Spores of Bacillus anthracis, the causative agent of anthrax, are enclosed by a prominent loose fitting layer called the exosporium. The exosporium consists of a basal layer and an external hairlike nap. The filaments of the nap are composed of a highly immunogenic glycoprotein called BclA, which has a long, central collagen-like region with multiple XG repeats. Most of the triplet repeats are PTG, and nearly all of the triplet repeats contain a threonine residue, providing multiple potential sites for O-glycosylation. In this study, we demonstrated that two O-linked oligosaccharides, a 715-Da tetrasaccharide and a 324-Da disaccharide, are released from spore- and exosporium-associated BclA by hydrazinolysis. Each oligosaccharide is probably attached to BclA through a GalNAc linker, which was lost during oligosaccharide release. We found that multiple copies of the tetrasaccharide are linked to the collagen-like region of BclA, whereas the disaccharide may be attached outside of this region. Using NMR, mass spectrometry, and other analytical techniques, we determined that the structure of the tetrasaccharide is 2-O-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-L-rhamnopyranose. The previously undescribed nonreducing terminal sugar (i.e. 2-O-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy-α-D-glucose) was given the trivial name anthrose. Anthrose was not found in spores of either Bacillus cereus and Bacillus thuringiensis, two species that are the most phylogenetically similar to B. anthracis. Thus, anthrose may be useful for species-specific detection of B. anthracis spores or as a new target for therapeutic intervention.

Bacillus anthracis is a Gram-positive, rod-shaped, aerobic soil bacterium that causes anthrax in humans and other mammals (1). Like other Bacillus species, B. anthracis forms endospores (or spores) when vegetative cells are deprived of an essential nutrient (2). The mature spore is dormant and highly resistant to extreme temperatures, radiation, harsh chemicals, desiccation, and physical damage (3). These properties allow the spore to persist in the soil for many years until encountering a signal to germinate (4). Anthrax is typically contracted by contact with spores (1).

Because of their ability to cause a potentially fatal disease and to withstand harsh conditions, spores of B. anthracis have been developed into weapons of mass destruction by numerous countries and terrorist groups (5). The effectiveness of B. anthracis spores as a biological weapon was demonstrated when letters laden with spores were mailed in the United States in the fall of 2001. In response to the threat of future releases of lethal spores, new research has been undertaken to enhance our knowledge of B. anthracis biology and pathogenesis. A major goal of such studies is to identify components of the B. anthracis spore that can serve either as molecular targets of spore inactivation or as unique markers that allow rapid and accurate spore detection (6).

Sporulation in the genus Bacillus begins in the starved vegetative cell with an asymmetric septation that produces large and small genome-containing compartments called the mother cell and forespore, respectively (7). The mother cell then engulfs the forespore and surrounds it with a layer of modified peptidoglycan called the cortex and a more external proteaceous layer called the coat. The spore coat, composed of three sublayers and many different proteins, forms the outermost detectable layer for spores of many species (e.g. B. subtilis) (8, 9). For other Bacillus species, such as B. anthracis, the spore coat is surrounded by another prominent layer called the exosporium, which is synthesized by the mother cell concurrently with the cortex and coat (10). After a final stage of maturation, during which covalent modifications occur in the outer layers of the spore, the mother cell lyses and releases the spore (9, 11).

Of particular interest in current studies of the B. anthracis spore is the exosporium, which is the primary permeability barrier of the spore and the source of spore surface antigens (10, 12). As the outermost surface of the spore, the exosporium interacts with the soil environment, detection devices, spore-binding cells in the mammalian host, and host defenses. Thus, it is likely that the exosporium plays an important role in spore survival and/or pathogenesis (12). To demonstrate such a role, it is necessary to characterize individual exosporium components.

Early studies revealed that spores of B. anthracis and closely related species (e.g. Bacillus cereus and Bacillus thuringiensis) possess an exosporium composed of a paracrystalline basal layer and an external hairlike nap, which exhibits a strain-specific length up to 600 Å (10, 13–16). The exosporium constitutes about 2% of the mass of the spore and contains approximately 50% protein, 20% lipid, 20% carbohydrate, and 10% other components (17). A recent proteomic analysis of the exosporium suggested that it contains at least 137 different proteins (18). However, this analysis was performed with an exo-
sporulation fraction prepared from spores that were not purified sufficiently to remove contaminating proteins released into the growth medium by lysed cells (44). Analyses of the exosporium prepared from highly purified spores indicates that about 20 different protein species are present in or tightly associated with the exosporium (12, 19, 44).

The first B. anthracis exosporium protein identified, and one of the most interesting, was a glycoprotein called BclA (for Bacillus collagen-like protein of anthracis) (12, 20). BclA is the structural component of the hairlike nap and contains multiple collagen-like-Xaa-Yaa-Gly (or XXY) repeats in its central region (20). The number of XXY repeats in BclA varies among strains (12, 21). This variation is responsible for the different lengths of the hairlike nap found on spores of different B. anthracis strains (21). BclA has also been shown to be the immunodominant protein on the B. anthracis spore surface, because most antibodies raised against spores react with this protein (12). Finally, most of the XXY repeats in the collagen-like region of BclA have the sequence PEGT, and nearly all of the XXY repeats contain a threonine residue, which may be a site of attachment of an O-linked oligosaccharide (22, 23).

In this report, we describe two O-linked oligosaccharides that are attached to BclA: a 715-Da tetrasaccharide and a 324-Da disaccharide. We show that multiple copies of the tetrasaccharide are linked to the collagen-like region of BclA, whereas the disaccharide may be attached outside of this region. The attachment of each oligosaccharide to BclA may occur through a GalNAc linker, which is lost during oligosaccharide release. Using several analytical techniques, we determine the complete structure of the tetrasaccharide. It contains a unique sugar residue that may be useful for species-specific detection of B. anthracis spores or even serve as a new target for preventing anthrax.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—The Sterne veterinary vaccine strain of B. anthracis along with B. cereus T and B. thuringiensis sp. kurstaki were obtained from John Ezzell (U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD). B. subtilis (typC2) A700 (originally designated 168) was obtained from the Bacillus Genetic Stock Center (Ohio State University, Columbus, OH). The Sterne strain of B. anthracis is not a human pathogen because it lacks plasmid pXO2, which carries the genes necessary to produce the protective poly-gamma-glutamic acid capsule of the vegetative cell. Spores of the Sterne strain were constructed by allelic exchange between the chromosome and a mutant locus carried by the shuttle vector pUTE29. A kanamycin resistance cassette from plasmid pUC18::H9024–2/H9024km into the multiple cloning site (between PstI and KpnI) of plasmid B. anthracis 1958/H9024–sp/H11001, which carries the genes necessary to produce the protective poly-gamma-glutamic acid capsule of the vegetative cell. Spores of the Sterne strain as described above. The mutant strain, designated CLT274, contains a deletion that removes codons 11–264 of the 284 rmlD codons, and the deleted codons are replaced by the Sp sp cassette.

**Preparation of Spores and Purified Exosporium**—Spores were prepared from cultures grown at 37 °C for 48–72 h on liquid or solid Difco sporulation medium, extensively washed in cold distilled water, and sedimented through 50% Renografin to remove vegetative cells and debris, and washed again in cold water (30, 31). Spores were stored in water at 4 °C (protected from light) and quantitated microscopically using a Petroff-Hausser counting chamber. The exosporium was removed from spores by passage through a French press and then highly purified by differential centrifugation as previously described (12).

**Monosaccharide Analysis by Gas Chromatography**—The monosaccharide compositions of spores, exosporium, and other samples were determined by gas chromatographic analysis of the trimethylsilyl derivatives of the sugar methyl glycosides. Samples were dried in a vacuum centrifuge, resuspended in 400 μl of 1.45 M methanolic HCl, and heated at 80 °C overnight. The methanolic HCl was removed by vacuum centrifugation, and the sample was resuspended in 200 μl of methanol, followed by the addition of 20 μl of acetic anhydride and 20 μl of pyridine. This mixture was allowed to react for 30 min at room temperature and then evaporated to dryness. The samples were then trimethylsilylated using 50 μl of Tri-Sil (Fischer), and the vials were sealed under argon. The trimethylsilylated glycosides were separated and quantitated on an HP 5890 gas chromatograph equipped with a 30-m HP-1 wide bore fused silica column coated with a 0.88-μm layer of cross-linked methyl silicone gum. Samples were applied to the column with an automatic injector, and sugars were detected by flame ionization.

**Determination of the Absolute Configuration of Rhamnose Residues**—To distinguish between the D- and L-forms of rhamnose residues, the (+)-2-butyl glycosides were prepared and analyzed by gas chromatography as previously described (32). However, HCl rather than trifluoroacetic acid was used as the catalyst, and rather than using acetate derivatives, as in the original procedure, trimethylsilyl derivatives were prepared as described above. The retention time of the uncommon D-form of rhamnose, for which a stable isotope was not available, was determined by chromatography of the (+)-2-butyl glycoside of l-rhamnose.

**Hydrazinolysis of Glycoproteins**—Selective hydrazinolysis was used to release O-linked oligosaccharides from spore glycoproteins. B. anthracis spores (1010) or exosporium samples were dried in a vacuum centrifuge and desiccated overnight over P2O5 under vacuum. Anhydrous hydrazine (1 ml) was added to each sample niche, which was flushed with argon and flame-sealed. The samples were heated at 60 °C for 5 h to specifically release O-linked oligosaccharides (33). The hydrazine was evaporated under vacuum and the residue was resuspended in 3 ml of water. The mixture was centrifuged at 14,000 ×
g for 10 min, and the supernatant containing oligosaccharides was collected.

**Gel Filtration Chromatography and Assay of Rhamnose-containing Oligosaccharides**—The supernatant obtained from the hydrazinolysis procedure was loaded onto a 170 × 2.2 cm Bio-Gel P4 (fine; Bio-Rad) column, and the oligosaccharides were eluted with 0.1 M acetic acid. Three-ml fractions were collected, and a 250-μl sample of each fraction was assayed for 6-deoxy sugars (e.g. rhamnose) using the Dische-Shettsel protocol (34).

**Mass Spectrometry**—Mass spectrometry was performed with a Micromass Q-TOF 2 mass spectrometer. Samples were introduced by flow injection into a stream of 50% acetonitrile containing 0.1% formic acid delivered by a Harvard model 22 syringe pump and were ionized by the electrospray mode.

**NMR Spectroscopy**—Approximately 750 μg of the purified tetrasaccharide was lyophilized, dissolved in 450 μl of Me$_2$SO-$d_6$ (99.99% deuterium), and transferred to a 5-mm NMR tube. NMR data were collected on a Bruker DRX-500 NMR spectrometer using a 5-mm TXI probe equipped with x, y, z gradients at a probe temperature of 25 °C. A few measurements were repeated at 600 MHz on an Avance DRX-600 NMR spectrometer. NMR experiments were performed on samples stored for 3–4 days at 4 °C. Standard Bruker pulse sequences were used, except for the 13C-coupled HSQC, where the program was modified to remove the 13C decoupling during acquisition. Proton and carbon chemical shifts were referenced to an internal Me$_2$SO peak (2.49 ppm for proton and 39.5 ppm for 13C).

In addition to standard 1H and 13C one-dimensional NMR spectra, a series of homo- and heteronuclear two-dimensional NMR data sets were obtained. DQF-COSY$^1$ was collected with 4096 data points and 0.409 s acquisition time in the F2 dimension with 800 increments in the indirect dimension. The data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 2048 points. The two-dimensional TOCSY experiments were performed with various spin lock times of 20, 40, 50, and 70 ms. The two-dimensional NOESY experiments were performed using 400- and 800-ms mixing times. The heteronuclear two-dimensional experiments HSQC, HMQC/TOCSY, and HMBC (with and without bilateral rotation decoupling filter) were performed using pulse field gradient programs. Data processing and plotting were performed using the Bruker Xwinplot program.

**RESULTS**

**Analysis of the Exosporium Monosaccharide Composition**—As the first step in the identification of BclA oligosaccharides, we analyzed the monosaccharide composition of exosporium preparations purified from spores of wild-type and ΔbclA strains of *B. anthracis* (Sterne). Equal amounts of each exosporium sample were subjected to methanolysis, and trimethylsilyl derivatives of the resulting methyl glycosides were analyzed by gas chromatography. Chiral 2-butyl glycosides of rhamnose were prepared, and the absolute configuration of the rhamnose in the exosporium. The minor peak was present in a chromatogram of a Δmld derivative of the Sterne strain that is unable to synthesize 1-rhamnose (data not shown). Based on retention times and isomeric ratios of sugar standards, the minor sugar was tentatively identified as ribose, which was previously reported to be present in low levels in the exosporium of *B. cereus* T (17) and in spores of *B. anthracis* (36). The only BclA-associated peak present in the chromatogram of the Δmld strain was that of GalNAc.

Although the four BclA-associated monosaccharides described above were major components of the exosporium carbohydrate, they were relatively minor components of the carbohydrate present in intact spores (data not shown). Thus, these monosaccharides appear to be components of glycoconjugates that are primarily or uniquely found in the exosporium.

**Isolation of Rhamnose-containing Oligosaccharides Associated with BclA**—The numerous threonine residues in the collagen-like region of BclA and the monosaccharide composition of the exosporium indicated that one or more rhamnose-containing oligosaccharides were O-linked to BclA. To isolate these oligosaccharides, 10$^{10}$ purified spores of the Sterne strain of *B. anthracis* were treated with anhydrous hydrazine under conditions that released only O-linked oligosaccharides. The free oligosaccharides were separated on a Bio-Gel P4 column (2.2 × 170 cm), and column fractions were assayed for rhamnose. Two oligosaccharide peaks were detected (Fig. 2). Based on the elution times of oligosaccharide standards, the larger peak corresponded to a tetrasaccharide, whereas the smaller peak corresponded to a disaccharide. Analysis of individual column fractions by ESI-Q-TOF mass spectrometry indicated that each oligosaccharide peak was essentially homogeneous and that the tetrasaccharide and disaccharide had masses of 715 and 324 Da, respectively. Both oligosaccharides were also isolated from purified exosporium of the Sterne strain following hydrazinolysis and gel filtration chromatography as described above. In addition, two minor peaks corresponding to a pentasaccharide and a trisaccharide were observed with masses of

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1 The abbreviations used are: DQF-COSY, double quantum filtered-correlated spectroscopy; HSQC, heteronuclear single quantum coherence; TOCSY, total correlation spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; HMBC, heteronuclear multiple bond correlation; ESI-Q-TOF, electrospray ionization-quadrupole-time of flight; Ant, anthrose.

**FIG. 1.** Monosaccharide composition of exosporium samples from wild-type and ΔbclA strains of *B. anthracis*. Exosporium samples were prepared from wild-type (A) and ΔbclA (B) strains of *B. anthracis* (Sterne), subjected to methanolysis, and analyzed by gas chromatography. Gas chromatographic peaks corresponding to the methyl glycosides of relevant sugars are labeled.
918 and 527 Da, respectively (data not shown). These masses are equal to those of the tetrasaccharide and disaccharide, respectively, with the addition of a GalNAc residue. The significance of the minor oligosaccharides detected in the exosporium is discussed below.

To determine whether the two major oligosaccharides were present in spores lacking BclA, we subjected 10^10 spores of a ΔbclA derivative of the Sterne strain to hydrazinolysis and assayed for oligosaccharides as described above. No oligosaccharides were detected in the column fractions (Fig. 2). This result indicated that the tetrasaccharide and disaccharide found in wild-type spores were attached to or at least associated with BclA.

Each purified oligosaccharide from wild-type spores was then analyzed for monosaccharide content by methanolysis and gas chromatography as described above. The data indicated that the 715-Da tetrasaccharide was composed of three rhamnose residues and an unusual residue initially called component A (Fig. 3). The 324-Da disaccharide was composed of one residue each of rhamnose and component B, which was tentatively identified as 3-O-methyl rhamnose (data not shown). Further details of the structure of the disaccharide will be presented in another communication.

Attachment of the Tetrasaccharide to the Collagen-like Region of BclA—To determine whether the two rhamnose-containing oligosaccharides were attached to the collagen-like region of BclA, we analyzed the oligosaccharide content of spores producing either wild-type BclA or mutant BclA proteins in which the collagen-like region was shortened. The shortened BclA proteins were produced by spores of two mutant Sterne strains, in which deletions in the bclA gene reduced the number of XXG repeats from the normal 76 to either 37 or 17 (12). The mutant BclA proteins were designated 1/2 CLR and 1/5 CLR, respectively. The shortened BclA proteins contain 400 amino acids (aa), with residues 39–266 included in the collagen-like region. The 1/2 CLR and 1/5 CLR deletions result in BclA proteins with collagen-like regions that are about one-half and one-fifth as long as that of the wild-type protein, respectively. The number and sequences of the XXG repeats in each BclA protein are shown.

FIG. 4. BclA collagen-like regions of the wild-type Sterne strain and mutant strains with internal bclA deletions. Wild-type BclA contains 400 amino acids (aa), with residues 39–266 included in the collagen-like region. The 1/2 CLR and 1/5 CLR deletions result in BclA proteins with collagen-like regions that are about one-half and one-fifth as long as that of the wild-type protein, respectively. The number and sequences of the XXG repeats in each BclA protein are shown.

FIG. 5. Gel filtration chromatography of rhamnose-containing oligosaccharides attached to wild-type and shortened BclA proteins. An equal number of spores of the wild-type strain of B. anthracis (Sterne) and of two mutant strains carrying either the 1/2 CLR or 1/5 CLR deletion were subjected to hydrazinolysis, and released oligosaccharides were separated on a Bio-Gel P4 column. Column fractions were assayed for rhamnose content. This result indicated that the disaccharide might be linked to BclA outside of the collagen-like region.

Analysis of the Tetrasaccharide by Mass Spectrometry—The ESI-Q-TOF mass spectrum (positive ion mode) of the tetrasaccharide indicated a molecular mass of 715 Da (Fig. 6). Fragment ion masses were consistent with the loss of rhamnose residues. There was a fragment peak of 552 m/z that corresponded to the loss (from 716 m/z) of a 164-Da terminal reducing end rhamnose and a fragment peak of 406 m/z that corresponded to the loss of two rhamnose residues. The peak at 698 m/z probably resulted from the loss of water from the tetrasaccharide.

Component A of the tetrasaccharide was analyzed separately.
by mass spectrometry. To purify component A, a 0.1-mg sample of the tetrasaccharide was treated with 1.45 M methanolic HCl at 80 °C overnight, and the digest was evaporated to dryness. The resulting methyl glycosides were separated by thin layer chromatography on a 5 × 20-cm silica gel plate (Merck), which was developed in ethyl acetate/pyridine/water (8:3:1). Fractions were eluted from the silica gel with methanol and identified by gas chromatography of their trimethylsilyl derivatives. Mass spectrometry in the positive ion mode of component A gave a peak of 292 m/z, indicating a molecular mass of 291 Da (data not shown). Assuming the sugar had been converted to a methyl glycoside, the mass of the free monosaccharide would be 277 Da. Collision-induced dissociation tandem mass spectrometry was then carried out on the 292 m/z parent peak, yielding the daughter ions shown in Fig. 7. The base peak of 260 m/z was probably due to the loss of methanol from the 292 m/z parent peak. Several fragment ions appeared to be derived from the loss of neutral fragments from the side chain(s) of the sugar. A loss of 58 Da was observed twice, from 260 to 292 m/z and from 242 to 184 m/z, which might indicate the loss of acetone. The peak at 168 m/z might result from the loss of 74 Da from the 242 m/z fragment ion, which could indicate the loss of 2-methylpropan-2-ol. There were two apparent losses of 100 Da: 260 to 160 m/z and 242 to 142 m/z. These losses could be explained by the removal of 3-hydroxy-3-methylbut-1-ene-1-one. Other peaks were probably derived from the degradation of the sugar ring. The predominantly even mass fragments suggested that the sugar ring retained a positively charged nitrogen atom. The above interpretation of the mass spectral data was consistent with the structure obtained by NMR analyses.

**NMR Analysis and Structure of the Tetrasaccharide**—A sample of purified tetrasaccharide was analyzed by NMR. 1H and 13C chemical shifts were fully assigned by a combination of homo- and heteronuclear methods, including TOCSY, DQF-COSY, NOESY, HSQC, HMBC, and two-dimensional HSQC-TOCSY (Table I). Starting from each anomeric proton, the 1H spin system of each sugar residue was assigned by using TOCSY with various mixing times and two-dimensional DQF-COSY, and values were confirmed by HSQC-TOCSY in case there were overlaps of proton chemical shifts. For the purposes of assignment, the sugar residues of the tetrasaccharide were labeled as A (nonreducing end), B, C, and D (reducing end). The complete structure of the tetrasaccharide is shown in Fig. 8 (with the unique residue A designated Ant for anthrose; see below).

**Characterization of the Modified Sugar at the Nonreducing End**—The spin system of residue A shows an anomeric proton resonance at 4.582 ppm, which gave cross-peaks to H-2 (2.850 ppm), H-3 (3.301 ppm), H-4 (3.401 ppm), H-5 (3.278 ppm), and H-6 (a methyl group, 1.082 ppm) in two-dimensional TOCSY (Table I). The strong NOE cross-peaks between the H-1, H-3, and H-5 protons and between the H-2 and H-4 protons, along with the large 3JH,H coupling of 8.5 Hz and the small one-bond 1JCH coupling of 161 Hz obtained from 13C-coupled HSQC (Fig. 9) confirmed the 4C1 chair conformation and β-configuration of the sugar residue (37, 38). A chemical shift of 84.3 ppm for the C-2 carbon was characteristic of an O-substitution (e.g., OMe). The OMe resonance was easily identified from its chemical shift of 3.526 ppm. The NOE cross peaks from the 2-OMe protons to the H-2 (very strong) and the H-1 (medium) protons and the long range HMBC cross-peaks from the H-2 proton to the methoxy carbon at 59.9 ppm and from the methoxy protons to the C-2 carbon indicated an OMe substitution at the C-2 position.

The shift of 56.4 ppm for the C-4 carbon indicated the presence of an amido group on the carbon. The amide proton at 7.754 ppm gave a cross-peak to H-4 in DQF-COSY and showed spin connectivity to all ring protons in residue A in two-dimensional TOCSY, confirming that the amide group was bonded to C-4 of sugar residue A. A singlet at 2.211 ppm, which integrated and normalized to two CH2 protons, showed NOE cross-peaks to the amide proton at 7.754 ppm and to the CH2 protons around 1.15 ppm. However, this CH2 resonance did not show J-coupling spin connectivity to the amide proton or other protons of residue A in TOCSY or DQF-COSY spectra. Two singlet proton resonances at 1.160 and 1.148 ppm, each corresponding to three protons in intensity, could have arisen from degenerate (CH3)2 resonances centered at 1.154 ppm with a vicinal coupling to a CH proton or from two nondegenerate CH2 groups at the respective positions without coupling to a CH proton. This ambiguity was resolved in the HMBC spectrum that showed the connectivity of the CH2 protons to the C=O carbon at 171.4 ppm, to the two CH3 carbons at 29.4 and 29.5 ppm, and to the COH carbon at 68 ppm (Fig. 10). This suggested that the CH2 group is close to a C=O, two CH3 groups, and another, possibly tertiary, carbon. In addition, the CH3 protons showed cross-peaks to each other in HMBC, which suggested that the CH2 groups were attached to the same carbon. The cross-peak at 68.0 ppm in HSQC-TOCSY was not seen in the standard HSQC spectrum, suggesting that this carbon was a tertiary carbon. The 13C chemical shift of this carbon indicated that it was linked to an oxygen-containing group. The 13C chemical shift of the CH2 carbon at 48.6 ppm indicated that it was not linked to oxygen. Based on this evidence, we unambiguously assigned the structure of the side chain as (CH3)2C(OH)CH2CONH and not (CH3)2CH-O-CH2CONH, both of which were in agreement with data from mass spectrometry (Fig. 7). Thus, the modified sugar at the nonreducing terminus of the tetrasaccharide was 2-O-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxyglucose (Fig. 8). We gave this novel sugar the trivial name anthrose (Ant).
Oligosaccharides of B. anthracis Exosporium Glycoprotein BclA

They showed small $^3J_{HH}$ values (2.4–2.5 Hz), large $^3J_{CC}$ values (166–171 Hz), and H-6/C-6 chemical shifts for $^1H$ (1.08–1.14 ppm) and $^{13}C$ (17.6–18.1 ppm) characteristic of an α-linked 6-deoxyhexose. The assignment of proton resonances in sugars B and C was completed using TOCSY. The anomeric proton of residue B at 4.876 ppm gave a cross-peak to the H-2 proton at 3.880 ppm, which in turn gave cross-peaks to H-3 (3.760 ppm), H-4 (3.404 ppm), and the H-6 methyl group (1.142 ppm). Similarly, the anomeric proton of residue C at 4.848 ppm gave a cross-peak to the H-2 proton at 3.783 ppm, which in turn gave cross-peaks to H-3 (3.592 ppm), H-4 (3.339 ppm), and the H-6 methyl group (1.103 ppm).

The anomeric proton resonances of residues D and B overlapped at 4.870 ppm in the one-dimensional $^1H$ spectrum but were resolved using two-dimensional TOCSY and NOESY. However, all protons from residue D could not be assigned using homonuclear methods due to overlapping of H-2, H-3, and H-5 proton chemical shifts. In two-dimensional TOCSY, the anomeric proton of residue D at 4.870 ppm gave a broad cross-peak to the H-2 proton at 3.601 ppm.
ppm, near the chemical shifts of H-3 (3.615 ppm) and H-5 (3.592 ppm). The broad H-2/H-3/H-5 peak showed cross-peaks to H-4 (3.144 ppm) and the H-6 methyl group (1.114 ppm). The two-dimensional HSQC-TOCSY identified the H-2, H-3, and H-5 resonances based on the chemical shifts of their carbons (Fig. 10). H-1/C-1 (4.870/92.8 ppm) showed a cross-peak at 3.601 ppm in the F2 dimension, which correlated to a normal HSQC peak at 3.601/77.7 ppm. This HSQC peak in turn showed connectivity to H-1 at 4.870 ppm in the F2 dimension. Thus, the peak at 3.601/77.7 ppm was assigned as H-2/C-2.

In addition to the cross-peak to H-1, H-2/C-2 also showed a cross-peak at 3.615 ppm in the F2 dimension, which correlated to a normal HSQC peak at 3.615/70.1 ppm. Thus, the peak at 3.615/70.1 ppm was assigned as H-3/C-3. Similarly, the HSQC peak at 3.592/67.7 ppm was identified as H-5/C-5. Interestingly, the H-2/C-2 (3.601/77.7) HSQC cross-peak for residue D appeared in the vicinity of the H-3/C-3 HSQC cross-peaks of residues B and C sugars, which suggested similar environments for these carbons (see below).

**Determination of Glycosidic Linkages**—The sequence of the sugar residues of the tetrasaccharide and their anomeric linkages were identified from a combination of interresidue NOEs across the glycosidic linkages, long range proton-carbon correlation data obtained from two-dimensional HMBC, and characteristic chemical shifts of carbons involved in linkages (i.e. C-3 carbons for B and C and C-2 carbon for the D sugar). The following interresidue NOEs were observed between (i) β-Antp (A) H-1 and α-Rhap (B) H-3 (very strong) and H-2, (ii) α-Rhap (B) H-1 and α-Rhap (C) H-3, and (iii) α-Rhap (C) H-1 and α-Rhap (D) H-2 (data not shown). Pairs of interresidue carbon-proton cross-peaks of (i) A/H-1 → B/C-3 and B/H-3 → A/C-1, (ii) B/H-1 → C/C-3 and C/H-3 → B/C-1, and (iii) C/H-1 → D/C-2 and D/H-2 → C/C-1 in HMBC spectra were also observed. Taken together, these observations indicated the linkages as Antp(A)β-(1→3)-Rhap(B)α-(1→3)-Rhap(C)α-(1→2)-α-Rhap(D).

**Absence of Anthrose in Other Spores**—B. cereus and B. thuringiensis are the species most closely related to B. anthracis (2, 24). To determine whether anthrose was a common component of spores of all three species, spores (10⁹) of each species were subjected to methanolyis, and the methyl glycosides of total spore sugars were analyzed by ESI-Q-TOF mass spectrometry in the positive ion mode. As controls, spores (10⁹) of aΔ mID strain of B. anthracis, which does not contain anthrose, and a purified sample of the 715-Da tetrasaccharide were hydrolyzed and analyzed in the same manner. The mass spectra for the four spore samples were normalized by assigning the generally invariant peak at 280 m/z, which is unrelated to anthrose, as 100% relative intensity. The mass spectra were then examined for parent and fragment ion peaks that were characteristic of anthrose, such as those at 292, 260, and 242 m/z (Fig. 11). Signals for these ions in the B. cereus and B. thuringiensis spectra were not above the low background levels of the ions in the ΔmID spectrum. In contrast, the signals for these ions were prominent in the wild-type B. anthracis spectrum. Furthermore, analysis of the methyl glycosides of total spore sugars by gas chromatography indicated that anthrose was present in spores of B. anthracis but absent in spores of B. cereus, B. thuringiensis, and B. subtilis (data not shown). Thus, anthrose appeared to be unique to B. anthracis spores.

**Discussion**

The glycoprotein BclA appears to form the outermost surface of the B. anthracis spore, which directly interacts with the soil environment and mammalian host defenses. These interac-

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**Fig. 10.** NMR analysis of anthrose. Shown is a portion of a gradient-HMBC spectrum showing the connectivities of the amido group side chain (CH₃)₂COH/CH₂CONH for the residue Antp. The spectrum was obtained without a low pass filter and without ¹³C decoupling during acquisition. See "Results" for additional details.

**Fig. 11.** Positive ion ESI-Q-TOF mass spectrum of the methyl glycosides of Bacillus spore sugars. Methyl glycosides were prepared from spores of the indicated strains and the 715-Da tetrasaccharide. Peaks correspond to anthrose-specific ions (e.g. 242, 260, and 292 m/z) and ions derived from other spore sugars (e.g. 248, 280, and 290 m/z). Based on NMR observations, we noticed that the tetrasaccharide appeared to undergo slow mutarotation in Me₂SO at its reducing terminal rhamnose residue (D) when left at 25 °C. Lowering the temperature (4 °C) favored the α anomer, and increasing the temperature shifted the equilibrium toward the β anomer.
tions are likely to be important for spore survival and pathogenicity, and understanding them will be necessary to develop improved methods for the prevention and treatment of anthrax. Presumably, the carbohydrate components of BclA contribute to the proper functioning of the spore surface. In this study, we identified two of the BclA carbohydrate components: a 715-Da tetrasaccharide and a 324-Da disaccharide. Multiple copies of the tetrasaccharide were shown to be O-linked to the collagen-like region of BclA, apparently through threonine residues present in the many XXQ repeats in this region. The attachment site of the disaccharide appears to be outside of the collagen-like region. The exact number of each oligosaccharide attached to BclA and the precise attachment sites on the protein remain to be determined. The BclA-associated oligosaccharides account for most of the carbohydrate in the exosporium, and they apparently are not linked to other spore proteins.

We also found that GalNAc is attached to BclA, although this sugar is not a component of the tetrasaccharide or disaccharide. We suspect that GalNAc serves as a linker between BclA and the two oligosaccharides. This possibility is consistent with the following observations. GalNAc is found on spores of a ΔrmlD mutant strain of *B. anthracis*, which is unable to synthesize the tetrasaccharide and disaccharide. We observed small amounts of a pentasaccharide and a trisaccharide following hydrazinolysis of purified exosporium of wild-type *B. anthracis* spores. The masses of these oligosaccharides are equal to those of the tetrasaccharide and disaccharide, respectively, with the addition of one GalNAc residue. The reducing end residue of an oligosaccharide can be lost by a “peeling” reaction during hydrazinolysis (39). This reaction is promoted by water and salt (40), both of which are present in spores and, to a lesser extent, in exosporium preparations. Thus, it appears reasonable that the tetrasaccharide and disaccharide described here were derived from a pentasaccharide and a trisaccharide containing GalNAc at their reducing ends.

A major goal of this study was to determine the complete structure of the BclA-associated tetrasaccharide. By using NMR, mass spectrometry, and other analytical methods, we showed that the structure is 2-O-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl. To our knowledge, the nonreducing terminal sugar of the tetrasaccharide (i.e. 2-O-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy-β-D-glucose) has not been described previously. We gave this novel sugar the trivial name anthrose. Anthrose was not found in previous studies of the monosaccharide composition of *B. anthracis* spores, because it was destroyed by the acid hydrolysis used to depolymerize glycoconjugates (36). We used methanolation rather than acid hydrolysis for this purpose, because methanolation is a milder procedure that converts anthrose to its methyl glycosides without degrading it.

The biosynthetic pathway of anthrose, including the origin of the 3-hydroxy-3-methyl-butaryl substituent has yet to be elucidated; however, one plausible biosynthetic scheme has the substituent derived from leucine. In this scheme, leucine is first converted to 3-hydroxy-3-methylbutyl-CoA through the sequential action of branched-chain amino acid transferase, branched-chain 2-oxo-acid dehydrogenase, isovaleryl-CoA dehydrogenase, and enoyl-CoA hydratase. This CoA carrier then transfers its substituent to dTDP-4-amino-4,6-dideoxy-α-D-glucose, derived from dTDP-4-keto-6-deoxy-α-D-glucose, an intermediate in the biosynthesis of rhamnose. All of the genes for the enzymes required for anthrose biosynthesis in this proposed pathway and also for the biosynthesis of the entire tetrasaccharide are present in the genome of *B. anthracis* (24). Many of these genes are located near bclA (12, 41). Several of the putative biosynthetic enzymes, including enoyl-CoA hydratase, the four L-rhamnose biosynthetic enzymes (encoded by rmlACBD), and numerous glycosyl transferases, are expressed during phase IV of sporulation, when the exosporium is synthesized (18).

The function of glycosylation of BclA remains to be determined. For other prokaryotic glycoproteins, several different functions for glycosylation have been proposed, including the maintenance of protein conformation, heat stability, surface recognition, resistance to proteolysis, enzymatic activity, cell adhesion, agglutination, ice nucleation, and immune evasion (22, 42). In the case of BclA, the proposed extensive glycosylation, especially within the collagen-like region, could contribute to the formation of an extended conformation that determines the length of the hairlike nap (23, 43). However, spores of a ΔrmlD derivative of the *B. anthracis* Sterne strain, which do not contain the tetrasaccharide or disaccharide, were shown by electron microscopy to have a hairlike nap that is equal in length to that of isogenic wild-type spores. Although glycosylation of BclA may not affect the length of the hairlike nap, it does appear to affect its porosity. Antibodies and peptide ligands that bind to elements of the basal layer of the exosporium, bind to ΔrmlD spores much better than they bind to wild-type spores. The effects of glycosylation on other properties of BclA and the *B. anthracis* spore are presently under investigation. Because a large number of genes and enzymes are devoted to BclA glycosylation and this extensive glycosylation occurs when the cell is starved for nutrients, it can be assumed that BclA is glycosylated for an important reason.

Finally, anthrose was not detected in spores of other *Bacillus* species, including those most closely related to *B. anthracis*. Thus, anthrose may be a useful marker for the rapid and specific identification of *B. anthracis* spores. This unique sugar may also provide a new target for spore inactivation by chemo-therapeutic agents, or it could be included in a vaccine designed to elicit an immune response to *B. anthracis* spores.

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**REFERENCES**

1. Mock, M., and Fouet, A. (2001) *Annu. Rev. Microbiol.* 55, 647–671
2. Priest, F. G. (1993) in *Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology, and Molecular Biology* (Sonenshein, A. L., Hoch, J. A., and Losick, R., eds) pp. 3–16, American Society for Microbiology, Washington, D. C.
3. Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J., and Setlow, P. (2000) *Microbiol. Mol. Biol. Rev.* 64, 548–572
4. Paidhungat, M., and Setlow, P. (2002) in *Bacillus subtilis and Its Closest Relatives: From Genes to Cells* (Sonenshein, A. L., Hoch, J. A., and Losick, R., eds) pp. 537–548, American Society for Microbiology, Washington, D. C.
5. Webb, G. F. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 4353–4356
6. Williams, D. D., Benedek, D., and Turnbough, C. L., Jr. (2003) *Appl. Environ. Microbiol.* 69, 6288–6293
7. Errington, J. (1993) *Microbiol. Rev.* 57, 1–33
8. Lai, E. M., Phadke, N. D., Kachman, M. T., Giorno, R., Vazquez, S., Vazquez, J. A., Maddock, J. R., and Driks, A. (2003) *J. Bacteriol.* 185, 1443–1454
9. Henriques, A. O., and Moran, C. P., Jr. (2000) *Methods* 29, 95–110
10. Gerhardt, P. (1967) *Fed. Proc.* 26, 1510–1511
11. Roels, S., and Losick, R. (1995) *J. Bacteriol.* 177, 6263–6275
12. Steichen, C., Chen, P., Kearney, J. F., and Turnbough, C. L., Jr. (2003) *J. Bacteriol.* 185, 1903–1910
13. Gerhardt, P., and Ribi, E. (1984) *J. Bacteriol.* 160, 1774–1789
14. Hashiba, Y., Kojima, K., and Sato, T. (1986) *J. Bacteriol.* 164, 2382–2384
15. Beaman, T. C., Pankratz, H. S., and Gerhardt, P. (1971) *J. Bacteriol.* 107, 320–324
16. Kramer, M. J., and Roth, I. L. (1968) *Can. J. Microbiol.* 14, 1299–1297
17. Mata, L. L., Beaman, T. C., and Gerhardt, P. (1970) *J. Bacteriol.* 101, 196–201
18. Liu, H., Bergman, N. H., Thomasen, B., Shallow, S., Hazen, A., Crosno, J., Ricke, D. A., Ravel, J., and Petren, S. N., Yates, J. I., and Hanna, P. C. (2004) *J. Bacteriol.* 186, 164–178

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*Oligosaccharides of B. anthracis Exosporium Glycoprotein BclA*
19. Todd, S. J., Moir, A. J. G., Johnson, M. J., and Moir, A. (2003) J. Bacteriol. 185, 3373–3378
20. Sylvestre, P., Couture-Tosi, E., and Mock, M. (2002) Mol. Microbiol. 45, 169–178
21. Sylvestre, P., Couture-Tosi, E., and Mock, M. (2003) J. Bacteriol. 185, 1555–1563
22. Schmidt, M. A., Riley, L. W., and Benz, I. (2003) Trends Microbiol. 11, 554–561
23. Jentoft, N. (1990) Trends Biochem. Sci. 15, 291–294
24. Read, T. D., Peterson, S. N., Tourasse, N., Bailie, L. W., Paulsen, I. T., Nelson, K. E., Tettelin, H., Fouts, D. E., Eisen, J. A., Moir, A. J. G., Johnson, M. J., and Moir, A. (2003) J. Bacteriol. 185, 3373–3378
25. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1989) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York
26. Koehler, T. M., Dai, Z., and Kaufman-Yarbray, M. (1994) J. Bacteriol. 176, 586–595
27. Sule, E., and Koehler, T. M. (2002) J. Bacteriol. 184, 370–380
28. Dai, Z. J.-C., Sirard, M., Mock, M., and Koehler, T. M. (1995) Mol. Microbiol. 16, 1171–1181
29. Perez-Casal, J., Caparon, M. G., and Scott, J. R. (1991) J. Bacteriol. 173, 2617–2624
30. Knurr, J., Benedek, O., Heslop, J., Vinson, R. B., Boydston, J. A., McAndrew, J., Kearney, J. P., and Turnbough, C. L., Jr. (2003) Appl. Environ. Microbiol. 69, 6841–6847
31. Nicholson, W. L., and Sethow, P. (1990) in Molecular Biological Methods for Bacillus (Harwood, C. R., and Cutting, S. M., eds) pp. 391–450, John Wiley & Sons, Ltd., West Sussex, UK
32. Leontein, K., Lindberg, B., and Lonngren, J. (1978) Carbohydr. Res. 62, 359–362
33. Patel, T., Bruce, J., Merry, A., Bigge, C., Wormald, M., Jaques, A., and Parekh, R. (1993) Biochemistry 32, 679–693
34. Duseh, Z., and Shettles, L. B. (1948) J. Biol. Chem. 175, 595–603
35. Bhatti, T., Chambers, R. E., and Clamp, J. R. (1970) Biochim. Biophys. Acta 223, 339–347
36. Fox, A., Black, G. E., Fox, K., and Rostovtseva, S. (1993) J. Clin. Microbiol. 31, 887–894
37. Kasai, R., Ohkura, J., Asakura, J., Mizutani, K., and Tanaka, O. (1979) Tetrahedron 35, 1427–1432
38. Altona, C., and Haasnoo, C. A. G. (1980) Org. Magn. Reson. 40, 417–429
39. Suzen, S., and Williams, M. (1999) J. Pept. Sci. 5, 283–286
40. Patel, T. P., and Parekh, R. B. (1994) Methods Enzymol. 230, 57–66
41. Fox, A., Stewart, G. C., Waller, L. N., Fox, K. F., Harley, W. M., and Price, R. L. (2003) J. Microbiol. Methods 54, 143–152
42. Moens, S., and Vanderleyden, J. (1997) Arch. Microbiol. 168, 169–175
43. Rasmussen, M., Jacobsen, M., and Bjørk, L. (2003) J. Bact. 186, 3213–3216
44. Williams, D. D., and Turnbough, C. L., Jr. (2004) J. Bacteriol. 186, 566–569
Novel Oligosaccharide Side Chains of the Collagen-like Region of BclA, the Major Glycoprotein of the Bacillus anthracis Exosporium

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