The Structure of the Major Cell Wall Polysaccharide of *Bacillus anthracis* Is Species-specific*

Biswa Choudhury‡, Christine Leoff§, Elke Saile*, Patricia Wilkins*, Conrad P. Quinn*, Elmar L. Kannenberg‡, and Russell W. Carlson‡

From the ‡Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602, the §Centers for Disease Control and Prevention, Atlanta, Georgia 30333, and the ¶Departments of Microbiology and Biotechnology, University of Tübingen, D72076 Tübingen, Federal Republic of Germany

Received for publication, June 15, 2006. Published, JBC Papers in Press, July 26, 2006, DOI 10.1074/jbc.M605768200

In this report we describe the structure of the polysaccharide released from *Bacillus 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 *Bacillus cereus* ATCC 10987 strain, and from the *B. cereus* ATCC 14579 type strain and compared with 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, and one- and two-dimensional nuclear magnetic resonance 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 O-3 and O-4, respectively, and the β-GlcNAC substituted with α-Gal at O-3. 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, furthermore, 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 *Bacillus* species.

*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. Whereas 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-δ-glutamate capsule and not by a polysaccharide capsule. Second, their cell walls do not contain teichoic acid (3), and last, their S-layer proteins are not glycosylated (1, 4). However, glycosyl composition comparisons of the cell walls of *B. anthracis*, *Bacillus cereus*, and *Bacillus 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 (6–8). All of these carbohydrates are rich in amino glycosyl residues but have variations in the type and amounts of these residues. The study of Ekwnife *et al.* (6) focused on the glycosyl composition of a carbohydrate polymer released from the cell wall through hydrogen fluoride (HF) treatment (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 Mesnagne *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.* (7) 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 *Bacillus* species 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

---

*This work was supported in part by National Institutes of Health Grant NIAID Grant R21 AI059577 (to R. W. C.) and Department of Energy Grant DE-FG09-93ER20097 to the Complex Carbohydrate Research Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Both authors contributed equally to this manuscript.

§ To whom correspondence should be addressed. Tel.: 706-542-4439; Fax: 706-542-4412; E-mail: rcarlson@ccrc.uga.edu.

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
A Species-specific B. anthracis Vegetative Cell Wall Polysaccharide

matter of debate (9, 10) 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, which is found also in other S-layer proteins from Gram-positive bacteria and in cell wall enzymes, such as xylanase and pullanase from Thermoaerobacterium thermohydrodsulfurigenes (Clostridium thermosulfurogenes) (11). It is thought that S-layer homology domains bind to secondary cell wall carbohydrates that are covalently linked to the cell wall peptidoglycan via HF-labile phosphate bridges (4) 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 (8, 12, 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 (13), whereas the acidic carbohydrate was composed of GlcNAc, GalNAc, and Glc 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 34F₂. 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 that differed from that of B. cereus ATCC 10987, which, in turn, differed from that of B. cereus ATCC 14579.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—B. anthracis Ames, B. anthracis Pasteur, B. anthracis Sterne 34F₂, and B. cereus strains ATCC 10987, and ATCC 14579 were provided from the Center for Disease Control culture collection. The mutant UT60 (i.e. Sterne 7702 ΔatxA) was provided by Dr. Theresa Kohler, University of Texas, Houston. Cultures were grown overnight (16 h, 37 °C) in 100 ml of brain heart infusion medium (BD BBL, Sparks, MD) containing 0.5% glycerol at 37 °C, 200 rpm. In the morning, 4 × 1.5 ml of the overnight cultures were pelleted (10,000 × g at room temperature for 5 min), the supernatants were discarded, the pellets resuspended in 500 μl of brain heart infusion and these four cell suspensions were inoculated into four 250 ml volumes of brain heart infusion medium in 1-liter Erlenmeyer flasks. Incubation was carried out at 37 °C (B. anthracis) or 30 °C (B. cereus and 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 at 4 °C for 15 min), washed two times in sterile saline and enumerated by serial dilution and surface spread counts on brain heart infusion agar plates. Cultures were sterilized by autoclaving for 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 × 10¹⁰ to 1 × 10¹¹ CFU) were disrupted in 40-ml sterile saline on ice by four 10-min sonication cycles, using a Branson Sonifier (Type 450, Branson Ultrasronics Corp., Danbury, CT) with a ½ inch probe, operating 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 at 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 (~30%) in 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 detector was used to monitor the sample eluting from the column. The fractions that gave a positive response in the refractive index 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 analysis of trimethylsilyl methylglycosides (15). The trimethylsilyl methylglycosides were identified and 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 the presence of methanol at 100 °C for 1 h. After removing the reagents by flushing with dry nitrogen, the methylglycosides were treated with Tri-Sil reagent (Pierce) at 80 °C for 30 min to form trimethylsilyl methylglycosides. The TMS methylglycosides were dissolved in hexane and analyzed on a gas chromatography-mass spectrometric using HP-1MS column (30 m × 0.25 mm × 0.25 μm). 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 (17). Briefly, the samples were dissolved in
dry dimethyl sulfoxide (0.250 μl) overnight with stirring, followed by addition of a Me₂SO/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. The partially methylated polysaccharide was methanolyzed to monomers using methanolic 1M HCl at 80 °C for 16 h followed by hydrolysis with 4M trifluoroacetic acid 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 partially methylated alditol acetates were dissolved in dichloromethane and analyzed by gas chromatography-mass spectrometric 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 partially methylated alditol acetate 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 of 100% D₂O (100% D; Cambridge Isotope Laboratories) and transferred to a 5-mm NMR tube. All one- and two-dimensional 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 ms, 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 96 scans were used in collecting the gHSQC spectra. All the NMR data were processed and analyzed using NMR processing software Mest-Rec version 4.7.5.0 for Windows.

**Mass Spectroscopy**—A matrix-assisted laser desorption ionization-time of flight mass spectrometer model Voyager-DE Biospectrometry Work station (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-dihydroxybenzoic acid as the matrix. About 0.7 μl of this mixture was loaded on each spot on a stainless steel matrix-assisted laser desorption plate and air-dried. The spectra were acquired in delayed, linear and positive mode using a 337-nm N₂ laser with acceleration voltage of 20 kV.

**RESULTS**

**Isolation and Initial Analysis**—Glycosyl composition analysis, as described elsewhere, showed that the HF-PSs from

---

**FIGURE 1.** Matrix-assisted laser desorption ionization-time of flight mass spectrometer spectrum (positive mode) of the HF-PS from *B. anthracis* Ames. The spectra of the HF-PSs from *B. anthracis* Sterne, UT60, and Pasteur were all identical to this spectrum.
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) of pyruvic acid as determined by the colorimetric method of Katsuki (16).

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 spectrometric analysis using a matrix-assisted laser desorption ionization-time of flight mass spectrometer 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 Fig. 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-acetylatedhexosaminosyl residues; e.g. Gal₆GlcNAc₄ManNAc₂Na. 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

| m/z    | Proposed composition                                       |
|--------|-----------------------------------------------------------|
| 2232   | Gal₆GlcNAc₄ManNAc₂Na⁻                                    |
| 2069   | Gal₅GlcNAc₄ManNAc₂Na⁻                                    |
| 1907   | Gal₄GlcNAc₄ManNAc₂Na⁻                                    |
| 3327   | Gal₅GlcNAc₄ManNAc₂Na⁺                                    |
| 3165   | Gal₄GlcNAc₄ManNAc₂Na⁺                                    |
| 3003   | Gal₃GlcNAc₄ManNAc₂Na⁺                                    |
| 4422   | Gal₄GlcNAc₄ManNAc₂Na⁺                                    |
| 4260   | Gal₃GlcNAc₄ManNAc₂Na⁺                                    |
| 4098   | Gal₂GlcNAc₄ManNAc₂Na⁺                                    |
| 5517   | Gal₃GlcNAc₄ManNAc₂Na⁺                                    |
| 5355   | Gal₂GlcNAc₄ManNAc₂Na⁺                                    |
| 5193   | Gal₁GlcNAc₄ManNAc₂Na⁺                                    |
| 6612   | Gal₁GlcNAc₄ManNAc₂Na⁺                                    |
| 6450   | Gal₁GlcNAc₄ManNAc₂Na⁺                                    |
| 6288   | Gal₁GlcNAc₄ManNAc₂Na⁺                                    |

FIGURE 2. The proton NMR spectra for the HF-PSs. The spectra for the HF-PSs are shown for B. anthracis Ames (A), B. cereus ATCC 10987 (B), and B. cereus ATCC 14579 (C).
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 \([\text{Gal}_3\text{GlcNAc}_2\text{ManNAc}_2]_5\) \(\text{Gal}_3\text{GlcNAc}_2\text{ManNAc}_1\), \([\text{Gal}_3\text{GlcNAc}_2\text{ManNAc}_1\text{GlcNAc}_2\text{ManNAc}_1]_5\text{Gal}_3\text{GlcNAc}_2\text{ManNAc}_1\), and \([\text{Gal}_3\text{GlcNAc}_2\text{ManNAc}_1\text{GlcNAc}_2\text{ManNAc}_1\text{GlcNAc}_2\text{ManNAc}_1]_5\), respectively.

**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 ATCC 14579. The proton NMR spectra comparing HF-PS from *B. anthracis* Ames with the two *B. cereus* strains is shown in Fig. 2. Each spectrum clearly differs from the other in the pattern of resonances for their glycosyl anomic 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, furthermore, that the *B. cereus* ATCC 10987 structure differs from that of strain ATCC 14579. The spectra comparing the HF-PSs of *B. anthracis* Ames, *B. anthracis* Sterne, and *B. anthracis* Pasteur are shown in Fig. 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.

The proton spectrum given in Fig. 3A for the *B. anthracis* Ames polysaccharide shows it contains six anomic signals at \(\delta 5.64, 5.53, 5.22, 4.91, 4.67 (J_{1,2} = 7.2 \text{ Hz}), \) and \(4.44 (J_{1,2} = 7.8 \text{ Hz})\) 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 \(J_{1,2}\) coupling constants of these anomeric protons indicate that three of these glycosyl residues are \(\alpha\)-anomers, and at least two are \(\beta\)-anomers, whereas the anomic configuration of the remaining glycosyl residue (i.e., the glycosyl residue with \(H_1\) at \(\delta 4.91\)) cannot be deduced from the one-dimensional proton spectrum due to its small \(J_{1,2}\) 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 (Fig. 4), which clearly shows six anomic proton/carbon resonances. We also observed a resonance at \(\delta 1.48\) (Fig. 3, arrows) that is consistent with the methyl protons of a pyruvyl substituent as reported by Mesnage *et al.* (4). However, the relatively low intensity of this
A Species-specific B. anthracis Vegetative Cell Wall Polysaccharide

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.

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 (Fig. 4), TOCSY (Fig. 5), and NOESY (Fig. 6) analyses. The rationale for these assignments (given in Table 2) is described in the following paragraphs.

Residue A contains an anomic proton, H1, resonating at δ 5.64. The H1 through H4 assignments are readily assigned from the COSY and TOCSY (Fig. 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. $J_{3,4} + J_{4,5} < 9.6$ Hz) supporting the conclusion that A has a galacto configuration, and, therefore, is an α-Gal residue. To assign H5 and H6, it was necessary to determine, using the TOCSY data, the resonances of the protons coupled to H4. This rationale for these assignments (given in Table 2) is described in the following paragraphs.

Residue B has an anomic H1 at δ 5.53. As with residue A, the H1 through H4 resonances are readily assigned from the COSY and TOCSY (Fig. 5) data, and the small overall $J_{3,4}$ and $J_{4,5}$ 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 C-6 carbons that resonate at about δ 62 (the HSQC spectrum, Fig. 4) supporting that these are H6 protons.

The anomic proton of residue C has a chemical shift of δ 5.22. The H1 through H5 assignments were made from the COSY and TOCSY (Fig. 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 (Fig. 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 C-6. 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. Because 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 an α-GlcNAc residue.

The anomic proton H1 of residue D has a chemical shift of δ 4.91. The COSY and TOCSY (Fig. 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 $J_{1,2}$ 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 C-6 carbon. The anomic configuration of a manno residue is difficult to determine because both α- and β-anomers have small \( J_{1,2} \) coupling constants. However, the NOESY spectrum (Fig. 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 \( J_{1,2} \) value of 7.2 Hz show that it has a β-configuration. The COSY and TOCSY (Fig. 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 because these protons are coupled to a C-6 carbon. The anomeric configuration of a residue is difficult to determine because both α- and β-manno residues have small \( J_{1,2} \) coupling constants. However, the NOESY spectrum (Fig. 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 \( J_{1,2} \) value of 7.2 Hz show that it has a β-configuration. The COSY and TOCSY (Fig. 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 because these protons are coupled to a C-6 carbon. The anomeric configuration of a residue is difficult to determine because both α- and β-manno residues have small \( J_{1,2} \) coupling constants. However, the NOESY spectrum (Fig. 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.

Residue F has an anomeric H1 with a chemical shift of δ 4.44 and a \( J_{1,2} \) coupling of 7.8 Hz showing that it has a β-configuration. The COSY and TOCSY (Fig. 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 \( J_{3,4} \) plus \( J_{4,5} \) 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 (Fig. 4) showed that these protons are coupled to a C-6 carbon at δ 61.9. Thus, F is a β-galactose 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 anomic proton at δ 5.27 (residue C'), and another minor glycosyl ring system at δ 5.14 (residue C") (Fig. 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-galactose 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 by NOESY analysis (Fig. 6). Residue A, α-galactose, 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-galactose, 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 A described above shows that the oligosaccharide repeating unit has a partial sequence (Structure 1).
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 because, as described above, it is already occupied by a α-galactose residue (residue A). 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 spatial 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 a partial sequence structure (Structure 2).

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. Because 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 C-3 of the β-GlcNAc residue is in close enough proximity to the ManNAc residue 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-GlcNAc residue, is attached...
to position 6 of residue C, the α-GlcNAc residue, indicating the a partial sequence for this repeating oligosaccharide (Structure 3).

\[
\begin{align*}
\rightarrow 6) & - \alpha-\text{GlcNAc}-(1\rightarrow 4) - \beta-\text{ManNAc}-(1\rightarrow 4) - \beta-\text{GlcNAc}-(1\rightarrow 3) \quad \alpha-\text{Gal}-(1\rightarrow 3) \quad \alpha-\text{Gal}-(1\rightarrow 3).
\end{align*}
\]

STRUCTURE 3

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

The remaining residue, F (β-galactose), 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 β-galactose residue F is attached to α-GlcNAc residue C at C-4. 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 mass spectrometer, glycosyl composition, and linkage data show that the polysaccharide contains an overall repeating unit sequence (Structure 4).

\[
\begin{align*}
\rightarrow 6) & - \alpha-\text{GlcNAc}-(1\rightarrow 4) - \beta-\text{ManNAc}-(1\rightarrow 4) - \beta-\text{GlcNAc}-(1\rightarrow 3) \quad \alpha-\text{Gal}-(1\rightarrow 3) \quad \alpha-\text{Gal}-(1\rightarrow 3).
\end{align*}
\]

STRUCTURE 4

**DISCUSSION**

We report in this study the structure of the HF-PS from *B. anthracis* (Fig. 7) and demonstrate that this structure is the same for *B. anthracis* Ames, *B. anthracis* Sterne, *B. anthracis* UT60, and *B. anthracis* Pasteur. In addition, a proton NMR comparison, as well as composition analysis, shows that this structure is different from the HF-PS of 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 (4, 6) reported the composition of the cell wall polysaccharide from *B. anthracis* and that of the major polysaccharide released from the cell wall by treatment with aqueous HF. 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 S-layer homology 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 O-3 with a β-Glc residue and the ManNAc residue is substituted at O-3 with a β-GlcNAc residue (19). The major polysaccharide for the cell walls of *Paenibacillus polymyxa* AHU 1385 (formerly *B. polymyxa*) consists of →3)-β-ManNAc-(1→4)-β-GlcNAc-(1→ backbone in which the ManNAc residue is substituted with a pyruvyl residue at the O-4/O-6 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→ backbone in which the GalNAc residue is substituted with an α-Glc at O-6, the ManNAc with a β-GlcNAc at O-3, and the GlcNAc residue with a →6)-β-GlcNAc-(1→6)-β-GlcNAc-(1→ disaccharide at O-6 (8). The structure we report here for the HF-PS from the *B. anthracis*, as shown in Fig. 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-galactose and β-galactose at O-3 and O-4, respectively, and the β-GlcNAc substituted with α-galactosidase at O-3. 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 *Bacillus* species.

That the HF-PS from *B. anthracis* Sterne had essential functions is supported in a report by Mesnage *et al.* (4). Whereas Mesnage *et al.* (4) did not determine the structure of this polysaccharide, the proton NMR spectrum they show of the HF-PS for *B. anthracis* Sterne is identical to the spectra we show (Fig. 3) for the HF-PS isolated from *B. anthracis* Ames, *B. anthracis* Sterne, and *B. anthracis* Pasteur. Mesnage *et al.* (4) state that pyruvate is a component of the HF-PS; however, whereas our NMR spectra showed a resonance consistent with a pyruvyl methyl group (the resonance at δ 1.48, see Fig. 2) as reported by Mesnage *et al.* (4), colorimetric analysis failed to detect pyruvate in any of the *B. anthracis* HF-PSs.
A Species-specific B. anthracis Vegetative Cell Wall Polysaccharide

The reason for this discrepancy is unknown at this time. However, because 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. Because 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 and, therefore, its synthetic mechanism is a potential target for novel therapeutics.

In addition to the function of the HF-PS in B. anthracis autolysis and cell division just described, a recent report by Mayer-Scholl et al. (21) present data that 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. Because 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 species 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.4 These composition differences reflect structural variation in the B. cereus group as demonstrated by the different NMR spectra (Fig. 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. (18) 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, whereas it is 90.9% similar to B. cereus ATCC 14579 (18). 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 (23). 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.4 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.

Acknowledgment—We thank Dr. Theresa Koehler for providing B. anthracis UT60.

REFERENCES

1. Mock, M., and Fouet, A. (2001) Annu. Rev. Microbiol. 55, 647–671
2. Messner, P. (1997) Glycoconj. J. 14, 3–11
3. Molnar, J., and Pragai, B. (1971) Acta Microbiol. Acad. Sci. Hung. 18, 105–108
4. Mesnage, S., Fontaine, T., Mignot, T., Delepierre, M., Mock, M., and Fouet, A. (2000) EMBO J. 19, 4473–4484
5. Fox, A., Stewart, C. G., Waller, L. N., Fox, K. F., Harley, W. M., and Price, R. L. (2003) J. Microbiol. Methods 54, 143–152
6. Ekwunife, F. S., Singh, J., Taylor, K. G., and Doyle, R. J. (1991) FEMS Microbiol. Lett. 82, 257–262
7. Fox, A., Steward, G. C., Fox, K., and Rostovtseva, S. (1993) J. Clin. Microbiol. 31, 887–894
8. Amano, K., Hazama, S., Araki, Y., and Ito, E. (1977) Eur. J. Biochem. 75, 513–522
9. Priest, F. G., Barker, M., Bailie, L. W. J., Holmes, E. C., and Maiden, M. C. J. (2004) J. Bacteriol. 186, 7959–7970
10. Han, C. S., Xie, G., Challacombe, J. F., Altherr, M. R., Bhotika, S. S., Bruce, D., Campbell, C. S., Campbell, M. L., Chen, J., Chertkov, O., Cieland, C., Dimitrijevic, M., Doggett, N. A., Fawcett, J. I., Glaiving, T., Goodwin, L. A., Hill, K. K., Hitchcock, P., Jackson, I., Koli, P., Kewalramani, A. R., Longmire, J., Lucas, S., Malafatti, S., McMurry, K., Meinke, L. J., Misra, M., Moseman, B. L., Mundt, M., Munk, A. C., Okinaka, R. T., Parson-Quinanta, B., Reilly, L. P., Richardson, P., Robinson, D. L., Rubin, E., Saunders, E., Tapia, R., Tesmer, J. G., Thayer, N., Thompson, L. S., Tice, H., Ticknor, L. O., Wills, P. L., Brettin, T. S., and Gilna, P. (2006) J. Bacteriol. 188, 3382–3390
11. Brechtel, E., and Bahl, H. (1999) J. Bacteriol. 181, 5017–5023
12. Kojima, N., Araki, Y., and Ito, E. (1985) Eur. J. Biochem. 148, 479–484
13. Murazumi, N., Araki, Y., and Ito, E. (1986) Eur. J. Biochem. 161, 51–59
14. Brown, W. C. (1973) J. Bacteriol. 125, 295–300
15. York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T., and Albersheim, P. (1995) Methods Enzymol. 234, 1–30
16. Katsuki, H., Yoshida, T., Tanegashima, C., and Tanaka, S. (1971) Anal. Biochem. 43, 349–356
17. Ciucanu, I., and Kerek, F. (1984) Carbohydr. Res. 131, 209–217
18. Rasko, D. A., Ravel, J., Okstad, O. A., Helgason, E., Czer, B. R., Jiang, L., Shores, K. A., Fouts, D. E., Tourseas, N. I., Angiuoli, S. V., Kolonay, J., Nelson, W. C., Kolsto, A. B., Fraser, C. M., and Read, T. D. (2004) Nucleic Acids Res. 32, 977–988
19. Iwasaki, H., Araki, Y., Kaya, S., and Ito, E. (1989) Eur. J. Biochem. 188, 635–641
20. Kojima, N. Kaya, S., Araki, Y., and Ito, E. (1988) Eur. J. Biochem. 174, 255–260
21. Mayer-Scholl, A., Hurwitz, R., Brinkmann, V., Schmid, M., Jungblut, P., Weinrauch, Y., and Zychlinsky, A. (2005) PLoS Path. 1, 179–186
22. Wang, W., Owen, S. M., Rudolph, D. L., Cole, A. M., Hong, T., Waring, A. J., Lal, R. B., and Lehrer, R. I. (2004) J. Immunol. 173, 515–520
23. Hoffman, A. R., Ravel, J., Rasko, D. A., Chapman, G. D., Chute, M. D., Marston, C. K., De, B. K., Sap, C. T., Fritzgerald, C., Mayer, L. W., Rasko, D. A., Chapman, G. D., Tomasz, A., and Cucu, M. (2004) Proc. Natl. Acad. Sci. USA 101, 8449–8454