

Identification of a Second Collagen-Like Glycoprotein Produced by *Bacillus anthracis* and Demonstration of Associated Spore-Specific Sugars

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Certain carbohydrates (rhamnose, 3-O-methyl rhamnose, and galactosamine) have been demonstrated to be present in *Bacillus anthracis* spores but absent in vegetative cells. Others have demonstrated that these spore-specific sugars are constituents of the glycoprotein BclA. In the current work, spore extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A second collagen-like glycoprotein, BclB, was identified in *B. anthracis*. The protein moiety of this glycoprotein was identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MS) and the carbohydrate components by gas chromatography-mass spectrometry and tandem mass spectrometry. Spore-specific sugars were also demonstrated to be components of BclB.

Anthrax is a fatal disease caused by the gram-positive, rod-shaped bacterium *Bacillus anthracis*. The most lethal form of human anthrax is pulmonary (26). Inhaled spores are transmitted through the air and are deposited in the lungs. The spore is resistant to harsh environmental conditions, including exposure to ultraviolet light, heat, desiccation, and organic solvents (39). In vitro spores are engulfed by alveolar macrophages, where they germinate, although it remains to be demonstrated whether germination must occur within macrophages for induction of anthrax in vivo (5, 12, 13, 26). The outer surfaces surrounding the spore, including the coat and exosporium (1, 16, 19, 24, 29, 30), might play a role in environmental protection and/or pathogenesis.

During sporulation, the vegetative cell divides asymmetrically and the mother cell engulfs the smaller cell that becomes the forespore. The spore cortex, unlike vegetative peptidoglycan, has not been shown to possess a covalently bound polysaccharide. Furthermore, muramic acid is found in both the cortex and vegetative peptidoglycan, but muramic acid delta lactam is exclusive to the spore (34). In the vegetative cell, S protein and poly-D-glutamic acid capsule constitute the outermost layers. However, in the spore, the coat and exosporium surround the cortex (6). After the spore is formed, autolytic enzymes digest the vegetative mother cell and the spore is released.

The spore surface is characterized by an external exosporium consisting of a basal layer surrounded by a nap of hair-like projections (14, 15). The first spore surface protein, BclA (*Bacillus*, collagen-like protein of *anthracis*), was discovered in *B. anthracis* (29, 30). BclA is localized to the exosporium nap

as demonstrated by monoclonal antibody labeling. The peptide backbone has a predicted size of approximately 39 kDa, but the intact protein migrates with an apparent mass of >250 kDa for the Sterne strain, which is consistent with its being heavily glycosylated (30). It is entirely possible that there may be additional protein modifications. The genetic region coding for the peptide backbone varies in length among strains since it codes for variable numbers of collagen-like tandem repeats (31).

Other exosporium glycoproteins, ExsJ and ExsH, have been identified in *Bacillus thuringiensis* (10) and *Bacillus cereus* (3, 18, 32). Both ExsJ and ExsH have both been reported to migrate, during electrophoresis, as 205-kDa species. Within the spores of *B. thuringiensis*, rhamnose, galactosamine, and a third unidentified sugar were shown to be components of ExsJ. ExsH has a region of collagen-like repeats, like ExsJ, but the carbohydrate composition of ExsH has not been determined. ExsH-like proteins have not been previously identified in *B. anthracis*.

The spores of *B. anthracis* (8) and related bacilli (38) contain carbohydrate monomers that are absent in vegetative cells. Spores and isolated exosporium contain two rhamnose oligosaccharides, penta- and trisaccharides, respectively (4). Both contain the spore-specific sugars 3-O-methyl rhamnose, rhamnose, galactosamine, and anthrose (4). The exosporium of BclA⁻ mutants contains much lower levels of these carbohydrates. This clearly demonstrates that BclA is the major spore glycoprotein. However, it is entirely possible that another minor glycoprotein may have not been detected in these studies. Mutants that have *BclA* genes coding for BclA with shortened tandem repeat regions contain proportionately less of the pentasaccharide in the exosporium, although the amount of the trisaccharide is unaffected (4). It has been reported that the trisaccharide may not be linked to the repeat region (4) or alternatively it is suggested here that it may be present in a second glycoprotein.

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In the current report, it is demonstrated that the anthrax spore contains a second collagen-like glycoprotein, having tandem repeats as are found in BclA and ExsH. Since it is demonstrated here that spore-specific sugars previously shown to be a component of BclA of *B. anthracis* are also found in this newly discovered protein, we have chosen to name it BclB.

MATERIALS AND METHODS

Growth conditions and preparation of cells for analysis. *Bacillus anthracis* strain ΔSterne-1 was a gift from S. H. Leppla (National Institutes of Health, Bethesda, MD). ΔSterne-1 is a strain derived from Sterne and lacking pXO1 and pXO2. Generally growth was on nutrient agar plates (Difco, Detroit, MI), and vegetative cells were harvested within 20 h before sporulation occurred (i.e., >99% vegetative). Sporulation was essentially complete after 48 h (>95%). Samples were stored frozen overnight and then thawed. The spores were then incubated at room temperature for 1 to 2 h to lyse remaining vegetative cells, when preparations were >99% spores. The degree of sporulation was assessed by either malachite green staining or phase contrast microscopy.

The spores or vegetative cells were harvested by centrifugation, washed three times in distilled water, and stored at -70°C . For additional purification of spores, primarily to remove vegetative debris, the procedure described by Nicholson and Setlow (20) was used with modification (33). Washed spores (25 mg) were suspended in a 70% Renografin (Sigma Aldrich, St. Louis, MO) solution to a final concentration of 50%. The Renografin spore suspension was placed on ice for 1 h then layered on top of the thawed gradient and centrifuged at $5,000 \times g$ for 45 min. A stock solution (75% solution of Renografin) was first used to generate a series of dilutions (varying from 45% to 55%). Each layer was frozen at 70°C for 10 min before the next layer was applied. In early experiments, cells were grown in nutrient broth (Difco) and shaken at 37°C . Vegetative cells were harvested after 6 to 12 h growth and spores were essentially absent. Spores were harvested after 7 to 10 days growth, when cultures were 90 to 95% spores.

Ultrastructural characterization of exosporium. To the buffer-washed spores or urea-extracted spores (see section below for details), 1 ml of a 2.5% glutaraldehyde, 0.1 M sodium cacodylate solution containing 0.1% ruthenium red (Electron Microscopy Sciences, Fort Washington, PA) was added and incubated for 1 hour at 37°C . Each pellet was then washed in sodium phosphate buffer (PBS, Fisher Scientific, Norcross, GA) and fixed for 3 h at room temperature in a 2% osmium tetroxide (Electron Microscopy Sciences), 0.1 M sodium cacodylate solution containing 0.1% ruthenium red. A negative control was treated identically, but ruthenium red was omitted from these two steps.

Spores were washed in buffer and embedded in 3% agar (EM Science, Gibbstown, NJ). Dehydration involved sequential treatment with 25, 50, 75, 95, and 100% ethanol (AAPER Alcohol & Chemical Co., Shelbyville, KY). Afterwards, cells were placed sequentially in propylene oxide (Electron Microscopy Sciences), propylene oxide/Polybed 812 (2:1) and pure Polybed 812 (Polysciences, Warrington, PA). Polymerization was carried out at 60°C in pure Polybed. Specimen blocks were thin-sectioned (≈ 120 nm thick) and then collected on 200-mesh copper grids, and electron microscopy sections were cut and then stained with a 2% uranyl acetate solution (Electron Microscopy Sciences) for 40 min at 37°C . The sections were then treated with Hanaichi lead citrate (0.15% lead nitrate, 0.15% sodium acetate, 1% sodium citrate dissolved in 41 ml water and 9 ml of 1 N sodium hydroxide [Fisher Scientific]) for 2 min. Spores were observed by transmission electron microscopy (33).

Analysis of glycoproteins using SDS-PAGE. We extracted 50 mg (wet weight) of *B. anthracis* spores with a urea buffer (50 mM Tris-HCl, pH 10, 8 M urea, 2% 2-mercaptoethanol) (Fisher Scientific) for 15 min at 90°C . The extracted spores were centrifuged at $13,000 \times g$ for 10 min at room temperature. The supernatant was removed and filtered by centrifugation through Microcon filters (>100 -kDa cutoff, Millipore, Bedford, MA). The Microcon filter holders were filled with PBS and centrifuged almost to dryness. This was repeated two additional times, and the retentate was stored for protein analysis. In some experiments, glycoproteins were additionally deglycosylated with trifluoromethanesulfonic acid (TFMS). Glycoproteins were lyophilized, and anisole:TFMS (1:9 ratio) (Fisher Scientific) was added to the samples. Samples were flushed with N_2 and vacuum in order to provide an anhydrous environment for deglycosylation.

After 72 h deglycosylation at 4°C , the reaction was quenched by adding a twofold excess of ice-cold ether (Fisher Scientific) followed by dropwise addition of an equal volume of ice-cold 50% aqueous pyridine (Alltech, Deerfield, IL). In order to recover the deglycosylated proteins, the ether layer was discarded, and the ether extraction was repeated in order for further cleanup of samples. Spore protein extracts were combined with loading buffer (50 mM Tris-HCl, pH 6.8,

4% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β -mercaptoethanol, 0.02% bromophenol blue [Fisher Scientific]), loaded onto each lane of 4 to 15% gradient polyacrylamide gels (Jule, Inc., Milliford, CT), and electrophoresed in Tris-glycine-SDS buffer (Fisher Scientific). The gels were stained with a Gelcode Blue staining kit (Pierce, Rockford, IL).

Prior to glycoprotein analysis, gels were equilibrated in cathode buffer (25 mM Tris, pH 9.4, 40 mM glycine, 0.01% SDS, 20% methanol) (Fisher Scientific). For glycoprotein staining, the proteins in the gel were transferred onto a polyvinylidene difluoride membrane (Millipore) using a semidry transfer apparatus (Millipore). The membranes were processed using the ECL glycoprotein detection system (Amersham Biosciences, Piscataway, NJ) to identify glycoprotein bands. In preparative experiments, one gel lane was processed using the ECL glycoprotein detection system (Amersham Biosciences) to identify the glycoprotein band and the other eight lanes left unstained. The membrane was incubated in PBS for 10 min and then 20 min in the dark in 100 mM sodium metaperiodate (Amersham Biosciences) and 100 mM acetate buffer, pH 5.5 [Fisher Scientific]. The membrane was washed and then incubated for 60 min in a 24 nM solution of biotin hydrazide in 100 mM acetate buffer, pH 5.5. The membrane was washed again in PBS and then placed in blocking agent overnight at 4°C . The blocked membrane was washed in PBS and incubated in a 1:6,000 dilution of streptavidin-horseradish peroxidase conjugate for 30 min. The filter was then washed with PBS, exposed to enzyme substrate (ECL Western Blotting Detection System) for 1 min, and placed against autoradiographic film (Hyperfilm ECL, Amersham Biosciences) and exposed for 2 min.

Analysis of the protein moiety of glycoproteins using MALDI-TOF MS. After Coomassie blue staining of gels as described above, the appropriate bands were excised from the gel with a razor blade and destained with a 50:50 mixture of acetonitrile (Fisher Scientific) and 25 mM ammonium bicarbonate (Sigma). A blank gel piece was similarly treated as a negative control. After destaining the gel pieces were dehydrated with 100% acetonitrile for 10 min and then dried under N_2 . Next, the gel pieces were incubated for 30 min in 10 mM dithiothreitol (Sigma Aldrich). The dithiothreitol solutions were removed and the pieces were incubated in iodoacetamide (Sigma Aldrich) for 30 min. The gel pieces were then washed with 100 mM ammonium bicarbonate solution for 10 min followed by two dehydrations with acetonitrile. The gels were rehydrated with 100 mM ammonium bicarbonate for 10 min. Finally, the gel slices were again dehydrated twice using acetonitrile and allowed to dry under N_2 .

The gel slices were prepared for enzymatic digestion by rehydration with 100 mM ammonium bicarbonate for 10 min. The rehydration solution was removed and then a solution of 0.4% pepsin (Sigma Aldrich) in 10 mM HCl was added. The digestion occurred for approximately 90 min at 37°C . Trypsin is the most commonly used enzyme for protein digestion prior to mass spectrometry (MS) analysis. However, there are very few trypsin sites in ExsH-like proteins. After digestion the samples were then spotted on a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) target plate along with the MALDI matrix α -cyano-4-hydroxycinnamic acid (HCCA) (Sigma Aldrich). A Micromass Biolyne (Waters Corporation, Beverly, MA) linear time-of-flight instrument was used for mass spectrometry analysis. The resulting mass spectra were interpreted using Protein Prospector software (<http://prospector.ucsf.edu>).

Analysis of the carbohydrate portion of glycoproteins using GC-MS and GC-MS-MS. Polyvinylidene difluoride membranes were washed three times in PBS and then water. The BclA and other bands were excised with a scalpel blade and placed in 2 N sulfuric acid (Fisher Scientific). Four lanes were pooled (140 μg) and analyzed in duplicate. For comparison, 10 mg of spores was also analyzed in duplicate. The samples were then heated at 100°C for 3 h. Arabinose and methylglucamine (Sigma Aldrich) were used as internal standards (500 ng for bands, 50 μg each for spores). For neutralization and hydrophobic prederivatization clean-up, samples were neutralized with 50% *N,N*-diethylmethylamine (Sigma Aldrich) in chloroform, followed by aqueous phase extraction using C-18 columns (Agilent Technologies, Wilmington, DE).

Samples were reduced overnight with sodium borodeuteride (Sigma Aldrich) at 4°C . Borodeuteride was removed, as tetramethylborate gas, by multiple methanol-acetic acid (200:1) (Fisher Scientific) evaporations under nitrogen. Samples were then dried under vacuum for 3 h at 60°C . The alditols were acetylated by heating at 100°C for 15 h with acetic anhydride (Alltech, Deerfield, IL). Hydrophilic postderivatization clean-up included acid and alkaline extractions. Gas chromatography-mass spectrometry (GC-MS) analysis was carried out with a mass-selective detector in electron impact mode (Hewlett Packard model 5970; Agilent) equipped with an automated sample injector and a DB5ms fused-silica capillary column (Agilent). Gas chromatography-tandem mass spectrometry (GC-MS-MS) was performed using an ion trap tandem mass spectrometer (GCQ, Finnigan, Atlanta, GA). Ionization was performed in electron impact mode followed by collision-induced dissociation and multiple reaction monitoring (8).

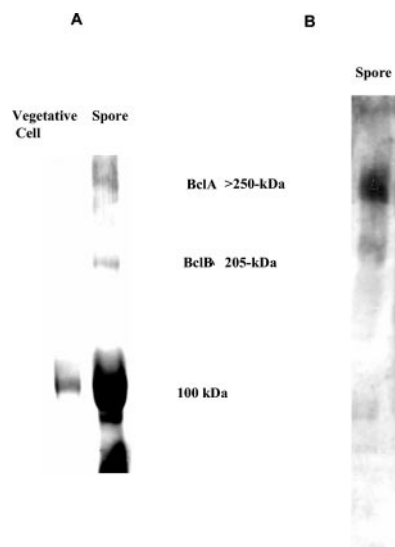


FIG. 1. (A) Vegetative cell and spore proteins separated by electrophoresis and stained for proteins with Gel Code Blue reagent. (B) The gel shows spore extracts separated by electrophoresis and stained for glycoproteins.

RESULTS

The primary goal was to identify a second glycoprotein produced by *B. anthracis*. Once this was established, then the second goal was to determine whether spore-specific sugars (rhamnose, 3-*O*-methyl rhamnose, and galactosamine) (8, 38) are components of this glycoprotein. Others have determined the carbohydrate composition of BclA, the first glycoprotein discovered in *B. anthracis* (4), but a second collagen-like glycoprotein has not previously been identified in this organism.

On electron microscopy observation of untreated spores, after ruthenium red staining, the exosporium is observed as a distinct carbohydrate-rich layer surrounding the underlying coat. After urea treatment the exosporium layer was disrupted but the spore remained otherwise intact. In these studies, cells were grown in nutrient broth. This avoided the possibility of agar, which consists largely of carbohydrate, adhering to spore surfaces and affecting electron microscopy morphology.

An approach was taken to focus on spore-specific glycoproteins in the urea extract. Spores and vegetative cells (each >99%, agar grown) were extracted with urea and their protein and glycoprotein profiles compared. In each case, bands of >250 kDa (previously identified as BclA) (29, 30) and 205 kDa (not previously identified in *B. anthracis*) were observed in spores but were absent in vegetative cells. The 250-kDa species was by far the most abundant spore protein (see Fig. 1). The gels were stained with a Gelcode Blue staining kit that stained both BclA and the 205-kDa bands. BclA does not stain with conventional Coomassie blue, as noted by others (29). Spores were further purified using Renografin gradients and both bands were still present. On deglycosylation the two bands disappeared and were replaced by two new smaller bands (83 and 71 kDa, respectively; data not shown).

Others have previously identified the >250-kDa band by N-terminal sequencing as BclA (30). The excised 205-kDa bands were subjected to in situ digestion with pepsin and re-

leased peptides were analyzed by MALDI-TOF MS. Five peptides had measured masses predicted to be generated from an ExsH-like protein coded by the genome of *B. anthracis* strain Sterne (hypothetical protein BAS2281); measured: 991.5, 1034.1, 1194.8, 1222.7; 1264.3; predicted: 992.0, 1034.2, 1192.4, 1221.5, 1263.4 (see Fig. 2). These masses would be consistent with peptide sequences TGPTGTTGATGA or TGATGPTGTTGA, TILPGVVVDY, MKQNDKLWL (methionine oxidized), TPVQIQMQIF (methionine oxidized), and TGVGTGA TGITGVTGA, respectively.

Using GC-MS, 3-*O*-methyl rhamnose, rhamnose, and galactosamine were confirmed to be components of isolated BclA, as demonstrated by others (4). GC-MS-MS was needed for definitive identification of sugars in ExsH-like bands, as BclB is present at such low concentrations. The parent ion (MS-MS spectrum) for rhamnose is mass 231 and the major fragments are 189 (loss of ketene, mass 42) and 129 (loss of 60). The presence of 3-*O*-methyl rhamnose in spore 205-kDa bands was also confirmed. The parent ion for 3-*O*-methyl rhamnose is 203 kDa and the major fragments are 143 kDa (loss of acetic acid) and 101 kDa (loss of ketene). The major masses, 143 and 101 Da, were prominent (in the peak at the retention for 3-*O*-methyl rhamnose) in the hydrolysate of the 205-kDa bands. Galactosamine was also demonstrated to be present in the 205-kDa bands; 53 ng rhamnose, 4 ng of 3-*O*-methyl rhamnose and 42 ng of galactosamine were present in the combined 205-kDa bands, an order of magnitude lower than in the BclA bands. Thus, we can clearly state that these spore-specific sugars are also derived from the 205-kDa species. In conclusion, rhamnose, 3-*O*-methyl rhamnose, and galactosamine are also components of the 205-kDa bands (ExsH-like protein).

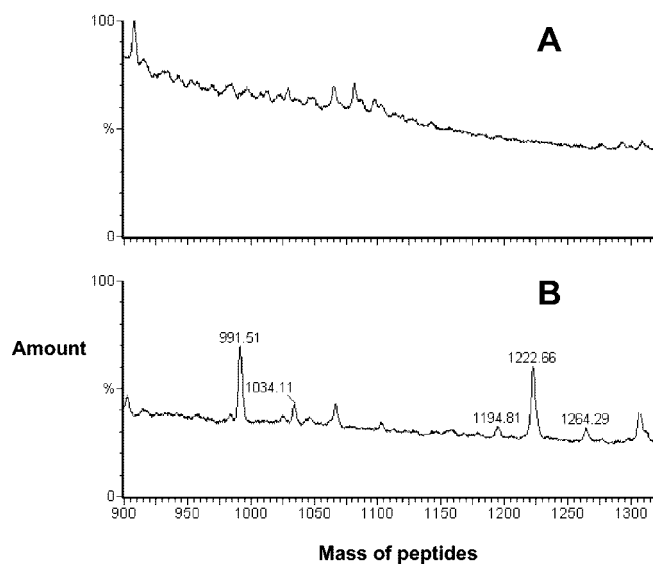


FIG. 2. Size of pepsin-digested bands in (A) control and (B) 205-kDa protein. The analysis was performed by MALDI-TOF MS. Five peptides are present that have measured masses similar to those predicted to be produced from the anthrax ExsH-like protein (BclB). Measured: 991.5, 1034.1, 1194.8, 1222.7; 1264.3; predicted: 992.0, 1034.2, 1192.4, 1221.5, 1263.4.

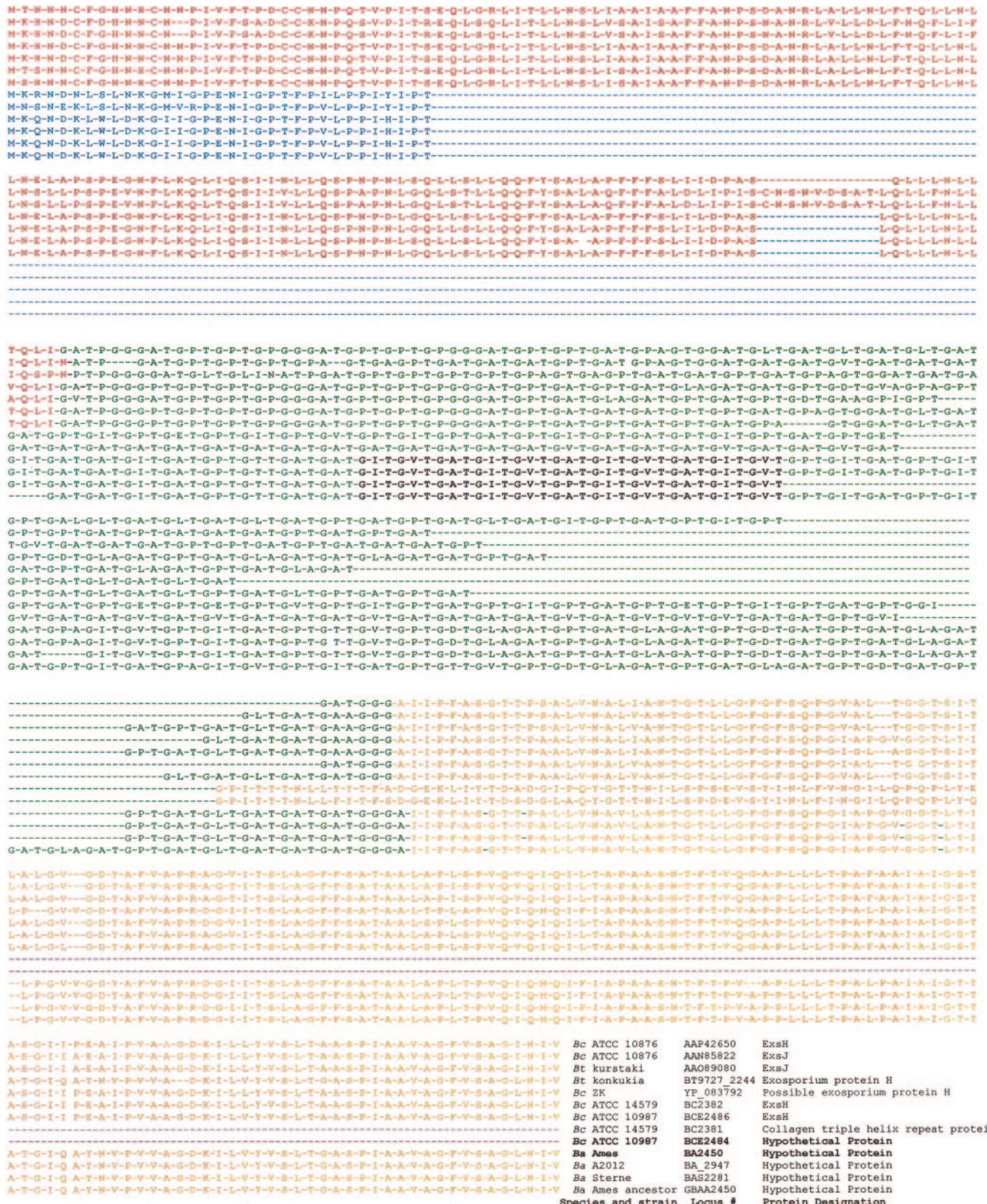


FIG. 3. Sequences of ExsH-like proteins, including BclB, of *B. anthracis* (*Ba*), *B. cereus* (*Bc*), and *B. thuringiensis* (*Bt*). The N terminus is marked in blue, the GXX repeat region is in green, and the C terminus is in orange. BclB repeat regions (GITGVIGAT) are marked in black.

DISCUSSION

In the current report, it is demonstrated that the *B. anthracis* spore contains a second collagen-like glycoprotein, BclB. Furthermore, it is demonstrated that spore-specific sugars are also components of this glycoprotein. We have previously shown that rhamnose, 3-*O*-methyl rhamnose, and galactosamine are readily detected in spores but not vegetative cells of *B. anthracis* (8). Others subsequently demonstrated that the spore-specific sugars (galactosamine, rhamnose, and 3-*O*-methyl rhamnose, along with anthrose) are components of BclA (4).

BclA from the Sterne strain migrates in SDS-PAGE gels with an apparent size of >250 kDa (29, 30). Previously, others reported, after partial deglycosylation of BclA, an 80-kDa band was observed (30). The presence of a second partially deglycosylated band of 71 kDa is described here and presumably derived from BclB. BclA and BclB (hypothetical protein BAS2281 of the Sterne strain) would be predicted, from their sequences, to have protein backbones 39 and 32 kDa in size, respectively. There is considerable size heterogeneity among the BclA proteins due to different numbers of GPT repeats and [GPT]₅GDTGTT repeats in the protein. The 21-amino-acid repeat has been named the BclA repeat (31). These repeats are believed to be the primary anchor point for rhamnose oligosaccharides within BclA (4). Size diversity in BclB proteins has not been studied, but from the genomic sequences, BclB coded by the Ames strain (hypothetical protein GBBA2450) contains six GXX repeats not present in Sterne (hypothetical protein BAS2281).

A *B. anthracis* 205-kDa glycoprotein has not been previously identified. A 205-kDa glycoprotein isolated from the exosporium of *B. thuringiensis* has the N-terminal sequence MKHN DXF (10). A 205-kDa glycoprotein has also been identified in *B. cereus* with the N-terminal sequence MKHNDCFXHNCC NPIVF (32). The region referred to as *exsH* was sequenced and codes for a collagen-like domain primarily of GATs, with a few GPT repeats (32).

Using the sequences of Todd and coworkers (32), a BLAST search was performed for all additional related sequences coded by the genomes of *B. anthracis*, *B. cereus*, and *B. thuringiensis* (see Fig. 3). Thirteen proteins were found in all which contained GXX repeat sequences related to ExsH. The N termini of seven Exs-H like proteins (including the four found in *B. anthracis*) showed homology. The C termini of 11/13 proteins (including all four *B. anthracis* proteins) also shared homology. There is also a BclB repeat (in analogy to the BclA repeat), GITGVTGAT, not found in *B. cereus* or *B. thuringiensis* proteins. It appears that the anthrax ExsH-like protein might have initially resulted from a recombination event which merged the N and C termini from the two types of *exs-H* like genes derived from a strain of *B. cereus* or *B. thuringiensis* and created a unique gene, *bclB* (*Bacillus* collagen-like protein gene B). In the current work, using MALDI TOF MS analysis, five peptides were identified in pepsin digests of the 205-kDa band (Sterne strain) that would be consistent with masses predicted to be generated from an ExsH-like protein coded by the genome of *B. anthracis* strain Sterne (protein BAS2281).

A gene cluster consisting of 11 open reading frames has been identified that contains the BclA glycoprotein structural protein (*orf4*) (9). Several of these *B. anthracis* genes show homol-

ogy with those of *Aneurinibacillus thermoaerophilus* which are involved in synthesis of rhamnose and 3-*O*-methyl rhamnose, which are also components of a cell surface glycoprotein (27). An ExsH glycoprotein gene synthesis cluster has not yet been identified.

The two well-established anthrax pathogenesis factors are the tripartite toxin (22) and the vegetative cell polyglutamic acid capsule (11, 21). It has been suggested that the current vaccine against the protective antigen component of lethal toxin may not be fully protective (2, 7, 17, 35–37). Recent reports have focused on the need for a conjugate vaccine, additionally targeting the vegetative cell (23, 28). Indeed, a possible candidate conjugate vaccine consisting of polyglutamic acid and protective antigen is quite reasonable and deserves exploration. However, there is good evidence that the protective antigen vaccine does not provide complete protection in animal models and survival is substantially increased by the inclusion of formalin-fixed spores on immunization (2). Thus, other candidates for inclusion in a conjugate vaccine might be spore proteins, including BclB.

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