualitatively, the sugar profiles of the different cell walls were not affected by the plasmid content. Quantitatively, the glycosyl composition of the cell wall from *B. anthracis* Pasteur which lacks pXO1 was the same as that of *B. anthracis* Ames which contains both pXO1 and pXO2. This finding suggests that the plasmid pXO1 has no impact on sugar composition of the cell wall. In contrast, pXO2-minus *B. anthracis* strains (Sterne 34F2 and 7702), have cell walls with reduced amounts of ManNAc and increases in Glc relative to Gal suggesting that the absence of the pXO2 plasmid impacts cell wall glycosyl composition. While the increase in Glc was modest for *B. anthracis* Sterne 34F2 cell walls, *B. anthracis* 7702 cell walls displayed a 3-fold increase (relative to Gal) in Glc levels compared to the cell wall from *B. anthracis* Ames. The increase in the amounts of cell wall Glc was even more pronounced in *B. anthracis* UT60, a derivative of *B. anthracis* 7702 that has a deletion mutation in the *atxA* regulatory gene on pXO1 in addition to lacking pXO2 (10). In this strain we

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observed approximately a 5-fold increase in Glc compared to the cell wall from *B. anthracis* Ames and about a 60% increase in Glc as compared to the amounts in the parent strain *B. anthracis* 7702. These data indicate that the absence of pXO2 in combination with the deletion of the regulatory gene *atxA* from pXO1 result in detectable changes in the cell wall glycosyl composition of *B. anthracis*.

Strain	Plasmid content	Relative sugar composition			
		Glc	Gal	ManNAc	GlcNAc
B. anthracis Ames	(pXO1+, pXO2+)	0.10	1.0	0.25	0.50
B. anthracis Pasteur 4229	(pXO1-, pXO2+)	0.10	1.0	0.30	0.50
B. anthracis Sterne 34F2	(pXO1+, pXO2-)	0.15	1.0	0.10	0.40
B. anthracis 7702	(pXO1+, pXO2-)	0.30	1.0	0.15	0.40
B. anthracis UT60 (*atxA* deletion mutant of strain 7702)	(pXO1+ΔatxA, pXO2-)	0.50	1.0	0.15	0.50

Table 3: Effect of different plasmid combinations on the sugar composition (normalized to the amount of Gal) of the *B. anthracis* cell walls.

Composition of HF released polysaccharides.

Polysaccharides that are attached to the bacterial cell walls through phosphate bonds can be released through HF treatment (28). This procedure was used in other studies to obtain the cell wall polysaccharide from *B. anthracis* which is thought to anchor the S-layer protein to the peptidoglycan (14, 33). The glycosyl residue compositions of the HF-PSs from the investigated strains of *B. anthracis* (Clade 1/Anthracis) and *B. cereus* (Clade 1/Cereus I, III, IV, and Clade 2/Tolworthi) are presented in Table 4, and GC-MS profiles of the cell wall compositions compared with the HF-PSs for one preparation each from the pXO2-minus *B. anthracis* Sterne 34F2 and *B. cereus* ATCC 10987 are shown in Figures 2A and 2B, respectively. These results show that the HF-PSs from *B. anthracis* Ames, *B. anthracis* Pasteur, *B.*

anthracis Sterne 34F2, and *B. anthracis* UT60 all have the same glycosyl residue composition, both qualitatively and quantitatively; they all contain Gal, ManNAc, and GlcNAc in approximately a 3:1:2 ratio as previously reported by Choudhury *et al.* (9). Each of these polysaccharides has a small amount of Glc, but further structural analysis has shown (9) that this is due to contamination by a Glc-rich component that is not part of this polysaccharide. The presence of a Glc-rich polysaccharide in *B. anthracis* cell walls that is not part of the HF-PS was most obvious for the preparation of one culture of *B. anthracis* Sterne 34F2 (Figure 1). In that preparation, the cell wall had a relatively large Glc content (Figure 1) while the HF-PS was greatly reduced in Glc. The "missing" Glc was found in the cell wall debris after HF-treatment and, therefore, *B. anthracis* Sterne 34F2 apparently has a Glc-rich component in the cell wall that is not released by HF-treatment. This relatively large amount of Glc-rich cell wall component was only observed in one of three *B. anthracis* Sterne 34F2 cultures. Because of the variability of the increased level of Glc from different cultures, it is not clear what actually governs the different levels of the possible Glc-rich cell wall component observed in the various Sterne 34F2 cell wall preparations. However, the increased cell wall Glc level, even though variable from different Sterne 34F2, cultures, was observed only in the pXO2-minus *B. anthracis* strains. The relationship of this possible Glc-rich component with the loss of the pXO2 plasmid requires further investigation.

The finding that the cell walls of several pXO2-minus *B. anthracis* strains may contain a Glc-rich component that is not released by treatment with aqueous HF was also observed in one cell wall preparation for *B. cereus* ATCC 10987 (Figure 2B). This result, as with results for extracts from pXO2-minus *B. anthracis* strains, indicates that *B. cereus* ATCC 10987 contains a Glc-rich polysaccharide that is not released by HF-treatment. As with the pXO2-minus *B. anthracis* strains, the Glc-rich cell wall component was found in the cell wall debris after HF-treatment. Further structural analysis (manuscript in preparation) shows that the relatively small amount of Glc found in the *B. cereus* 10987 HF-PS is due to residual contamination by a 4-linked glucose-containing component. Thus, we conclude that the minor amount of Glc found in the *B. cereus* ATCC 10987 HF-PS is not part of this polysaccharide and, therefore, this HF-PS consists of Gal, ManNAc, GlcNAc and GalNAc in a 1:1:1:1 ratio. It clearly has a different structure than the *B. anthracis* HF-PS. This structural difference was also

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supported by a comparison of the proton NMR spectra of these HF-PSs in the report by Choudhury *et al.* (9). The HF-PS isolated from strain *B. cereus* F666 (this strain is in the same Clade 1/Cereus I lineage as strain *B. cereus* ATCC 10987) has a glycosyl composition that resembles the HF-PS of strain *B. cereus* ATCC 10987 but with a significantly increased amount of Glc. In fact, the HF-PS from strain F666 showed three times the amounts of Glc compared to the HF-PS from *B. cereus* ATCC 10987. This result suggests that Glc is a part of the F666 HF-PS and; therefore, this HF-PS likely consists of Glc, Gal, ManNAc, GlcNAc, and GalNAc in a 1:1:1:1:1 ratio.

The strains that belong to the *B. cereus* group Clade 1/Cereus III, strains B5780 and 03BB102, showed more pronounced differences from one another in their HF-PS sugar compositions. Both strains contained a small amount of Man in their isolated HF-PS fractions, which was not observed in the other HF-PSs examined. In addition, strain B5780 HF-PS contained larger amounts of Glc and lower amounts of Gal and ManNAc compared to the HF-PS from strain 03BB102 HF-PS which contained a small amount of Glc and larger amounts of Gal and ManNAc (Table 4). It is possible that the glycosyl residues present in small amounts are due to low levels of contaminating carbohydrates that are not part of the HF-PS structures. If this were the case, then the HF-PS of strain *B. cereus* B5780 would be composed of Glc and GlcNAc in a 2:1 ratio, and the HF-PS of *B. cereus* 03BB102 would be composed of Gal, ManNAc, and GlcNAc in a 6:1:2 ratio. Further structural investigation of these HF-PSs is in progress.

The HF-PS preparations that were most similar to the *B. anthracis* HF-PSs were from the *B. cereus* group strains that belong to the Clade 1/Cereus IV lineage, G9241 and 03BB87. These strains, as with strain 03BB102, are pathogens that caused severe pneumonia (21). The HF-PSs from strains G9241 and 03BB87 consisted of Gal, ManNAc, and GlcNAc in a 3:1:1 (or 3:1:2) ratio. The ratio of these glycosyl residues in the *B. anthracis* HF-PS is 3:1:2. We are currently characterizing the structures of the 03BB87 and G9241 HF-PSs to determine if they are the same or different from the *B. anthracis* HF-PS structure.

The HF-PS from the type strain *B. cereus* ATCC 14579 (Clade 2/Tolworthi) also showed a consistant small decrease in Glc content compared to its cell wall (compare Tables 2 and 4). This result indicates that the *B. cereus* ATCC 14579 cell wall contained a Glc-rich component that was not released from the cell wall by HF-

treatment. Unlike the HF-PSs from the *B. anthracis* strains and from *B. cereus* ATCC 10987, Glc is also a major glycosyl residue in the *B. cereus* ATCC 14579 HF-PS and, therefore it is likely that Glc is a component of this polysaccharide. This was verified by further structural analysis of this HF-PS (manuscript in preparation). The components of the HF-PS from *B. cereus* ATCC 14579 are Glc, ManNAc, GlcNAc, and GalNAc in approximately a 1:1:2:1 ratio.

MLST Clade, Lineage	Strain	Sugar Composition					
		Man	Glc	Gal	ManNAc	GlcNAc	GalNAc
Clade 1 Anthracis	*B. anthracis* Ames	n.d.	2.7 ± 0.10	57.0 ± 2.8	19.3 ± 1.0	21.1 ± 2.1	n.d.
	B. anthracis Pasteur 4229	n.d.	0.50	53.4	15.7	30.4	n.d.
	B. anthracis Sterne 34F2	n.d.	4.2	52.7	13.7	29.5	n.d.
	B. anthracis UT60	n.d.	3.8	56.5	18.1	21.7	n.d.
Clade1 Cereus I	*B. cereus* F666 B. cereus ATCC 10987	n.d. n.d.	25.9 8.6	21.4 26.7	19.1 25.3	18.0 16.4	15.7 23.0
Clade 1 Cereus III	*B. cereus* B5780 *B. cereus* 03BB102	1.1 2.2	65.6 3.1	0.9 65.5	3.6 11.4	28.8 17.8	n.d. n.d.
Clade 1 Cereus IV	*B. cereus* G9241 *B. cereus* 03BB87	n.d. n.d.	1.3 0.90	55.8 61.8	19.3 14.2	23.6 23.2	n.d.
Clade2 Tolworthi	*B. cereus* ATCC 14579	n.d.	25.3	n.d.	15.4	44.9	14.4

Table 4: Sugar composition of isolated polysaccharides released from the *Bacillus* cell walls through HF treatment. Compositions are given as relative percent of total carbohydrate after HF treatment. n.d. = none detected (i.e., relative percentage levels below 0.5 %); Man = mannose; Glc = glucose; Gal = galactose; ManNAc = *N*-acetylmannosamine; GlcNAc = *N*-acetylglucosamine; GalNAc = *N*-acetylgalactosamine. For MLST classification, see Table 1
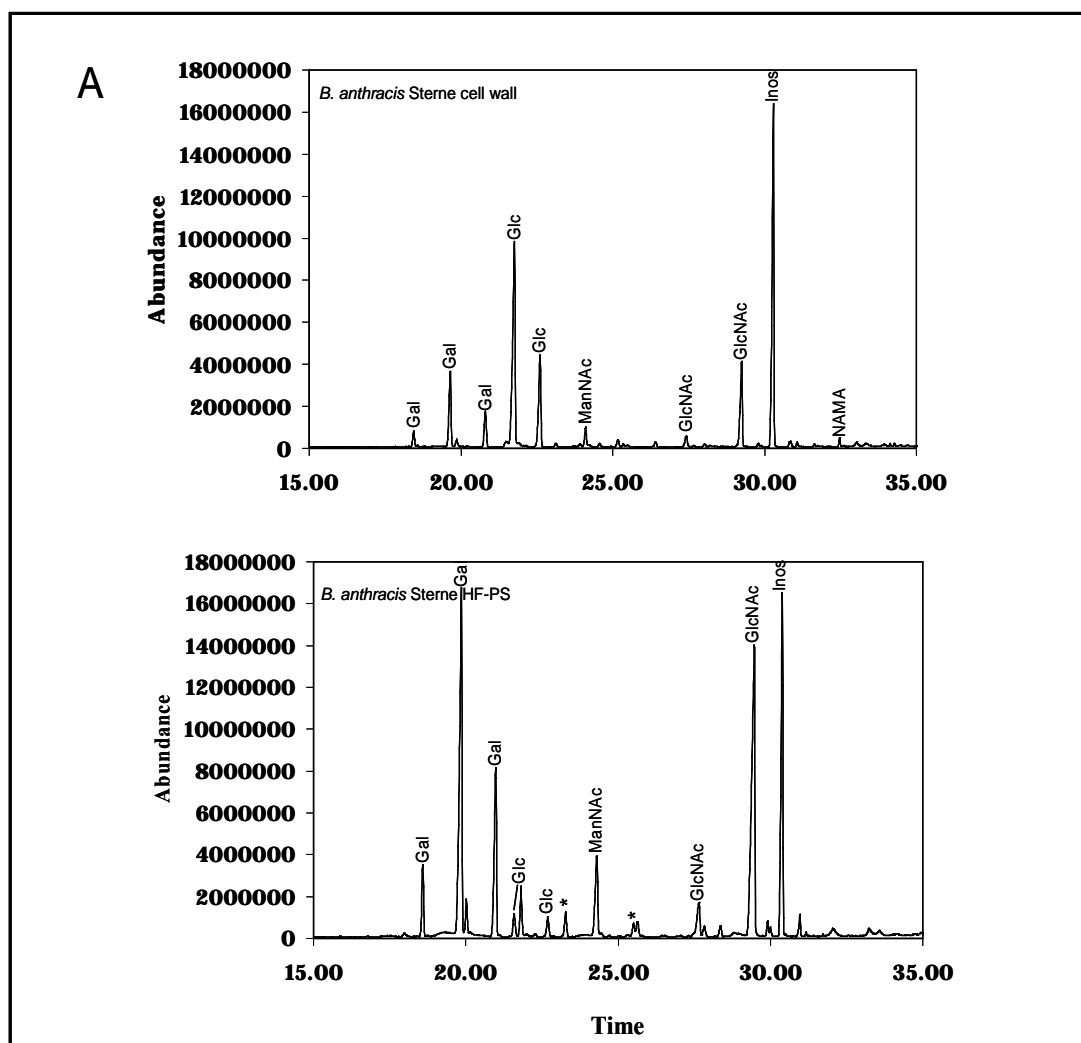
Figure 2 (A): Gas chromatographic-mass spectrometric (GC-MS) sugar profiles obtained from *B. anthracis* Sterne 34F2 vegetative cell walls after hydrolysis of the total cell wall preparations and derivatisation into trimethylsilyl (TMS) methylglycosides. Sample origins are indicated in the profiles. HF-PS samples were released from cell walls through HF treatment and purified on BioGel P2 columns. Gal, galactose; Glc, glucose; ManNAc, *N*-acetylmannoseamine; GlcNAc, *N*-acetylglucose-amine; GalNAc, *N*-acetylgalactoseamine; Inos, inositol (internal standard); MurNAc, *N*-acetylmuramic acid; *, non-carbohydrate component (not further investigated).

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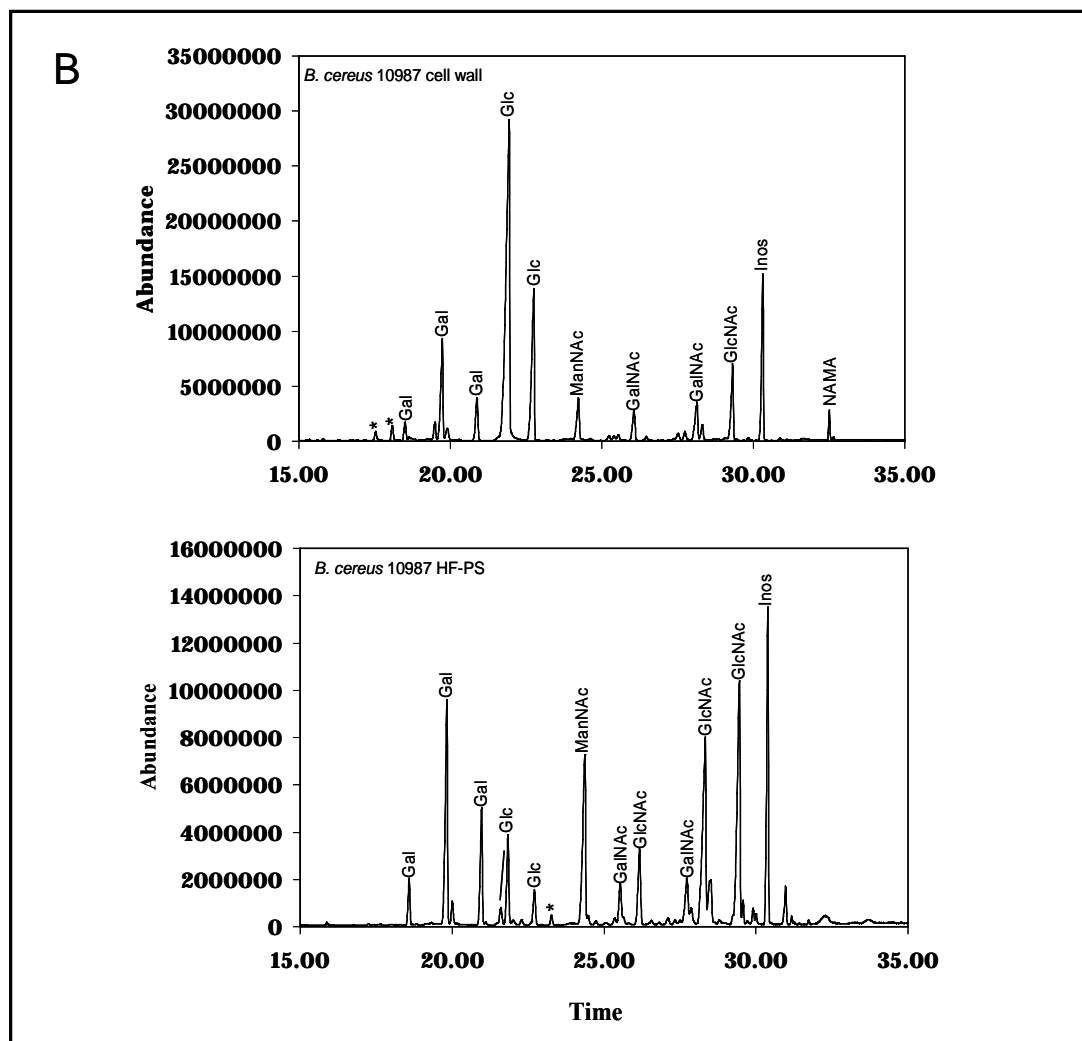
Figure 2 (B): Gas chromatographic-mass spectrometric (GC-MS) sugar profiles obtained from *B. cereus* ATCC 10987 vegetative cell walls after hydrolysis of the total cell wall preparations and derivatisation into trimethylsilyl (TMS) methylglycosides. Sample origins are indicated in the profiles. HF-PS samples were released from cell walls through HF treatment and purified on BioGel P2 columns. Gal, galactose; Glc, glucose; ManNAc, *N*-acetylmannoseamine; GlcNAc, *N*-acetylglucose-amine; GalNAc, *N*-acetylgalactoseamine; Inos, inositol (internal standard); MurNAc, *N*-acetylmuramic acid; *, non-carbohydrate component (not further investigated).

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DISCUSSION

We investigated cell wall compositions from a selection of strains belonging to the *B. cereus* group species, *B. anthracis, B. cereus,* and *B. thuringiensis*. Recent investigations into the phylogenetic relatedness of these *B. cereus* group strains, e.g. multilocus sequence typing, offer a more differentiated picture than previous classification schemes and resulted in separating these strains into two clades and several lineages (Table 1) (2, 39). Here we showed that the glycosyl residue composition of the cell walls varied significantly both qualitatively and quantitatively among the investigated strains in a manner that reveals possible correlations with their phylogenetic relatedness. In summary, we observed the following: (1.) *B. cereus* strains that are closely related had cell wall glycosyl compositions that qualitatively varied from one another in a clade/lineage-specific manner. (2.) Quantitative glycosyl analysis showed that strains belonging to the same lineage vary from one another in the amounts of various glycosyl residues indicating the presence of strain-specific cell wall carbohydrates. (3.) Analysis of the cell walls from recently discovered pathogenic *B. cereus* strains that caused severe pneumonia, i.e. strains 03BB102, 03BB87, and G9241 (21, 22), showed that they have glycosyl compositions that were most similar to the cell walls of the *B. anthracis* strains. (4.) The plasmid content of *B. anthracis* strains appeared to affect cell wall glycosyl compositions, i.e. the amounts of ManNAc and Glc were lower and higher, respectively, in the cell walls from strains that lacked the pXO2 virulence plasmid, and the amount of a possible Glc-rich non-HF-PS cell wall was particularly increased in an *atxA* mutant of a pXO2-minus *B. anthracis* strain. (5.) The HF-PSs released from the cell walls of the different *B. anthracis* strains all had the same Gal:ManNAc:GlcNAc ratio, 3:1:2 as previously reported (9), consistent with the fact that they have the same structure. (6.) The HF-PSs from strains of the *B. cereus group* Clade 1/Cereus I (i.e. *B. cereus* ATCC 10987 and F666), Clade 1/Cereus III (i.e. *B. cereus* B5780 and 03BB102), and Clade 2/Tolworthi (i.e. the type strain *B. cereus* ATCC 14579) each had a unique glycosyl composition that was different from the *B. anthracis* HF-PSs indicating that they had different structures from one another and from the *B. anthracis* HF-PS structure.

To our knowledge, this is the first report that compares, in a systematic manner, the cell wall carbohydrates of several pathogenic and nonpathogenic members of the *B.*

cereus with known phylogenetic grouping based on the MLST analysis (2, 39). Earlier studies by Fox *et al.* (16, 50) determined carbohydrate profiles from vegetative cells and spores of a number of *B. cereus* and *B. anthracis* strains that had less clearly defined relationships. As expected, our findings corroborate some of those reported by Fox *et al.*(16, 50). The reports by Fox *et al.* showed that, in addition to the major glycosyl residues, minor amounts of rhamnose, ribose, and methylated sugars were present in the cell wall preparations; however, these glycosyl residues were attributed to contamination from spore components and RNA (16, 50). It is known that the exosporium BclA protein is glycosylated by a rhamnose-containing oligosaccharide (11).

Our comparative analyses of the cell walls from MLST-defined *Bacillus* strains provide new information that correlates with their phylogenetic relatedness. Even though our study involved a limited number of strains, the qualitative glycosyl residue differences suggest that cell wall compositions qualitatively varied in a clade/lineage-specific manner. In addition, comparison of two *B. cereus* strains, B5780 and 03BB102, both belonging to linage Cereus III of Clade 1 showed that, while they contain the same glycosyl residues, these residues are present at very different levels (Table 2). This result suggests the possibility of strain-specific quantitative differences that could, in some cases, allow identification of strains within a single *B. cereus* lineage. However, a larger sample of *Bacillus* strains is needed to determine breadth and consistency of these qualitative and quantitative differences.

Glycosyl compositions of the cell walls of *B. anthracis* strains before and after treatment with HF revealed that the absence of plasmid pXO2 may have some impact on cell wall glycosyl composition. While the plasmid effects on cell wall carbohydrates are preliminary and in need of confirmation by examining genetically better defined strains, it is worth noting that we observed consistently decreased relative amounts of ManNAc and variably increased levels of Glc (relative to the amounts of Gal) in the cell walls of all *B. anthracis* strains missing pXO2. The fact that the HF-PS from all the pXO2-minus *B. anthracis* strains had the same glycosyl composition and structure (9) as the HF-PSs from *B. anthracis* Ames and Pasteur suggests that the lower level of ManNAc and increased level of Glc in the cell walls reflect changes in carbohydrate structures that are not part of the HF-PS. An additional effect on cell wall glycosyl

composition was detected in *B. anthracis* UT60; namely the deletion of *atxA* from pXO1 results in higher levels of Glc in the cell wall (as compared to its parent strain, *B. anthracis* 7702), presumably due to larger amounts of the Glc-rich non-HF-PS component in its cell wall. Taken together, these results indicate that the pXO1 and pXO2 plasmids may have a role in determining the presence or absence of a Glc-rich component in some cell walls even though there are no known carbohydrate synthesis-related genes on pXO1 or pXO2 that could easily explain the observed glycosyl changes. The gene products of the majority of open reading frames (ORFs) predicted on the pXO1 and pXO2 virulence plasmids are still unidentified (41). It may well be that there are ORFs that encode as yet unidentified carbohydrate synthesis-related genes. In the case of *B. anthracis* UT60, the deleted *atxA* gene located on virulence plasmid pXO1 encodes a global regulator and the major transcriptional activator of the pXO1-borne anthrax toxin genes (4). In a genetically complete strain, containing both pXO1 and pXO2, *atxA* has also been shown to be indirectly involved in the regulation of the capsule biosynthesis operon *capBCAD* located on pXO2 (13). The *cap* genes are essential for the encapsulation of *B. anthracis* cells by a poly-γ-D-glutamic acid, one of the identified *B. anthracis* virulence factors necessary for the protection of *B. anthracis* cells inside the host (24, 31). The stimulating effect on the Glc level and the relatively lower amount of ManNAc in *B. anthracis* UT60 (and the other pXO2-minus *B. anthracis* strains) may indicate additional and previously unknown regulatory roles of *atxA* and of pXO2-encoded genes in cell wall polysaccharide biosynthesis. Further work using isogenic strains of *B. anthracis* is required to determine the significance and role of these plasmid effects on the cell wall carbohydrates.

As a first approach to determining the cell wall polysaccharide structures underlying the observed sugar composition profiles, phosphate-bound cell wall polysaccharides were released by HF treatment of the cell walls and purified. This procedure was used to purify the cell wall from *B. anthracis* that is thought to anchor the S-layer protein to the peptidoglycan (33). Composition analysis of these HF-PSs from the different *B. anthracis* strains revealed that all had the same 3:1:2 Gal:ManNAc:GlcNAc ratio, reflecting the identical structures of these polysaccharides (9). Since the HF-PS compositions and structures from all the *B. anthracis* strains were the same, it is likely that their structures are not depending on the presence of the

virulence plasmids, pXO1 or pXO2. The *B. anthracis* HF-PSs were clearly different in glycosyl composition from the HF-PSs from the cell walls of other *B. cereus* group members which differed from one another. While it is possible that the HF-PSs from the *B. cereus* strains vary in a manner that correlates with clade or lineage, further work on more strains would be required to determine the validity of this possibility.

An interesting observation is the similarity of glycosyl compositions among the cell walls of *B. cereus* strains that have recently been shown to cause severe pneumonia in humans (21, 22) with those of *B. anthracis* (Table 2). These clinical strains, namely *B. cereus* G9241, 03BB102, and 03BB87 belong to Clade 1, lineage Cereus III or IV (37). In comparing small acid soluble proteins in *B. anthracis* with those in strain *B. cereus* G9241, a recent report noticed that the acid soluble proteins of G9241 fell into a more distantly related protein cluster and stated that, on the basis of this criterion, pathogenicity and phylogenicity are not necessarily correlated features (7). Our result indicates that the cell walls of these pathogenic *B. cereus* strains may contain carbohydrates that have common structural features with each other and with those of *B. anthracis*. The HF-PS preparations of these *B. cereus* strains displayed glycosyl compositions that were relatively similar to one another and to the HF-PSs from the *B. anthracis* strains (Table 4). This finding could be taken as an indication for functional importance of the HF-PS (and the S-layer anchoring mechanism) in virulence and, possibly, of its relative independence from phylogenetic strain relatedness in pathogenic *B. cereus* strains.

Bacillus cereus strains G9241, 03BB87 and 03BB102 all contain at least considerable numbers of genes with high similarity to genes of the virulence plasmid pXO1 of *B. anthracis* (e.g. *B. cereus* G9241 carries a plasmid that is almost identical to *B. anthracis* pXO1) (21, 22). Recently "*Bacillus anthracis*-like" isolates were obtained from chimpanzees and gorillas from Cote d'Ivoire and Cameroon that were thought to have died from anthrax-like disease (26, 29). Interestingly, based on molecular analyses (MLST and others), these strains fell outside the well-supported cluster of classic *B. anthracis* strains and instead clustered with *B. cereus* and *B. thuringiensis* strains, most closely with a recently described atypical and pathogenic *B. thuringiensis* (26, 29). These *B. anthracis*-like isolates from great apes reportedly contain both pXO1 and pXO2 plasmids, while the pathogenic *Bacillus cereus* strains 03BB102, G9241,

03BB87 all contain a pXO1-like plasmid but not pXO2 (21). The similarity of the HF-PS compositions for strains 03BB87, 03BB102, and G9241 with those observed for the *B. anthracis* HF-PSs suggests that the underlying HF-PSs in these strains may be structurally related. Perhaps, the HF-PS structure found in *B. anthracis* and, possibly, the related HF-PS structures of the pathogenic *B. cereus* strains are necessary for virulence and/or are a characteristic of *B. cereus* strains that were able to acquire one or both of the *B. anthracis* virulence plasmids. It is not known whether the African gorilla isolates contain HF-PSs that corroborate these suspicions. To date, these strains have not yet been characterized with regard to their cell wall carbohydrates. The HF-PS structures of the *B. cereus* strains causing severe pneumonias are currently being investigated in our laboratory.

Much remains to be determined regarding the genetic basis for synthesis of cell wall carbohydrates, the exact location of these molecules in the cell wall, and their functions. However, the results described here suggest that the description of the cell wall carbohydrates of the *B. cereus* group strains will eventually be useful for strain classification and, therefore, for the development of diagnostic and vaccine applications. In addition, the functional importance of these molecules with regard to virulence and pathogenicity requires further structural analysis which is currently underway in our laboratory.

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THE STRUCTURE OF THE MAJOR CELL WALL POLYSACCHARIDE OF *BACILLUS ANTHRACIS* IS SPECIES SPECIFIC

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Running Title: A species specific *Bacillus anthracis* vegetative cell wall polysaccharide.

Contribution to this publication

I prepared the first draft of this manuscript and was involved in the design and configuration of tables and figures. The isolation of the cell walls and purification of the HF-PS as well as their analyses by gas chromatography mass spectrometry, MALDI-TOF analysis and glycosyl linkage analysis was performed by me. I assisted Biswa Choudhury with the NMR spectroscopy as well as with the assignment of the 2D NMR signals. Elke Saile was responsible for growing the bacterial cultures used in the experiments and preparing them for shipment. The bacterial strains required cultivation in BSL2 and BSL3 laboratories at the Center for Disease Control (CDC).

Key findings and conclusions

- The HF-PS of *B. anthracis* is composed of hexasaccharide repeating units with the following structure:

  β- Gal-(1→4)┐
       →6)-α-GlcNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc-(1→
  α-Gal-(1→3)┘   α-Gal-(1→3)┘
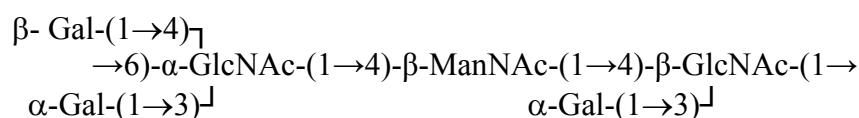
- This structure was species specific and identical in all investigated *B. anthracis* strains (Ames, Pasteur, and Sterne).

- Comparison with the HF-PSs from closely related *B. cereus* ATCC 10987 and *B. cereus* ATCC 14579 showed that the *B. anthracis* structure was different from each of these HF-PSs.

- The HF-PS structure from *B. cereus* ATCC 10987 was also different from that of *B. cereus* ATCC 14579.

ABSTRACT

In this report we describe the structure of the polysaccharide released from *B. anthracis* vegetative cell walls by aqueous hydrogen fluoride (HF). This HF-released polysaccharide (HF-PS) was isolated and structurally characterized from the Ames, Sterne, and Pasteur strains of *B. anthracis*. The HF-PSs were also isolated from the closely related *B. cereus* ATCC 10987 strain and from the *B. cereus* ATCC 14579 type strain and compared to those of *B. anthracis*. The structure of the *B. anthracis* HF-PS was determined by glycosyl composition and linkage analyses, matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS), and 1- and 2-D nuclear magnetic resonance (NMR) spectroscopy. The HF-PSs from all of the *B. anthracis* isolates had an identical structure consisting of an amino sugar backbone of →6)-α-GlcNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc-(1→ 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. There is some variability in the presence of two of these three Gal substitutions. Comparison with the HF-PSs from *B. cereus* ATCC 10987 and *B. cereus* ATCC 14579 showed that the *B. anthracis* structure was clearly different from each of these HF-PSs and, further, that the *B. cereus* ATCC 10987 HF-PS structure was different from that of *B. cereus* ATCC 14579. The presence of a *B. anthracis*-specific polysaccharide structure in its vegetative cell wall is discussed with regard to its relationship to those of other *Bacilli*.

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INTRODUCTION:

Bacillus anthracis is a gram-positive, spore-forming bacterium that causes anthrax (1). Cell wall carbohydrates such as capsular polysaccharides are well known virulence factors with regard to numerous bacterial pathogens, both gram-negative and gram-positive. However, relatively little is known about the carbohydrates in the vegetative cell walls of *B. anthracis* as well as other members of the *B. cereus* group of bacteria. While there have been some glycosyl composition analyses, there have been no reported structures for carbohydrates from the vegetative cell wall of *B. anthracis*.

Generally, the carbohydrate-containing components of the vegetative cell walls of gram-positive bacteria consist of the extensive peptidoglycan layer, teichoic acids, lipoteichoic acids, capsular polysaccharides, and crystalline cell surface proteins known as S-layer proteins that are often glycosylated (2). However, the *B. anthracis* cell wall differs in several aspects from this generalized description. First, *B. anthracis* cells are surrounded by a poly-γ-D-glutamate capsule and not by a polysaccharide capsule. Second, their cell walls do not contain teichoic acid (3), and lastly, their S-layer proteins are not glycosylated (1,4). However, glycosyl composition comparisons of the cell walls of *B. anthracis*, *B. cereus*, and *B. thuringiensis* show that they do contain glycosyl residues and that they differ from one another in their glycosyl compositions (5).

To date, cell wall carbohydrates from the vegetative cells of members of the *B. cereus* group have been addressed only to a limited extent (5-7). All of these carbohydrates are rich in amino glycosyl residues but have variations in the type and amounts of these residues. The study of Ekwunife *et al.* (6) focused on the glycosyl composition of a carbohydrate polymer released from the cell wall through HF-treatment (the HF-treatment releases wall polysaccharides covalently bound via a phosphate bond to the peptidoglycan) of *B. anthracis* (Δ Sterne) and found that the HF-released polysaccharide (HF-PS) contained Gal, GlcNAc, and ManNAc in an approximate ratio of 3:2:1. This HF-PS was also further investigated by Mesnage *et al.* (4). They reported the importance of a pyruvyl substituent with regard to the function of this polysaccharide in anchoring the S-layer proteins to the cell wall.

Fox *et al.* (5) investigated a number of *B. anthracis* and *B. cereus* strains for their total cell glycosyl compositions, which showed interesting differences between the

different strains. For example, in contrast to the *B. anthracis* strains, all *B. cereus* strains investigated contained GalNAc, suggesting possible differences in cell wall architecture in the different *Bacilli* cell walls and, possibly, the occurrence of strain- or species-specific carbohydrates. The possibility of species/strain-specific structures is of interest for at least two reasons: the taxonomy within the *B. cereus* group has recently become a matter of debate (8,9) and investigations into cell wall carbohydrates of *B. cereus* group members may hold additional clues to their phylogenetic relatedness. In addition, the identification of specific cell wall carbohydrate structures could provide valuable leads in the elucidation of their functional importance in pathogenic interactions.

The function of one *B. anthracis* cell wall polysaccharide has been addressed in the literature. This function is its role for anchoring the S-layer proteins to the vegetative cell wall (4). The S-layer proteins contain a S-layer homology domain (SLH) which is found also in other S-layer proteins from gram-positive bacteria and in cell wall enzymes, such as xylanase and pullanase from *Thermoanaerobacterium thermohydrosulfurigenes* (*Clostridium thermosulfurogenes*) (10). It is thought that SLH domains bind to secondary cell wall carbohydrates that are covalently linked to the cell wall peptidoglycan via HF-labile phosphate bridges and thus anchor the S-layer proteins to the bacterial cell walls. This function has been investigated in greatest detail for *B. anthracis* (4).

Thus far, only a series of older reports about an isolated strain, namely *B. cereus* AHU 1356, addressed the question of a *Bacillus* species cell wall carbohydrate structure directly (11-13). The structures of neutral and acidic cell wall carbohydrates have been described for that strain. The neutral carbohydrate was composed of GlcNAc, ManNAc, GalNAc, and Glc in ratios of 4:1:1:1 (Murazumi, 1986 #10208}, while the acidic carbohydrate was composed of GlcNAc, Gal, Rha, glycerol and phosphorus in ratios of 1:1:2:1:1 (12).

As a first step in addressing cell wall carbohydrate structure/function relationships within members of the *B. cereus* group, we here report the structures of the HF-PSs of a number of *B. anthracis* and *B. cereus* strains. Structures were determined for these polysaccharides from *B. anthracis* Ames, *B. anthracis* Pasteur, and *B. anthracis* Sterne 34F$_2$. We also compared these structures with those from a closely related *B. cereus* strain, ATCC 10987 and from the *B. cereus* type strain, ATCC 14579.

The results showed that all three *B. anthracis* strains contained the same HF-PS structure which differed from that of *B. cereus* ATCC 10987, which, in turn, differed from that of *B. cereus* ATCC 14579.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Bacillus anthracis Ames, *B. anthracis* Pasteur, *B. anthracis* Sterne 34F2, and *B. cereus* strains ATCC 10987, and ATCC 14579 were provided from the CDC culture collection. The mutant UT60 (i.e. Sterne 7702 Δ*atxA*) was provided by Dr. Theresa Kohler, University of Texas Houston. Cultures were grown over night (16 h, 37 °C) in 100 ml brain heart infusion medium (BHI) (BD BBL, Sparks, MD) containing 0.5 % glycerol at 37 °C, 200 rpm. In the morning, 4 x 1.5 ml of the overnight cultures were pelleted (10,000 x g, room temperature, 5 min), the supernatants discarded, the pellets resuspended in 500 μl BHI and these four cell suspensions used to inoculate four 250 mL volumes of BHI medium in 1 L Erlenmeyer flasks. Incubation was carried out at 37 °C (*B. anthracis*) or 30 °C (*B. cereus, B. thuringiensis*) on a shaker at 200 rpm. Growth was monitored by measuring the optical density at 600 nm. Cells were harvested in mid-log phase by centrifugation (8,000 × g, 4 °C, 15 min), washed two times in sterile saline and enumerated by serial dilution and surface spread counts on BHI agar plates. Cultures were sterilized by autoclaving, 1 h at 121 °C prior to further processing and carbohydrate analysis.

Preparation of cell wall extracts

The bacterial cells were grown as described above and cell walls were prepared by modification of a previously described procedure (14). Briefly, the autoclaved bacterial cells (1 x 1010 to 1 x 1011 CFU) were disrupted in 40 ml sterile saline on ice by four 10-minute sonication cycles, using a Branson Sonifier (Type 450, Branson Ultrasonics Corporation, Danbury, CT) with a 1/2 inch probe, operating it at a frequency of 20 kHz. The complete or near complete disruption of cells was checked microscopically. Unbroken cells were removed by centrifugation (8,000 × g, 4 °C, 15 min). The separated pellet and supernatant fractions were stored at -80 °C. Cell wall materials were sedimented by ultracentrifugation at 100,000 × g, 4 °C for 4 h (Optima L-90K Ultracentrifuge, Beckman). The resulting cell wall pellets were washed by suspension in cold, deionized water followed by an additional ultracentrifugation as above and lyophilization.

Isolation and purification of the cell surface polysaccharide

Cell wall materials from the *B. anthracis* Ames, *B. anthracis* Pasteur, *B. anthracis* Sterne 34F2 and UT60, the *atxA* deletion mutant of *B. anthracis* Sterne 7702, were treated with 48 % HF at 4 °C for 48 h. The HF treated material was neutralized by ice-cold ammonium hydroxide solution (approximately 30%) on an ice-water bath. The neutralized material was desalted by gel permeation chromatography using fine grade Bio-Gel P2 (Bio-Rad). Water was used as the eluent and an online refractive index (RI) detector was used to monitor the sample eluting from the column. The fractions which gave a positive response in the RI-detector were collected, pooled, lyophilized and used for further analysis.

Composition analysis

Glycosyl composition analysis was done by the preparation and gas chromatography-mass spectrometric (GC-MS) analysis of trimethylsilyl (TMS) methyl glycosides (15). The TMS methyl glycosides were identified and were quantified by comparison to authentic standards. In brief, the samples were methanolyzed using 1 M methanolic HCl at 80 °C for 18 h to form the monomeric methylglycosides, followed by N-acetylation using pyridine and acetic anhydride (1:1) in presence of methanol at 100 °C for 1 h. After removing the reagents by flushing with dry nitrogen, the methyl glycosides were treated with Tri-Sil reagent (Pierce, Rockford, IL) at 80 °C for 30 min to form TMS methyl glycosides. The TMS methyl glycosides were dissolved in hexane and analyzed on a GC-MS using HP-1MS column (30 m × 0.25 mm × 0.25μm). Pyruvic acid content was measured according to the method of Katsuki (16). This method can detect less than 2 μg of pyruvic acid, and 200 μg of the isolated polysaccharides were assayed.

Glycosyl linkage analysis

The linkage analysis was performed according to a modification of the method of Ciucanu and Kerek (16). Briefly, the samples were dissolved in dry dimethylsulfoxide (DMSO, 0.250 μL) overnight with stirring, followed by addition of a DMSO/sodium hydroxide slurry (0.250 μL) and stirring for 2 h at room temperature. Methyl iodide was added to the sample and stirred for 40 min. Another aliquot of

methyl iodide was added and stirred for another 30 min. The reaction was cooled on an ice-bath and the partially methylated polysaccharide was extracted by partitioning between chloroform and water. The partially methylated sample in the chloroform layer was dried and used for the preparation of partially methylated alditol acetates (PMAAs). The partially methylated polysaccharide was methanolyzed to monomers using 1 M methanolic HCl at 80 °C for 16 h followed by hydrolysis with 4 M trifluoroacetic acid (TFA) at 100 °C for 4 h. The aldoses were reduced to their corresponding alditols by sodium borodeuteride (NaBD4) overnight at room temperature. The excess borodeuteride was neutralized using 30 % acetic acid solution and boric acid was removed as methyl borates by repeated refluxing and evaporation with acidified methanol and methanol respectively. The partially methylated alditols were then acetylated using a pyridine:acetic anhydride (1:1) solution at 100 °C for 1 h. Pyridine and acetic anhydride were removed by flushing with dry nitrogen and the PMAAs were dissolved in dichloromethane and analyzed by GC-MS using a HP-1 MS column. The linkage positions of each monosaccharide were identified from its mass fragmentation pattern and by matching its retention time to that of authentic PMAA standards.

NMR analysis

The polysaccharide samples (2-3 mg) were dissolved in 0.5 mL of regular grade deuterium oxide (D2O) (99.8% Aldrich), and lyophilized; this process was repeated to exchange the hydroxyl and amide protons with deuterium. The sample was finally dissolved in 0.5 mL 100 % D2O (100 % D; Cambridge Isotope Laboratories) and transferred to a 5 mm NMR tube. All 1- and 2-D NMR spectra were acquired at 25 °C on a 600 MHz Varian Inova instrument using the standard software supplied by Varian. Proton NMR spectra were measured using a spectral width of 8 kHz and the data were processed with HOD signal referenced to δ 4.78 ppm (the chemical shift of HOD relative to that of acetone at 25 °C). Gradient correlated spectra (gCOSY) were measured over a spectral width of 2.25 kHz in both dimensions using a dataset of (t1 × t2) of 256 × 1024 points with 16 scans. Homonuclear total correlated (TOCSY) spectra and through space nuclear Overhauser effect correlation spectra (NOESY) were collected using a dataset of (t1 × t2) of 256 × 1024 points and acquired over 32 scans. The mixing time used for TOCSY and NOESY experiments were 80 and 300 msec,

respectively. To determine the carbon chemical shift a gradient 1H-13C single quantum coherence experiment (gHSQC) was done. Spectral widths with proton and carbon dimensions of 2.25 and 13.9 kHz, respectively and a dataset of (t1 × t2) 128 × 512 with of 96 scans were used in collecting the gHSQC spectra. All the NMR data were processed and analyzed using an NMR processing software Mest-Rec version 4.7.5.0 for Windows.

Mass Spectroscopy

Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer model/type Voyager-DE BioSpectrometry Workstation (Applied Biosystems, Foster City, CA) was used to obtain the mass spectrum for each polysaccharide sample. Each sample was dissolved in 1:1 mixture of methanol:water and mixed at equal proportion (v/v) with 0.5 M 2,5-dihydroxy benzoic acid (DHB) as the matrix. About 0.7 μl of this mixture was loaded on each spot on a stainless steel MALDI plate and air-dried. The spectra were acquired in delayed, linear and positive mode using 337 nm N2 laser with acceleration voltage of 20 kV.

RESULTS

Isolation and initial analysis

Glycosyl composition analysis, as described elsewhere (17), showed that the HF-PSs from *B. anthracis* strains Ames, *B. anthracis* Pasteur, *B. anthracis* Sterne 34F2, and *B. anthracis* UT60 all had the same composition: namely, galactose (Gal), N-acetylglucosamine (GlcNAc), and N-acetylmannose (ManNAc) in an approximate 3:2:1 ratio. The composition of the HF-PS from *B. cereus* ATCC 10987 consisted of Gal, ManNAc, GlcNAc and GalNAc in a 1:1:1:1 ratio, and that of *B. cereus* ATCC 14579 of Glc, ManNAc, GlcNAc, and GalNAc in approximately a 1:1:2:1 ratio. None of the *B. anthracis* HF-PSs contained detectable levels (above 0.5 % of the sample mass) pyruvic acid as determined by the colorimetric method of Katsuki (18). Methylation analysis of the *B. anthracis* HF-PSs showed that all of these polysaccharides contained the same glycosyl linkages: namely, terminally linked Gal, 4- linked GlcNAc, 6-linked GlcNAc, 4,6-linked GlcNAc, 3,4-linked GlcNAc, 3,4,6-linked GlcNAc, and 4-linked ManNAc. The variation in the GlcNAc linkages in these polysaccharides indicated that there is heterogeneity in the substitution of the GlcNAc residues.

Mass spectrometry

Mass spectrometric analysis using MALDI-TOF MS confirmed that the *B. anthracis* HF-PSs were heterogeneous in the number of hexosyl (in this case, Gal) residues. The mass spectrum of the *B. anthracis* Ames polysaccharide is shown in Figure 1 and the proposed compositions for the various ions are given in Table 1. The mass spectrum shows a series of ion clusters. The mass ions observed in each ion cluster differ from those in the adjacent cluster by *m/z* 1095, a mass that is consistent with a hexasaccharide repeating oligosaccharide comprised of three hexosyl and three *N*-acetylhexosaminosyl residues; e.g. Gal3GlcNAc2ManNAc1. Each ion cluster contains three major ions that differ from one another by a single hexosyl unit, which, in this case, would be Gal. For example (see Table 1), *m/z* 2232 is consistent with a composition of Gal6GlcNAc4ManNAc2; 2069 contains one less Gal residue, and *m/z* 1907 contains two less Gal residues. This heterogeneity in Gal residues, together with the variation in substitution pattern of the GlcNAc residues suggests that the molecular heterogeneity in these polysaccharides is due to variation in substitution of one or more

of the GlcNAc residues by Gal residues. The fact that each ion cluster contains variation in only one or two less Gal residues indicates that these changes may occur in only one of the multiple oligosaccharide repeating units for each ion cluster; _e.g._ the _m/z_ ions 6612, 6450, and 6288 are due to [Gal3GlcNAc2ManNAc2]5**Gal3**GlcNAc2ManNAc1, [Gal3GlcNAc2ManNAc1]5**Gal2**GlcNAc2ManNAc1, and [Gal3GlcNAc2ManNAc1]5**Gal1**GlcNAc2ManNAc1, respectively.

Observed mass (m/z)	Proposed Composition
2232	$Gal_6GlcNAc_4ManNAc_2Na^+$
2069	$Gal_5GlcNAc_4ManNAc_2 Na^+$
1907	$Gal_4GlcNAc_4ManNAc_2 Na^+$
3327	$Gal_9GlcNAc_6ManNAc_3Na^+$
3165	$Gal_8GlcNAc_6ManNAc_3Na^+$
3003	$Gal_7GlcNAc_6ManNAc_3Na^+$
4422	$Gal_{12}GlcNAc_8ManNAc_4Na^+$
4260	$Gal_{11}GlcNAc_8ManNAc_4Na^+$
4098	$Gal_{10}GlcNAc_8ManNAc_4Na^+$
5517	$Gal_{15}GlcNAc_{10}ManNAc_5Na^+$
5355	$Gal_{14}GlcNAc_{10}ManNAc_5Na^+$
5193	$Gal_{13}GlcNAc_{10}ManNAc_5Na^+$
6612	$Gal_{18}GlcNAc_{12}ManNAc_6Na^+$
6450	$Gal_{17}GlcNAc_{12}ManNAc_6Na^+$
6288	$Gal_{16}GlcNAc_{12}ManNAc_6Na^+$

**Table 1:** Mass spectral data of de-O-acetylated polysaccharide from _B. anthracis_ Ames.
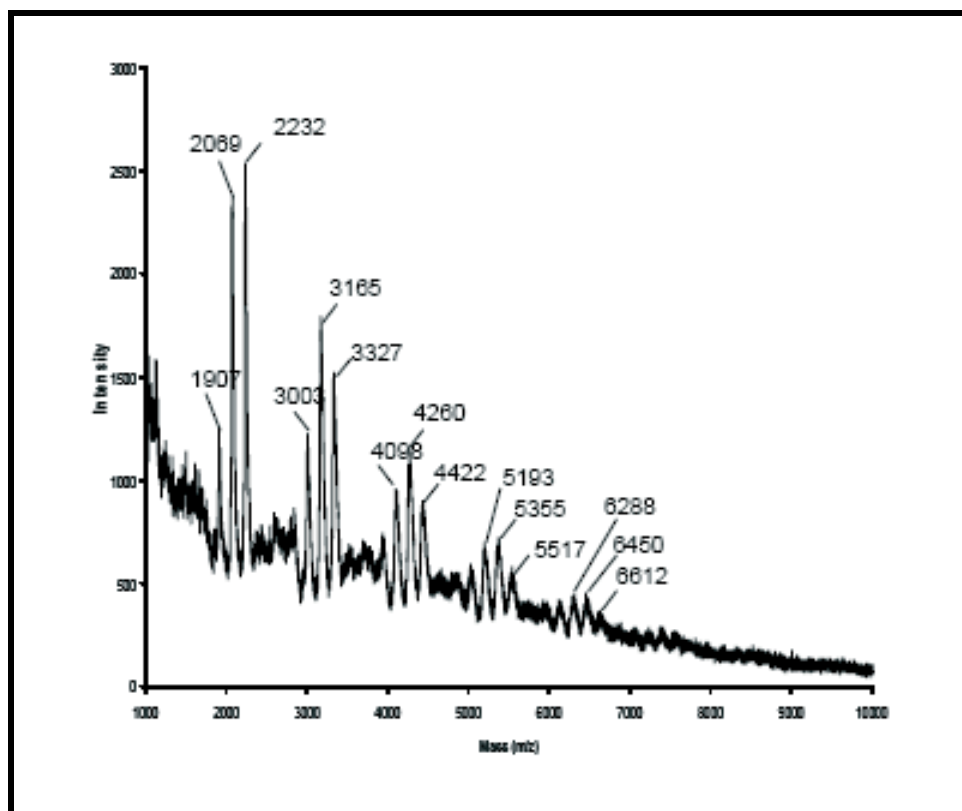
Figure 1. MALDI-TOF MS spectrum (positive mode) of the HF-PS from *B. anthracis* Ames. Analysis of the HF-PSs from *B. anthracis* Sterne, UT60, and Pasteur were all identical to this spectrum.

NMR analysis of the **B. anthracis** *polysaccharides*

Glycosyl residue compositions of the HF-PSs from *B. anthracis* are different from those for *B. cereus* ATCC 10987, and the *B. cereus* type strain ATTC 14579 (17). The proton NMR spectra comparing HF-PS from *B. anthracis* Ames with the two *B. cereus* strains is shown in Figure 2. Each spectrum clearly differs from the other in the pattern of resonances for their glycosyl anomeric and ring protons. These results show, as is indicated by the composition differences, that the structure of the HF-PS from the *B. anthracis* is different from those of *B. cereus* ATCC 10987 and ATCC 14579 and, further, that the *B. cereus* ATCC 10987 structure differs from that of strain ATCC 14579.

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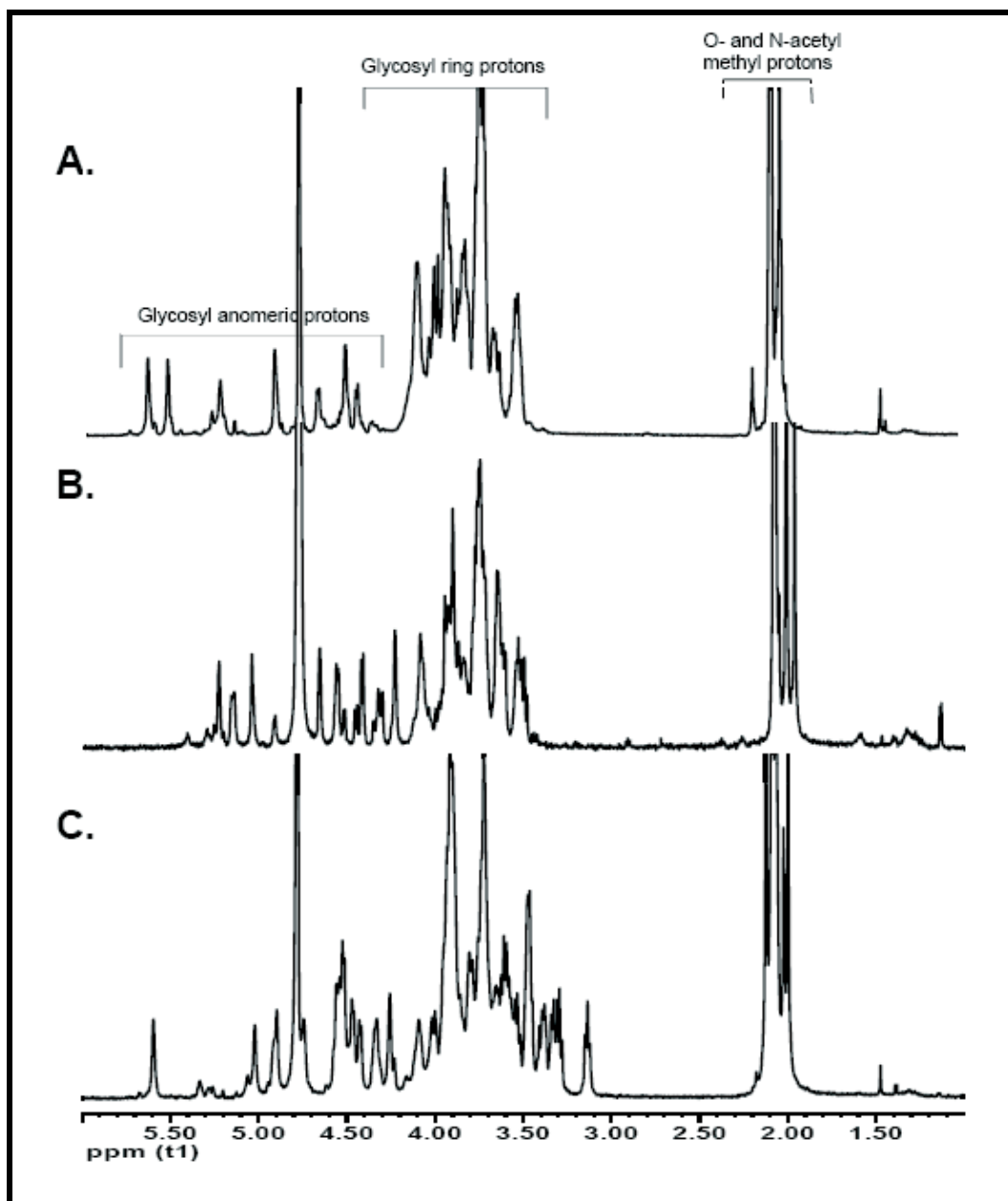
Figure 2. The proton NMR spectra for the HF-PSs. The spectra for the HF-PSs are shown for (A.) *B. anthracis* Ames, (B.) *B. cereus* ATCC 10987, and (C.) *B. cereus* ATCC 14579.

The spectra comparing the HF-PSs of *B. anthracis* Ames, *B. anthracis* Sterne and *B. anthracis* Pasteur are shown in Figure 3. These spectra are identical to one another and support the conclusion that these polysaccharides all have the same structure. Further NMR analyses (gCOSY, TOCSY, NOESY, and gHSQC) also gave identical spectra for

the HF-PSs from the *B. anthracis* strains. Because of the identical nature of these NMR analyses of the *B. anthracis* HF-PSs, the structural details are described below for the HF-PS isolated from *B. anthracis* Ames.
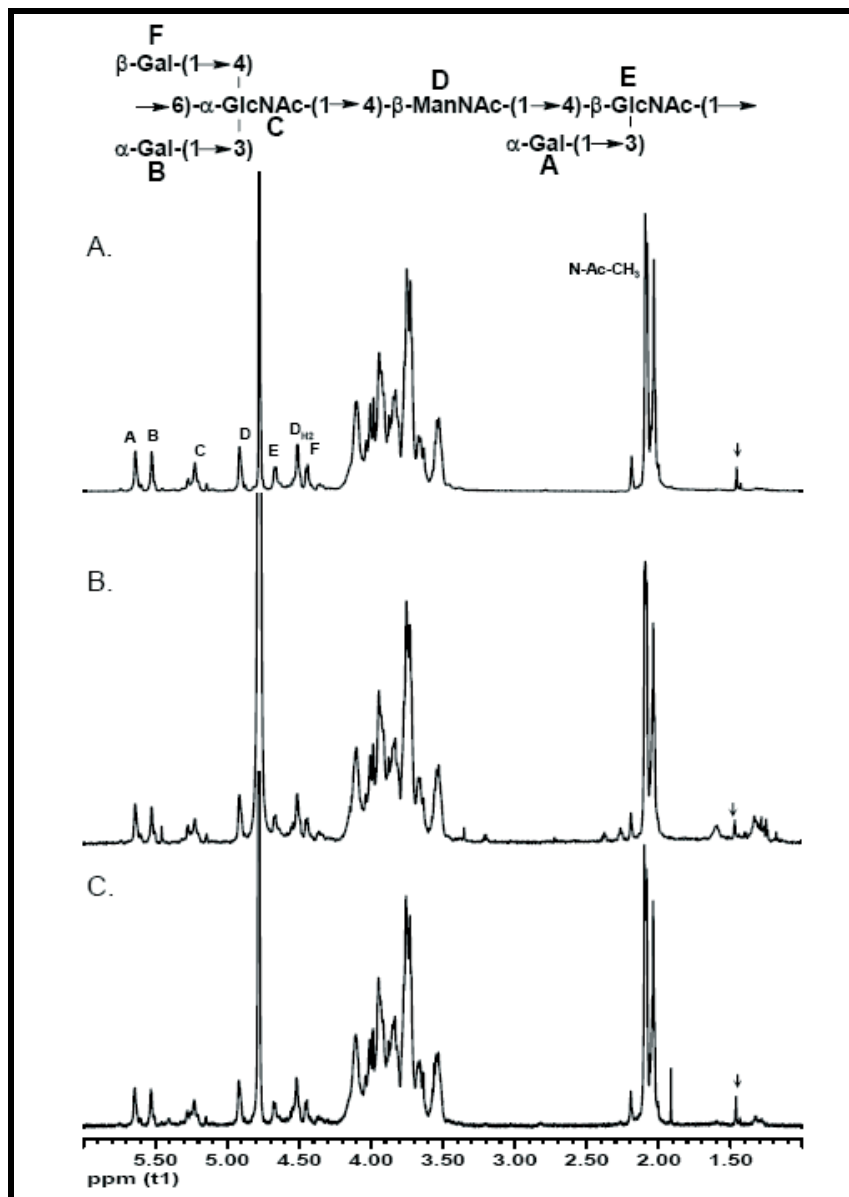

Figure 3. The proton NMR spectra for the HF-PSs from the various *B. anthracis* strains. The spectra are shown for the HF-PSs from (A.) *B. anthracis* Aims, (B.) B. anthracis Sterne, and (C.) *B. anthracis* Pasteur. The spectrum for *B. anthracis* UT60 was identical to those shown in this figure.

The proton spectrum given in Figure 3A for the *B. anthracis* Ames polysaccharide shows it contains six anomeric signals at δ 5.64, δ 5.53, δ 5.22, δ 4.91, δ 4.67 (J1,2 = 7.2 Hz), δ 4.44 (J1,2 = 7.8 Hz) supporting the conclusion that this

polysaccharide consists of a hexasaccharide repeat unit a result that is consistent with the composition and mass spectrometric data described above. Furthermore, the chemical shifts and J1,2 coupling constants of these anomeric protons indicate that three of these glycosyl residues are α-anomers, and at least two are β-anomers, while the anomeric configuration of the remaining glycosyl residue (i.e. the glycosyl residue with H1 at δ 4.91) can not be deduced from the 1-D proton spectrum due to its small J1,2 coupling which indicates that this residue is in the *manno* configuration. The presence of a repeating unit of six glycosyl residues is also supported by the HSQC spectrum (Figure 4) which clearly shows six anomeric proton/carbon resonances.
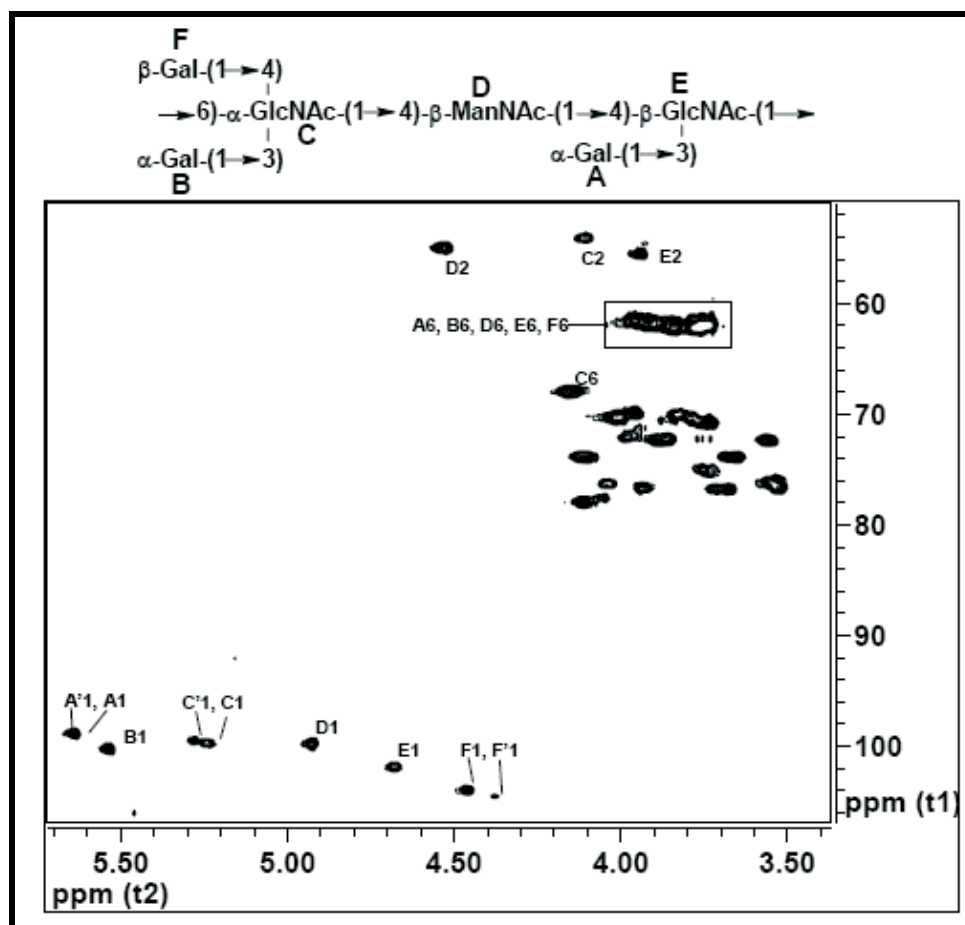
Figure 4. The HSQC spectrum of the HF-PS from *B. anthracis* Ames. The structure and the assigned proton/carbon correlations are as shown. The complete NMR assignments are given in Table 2. The HSQC spectra of the HF-PSs from *B. anthracis* Sterne, UT60, and Pasteur are identical to this spectrum.

Residue	H1(C1)	H2(C2)	H3(C3)	H4(C4)	H5(C5)	H6(C6)
A. α-D-Gal	5.64 (98.8)	3.82 (70.0)	3.74 (70.6)	4.00 (72.2)	3.84 (72.2)	≈ 3.76 (61.9)
B. α-D-Gal	5.53 (100.3)	3.77 (70.3)	3.72 (70.6)	3.98 (70.3)	3.87 (72.2)	≈ 3.73 (61.9)
C. α-D- GlcNAc	5.22 (99.7)	4.09 (54.1)	4.02 (76.3)	4.03 (77.5)	3.94 (71.9)	4.12/4.07 (68.1)
D. β-D-ManNAc	4.91 (99.7)	4.51 (55.0)	4.10 (73.8)	3.74 (75.3)	3.51 (76.3)	3.84/3.77 (62.2)
E. β-D- GlcNAc	4.67 (101.9)	3.92 (55.3)	3.92 (76.6)	4.10 (78.1)	3.54 (76.3)	3.84/3.77 (61.6)
F. β-D-Gal	4.44 (104.1)	3.54 (72.5)	3.64 (73.8)	3.94 (70.0)	3.63 (73.8)	3.84/3.77 (61.9)

Table 2: 1H and 13C chemical shift values for the *B. anthracis* Ames cell wall polysaccharide.

We also observed a resonance at δ 1.48 which is consistent with the methyl protons of a pyruvyl substituent as reported by Mesnage *et al.* (4). However, the relatively low intensity of this resonance indicates that the putative pyruvyl component is present in non-stoichiometric and low amounts. Pyruvate is an acid labile component and it is very likely that the majority of this component was removed during the aqueous HF treatment of the cell walls. The assignments of the proton and carbon resonances for the polysaccharides were determined by a series of two-dimensional NMR experiments; COSY (not shown), HSQC (Figure 4), TOCSY (Figure 5), and NOESY (Figure 6) analyses. The rationale for these assignments (given in Table 2) is described in the following paragraphs.

Residue A contains an anomeric proton, H1, resonating at δ 5.64. The H1 through H4 assignments are readily assigned from the COSY and TOCSY (Figure 5) data. It is apparent from the TOCSY spectrum that the H4 resonance at δ 4.00 has a small overall coupling to the adjacent H3 and H5 protons (i.e. J3,4 + J4,5 < 9.6 Hz) supporting the conclusion that **A** has a *galacto* configuration, and, therefore, is an α-Gal

residue. In order to assign H5 and H6, it was necessary to determine, using the TOCSY data, the resonances of the protons coupled to H4. This analysis showed that H4 is coupled to protons at δ 3.74 (H3), δ 3.82 (H2), and another proton at δ 3.84 which was assigned as H5. The H5 resonance was, in turn coupled to protons in the δ 3.76 range which are likely the H6 protons. The HSQC spectrum (Figure 4) shows that protons at δ 3.76 are coupled to a C6 at δ 62 which supports the presence of H6 protons at this chemical shift. The remaining carbon chemical shifts for this residue, and for the following residues were also obtained from the HSQC spectrum (Figure 4).

Residue B has an anomeric H1 at δ 5.53. As with residue **A**, the H1 through H4 resonances are readily assigned from the COSY and TOCSY (Figure 5) data, and the small overall J3,4 and J4,5 coupling constants of H4 (< 9.6 Hz) show that residue **B** has a *galacto* configuration and is a second α-Gal residue. Further analysis of the TOCSY data show that H4 (δ 3.98) is coupled to H3 (δ 3.72), H2 (δ 3.77), and a proton resonating at δ 3.87 which was assigned to H5. This proton was coupled to H6 protons with chemical shifts in the δ 3.73 range. As with residue **A**, due to overlapping resonances in this range, it was not possible to determine the exact chemical shifts of the H6 protons. However, the protons in this range are coupled to C6 carbons that resonate at about δ 62 (the HSQC spectrum, Figure 4) supporting that these are H6 protons.

The anomeric proton of **residue C** has a chemical shift of δ 5.22. The H1 through H5 assignments were made from the COSY and TOCSY (Figure 5) data. Further analysis of the TOCSY data showed that H3 (δ 4.02) was coupled to H4 (δ 4.03), H5 (δ 3.94) and to protons at δ 4.07 − 4.12 which were assigned as the H6 protons. The HSQC spectrum (Figure 4) showed that protons at δ 4.07 − 4.12 were coupled to a carbon at δ 68.1 which is consistent with a glycosyl residue that is substituted at position C6. The HSQC spectrum also showed that H2 (δ 4.09) was coupled to a carbon at δ 54.1 consistent with this carbon having an attached nitrogen and, therefore, supporting the conclusion that this is a glycosaminosyl residue. Since composition analysis shows the presence of only GlcNAc and ManNAc residues, the TOCSY proton interactions from H1 through H5 supports the conclusion that this residue has large glycosyl ring proton-proton coupling constants that are consistent with a *gluco* configuration and, therefore, residue **C** is identified as a α-GlcNAc residue.

The anomeric H1 of **residue D** has a chemical shift of δ 4.91. The COSY and TOCSY (Figure 5) data show that H1 is coupled to H2 at δ 4.51. The TOCSY spectrum shows that only H2 can be observed via H1 indicating that residue **D** has a very small J1,2 coupling and, therefore, has a *manno* configuration. The HSQC spectrum shows that H2 is coupled to a nitrogen-bearing carbon at δ 55.0 supporting the conclusion that residue **D** is a glycosaminosyl residue. Examination of the protons coupled to H2 from the TOCSY data allowed assignment of H3 (δ 4.09), H4 (δ 3.74), and H5 (δ 3.51). The TOCSY spectrum also showed that H5 is coupled to H3, H4, and to protons with chemical shifts at δ 3.84/3.77 which were assigned as H6 protons. These protons were coupled to a carbon resonating at δ 62.2 consistent with a C6 carbon. The anomeric configuration of a *manno* residue is difficult to determine since both α- and β-anomers have small J1,2 coupling constants. However, the NOESY spectrum (Figure 6, discussed further below) shows NOEs between H1, H3, and H5 supporting the conclusion that these protons all have axial positions and, therefore, that this residue has a β-configuration. Thus, **D** is a β-ManNAc residue.

The H1 of **residue E** has a chemical shift of δ 4.67 and, as described above, both this chemical shift and the J1,2 value of 7.2 Hz show that it has a β-configuration. The COSY and TOCSY (Figure 5) data show that H1 is coupled to H2 at δ 3.92, H3 also at δ 3.92, H4 at δ 4.10, and H5 at δ 3.54. Further analysis of the TOCSY data showed that H5 is coupled to protons at δ 3.77 and 3.84 which were assigned as H6 protons, since these protons are coupled to a C6 with a chemical shift of δ 61.6 (HSQC analysis, Figure 4). The HSQC spectrum also shows that H2 (δ 3.92) is coupled to a nitrogen-bearing carbon at δ 55.3. These results show that residue **E** is the remaining glycoaminosyl residue, a β-GlcNAc residue.

Residue F has an anomeric H1 with a chemical shift of δ 4.44 and a J1,2 coupling of 7.8 Hz showing that it has a β-configuration. The COSY and TOCSY (Figure 5) data allow assignment from H1 to H2 (δ 3.54), H3 (δ 3.64), and H4 (δ 3.94). The TOCSY data also revealed that H4 has a small total J3,4 plus J4,5 coupling of less than 9.6 Hz showing that residue **F** has a *galacto* configuration. The TOCSY data also show that H4 is coupled to H2 and H3 as expected, and also to a proton with a chemical shift of δ 3.63 which was assigned to H5. This H5 was, in turn coupled to protons at δ 3.77 to 3.84 which were assigned as the H6 protons. The HSQC spectrum (Figure 4)

showed that these protons are coupled to a C6 carbon at δ 61.9. Thus, **F** is a β-Gal residue.

The COSY and TOCSY spectra, as with the methylation and mass spectrometric data, also suggest heterogeneity in the polysaccharide. There are multiple versions of residue **C** (the α-GlcNAc residue) as evidenced by an additional minor glycosyl ring system connected to an anomeric proton at δ 5.27 (residue **C'**), and another minor glycosyl ring system at δ 5.14 (residue **C''**), Figure 4. Similarly, there is an additional version of residue **A**, **A'**, as evidenced by another ring system through an H1 at δ 5.60, and an additional version of residue **F**, **F'**, via a ring system through H1 at δ 4.36. These additional terminal α- and β-Gal glycosyl ring systems (**A'** and **F'**) as well as the additional α-GlcNAc residues (**C'** and **C''**) support the above methylation and mass spectrometric data that show heterogeneity in the GlcNAc substitution pattern, and heterogeneity in the level of hexose (i.e. in this case, Gal) addition, likely due to variable substitution of the α-GlcNAc residue **C** by the Gal residues **A** and **F**.

The sequence of the glycosyl residues was determined from by NOESY analysis, Figure 6. Residue **A**, α-Gal, has a strong inter-residue NOE from H1 at δ 5.64 to H3 (δ 3.92) of residue **E**, β-GlcNAc, supporting a α-Gal-(1→3)-β-GlcNAc sequence. There is also a weak inter-residue NOE to H4 (δ 4.10) of the β-GlcNAc residue and strong and weak intra-residue NOEs to H2 at δ 3.82 and H3 at δ 3.74, respectively.

Residue **B**, the second α-Gal, has a strong inter-residue NOE from H1 at δ 5.53 to H3 (δ 4.02) of residue **C**, α-GlcNAc, supporting a α-Gal-(1→3)-α-GlcNAc sequence. A weak inter-residue NOE to H2 (δ 4.09) of residue **C** was also present. Strong and weak intra-residue NOEs to H2 and H3 at δ 3.77 and 3.72, respectively were also observed.

Residue **C,** α-GlcNAc**,** has a strong inter-residue NOE from H1 (δ 5.22) to the H4 (δ 3.74) of residue **D**, β-ManNAc, which supports a α-GlcNAc-(1→4)-β-ManNAc sequence. This information combined with inter-residue NOE for residue **B** described above shows that the oligosaccharide repeating unit has the following partial sequence:

α-GlcNAc-(1→4)-β-ManNAc
α-Gal-(1→3)⎦
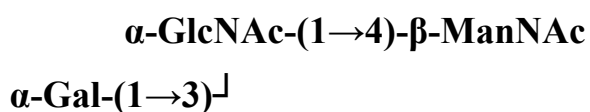
There is also a strong intra-residue NOE from the H1 of residue **C** to its H2 at δ 4.09.

Residue **D**, β-ManNAc, has NOEs to protons at δ 4.51, 4.10, 3.92, 3.74, and 3.51. The NOEs at δ 4.51 and 3.51 are due to intra-residue interactions with H2 and H5, respectively, as expected for a β-linked ManNAc residue. The NOE to the proton at δ 3.92 is an inter-residue NOE to H3 of residue **E**, β-GlcNAc. However, it is unlikely that the ManNAc residue is attached to this position of β-GlcNAc since, as described above, it is already occupied by a α-Gal residue (residue **B**). However, there is also a strong NOE to a proton at δ 4.10. It is likely that this NOE is due to a combination of an intra-residue NOE to H3 (δ 4.10) and an inter-residue NOE to H4 (δ 4.10) of residue **E**, β-GlcNAc. The placement of β-ManNAc at this position on the β-GlcNAc likely results in a close spacial arrangement the β-ManNAc H1 to the H3 of the β-GlcNAc residue accounting for the NOE between these two protons. Therefore, these data indicate the presence of a β-ManNAc-(1→4)-β-GlcNAc sequence and, together with the inter-residue NOEs described above for residues **A**, **B** and **C** indicate that the polysaccharide contains the following partial sequence:

α-GlcNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc

α-Gal-(1→3)⌐   **α-Gal(1→3)⌐**
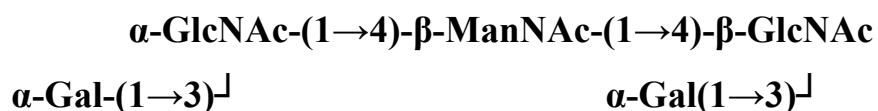
The presence of a 3,4-linked GlcNAc residue is also consistent with the methylation data described earlier. The β-ManNAc residue also has an NOE from H1 to a proton at δ 3.74. Since this residue is a β-linked ManNAc, it is unlikely that this proton is the intra-residue H4 as that proton would not be in close proximity to H1. However, it is possible that one of the H6 protons of the α-Gal residue (**A**) (in the δ 3.74 to δ 3.77 range) linked to position C3 of the β-GlcNAc residue is in close enough proximity to the ManNAc H1 to account for this NOE.

Residue **E**, the β-GlcNAc residue, has NOEs to protons at δ 4.12, 3.92, and 3.54. The NOEs to δ 3.92 and 3.54 are intra-residue contacts to H3 and H5, respectively which would be expected for a β-linked GlcNAc residue. The contact at δ 4.12 is due to an inter-residue NOE to H6 of residue **C**, the α-GlcNAc residue. Thus, residue **E**, the β-

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GlcNAc residue is attached to position 6 of residue **C**, the α-GlcNAc residue, indicating the following partial sequence for this repeating oligosaccharide:

→6)-α-GlcNAc-(1→4)-β-ManNAc-(1→4)-β−GlcNAc-(1→

α-Gal-(1→3)⌐   **α-Gal-(1→3)⌐**

An inter-residue NOE with the H1 (δ 4.44) of residue **F**, β-Gal, was also observed indicating that the anomeric protons of residues **E** and **F** are in close proximity (discussed further below).

The remaining residue, **F** (β-Gal), has a strong inter-residue NOE to H4 (δ 4.03) of residue **C** as well as intra-residue NOEs to H2, H3, and H4 at δ 3.54, 3.64, and 3.94, respectively. The NOE at δ 3.64 could also overlap somewhat with an intra-residue NOE to H5 at δ 3.63. These results indicate that the β-Gal residue **F** is attached to the α-GlcNAc residue **C** at C4. As described above for residue **E**, a NOE between the anomeric protons of residues **F** and **E** were also observed supporting that the anomeric protons of these two residues are in close proximity. Therefore, these NMR data together with the MS, glycosyl composition and linkage data show that the polysaccharide contains the following overall repeating unit sequence:

β-Gal-(1→4)⌐

  →6)-α-GlcNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc-(1→

α-Gal-(1→3)⌐   **α-Gal-(1→3)⌐**
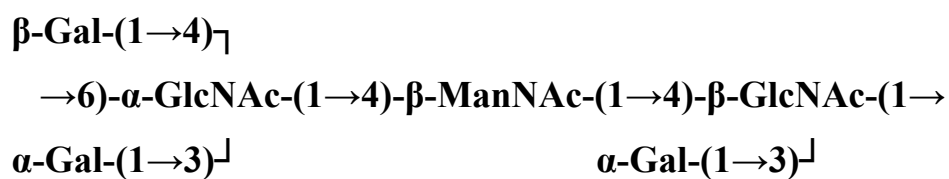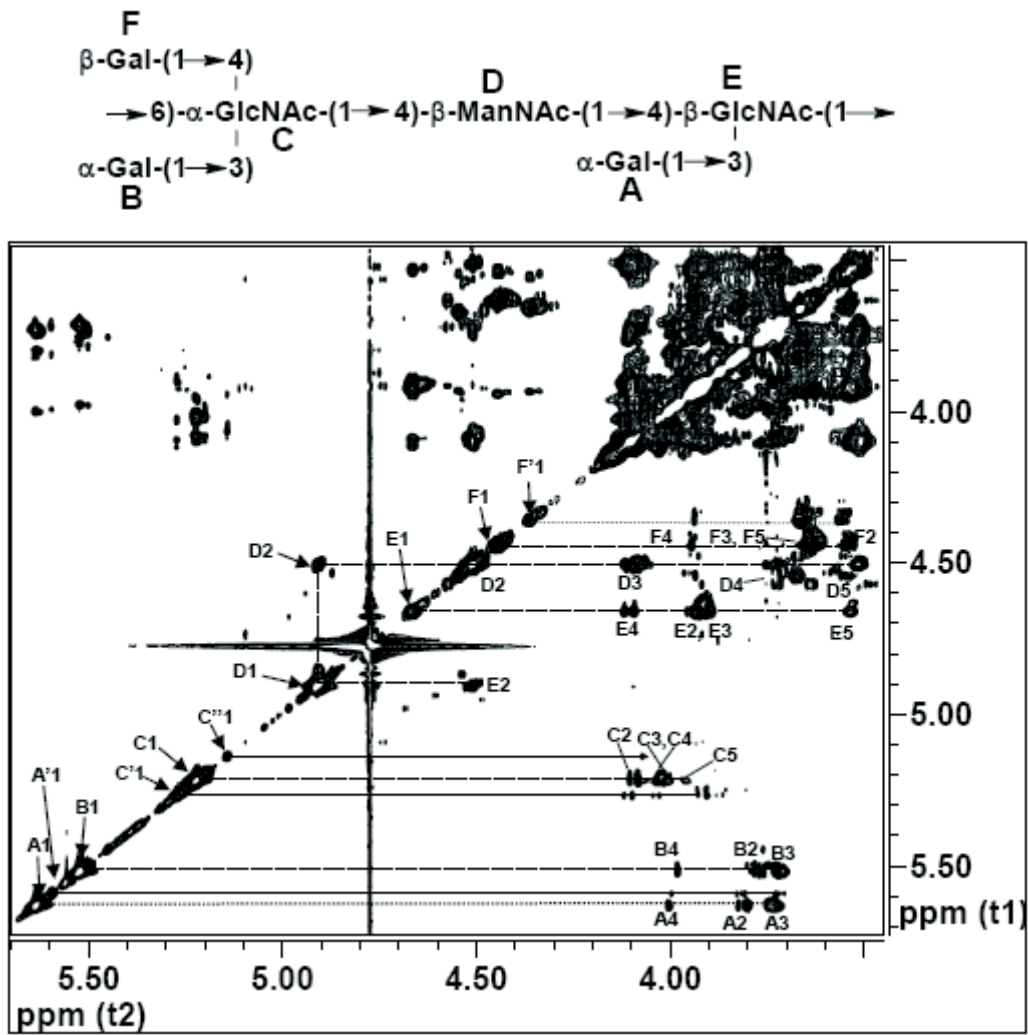
Figure 5. The TOCSY spectrum of the HF-PS from *B. anthracis* Ames. The structure and the assigned proton resonances are as shown. The complete NMR assignment is given in Table 2. The TOCSY spectra of the HF-PSs from *B. anthracis* Sterne, UT60, and Pasteur are identical to this spectrum.
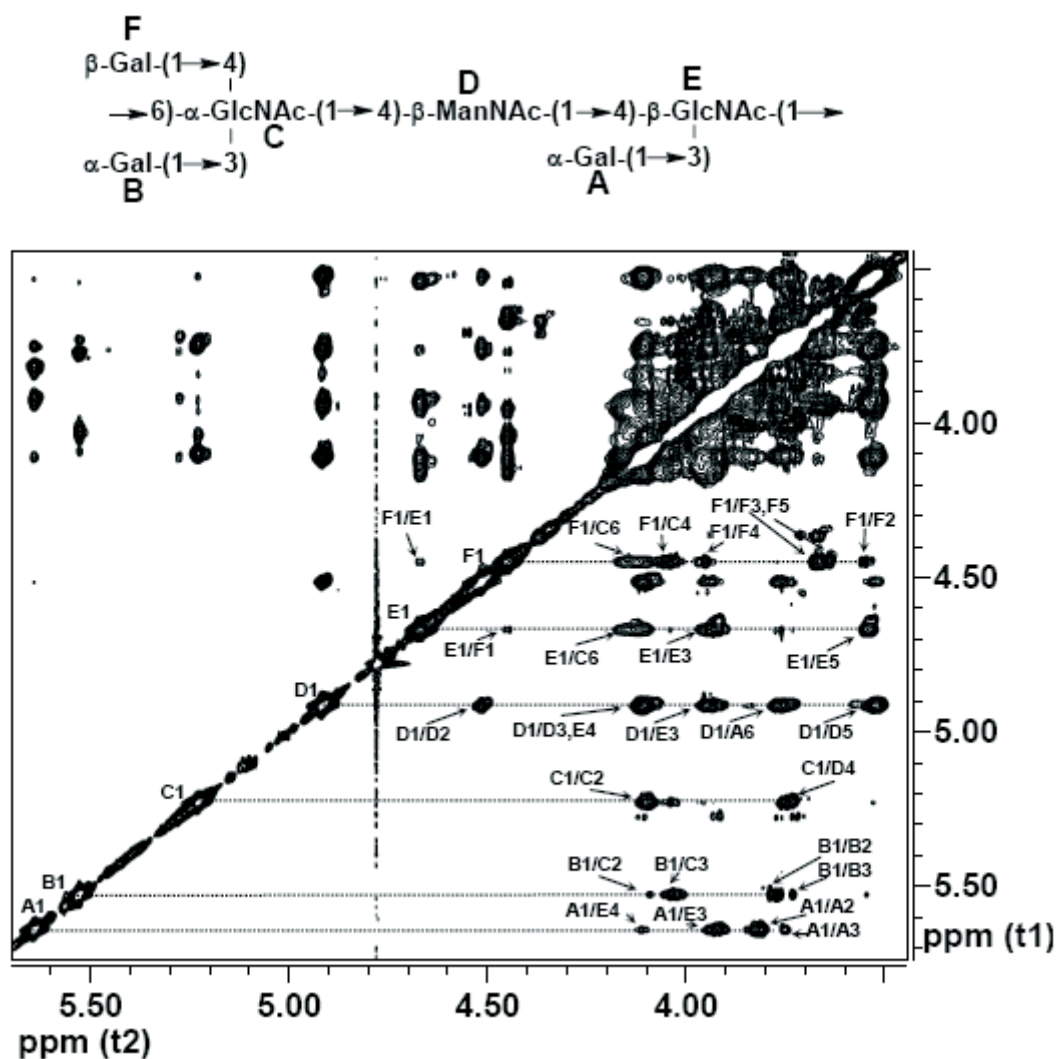
Figure 6. The NOESY spectrum of the HF-PS from *B. anthracis* Ames. The structure and the inter- and intra-residue NOEs are indicated. The NOESY spectra of the HF-PSs from *B. anthracis* Sterne, UT60, and Pasteur are identical to this spectrum.

DISCUSSION

HF-PS from *B. anthracis* (Figure 7) and demonstrate that this structure is the same for *B. anthracis* Ames, *B. anthracis* Sterne, *B. anthracis* UT60, and *B. anthracis* Pasteur.
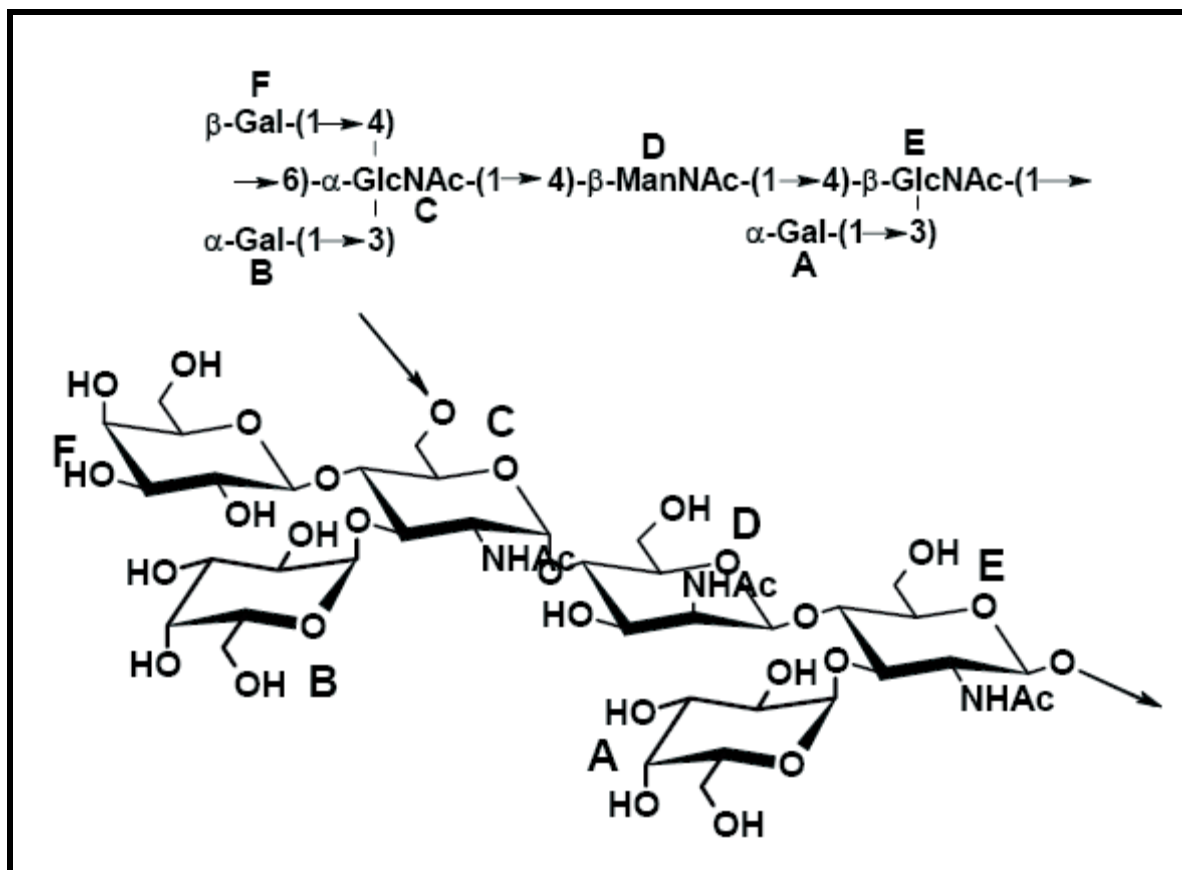
Figure 7. The structure of the HF-PS repeating oligosaccharide from *B. anthracis*.

In addition, a proton NMR comparison, as well as composition analysis, shows that this structure is different from the HF-PS from a strain of *B. cereus* ATCC 10987 that is closely related to *B. anthracis* (18) and from the HF-PS of *B. cereus* ATCC 14597 (the type strain). Earlier publications reported the composition of the cell wall polysaccharide from *B. anthracis* and that of the major cell wall polysaccharide released from the cell wall by treatment with aqueous HF (4,6). This HF treatment disrupts the phosphate bridge of the polysaccharide to the cell wall peptidoglycan. The composition

of the HF-PS was reported to consist of Gal, ManNAc, and GlcNAc in a 3:1:2 ratio (6), or in a 10:3:1 ratio (4). Our work, as described in this report, is consistent with the earlier report of Ekwunife _et al._ (6) showing that the _B. anthracis_ HF-PS consists of these glycosyl residues in a 3:1:2 ratio. It was also reported that the HF-PS was pyruvylated, (4) and that the pyruvyl substituent is required for the function of the HF-PS; acting as the ligand for the SLH domain of S-layer proteins (4). From our results, the presence of a pyruvyl substituent is uncertain (described further below).

It is worth noticing that a number of reported polysaccharide structures from related _Bacilli_ strains have the common feature of an amino sugar backbone substituted by branching glycosyl residues and non-carbohydrate substituents. The major polysaccharide from _B. subtilis_ AHU 1219 cell walls consists of a →6)-α-GalNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc-(1→ backbone in which the GalNAc residue is substituted at O3 with a β-Glc residue and the ManNAc residue is substituted at O3 with a β-GlcNAc residue (19). The major polysaccharide for the cell walls of _Paenibacillus polymyxa_ AHU 1385 (formerly _B. polymyxa_) consists of a →3)-β-ManNAc-(1→4)-β-GlcNAc-(1→ in which the ManNAc residue is substituted with a pyruvyl residue at the O4/O6 positions (20). A major _B. cereus_ cell wall polysaccharide from strain AHU 1356 is reported to have a →3)-α-GalNAc-(1→4)-β-ManNAc-(1→3)-α-GlcNAc-(1→ in which the GalNAc residue is substituted with an α-Glc at O6, the ManNAc with a β-GlcNAc at O3, and the GlcNAc residue with a →6)-β-GlcNAc-(1→6)-β-GlcNAc-(1→ disaccharide at O6 (11). The structure we report here for the HF-PS from the _B. anthracis_, as shown in Figure 7, also consists of an amino sugar backbone of →6)-α-GlcNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc-(1→ 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. The data also suggest that there is variability in the presence of the two Gal substitutions on the α-GlcNAc residue. A common feature in the backbone structure of all of these polysaccharides, including the _B. anthracis_ structure, seems to be the presence of a ManNAc-GlcNAc disaccharide component. The commonality of the amino sugar backbone in all of these _Bacilli_ cell wall polysaccharides may indicate that this molecule has an essential function for the viability of _Bacilli_ species.

That the HF-PS from *B. anthracis* Sterne had essential functions is supported in a report by Mesnage *et al*. (4). While Mesnage *et al*. did not determine the structure of this polysaccharide, their proton NMR spectrum for *B. anthracis* Sterne is identical to our spectra for the HF-PSs isolated from *B. anthracis* Ames, *B. anthracis* Sterne and *B. anthracis* Pasteur (Figure 3). Mesnage *et al*. state that pyruvate is a component of the HF-PS; however, while our NMR spectra showed a resonance consistent with a pyruvyl methyl group (the resonance at δ 1.48, see Figure 2) as reported by Mesnage *et al*. (4), colorimetric analysis failed to detect pyruvate in any of the *B. anthracis* HF-PSs. The reason for this discrepancy is unknown at this time. However, since pyruvate substituents are labile to mild acid, it is possible that the majority of these substituents were removed by HF-treatment and, in our work, resulted in lowering the pyruvate content below detectable levels when using the colorimetric assay. Mesnage *et al*. (4) reported that a *csaB* mutant was affected in the addition of the pyruvyl substituent to the polysaccharide in that it lacked the resonance at δ 1.48. The *csaB* mutant was shown to be defective in locating the S-layer proteins, EA1 and Sap, to its surface. The mutant also showed an increase in sedimentation when grown in liquid medium, formation of aberrant colonies on solid medium, and on microscopic examination, a defect in cell separation. Furthermore, the *csaB* mutant failed to undergo autolysis. Since all of these effects were produced by the failure to substitute the otherwise normal HF-PS with pyruvate it seems likely that the ability to produce the entire polysaccharide is essential for the viability of *B. anthracis;* therefore, its synthetic mechanism is a potential target for novel therapeutics.

In addition to the above described possible function of the HF-PS in *B. anthracis* in autolysis and cell division, a recent report by Mayer-Scholl *et al*. (21) presents data which indirectly indicate a role for a cell wall polysaccharide in the defense response of the host. They showed that the active component from the neutrophil granule that killed vegetative *B. anthracis* cells were α-defensins. The α-defensins are cationic peptides that are part of the innate immune system and are involved in the resistance of a host toward both Gram-negative and Gram-positive infections. Since α-defensins are lectin-like and likely function by binding to carbohydrate components on the surface of the pathogen (22), it seems quite possible that the ability of neutrophils to kill vegetative *B. anthracis* cells depends on the binding of α-defensins to a carbohydrate component of

the vegetative cell wall. It is tempting to speculate that the α-defensin ligand may be the HF-PS. The merits of this speculation obviously require investigation.

As described above, previous composition analysis and structural determination have shown that the major polysaccharide from *Bacillus* cell walls varies depending on the species being examined. We have shown that this variation is even more refined in that glycosyl compositions vary among *B. cereus* clades and also among lineages within a single clade (17). These composition differences reflect structural variation in the *B. cereus* group as demonstrated by the different NMR spectra (Figure 2) of the HF-PSs from *B. anthracis*, *B. cereus* ATCC 10987, and *B. cereus* ATCC 14579. The *B. cereus* ATCC 10987 strain is quite closely related to *B. anthracis* strains as reported by Rasko *et al.* (23) in that it contains a plasmid that is similar to pXO1 but lacks the pathogenicity island that encodes for the toxin components. In fact, the genome of *B. cereus* ATCC 10987 is 93.7% similar to *B. anthracis* while it is 90.9% similar to *B. cereus* ATCC 14579 (23). Thus, these results support the conclusion that the *B. anthracis* structure we report here is specific to *B. anthracis* and different from that of even closely related *B. cereus* strains.

An exception to this conclusion may be several pathogenic isolates of *B. cereus* which are able to cause pneumonia in humans (24). Ongoing work in our laboratory shows that in these *B. cereus* strains, the cell wall polysaccharide compositions are very close to those of the *B. anthracis* strains (17). These results suggest that the major cell wall polysaccharide may have a function in determining the virulence of *B. anthracis* as well as of these pathogenic *B. cereus* strains. Current work in our laboratory is focused on more detailed structural comparisons of the HF-PSs from the pathogenic *B. cereus* strains that are closely related to *B. anthracis*.

In conclusion, we have described the structure of the predominant cell wall carbohydrate of *B. anthracis*. We propose that this cell wall carbohydrate is critical for viability and for pathogenicity of *B. anthracis* and therefore, may be a target for development of specific antimicrobials against anthrax.

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FOOTNOTES

4

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Structural elucidation of the non-classical secondary cell wall polysaccharide from *B. cereus* ATCC 10987 and comparison with the polysaccharides from *B. anthracis* and *B. cereus* type strain ATCC 14579 reveals a common structural theme with varying modifications.

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Running Title: *Bacillus cereus* cell wall carbohydrate structures

Contribution to this publication

I prepared the first draft of this manuscript and was involved in the design and configuration of tables and figures. The isolation of the cell walls and purification of the HF-PS as well as their analyses by gas chromatography mass spectrometry, MALDI-TOF analysis and glycosyl linkage analysis was performed by me. I assisted Biswa Choudhury with the NMR spectroscopy as well as with the assignment of the 2D NMR signals. Elke Saile was responsible for growing the bacterial cultures used in the experiments and preparing them for shipment. The bacterial strains required cultivation in BSL2 and BSL3 laboratories at the Center for Disease Control (CDC).

Key findings and conclusions

- The HF-PS from *B. cereus* ATCC 10987 was determined to be a O-acetylated tetrasaccharide repeating unit with the following structure:

$\rightarrow$6)-α-GalNAc-(1$\rightarrow$4)-β-ManNAc-(1$\rightarrow$4)-β-GlcNAc-(1$\rightarrow$

β-Gal-(1$\rightarrow$3)$\lrcorner$ Ac$_{(0.6)}$-($\rightarrow$3)$\lrcorner$

- Structural data of the HF-PS from *B. cereus* ATCC 14579 type strain indicate a [-[Hex]-HexNAc-HexNAc-HexNAc-] structural theme in common with the HF-PS from *B. cereus* ATCC 10987.

- Composition analysis showed the absence of galactose in *B. cereus* ATCC 14579 but the presence of greater amounts of glucose as compared to the HF-PS from *B. cereus* ATCC 10987.

- Significant differences of the *B. cereus* ATCC 14579 HF-PS to the HF-PS form *B. anthracis* include the presence of N-acetylgalactosamine and glucose which are both not found in the HF-PS of latter strain.

ABSTRACT

Non-classical secondary cell wall polysaccharides constitute a major cell wall structure in the *Bacillus cereus* group of bacteria. The structure of the secondary cell wall polysaccharide from *Bacillus cereus* ATCC 10987, a strain that is closely related to B. anthracis, was determined. The polysaccharide was released from the cell wall with aqueous hydrogen fluoride (HF) and purified by gel filtration chromatography. The purified polysaccharide, HF-PS, was characterized by glycosyl composition and linkage analyses, mass spectrometry, and 1- and 2-D NMR analysis. The results showed that the *B. cereus* ATCC 10987 HF-PS has the following structure:

$$\rightarrow 6)\text{-}\alpha\text{-GalNAc-}(1\rightarrow 4)\text{-}\beta\text{-ManNAc-}(1\rightarrow 4)\text{-}\beta\text{-GlcNAc-}(1\rightarrow$$

$$\beta\text{-Gal-}(1\rightarrow 3)^\rfloor \qquad Ac_{(0.6)}\text{-}(\rightarrow 3)^\rfloor$$
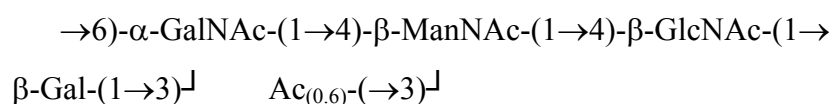
Comparison of this structure with that of the *B. anthracis* HF-PS and with structural data obtained for the HF-PS from *B. cereus* type strain ATCC 14579 revealed that each HF-PS had the same general structural theme of -[Hex]-HexNAc-HexNAc-HexNAc-. A common structural feature is that all of the HF-PSs contain a repeating unit consisting of a $HexNAc_3$ trisaccharide backbone in which two of the three HexNAc residues are GlcNAc and ManNAc, and the third can be either GlcNAc or GalNAc. The implications of these results with regard to the possible functions, and characterization of those functions, are discussed.

INTRODUCTION

Bacillus cereus ATCC 10987, *B. cereus* ATCC 14579, and *B. anthracis* strains belong to the *Bacillus cereus* group of strains, i.e. a group of Gram-positive, phylogenetically closely related bacteria. *Bacillus cereus* strains are best known as opportunistic pathogens causing a range of conditions including severe pneumonia, sepsis, endophthalmitis and emetic food poisoning. *B. anthracis* causes anthrax and is considered a high threat bioterrorism agent.

Comparatively little is known about the cell wall polysaccharide structures in strains of the *Bacillus cereus* group. In Gram-positive bacteria, the cell walls are generally comprised of a number of polysaccharides and polysaccharide conjugate classes that are specific for these bacteria. This specific molecular repertoire includes capsule polysaccharides, peptidoglycan-anchored teichoic- and teichuronic acids, lipoteichoic acids or polysaccharides that anchor crystalline cell surface proteins (S-layer proteins) to the cell wall (20). There are different classes of these polysaccharides that can vary between species and much remains to be learned about their structure-function relationships. For example, their involvement in pathogenicity is still a matter of debate. Only recently have bacterial mutants become available that allow the function of cell wall components to be addressed and to demonstrate the importance of teichoic and lipoteichoic acids in the virulence of pathogenic strains (7, 9, 24). The cell wall architecture of *B. anthracis* has a number of structural features that are an exception to the general picture of Gram-positive cell walls. It was reported that *B. anthracis* lacks teichoic and teichuronic acids (14) and that it displays a non-carbohydrate capsule consisting of poly-γ-D-glutamate (13).

An earlier report established that differences exist in the glycosyl composition of cell walls from *B. anthracis*, *B. cereus* and *B. thuringiensis* (11). In a recent report it has been shown that the differences relate, at least in part, to a polysaccharide that is released from the cell walls through treatment with aqueous hydrofluoric acid (HF), indicating the involvement of a HF labile phosphate bond in the anchoring of a glycosyl polymer to the bacterial cell wall (17). In *B. anthracis*, recent structural characterization of this HF-released polysaccharide (HF-PS) showed that it comprises a trisaccharide backbone consisting of two *N*-acetylglucosamine (GlcNAc) and one *N*-

acetylmannosamine (ManNAc) residue, and that this backbone is variably substituted with terminal galactose (Gal) residues (4, 17). This structure falls into the class of non-classical secondary cell wall polymers (SCWP) of Gram-positive bacteria, as defined by Schäffer *et al.* (22).

In *B. anthracis,* the HF-PS is thought to anchor the S-layer proteins to the wall of the vegetative cells (19). The S-layer proteins form a paracrystalline protein sheath on the surface of the vegetative bacilli. They contain a S-layer homology (SLH) domain that is found in a number of other *Bacillus* species proteins of putative or unclear function (21), but also in S-layer proteins of other Gram-positive bacteria as well as in a number of cell wall-associated enzymes, such as xylanase and pullanase from *Thermoanaerobacterium thermohydrosulfurigenes,* and *Clostridium thermosulfurogenes*, respectively (2). It is considered that SLH domains constitute carbohydrate-binding sites that bind to the SCWP polysaccharide, which, in turn, is linked to the cell wall peptidoglycan via a acid-labile phosphate bond (19). Composition analysis of the HF-PSs in a number of *B. cereus* strains with known phylogenetic relatedness to *B. anthracis* revealed that *B. cereus* strains display both differences and similarities that correlate with the phylogenetic relatedness of these strains (17). A particularly interesting finding was that some *B. cereus* strains which are phylogenetically more distantly related to *B. anthracis* but capable of causing fatal pneumonia in humans, e.g. strain *B. cereus* G9241, have HF-PS compositions that are very similar to that of the *B. anthracis* HF-PS, indicating that, besides phylogenetic relatedness, functional aspects of pathogenicity could also play a role in HF-PS structural conservation (17).

Secondary cell wall polysaccharide structures have been determined for a number of Gram-positive bacteria including some *B. cereus* strains (22). Older reports described that *B. cereus* AHU 1356 produced a neutral carbohydrate composed of GlcNAc, ManNAc, *N*-acetylgalactosamine (GalNAc), and glucose (Glc) (1). In addition, an acidic carbohydrate composed of GlcNAc, Gal, rhamnose (Rha), glycerol and phosphate was also identified in this strain (16). Because of the considerable importance of bacilli with regard to public health, a more complete picture of these carbohydrates is needed. Structural determination of the HF-PSs is necessary to lay the groundwork for structure-function studies of these SCWP polysaccharides as well as to

answer questions about their suitability for developing new and/or improved vaccines and diagnostic agents.

MATERIAL AND METHODS

Bacterial strains and cultural conditions.

Bacillus anthracis Ames, *B. cereus* ATCC 10987, and *B. cereus* ATCC 14579 were provided from the CDC culture collection. Cultures were grown overnight (16 h) at 30 °C in 100 mL of brain heart infusion medium (BHI) (BD BBL, Sparks, MD) containing 0.5 % glycerol. The next morning, 4×1.5 mL of the overnight cultures were centrifuged at $10,000 \times g$ and room temperature for 5 min, the supernatants discarded, the pellets resuspended in 500 µL BHI; the four cell suspensions were used to inoculate four 250 mL volumes of BHI medium in 1 L Erlenmeyer flasks. The cultures were incubated at 30 °C under shaking at 200 rpm. Growth was monitored by measuring the optical density at 600 nm. The cells, growing at mid-log phase, were harvested through centrifugation at $8,000 \times g$ and 4 °C for 15 min and washed two times in sterile saline. The cultures were enumerated by serial dilution and surface spread counts on BHI agar plates and sterilized by autoclaving (1 h at 121 °C) prior to further processing and carbohydrate analysis.

Preparation of **Bacillus** *cell wall extract.*

The bacterial cell walls were prepared in a modified procedure described by Brown (3). Briefly, the autoclaved bacterial cells (1×10^{10} to 1×10^{11} CFU) were disrupted by sonication in 40 mL sterile saline on ice by four 10 min sonication cycles using a Branson Sonifier (Type 450, Branson Ultrasonics Corporation, Danbury, CT) with a 1/2 inch probe, operating it at a frequency of 20 kHz. The complete or near complete disruption of cells was checked microscopically. Unbroken cells were removed by centrifugation ($8,000 \times g$, 4 °C, 15 min). The separated pellet and supernatant fractions were stored at -80 °C. Cell wall material was sedimented from the supernatant fractions by ultracentrifugation at $100,000 \times g$ and 4 °C for 4 h (Optima L-90K Ultracentrifuge, Beckman). The resulting cell wall pellets were washed by suspension in cold, deionized water followed by an additional ultracentrifugation under conditions described above and lyophilization.

Isolation and purification of the cell wall polysaccharide

Cell wall material from *B. anthracis* Ames, *B. cereus* ATCC 10987, and *B. cereus* ATCC 14579 were treated with aqueous hydrofluoric acid (HF) under stirring (48 % HF) at 4 °C for 48 h. The HF treated material was neutralized by ice-cold ammonium hydroxide solution (approximately 30 %) on an ice-water bath. The neutralized material was then centrifuged at low speed for 10 min at 3500 × *g*. The supernatant was removed, the remaining pellet resuspended and washed in cold deionized water followed by an additional low speed centrifugation as described above. The supernatants were collected, and concentrated in a SpeedVac. The concentrated sample was then desalted by gel filtration chromatography (GPC) using fine grade Bio-Gel P2 (Bio-Rad). Water was used as the eluent and an online refractive index (RI) detector was used to monitor the sample eluting from the column. The fractions which gave a positive response on the RI-detector were collected, pooled, lyophilized and used for further analysis.

Composition analysis

Glycosyl composition analysis was done by the preparation and gas chromatography-mass spectrometric (GC-MS) analysis of trimethylsilyl (TMS) methyl glycosides (25). The TMS methyl glycosides were identified and were quantified by comparison to authentic standards. In brief, the samples were methanolyzed using methanolic 1 M HCl at 80 °C for 18 h to form the monomeric methylglycosides, followed by *N*-acetylation using pyridine and acetic anhydride (1:1) in presence of methanol at 100 °C for 1 h. After removing the reagents by flushing with dry nitrogen, the methyl glycosides were treated with Tri-Sil reagent (Pierce, Rockford, IL) at 80 °C for 30 min to form TMS methyl glycosides. The TMS methyl glycosides were dissolved in hexane and analyzed on a GC-MS using HP-1MS column (30 m × 0.25 mm × 0.25 μm).

Glycosyl linkage analysis

The linkage analysis was performed according to a modification of the method of Ciucanu and Kerek (5). Briefly, the samples were dissolved in dry dimethylsulfoxide (DMSO, 0.250 μL) overnight with stirring, followed by addition of a DMSO/sodium

hydroxide slurry (0.250 μL) and stirring for 2 h at room temperature. Methyl iodide was added to the sample and stirred for 40 min. Another aliquot of methyl iodide was added and stirred for another 30 min. The reaction was cooled on an ice-bath and the partially methylated polysaccharide was extracted by partitioning between chloroform and water. The partially methylated sample in the chloroform layer was dried and used for the preparation of partially methylated alditol acetates (PMAAs). The partially methylated polysaccharide was methanolyzed to monomers using methanolic 1 M HCl at 80 °C for 16 h followed by hydrolysis with 2 M trifluoroacetic acid (TFA) at 100 °C for 2 h. The aldoses were reduced to their corresponding alditols by sodium borodeuteride (NaBD$_4$) overnight at room temperature. The excess borodeuteride was neutralized using 30 % acetic acid solution and boric acid was removed as methyl borates by repeated refluxing and evaporation with acidified methanol and methanol respectively. The partially methylated alditols were then acetylated using a pyridine:acetic anhydride (1:1) solution at 100 °C for 1 h. Pyridine and acetic anhydride were removed by flushing with dry nitrogen and the PMAAs were dissolved in dichloromethane and analyzed by GC-MS using a HP-1 MS column. The linkage positions of each monosaccharide were identified from its mass fragmentation pattern and by matching its retention time to that of authentic PMAA standards.

NMR analysis

The HF-PS sample (2-3 mg) was dissolved in 0.5 mL of regular grade deuterium oxide (D$_2$O 98.5 %) and lyophilized. This process was done twice to exchange the hydroxyl and amide protons with deuterium. The sample was finally dissolved in 0.5 mL of 100 % D$_2$O (Cambridge Isotopes) and transferred to 5 mm NMR tube. All 1-D and 2-D NMR spectra were acquired with a 600 MHz Varian Inova instrument using the standard software supplied by Varian. [1]H-^{1}H homonuclear 2-D experiments were done after perfect 90° pulse calibration and 3.5 K spectral width in both dimensions; however ^{1}H-^{13}C HSQC data was acquired taking 3.5 K and 12 K in direct and indirect dimensions respectively.

Mass Spectroscopy

Mass spectral analysis of the isolated HF-PS was determined using a matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer from Applied Biosystems. The HF-PS was dissolved in 1: 1 mixture of methanol: water and mixed in equal proportion (v/v) with 0.5 M 2,5-dihydroxy benzoic acid (DHB) as the matrix. About 0.7 µL of the mixture was loaded on each spot on a stainless steel MALDI plate and air dried. The spectra were acquired in the linear and positive mode using 337nm N_2 laser with acceleration voltage of 20 kV.

RESULTS

Isolation and initial analysis - Glycosyl composition and linkage analysis

The HF-PS from the investigated *B. cereus* strains eluted as a single major peak within the void volume of the Bio-Gel P2 column; the peak fractions were collected, lyophilized and used for detailed structural analysis. The HF-PS composition from *B. anthracis* Ames was the same as previously described for four *B. anthracis* strains (Ames, Pasteur, Sterne 34F$_2$, and UT60) (4, 17) and consisted of Gal, GlcNAc, and ManNAc in an approximate 3:2:1 ratio. The composition of the HF-PS from *B. cereus* ATCC 10987 consisted of Gal, ManNAc, GlcNAc and GalNAc in a 1:1:1:1 ratio (Table 1).

Strain	Sugar Composition					
	Man	**Glc**	**Gal**	**ManNAc**	**GlcNAc**	**GalNAc**
***B. cereus* ATCC 10987**	n.d.	8.6	26.7	25.3	16.4	23.0
***B. cereus* ATCC 14579**	n.d.	25.3	n.d.	15.4	44.9	14.4

Table 1. The glycosyl compositions of the HF-PS preparations from *B. cereus* strains ATCC 10987 and ATCC 14579. The compositions are given as relative mass percent of total carbohydrate.

A minor amount of Glc was present due to contamination of the HF-PS preparation with a Glc-rich polysaccharide as previously reported (17). The composition of *B. cereus* ATCC 14579 HF-PS consisted of Glc, ManNAc, GlcNAc, and GalNAc in approximately a 2:1:3:1 ratio (Table 1). Thus, the differences in glycosyl compositions of these two *B. cereus* HF-PSs shows that their structures are not identical to each other and that they also differ from that of the *B. anthracis* HF-PS. Moreover, these two *B. cereus* HF-PSs contain GalNAc which is not present in the *B. anthracis* HF-PS, and the *B. cereus* ATCC 14579 HF-PS contains Glc which is not found in either the *B. cereus* ATCC 10987 or in the *B. anthracis* HF-PSs.

Methylation analysis showed that the *B. cereus* ATCC 10987 HF-PS consisted of terminally linked Gal, 3,6-linked GalNAc, 4-linked ManNAc and 4-linked GlcNAc. Minor components included terminally linked GlcNAc, ManNAc and GalNAc indicating the possibility of molecular microheterogeneity within the HF-PS preparation. For the *B. cereus* ATCC 14579 HF-PS, methylation analysis showed terminally linked Glc, terminally linked GlcNAc, 4-linked Glc, 4-linked GlcNAc, 4-linked ManNAc, 6-linked GalNAc, 3,4-linked ManNAc and 3,4,6-linked GlcNAc. Exact quantification was not possible due to the resistance of the aminoglycosyl glycoside bonds in the permethylated HF-PS to acid hydrolysis.

Structural determination of the *B. cereus* ATCC 10987 HF-PS.

The MALDI-TOF/TOF MS mass spectrum of the *B. cereus* ATCC 10987 HF-PS is shown in Figure 1.
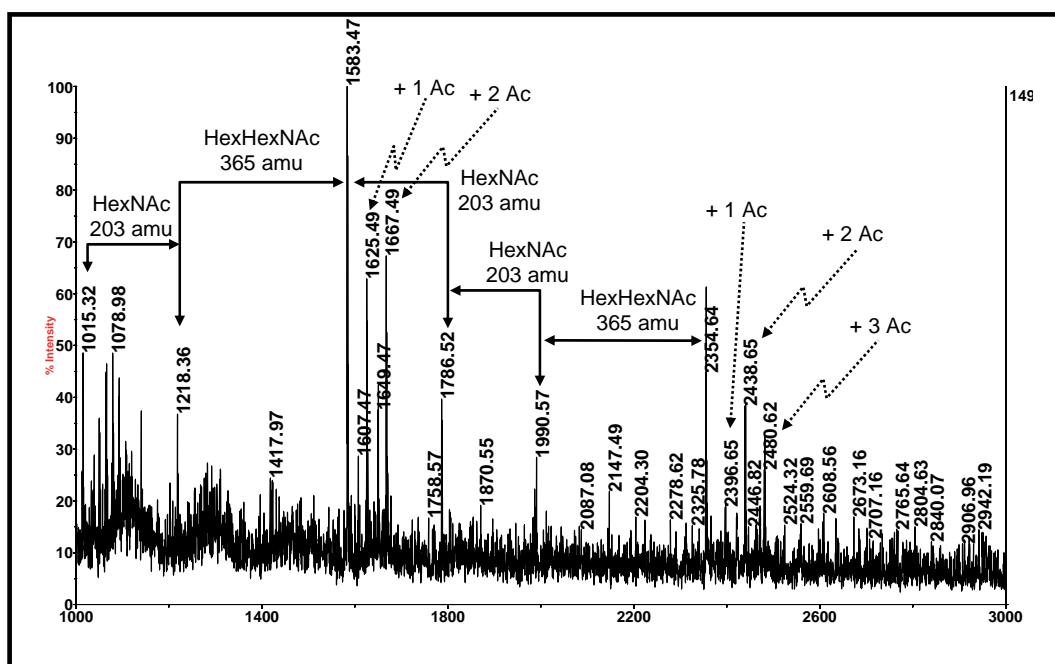
Figure 1. MALDI-TOF MS spectra of the HF-PS from *B. cereus* ATCC 10987. The spectrum shown was acquired in the positive mode and the proposed structures for the various ions are given in Table 2.

The results show that this HF-PS preparation consists of a microheterogeneous mixture of molecules. Two ion clusters were observed with the most intense ion in each cluster being the $[M+Na]^+$ ion observed at *m/z* 1583.5 and at *m/z* 2354.6, respectively. The difference between these ions, 772 mass units, exactly matches the mass of a $HexNAc_3Hex_1$ tetramer. These results suggest that the *m/z* = 1583.5 ion is due to an octasaccharide composed of two $HexNAc_3Hex_1$ tetrameric repeating units, and that the ion at *m/z* = 2354.6 corresponds to a molecule with three tetrameric repeat units. Probable structures for these masses are given in Table 2.

Repeat Units	Proposed Structure	Observed Ion [M+Na]+
$n_1 + 1$	⌈ManNAc-GlcNAc-GalNAc⌉-ManNAc 　　　　　　　　Gal⌋	1015.3
$n_1 + 2$	⌈ManNAc-GlcNAc-GalNAc⌉-ManNAc-GlcNAc 　　　　　　　　Gal⌋	1218.4
$n_2 + 0$	⌈ManNAc-GlcNAc-GalNAc⌉-⌈ManNAc-GlcNAc-GalNAc⌉ 　　　　　　　　Gal⌋　　　　　　　　　　Gal⌋	1583.5
$^2n_2 + 0 + 1Ac$	⌈ManNAc-GlcNAc-GalNAc⌉⌈ManNAc-GlcNAc-GalNAc⌉ Acetyl⌋　　　　Gal⌋　　　　　　　　Gal⌋	1625.5
$^2n_2 + 0 + 2Ac$	⌈ManNAc-GlcNAc-GalNAc⌉ManNAc-GlcNAc-GalNAc⌉ Acetyl⌋　　Gal⌋ Acetyl⌋　　　Gal⌋	1667.5
$n_2 + 1$	⌈ManNAc-GlcNAc-GalNAc⌉-ManNAc-GlcNAc-GalNAc⌉-ManNAc 　　　　　　　Gal⌋　　　　　　　　Gal⌋	1786.5
$n_2 + 2$	⌈ManNAc-GlcNAc-GalNAc⌉ManNAc-GlcNAc-GalNAc⌉-ManNAc-GlcNAc 　　　　　Gal⌋　　　　　　　　Gal⌋	1990.6
$n_3 + 0$	⌈ManNAc-GlcNAc-GalNAc⌉ManNAc-GlcNAc-GalNAc⌉ManNAc-GlcNAc-GalNAc⌉ 　　　　　Gal⌋　　　　　　　Gal⌋　　　　　　　Gal⌋	2354.6
$^2n_3 + 0 + 1Ac$	⌈ManNAc-GlcNAc-GalNAc⌉ManNAc-GlcNAc-GalNAc⌉ManNAc-GlcNAc-GalNAc⌉ Acetyl⌋　　　Gal⌋　　　　　　Gal⌋　　　　　　Gal⌋	2396.7
$^2n_3 + 0 + 2Ac$	⌈ManNAc-GlcNAc-GalNAc⌉ManNAc-GlcNAc-GalNAc⌉ManNAc-GlcNAc-GalNAc⌉ Acetyl⌋　　　Gal⌋ Acetyl⌋　　Gal⌋　　　　　Gal⌋	2438.7
$n_3 + 0 + 3Ac$	⌈ManNAc-GlcNAc-GalNAc⌉ManNAc-GlcNAc-GalNAc⌉ManNAc-GlcNAc-GalNAc⌉ Acetyl⌋　　Gal⌋ Acetyl⌋　　Gal⌋ Acetyl⌋　　Gal⌋	2480.6

[1]Proposed structures are also consistent with the glycosyl composition, linkage and NMR data described in this paper. [2]The location of these acetyl groups could be on any of one, two, or three of the various ManNAc residues. n = the number of repeating units and the number following "n" = the number of added HexNAc residues.

Table 2: Proposed structures for the various molecular ions observed on MALDI-TOF MS analysis of *B. cereus* ATCC 10987 HF-PS

These structures are based on the 1: 1: 1: 1 GlcNAc: GalNAc: ManNAc: Gal composition ratio and the fact that the Gal is terminally linked and that the only branching HexNAc is GalNAc. In addition to these major ions for the two ion clusters,

several other ions are observed. The ions at *m/z* 1625.5 and 1667.5 are 42 and 84 mass units greater, respectively, than the *m/z* 1583.5 ion indicating that these ions are due to mono- and di-O-acetylation, respectively, of the di-tetrasaccharide repeat structure. The structures shown in Table 2 place the O-acetyl group on the ManNAc residues since this location was determined by NMR analysis, which is described below. The ions at *m/z* 1607.5 and *m/z* 1649.5 are due to minor amounts of dehydrated (- 18 mass units) versions of these acetylated molecules. We also observe ions at *m/z* 1786.5 and 1990.6 which are consistent with one and two additional HexNAc residues, respectively, to the *m/z* 1583.5 ion. The difference between the *m/z* 1990.6 ion and the *m/z* 2354.6 ion is 365 mass units which is consistent with the addition of a HexHexNAc unit to the 1990.6 molecule. The ions at *m/z* 2396.7, 2438.7, and 2480.6 are 42, 84, and 126 mass units, respectively, greater than the 2354.64 ion and account for mono-, di-, and tri-O-acetylated versions of the *m/z* 2354.64 structure. Again, proposed structures for these ions are given in Table 2.

The anomeric configuration and the sequence of the glycosyl residues in the *B. cereus* ATCC 10987 were determined by both 1- and 2-D NMR analysis of the HF-PS before and after removal of the O-acetyl groups. The complete proton and carbon assignments of this HF-PS were obtained by 1-D proton NMR (Figure 2) and by 2-D gCOSY, TOCSY (Figure 3) and gHSQC (Figure 4) experiments. The proton spectrum shows four major anomeric H1 signals at δ 5.23, δ 5.04, δ 4.57, and δ 4.45 and a minor anomeric H1 signal at δ 4.92.
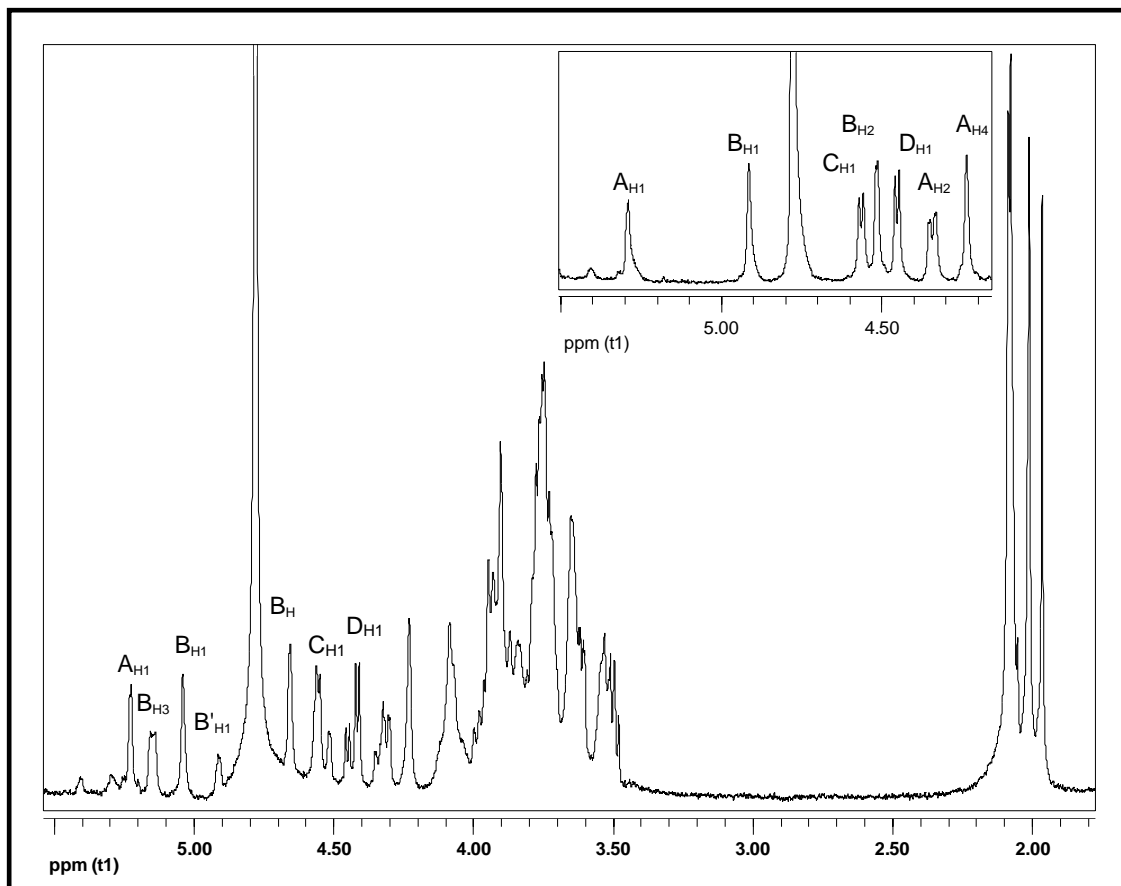
Figure 2. The proton NMR spectrum of the HF-PS from *B. cereus* ATCC 10987. The resonances A-D are the indicated respective resonances for the GalNAc, ManNAc, GlcNAc, and Gal residues of the HF-PS. The inset shows the anomeric region of spectrum of the HF-PS after de-O-acetylation.

Each of these anomeric protons is due to a unique glycosyl ring system in this HF-PS, and the gCOSY, TOCSY (Figure 3) and gHSQC (Figure 4) experiments allowed the proton and carbon assignments of each of these ring systems as explained in the following paragraphs and given in Table 3.
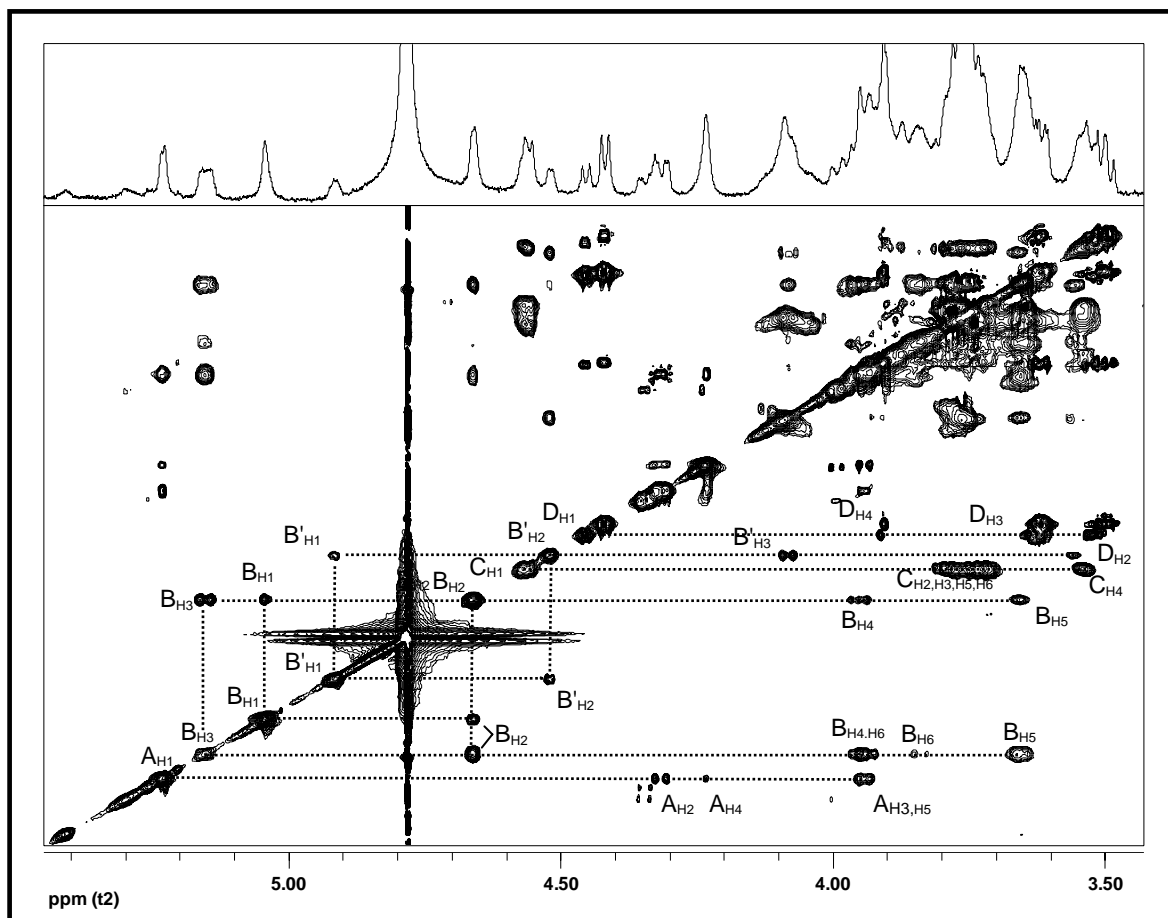
Figure 3. The TOCSY spectrum of the HF-PS from *B. cereus* ATCC 10987. The protons for the various glycosyl ring systems are as labeled. The residues are labeled as defined in the legend to Figure 2. The complete proton assignments were made from the TOCSY and COSY (spectrum not shown) data and are given in Table 3.
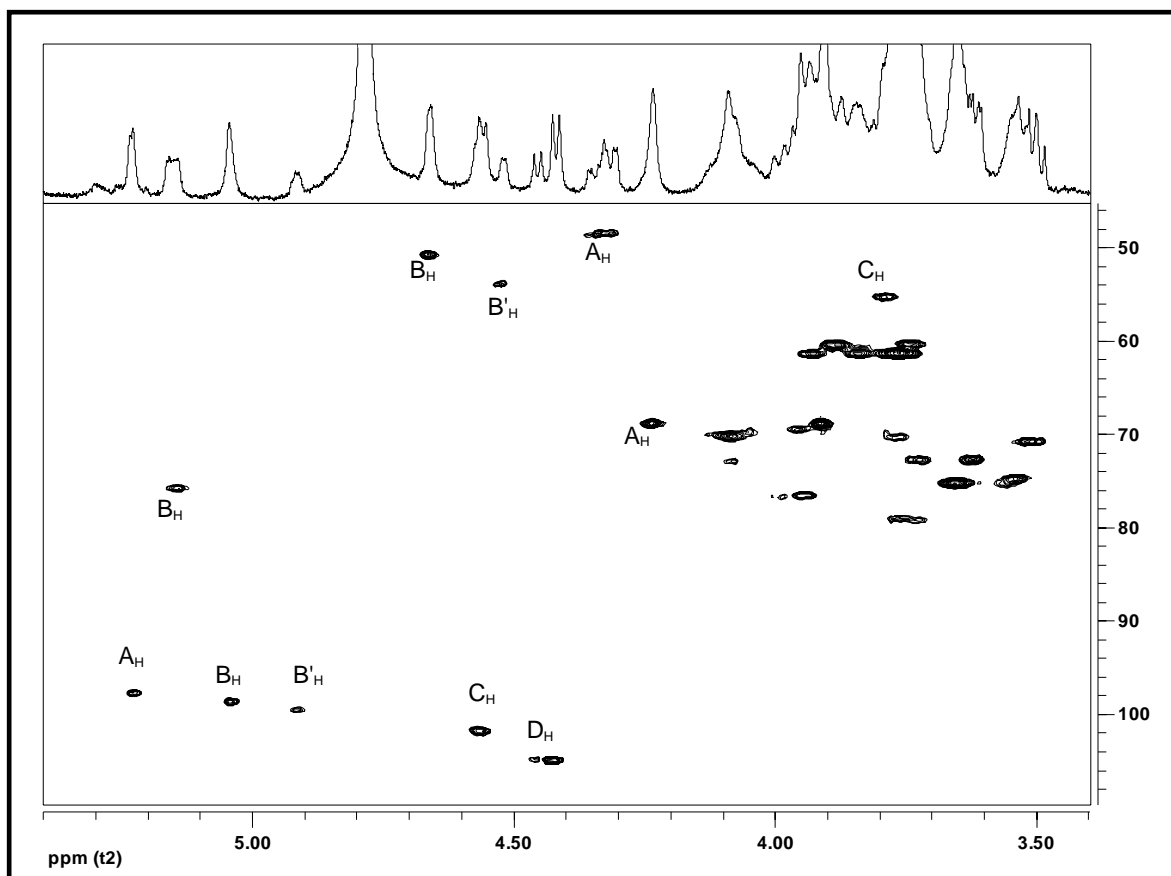
Figure 4. The HSQC spectrum of the HF-PS from *B. cereus* ATCC 10987. The assignments are as indicated and the labeling for the various glycosyl residues is as defined in the legend to Figure 2. The carbon chemical shift assignments for the various glycosyl residues are given in Table 3.

The H1 signal at δ 5.23 (1H, s) is the most downfield signal in the spectrum and was assigned to the anomeric proton of an α-glycosyl residue (**A**). The H2 of residue **A** resonates at δ 4.32, as was confirmed from the gCOSY experiment. The chemical shift of the carbon to which this proton is attached was determined by gHSQC (Figure 4) to be δ 48.2 indicating it to be nitrogen-bearing carbon consistent with a HexNAc residue. In addition, the downfield chemical shifts of C3 and C6 of this residue at δ 76.4 and 69.5, respectively, indicate that residue **A** is substituted at O3 and O6. The gCOSY and TOCSY (Figure 3) spectra showed that H3 and H4 resonate at δ 3.94 and δ 4.23, respectively, and that the small overall $J_{3,4}$ and $J_{4,5}$ coupling constant of H4 (< 9.6 Hz) indicates that residue **A** has a *galacto*- configuration. Therefore, residue **A** was assigned to be O3 and O6 substituted α-GalNAc, which is consistent with the glycosyl

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composition and linkage analysis described above showing the presence of a 3,6-linked GalNAc residue.

The next upfield anomeric proton signal at δ 5.04 (s, 1H) had a corresponding C1 chemical shift of δ 98.56. The characteristic downfield chemical shift of H2 at δ 4.66 with small $J_{1,2}$ and $J_{2,3}$ coupling constants indicated that this residue has a *manno*-configuration.

Residue	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6$_a$/C6	H6$_b$
α-D-GalNAc (**A**)	5.23	4.32	3.94	4.23	3.93	4.06	3.78
	97.57	48.2	76.35	68.7	70.0	69.5	(3.78)
	(5.29)	(4.35)	(3.99)	(4.23)	(3.97)	(4.04)	
	(98.85)	(48.8)	(76.9)	(69.1)	(70.0)	(69.6)	
β-D-ManNAc (**B**)	5.04	4.66	**5.15**	3.94	3.65	3.92	3.83
	98.56	50.76	**75.8**	75.5	75.6	61.4	(3.83)
	(4.92)	(4.52)	**(4.08)**	(3.66)	(3.55)	(3.91)	
	(99.48)	(53.9)	**(73.0)**	(75.5)	(75.6)	(61.4)	
β-D-GlcNAc (**C**)	4.57	3.78	3.73	3.53	3.71	3.88	3.74
	101.88	55.1	70.9	79.3	74.9	60.5	(3.74)
	(4.57)	(3.77)	(3.72)	(3.51)	(3.70)	(3.88)	
	(101.6)	(55.4)	(70.9)	(79.3)	(74.9)	(60.5)	
β-D-Gal (**D**)	4.45	3.52	3.62	3.91	3.63	3.92	3.92
	104.8	71.0	72.8	69.0	72.8	61.3	(3.92)
	(4.46)	(3.52)	(3.62)	(3.91)	(3.60)	(3.92)	
	(105.0)	(71.0)	(72.8)	(69.0)	(72.8)	(61.3)	

The values in parentheses are for the de-O-acetylated HF-PS.

Table 3: ^{1}H and ^{13}C chemical shift values for the *B. cereus* ATCC 10987 HF-PS

The chemical shift of C2 is at δ 50.8 and shows that C2 is a nitrogen-bearing carbon. Therefore, residue **B** was assigned as ManNAc. Since both α/β-anomeric configurations of *manno*-glycosyl residues have low coupling constants, the β-anomeric configuration of residue **B** was confirmed by comparing the TOCSY with NOESY spectrum. The NOESY spectrum (not given) showed strong intra-residue NOE interactions of H1 at δ 5.04 to H2, H3 and H5 at δ 4.66, δ 5.15 and δ 3.65, respectively. These NOE interactions are consistent with the axial positions of H1, H3 and H5 of a β-

anomeric configuration for residue **B** (the NOESY spectrum for the de-O-acetylated HF-PS is shown in Figure 5 and also shows the H1/H3/H5 interactions).
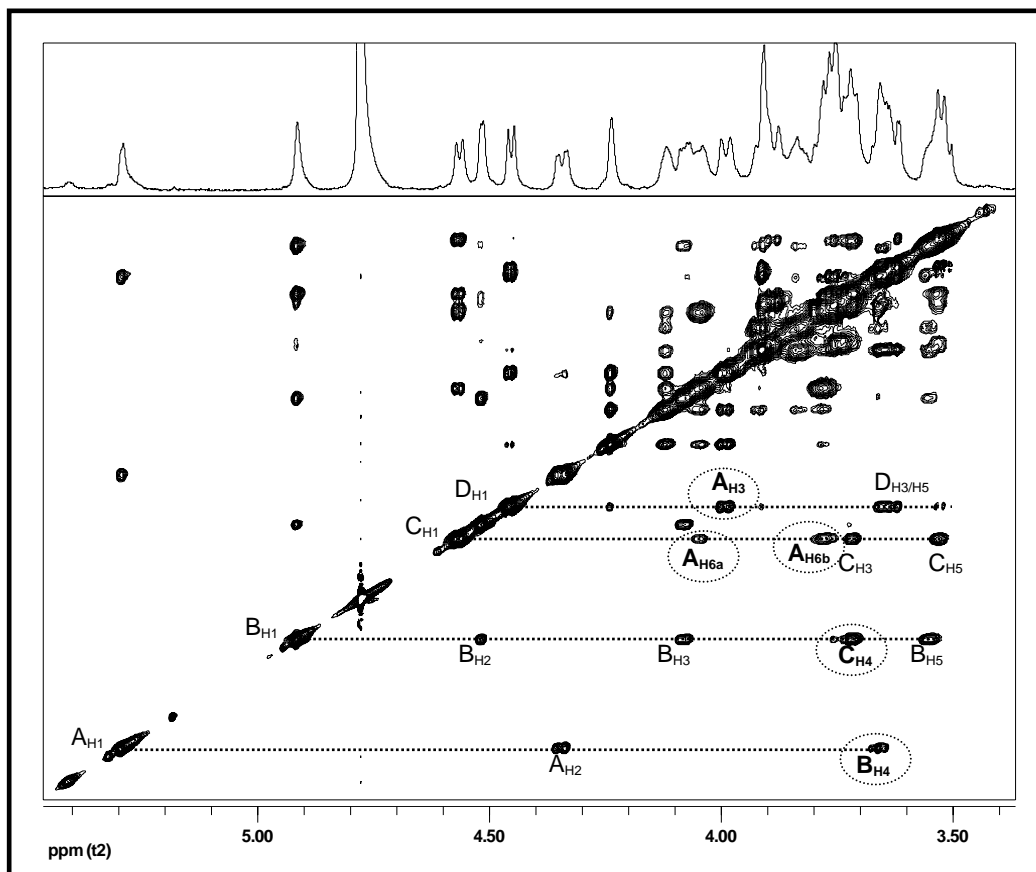
Figure 5. The NOESY spectrum of the de-O-acetylated HF-PS from *B. cereus* ATCC 10987. The assignments for the various NOE contacts are as indicated in the figure. The labeling of the various glycosyl residues are as defined in the legend to Figure 2. The inter-residue NOE contacts are circled and in bold-face font.

The downfield chemical shift of H3 at δ 5.15, which is attached to a carbon with a chemical shift of δ 75.8 is consistent with O-acetylation at O3 of this β-ManNAc residue. The presence of O-acetyl groups on this HF-PS is also consistent with the mass spectrometric data as described above.

The minor H1 anomeric signal at δ 4.92 was identified to be another β-ManNAc residue (**B'**) that does not bear an O-acetyl ester group on O3. The upfield chemical shifts of H2 at δ 4.53 (compared to δ 4.66 for residue **B**) and H3 at δ 4.08 (compared to δ 5.15 for residue **B**), respectively, are consistent with the lack of an O-acetyl group at

O3 of this residue. The relative quantification of HF-PS bearing O-acetyl group was done by comparing the H1 integral values of residues **B** and **B'** from proton NMR spectrum and it was calculated that about 60% of the HF-PS was O-acetylated at O3 of the ManNAc residue. It is quite possible that the polysaccharide as found in the cell wall is completely O-acetylated and that the 60% value is due to partial removal of O-acetyl groups during its release by aqueous HF.

The next upfield signal at δ 4.57 is a doublet with a $J_{1,2}$ coupling constant of 7.8 Hz indicating it to have a β-anomeric configuration. The C1 chemical shift of δ 101.88 also supports the β-anomeric configuration. The H2 of this residue resonates at δ 3.65 and is attached to a nitrogen-bearing carbon at δ 55.1 (see Figure 4) showing that this is a β-HexNAc residue. The TOCSY spectrum showed relatively large $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ coupling constants which is consistent with a *gluco*-configuration; therefore, this residue (**C**) was assigned as β-GlcNAc. The downfield chemical shift of C4 at δ 79.3 indicates that this residue is 4-substituted β-GlcNAc.

The remaining H1 anomeric signal has a proton chemical shift at δ 4.45 (1H, $J_{1,2}$ = 7.8 Hz) and a C1 chemical shift at δ 104.75. These H1 and C1 chemical shifts and the large $J_{1,2}$ coupling constant are consistent with this glycosyl residue (**D**) having a β-anomeric configuration. By comparing gCOSY and TOCSY(Figure 3) spectra it was found that the H4 signal resonates at $δ_H$ 3.91 and has small $J_{3,4}$ and $J_{4,5}$ coupling constants (< 9.6 Hz) showing that this residue has a *galacto*-configuration. Therefore, this residue (**D**) was assigned as Gal.

The NMR and mass spectral data just described indicate that this HF-PS preparation consists of a microheterogeneous mixture of molecules due to non-stoichiometric amounts of O-acetyl groups on O3 of the ManNAc residue, to a varying number of tetrasaccharide repeating units, and to the addition of HexNAc and HexNAcHex saccharides. This microheterogeneity impacts the chemical shift values which makes determining the glycosyl sequence by NMR analysis difficult. Therefore, in order to reduce the molecular heterogeneity, the sample was treated with anhydrous hydrazine which removes the O-acetyl groups while leaving the rest of the structure untouched. The removal of O-acetyl groups was confirmed in the NMR experiments, and the proton and carbon chemical shifts were determined by 1-D proton NMR analysis, and 2-D gCOSY, TOCSY and gHSQC NMR experiments (spectra not shown).

These assignments are given in Table 3. The insert in Figure 2 shows the proton spectrum of the anomeric and *N*-acetyl methyl regions of the de-*O*-acetylated HF-PS. After hydrazine treatment, NMR analysis showed the presence of four glycosyl residues between δ 4.4 and δ 6.0 and three *N*-acetyl methyl protons near δ 2.0 indicating the presence of three *N*-acetylamino sugars. The H1 (δ 5.04) and the H3 (δ 5.15) resonances due to the presence of an acetylated β-ManNAc residue are both absent in the de-*O*-acetylated HF-PS.

Due to spectral simplicity of the de-*O*-acetylated HF-PS, the glycosyl sequence was determined from a NOESY experiment. The NOE spectrum (Figure 5) showed a through space inter-residue connectivity between H1 of residue **A** (δ 5.29) to H4 of residue **B** (δ 3.66) along with an intra-residue NOE with H2 at δ 4.35. This indicates that α-GalNAc (**A**) is linked to the 4-position of β-ManNAc residue (**B**); i.e. α-GalNAc-(1→4)-β-ManNAc. This sequence is consistent with the methylation analysis which shows that the β-ManNAc residue is 4-linked. The H1 of residue **B** (δ 4.92) showed an inter-residue NOE with H4 of residue **C** (δ 3.72) along with a number of intra-residue NOE contacts. Hence β-ManNAc (**B**) is connected to the 4-position of the β-GlcNAc residue (**C**); i.e. α-GalNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc. This result is consistent with the methylation data showing that the GlcNAc residue is 4-linked. The H1 of residue **C** had an inter-residue NOE with H6$_a$ and H6$_b$ of residue **A** indicating β-GlcNAc (**C**) to be linked to the O6 of α-GalNAc (residue **A**); i.e. →6)-α-GalNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc-(1→. The most upfield H1 signal of the β-Gal residue (**D**) at δ 4.46 has an inter-residue NOE to H3 of residue **A**, indicating that β-Gal residue is connected to the O3 of the α-GalNAc residue; i.e. →6)-[β-Gal-(1→3)]α-GalNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc-(1→ The NOE contacts of the GlcNAc (**C**) and Gal (**D**) residues to O6 and O3 respectively of the GalNAc residue (**A**) are consistent with the methylation data showing that the GalNAc residue is 3,6-linked. The NOESY experiment indicates that there are no NOE contacts to the Gal residue from any of the H1 protons of the other glycosyl residues indicating that Gal is a terminally-linked glycosyl residue, a result that is also consistent with the methylation data. The combination of the composition, methylation, mass spectrometry, and NMR

analyses all support that the repeating unit of the *B. cereus* ATCC 10987 HF-PS has the following structure:

$$\rightarrow 6)\text{-}\alpha\text{-GalNAc-}(1\rightarrow 4)\text{-}\beta\text{-ManNAc-}(1\rightarrow 4)\text{-}\beta\text{-GlcNAc-}(1\rightarrow$$

$$\beta\text{-Gal-}(1\rightarrow 3)^{\lrcorner} \qquad Ac_{(0.6)}\text{-}(\rightarrow 3)^{\lrcorner}$$
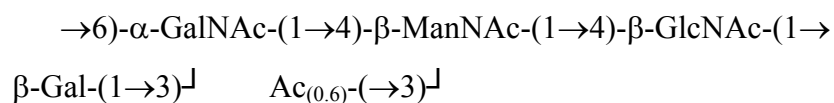
Comparison of the **B. cereus** *ATCC 10987 HF-PS structure with the structural features of the* **B. cereus** *ATCC 14579 HF-PS*

Previously reported composition analysis (17) and proton NMR spectroscopy (4), as well as the composition and methylation analysis described above show that the HF-PS from the *B. cereus* type strain, ATCC 14579, has a different structure than the HF-PSs of *B. cereus* ATCC 10987 and *B. anthracis*. For example, it contains GalNAc as is found in *B. cereus* ATCC 10987 HF-PS but not found in *B. anthracis* HF-PS, and it contains Glc rather than the Gal that is found in both *B. cereus* ATCC 10987 and in *B. anthracis* HF-PSs. Mass spectrometric analysis of the *B. cereus* ATCC 14579 HF-PS (Figure 6) shows that its spectrum has a pattern of ions very similar to that obtained for the *B. cereus* ATCC 10987 HF-PS (Figure 1).
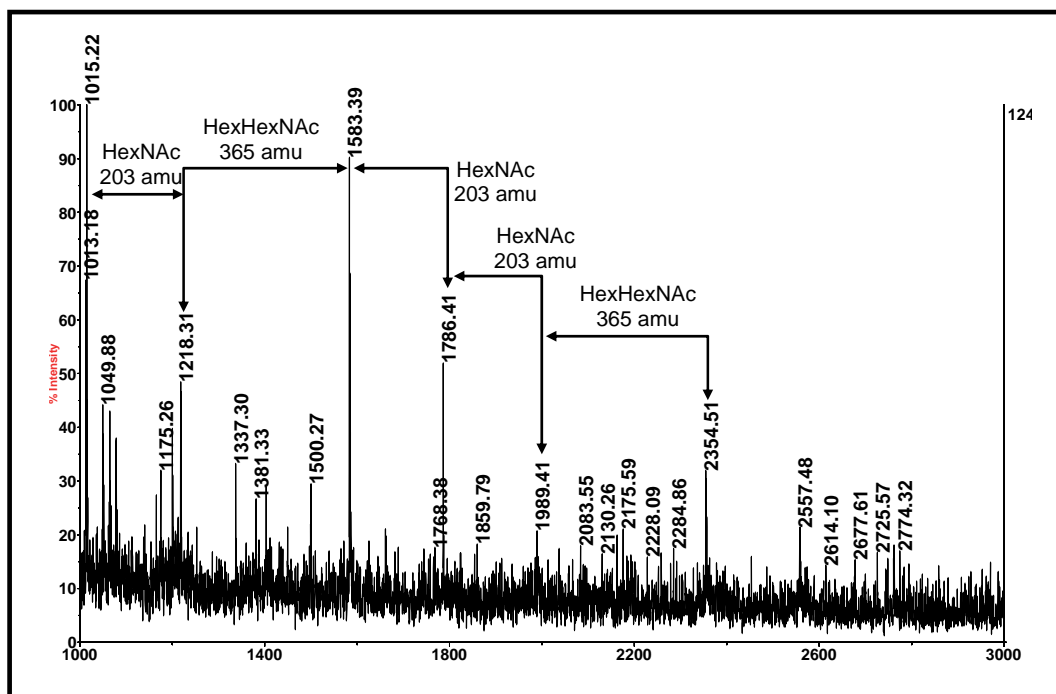
Figure 6. MALDI-TOF MS spectra of the HF-PS from *B. cereus* ATCC 14579. The spectrum shown was acquired in the positive mode and the proposed structures for the various ions are given in Table 4.

The ion with the greatest intensity is the [M+Na]$^+$ ion at *m/z* 1583.4 as is the case for the *B. cereus* ATCC 10987 HF-PS. The ion at *m/z* 1786.4 is consistent with an added HexNAc residue (i.e. + 203 mass units), and the ion at *m/z* 1989.6 is likely due to the addition of a second HexNAc residue. The ion at *m/z* 2354.6 is 365 mass units greater than the 1989.6 ion and is consistent with an added HexHexNAc disaccharide. There are also ions consistent with the loss of a HexHexNAc from the 1583.5 ion (i.e. that at *m/z* 1218.4) and an ion at *m/z* 1015.3 which is consistent with the loss of a HexNAc from the 1218.4 ion. The ions and the proposed pattern of structures are shown in Table 4. This pattern indicates that this HF-PS preparation consists of one, two, and three HexNAc$_3$Hex tetrasaccharide repeat units as observed for the *B. cereus* ATCC 10987 HF-PS, except that there is no evidence that the *B. cereus* ATCC 14579 HF-PS is acetylated since ions with added increments of 42 mass units are not observed.

Figure 7 compares the structures of the HF-PSs from *B. anthracis* (4) with the HF-PS structure of *B. cereus* ATCC 10987 (this paper), and the proposed HF-PS structure for *B. cereus* ATCC 14579 (this paper).
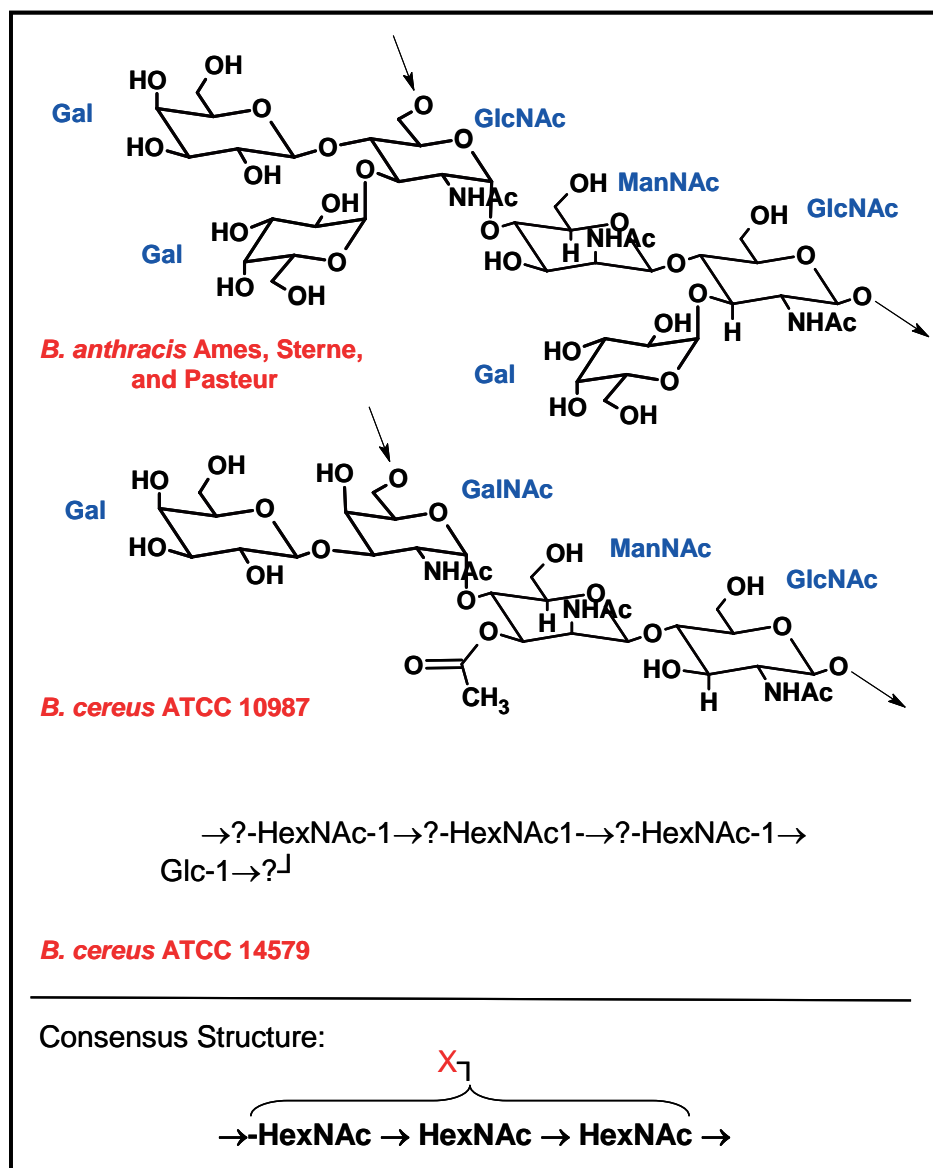
Figure 7. A summary comparing the structure and structural data for *B. cereus* ATCC 10987 and ATCC 14579 with the published structure (4) for the HF-PSs from *B. anthracis* Ames, Sterne, and Pasteur. A proposed consensus structure based on these structures is also given in which "X" stands for substitution of the aminoglycosyl backbone with other glycosyl residues such as Gal or Glc, or with non-carbohydrate substituents such as an O-acetyl group.

From this comparison a consensus HF-PS structure is indicated in which the repeating unit backbone consists of a $HexNAc_3$ trisaccharide. At this point, it is not known how common this consensus structure is among the bacilli. The evidence indicates that there is a ManNAc-GlcNAc disaccharide component in this $HexNAc_3$

trisaccharide backbone, and that the third HexNAc is either GalNAc or a second GlcNAc residue. This HexNAc$_3$ trisaccharide backbone can be substituted by other glycosyl residues such as Glc or Gal, as well as by non-carbohydrate components such as the O-acetyl substituent in the case of the *B. cereus* ATCC 10987 HF-PS, and a possible pyruvyl component reported for the *B. anthracis* HF-PS (19).   In the case of the *B. cereus* ATCC 14579 HF-PS structure, the complexity of the methylation profile with regard to the aminoglycosyl substitution pattern and the presence of 4-linked and terminally linked Glc residues, indicates more structural complexity than is accounted for by the mass spectrometric data. This inconsistency between the mass spectral and methylation data could be due to undermethylation, or to as yet unresolved structural features. The structural heterogeneity of this *B. cereus* ATCC 14579 HF-PS is being investigated further.

DISCUSSION

We have previously reported the structure of the HF-PS from *B. anthracis* and that comparison of NMR spectra showed that its structure varied from the HF-PS of *B. cereus* ATCC 10987 and ATCC 14579 (4). Here, we have reported the structure of a non-classical SCWP polysaccharide isolated from *B. cereus* ATCC 10987 as well as structural data on this polysaccharide from the *B. cereus* type strain, ATCC 14579, and we have compared those structural data with the reported *B. anthracis* HF-PS structure (4). The results are summarized as follows: (i.) The HF-PS from *B. cereus* ATCC 10987 is composed of a tetrasaccharide repeat unit consisting of a →6)-α-D-GalNAc-(1→4)-β-D-ManNAc-(1→4)-β-D-GlcNAc-(1→ trisaccharide in which the GalNAc residue is substituted at O3 with β-D-Gal, and the ManNAc residue is 3-O-acetylated. (ii.) There is heterogeneity in this HF-PS polysaccharide due to variation in the number of repeating units, the presence of O-acetyl groups, and addition of ManNAc, GlcNAc, and GalGalNAc, respectively, to the di-, and, possibly, mono- and tri-repeat unit structures. (iii.) In the *B. cereus* and *B. anthracis* strains that we have examined, there is a consensus HF-PS structural feature in that the repeating unit consists of a trisaccharide aminoglycosyl backbone in which two of the three aminoglycosyl residues are ManNAc and GlcNAc, while the third is either GlcNAc or GalNAc. Variability between the various HF-PS structures occurs in the substitution pattern of this trisaccharide with regard to both glycosyl and non-glycosyl substituents.

The structures of non-classical SCWP polysaccharides from bacilli have been described in a recent review by Schäffer and Messner (22). In that review, both common and variable structural features of these polysaccharides were discussed. The general structural feature for all of these polysaccharides is that they have repeating oligosaccharide units that contain aminoglycosyl residues and, further, that at least one of these residues has a *manno* configuration and a second has a *gluco* configuration. As we have shown here, this general structural theme is also present in the *B. cereus* ATCC 10987 HF-PS, *B. cereus* ATCC 14579 HF-PS, as well as in the previously reported structure of *B. anthracis* HF-PS (4). However, in these latter HF-PSs, the structural theme is a bit more specific in that the repeating unit consists of an aminoglycosyl trisaccharide backbone. While it has been reported (19) that these HF-PSs are involved

in anchoring and/or targeting proteins to the cell surface, including the S-layer proteins, it is not known if they have essential functions for viability or virulence. Nevertheless, some indirect evidence for their involvement in infection processes has already emerged. During *B. cereus* infections of insects, it has been noticed that the expression of an internalin-like protein is induced (8). This internalin-like protein is a candidate virulence protein that contains at its C-terminal end a SLH domain that presumably anchors the protein to the peptidoglycan via secondary cell wall carbohydrates (8). In the case of *B. anthracis*, it was reported that a mutant that is unable to add a pyruvate substituent to the HF-PS is defective in targeting the S-layer protein to the cell surface and is also adversely affected in cell division (19). It is also known that phage endolysins have, in addition to a catalytic domain, a carbohydrate-binding domain (CBD) that is thought to bind to a cell wall polysaccharide (18). The binding of the CBD domain of the endolysin to the polysaccharide is proposed to be required for the lytic activity, and is also thought to determine the specificity of the endolysin for a particular bacterial host (23) Thus, structural specificity in the host bacterial cell wall polysaccharide would account for the specificity of the endolysins; i.e. the endolysin CBD binds to specific host cell wall polysaccharides. This type of specificity was reported for the interaction of PlyG with strains of *B. anthracis* (23). Since the HF-PS is the major cell wall polysaccharide of *B. anthracis*, as well as of the *B. cereus* strains ATCC 10987 and ATCC 14579 and displays structural specificity it seems likely that it is the carbohydrate ligand for the endolysins and accounts for their specificity. In fact, PlyG specifically lyses *B. anthracis* strains, but is unable to lyse either *B. cereus* ATCC 10987 or type *B. cereus* strain ATCC 14579. Therefore, determining if the HF-PS is the endolysin receptor and characterizing the structural features required for binding of the endolysin has important implications with regard to diagnostic and therapeutic application of such phages (10, 23).

The structure of the HF-PS and the available genome sequence allows one to identify putative genes that may be involved in HF-PS synthesis. For example, a recent survey of gene differences in *B. anthracis* and *B. cereus* using suppression subtractive hybridization and bioinformatic analysis of *Bacillus* whole genome sequences found a glycosyltransferase group 1 family gene apparently specific to *B. anthracis* (15) While the involvement of the gene in question in HF-PS biosynthesis is at this point not clear

and currently under investigation in our laboratory, it is worth mentioning that from the few currently characterized bacterial galactosyltransferases some belong to the group family 1 glycosyltransferases (6, 12). Moreover, since, all of the HF-PSs have ManNAc, mutation of the gene that encodes a protein required for ManNAc synthesis should prevent the synthesis of the HF-PS and allow one to determine whether or not the HF-PS has essential functions for growth, anchoring/exporting S-layer and other surface proteins, interacting with endolysins, or in the case of a pathogen such as *B. anthracis*, for virulence. The preparation and analysis of such *B. anthracis* mutants is in progress.

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5

Secondary cell wall polysaccharides in *B. anthracis* and pathogenic *Bacillus cereus* strains are structurally closely related.

INTRODUCTION

Bacillus cereus is known as food pathogen. Between 1996 and 1997 an estimated 27,360 cases of food poisoning are reported to be due to the consummation of *B. cereus* contaminated food (3, 4). This does not seem a lot compared to the estimated 248,520 cases of *Clostridium perfringens* or 1,412,498 non-typhoidal *Salmonella* infections (13). However, the report of three human cases of fatal pneumonia due to a *B. cereus* infection was alarming. The first case was reported in 1995 where a Louisiana welder was admitted to the hospital with cough congestion, chills and fever, symptoms similar to inhalation anthrax, all of which he developed within 24h. *B. cereus* was identified in pathology samples of the lung tissue (15). The strain identified as *B. cereus* G9241 was after multi locus sequence typing (MLST) determined to be a close relative to *B. anthracis* (8). Two additional cases were reported in October and November of 2003 in Texas. The apparently healthy metal workers died of *B. cereus* infections that exhibited similar symptoms to inhalation anthrax (1). Isolates were collected from patients and screened for pathogenic bacteria. This resulted in the identification of two *B. cereus* strains (03BB87 and 03BB102) that were also confirmed by MLST analysis to be closely related to *B. anthracis* (1). Molecular sub-typing also confirmed that the two cases were, in fact, caused by two separate bacterial strains (7); one (03BB87) of which was indistinguishable from the previously isolated strain, *B. cereus* G9241 (8). Detailed genetic characterization of the isolates unveiled that all three strains harbored most of the *B. anthracis* pXO1 virulence plasmid including the toxin genes *pagA*, *lef* and *cya* that encode for the toxins protective antigen (PA), lethal factor (LF), and edema factor (EF) (7). It was found that *B. cereus* 03BB87 additionally carried the circular plasmid pBC218 which was first described in *B. cereus* G9241 and is thought to be involved in the production of a polysaccharide capsule (8). This finding further supported the high similarity of *B. cereus* G9241 and *B. cereus* 03BB87 (8). Using primers designed to identify pBC218-specific gene sequences, PCR analysis did not reveal any pBC218-indicative amplifications in *B. cereus* 03BB102. The genes *capA*, *capB* and *capC* that are required for the poly-D-γ-glutamic acid capsule synthesis in *B. anthracis* were detected in *B. cereus* 03BB102. However, while capsule production could be demonstrated in all three clinical isolated, none of them produced poly-D-γ-glutamic

acid (7, 8). Thus far, the one case in Louisiana and the two cases in Texas are the only reports where confirmed *B. cereus* strains caused anthrax-like symptoms in humans.

Recently, we compared the cell wall glycosyl composition of a variety of *B. anthracis* and *B. cereus* strains including the above described clinical isolates and demonstrated that a compositional variation correlates with differences in phylogenetic relatedness (**Chapter 1/(12)**). We also subsequently isolated secondary cell wall polysaccharides from *B. anthracis* and *B. cereus* strains and described their structures (hereafter called HF-PS) (5, 11). The HF-PSs are species-specific cell wall structures composed of repeating units where each repeating unit has as consensus structure a trisaccharide aminoglycosyl backbone in which two of the three aminoglycosyl residues are ManNAc and GlcNAc while the third is either GlcNAc or GalNAc. Additionally, we showed that the HF-PS from *B. anthracis* is immunogenic and that antisera generated from live and killed *B. anthracis* Sterne 34F$_2$ spore preparations react specifically with the HF-PS from *B. anthracis* and not with the HF-PS from *B. cereus* ATCC 10987 or ATCC 14579 (**Chapter 6)**. However, these *B. anthracis* antisera did show some cross-reactivity against the HF-PSs of the pathogenic *B. cereus* strains G9241, 03BB87 and 03BB102 (Chapter 6) indicating, as with the composition data, that their structures may be similar to that of *B. anthracis*.

While it has been reported (14) that these HF-PSs are involved in anchoring and/or targeting proteins to the cell surface, including the S-layer proteins, it is not known if they have essential functions for viability or virulence. Nevertheless, the intriguing fact that the HF-PSs from *B. anthracis* strains were all identical to each other but differed from the HF-PSs from investigated *B. cereus* strains prompted us to investigate the HF-PS structures of the *B. cereus* strains isolated from the fatal pneumonia infections mentioned above.

In this paper the HF-PS structure of *B. anthracis* Ames (5) is compared with the HF-PSs isolated from *B. cereus* G9241, *B. cereus* 03BB87 and *B. cereus* 03BB102 using NMR, mass spectrometric and gas chromatographic analyses.

MATERIAL AND METHODS

Bacterial strains and cultural conditions.

  Bacillus anthracis Ames, *B. cereus* G9241, *B. cereus* 03BB87 and *B. cereus* 03BB102 were provided from the CDC culture collection. Cultures were grown overnight (16 h) at 30 °C in 100 mL of brain heart infusion medium (BHI) (BD BBL, Sparks, MD) containing 0.5 % glycerol. The next morning, 4×1.5 mL of the overnight cultures were centrifuged at $10,000 \times g$ and room temperature for 5 min, the supernatants discarded, the pellets resuspended in 500 μL BHI; the four cell suspensions were used to inoculate four 250 mL volumes of BHI medium in 1 L Erlenmeyer flasks. The cultures were incubated at 30 °C under shaking at 200 rpm. Growth was monitored by measuring the optical density at 600 nm.. The cells, growing at mid-log phase, were harvested through centrifugation at $8,000 \times g$ and 4 °C for 15 min, washed two times in sterile saline. The cultures were enumerated by serial dilution and surface spread counts on BHI agar plates and sterilized by autoclaving (1 h at 121 °C) prior to further processing and carbohydrate analysis.

Preparation of* Bacillu*s cell wall extract.

  The bacterial cell walls were prepared by modification of a previously described procedure (2). Briefly, the autoclaved bacterial cells (1×10^{10} to 1×10^{11} CFU) were disrupted by sonication in 40 ml sterile saline on ice by four 10-minute sonication cycles, using a Branson Sonifier (Type 450, Branson Ultrasonics Corporation, Danbury, CT) with a 1/2 inch probe, operating it at a frequency of 20 kHz. The complete or near complete disruption of cells was checked microscopically. Unbroken cells were removed by centrifugation ($8,000 \times g$, 4 °C, 15 min). The separated pellet and supernatant fractions were stored at -80 °C. Cell wall materials were sedimented by ultracentrifugation at $100,000 \times g$, 4 °C for 4 h (Optima L-90K Ultracentrifuge, Beckman). The resulting cell wall pellets were washed by suspension in cold, deionized water followed by an additional ultracentrifugation as above and lyophilization.

Isolation and purification of the cell wall polysaccharide

Cell wall materials from all strains were treated with aqueous hydrofluoric acid (HF) according to a modification of the procedure described by Ekwunife *et al*. (6). Briefly, the cell walls are subjected to 47% hydrofluoric acid under stirring at 4 °C for 48 h. The reaction mixture was neutralized with ice-cold ammonium hydroxide solution (approximately 30%) on an ice-water bath. The neutralized material was then centrifuged at low speed for 10 min. at 3500 x *g*. The supernatant was removed, the remaining pellet resuspended and washed in cold deionized water, followed by an additional low speed centrifugation as described above. The supernatants were collected and concentrated in a SpeedVac. The concentrated sample was then desalted by gel filtration chromatography (GPC) using fine grade Bio-Gel P2 (Bio-Rad, Hercules, CA). Water was used as the eluent and an online refractive index (RI) detector was used to monitor the sample eluting from the column. The fractions which gave a positive response on the RI-detector were collected, pooled, lyophilized and used for further analysis.

Composition analysis

Glycosyl composition analysis was done by the preparation and gas chromatography-mass spectrometric (GC-MS) analysis of trimethylsilyl (TMS) methyl glycosides (17). The TMS methyl glycosides were identified and quantified by comparison to authentic standards. In brief, the samples were methanolyzed using 1 M methanolic HCl at 80 °C for 18 h to form the monomeric methylglycosides, followed by *N*-acetylation using pyridine and acetic anhydride (1:1) in presence of methanol at 100 °C for 1 h. After removing the reagents by flushing with dry nitrogen, the methyl glycosides were treated with Tri-Sil reagent (Pierce, Rockford, IL) at 80 °C for 30 min to form TMS methyl glycosides. The TMS methyl glycosides were dissolved in hexane and analyzed on a GC-MS using HP-1MS column (30 m × 0.25 mm × 0.25 μm).

Mass Spectroscopy

Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer model/type Voyager-DE BioSpectrometry Workstation (Applied Biosystems, Foster City, CA) was used to obtain the mass spectrum for each

polysaccharide sample. Each sample was dissolved in 1:1 mixture of methanol:water and mixed at equal proportion (v/v) with 0.5 M 2,5-dihydroxy benzoic acid (DHB) mixed with 0.1M 5-methoxysialicylic acid (Aldrich, St. Louis, MO) as the matrix. About 0.7 µl of this mixture was loaded on each spot on a stainless steel MALDI plate and air-dried. The spectra were acquired in delayed, linear and positive mode using 337 nm N2 laser with acceleration voltage of 20 kV.

NMR analysis

The HF-PS sample (2-3 mg) was dissolved in 0.5 mL of regular grade deuterium oxide (D_2O 98.5 %) and lyophilized. This process was done twice to exchange the hydroxyl and amide protons with deuterium. The sample was finally dissolved in 0.5 mL of 100 % D_2O (Cambridge Isotopes, Andover, MA) and transferred to 5 mm NMR tube. All 1D and 2D NMR spectra were acquired with a 600 MHz Varian Inova instrument using the standard software supplied by Varian. ^1H-^1H homonuclear 2-D experiments were done after perfect 90° pulse calibration and 3.5 K spectral width in both dimensions; however, ^1H-^{13}C HSQC data was acquired taking 3.5 K and 12 K in direct and indirect dimensions respectively.

RESULTS

Isolation and glycosyl composition of the HF-PS

The HF-PS from the investigated *B. cereus* strains eluted as a single major peak within the void volume of the Bio-Gel P2 column; the peak fractions were collected, lyophilized and used for detailed structural analysis. The glycosyl composition of the HF-PS from *B. anthracis* Ames was the same as previously described for four *B. anthracis* strains (Ames, Pasteur, Sterne 34F2, and UT60) (5, 12). Our previous structural determination showed that the *B. anthracis* HF-PSs all have structure(5, 12) consisting of galactose (Gal), *N*-acetylglucosamine (GlcNAc), and *N*-acetylmannose (ManNAc) in an approximate 3:2:1 ratio (5); i.e. the structure having a repeating unit consisting of a →6)-α-GlcNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc-(1→ backgone in which the α-GlcNAc residue was variably substituted with α- and β-Gal at the O3 and O4, respectively, and the β-GlcNAc with α-Gal at O3. The compositions of the HF-PS from *B. anthracis* Ames, *B. cereus* 03BB87, *B. cereus* G9241 and *B. cereus* 03BB102 are given in table 1.

Strain	Sugar Composition (in relative %)					
	Man	**Glc**	**Gal**	**ManNAc**	**GlcNAc**	**GalNAc**
***B. anthracis* Ames**	n.d.	2.7	57.0	19.3	21.1	n.d.
***B. cereus* G9241**	n.d.	1.3	55.8	19.3	23.6	n.d.
***B. cereus* 03BB87**	n.d.	0.9	61.8	14.2	23.2	n.d.
***B. cereus* 03BB102**	2.2	3.1	65.5	11.4	17.8	n.d.

Table 1. The glycosyl compositions of the HF-PS preparations from *B. anthracis* and the various *B. cereus* strains. The compositions are given as relative mass percent of total carbohydrate. Man =mannose, Glc =glucose, Gal =galactose, ManNAc =*N*-acetylmannosamine, GlcNAc =*N*-acetylglucosamine, GalNAc =*N*-acetylgalactosamine

The composition of the HF-PS from *B. cereus* G9241 and *B. cereus* 03BB87 were very similar and consisted of Gal, GlcNAc, and ManNAc in a 3:2:1 and 4:2:1 ratio, respectively. The minor amount of Glc was due to contamination of the HF-PS preparation with a Glc-rich polysaccharide as previously reported (12). The composition of *B. cereus* 03BB102 HF-PS showed the presence of Gal, GlcNAc and ManNAc in approximately a 6:2:1 ratio with small amounts of Man and Glc. These results indicated that, in comparison to the HF-PS from *B. anthracis*, the HF-PSs from *B. cereus* 03BB87 and 03BB102 contain increased levels of Gal.

Mass spectrometric analysis:

The mass spectra acquired using MALDI-TOF analysis of the HF-PSs from three pathogenic *B. cereus* strains compared with that of the *B. anthracis* HF-PS showed that all of the HF-PSs contained similar ion clusters. The ions and proposed compositions are given in Table 2. The ions of each cluster differed from those in the adjacent cluster by *m/z* 1095, a mass that was shown to be the hexasaccharide repeating unit comprised of three Gal and three *N*-acetylhexosaminosyl (HexNAc) residues in the *B. anthracis* HF-PS; e.g. $Gal_3GlcNAc_2ManNAc_1$ (5). The mass spectra for the HF-PSs from the three pathogenic *B. cereus* strains showed more heterogeneity than observed for *B. anthracis* HF-PS. This variability existed entirely in the number of Gal substitutions present on each repeating unit.

Strains	Single Repeating Unit		Double Repeating Unit	
	No. of Gal	^{2}[M+Na]$^+$ (MALDI-TOF)	No. of Gal	^{2}[M+Na]$^+$
B. anthracis	2 3	974.3 1136.4	4 5 6	1908.6 2069.7 2231.7
B. cereus G9241	3 4	1136.4 1298.6	3 4 5 6 7 8	1745.9 1907.9 2069.9 2231.9 2394.0 2556.1
B. cereus 03BB87	1 2 3 4	812.3 974.4 1136.5 1298.5	4 5 6 7 8	1907.8 2069.8 2231.9 2393.9 2556.9
B. cereus 03BB102	1 2 3 4 5	812.3 974.4 1136.5 1298.5 1460.7	2 5 6 7 8 9 10	1583.7 2069.8 2231.9 2393.9 2556.0 2718.0 2880.1
$m/z = 1095 = \mathbf{\textcolor{red}{[Gal_3GlcNAc_2ManNAc_1]_n}}$ **For a single repeat unit: 1095 + 18 + 23 = 1136**				

Table 2: Mass spectral data obtained by MALDI-TOF analysis (positive mode) of the HF-PS from *B. anthracis* Ames, *B. cereus* G9241, *B. cereus* 03BB87 and *B. cereus* 03BB102. The fractions containing single and double repeating units of the HF-PSs were obtained by size exclusion chromatography as described in the methods section. Analysis of the HF-PSs from *B. anthracis* Sterne, UT60, and Pasteur were all identical to this spectrum.

For example, in the case of the *B. cereus* 03BB87 HF-PS, the *m/z* 2231.9 ion is consistent with the composition of two repeating units composed of $Gal_6GlcNAc_4ManNac_2$, while the *m/z* 2394 contains one more Gal residue, *m/z* 2556.1 contains two more Gal residues, and the ion at *m/z* 2069.9 is consistent with two

repeating units where one repeating unit has 3 Gal residues and the second repeating unit has only 2 Gal attached. The HF-PS of *B. cereus* 03BB87 also showed the single repeating unit with greater heterogeneity with regard to the attached Gal residues compared to *B. anthracis* or *B. cereus* G9241 HF-PS. The single repeating unit had structures with 1, 2, 3, or 4 Gal residues attached whereas for *B. cereus* G9241 HF-PS only ions due to a repeat unit with 3 or 4 Gal residues attached, and for *B. anthracis* HF-PS, a single repeat unit with 2 or 3 attached Gal residues. The mass spectrometric data for the HF-PS of *B. cereus* 03BB102 had the highest heterogeneity with between 1 and 5 Gal residues attached to a single repeat unit. These mass spectral data are consistent with the composition data showing that the HF-PSs from the *B. cereus* strains contain increased levels of Gal compared to *B. anthracis* HF-PS.

NMR analysis:

Figure 1 compares the proton NMR spectra of the HF-PS from *B. anthracis* Ames with the HF-PSs from the three pathogenic *B. cereus* strains.
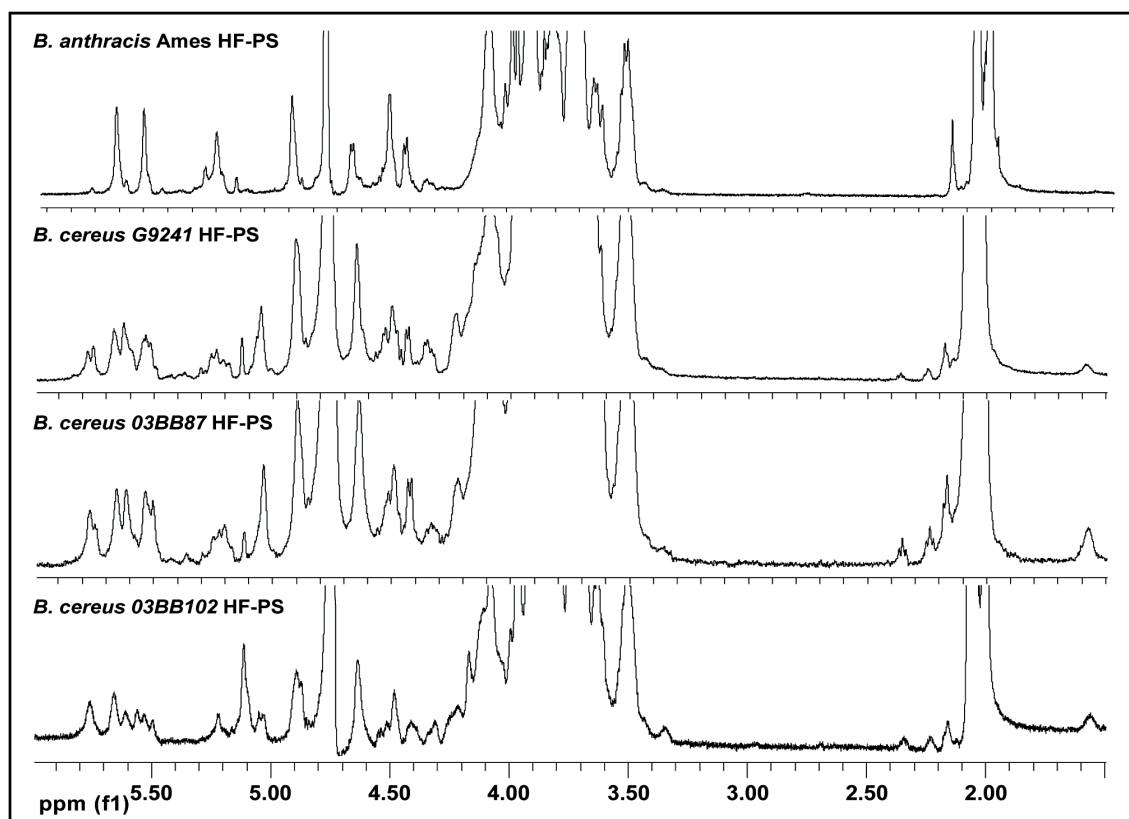
Figure 1: Proton NMR spectra of the HF-PSs from *B. anthracis* Ames, *B. cereus* G9241, *B. cereus* 03BB87 and *B. cereus* 03BB102

The comparison clearly shows that the spectra of the *B. cereus* HF-PS differ from that of the *B. anthracis* HF-PS in the anomeric resonance region from δ 4.4 to 6.0 in that there is increased complexity in *B. cereus* spectra. This increased complexity is likely due to the increased level and variability of the Gal substitutions as indicated by the composition and mass spectra data described above. The spectra of the *B. cereus* 03BB87 and G9241 HF-PSs are very similar indicating that these two HF-PSs are very similar structures. This structural similarity is consistent with the observed genetic similarity as determined by MLST (7).
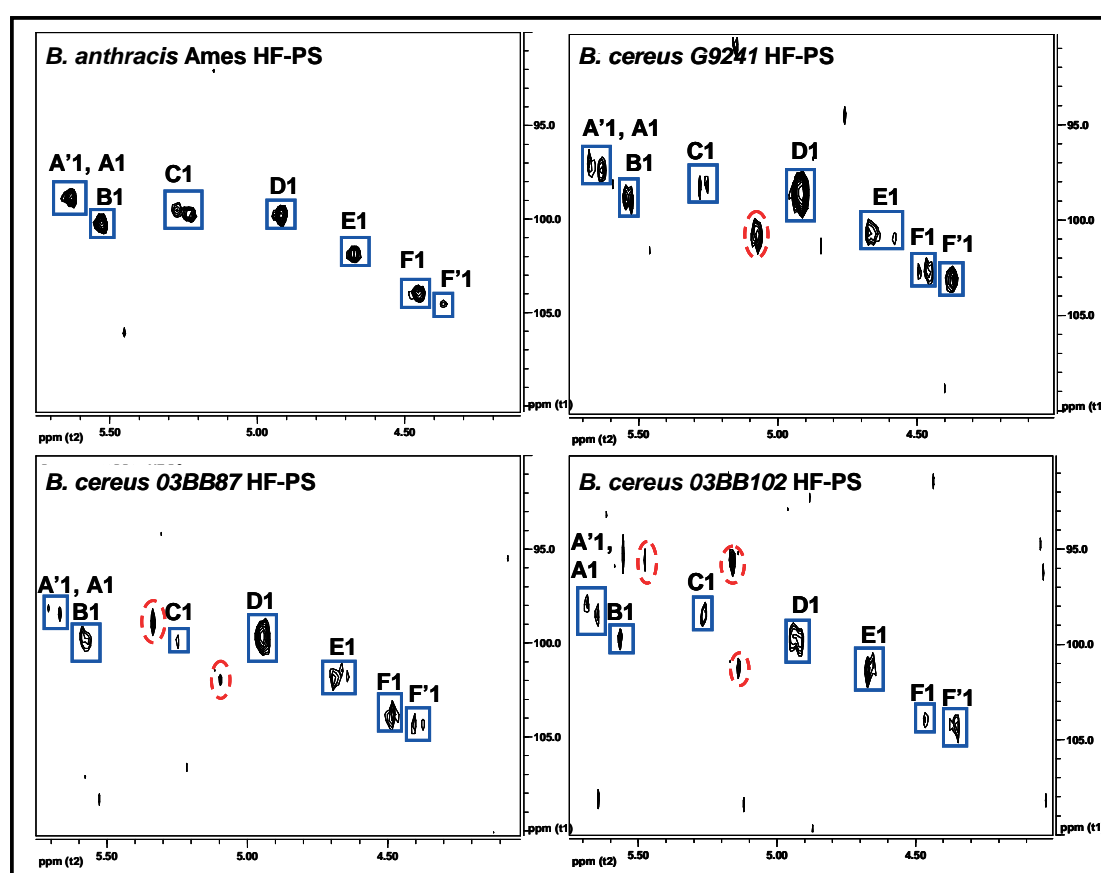
Figure 2: **The HSQC spectrum of the anomeric region of the HF-PS from *B. anthracis* Ames, *B. cereus* G9241, *B. cereus* 03BB87 and *B. cereus* 03BB102.** The structure and the assigned proton/carbon correlations are as shown. Signals in blue boxes were identical in all four spectra. Signals circled red were additional signals not found in the spectrum of *B. anthracis* HF-PS. The NMR assignments of the anomeric regions are given in Table 3. The HSQC spectra of the HF-PSs from *B. anthracis* Sterne, UT60, and Pasteur are identical to the spectrum from *B. anthracis* Ames.

A further NMR comparison of these HF-PSs was made by ^{1}H/^{13}C HSQC NMR analysis. The results for the ^{1}H/^{13}C anomeric signals are shown in **Figure 2**, listed in **Table 3**, and confirm the structural similarity of all of these HF-PSs. The major anomeric cross peaks were identical for the HF-PSs from *B. anthracis* and the three *B. cereus* strains. However there were additional cross peaks for the B. *cereus* HF-PSs. Compared to the HF-PS from *B. anthracis*, the HSQC data from *B. cereus* G9241 HF-PS showed one additional signal at δ 5.08/101.0, two additional signals at δ 5.01/102 and δ 5.03/99 in *B. cereus* 03BB87 HF-PS and three additional signals at δ 5.13/101.4, δ 5.15/95.8 and δ 5.48/95.8 for *B. cereus* 03BB102. These additional signals are likely due to the increased complexity of the anomeric region caused by the additional Gal substitutions. The fact that all of the remaining ^{1}H/^{13}C signals of the *B. cereus* HF-PSs have a very similar pattern and values to those of the *B. anthracis* HF-PS support the conclusion that these *B. cereus* and *B. anthracis* HF-PSs have the same backbone structure with the same Gal substitutions but also with additional Gal residues.

	B. anthracis Ames HF-PS	*B. cereus* G9241 HF-PS	*B. cereus* 03BB87 HF-PS	*B. cereus* 03BB102 HF-PS
A. α-D-Gal	5.64 (98.8)	5.64 (97.2)	5.66 (98.3)	5.65 (98.5)
B. α-D-Gal	5.53 (100.3)	5.53 (98.9)	5.58 (100.0)	5.56 (100.0)
C. α-D-GlcNAc	5.22 (99.7)	5.25 (98.2)	5.25 (99.7)	5.26 (98.4)
D. β-D-ManNAc	4.91 (99.7)	4.92 (98.6)	4.95 (99.7)	4.93 (99.7)
E. β-D-GlcNAc	4.67 (101.9)	4.67 (101.0)	4.67 (102.0)	4.67 (101.4)
F. β-D-Gal	4.44 (104.1)	4.46 (102.5)	4.41 (104.3)	4.47 (104.1)
X1		5.08 (101.0)	5.01 (102.0)	5.13 (101.4)
X2			5.33 (99.0)	5.15 (95.8)
X3				5.48 (95.8)

Table 3: 1H and 13C chemical shift values for the glycosyl residues found in the anomeric region from HF-PSs of *B. anthracis* Ames, *B. cereus* G9241, *B. cereus* 03BB87 and *B. cereus* 03BB102. The glycosyl residues were first assigned in the structural analysis of *B. anthracis* HF-PS (5) (boxed blue in Figure 2). X1, X2, and X3 are the additional anomeric signals found in the *B. cereus* strains that were not present in the *B. anthracis* HF-PS. These signals are circled red in Figure 2.

DISCUSSION

A recent review by Schäffer and Messner (16) addressed the structures of non-classical SCWP polysaccharides from bacilli. The general structural feature for all of these polysaccharides is that they have repeating oligosaccharide units that contain aminoglycosyl residues and, further, that at least one of these residues has a *manno* configuration and a second has a *gluco* configuration. This general structure theme was also present in the HF-PS structures of *B. anthracis, B. cereus* ATCC 10987 and *B. cereus* ATCC 14579 (5, 11). These reported structures were consistently composed of an aminoglycosyl trisaccharide backbone. While it has been reported (14) that these HF-PSs are involved in anchoring and/or targeting proteins to the cell surface, including the S-layer proteins, it is not known if they have essential functions for viability or virulence. In this chapter, we compared the known structure of the HF-PS from *B. anthracis* which was determined to be a hexasaccharide repeat unit composed of $Gal_3GlcNAc_2ManNAc_1$, with data from the structural analysis of the HF-PSs from three pathogenic *B. cereus* strains *B. cereus* G9241, *B. cereus* 03BB87 and *B. cereus* 03BB102. The comparison of composition, mass spectral, and the HSQC data suggested that the HF-PSs from the pathogenic *B. cereus* strains have the same amino-sugar backbone [$\rightarrow$6)-α-D-GlcNAc-(1$\rightarrow$4)-β-D-ManNAc-(1$\rightarrow$4)-β-D-GlcNAc-(1$\rightarrow$] substituted with Gal residues at both the α- and β-GlcNAc residues but with additional Gal substitutions that are not present in the *B. anthracis* HF-PS. This structural similarity is unlike that observed for the HF-PSs from closely related non-pathogenic *B. cereus* strains ATCC 10987 and *B. cereus* ATCC 14579 which had structures that differed more significantly from *B. anthracis* HF-PS (11).

The three pathogenic *B. cereus* strains not only have HF-PS structures similar to that of *B. anthracis*, but it was also found that there was some genetic relatedness to *B. anthracis* (1). It was shown that two of these strains harbor the entire *B. anthracis* pXO1 virulence plasmid (e.g. *B. cereus* G9241and 03BB87), and *B. cereus* 03BB102 contains only the *B. anthracis* virulence genes (1, 9). At this time it is not known whether or not the genetic similarities are responsible for the anthrax-like symptoms caused by these *B. cereus* strains, or if the HF-PS plays a role (directly/indirectly) in their pathogenicity.

Case reports about anthrax infections in great apes at the Côte d'Ivoire and in Cameroon could however shed some light on this matter since these isolates from primates showed some very interesting parallels to the human pathogenic *B. cereus* strains. Initial identification of the bacterial strains isolated from four chimpanzees and one gorilla that presumably died of anthrax through microbiological features would suggest that the isolates are not *B. anthracis* species based on their high motility, resistance to gamma-phages and penicillin G (10). However, southern blot analysis and RT-PCR confirmed the presence of *B. anthracis* virulence plasmids pXO1 and pXO2. MLST analysis classified these strains as sequence type 1 and 2, the same sequence type as 'classic' *B. anthracis* strains (10). This illustrates very well how difficult the identification of *B. cereus* group members is and how fine the line is especially between *B. anthracis* and *B. cereus* strains is.

The analysis of the HF-PS from these newly identified *B. anthracis* strains could put the hypothesis of a common structural pattern in the HF-PS structure of anthrax and anthrax-like infection causing strains to a test. Confirmed structural similarities in the HF-PS structures of such strains could aid in the development of specific antimicrobials against anthrax and anthrax-like infections. The serological analysis of the reactivity of rabbit anti-*B. anthracis* Sterne spore antiserum against the HF-PS from several *B. cereus* strains already showed discrimination between non-pathogenic and pathogenic *B. cereus* strains. The antiserum recognized the HF-PS from *B. anthracis* and from *B. cereus* G9241, *B. cereus* 03BB87 and *B. cereus* 03BB102, but not from the non-pathogenic *B. cereus* ATCC 10987 and *B. cereus* ATCC 14579 type strain. The details of these experiments are described in Chapter 6.

Overall, the structural similarities of the HF-PSs from pathogenic *B. anthracis* and *B. cereus* strains still need to be confirmed on a larger group of bacteria. However, if this structural trend can be confirmed throughout a group of pathogenic *B. cereus* group strains, the HF-PS could represent a valuable candidate for the development of a broad range vaccine against *Bacillus* strains causing anthrax and anthrax-like infections.

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Secondary cell wall polysaccharide of *Bacillus anthracis* and *Bacillus cereus* strains are antigens that display both common and strain-specific epitopes

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Running Title: *Bacillus cereus* cell wall carbohydrate structures

This chapter is a manuscript in preparation

Contributions to this manuscript:

I prepared the first draft of this manuscript and was involved in the design and configuration of tables and figures. The isolation and purification of the HF-PS, the conjugation of the HF-PSs to BSA, the immuno dot blot and all ELISA experiments (with the exeption of the experiments that lead to Figure 7) were performed by me. Elke Saile was responsible for growing the bacterial cultures used in the experiments and preparing them for shipment. The bacterial strains required cultivation in BSL2 and BSL3 laboratories at the Center for Disease Control (CDC). The rabbits were immunized and the sera were collected at the CDC animal facility and then shipped to me.

Key findings and conclusions

- Immunization of rabbits with live or irradiated *B. anthracis* Sterne spores induces the production of antibodies specific to BSA-*B. anthracis* HF-PS and to BSA conjugates with HF-PS from pathogenic *B. cereus* strains.
- HF-PS or a HF-PS like immunodominant structure is expressed on the spore surface visible to the immune system and elicits a specific antibody response.
- Immunization of rabbits with KLH-*B. anthracis* HF-PS induces the production of unspecific antibodies that recognize HF-PS from *B. anthracis* and all *B. cereus* strains.
- Isolation or conjugation of the HF-PS exposes a different, unspecific epitope to the immune system.
- The HF-PS has a specific and a common epitope since anti KLH-*B. anthracis* HF-PS antiserum was specific towards cell wall or cell preparations.
- The specific epitope in question is accessible to the immune system as long as it is attached to the cells or cell walls. It becomes inaccessible when the HF-PS is released.

ABSTRACT

The polysaccharides (PSs) from _B. anthracis_ and _B. cereus_ strains released by treating the cell walls with aqueous hydrogen fluoride (HF-PSs) were compared with regard to their immunochemical reactivity. Antiserum to _B. anthracis_ live or killed spores was measured for HF-PS-binding IgG antibodies. It was found that antiserum to live or killed _B. anthracis_ spores both bound the HF-PS from _B. anthracis_ but not the HF-PSs from the closely related _B. cereus_ ATCC 10987 or from _B. cereus_ type strain ATCC 14579. However, both antisera bound the HF-PSs of three pathogenic _B. cereus_ strains, _B. cereus_ strains G9241, 03BB87, and 03BB102, which caused severe or fatal pneumonia in humans, indicating that these HF-PSs and the _B. anthracis_ HF-PS share structural epitopes. Antiserum against the _B. anthracis_ HF-PS-keyhole limpet hemocyanine (KLH) conjugate was prepared and contained IgG antibodies that bound spores, HF-PSs, cells and cell walls of _B. anthracis_ strains but not of the _B. cereus_ type strain ATCC 14579 or of the closely related _B. cereus_ ATCC 10987 strain. However, as with the _B. anthracis_ spore antiserum, the HF-PS-KLH conjugate antiserum did bind the HF-PSs, cells and cell walls of the _B. cereus_ strains G9241, 03BB87, and 03BB102. Unlike the spore antisera, the HF-PS-KLH conjugate antiserum contained cross-reactive antibodies that bound to the HF-PS-BSA conjugates of all _B. cereus_ strains tested, including _B. cereus_ type strain ATCC 14579. This result indicated that the HF-PS-protein conjugates, but not unconjugated HF-PSs, cells, or cell walls have common immunodominant epitope.

INTRODUCTION

Anthrax is mainly a disease in herbivores although humans can be infected through exposure to *B. anthracis* spores. The three clinical forms of anthrax; cutaneous, gastrointestinal and inhalation anthrax are, if untreated, potentially fatal. Within the last few years *Bacillus anthracis*, the causal agent of anthrax, was used to make bioterroristic threats. Consequently, it became important to improve diagnostics and vaccination methods. Therefore, we became interested in determining if *B. anthracis* contained carbohydrates either on the spores or vegetative cells that are antigenic and have structural and immunochemical properties that would 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 potential for such uses. One of these carbohydrates is an oligosaccharide that is part of the collagen-like protein, BclA, on the spore exosporium (9, 23), and the second is a non-classical secondary cell wall polysaccharide found in the vegetative cell wall (7).

The BclA of *B. anthracis* forms a uniform hair-like nap that surrounds the spore (31). The glycan part of BclA was structurally characterized and consists of a pentasaccharide containing a single galactose (Gal), four rhamnosyl (Rha) residues, and a previously unknown sugar which was named anthrose (Ant) (9). The importance of the *B. anthracis* specific glycoprotein in the infection process is still unclear. There are reports that BclA is not required for the pathogenicity of fully virulent *B. anthracis* strains (4). However, immunochemical experiments showed that the BclA glycoprotein was recognized by monoclonal antibodies raised against spores (31) and that it was an effective boost for mice previously immunized with protective antigen (PA) (5). Mehta *et al.* (23) chemically synthesized an AntRha$_2$ trisaccharide and various structural analogues, and demonstrated that anti-AntRha$_2$ antibodies were present in the serum from animals inoculated with either live or killed *B. anthracis* spores. Examination of post-infection sera of both naïve and vaccinated *Rhesus macaques* that survived inhalation anthrax as well as serum from human cases of inhalation or cutaneous anthrax also contain antibodies that bind the chemically synthesized AntRha$_2$ structure {Saile, *et al.*, manuscript in preparation}. Further, it has been shown that a monoclonal antibody raised against a chemically synthesized AntRha$_3$ tetrasaccharide is able to

specifically bind *B. anthracis* spores (32). Thus, this *B. anthracis* spore carbohydrate has both structural and immunochemical reactivity properties that warrant further investigation into its possible use for diagnostic methods and as a vaccine antigen.

The research described in this report focuses on the secondary cell wall polysaccharide (PS) of the vegetative cell wall of *B. anthracis* that is released from the cell wall by aqueous hydrogen fluoride (HF), HF-PS. For *B. anthracis,* it was shown that the HF-PS anchors cell surface proteins, such as S-layer proteins, to the peptidoglycan. It is thought that the PS is binding to the carbohydrate-binding SLH-domain of the surface protein while a HF-labile phosphate bond anchors the PS to the peptidoglycan (24). We have previously shown, by examining the cell walls of *B. anthracis* and related *B. cereus* strains, that *B. anthracis* produces a specific HF-PS structure that is identical for investigated *B. anthracis* strains; i.e. Ames, Sterne, and Pasteur, but different from that of *B. cereus* cell walls (7, 22). The *B. anthracis* HF-PS is comprised of an amino sugar backbone of   →6)-α-GlcNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc-(1→ 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 (7). We have also recently determined the structure of the HF-PS from the closely related *B. cereus* ATCC 10987 which consists of a →6)-α-GalNAc-(1→4)-β-ManNAc-(1→4)-β-GlcNAc-(1→ backbone in which the α-GalNAc is substituted at O3 with a β-Gal residue and the β-ManNAc is acetylated at O3 (Leoff *et al.*, submitted). Comparison of structural data for the HF-PS from the *B. cereus* type strain ATCC 14579 with those for the *B. anthracis* and *B. cereus* ATCC 10987 HF-PSs showed that all have a common HexNAc-HexNAc-HexNAc trisaccharide structural component, but that each is differently substituted with glycosyl residues and/or non-carbohydrate substituents such as acetyl groups.

The strain-specific structural features of the HF-PSs from *B. anthracis* and *B. cereus* strains made them attractive candidates to determine if they have immunochemical properties that would make them useful vaccine and diagnostic antigens. Therefore, 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 sera of rabbits inoculated with *B. anthracis* live or killed spores and in the sera of *Rhesus macaques* that survived exposure to *B. anthracis* spores contain HF-PS-binding IgG antibodies. Further, we

investigated the immunochemical specificity of the HF-PSs from *B. cereus* group strains such as the closely related *B. cereus* ATCC 10987, and *B. cereus* type strain ATCC 14579. Also, since our previous work indicated that the *B. cereus* strains G9241, 03BB87, and 03BB102 which were isolated from cases of severe or fatal pneumonia (1, 17, 18), have HF-PSs that are very similar in their glycosyl compositions to that of the *B. anthracis* HF-PS, we investigated the immunochemical specificities of these HF-PSs. We also examined the presence of *B. anthracis* HF-PS structural epitopes in the cells and cell walls of *B. anthracis* and *B. cereus* strains using antiserum prepared against the keyhole limpet hemocyanin (KLH) conjugate of *B. anthracis* HF-PS.

MATERIAL AND METHODS

Bacterial strains and culture conditions.

The strains used in this work are listed in Table 1. Most *B. anthracis* strains were obtained from the CDC culture collection. Strain *B. anthracis* UT60 was obtained from T. Koehler, University of Texas/Houston Health Science Center. Cells cultured over night in 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 (*B. anthracis*) or 30 °C (*B. cereus*) shaking at 200 rpm. Growth was monitored by measuring the optical density of the cultures at 600 nm. In mid-log phase, cells were harvested by centrifugation (8,000 × *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.

Preparation of bacterial cell walls.

The bacterial cell walls were prepared from autoclaved bacterial cells (3×10^8 to 3×10^9 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 (8,000 × *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 × *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 × *g*, 4 °C for 4 h, and lyophilized.

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 a modification of the procedure described by Ekwunife *et al.*(10). Briefly, the cell walls were subjected to 47% hydrogen fluoride (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 BioGel 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 (7).

Preparation of Spores.

Spores of *B. anthracis* were prepared from liquid cultures of PA medium (phage assay medium (15)) grown at 37 ºC, 200 rpm for six days. Spores of *B. cereus* ATCC 14579 were prepared from liquid cultures of PA medium grown at 30 ºC, 200 rpm for six days. Spores were harvested by centrifugation and washed two times by suspension in cold (4 ºC) 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 ºC) and washed a further four times in cold sterile deionized water. After suspension in sterile deionized water, spores were quantified by surface spreading on brain heart infusion (BHI) agar plates (BD BBL, Sparks, MD) and counting the colony forming units (cfu). Spore suspensions were stored in water at -80 ºC.

For the preparation of killed spores, 500 µL aliquots of spore suspensions in water, prepared as described above and containing approximately 3 x 10^8 CFU/mL, were irradiated in 200 mL Sarstedt freezer tubes (Sarstedt, Newton, NC) in a gamma cell irradiator with an absorbed dose of 2 million rads. Sterility after irradiation was monitored 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. Absence of growth was taken as an indicator of sterility.

Preparation of spore antiserum.

Each spore antiserum was prepared in female New Zealand White rabbits (2.0 - 3.5 kg) purchased from Myrtle's Rabbitry (Thompson Station, TN). For antiserum production each of two rabbits were inoculated intramuscularly at two sites in the dorsal hind quarters with 0.5 mL of washed live-spore or irradiated killed-spore inoculum (3 × 10^6 total spores). Rabbits were vaccinated at 0, 14, 28, and 42 days. Serum was

collected prior to the first immunization (pre-immune 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.

Conjugation of HF-PSs to BSA or KLH

Conjugation was performed by modification of a previously described method (6, 28). Approximately 1 mg of freeze dried polysaccharide was dissolved in 90 μL of 0.15 M HEPES buffer, pH 7.4. While stirring, 4 mg of 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) in of acetonitrile (90 μL) were slowly added to a solution of the polysaccharide to avoid precipitation. After activation of the polysaccharide (30 sec), 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 KLH (Sigma, St. Louis, MO) were dissolved in 348 μL 0.01 M phosphate buffered saline (PBS) buffer, pH 7.4 and added to the reaction mixture. After stirring for 18 h at 4 °C, the reaction mixture was quenched with addition of 120 μL of 0.5 M ethanolamine in 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 x g using a centrifugal filter device (Centriplus YM-10, Millipore, Billerica, MA). The conjugate was freeze dried 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 (34). Briefly, 200 μg of the HF-PS-KLH or -BSA conjugate were methanolyzed in 1 M methanolic 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.*

Antiserum to *B. anthracis* HF-PS-KLH conjugate was prepared in female New Zealand White rabbits (2.0–3.5 kg) purchased from Myrtle's Rabbitry (Thompson

Station, TN). For antiserum production each of two rabbits 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, St. Louis, MO) with 500 μg of the HF-PS-KLH conjugate were divided into two injections per rabbit. For the booster shots 1.0 mL of MPL + TDM + CWS Adjuvant System with 250 μg of the HF-PS-KLH conjugate were used. Rabbits were immunized at 0, 14, 28, and 42 days. Serum was collected prior to the first immunization (pre-immune serum) and at day 7 and day 14 after each injection of antigen. Terminal bleeds were collected 14 days 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.

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

The immunoreactivity of serum from rabbits inoculated with *B. anthracis* Sterne spores and 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 14597 by enzyme linked immunosorbent assay (ELISA). Slightly different protocols were used to examine these antisera. The *B. anthracis* spore antiserum was 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 over night at 4°C.. The next day, the plates were washed 3 times with wash buffer (0.01 M PBS, pH 7.4, 0.1% Tween-20) followed by blocking buffer (5% non fat dry milk in 0.01 M PBS, pH 7.4, 0.5% Tween-20) for 1h at room temperature. The plates were then washed again, and serial dilutions (100 μL per well) of spore rabbit antiserum in blocking buffer were added and the plates incubated for 1 h at room temperature. The plates were then washed three times with wash buffer. Horseradish peroxidase 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 wash buffer before adding 100 μL of ABTS 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 using Immulon 2 HB flat bottom 96-well microtiter plates (Thermo Labsystems, Franklin, MA) in which each well was coated with 100 µl of the *B. anthracis* HF-PS KLH conjugate at a concentration of 0.5 µg per ml in coating buffer (0.01M PBS, pH 7.4). Plates were covered with plastic wrap, stored at +4°C and used within seven days. For ELISA, plates were washed three times in wash buffer (0.01M PBS, pH 7.4, 0.1% Tween-20) using an ELX405 microplate washer (BioTek Instruments Inc., Winooski, VT). Serial dilutions (100 µl per well) of serum in dilution buffer (0.01M PBS, pH 7.4, 5% skim milk, 0.5% Tween-20) were added and plates incubated for 60 min at 37ºC. After plates were washed three times in wash buffer, depending on the serum used, HRPO-conjugated goat anti-Rhesus monkey IgG or HRPO-conjugated goat anti-rabbit IgG was added (100µl/well) at a dilution of 1:10,000 and the incubation continued for 60 min at 37ºC. Plates were washed three times in wash buffer and bound conjugate was detected colorimetrically by using 100 µl per well ABTS/H_2O_2 substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Color development was for 15 min and was stopped by addition of 100 µl of Peroxidase Stop Solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD) to each well. OD values were read within 15 min of addition of the stop solution with a MRX Revelation microtiter plate reader (Thermo Labsystems, Franklin, MA) at a wavelength of 410 nm with a 490-nm reference filter. Samples were tested three times and average OD and standard deviation were calculated.

Inhibition ELISA analysis.

To test the ability of the various HF-PS samples to inhibit the binding of *B. anthracis* spore antiserum to the *B. anthracis* HF-PS-BSA conjugate, 100 µL of rabbit anti live Sterne spore serum (1:1600 dilution in blocking buffer, this gives an OD of about 0.7 in the reactivity of the antiserum with the HF-PS-BSA conjugate) were added to the coated microtiter plate wells together with 0-, 5-, 10-, 25-, or 50-fold excess unconjugated HF-PS (i.e. fold excess relative to the 0.35 µg of carbohydrate equivalent

of the *B. anthracis* HF-PS-BSA conjugate coating each well of the microtiter plate). The HF-PS samples tested for inhibition were: *B. anthracis* HF-PS, *B. cereus* ATCC 10987 HF-PS, *B. cereus* ATCC 14579 HF-PS, and *B. cereus* G9241 HF-PS. In addition, BSA was used as the inhibition negative control. Each HF-PS inhibitor was diluted in blocking solution. Inhibitor and serum were briefly mixed in an uncoated microtiter plate followed by immediate transfer to the coated plate. Plates were incubated for 1 h at room temperature followed by washing with wash buffer three times. The microtiter plates were incubated with horseradish peroxidase labeled anti-rabbit IgG and developed as described above.

The binding of anti* B. anthracis *HF-PS-KLH antiserum to the cells, cell walls, and spores of* B. anthracis *and* B. cereus *strains.

Immuno dot-blot assays were used to measure the binding of *B. anthracis* HF-PS-KLH to cells, cell walls and spores. Cells, cell walls, or spores were suspended in distilled water and blotted onto a nitrocellulose membrane. The spore suspension was prepared so that it had a slightly hazy appearance. Samples with a volume > 5 μL were taken from 1mg/mL of cell or cell wall stock preparations, dried in a speed-vac and re-dissolved in 3 μL of distilled water before they were blotted onto the membrane. Samples with volumes <5μL were taken from the above mentioned stock preparations and directly blottet onto the membrane without prior reduction of the volume. BSA, maltoheptaose, and chemically synthesized *B. anthracis* BclA AntRha$_2$ trisaccharide were blotted as controls. The membrane was allowed to dry over night before blocking with blocking buffer for 1 h. The membrane was then incubated at room temperature for 1 h with antiserum to *B. anthracis* HF-PS-KLH conjugate that had been diluted 1:1600 in blocking buffer. After washing three times with wash buffer the membrane was incubated with a 1:1000 dilution of mouse anti-rabbit IgG linked to alkaline phosphatase in 0.01 M PBS buffer, pH 7.4 for 1 h at room temperature. After washing five times, the membrane was developed using Nitro Blue Tetrazolium (0.3 mg/mL in 0.1 M NaCl, 0.1 M trishydroxymethylaminomethane (Tris), 5mM MgCl$_2$ of 0.15 mg/mL of 5-bromo-4-chloro-indolyl-phosphate). The reaction was stopped by washing in tap water.

RESULTS

Reactivity of anti- B. anthracis *HF-PS-KLH conjugate antiserum with HF-PS from* B. anthracis *and* B. cereus.

An ELISA experiment was used to determine if the *B. anthracis* HF-PS was antigenic. The results, Figure 1, show that antiserum to both live and killed *B. anthracis* spores contain IgG antibodies that recognize *B. anthracis* HF-PS, with the live-spore antiserum having a significantly greater HF-PS-specific antibody titer.
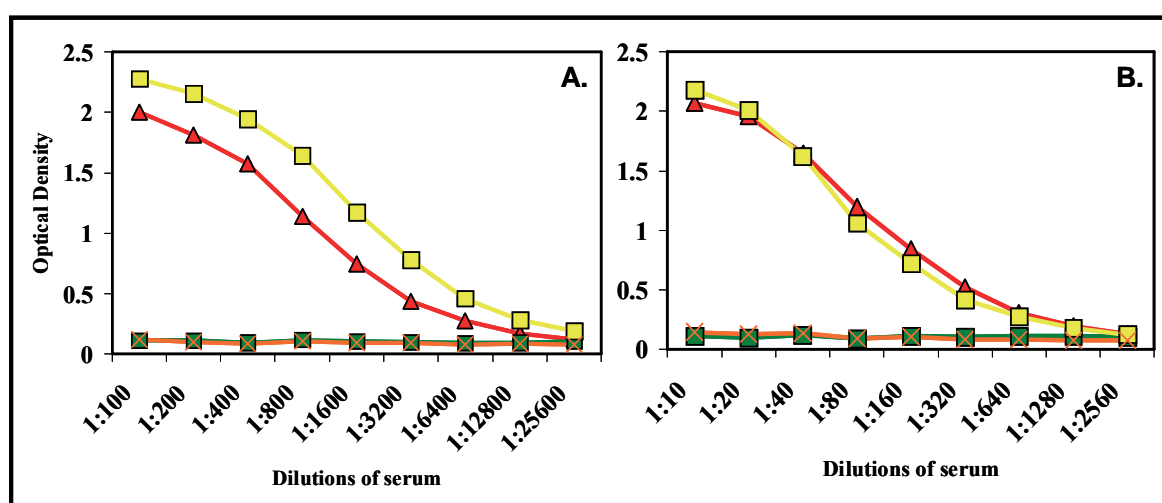
Figure 1. Immunoreactivity of the HF-PSs to antisera raised in rabbits against *B. anthracis* Sterne (A) live spores and (B) irradiated killed spores. The microtiter plate wells were coated with *B. anthracis* Pasteur HF-PS-BSA (▲), the chemically synthesized AntRha$_2$-BSA conjugate (23) as found in the BclA glycoprotein of the spore exosporium as a positive control (■), and maltoheptaose-BSA (■) and BSA (×) alone as negative controls. Even though the antisera were raised against spores from *B. anthracis* Sterne and the HF-PS-BSA conjugate was prepared from *B. anthracis* Pasteur HF-PS, it should be noted that the HF-PSs from *B. anthracis* Ames, Pasteur, and Sterne all have identical structures (7, 22).

In the positive control, binding was also observed to wells coated with the synthetic AntRha$_3$-BSA conjugate. No binding was observed to the negative controls BSA and maltoheptaose-BSA conjugate. Thus, the *B. anthracis* HF-PS is an antigen that is recognized by animals inoculated with either live or killed spores.

The specificity of this binding was determined using either HF-PS-BSA conjugates from other *B. cereus* strains and by measuring the ability of various HF-PSs

to inhibit binding of antibodies to *B. anthracis* HF-PS-BSA in an ELISA inhibition assay. These results are shown in Figures 2 and 3. In Figure 2, an ELISA experiment was done with microtiter wells coated with the BSA-conjugates of the HF-PSs from *B. anthracis* Pasteur, and *B. cereus* strains ATCC 14579, G9241, 03BB87, and 03BB102.
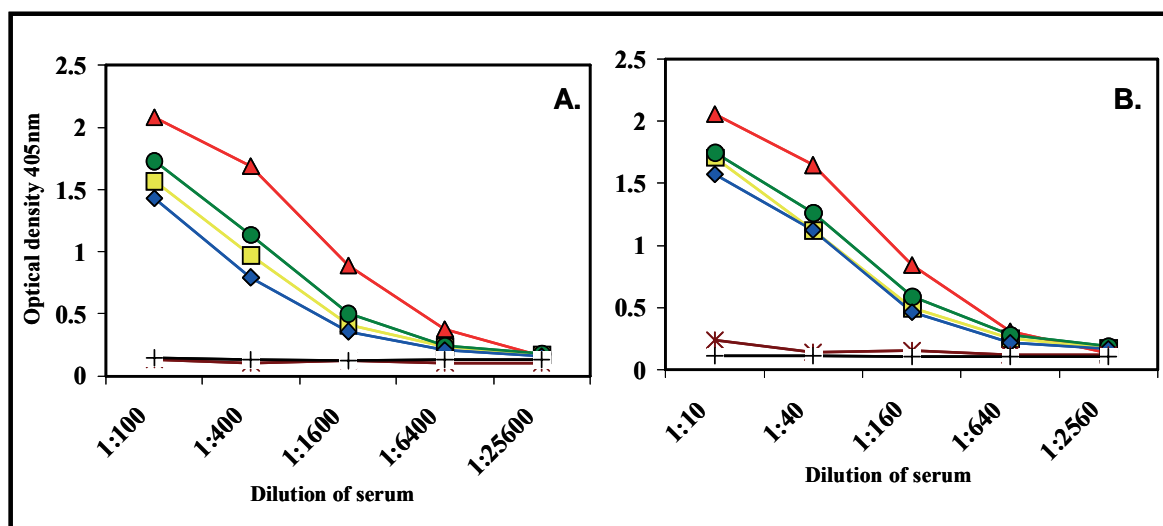
Figure 2. An ELISA experiment showing the binding of antiserum to (A.) live *B. anthracis* Sterne spores and (B.) killed *B. anthracis* spores to HF-PS-BSA conjugates from .*B. anthracis* (▲), *B. cereus* G9241 (■), *B. cereus* 03BB87 (●), *B. cereus* 03BB102 (◆), *B. cereus* ATCC 14579 (×), and BSA only (+).

As with Figure 1, antiserum to *B. anthracis* live or killed spores contained IgG antibodies that bound to the *B. anthracis* HF-PS-BSA conjugate. Binding was not observed to the *B. cereus* ATCC 14579 HF-PS-BSA conjugate. However, there were IgG antibodies that bound to the BSA conjugates of the HF-PSs from *B. cereus* strains G9241, 03BB87, and 03BB102; the strains which caused severe or fatal pneumonia. Figure 3 gives the results of ELISA inhibition assays which show that the HF-PSs from *B. anthracis* Pasteur, *B. anthracis* Ames inhibit binding of *B. anthracis* spore antiserum to the BSA conjugate of *B. anthracis* HF-PS.
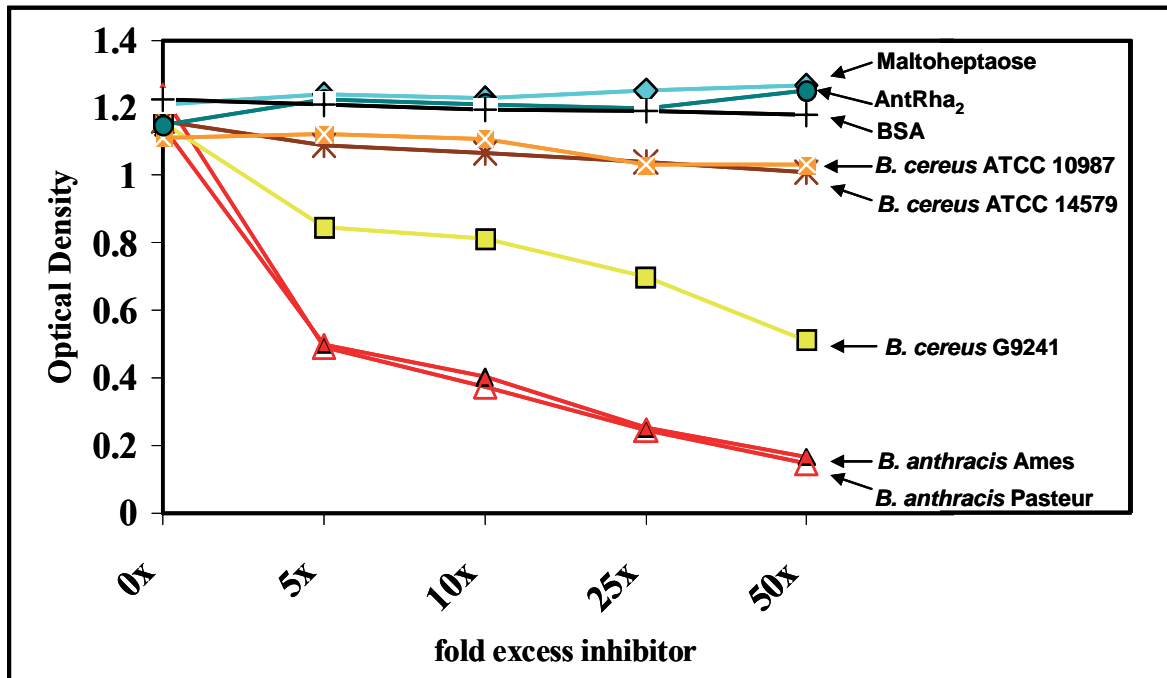
Figure 3. An ELISA inhibition assay showing the ability of various HF-PSs (as indicated) to inhibit the binding of *B. anthracis* spore antiserum to microtiter wells coated with the BSA-conjugate of *B. anthracis* Pasteur. As controls, inhibition by maltoheptaose, the chemically synthesized AntRha$_2$ trisaccharide component of the BclA oligosaccharide, and BSA.

However, the HF-PS from *B. cereus* ATCC 14579 and from the closely related *B. cereus* ATCC 10987 does not inhibit binding. The HF-PS from *B. cereus* G9241 is able to inhibit binding but less so than the *B. anthracis* HF-PSs. These results confirm those shown in Figure 2, and also show that *B. anthracis* spore antiserum does not contain antibodies that bind to the HF-PS from *B. cereus* ATCC 10987. No inhibition was observed when using the chemically synthesized AntRha$_2$ trisaccharide, BSA, or maltoheptaose.

As shown above, *B. anthracis* spore antiserum does not cross-react with the *B. cereus* ATCC 14579 HF-PS. It seemed of interest, therefore, to determine whether antiserum prepared against *B. cereus* ATCC 14579 spores would specifically react with its HF-PS. The results are shown in Figure 4.

Figure 4. **An ELISA experiment showing the binding of antiserum to *B. cereus* ATCC 14579 spores to HF-PS-BSA conjugates** from *B. anthracis* (▲) **,** *B. cereus* G9241 (▪), *B. cereus* 03BB87 (●), *B. cereus* 03BB102 (♦), *B. cereus* ATCC 14579 (×), and BSA only (+).
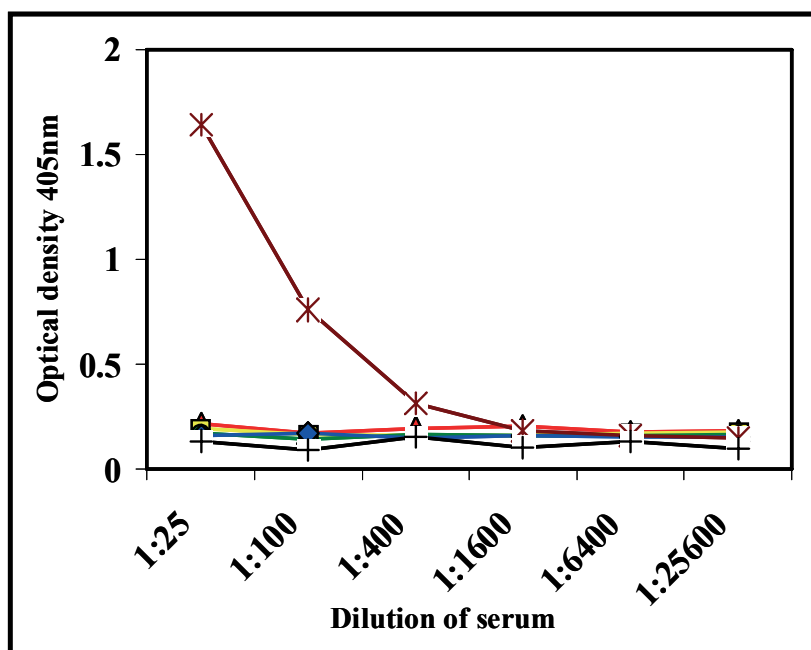
The *B. cereus* ATCC 14579 spore antiserum binds its own HF-PS-BSA conjugate but is unable to bind to the HF-PS-BSA conjugates from *B. anthracis*, or the *B. cereus* G9241, 03BB87, and 03BB102 strains.

The binding of antiserum from rabbits injected with B. anthracis *HF-PS-KLH conjugate to the HF-PS-BSA conjugates, HF-PSs, cell preparations, and cell walls from* B. anthracis *and* B. cereus *strains.*

Two rabbits were immunized with *B. anthracis* HF-PS-KLH conjugate and the presence of *B. anthracis* and *B. cereus* ATCC 14579 HF-PS-binding antibodies was measured using an ELISA assay. The results, Figure 5, show that equivalent binding was observed to both *B. anthracis* and *B. cereus* ATCC 14579 HF-PS-BSA conjugates.
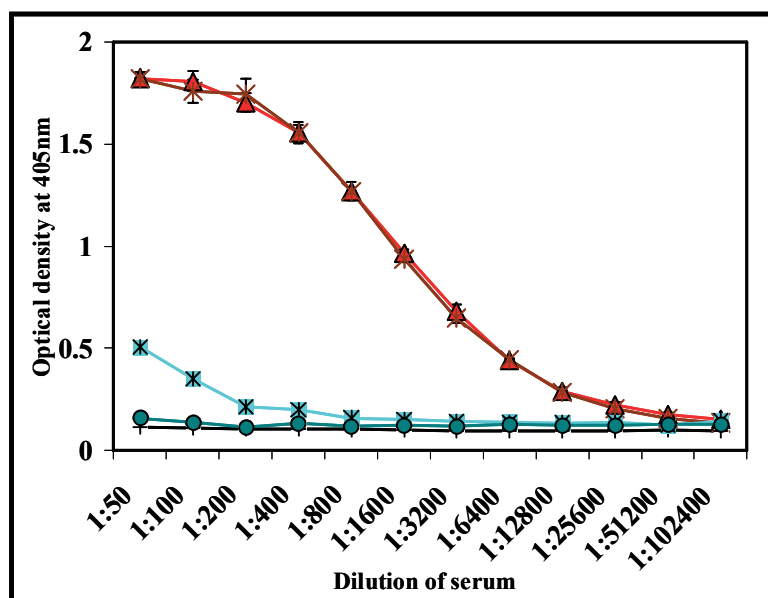
Figure 5. An ELISA experiment showing the binding of antiserum to *B. anthracis* HF-PS-KLH conjugate to the BSA conjugates of *B. anthracis* HF-PS (▲), *B. cereus* ATCC 14579 HF-PS (×), chemically synthesized AntRha$_2$ trisaccharide (●), maltoheptaose (◆) , and BSA only (+).

No binding was observed to wells coated with BSA alone or with the AntRha$_3$-BSA conjugate, and only slight binding was observed to wells coated with a maltoheptaose-BSA conjugate.

The ability of antiserum raised against the *B. anthracis* HF-PS-KLH conjugate to bind *B. cereus* and *B. anthracis* HF-PSs, HF-PS-BSA conjugates, cells and cell walls, and to *B. anthracis* spores was determined using an immuno dot-blot assay. The immuno dot-blot assay using nitrocellulose membrane was used instead of an ELISA assay since cell and cell wall preparations did not bind well to the microtiter plate wells. Panel A of Figure 6 shows that binding was observed to *B. anthracis* Sterne spores, and not to the AntRha$_3$ trisaccharide or to the maltoheptaose or BSA controls indicating that HF-PS structural epitopes are present on spores. The dilution of serum used was 1:1600; this dilution also showed no binding to the trisaccharide-BSA or maltoheptaose-BSA conjugates in the ELISA assay shown in Figure 5. This result is consistent with our observation that antiserum to killed *B. anthracis* spores contained antibodies that bind the *B. anthracis* HF-PS indicating that spore preparations contain HF-PS epitopes.
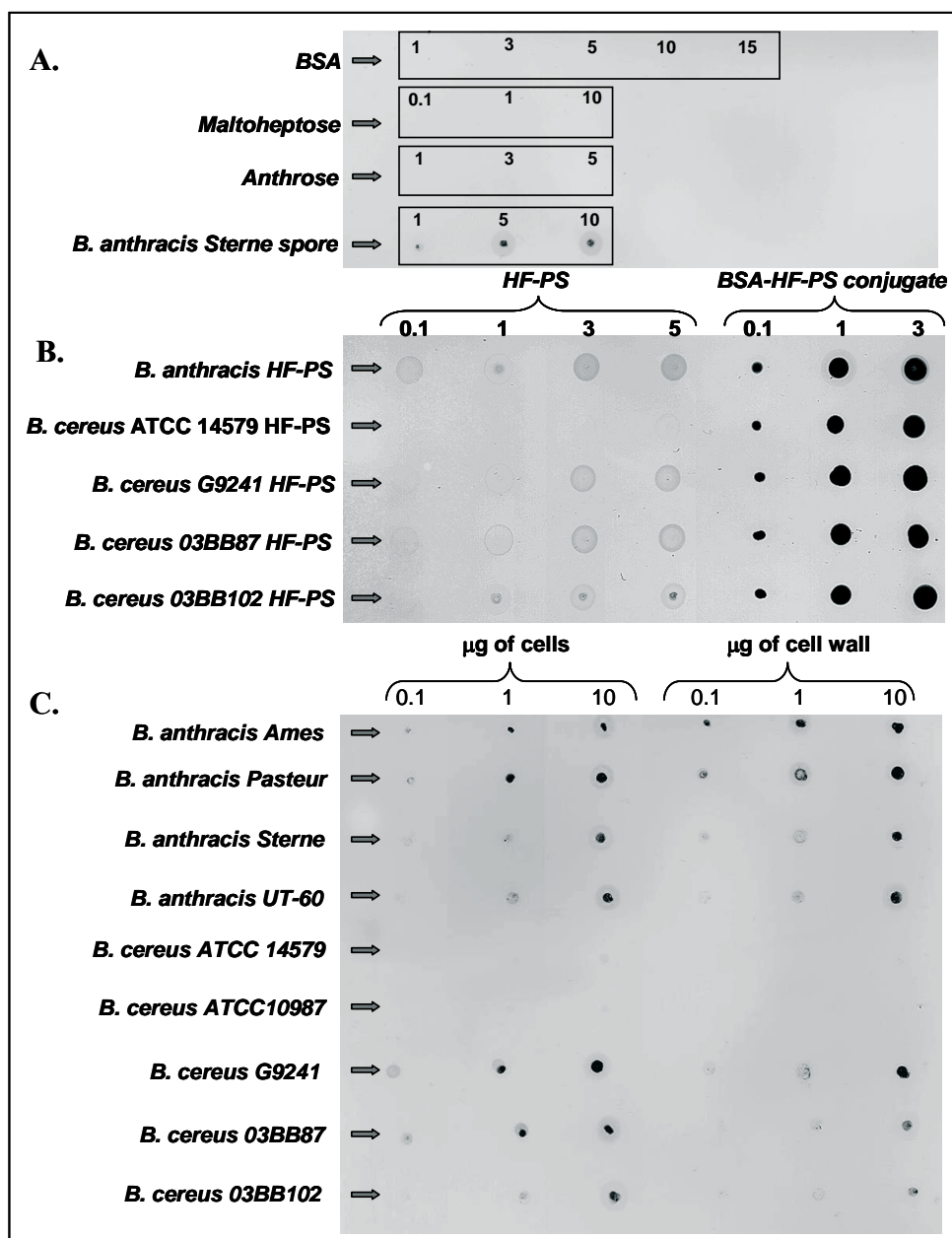
Figure 6. **An immuno dot-blot assay shown the binding of antiserum to *B. anthracis* HF-PS-KLH conjugate to**: (A.) BSA, maltoheptaose, chemically synthesized AntRha2 trisaccharide (labeld as Anthrose), and *B. anthracis* Sterne spores; (B.) unconjugated and BSA-conjugated HF-PS from the indicated *B. anthracis* and *B. cereus* strains; and (C.) cells and cell walls from the indicated *B. anthracis* and *B. cereus* strains.

Panel B of Figure 6 shows that the *B. anthracis* HF-PS-KLH antiserum binds to the isolated HF-PSs from *B. anthracis*, *B. cereus* G9241, *B. cereus* 03BB87, and *B. cereus* 03BB102, but not to the HF-PS from *B. cereus* ATCC 14579.

However, a different reactivity pattern was observed with the HF-PS-BSA conjugate antigens. The antiserum strongly bound the HF-PS-BSA conjugates from all strains, including that of *B. cereus* ATCC 14579. This result is consistent with the results described above for the ELISA experiment shown in Figure 5. The fact that there was no cross reactivity between the HF-PS from *B. cereus* ATCC 14579 and that of *B. anthracis*, but that there is significant cross-reactivity of their protein conjugates indicates that there is an common immunodominant structural epitope after, but not before conjugation. The cross-reactivity of the HF-PS from *B. anthracis* with the HF-PSs from *B. cereus* strains that caused fatal pneumonia indicates that the latter HF-PSs have closely related structural features with that of *B. anthracis*, a conclusion that is consistent with the similar glycosyl composition of these HF-PSs (22).

Panel C of Figure 4 shows that cells and cell walls of all *B. anthracis* strains used in this experiment as well as cells and cell walls from *B. cereus* strains G9241, 03BB87, and 03BB102 bind to the *B. anthracis* HF-PS-KLH antiserum while the cells and cell walls of *B. cereus* ATCC 14579 and ATCC 10987 do not bind this antiserum. The detection limit for the cell recognition was between 0.1 and 1.0 μg for the *B. anthraci*s cells, and 0.1 μg for the *B. anthracis* cell walls. The serum reactivity for cells and cell walls of *B. cereus* strains G9241 and 03BB87 was somewhat weaker compared to that of *B. anthracis*, and even weaker for strain *B. cereus* 03BB102 which required a minimum amount 1 μg cells or cell walls for detection. The inability of this antiserum to bind the cells and cell walls of *B. cereus* ATCC 14579 and *B. cereus* ATCC 10987 is consistent with our result showing that *B. anthracis* spore antiserum specifically binds the *B. anthracis* HF-PS but not the HF-PSs from these *B. cereus* strains. The cross-reactivity of the HF-PS from *B. anthracis* with the HF-PS from *B. cereus* strains that caused fatal pneumonia is intriguing and indicative of a common structural motif across these pathogenic bacilli, a conclusion that is consistent with the similar glycosyl compositions of these HF-PS (22). Whether this important observation extends to all pathogenic bacilli in this group remains to be explored.

Reactivity of Rhesus macaque anti-AVA and post-infection 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 inhalation infection with *B. anthracis* (C.P. Quinn, unpublished data).
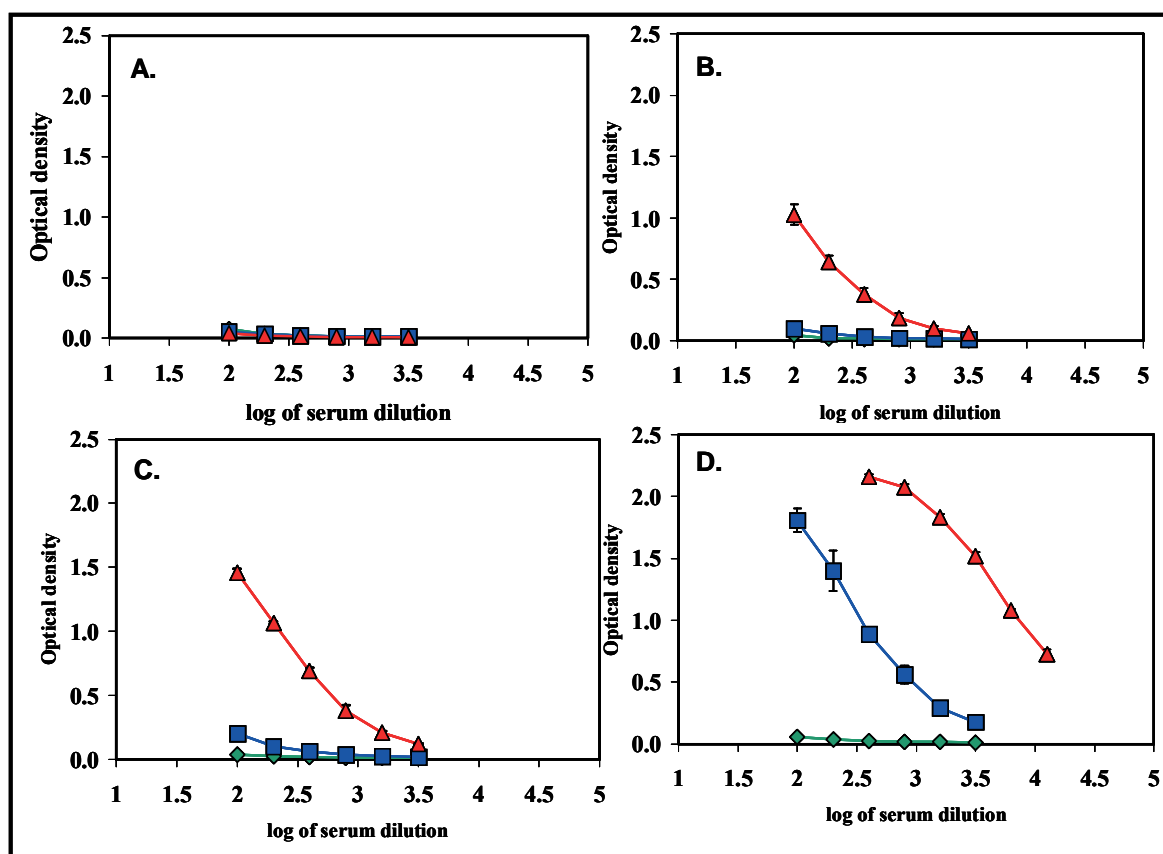
Figure 7. The binding of sera from Rhesus macaques to the *B. anthracis* HF-PS-KLH conjugates. (A.) Sera from two representative (as explained in the text) unvaccinated animals: Sera from the animal at time of arrival (♦), just prior to exposure to *B. anthracis* spores (■), and at 14 days after exposure to the spores (▲). (B.) Sera from two representative (as explained in the text) AVA-vaccinated animals: Sera from the animal at time of arrival (♦), just after the last AVA-vaccination (i.e. at 30 weeks after initial vaccination) and before exposure to spores (■), and 14 days after exposure to spores (▲).

Pre- and post-infection sera were evaluated by ELISA using the *B. anthracis* Pasteur HF-PS-KLH conjugate as the capture antigen. The results for serum from two non-vaccinated NHP that survived aerosol exposure to *B. anthracis* spores are shown in Figure 7A & 7B. The serum from a third unvaccinated animal was also tested and gave the same results as shown in Figure 7A. Sera from all three animals taken on the day of arrival as well as just before exposure to *B. anthracis* spores show no binding to the *B. anthracis* HF-PS-KLH coated microtiter plate. At 14 days after spore exposure, one of the three animals produced serum that bound the HF-PS-BSA conjugate. The results for AVA vaccinated animals are shown in Figure 7C & 7D. Five animals were evaluated and two of the five animals had the response shown in Figure 7C, while the remaining three animals gave the response shown in Figure 7D. Serum samples were taken at the time the animals arrived in the facility, at 30 weeks just after the completion of the AVA vaccination regime but before exposure to spores, and at 14 days after exposure to spores. The results show that none of the animals produced antibodies that bound the HF-PS-KLH conjugate at their time of arrival. Three out of five animals produced antibodies that showed binding to the *B. anthracis* HF-PS-KLH after AVA vaccination but before spore exposure (Figure 7D). All five animals produced antibodies with a strong binding capacity to *B. anthracis* HF-PS-KLH 14 days after exposure to spores

DISCUSSION

In this report, we have demonstrated that the major cell wall polysaccharides from *B. anthracis* and *B. cereus* strains are antigenic and also that they contain both specific and common structural epitopes.

Antiserum to *B. anthracis* or *B. cereus* ATCC 14579 spores specifically bound to the HF-PS-BSA conjugates of *B. anthracis* or *B. cereus* ATCC 14579, respectively. Further, *B. anthracis* spore antiserum did not contain antibodies that bound the HF-PS from the closely related *B. cereus* ATCC 10987. The lack of binding to the HF-PSs from *B. cereus* strains ATCC 10987 and ATCC 14579 is consistent with the fact that, while these HF-PSs share a common general structural theme with each other and with *B. anthracis* HF-PS, they have significant structural differences (7) (Leoff *et al.*, submitted). Thus, the vegetative cell wall HF-PS for *B. anthracis* and for *B. cereus* ATCC 14579 is an immunogenic antigen of spore preparations and has species-specific structural features that are recognized by the host's immune system. These structural features are present even in killed spore preparations since antiserum to killed spores also contains HF-PS antibodies, which supports the suggestion that the HF-PS could prove to be a vaccine antigen that may be effective against both spores and vegetative cells.

An exception to the specificity of the HF-PS immunochemical reactivity was that *B. anthracis* spore antiserum also bound, to some extent, the HF-PS-BSA conjugates of *B. cereus* strains G9241, 03BB87, and 03BB102, which are strains isolated from cases of severe or fatal pneumonia (1, 16, 17). The glycosyl compositions of the HF-PS from these *B. cereus* strains very closely match that of the *B. anthracis* HF-PS (20). This suggests the possibility that there may be a correlation between the structure of the HF-PS and the ability of these strains and *B. anthracis* to cause severe illness. At this time, however, only indirect evidence is available for the involvement of HF-PS in the infection process. It has been reported that an internalin-like protein is expressed during the *B. cereus* infection of insects (11). This internalin-like protein is a candidate virulence protein that contains a SLH carbohydrate-binding domain at its C-terminal end that presumably anchors the protein via HF-PS to the peptidoglycan. However, a direct connection between the HF-PS with regard to the virulence of *B.*

anthracis or the pathogenic *B. cereus* strains has not yet been established. Therefore, it is not known if the HF-PS has a direct role with regard to the virulence of *B. anthracis* or these *B. cereus* strains. In addition, *B. cereus* G9241, 03BB87, and 03BB102 each harbor *B. anthracis* virulence genes in pXO1-like plasmids (16, 17). The presence of the pXO1 plasmid almost certainly contributes to the virulence of these strains.

Recently, there have been reports about the isolation of "*B. anthracis*-like" strains from chimpanzees and one gorilla at the Ivory Coast and Cameroon. Pathological and histological examinations determined anthrax as the cause of death (20, 21). Genetic analysis including specific amplification and sequencing of 16S RNA and RT-PCR revealed that these strains fell outside the *B. anthracis* cluster of strains as determined by various molecular analyses such as MLST; in fact, they clustered with *B. cereus* and *B. thuringiensis* strains (19, 20). However, as with the *B cereus* G9241, 03BB87 and 03BB102 strains, these strains contained the *B. anthracis* pXO1 plasmid, and additionally, the pXO2 plasmid. Plasmid transfer within the bacilli group is not unusual has been previously reported (2, 16). This could be a possible explanation of how these strains obtained the *B. anthracis* virulence plasmids. It seems likely, that the acquisition of pXO1 and pXO2 by these strains has affected their virulence. It may be that the HF-PS structure that we observe in *B. anthracis* and the possibly very similar structures of the *B. cereus* G9241, 03BB87, and 03BB102 HF-PSs are necessary for virulence and/or are a characteristic of strains that have the ability to acquire these *B. anthracis* virulence plasmids. Structural determination of the HF-PSs from *B. cereus* G9241, 03BB87 and 03BB102 will soon be completed and described in a subsequent manuscript; however, preliminary results verify that each of these HF-PSs has a structure that is very similar, but not identical, to that of the *B. anthracis* HF-PS. In addition, *B. anthracis* Sterne 34F$_2$ mutants that are defective in putative genes required for the synthesis of the HF-PS will be prepared and the effect of these mutations on the structure/presence of the HF-PS, on bacterial viability, and virulence will be determined. The fact that these *B. cereus* strains and *B. anthracis* have similar HF-PSs which have cross-reactive structural epitopes suggests the possibility that a broad spectrum vaccine antigen could be developed based on a shared structural region of the HF-PS.

An interesting observation was that rabbit antiserum generated against the *B. anthracis* HF-PS-KLH conjugate recognized, not only the *B. anthracis* HF-PS-BSA conjugate as expected, but equally well the HF-PS-BSA conjugates from all *B. anthracis* and *B. cereus* strains, including the type strain ATCC 14579. This indicated that all of these HF-PS-protein conjugates contain a common cross-reactive epitope. However, when the HF-PS-KLH antiserum was tested against unconjugated HF-PS, cell and cell wall preparations, it was specific for *B. anthracis* preparations and bound to those preparations from *B. cereus* strains G9241, 03BB87, and 03BB102, but did not bind *B. cereus* ATCC 10987 or *B. cereus* ATCC 14579 preparations. This result, as with those obtained with spore antiserum, showed that the HF-PS contains structural epitopes that are specific to the HF-PSs of *B. anthracis* and to the *B. cereu*s strains that caused severe or fatal pneumonia.

The combination of the non-specific binding of the HF-PS-KLH antibodies to the HF-PS-BSA conjugates and the specific binding to unconjugated HF-PSs, cells and cell walls suggest that, even though the HF-PS structures vary, the HF-PS-protein conjugates of *B. anthracis* and *B. cereus* strains ATCC 14579 and ATCC 10987*,* but not the unconjugated HF-PSs, have a common immunodominant structural epitope. This indicates that there may be a common structural feature in these different HF-PSs that, when conjugated to the protein, becomes an immunodominant epitope. At this point, the identity of this common structural feature is not known. However, structural work suggests that the HF-PSs from *B. anthracis* and all of the *B. cereus* strains examined have a consensus repeating unit structure consisting of –HexNAc-HexNAc-HexNAc-trisaccharide in which two of the three HexNAc residues are GlcNAc and ManNAc with the third residue being either GlcNAc or GalNAc, and that this trisaccharide backbone is substituted with Gal or Glc residues or non-carbohydrate substitutents such as acetyl groups (Leoff, *et al.*,submitted). It may be that conjugation to the proteins involves a ManNAc-GlcNAc- common structural motif of the HF-PS that, as conjugated to the protein, becomes an immunodominant epitope.

At this point, the identity of this common epitope is not known. However, structural work suggests that the HF-PSs from *B. anthracis* and all of the *B. cereus* strains examined have a consensus repeating unit consisting of –HexNAc-HexNAc-HexNAc- trisaccharide in which two of the three HexNAc residues are GlcNAc and

ManNAc with the third residue being either GlcNAc or GalNAc; whereas, this trisaccharide backbone is substituted with Gal or Glc residues or non-carbohydrate substitutents such as acetyl substituents (Leoff, *et al.*, submitted). It may be that conjugation to the proteins involves a ManNAc-GlcNAc- common structural motif of the HF-PS that, as conjugated to the protein, becomes an immunodominant epitope of the conjugate antigens.

Another possible common epitope of these HF-PSs may result from a highly conserved linkage group of these HF-PSs to the peptidoglycan (PG), e.g. if the HF-PSs of all of these *B. anthracis* and *B. cereus* strains were attached to the peptidoglycan (PG) via the same glycosyl-phosphate bridge, e.g. HF-PS-HexNAc-P-PG. In the cell wall, such a common –HexNAc-P-PG region in each of the polysaccharides (PS) would be in the innermost portion of the cell wall and not directly accessible to the immune system while the structurally variable portion of the polysaccharide is more external, less hidden, and, therefore, more prone to early recognition by the immune system. However, when the PSs are released by HF cleavage of the phosphate bridge, the common structural region that was linked to the PG is released and separated together with the HF-PS from the rest of the cell wall. Conjugation to the protein may in this case expose this very region which would explain a new immunodominant epitope compared to intact cells and cell walls. 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 phosphate) however, there is evidence that certain bacilli cell wall teichoic acid polymers are linked to the peptidoglycan through a common –ManNAc-GlcNAc-P-PG linkage (3, 13, 14). Investigation into the existence and structures of the PG linkage region of the *B. anthracis* and *B. cereus* HF-PSs is underway.

Finally, because the HF-PS was immunogenic, we tested the sera of *Rhesus macaques* that had been exposed to an aerosol of *B. anthracis* spores to determine if these sera had antibodies against the HF-PS. In fact, our findings confirmed that these sera contain IgG antibodies that bind the *B. anthracis* HF-PS. Our data also showed that AVA vaccinated animals contain low levels of HF-PS antibodies which increase to high antibody levels after exposure to spores. These results support the potential use of the HF-PS-conjugates to detect exposure of primates to *B. anthracis*, and for use as an

alternative antigen component for the development of a new vaccine or to improve the current vaccine.

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RESUME AND CONCLUSIONS

Differences in cell wall glycosyl (CWG) composition correlate with phylogenetic relatedness and, possibly, with pathogenic functions:

Up to now, a systematic comparison of cell wall carbohydrates in the *B. cereus* group of bacteria has not been done. Therefore, one focus of this thesis was to study the cell wall carbohydrates in a number of *B. cereus* group strains of characterized phylogenetic relatedness. We analyzed the glycosyl compositions of cell walls in a set of *B. cereus* group strains as well as the composition of a purified polysaccharide that was released from these cell walls by hydrofluoric acid treatment (hereafter called HF-PS). The cell walls of the investigated *B. anthracis* strains all had qualitatively the same glycosyl composition of Glc, Gal, ManNAc, and GlcNAc. The HF-PSs isolated from these cell walls were composed of Gal: GlcNAc: ManNAc in a 3:2:1 ratio. The composition was the same for all *B. anthracis* strains investigated (*B. anthracis* Ames, Sterne and Pasteur) indicating that it may be specific for *B. anthracis*. The glycosyl compositions of the cell walls and the HF-PSs from the *B. cereus* group strains showed qualitative and quantitative variations. However, strains of the same group and lineage had qualitatively similar cell wall and HF-PS compositions. With one exception, they were distinctly different from the cell walls and the HF-PSs of the *B. anthracis* strains. There was a noticeable similarity (but not identical) between the glycosyl compositions from the cell walls from *B. anthracis* strains and three *B. cereus* isolates that caused severe pneumonia in humans which lead, in some cases, to the patients death (1, 13). Analysis of the HF-PSs from these strains showed them to be comprised of the same glycosyl residues in approximately similar relative amounts as observed in *B. anthracis* HF-PS.

We also noticed an influence of the plasmid content of the *B. anthracis* strains on their CWG composition. The absence of plasmid pXO2 seems to have an impact on the quantity of the glycosyl residues (e.g. increase in Glc and decrease in Man).

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However, this observation is preliminary and needs to be verified in more *B. anthracis* strains that should include near-isogenic strain derivatives that have been cured differentially of one or both virulence plasmids, pXO1 and pXO2.

Interestingly, genetic analyses of the *B. cereus* isolates causing severe pneumonia, i.e. strains G9241, 03BB87 and 03BB102, revealed large numbers of genes with high similarities to genes of the *B. anthracis* virulence plasmids, e.g. the anthrax toxin genes and others (1, 12). In fact, the strains *B. cereus* G9241 and *B. cereus* 03BB87 carried almost the entire *B. anthracis* pXO1 plasmid (12). The striking similarity of cell wall and HF-PS glycosyl compositions in virulent *B. cereus* group strains (i.e. *B. anthracis* and the pneumonia causing *B. cereus* strains) taken together with the presence of virulence plasmid genes in all these strains raises the question, whether there is a correlation between genetic inventory (i.e. the plasmid genes) and the presence of certain HF-PS structures and whether the HF-PSs play a role in plasmid DNA distribution and spread. (**Chapter 2**/(23))

Our intention to use carbohydrate profiling for *Bacillus* strain characterization was not the first attempt of this kind. An older report investigated *B. anthracis* and *B. cereus* strains for their carbohydrates in intact cells and spores (8). However, the conclusions drawn in this paper could not be viewed in a systematic matter because the strains used in that investigation were of unclear phylogenetic relatedness. Recently, a previously unknown glycoprotein on *B. anthracis* spores is getting much attention for its potential use in strain and spore characterization as well as a vaccine component. In fact, an oligosaccharide (named anthrose) has been characterized that was thought to be expressed specifically on the surface of *B. anthracis* spores (3). Very recent evidence, however, showed through mutation of putative genes that encode anthrose biosynthetic enzymes that the biosynthesis of this carbohydrate is not limited to *B. anthracis* (5)

In conclusion, our comparison of cell wall and HF-PS compositions of strains from the *B. cereus* group suggests a correlation between the glycosyl profiles and the phylogenetic relatedness of the investigated strains. Based on the detection of strain-specific glycosyl profiles, we propose that the cell wall carbohydrate profiles could be useful for the taxonomic classification of strains from the *B. cereus* group as well as for diagnostic strain identification. These findings corroborate an earlier report on cell carbohydrate profiling in *B. anthracis* and *B. cereus* strains of unclear relatedness (8) In

particular, the observed correlations between the HF-PS compositional similarities in pathogenic *B. cereus* group members and the presence of toxin genes or virulence plasmids known from *B. anthracis* may provide fast and simple screening methods as part of the emergency response to anthrax and anthrax-like infections e.g. by using DNA-DNA hybridization methods (28) or monoclonal antibodies in the recognition of HF-PS as indicator for pathogenic bacilli.

The non-classical secondary cell wall polysaccharide of B. cereus *group members*

We determined the molecular structure of the HF-PS from *B. anthracis* (Clade 1/lineage Anthracis) strains and compared it with the HF-PSs isolated from the non-pathogenic strains *B. cereus* ATCC 10987 (Clade 1/lineage Cereus I) and *B. cereus* ATCC 14579 (Clade 2/lineage Tolworthi), and the pathogenic strains *B. cereus* G9241, *B. cereus* 03BB87 (Clade 1/lineage Cereus IV) and *B. cereus* 03BB102 (Clade 1/lineage Cereus III). The detailed structural analysis using glycosyl composition and linkage analyses, matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS), and 1- and 2-D nuclear magnetic resonance (NMR) spectroscopy showed that the HF-PS from *B. anthracis* is comprised of hexasaccharide repeating units with the following structure (**Figure 1**)
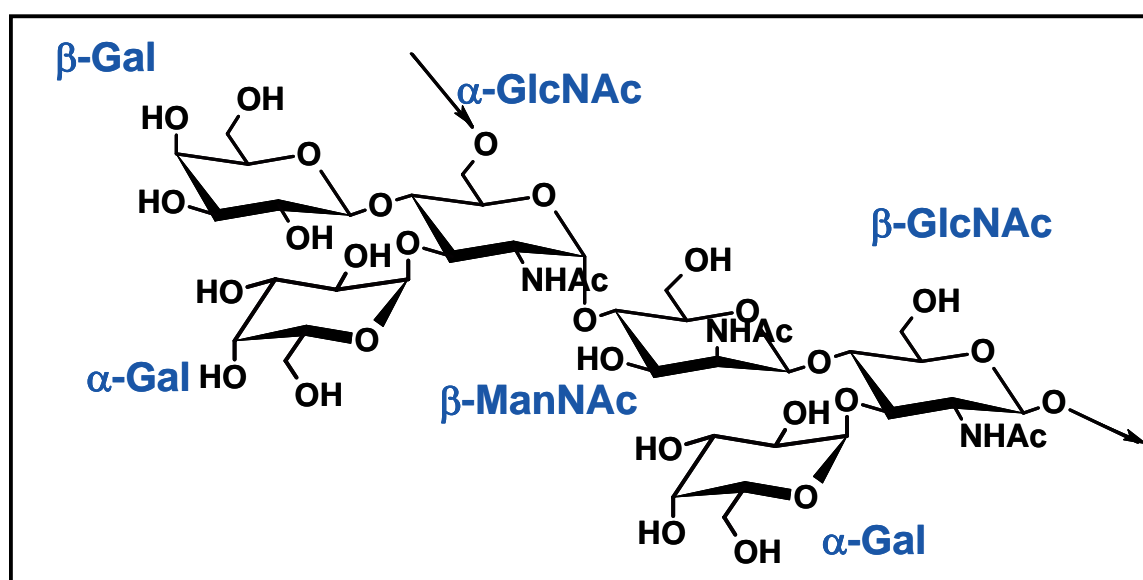
Figure 1: Structure of *B. anthracis* HF-PS repeating hexasaccharide. The arrows indicate the positions where additional units are attached in the complete polysaccharide

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structure. Gal – galactose, GlcNAc – *N*-acetylglucosamine, ManNAc – *N*-acetylmannosamine

This structure was the same for the HF-PS from *B. anthracis* Ames, *B. anthracis* Pasteur, *B. anthracis* Sterne or *B. anthracis* UT-60, corroborating that it is species-specific. Based on mass spectrometric data we detected molecular microheterogeneity in the polysaccharide due to a variation in substitution of one or more of the GlcNAc residues by Gal residues. We did not find any evidence that the minor amounts of Glc detected in the glycosyl composition analysis of the HF-PS preparation was part of the HF-PS structure. (**Chapter 3/(2)**).

In the structural analysis of the HF-PS isolated from cell walls of *B. cereus* ATCC 10987 we employed the same extensive range of analytical methods; this structure is shown in **Figure 2**.
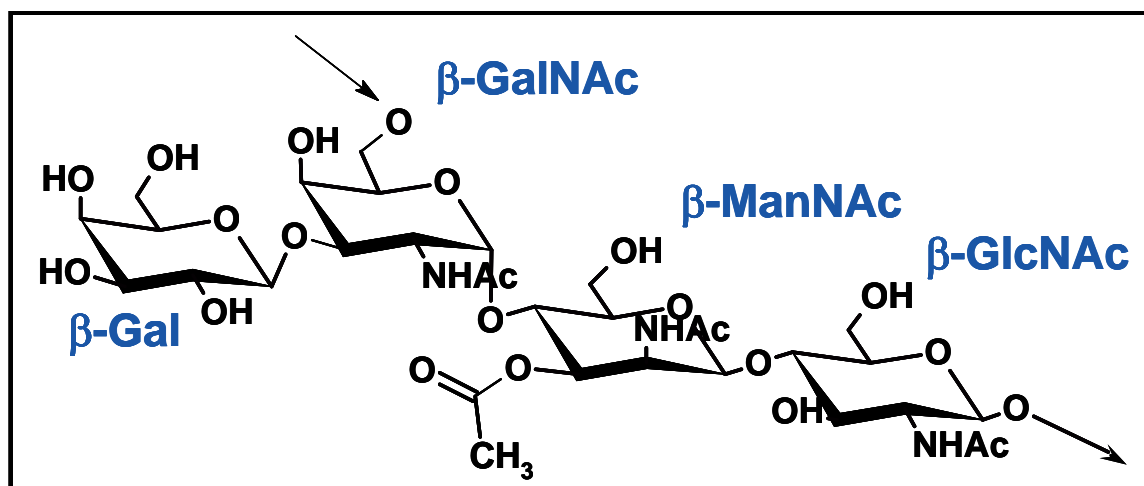
Figure 2: Structure of *B. cereus* ATCC 10987 HF-PS repeating tetrasaccharide. The arrows indicate the positions where additional units are attached in the complete polysaccharide structure. Gal – galactose, GalNAc – *N*-acetylgalactosamine, GlcNAc – *N*-acetylglucosamine, ManNAc – *N*-acetylmannosamine

The back bone of the HF-PS in this strain is formed of GalNAc, GlcNAc, and ManNAc. As observed for the HF-PS from *B. anthracis,* the HF-PS from *B. cereus* ATCC 10987 showed a degree of heterogeneity as well. This heterogeneity was due to variation in the number of repeating units (e.g., structures with di-, and, possibly, mono- and tri-repeat unit), and the non-stoichiometry amounts of O-acetyl groups in the

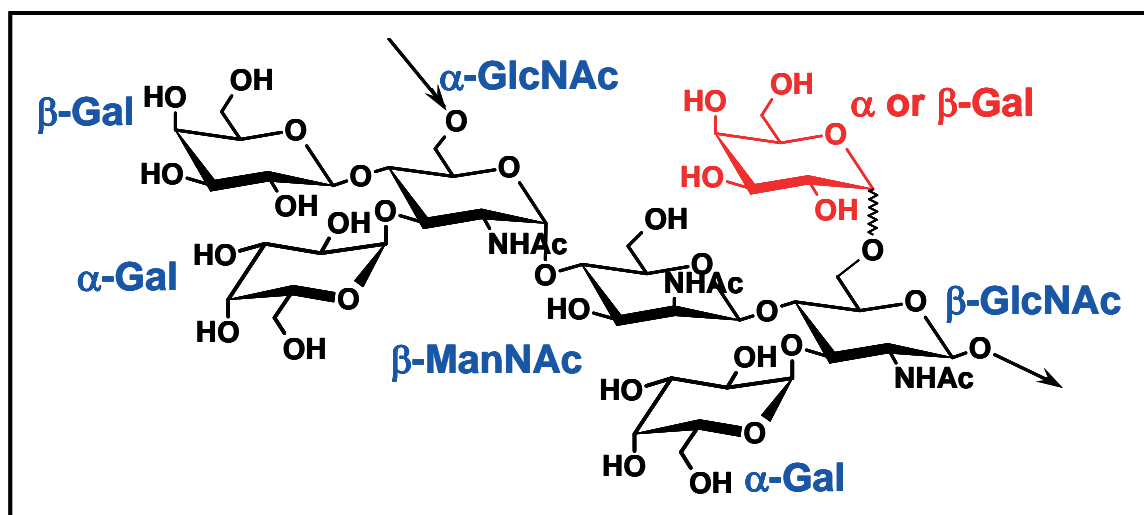polysaccharide structure. The purified HF-PS sample contained a relatively small amount of Glc compared to the other glycosyl residues. As already mentioned for the HF-PS of *B. anthracis*, we did not find any evidence that would support that Glc as part of the HF-PS structure. It is possible that there is a Glc-containing oligosaccharide present which is also linked to the cell wall via phosphate and was co-released with the HF-PS. However, as repeatedly only low amounts of glucose were detected in cultures of *B. cereus* ATCC 10987, we have to assume that the Glc-containing component constitutes a much smaller fraction of the cell wall than the HF-PS. (**Chapter 4/(21)**).

In order to further consolidate one of our main findings, i.e. that the HF-PSs are species-specific, we also analyzed the HF-PS from *B. cereus* ATCC 14579, the type strain of that species, and compared it with the HF-PS from *B. anthracis* and other *B. cereus* strains. Mass spectrometric data from the HF-PS of *B. cereus* ATCC 14579 showed very similar ion patterns to the mass spectrometric data for the HF-PS from *B. cereus* ATCC 10987. On the basis of our findings to date, the *B. cereus* ATCC 14579 HF-PS is composed of one, two, and three HexNAc$_3$Hex tetrasaccharide repeat units as observed for the *B. cereus* ATCC 10987 HF-PS, but with an altered composition. The HF-PS from the type strain is composed of Glc, ManNAc, GlcNAc and GalNAc instead of Gal, ManNAc, GlcNAc and GalNAc, as is the case for strain *B. cereus* ATCC 10987. Glucose was present in the type strain in large amounts and is therefore assumed to be part of the HF-PS. Another difference compared to the HF-PS of *B. cereus* ATCC 10987 was that we did not find any evidence that the *B. cereus* ATCC 14579 HF-PS is acetylated (**Chapter 4**). The detailed structural analysis of the HF-PS from *B. cereus* ATCC 14579 posed to be more challenging. The treatment with HF resulted in the release of multiple components from the cell wall as determined by mass spectrometry and NMR and made the identification of residues belonging to the HF-PS complicated. Further separation of the HF released components is necessary and is currently in progress.

So far, the evaluation of structural data from the severe pneumonia causing *B. cereus* strains, i.e. *B. cereus* G9241, *B. cereus* 03BB87 and *B. cereus* 03BB102, confirmed a much closer structural relatedness of their HF-PSs with that of *B. anthracis* than with that of other *B. cereus* strains. These observations were not unexpected considering the previously observed similarities of the HF-PS glycosyl compositions.

The HF-PSs of all three pathogenic *B. cereus* strains have the same amino-sugar backbone [→6)-α-D-GlcNAc-(1→4)-β-D-ManNAc-(1→4)-β-D-GlcNAc-(1→] as found in the *B. anthracis* HF-PS (see **Figure 1**). The 2D-NMR and mass spectrometric data along with composition and linkage analysis of constituent sugar residues showed only that the HF-PSs from the pathogenic *B. cereus* strains were slightly altered in structure compared to the *B anthracis* HF-PS. These HF-PSs have more galactosyl residues attached to the amino sugar back-bone at variable positions. For example, the HF-PS from *B. cereus* G9241 differs from the HF-PS of *B. anthracis* by only one additional Gal residue attached to the O6 position of the β-GlcNAc residue as shown in **Figure 3** (**Chapter 5**).

Figure 3: Structure of *B. cereus G9241* HF-PS repeating hexasaccharide. The arrows indicate the positions where additional units are attached in the complete polysaccharide structure. Gal – galactose, GlcNAc – *N*-acetylglucosamine, ManNAc – *N*-acetylmannosamine, the additional Gal residue that differentiates this structure from the *B. anthracis* HF-PS is shown in red. It is at the moment still unclear whether this residue is attached in the alpha or beta conformation (wavy line).

A comparison of the 2D-NMR data from our structure analysis of the HF-PS from *B. cereus* G9241 and *B. cereus* 03BB87 (two strains that were genetically indistinguishable (12) showed only minor differences from each other. At this point it is unclear whether these are actual strain-specific structural differences, or are simply

minor variations that would normally occur in different cultures, or perhaps during sample preparation for NMR analysis.

As already mentioned earlier, genetic characterization of these isolates showed that all three pathogenic *B. cereus* strains are closely related to *B. anthracis. T*he strains *B. cereus* G9241 and 03BB87 were found to harbor most of the *B. anthracis* pXO1 virulence plasmid,(12, 13), while the strain *B. cereus* 03BB102 only harbors most of the genes found in this plasmid's pathogenicity island. (12). However in this strain, Hoffmaster *et al*. additionally reported the finding of capsule biosynthesis genes *capA capC* and *capB* which are, in *B. anthracis*, located on the virulence plasmid pXO2 (12). The MLST analysis of strains *B. cereus* G9241 and *B. cereus* 03BB87 showed these strains to be genetically virtually identical. Hence, both strains were classified as serotype 78 (ST-78); whereas, *B. cereus* 03BB102 was classified as ST-11 (www.mlst.net). Their ability to cause anthrax-like symptoms correlates with the possession of *B. anthracis* toxin genes as well as with a close resemblance of their HF-PS structures with that of the *B. anthracis* HF-PS. This striking correlation could potentially be taken as an indication that either the HF-PSs play a direct and specific role in the pathogenicity of these *B. cereus* strains, e.g. in cell-cell attachment, or they play a more indirect role as would be the case if the have a function in bacterial conjugation and plasmid DNA exchange.

Recent reports about the isolation of *B. anthracis*-like bacteria from wild great apes at the Côte d'Ivoire and in Cameroon could be helpful in answering this question. The strains were isolated from four chimpanzees and one gorilla that evidently died from anthrax-like disease (19, 20). PCR and southern blot analysis gave results similar to those observed for the pathogenic *B. cereus* strains; that they contained virulence plasmids with similar gene content to *B. anthracis* pXO1 and pXO2. The bacterial isolates were subjected to MLST analysis and classified as ST-1 and ST-2, the same sequence type as 'classic' *B. anthracis* strains (16). The structural analysis of the HF-PS from these strains will clarify whether or not there is a functional connection of the HF-PS structure with pathogenicity, the presence of virulence plasmids, and the anthrax-like symptoms caused by these strains.

The immunochemical properties of the HF-PS structure

In order to investigate the antigenic properties of the HF-PS structures, we immunized rabbits with live or irradiated spores. Spores are of interest since *B. anthracis* enters the host in its spore form. After phagocytosis, the spores reach the mediastinal lymph nodes, where they respond to a still unknown signal and germinate into metabolically active vegetative bacterial forms (4, 10, 11). The bacteria, then producing large amounts of toxins, enter the systemic circulation and induce sepsis, edema, hemorrhage, necrosis, and septic shock (33). From an immunization of rabbits with live spores we would expect antibodies against spore components as well as against vegetative cell components. However, if the spores are killed through irradiation prior to the immunization of the rabbit, they loose the ability to germinate and, therefore, the sera derived from the administration of irradiated spores would be expected to contain antibodies only against spore components.

In our investigation we determined, therefore, whether antiserum from rabbits immunized with live or irradiated spores from *B. anthracis* Sterne contained antibodies that recognize the HF-PSs isolated from *B. anthracis* and the HF-PS isolated from a range of other *B. cereus* strains. The possibility of cross reactivity was addressed by generating antiserum against *B. cereus* ATCC 14579 live spores. Our experiments showed that the immunization of rabbits with live or irradiated spores from *B. anthracis* Sterne induced the production of antibodies that recognize HF-PS from *B. anthracis* and the HF-PS from the *B. cereus* strains that caused fatal pneumonia; but these spore-derived antisera did not react with the HF-PS from closely related *B. cereus* ATCC 10987 or *B. cereus* ATCC 14579. The *B. cereus* ATCC 14579 anti-spore antiserum in our cross reactivity test exclusively reacted with the HF-PS-BSA conjugate from *B. cereus* ATCC 14579 and not with the HF-PS conjugates derived from *B. anthracis* or *B. cereus* G9241, 03BB87, or 03BB102 strains (**Chapter 6**/(22)). The lack of binding from the *B. anthracis* anti-spore antisera to the *B. cereus* ATCC 14579 HF-PS or the *B. cereus* ATCC 10987 HF-PS seems consistent with the fact that the HF-PSs from these strains, while they share a common HexNAc-HexNAc-HexNAc trisaccharide backbone with each other, have additional significant structural differences that likely explain the differential reactivity of the sera (**Chapter 3**/(2) and **Chapter 4**/(21)**).

The conclusions drawn from these observations are: (i) the HF-PS fragment attached to the bacterial cell wall is recognized by the immune system of the host and is, therefore *per definitionem*, an antigen (www.nlm.nig.gov/mesh); (ii) The antisera raised against spores contained antibodies that were specific for cell wall attached HF-PS fragments of pathogenic *B. anthracis* and *B. cereus* strains, but did not contain antibodies for the HF-PSs from closely related, but non-pathogenic *B. cereus* strains. This suggests that besides phylogenetic relatedness, pathogenic functions may also play an important role in HF-PS structural conservation; (iii) The observed specific antibody recognition of HF-PSs from pathogenic *B. anthracis* and *B. cereus* strains suggests that the cell wall attached HF-PS fragments hold some promise for the development of improved vaccines and diagnostics.

The next question was whether a serum raised against a HF-PS protein conjugate would be specific to *B. anthracis* HF-PS or would cross-react with any of the HF-PS structures isolated from the different *B. cereus* group bacteria. For that purpose, rabbits were immunized with a KLH-conjugated HF-PS from *B. anthracis*. This *B. anthracis* HF-PS-KLH antiserum was unspecific and recognized not only the *B. anthracis* HF-PS-BSA conjugate as expected, but recognized equally well the HF-PS-BSA conjugates from all *B. cereus* strains, including the type strain ATCC 14579. However, when incubated with unconjugated HF-PSs the same antiserum recognized the HF-PS from *B. anthracis*, *B. cereus* G9241, *B. cereus* 03BB87, and *B. cereus* 03BB102, but did not recognize the HF-PS from *B. cereus* ATCC 14579, indicating a potentially specific reactivity towards HF-PSs from pathogenic strains. Moreover, the antiserum still reacted specifically to cells or cell walls from all investigated *B. anthracis* strains as well as to cells and cell walls from the pathogenic *B. cereus* strains (i.e. *B. cereus* G9241, 03BB87, and 03BB102). Of particular note was that the *B. anthracis* HF-PS-KLH antiserum did not recognize the cells and cell walls of *B. cereus* ATCC 14579 or *B. cereus* ATCC 10987. The antiserum also reacted with a purified spore preparation of strain *B. anthracis* Sterne.

Three main conclusions were drawn from these immunochemical results. **Conclusion 1**: Even though the HF-PS structures from the different bacilli vary, the HF-PS-protein conjugates of strains *B. anthracis, B. cereus* ATCC 14579, and ATCC 10987 contain at least one common epitope in their antigenic structure. The epitope of the

conjugated HF-PS has immunodominant properties. Surprisingly this epitope was not found in cell and cell wall preparations of these strains, as the antiserum derived from the *B. anthracis* HF-PS-KLH conjugate did not extensively cross-react with cells or cell walls from *B. cereus* ATCC 14579 and *B. cereus* ATCC 10987. The *B. anthracis* HF-PS-KLH antiserum was however specific for cells, cell walls and isolated HF-PSs from *B. anthracis* and for the same components from those *B. cereus* strains that had structurally related HF-PSs to the HF-PS from *B. anthracis* (i.e. the pathogenic *B. cereus* strains).

These findings indicate that the HF-PS conjugates from these strains may have a common structural feature that becomes an immunodominant epitope only when conjugated to the protein. The common epitope may be part of the HexNAc-HexNAc-HexNAc trisaccharide backbone as mentioned earlier. Another possibility would be a highly conserved linkage group common to all HF-PS structures, i.e. if all the HF-PSs from *B. anthracis* and the different *B. cereus* strains were attached to the peptidoglycan (PG) via the same glycosyl-phosphate bridge, e.g. HF-PS-HexNAc-P-PG. Such a common region would be in the innermost portion of the cell wall and not directly accessible to the immune system while the structurally variable portion of the polysaccharide is more external, less hidden, and, therefore, more prone to early recognition by the immune system. However, this common linkage group could be made accessible to the immune system when the HF-PS is released through breakage of the phosphate bridge by HF treatment (this would separate the common structural region that was linked to the PG together with the HF-PS from the rest of the cell wall) and subsequently conjugated to the KLH carrier protein. .

Conclusion 2: The similarities in sera reactivity towards pathogenic *Bacillus* strains further corroborated the correlation between the structure of the HF-PS and the ability of the pathogenic *B. cereus* strains and *B. anthracis* to cause severe illnesses. At this point, it is not known if the HF-PS has a direct role with regard to the virulence of *B. anthracis* or the pathogenic *B. cereus* strains. Genetic analysis of likely biosynthetic genes is currently underway and should help to clarify that aspect. Nevertheless, the fact that all pathogenic *Bacillus* strains investigated have similar HF-PSs with cross-reactive structural epitopes makes these HF-PSs potent candidates for the development of a broad spectrum vaccine based on a shared structural region of the HF-PS. With respect

to the development of a vaccine, the fact that the HF-PS was also recognized by anti-*B. anthracis* live and killed spore antiserum gives an additional advantage of being effective against both spores and vegetative cells.

Conclusion 3: The reactivity of the *B. anthracis* HF-PS-KLH antiserum with the *B. anthracis* spore preparation is an indication that the spore could also contain either the HF-PS cell wall fragment or a related carbohydrate fragment that contains a cross-reactive epitope. These spore observations are still tentative and in need to be corroborated through detailed biochemical analysis of the spore components and investigation of the reactivity of additional sera, e.g. the antisera derived from additional HF-PS conjugate antigens.

Implications of the results for the viability and virulence functions of the HF-PS, and future research perspectives

Taken together, the here presented work proved that the cell wall carbohydrate from *B. anthracis* and *B. cereus* strains investigated is antigenic and is immunochemically as well as structurally species-specific. Interestingly, a recent report using subtractive hybridization to compare DNA from *B. anthracis* with that of other *B. cereus* group strains came to a related conclusion and suggested that a specific glycosyl transferase from *B. anthracis* Ames (gene BA5519) (15) could have some value as molecular marker specific to *B. anthracis* (i.e. supporting our notion of species-specific cell wall carbohydrates in *B. anthracis* and *B. cereus* strains). We also showed that in strains from *B. anthracis* and *B. cereus* causing severe pneumonia, the structures of a main cell wall carbohydrate, the HF-PS, are closely related, but not identical. Also, the fact that the HF-PSs from pathogenic *B. cereus* strains and *B. anthracis* exhibit cross-reactive antigenic structures could provide a starting point for the development of a broad spectrum vaccine generated with a shared structural region of the HF-PSs. Based on our current findings, our expectations are that the HF-PS exhibits necessary properties for the development of future diagnostic applications, vaccines, and, possibly, therapeutics (see below) for *B. cereus* group members.

However, much remains to be learned about the cell wall carbohydrates from *B. cereus* group strains. For example, a research area of great functional importance that

has not yet been addressed is the localization of the HF-PS on cells and spores. Helpful in this quest will be the production of monoclonal antibodies (mAb) with specificity to the *B. anthracis* HF-PS. The production of mAb with HF-PS fragments isolated in the framework of this thesis has been started. Using light and electron microscopy, tagged mAb with specificity for the HF-PSs will be used to localize the HF-PS on vegetative cell walls and on the spore surface. Furthermore, mAb will be an ideal means to investigate the occurrence and distribution of the HF-PSs on vegetative cell walls and spores from *B. anthracis* and other members of the *B. cereus* group.

The cellular localization will be of critical importance to a future functional analysis. To date, the function of the cell wall carbohydrate and the HF-PS fragment is not well understood and a detailed functional analysis is missing. Therefore, a major future objective will also be to determine the importance of the HF-PS for vegetative cell growth and virulence. A previous report proposed that the HF-PS is involved in anchoring and exporting of S-layer proteins to the cell surface, e.g. in *B. anthracis* the S-layer proteins Sap and EA1 (29). As described earlier, it is thought that a specialized domain in these proteins, the S-layer homology (SLH) domain, binds to a cell wall carbohydrate (CWC) in the cell wall of *B. anthracis*. While still preliminary, the findings in this thesis strongly suggest that this CWC structure is likely to be the HF-PS. Investigations into the details of this carbohydrate-protein binding interaction and the functions of these SLH domain containing proteins will be an additional interesting future area of research. In this context, some of the important questions that need to be resolved include (a) whether the HF-PS is, indeed, the proposed carbohydrate anchor, (b) if more than one carbohydrate is involved in the anchoring of these proteins, (c) if SLH containing proteins play a role in bacterial viability and virulence, (d) if carbohydrate anchor expression depends on certain growth conditions or host interaction, and (e) what molecular mechanisms are involved in SLH mediated anchoring and export.

At the moment, arguably the most pressing question is, however, to show the direct functions of the HF-PS in *B. anthracis* and *B. cereus* strains with regard to bacterial cell viability and virulence. Some clues have already become available that point towards an important role for viability. The biosynthesis of the HF-PS most likely

requires the enzymatic activity of UDP-GlcNAc 2-epimerase which converts UDP-GlcNAc into UDP-ManNAc, as ManNAc is a constituent of HF-PS in all investigated strains. Recently, a *B. anthracis* Sterne mutant has been described carrying a mutation in a gene coding for the UDP-GlcNAc 2- epimerase. The knockout mutant of one of two functionally redundant copies of the epimerase gene in *B. anthracis* showed significant growth defects; attempts to create a double mutant failed completely (34). The authors of the paper therefore concluded that the double mutant was not viable. Reasons why this double mutant is not viable are not known. However, an attractive explanation could be that in a double mutant a complete block of ManNAc biosynthesis would lead to a incomplete HF-PS synthesis, which in turn leads to lethality. Taken together, these findings point (all-the-while indirectly) to a critical as yet unknown function of the HF-PS in cell viability (34). Unfortunately, neither the lethality was directly shown with conditional mutations in the respective genes, nor was the isolated single mutant investigated for structural alterations to the HF-PS.

Based on our HF-PS structure in conjunction with a bioinformatic analysis of publicly accessible genome data bases from *B. anthracis*, it was possible to construct likely biosynthetic pathways for the sugar nucleotides necessary for the synthesis of the HF-PS and identify corresponding putative genes in the *B. anthracis* genome. Candidate gene from that pathway (e.g the mentioned genes coding for UDP-GlcNAc 2-epimerase and others, including a number of glycosyl transferases) are currently being mutagenized in *B. anthracis* to assess their biochemical, chemical, physiological and pathogenic phenotype. The isolation of *B. anthracis* mutants with attenuated pathogenicity and/or an impaired bacterial growth in conjunction with a modified or missing HF-PS would be a big step towards establishing the role of HF-PS in the development of anthrax and in the elucidation of its function in bacterial cell viability.

The above described carbohydrate SLH domain-mediated anchoring mechanism of proteins to the cell surface was hypothesized to be wide spread among bacteria (37). Hence, the report of 22 *B. anthracis* proteins, along with EA1 and Sap, of known and unknown functions that have been predicted to contain SLH-domains was not surprising. Recently, one of these proteins, a third S-layer protein (BslA) was identified and maps to the pathogenicity island of the virulence plasmid pXO1 (14). The BslA

protein proved to have two functions, one as S-layer protein and one as a bacterial adhesin of *B. anthracis*. Adhesins have been implicated in cell division, adherence to epithelial cells as well as in bacteriophage attachment (26, 32, 35). In pathogenic host colonization, adherence to host epithelial cells is an important first step, suggesting that BslA could be involved with virulence and with the establishment of anthrax; therefore, it is expected that BslA is an additional virulence factor of *B. anthracis* (14). If our assumption that the HF-PS is part of the SLH-mediated general cell wall anchoring mechanism proves to be correct, then the HF-PS is likely to play an important role in the BslA S-layer protein translocation and in the adherence of the bacterial cells to host tissue cells. As a cell wall component of the *B. anthracis* cells, the HF-PS is probably *per se* not a virulence factor. However, the assumed involvement in targeting the BslA S-layer/adhesin protein (a putative virulence factor) as well as other proteins to the cell surface could make HF-PS a key factor in *B. anthracis* pathogenicity and the development of the anthrax disease.

There are additional roles for the HF-PS possible. Older reports showed that bacterial conjugation requires interaction between cell surface components such as lipoteichoic acids, glycoproteins (9, 17, 36). In particular lipoteichoic acids or teichoic acids were suggested to be involved in the formation of mating aggregates in *Streptococcus faecalis* (6, 31). As *B. anthracis* does not have any teichoic or lipoteichoic acids (18, 30), it seems feasible that the HF-PS functionally substitutes for teichoic or lipoteichoic acid in *B. anthracis* conjugation. This could be of particular interest if structural specificity is playing a role in the formation of mating aggregates. The pathogenic *B. cereus* strains that cause anthrax-like symptoms contain toxin genes and other genes known from the *B. anthracis* virulence plasmids (12). It has been suggested that these strains receive the toxin genes via conjugation (13), most probably from a *B. anthracis* strain. One plausible idea is, therefore, that the HF-PS is a specific mediator (either directly or indirectly via additional proteins) involved in the formation of these bacterial mating aggregates. This could be one explanation of why the *B. cereus* strains that caused severe pneumonia all contain HF-PSs that are structurally related to the *B. anthracis* HF-PS and also contain toxin genes, which until recently, were only found on the *B. anthracis* virulence plasmids. Our findings indicate that the closely

related HF-PS structures of *B. anthracis* and pathogenic *B. cereus* strains could be the primary reason why these and no other *B. cereus* strains acquired anthrax pathogenicity related genes and are now capable of causing severe pneumonia. If correct, investigating the HF-PS structure in *B. cereus* group strains could provide valuable information on the pathogenic potential of the various members of this group for causing severe pneumonia in humans.

In addition to the above possible functions, there are indications for an involvement of the HF-PS in bacteriophage lysin function. Bacteriophages are viruses that infect and kill bacteria during the course of their life cycle (27). Lysins commonly consist of a catalytic domain, capable of degrading cell wall components, and a domain that binds to the cell wall or cell surface molecules. The binding domain is highly specific and often attaches to species or strain specific cell wall carbohydrates (24, 25). Recently, the phage endolysins PlyL, and PlyG isolated from two prophages, the α- and γ-prophage respectively, have been described in the literature to affect *B. anthracis* and *B. cereus* cells (26, 32). The lysin activity of PlyG was demonstrated to be specifically directed towards *B. anthracis* cells, cell walls and spores, and against *B. cereus* strain RSVF1 which caused fatal disease in mice, and slightly directed against cells from the closely related *B. cereus* strain ATCC 10987 (26, 32). The exact structures to which PlyG binds to in the *B. anthracis* cell wall has not yet been identified; however, as already mentioned, they are known to be carbohydrate structures (24, 25). The second lysine PlyL originates from the *B. anthracis* genome and was analyzed for its structure and function (26). Low *et al*. reported the N-terminal domain to function as an amidase against the cell wall of several *Bacillus* species. The C-terminal binding domain was reported to have a dual function as a cell wall binding domain and as an inhibitor of the N-terminal domain's amidase activity whenever the enzyme's specific substrate was absent (26). The exact binding position of PlyL on the cell wall is also still unknown and is currently investigated in follow-up work to the here presented findings. As the phage lysin specificities correlate reasonably well with relatedness of the HF-PSs presented in this thesis, it is a possibility that the HF-PS confer the bacteriophage lysin binding to the cell wall, e.g. *for B. anthracis* cell walls. If the HF-PS is, in fact the receptor molecule for the lysins, it would also be involved in the activation of the

enzyme activity at the N-terminal end of the lysin proteins. This lysin cell binding and activation mechanism would have important implications for the development of both novel phage-based diagnostics and therapeutics (7, 26, 32)

In summary, although the functional importance of the HF-PS and its involvement in *B. anthracis* cell viability and virulence is at the molecular level still unclear and a work in progress, we suspect a possible involvement in anchoring and exporting of S-layer and other SLH-domain proteins of known and unknown functions, in bacteriophage endolysin acitivity, in cell-cell adhesion and aggregation, possibly in conjugation, and in pathogenicity. Moreover, if the structural similarities of the HF-PS from *B. anthracis* and the HF-PS from *B. cereus* strains causing severe pneumonia can be linked to the anthrax-like symptoms caused by latter strains, this could make the HF-PS an important future target for the development of new and improved therapeutics.

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