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Bacillus anthracis* Surface-Layer Proteins Assemble by Binding to the Secondary Cell Wall Polysaccharide in a Manner that Requires *csaB* and *tagO

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Abstract

Bacillus anthracis, the causative agent of anthrax, requires surface (S)-layer proteins for the pathogenesis of infection. Previous work characterized S-layer protein binding via the surface layer homology domain to a pyruvylated carbohydrate in the envelope of vegetative forms. The molecular identity of this carbohydrate and the mechanism of its display in the bacterial envelope are still unknown. Analyzing acid-solubilized, purified carbohydrates by mass spectrometry and NMR spectroscopy, we identify secondary cell wall polysaccharide (SCWP) as the ligand of S-layer proteins. In agreement with the model that surface layer homology domains bind to pyruvylated carbohydrate, SCWP was observed to be linked to pyruvate in a manner requiring *csaB*, the only structural gene known to be required for S-layer assembly. *B. anthracis* does not elaborate wall teichoic acids; however, its genome harbors *tagO* and *tagA*, genes responsible for the synthesis of the linkage unit that tethers teichoic acids to the peptidoglycan layer. The *tagO* gene appears essential for *B. anthracis* growth and complements the *tagO* mutant phenotypes of staphylococci. Tunicamycin-mediated inhibition of TagO resulted in deformed, S-layer-deficient bacilli. Together, these results suggest that *tagO*-mediated assembly of linkage units tethers pyruvylated SCWP to the *B. anthracis* envelope, thereby enabling S-layer assembly and providing for the pathogenesis of anthrax infections.

Keywords

Bacillus anthracis; secondary cell wall polysaccharide; surface-layer; pyruvylation; *tagO*

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Supplementary Data

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Introduction

Ingestion, inoculation, or inhalation of *Bacillus anthracis* spores can lead to anthrax disease in mammalian hosts.¹ Typically, anthrax is a disease of herbivorous mammals, capable of producing infection in humans exposed to spores via contaminated animal products or, more recently, by acts of bioterrorism.² The envelope of *B. anthracis* is a dynamic organelle composed of peptidoglycan, lipoteichoic acid, polysaccharides, proteins, and poly- γ -D-glutamic acid capsule.³ These surface molecules must interact directly with host cells and tissues during all stages of infection. Within the envelope, anthrax bacilli synthesize and assemble a surface (S)-layer, a paracrystalline lattice of protein that covers the entire cell surface.³ Although many microbes elaborate an S-layer, *B. anthracis* provides a model system for the study of S-layer assembly in Gram-positive bacteria, laying groundwork for understanding how these molecules are immobilized within the envelope.

S-layers in *B. anthracis* comprise two secreted proteins: Sap and EA1, hypothesized to bind the murein sacculus non-covalently by virtue of three conserved tandem repeats of a surface layer homology (SLH) domain,^{4,5} which specifically engages a pyruvylated cell wall polysaccharide.⁶ An N-terminal signal peptide and three tandem copies of the SLH domain are sufficient for the secretion and immobilization of proteins onto the surface of *B. anthracis* via this mechanism.⁷ In addition to the Sap and EA1 proteins, 22 other *B. anthracis* genes (*bslA*–*bslU*) encode both a signal peptide and three SLH domains and are therefore potentially surface-displayed by a similar mechanism.⁸

Directly adjacent to *sap* and *eag*, the structural genes for the Sap and EA1 S-layer proteins, is positioned a two-gene operon encoding *csaAB* (cell surface attachment). *csaB* is essential for S-layer assembly, as *csaB* mutants secrete both Sap and EA1 but fail to retain either on the cell surface.⁶ *csaB*-dependent binding of SLH domains to the cell wall has also been observed in *Thermus thermophilus*, another microbe that elaborates S-layers.⁹ Presumably, bacilli deficient in *csaB* are incapable of properly retaining any SLH protein and, consequently, secrete these molecules into the extracellular environment. The predicted primary translation product of the *csaB* open reading frame displays homology to exopolysaccharide pyruvyl transferases of other bacteria, suggesting a role for *csaB* in synthesizing the pyruvylated polysaccharides.⁶ Purified polysaccharides from *B. anthracis* Sterne bind the SLH domains of Sap and EA1, whereas analogous polysaccharides purified from *csaB* cells do not.⁶ When analyzed by NMR spectroscopy, the only differences between the two polymers were methyl proton chemical shifts correlated with ketal carbon and carboxylic carbon chemical shifts.⁶ Similar shifts are documented in spectra of polysaccharides purified from other S-layer-containing organisms, suggesting a conserved structure.^{10,11} These chemical shifts are diagnostic of ketal pyruvate modification of these polysaccharides and, by extension, assign a role for CsaB in this process.

To date, no further structural information regarding the pyruvylation of *B. anthracis* polysaccharides has been presented. However, the structure of the major secondary cell wall polysaccharide (SCWP) has been determined for various *B. anthracis* and *Bacillus cereus* strains via linkage analysis and NMR spectroscopy.^{12,13} Atomic-level resolution of the SCWP revealed structural differences, which confer the exquisite specificity observed

between the *B. anthracis* and *B. cereus* SCWP-binding phage lysins.¹⁴ Nevertheless, the published structure did not provide evidence of pyruvate modifications to these polysaccharides, leaving the identity of the polymeric SLH ligand unknown.

Given the published reports, placing ketal pyruvate on polysaccharides of various S-layer-containing bacteria^{6,9} and the similarity between the proposed structures of the SCWP from *Geobacillus stearothermophilus* PV72/p2 (including a ketal pyruvate) and *B. anthracis*,^{11,12} we sought to purify SLH-binding polysaccharides to test the hypothesis that these molecules contain pyruvate. Towards this end, we developed a fluorescence-based measurement of SLH protein deposition onto the cell walls of anthrax bacilli and used this system to assay purified components of the cell wall for their ability to bind SLH domains. This approach makes no assumption as to the composition of the SLH ligand, but rather examines a variety of constituent cell wall molecules for their ability to bind SLH domains. Hydrofluoric-acid-extractable polysaccharides (HF-PS) bind the SLH domain, which is consistent with previous studies. These SLH ligand polysaccharides exhibit mass spectra identical with the published structural models for the *B. anthracis* SCWP.¹² High-resolution mass measurement and tandem mass spectrometry (MS) of these carbohydrates unequivocally confirmed their identity as the SCWP and also provided evidence for greater structural homogeneity than previously appreciated. Importantly, the mass spectra of SCWP, purified from wild-type Sterne cells, contain multiple additional ions not observed in *csaB* strains. These ions arise from pyruvylated forms of the SCWP, providing the first direct evidence linking ketal pyruvate to the SCWP structure and unifying studies of S-layer assembly and SCWP structure. These data expand the roles of SCWP to include SLH protein retention and S-layer assembly in *B. anthracis*.

Many Gram-positive organisms are known to elaborate an SCWP and we sought to identify the genetic pathway responsible for SCWP production. TagA and TagO are glycosyl transferases that synthesize the cell wall linkage unit of wall teichoic acid (WTA).^{15,16} Genetic linkage between *tagA* and *csaB* homologs in multiple organisms and the lack of WTA in the *B. anthracis* envelope prompted the hypothesis that TagO and TagA play essential roles in the synthesis of SCWP. We demonstrate that *B. anthracis* cells grown in the presence of the TagO-specific inhibitor tunicamycin display aberrant forms and no longer bind SLH domains. This implies a novel function of the *tag* machinery in *B. anthracis*: synthesis of the SCWP. Taken together, these data promote understanding of S-layer assembly, SCWP function, and TagO activity in the assembly of SCWP molecules.

Results

csaB mutants no longer retain SLH proteins

Previous studies reported that *B. anthracis* strains deficient in *csaB* are incapable of retaining the Sap and EA1 S-layer proteins.⁶ To examine the contribution of *csaB* to S-layer assembly, we constructed an in-frame deletion of the entire *csaB* open reading frame in *B. anthracis* Sterne. As expected, strains lacking the *csaB* gene grew aberrantly, producing large masses of cells that rapidly settled out of liquid culture (data not shown).⁶ To determine if this retention-deficient phenotype includes all SLH proteins, we examined the entire protein profile of SDS-extracted culture sediments by SDS-PAGE and Coomassie

blue staining and by immunoblotting for BslA, an SLH adhesin.⁸ Whereas extracts of Sterne culture sediments contain abundant species corresponding to Sap, BslA, and presumably other SLH proteins, these species no longer sediment with bacilli in *csaB* cultures (Fig. 1a). Proteins precipitated from the supernatants of these cultures appeared similar (data not shown). Transformation of the *csaB* strain with *pcsaB*, a plasmid containing the *csaB* open reading frame, complements this defect in trans, restoring the profile of sedimented, SDS-extracted proteins whereas transformation with the empty vector, pLM5, does not (Fig. 1a). Immunoblots using BslA-specific antisera to probe proteins extracted from culture sediments and supernatants confirmed that these strains still produce and export SLH proteins. As expected, immunoreactive species are detected in the supernatant of all cultures (all SLH proteins are shed from the cell surface into the media supernatant over time) but only cells harboring the *csaB* gene contain immunoreactive species in the sediment (Fig. 1a).

As all previous measurements of SLH retention have relied on SDS-PAGE and immunoblot analysis of whole cultures,⁶ we engineered a reporter protein whose *in vitro* assembly onto the cell surface could be visualized microscopically and fluorometrically. To verify that fluorescent reporter hybrids to SLH proteins are tolerated for cell wall binding, we inserted the *mCherry* open reading frame directly before the TAA stop of *eag* on the *B. anthracis* chromosome, generating Sterne *eag-mCherry*. Fluorescent micrographs of late-exponential and stationary phase cultures of this strain stained with the double-stranded-DNA-specific dye Hoechst reveal abundant red fluorescence surrounding most cells (Fig. 1b). In contrast, *csaB* strains carrying this hybrid do not exhibit any red fluorescence on the cell surface (Fig. 1b). These microscopy data not only corroborate observations reported for whole culture extracts but also enable us to follow the trafficking of specific substrates to the cell envelope. More importantly, these data provide proof of principle for the use of fluorescence molecules as reporters of SLH–cell wall binding.

Recombinant Sap_{SLH}–mCherry binds cell walls from strains harboring *csaB*

Given that S-layer protein–mCherry hybrids are assembled on the cell surface *in vivo*, we sought to develop an *in vitro* assay to measure the deposition of fluorescent SLH proteins on the *B. anthracis* cell wall. Such an assay would allow screening of cell wall extracts for their ability to compete for SLH–cell wall binding and, consequently, identify fractions containing the SLH ligand. To accomplish this, we purified decahistidyl-tagged mCherry or Sap_{SLH}–mCherry, a translational hybrid between the SLH domains of the *B. anthracis* S-layer protein Sap and mCherry from *Escherichia coli*. Nickel affinity purification yields abundant quantities of both recombinant proteins with comparable fluorescence yields (Fig. 2a). To measure binding of the SLH domains to the cell wall, we incubated equimolar concentrations of mCherry or Sap_{SLH}–mCherry with equal numbers of *B. anthracis* cells, which had been stripped of all endogenous non-covalently bound proteins by boiling in urea. After incubation, the suspensions were centrifuged and the sediment was washed extensively and then imaged microscopically. Cells of *B. anthracis* Sterne incubated with Sap_{SLH}–mCherry were abundantly stained, whereas incubation of these walls with mCherry alone displayed no staining (Fig. 2b). In contrast, cell wall material from *B. anthracis csaB* showed no labeling by either fluorescent protein unless transformed with the complementing *pcsaB* plasmid (Fig. 2b). Thus, Sap_{SLH}–mCherry binds to the cell wall of bacilli *in vitro*

dependent on the presence of both the SLH domain and the *csaB* gene, recapitulating requirements known from *in vivo* studies and validating it as a measurement of SLH assembly in the *B. anthracis* envelope. Development of this assay provided a tool with which to quantify binding of the SLH domain to cell walls fluorometrically and allowing us to measure the ability of purified compounds to compete for this binding.

Mutanolysin and hydrofluoric acid cell wall extracts from *B. anthracis* Sterne, but not *csaB*, cell walls inhibit SLH binding to the cell wall

We next used our *in vitro* fluorescence assay of SLH assembly to score various extracts of the *B. anthracis* cell wall for inhibitory activity, aiming to characterize a purified SLH ligand. Sterne and *csaB* cell walls, free from protein, lipid, and nucleic acid contaminants, were then solubilized with mutanolysin or extracted with hydrofluoric acid, the former containing all components of the murein sacculus and the latter containing only cell-wall-linked polysaccharides (HF-PS).^{17,18} We measured the concentration of mutanolysin-solubilized peptidoglycan and HF-PS by the Morgan–Elson and Anthrone method, respectively.^{19,20} These two extracts, purified from either wild-type or *csaB* strains, were then included in the fluorescence binding assay described above. The ability of these extracts to inhibit SLH–cell wall binding is determined by the quantity of fluorescence that would co-sediment with the cell walls after incubation. As expected, mutanolysin-solubilized peptidoglycan from *B. anthracis* Sterne cells inhibited the binding of Sap_{SLH}–mCherry to the cell wall in a dose-dependent manner, whereas no inhibition by soluble peptidoglycan from the *csaB* strain or by our normalization standard *N*-acetyl-glucosamine (Sigma) was observed regardless of concentration (Fig. 3a). Micrographs of stained bacilli were collected from each reaction, qualitatively verifying the fluorescence measurements plotted in Fig. 3a (Fig. 3c). Similarly, neutralized HF-PS of *B. anthracis* Sterne cell walls inhibited Sap_{SLH}–mCherry binding to the cell wall. This inhibitory activity is *csaB*-dependent, as neither *csaB* cell walls nor a D-glucose normalization standard (Fisher) impacted Sap_{SLH}–mCherry binding (Fig. 3b). These measurements were also confirmed by microscopy (Fig. 3d).

Extracts were then subjected to *rp*HPLC in order to obtain chromatographically pure compounds for structural analysis. Crude, soluble peptidoglycan from *B. anthracis* Sterne eluted as many peaks over a 100-min gradient, most of which exhibited a low level of inhibitory activity (Fig. S1). These data suggested that the SLH ligand cannot be purified to homogeneity from mutanolysin-solubilized peptidoglycan. In contrast, HF-PS from both strains displayed similar chromatograms (A_{206}) with a few discrete peaks eluting with similar profiles (Fig. 4a). Each fraction was collected, neutralized, and scored for inhibitory activity in the *in vitro* Sap_{SLH}–mCherry cell wall binding assay as before. Fluorescence intensities were recorded in duplicate in 96- well format. Strikingly, all fractions containing significant inhibitory activity corresponded to a single, large elution peak (Fig. 4b). Although this peak is present in both the Sterne and *csaB* strains, only the eluant fraction from Sterne cell walls displayed inhibitory activity. This fraction represents a chromatographically pure HF-PS amenable to structural analyses.

HF-PS are pyruvylated in a *csaB*-dependant manner

Our *rp*HPLC purification of HF-PS from *B. anthracis* Sterne and *csaB* yielded approximately equal quantities of polysaccharides (as measured by A_{206}) with dramatically different inhibitory activity (Fig. 4). We reasoned, therefore, that these polysaccharides must contain important differences in structure. To test this hypothesis, we subjected the material purified by *rp*HPLC to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS and to ^1H nuclear magnetic resonance spectroscopy.

The spectra of ions we obtained by MALDI-TOF were remarkable for two reasons. First, both spectra contain the same pattern of major ions and repeating units as the SCWP of *B. anthracis*, whose structure has been determined (Fig. 5a).¹² This result identifies the major SCWP as the SLH ligand. The most abundant ion in these spectra occurred at $m/z \sim 1136$, which corresponds to a sodium adduct of the repeating SCWP oligosaccharide (Table 1). This oligosaccharide has a $\rightarrow 6$ - α -GlcNAc-(1 $\rightarrow$ 4)- β -ManNAc-(1 $\rightarrow$ 4)- β -GlcNAc-(1 $\rightarrow$ backbone, substituted with β -Gal via 1 $\rightarrow$ 3 and 1 $\rightarrow$ 4 linkages to the α -GlcNAc and with a β -Gal via 1 $\rightarrow$ 3 linkage to the β -GlcNAc.¹² In addition to the conservation of major peaks between these and other mass spectra,¹² we detected numerous additional ions, corresponding to structural permutation of the SCWP, indicating that this molecule displays significant heterogeneity in glycosyl and acetyl composition after extraction and purification (Table 1). Second, the wild-type spectrum contains a series of ions absent from the *csaB* spectrum whose mass-to-charge ratios differ from ions present in both samples by 70, the increase that can be expected from ketal pyruvate linked to a pyranose sugar (Fig. 5b, bold). The composition of these pyruvylated ions is summarized in Table 2.

These data led to the hypothesis that the SCWP is pyruvylated and that it serves as the SLH ligand polysaccharide. To test this, and to confirm the structural assignment of the polysaccharides, we subjected the SCWP and its pyruvylated forms to high-resolution MS to generate elemental compositional data and therefore provide a test of the hypothesis. Polysaccharides were purified from *B. anthracis* Sterne as before, and fractions were analyzed off-line by electrospray ionization interfaced with a Fourier transform mass spectrometer (ESI-FTMS). The 115 most intense monoisotopic species in the spectra (Fig. 6a) were within 4 mDa of the masses calculated for the corresponding predicted structures (Table 3); without exception, all of these masses could be explained as structural permutations of the SCWP.

The most abundant ion in the FTMS data set ($m/z=1114.4148$) corresponds to the repeating unit of the SCWP, a singly charged, protonated hexasaccharide (Fig. 6a, a); structural variants of this oligosaccharide comprise the remainder of the most abundant ions (**b, c, d, e, f, g, h, and i**). Interestingly, the ESI-FTMS spectra revealed an ion (Fig. 6a, z), the proposed structure of which includes two acetylated amino sugar residues and a ketal pyruvate. In total, 37 of the 115 ions identified in the ESI-FTMS spectra are proposed to contain a ketal pyruvate and two HexNAc residues in addition to the SCWP (Table 3, bold). These high-resolution data further support the hypothesis that pyruvylated SCWP is the SLH ligand. A representative structure for **z** is given as an inset in Fig. 6a. Although the pyruvylated SCWP ions were insufficiently intense for tandem mass spectrometric analysis, for every major

variant of the SCWP, we observed the same ion modified by the addition of a ketal pyruvate and two acetylated, amino sugars (Fig. 6a, **a+z**, **b+z**, **c+z**, **d+z**, **e+z**, **f+z**, **g+z**, **h+z**, **i+z**).

Collision-induced disassociation tandem mass spectra (CID-MS²) of ion **a** (m/z = 1114.4152, Fig. 6b) generated 15 major fragment ions (Fig. 6c), all of which could be plausibly assigned as arising from simple or known cleavages: loss of an acetyl residue, dehydration, cleavage across a glycosidic bond (**Y**, **C** ions), or cross-ring cleavages (**X** ions) (Fig. 6d). The most abundant fragment ion (**b**, m/z =952.3631), postulated to arise from loss of any one of three galactosyl residues, is also present in the full FTMS spectrum. This ion was isolated and analyzed by CID-MS² as well (Fig. S2), further supporting our assignment of the SLH ligand polysaccharide as the SCWP.

Finally, because the pyruvylated forms of the SCWP generated less intense signals than their unmodified counterparts, we performed a narrow select ion monitoring (nSIM) experiment to accurately measure and document the mass of these compounds. Four of the most abundant ions (m/z = 693.7611, 714.7668, 735.7722, and 816.7988) whose predicted structures included a ketal pyruvate were selected and analyzed. The nSIM spectra of these species produced an accurate and robust mass measurement, confirming their hypothesized structures (Fig. 7).

Initial work on SLH assembly concluded that a cell wall polysaccharide containing a ketal pyruvate served as the SLH ligand.^{6,9} This conclusion was based on the presence of chemical shifts in one- and two-dimensional NMR spectra diagnostic of a ketal pyruvate.^{6,9–11} These studies did not, however, identify the larger structure of this polysaccharide. ¹H NMR spectra from both *B. anthracis* Sterne and *csaB* polysaccharides were used to compare the polysaccharides we purified to those previously published (Fig. S3). The spectrum obtained from the Sterne strain differs from its isogenic *csaB* strain by a proton shift, δ 1.54 ppm, which arises from the methyl group attached to the ketal carbon. Our spectra bear striking resemblance to those of SCWP, further supporting the model that pyruvylated SCWP is the SLH ligand and confirming that the NMR spectra generated in these earlier studies arose from samples containing the SCWP.^{6,12}

Although several structural studies on the SCWP have been published, none of these studies demonstrated pyruvyl moieties in this polymer; neither have these studies identified other gene products required for its synthesis. Our mass spectra unequivocally identify this material as the SCWP, whose structure has been solved by NMR and linkage analysis, and provide the first direct evidence linking the SCWP structure and *csaB*-mediated pyruvylation. We observed a perfect correlation between pyruvylation of the SCWP and the addition of two acetylated amino sugars, demonstrating that the SCWP is modified by the addition of a unit structure in order to become an SLH ligand. Moreover, our purification strategy correlates structure (ketal pyruvate) with function (SLH binding) and advances testable hypotheses as to the attachment location of the ketal pyruvate to the polysaccharide (Fig. S4).

Tunicamycin inhibits TagO and subsequent SLH-binding, implicating *tagO* in the assembly of the SCWP

We aimed to identify the gene products required for the synthesis of the SLH ligand polysaccharide or S-layer assembly. With the exception of *csaB*, no other gene products have been assigned an essential role in the assembly of this polymer. Previous work suggested that SCWP is essential for growth of *B. anthracis*.¹³ We reasoned that because *csaB* modifies the SCWP, it might be genetically linked to genes required for its biosynthesis in SLH-domain-containing organisms. Members of the TagA/WecB/CpsF family of glycosyl transferases are frequently linked to *csaB* homologs that are themselves associated with SLH proteins in a variety of organisms, including *Geobacillus thermodenitrificans* NG80-2, *Desulfotomaculum reducens* MI-1, and *Alkaliphilus metalliredigens* QYMF. Members of the TagA/WecB/CpsF enzyme superfamily catalyze the transfer of nucleotide-activated sugars into growing polysaccharide chains.^{21–23} The enzymes with significant linkage to *csaB* had greatest homology to *tagA* of *Bacillus subtilis* and *Staphylococcus aureus*, an enzyme responsible for the addition of *N*-acetyl-mannosamine to undecaprenyl-pyrophosphate-*N*-acetyl-glucosamine, the second step in WTA synthesis.²²

B. anthracis does not synthesize glycerol or ribitol phosphate (RibP) WTA.²⁴ Moreover, it lacks homologs of *tarI*, *tarJ*, *tarL*, *tagB*, *tagD*, or *tagF*, genes whose products catalyze activation and polymerization of RibP and glycerol phosphate (GroP) monomers to teichoic acid linkage units. As such, cell walls of *B. anthracis* do not contain poly(RibP) or poly(GroP). However, the genome of *B. anthracis* does encode homologs of nearly every other *tag* gene, including *tagO*. In *S. aureus* and in *B. subtilis*, strains lacking *tagB*, *tagD*, *tagE*, *tagF*, *tagG*, *tagH*, or *tagX* display a synthetic lethal phenotype if they contain the *tagO* gene.^{25,26} These observations beg the question, what product, if any, do the *tag* genes synthesize in *B. anthracis*? The close linkage between *tagA* homologs and *csaB* homologs in various strains in conjunction with the similarity of the GlcNAc–ManNAc–GlcNAc backbone of the SCWP and the WTA linkage unit of GroP–ManNAc–GlcNAc led us to hypothesize that the *tag* genes of *B. anthracis* serve to synthesize and assemble the SCWP.

The ManNAc–GlcNAc moiety of the WTA linkage unit is synthesized by the sequential activity of TagO and TagA.^{15,16} To test our hypothesis that these enzymes are required for SCWP synthesis, we first sought to delete either *tagO* or *tagA*. We were unable to isolate a deletion of either *tagO* or *tagA* in the *B. anthracis* Sterne background, though both of these mutations are tolerated in *B. subtilis* and *S. aureus*.^{25,26} Our inability to mutate these genes is consistent with the nature of SCWP in *B. anthracis*. Because these efforts to generate conditional expression strains of *tagO* and *tagA* were unsuccessful, we applied a pharmacological approach to test our hypothesis.

Tunicamycin is a drug commonly used to inhibit the transfer of activated sugars to sites of protein glycosylation in eukaryotes, but it is also a potent inhibitor of WTA synthesis.^{27,28} Nanomolar concentrations of tunicamycin terminate WTA synthesis in *S. aureus* by inhibiting the activity of TagO.²⁹ To test the requirement of TagO in SCWP synthesis, and subsequent SLH protein binding to the cell wall, we sought to determine what effect

tunicamycin exerts on *B. anthracis* cultures. We grew cultures of *B. anthracis* with or without the addition of $1 \mu\text{g mL}^{-1}$ tunicamycin for 4 h, normalized by OD₆₀₀ (optical density at 600 nm) and then incubated cells with fluorescently labeled vancomycin [4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY)-vancomycin] (Invitrogen Molecular Probes) or with Sap_{SLH}-mCherry. Cultures grown in the presence of $1 \mu\text{g mL}^{-1}$ tunicamycin grew more slowly than untreated cultures. Further, the characteristic vegetative forms became coccoid in shape (Fig. 8a). Tunicamycin treatment did not inhibit Mray, which can be subject to inhibition by tunicamycin at high concentration,³⁰ as both treated and untreated bacilli stained abundantly with BODIPY-vancomycin. As expected, BODIPY-vancomycin labeled untreated bacilli most intensely at the division septa, correlated to the foci of peptidoglycan synthesis. In contrast, treated cells displayed increased, delocalized staining throughout the cell wall upon BODIPY-vancomycin labeling (Fig. 8a). Further, a subpopulation of treated cells seemed to exhibit multiple division planes, suggesting that tunicamycin serves to disorganize features of both cell division and peptidoglycan synthesis (arrows Fig. 8a).

Next, equal numbers of bacilli harvested from treated and untreated conditions were incubated with equimolar concentrations of Sap_{SLH}-mCherry as a proxy to measure SCWP production. We observed a dramatic reduction in the amount of staining seen on bacilli treated with tunicamycin (Fig. 8a). Increased exposure time of treated bacilli revealed an unusual staining pattern as compared to untreated cells. Whereas untreated bacilli when incubated with Sap_{SLH}-mCherry stain almost uniformly around the cell surface, tunicamycin-treated cells stain only at a few poles (arrows Fig. 8a). The paucity of staining on septa or lateral cell walls of bacilli treated with tunicamycin led us to speculate that envelope areas capable of binding Sap_{SLH}-mCherry represent portions of the bacilli that arose before tunicamycin treatment and therefore still contain the SCWP.

Based on these data, we hypothesized that tunicamycin specifically inhibits TagO of *B. anthracis*, resulting in aberrant forms and a deficiency in SLH protein binding. In order to test if the concentration of tunicamycin used to inhibit SLH binding to the cell wall is sufficient to inhibit the *B. anthracis tagO* gene product, we employed a heterologous system to measure TagO activity. As *tagO* and WTA are dispensable for growth in *S. aureus*, we asked if *tagO* from *B. anthracis* is capable of complementing the defect in WTA synthesis of an *S. aureus* RN4220 *tagO* strain. The *B. anthracis tagO* homolog is encoded by BAS5050, and its predicted translation product, TagO, is 40% identical with TagO of *S. aureus*. WTA was extracted and visualized via PAGE analysis according to previously published protocols.²⁹ Extracts of RN4220 wild-type strains contain abundant quantities of WTA, while the isogenic RN4220 *tagO* strain produces no WTA (Fig. 8b). This defect is complemented in trans when the *tagO* strain is transformed with either *ptagO^{S.aureus}*, a plasmid encoding the *S. aureus tagO* open reading frame, or *ptagO^{B. anthracis}*, a plasmid encoding *tagO* from *B. anthracis* Sterne (Fig. 8b). These data demonstrate that *tagO* from *B. anthracis* is sufficient to restore WTA synthesis in a RN4220 *tagO* strain and must therefore also catalyze the transfer of UDP-GlcNAc to undecaprenylphosphate.

Given that RN4220 *tagO ptagO^{B. anthracis}* produces WTA as a function of TagO, we asked if tunicamycin inhibits WTA synthesis in this strain and therefore, by extension, TagO

in *B. anthracis*. Cultures were grown in the presence of increasing concentrations of tunicamycin and normalized by optical density, and WTA extracts were subjected to PAGE analysis. Treatment with tunicamycin yielded a dose-dependent diminution of WTA production, with a concentration of $1 \mu\text{g mL}^{-1}$ completely ablating WTA synthesis (Fig. 8b). The production of lipoteichoic acid, an analogous polymer with a distinct synthetic machinery,³¹ was unaffected by genetic background or growth conditions, an indication of the specificity of tunicamycin for TagO (Fig. 8b). We conclude that TagO from *B. anthracis* is subject to inhibition by tunicamycin at $1 \mu\text{g mL}^{-1}$, and the defect in SLH binding to the cell wall is a direct result of this inhibition.

If, indeed, the TagO-synthesized linkage unit participates in the assembly of the SCWP of *B. anthracis*, then mass spectra of HF-extracted SCWP should contain ions representing portions of the SCWP attached to the ManNAc–GlcNAc linkage unit. Accordingly, our MALDI spectra contain a series of ions whose proposed structures contain repeats of the SCWP oligosaccharide with the addition of the ManNAc–GlcNAc linkage unit. These ions and their predicted composition are listed in bold in Table 3. Taken together, the data implicate a role for TagO in the synthesis of the SCWP of *B. anthracis* and potentially other organisms that elaborate similar envelope components. That the *tag* genetic machinery constitutes a multifunctional pathway, conserved in the synthesis of structurally distinct secondary wall polymers across various bacterial species, is an unexpected and exciting finding, which suggests that these molecules might play a much broader role in Gram-positive envelope biogenesis than previously appreciated.

Discussion

S-layers in Gram-positive eubacteria are often composed of proteins containing three tandem repeats of the SLH domain whose cell surface retention is predicated on an interaction with the cell wall.³² *B. anthracis* has served as a model system to understand SLH protein assembly onto the cell wall, leading to the identification of *csaB*, the first gene assigned an essential role in SLH protein retention.^{6,9} These studies provided evidence that strains harboring *csaB* pyruvylate a cell wall polysaccharide but did not identify the structure of this polysaccharide. Recently, a series of reports have elucidated the structure of the major SCWPs of the *B. cereus sensu lato* group, including *B. anthracis*.^{12,13} This work did report pyruvyl modifications on these molecules and therefore left unanswered the question, what, if any, pyruvylated polymer serves as the attachment site for S-layer proteins? Here, we report evidence that the SCWP of *B. anthracis* Sterne, but not *csaB* mutants, binds SLH domains and contains pyruvyl modifications. These data finally connect the activity of *csaB*, the structure of the SCWP, and S-layer assembly. Moreover, we have used tunicamycin for chemical genetic experiments to perturb the activity of TagO and, consequently, the retention of SLH proteins. On the basis of these results, we hypothesize that the *tag* pathway is required for the production of the SCWP in *B. anthracis*. Tunicamycin inhibition of TagO not only prevents binding of SLH domains to the cell wall but also induces aberrant, rounded forms sustaining multiple division septa and delocalized peptidoglycan synthesis. Studies in *B. subtilis* have shown that the WTA is a requirement for the bacillus shape and our data suggest that SCWP plays an analogous role.^{25,33} *B. anthracis* encodes homologs of the *tag* biosynthetic pathway with the exception of genes that are

required to produce poly(RibP) or poly(GroP). This genetic information is consistent with the observation that poly(GroP) polymers have not been observed in *B. anthracis* cell walls. That the *tag* pathway may synthesize the SCWP in *B. anthracis* is an unexpected finding with the obvious supposition that the genetic machinery for secondary wall polymers is highly conserved even if the structure of polymers is not. Interestingly, we have not been able to construct null mutants of *tagO* or *tagA*, two genes that are dispensable for growth in *B. subtilis* and *S. aureus*. It is possible that null mutations in these genes generate synthetic lethal phenotypes analogous to those observed in downstream *tag* genes in *B. subtilis* and *S. aureus*. It is also possible that the SCWP in *B. anthracis* serves an essential function under the conditions tested and deletion of the synthetic machinery yields non-viable strains. Whether the SCWP is truly an essential molecule remains an area of active investigation.

Identification of the structural requirements for S-layer assembly in Gram-positive bacteria may provide additional avenues for the development of therapeutic countermeasures against emerging pathogens. Furthermore, the ability to target and assemble arrays of proteins *in vitro*, exploiting the SLH domain and synthetic pyruvylated polysaccharides, would have numerous translational applications in industrial processes. This report provides data that the SLH ligand SCWP is synthesized using the *tag* pathway and that this pathway may produce an essential polymer in *B. anthracis* and other organisms. These findings extend the reported functionality of the *tag* pathway and identify the *tag* machinery as a target for the design of small-molecule inhibitors, as any such molecule could be another weapon for the arsenal against drug-resistant *B. anthracis*.

Materials and Methods

Bacterial strains and plasmids

B. anthracis strain Sterne 34F2 or its variants were grown in brain heart infusion broth (BHI) (Difco) or lysis broth (LB) (Difco) at 37 or at 30 °C when transformed with pLM4-derived plasmids. *E. coli* DH5 α and K1077 were grown in LB. *S. aureus* strain RN4220 and its variants were grown in tryptic soy broth (TSB). Media were supplemented with kanamycin (20 μ g mL⁻¹) to maintain plasmid selection in *B. anthracis* and *S. aureus* and with kanamycin (50 μ g mL⁻¹) and ampicillin (100 μ g mL⁻¹) in *E. coli* where appropriate. In all cases, plasmid DNA used to transform *B. anthracis* was purified from *E. coli* K1077 (*dcm*⁻/*dam*⁻). The *B. anthracis* strain Sterne *csaB* mutant was constructed by allelic replacement with pJK76. *B. anthracis* Sterne *eag-mCherry* and *csaB eag-mCherry* were constructed by allelic replacement with pJK96. pJK76 was generated by PCR amplifying 1-kb regions upstream (P243: 5'-

TTTCCCGGGAGATTTTAAAGATAATGCAAAAAAGATACT-3'/P244: 5'-

TTTGGTACCCCGCACTCTTAATCTCCTCCAA-3') and downstream (P245: 5'-

TTTGGTACCTCTTAATTTAAGAGGACATCCTCTTT-3'/P246: 5'

TTTGAATTCTGAATGGAGTGTACCATTGGC-3') of the *csaB* open reading frame, restricting these PCR products with XmaI-KpnI and KpnI-EcoRI, respectively, and ligating them with pLM4 vector, which had been previously restricted with XmaI-EcoRI. Ligation products were transformed into *E. coli* DH5 α , plated on selective media, and confirmed by PCR and sequencing analysis. pJK96 was constructed by PCR amplifying 1-kb regions

upstream (P289: 5'-GGAATTCCATATGGGTAAATCATTCCCAGACGTTC-3'/P290: 5'-TTTCTCGAGTTTTTTTCCATATTTTTATCTGTTAATG-3') and downstream (P305: 5'-TTTGGTACCGTCGATTATAGATAAAGTGAAAAATCAGTG-3'/P306: 5'-TTTGAATTCCTGATGATAAATGGCTCCTGAAA) of the TAA *eag* stop, restricting these PCR products with XmaI-KpnI and KpnI-EcoRI, respectively, and ligating them with pLM4 vector that had been restricted with XmaI-EcoRI. Ligation products were transformed into DH5a and confirmed by PCR to generate pJK85. pJK85 was then restricted with KpnI and ligated with PCR-amplified, KpnI-restricted mCherry (P283: 5'-TTTGGTACCAGCAAGGGCGAGGAGG-3'/P284: 5'-TTTGGTACCTTACTTGTACAGCTCGTCCATG-3'). Ligation products were transformed into *E. coli* DH5a and screened by PCR to generate pJK96. *B. anthracis* transformants of pJK76 and pJK96 were grown at 30 °C overnight and single recombinants were selected by plating at 43 °C overnight on LB agar containing kanamycin. Single colonies were then passed at 30 °C four times serially to induce a second recombination event and loss of pJK76 or pJK96. Mutations were confirmed by PCR. Complementation studies were preformed using *pcsB*, which was constructed by amplifying the *csaB* open reading frame from genomic DNA with P249: 5'-TTTTCTAGAGTGCGGTTAGTTTTATCAGGATATT-3'/P250: 5'-TTTGGTACCTTAAGATCCCATTCTCTTTTTTTG-3', restricting amplified DNA and pLM5 plasmid DNA with XbaI-KpnI, ligating restriction products, and transforming ligation products into *E. coli* DH5a. *ptagO^{S.aureus}* (pJK196) was constructed by amplifying *tagO* from *S. aureus* RN4220 chromosomal DNA using P618: 5'-TTTTCTAGATTAAAAACAATTAATATCGATGAAGGTG-3'/P619: 5'-TTTGGTACCCTATTCTCTTTATGAGATGACTTACG-3', restricting amplified DNA and pJK4 plasmid DNA with XbaI-KpnI, ligating the restriction products, and transforming the ligation products into *E. coli* DH5a. *ptagO^{B. anthracis}* (pJK156) was constructed by amplifying *tagO* (BAS5050) from *B. anthracis* Sterne genomic DNA using P470: 5'-TTTTCTAGAATGGACTCACAAGTGATTTATGC-3'/P471: 5'-TTTGGTACCTTAGTCTTCGCGTTTTTCACTT-3', restricting amplified DNA and pJK4 plasmid DNA with XbaI-KpnI, ligating the restriction products, and transforming the ligation products into *E. coli* DH5a. The mCherry expression vectors were constructed by amplifying mCherry with P287: 5'-TTTCTCGAGAGCAAGGGCGAGGAGG-3'/P288: 5'-TTTGGATCCTTACTTGTACAGCTCGTCCATG-3' and restricted along with pET16b vector DNA with XhoI-BamHI, ligated, and transformed into DH5a to generate pJK81. pJK81 was then restricted with NdeI-XhoI and ligated with the NdeI-XhoI-restricted, PCR-amplified portion of *sap* encoding the SLH domains (amino acids 30–210) (P281: 5'-GGAATTCCATATGGGTAAAACATTCCCAGACGTTC-3'/P282: 5'-TTTCTCGAGTTCTGTACCGAACTGCTTGTCAG-3') to generate pJK87. These plasmids were transformed into *E. coli* BL21 (DE3).

Immunoblotting

Media supernatant proteins were prepared by precipitating proteins from culture filtrates of *B. anthracis*, grown overnight in BHI supplemented with 0.8% NaHCO₃, by addition of trichloroacetic acid to a final concentration of 7.5% and incubation on ice for 20 min. Protein pellets were extracted with acetone, dried at room temperature, and dissolved in

sample buffer. Sediment proteins were extracted with sample buffer from washed sediments of *B. anthracis* cultures. These samples were subjected to SDS-PAGE and electrotransferred to polyvinylidene fluoride membranes, and protein species were detected by incubation with specific antisera raised in rabbits against rBsIA.⁸ *S. aureus* cultures grown overnight in TSB were centrifuged and bacteria were subjected to mechanical lysis and sedimented at 21,000g for 20 min. The sediment was boiled in sample buffer, subjected to SDS-PAGE, transferred to polyvinylidene fluoride, and blotted using a Hycult Clone 55 mouse monoclonal antibody α -lipoteichoic acid (Cell Sciences).

MALDI-TOF, ¹H NMR, and FTMS

*rp*HPLC eluate fractions were dried in a vacuum desiccator and dissolved in 30% acetonitrile/70% water/0.1% trifluoroacetic acid (TFA). These samples were co-spotted with matrix (0.5 μ L α -cyano-4-hydroxycinnamic acid) prepared at 10 mg mL⁻¹ in the same solvent supplemented with 4 mM (NH₄)₂HPO₄. MALDI-MS spectra were obtained in a reflectron TOF instrument (ABI Biosystems MALDI 4700) in positive ion, reflectron mode. ¹H NMR spectra were collected by the University of Chicago Biological Sciences NMR facility. *rp*HPLC eluents of HF-PS were dried in a vacuum desiccator and suspended in ²H₂O at 100 mg mL⁻¹, and spectra were collected overnight at 600 MHz.

For accurate molecular weight measurements, HF-PS, eluted in 20% acetonitrile/80% water/0.1% TFA from a C18 Sep-Pak cartridge (Waters), were lyophilized and then re-dissolved to 20 mg/mL in 50% acetonitrile/50% water/0.1% TFA and analyzed on a hybrid linear ion-trap 7-T FT-ICR mass spectrometer (LTQ-FT Ultra; Thermo Fisher Corp., San Jose, CA, USA) fitted with an off-line nanospray source. Samples were individually loaded into 2- μ m-internal-diameter, externally coated nanospray emitters (Proxeon, Cambridge, MA, USA) and desorbed using a spray voltage of 1.8 kV (*versus* the inlet of the mass spectrometer). These conditions produced a flow rate of 20–50 nL/min. Ion transmission into the linear trap and further to the ICR cell was automatically controlled, with ion count targets for both full-scan and MS² experiments of 2 $\times$ 10⁶. The ion trap was operated in the standard mass range (m/z =300–2000), and the resolving power of the FTMS was set at 100,000 (defined by m/m 50% at m/z = 400). For MS² experiments, precursor ions were selected (isolation width, m/z =4–8) and fragmented (collision energy settings, 12–15 at the default activation q -value of 0.25) using helium as the collision gas in the linear ion trap, before transmission to and recording from the ICR cell.

All FT-ICR spectra were obtained by averaging between 50 and 200 transient signals. Precursor masses were calculated using Xtract version 3.0.1.1 (Thermo Scientific, Bremen, Germany) with an S/N threshold of 2, a minimum intensity of 2, a minimum fit of 30, and a remainder threshold of 3. Product ion spectra were processed using ProSight PC version 2 (Thermo Scientific) to produce monoisotopic mass lists (S/N=2; minimum RL value=0.9).

Purification of Saps_{SLH}-mCherry and mCherry

E. coli BL21 (DE3) carrying pJK81 or pJK87 were grown in 1-L cultures to an OD₆₀₀ of 1 and induced with 1 mM IPTG for 3 h at 37 °C, harvested by centrifugation, and suspended in TN buffer (50 mM Tris-HCl, pH 7.5, and 150 mM NaCl). Cell pellets were disrupted by

French press and separated into soluble and insoluble fractions by centrifugation. mCherry was purified by affinity chromatography over Ni-NTA beads (Qiagen) from the soluble fraction of lysates from pJK81 cultures according to the manufacturer's recommendations under native conditions. The insoluble portion of pJK87 cultures was dissolved in Buffer A (6 M guanidine HCl, 10 mM Tris-HCl, and 100 mM NaH₂PO₄, pH 8.0) and applied to Ni-NTA beads equilibrated with Buffer A. The column was washed with Buffer A and then washed extensively with TN buffer and protein purified according to the manufacturer's recommendations under native conditions.

Fluorescence micrographs

B. anthracis Sterne and *csaB eag-mCherry* strains were grown in BHI overnight at 37 °C with shaking and then harvested by centrifugation, washed with phosphate-buffered saline (PBS), and stained with 1 µg·mL⁻¹ Hoechst in PBS. Digital, confocal images were collected on a Nikon TE-2000 inverted scope with a 100× objective and a BD-CARV II spinning disk confocal unit. *B. anthracis* cells labeled with Sap_{SLH}-mCherry or mCherry were visualized with an Olympus PROVIS microscope with a 100× objective and digital images were collected. *B. anthracis* cells grown in the presence or absence of 1 µg·mL⁻¹ tunicamycin were harvested by centrifugation, normalized by optical density, and then treated either with 1 µg·mL⁻¹ vancomycin-BODIPY conjugate (Invitrogen) or with purified, recombinant Sap_{SLH}-mCherry, and images were collected digitally on an Olympus PROVIS microscope with a 100× objective.

SLH binding assays

B. anthracis Sterne or *csaB* cells were harvested by centrifugation and resuspended in 3 M urea and heated at 95 °C for 10 min. Cells were collected by centrifugation and then washed extensively with water, resuspended in PBS, and normalized by optical density (OD₆₀₀). Equal numbers of these cells were then incubated with equimolar quantities of purified mCherry or Sap_{SLH}-mCherry for 10 min at room temperature in the dark. After incubation, cells were harvested by centrifugation, washed extensively with PBS, and either imaged or fluorescence intensity measured in 96-well format with a Tecan plate reader.

Purification of peptidoglycan, HF-PS, rPHPLC

B. anthracis Sterne peptidoglycan was purified from cells scraped from 100 mm×100 mm BHI agar plates and resuspended in water. Cells were harvested by centrifugation and resuspended in 500 mL 4% SDS and then submerged in a boiling water bath for 30 min. After cooling to room temperature, the cells were harvested by centrifugation (9000g, 10 min) and washed repeatedly with water until no trace of SDS remained. The cells were then suspended in 80 mL of water and subjected to mechanical lysis with a Bio-Spec bead beater and 0.1 mm glass beads for 10 pulses of 1 min, each pulse separated by a 5-min rest on ice. Cell walls were then harvested by centrifugation (8000g, 15 min) and washed once with water. This material was treated for 4 h at 37 °C with 10 µg mL⁻¹ RNase and DNase (Roche) in 100 mM Tris-HCl, pH 7.5, supplemented with 20 mM MgSO₄ and then for 16 h at 37 °C with 10 µg mL⁻¹ trypsin (Sigma) in 100 mM Tris-HCl, pH 7.5, supplemented with 10 mM CaCl. Insoluble material was then harvested by centrifugation at 32,000g for 15 min

and washed once with water and sedimented again. The pellet was resuspended in 1% SDS and submerged in a boiling water bath for 30 min. SDS was removed by repeated washing with water and centrifugation (15 min, 32,000g) until all traces of SDS were absent. This material was then washed once with 100 mM Tris-HCl, pH 8.0, once with water, once with 100 mM ethylenediaminetetraacetic acid, pH 8.0, once again with water, once with acetone, and then twice more with water; insoluble material was collected between each step by centrifugation for 15 min at 32,000g. After the final wash, the pellet was lyophilized and stored at -20 °C. Soluble peptidoglycan was prepared by suspending lyophilized wall material in water to an OD₆₀₀=40 and treating 2 mL of this material in 50 mM Tris-HCl, pH 6.3, and 1.5 mM MgCl₂ with 5000 µg mL⁻¹ mutanolysin (Sigma). After incubation with tumbling overnight at 37 °C, the reaction was incubated at 95 °C for 30 min, and insoluble material was removed by centrifugation for 15 min at 20,000g. The supernatant was stored at -20 °C for future use or used immediately to measure GlcNAc concentration by the method of Morgan-Elson.¹⁹ HF-PS were removed by resuspending lyophilized peptidoglycan in 10 mL of 48% HF (Acros) at an OD₆₀₀ of 25 and incubating with shaking for 18 h at 4 °C. After incubation, insoluble material was removed by centrifugation at 20,000g for 15 min and the supernatant was precipitated by adding 5 volumes of ethanol at -20 °C and storing at -20 °C for 1 h. Precipitated polysaccharides were harvested by centrifugation at 32,000g for 15 min and washed five times with ethanol at -20 °C. The pellet was then suspended in 5 mL water and applied to a Sep-Pak Plus C18 column (Waters) equilibrated with water containing 0.1% TFA. The column was washed twice with 10 mL water and 0.1% TFA and eluted with 20% acetonitrile/80% water with 0.1% TFA. The eluted material was neutralized with Tris-HCl, pH 8.0, buffer (its concentration measured by the Anthrone method) and stored at -20 °C. *rp*HPLC was used to further purify the HF-PS over a 250 mm×4.6 mm C18 ODS hypersile 3-µm-particle-size guard column (Thermos) to separate peptidoglycan or HF-PS with H₂O-0.1% TFA (Buffer A) and acetonitrile-0.1% TFA (Buffer B) gradients as follows: 10 min, 0% Buffer B; 5 min, linear gradient 0-10% Buffer B; 20 min, linear gradient 10-20% Buffer B; 65 min, linear gradient 20-100% Buffer B; 10 min, 100% Buffer B at a flow rate of 0.5 mL min⁻¹. The HF-PS eluted at ~30 min, 15-16% acetonitrile routinely.

Extraction of *S. aureus* WTA

Extraction of WTA of *S. aureus* strain RN4220 and its variants was carried out according to previously published protocols.²⁹ *S. aureus* strains were grown as overnight cultures in 100 mL of TSB (supplemented with the appropriate antibiotic/tunicamycin concentration and 1 mM IPTG) and normalized by optical density (OD₆₀₀). Overnight culture (30 mL) at OD₆₀₀=6 was harvested by centrifugation (10 min, 6000g), washed once with 30 mL buffer 1 [50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.5], and suspended in 30 mL of buffer 2 (4% SDS and 50 mM Mes, pH 6.5). Cell suspensions were submerged in a boiling water bath for 1 h and collected by centrifugation (10,000g, 10 min). Insoluble material was suspended in 1 mL of buffer 2 and transferred to a 2-mL microcentrifuge tube, sedimented (21,000g, 10 min), and washed once with 1.5 mL buffer 2, once with 1.5 mL buffer 3 (2% NaCl and 50 mM Mes, pH 6.5), and finally with 1.5 mL buffer 1. After the last wash, samples were incubated with 20 µg proteinase K in 20 mM Tris-HCl, pH 8.0, and 0.5% SDS at 50 °C for ~4 h. Following digestion, wall material was collected by centrifugation

(21,000g, 10 min) and washed once with 1.5 mL buffer 3 and then at least three times with distilled H₂O to remove the SDS. WTA samples were then extracted with 1 mL of 0.1 M NaOH by tumbling at room temperature for 16 h. Insoluble material was removed by centrifugation (21,000g, 10 min), and the supernatant, containing hydrolyzed WTA, was neutralized by the addition of 50 μ L of a 0.1-N acetic acid solution and of Tris-HCl, pH 8.5, to 100 mM.

PAGE analysis of WTA

The PAGE analysis of the WTA method was adapted from previously published protocols.²⁹ A Bio-Rad Protean II xi electrophoresis cell (20 cm×16 cm×0.75 mm) was used for separation. A solution of 19:1 (5% C) acrylamide was supplemented with bisacrylamide to obtain a final solution of 30% T (6% C). The separating gel was 20% total acrylamide, 6% of which was the cross-linker bisacrylamide in 1 M Tris-HCl, pH 8.5. The gel was prepared by mixing 10 mL of gel buffer (3 M Tris-HCl, pH 8.5) with 20 mL of an acrylamide stock solution (30% T, 6% C). The gel was polymerized using 300 μ L of 10% ammonium persulfate along with 30 μ L of *N,N,N',N'*-tetramethylethylenediamine. The stacking gel (4% T, 5% C) was cast using a mixture of 1 mL acrylamide stock solution (40% T, 5% C), 3 mL of gel buffer (3 M Tris-HCl, pH 8.5), and 6 mL water, polymerized with 100 μ L of 10% ammonium persulfate and 10 μ L of *N,N,N',N'*-tetramethylethylenediamine. WTA samples were diluted 1:3 in loading buffer (50% glycerol in running buffer with a trace of bromophenol blue) and 20 μ L was loaded in each well. Tris-*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] glycine running buffer {0.1 M Tris base and 0.1 M *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine, pH 8.2} was used to run the gel for 16 h at 20 mA per gel. WTA bands were visualized using the alcian blue-silver staining protocol.³⁴ Briefly, the gel was submerged in 1 mg/mL alcian blue in water and rotated slowly at room temperature for 30 min. The alcian blue solution was replaced after 30 min, and the gel was allowed to stain for another 30 min, washed twice with water and destained with 45% methanol and 10% acetic acid in water for 30 min, and washed twice more with water. WTA bands were developed via the Bio-Rad silver staining kit according to the manufacturer's recommendations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SCWP	secondary cell wall polysaccharide
SLH	surface layer homology
MS	mass spectrometry
HF-PS	hydrofluoric-acid-extractable polysaccharides
WTA	wall teichoic acid
MALDI-TOF	matrix-assisted laser desorption/ionization time of flight
ESI	electrospray ionization
FTMS	Fourier transform mass spectrometer
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza- <i>s</i> -indacene
nSIM	narrow select ion monitoring
RibP	ribitol phosphate
GroP	glycerol phosphate
BHI	brain heart infusion broth
LB	lysis broth
TSB	tryptic soy broth
TFA	trifluoroacetic acid
PBS	phosphate-buffered saline
Mes	2-(<i>N</i> -morpholino)ethanesulfonic acid

References

1. Koch R. Die Ätiologie der Milzbrand-Krankheit, begründet auf die Entwicklungsgeschichte des *Bacillus anthracis*. Beitr Biol Pflanz. 1876; 2:277–310.
2. Mock M, Fouet A. Anthrax. Annu Rev Microbiol. 2001; 55:647–671. [PubMed: 11544370]
3. Fouet A. The surface of *Bacillus anthracis*. Mol Aspects Med. 2009; 30:374–385. [PubMed: 19607856]
4. Etienne-Toumelin I, Sirard JC, Duflot E, Mock M, Fouet A. Characterization of the *Bacillus anthracis* S-layer: cloning and sequencing of the structural gene. J Bacteriol. 1995; 177:614–620. [PubMed: 7836294]
5. Farchaus JW, Ribot WJ, Downs MB, Ezzell JW. Purification and characterization of the major surface array protein from a virulent *Bacillus anthracis* Delta Sterne-1. J Bacteriol. 1995; 177:2481–2489. [PubMed: 7730281]
6. Mesnage S, Fontaine T, Mignot T, Delepierre M, Mock M, Fouet A. Bacterial SLH domain proteins are non-covalently anchored to the cell surface via a conserved mechanism involving wall polysaccharide pyruvylation. EMBO J. 2000; 19:4473–4484. [PubMed: 10970841]
7. Mesnage S, Tosi-Couture E, Fouet A. Production and cell surface anchoring of functional fusions between the SLH motifs of the *Bacillus anthracis* S-layer proteins and the *Bacillus subtilis* levansucrase. Mol Microbiol. 1999; 31:927–936. [PubMed: 10048035]
8. Kern JW, Schneewind O. BslA, a pXO1-encoded adhesin of *Bacillus anthracis*. Mol Microbiol. 2008; 68:504–515. [PubMed: 18366441]

9. Cava F, de Pedro MA, Schwarz H, Henne A, Berenguer J. Binding to pyruvylated compounds as an ancestral mechanism to anchor the outer envelope in primitive bacteria. *Mol Microbiol.* 2004; 52:677–690. [PubMed: 15101975]
10. Ilk N, Kosma P, Puchberger M, Egelseer EM, Mayer HF, Sleytr UB, Sára M. Structural and functional analyses of the secondary cell wall polymer of *Bacillus sphaericus* CCM 2177 that serves as an S-layer-specific anchor. *J Bacteriol.* 1999; 181:7643–7646. [PubMed: 10601228]
11. Petersen BO, Sára M, Mader C, Mayer HF, Sleytr UB, Pabst M, et al. Structural characterization of the acid-degraded secondary cell wall polymer of *Geobacillus stearothermophilus* PV72/p2. *Carbohydr Res.* 2008; 343:1346–1358. [PubMed: 18420185]
12. Choudhury B, Leoff C, Saile E, Wilkins P, Quinn CP, Kannenberg EL, Carlson RW. The structure of the major cell wall polysaccharide of *Bacillus anthracis* is species-specific. *J Biol Chem.* 2006; 281:27932–27941. [PubMed: 16870610]
13. Leoff C, Choudhury B, Saile E, Quinn CP, Carlson RW, Kannenberg EL. Structural elucidation of the nonclassical secondary cell wall polysaccharide from *Bacillus cereus* ATCC 10987. Comparison with the polysaccharides from *Bacillus anthracis* and *B cereus* type strain ATCC 14579 reveals both unique and common structural features. *J Biol Chem.* 2008; 283:29812–29821. [PubMed: 18757856]
14. Schuch R, Fischetti VA, Nelson DC. A genetic screen to identify bacteriophage lysins. *Methods Mol Biol.* 2009; 502:307–319. [PubMed: 19082564]
15. Brown S, Zhang YH, Walker S. A revised pathway for *Staphylococcus aureus* wall teichoic acid biosynthesis based on *in vitro* reconstitution of the intracellular steps. *Chem Biol.* 2008; 15:12–21. [PubMed: 18215769]
16. Xia G, Kohler T, Peschel A. The wall teichoic acid and lipoteichoic acid polymers of *Staphylococcus aureus*. *Int J Med Microbiol.* 2010; 300:148–154. [PubMed: 19896895]
17. Ekwunife FS, Singh J, Taylor KG, Doyle RJ. Isolation and purification of cell wall polysaccharide of *Bacillus anthracis*. *FEMS Microbiol Lett.* 1991; 66:257–262. [PubMed: 1769521]
18. Kojima N, Araki Y, Ito E. Structural studies on the acidic polysaccharide of *Bacillus cereus* AHU 1356 cell walls. *Eur J Biochem.* 1985; 148:479–484. [PubMed: 2986963]
19. Elson LA, Morgan WT. A colorimetric method for the determination of glucosamine and chondrosamine. *Biochem J.* 1933; 27:1824–1828. [PubMed: 16745305]
20. Morris DL. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. *Science.* 1948; 107:254–255. [PubMed: 17814729]
21. Alexander DC, Valvano MA. Role of *rfe* gene in the biosynthesis of the *Escherichia coli* O7-specific lipopolysaccharide and other O-specific polysaccharides containing *N*-acetylglucosamine. *J Bacteriol.* 1994; 176:7079–7084. [PubMed: 7525537]
22. D'Elia MA, Henderson JA, Beveridge TJ, Heinrichs DE, Brown ED. The *N*-acetylmannosamine transferase catalyzes the first committed step of teichoic acid assembly in *Bacillus subtilis* and *Staphylococcus aureus*. *J Bacteriol.* 2009; 191:4030–4034. [PubMed: 19376878]
23. Haft RF, Wessels MR, Mebane MF, Conaty N, Rubens CE. Characterization of *cpsF* and its product CMP-*N*-acetylneuraminic acid synthetase, a group B streptococcal enzyme that can function in K1 capsular polysaccharide biosynthesis in *Escherichia coli*. *Mol Microbiol.* 1996; 19:555–563. [PubMed: 8830246]
24. Molnár J, Prágai B. Attempts to detect the presence of teichoic acid in *Bacillus anthracis*. *Acta Microbiol Acad Sci Hung.* 1971; 18:105–108. [PubMed: 5004640]
25. D'Elia MA, Millar KE, Beveridge TJ, Brown ED. Wall teichoic acid polymers are dispensable for cell viability in *Bacillus subtilis*. *J Bacteriol.* 2006; 188:8313–8316. [PubMed: 17012386]
26. D'Elia MA, Pereira MP, Chung YS, Zhao W, Chau A, Kenny TJ, et al. Lesions in teichoic acid biosynthesis in *Staphylococcus aureus* lead to a lethal gain of function in the otherwise dispensable pathway. *J Bacteriol.* 2006; 188:4183–4189. [PubMed: 16740924]
27. Duksin D, Bornstein P. Changes in surface properties of normal and transformed cells caused by tunicamycin, an inhibitor of protein glycosylation. *Proc Natl Acad Sci USA.* 1977; 74:3433–3437. [PubMed: 198786]
28. Hancock IC, Wiseman G, Baddiley J. Biosynthesis of the unit that links teichoic acid to the bacterial wall: inhibition by tunicamycin. *FEBS Lett.* 1976; 69:75–80. [PubMed: 825388]

29. Meredith TC, Swoboda JG, Walker S. Late-stage polyribitol phosphate wall teichoic acid biosynthesis in *Staphylococcus aureus*. J Bacteriol. 2008; 190:3046–3056. [PubMed: 18281399]
30. Ikeda M, Wachi M, Jung HK, Ishino F, Matsuhashi M. The *Escherichia coli* mraY gene encoding UDP-*N*-acetylmuramoyl-pentapeptide: undecaprenyl-phosphate phospho-*N*-acetylmuramoyl-pentapeptide transferase. J Bacteriol. 1991; 173:1021–1026. [PubMed: 1846850]
31. Gründling A, Schneewind O. Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus*. Proc Natl Acad Sci USA. 2007; 104:8478–8487. [PubMed: 17483484]
32. Sleytr UB, Beveridge TJ. Bacterial S-layers. Trends Microbiol. 1999; 7:253–260. [PubMed: 10366863]
33. Schäffer C, Messner P. The structure of secondary cell wall polymers: how Gram-positive bacteria stick their cell walls together. Microbiology. 2005; 151:643–651. [PubMed: 15758211]
34. Pollack JH, Neuhaus FC. Changes in wall teichoic acid during rod-sphere transition of *Bacillus subtilis*. J Bacteriol. 1994; 176:7252–7259. [PubMed: 7961496]

Fig. 1.

csaB is required for SLH protein retention on the *B. anthracis* cell wall. (a) Coomassie-blue-stained SDS-PAGE of protein extracts from culture sediments (upper panel). Strains harboring *csaB* contain numerous protein species that no longer sediment when the *csaB* gene is deleted. Immunoblots of these samples (middle panel) using antiserum against the SLH protein BslA suggest that SLH proteins are no longer retained even though all strains produce BslA, as total precipitated proteins from culture filtrate contain specific signals (bottom panel). (b) Genetic organization of *B. anthracis* Sterne *eag-mCherry* and $\Delta csaB$ *eag-mCherry* strains. (c) Fluorescent micrographs of these cells harvested in late-exponential phase stained with Hoechst (blue). *B. anthracis* Sterne *eag-mCherry* assemble the EA1-mCherry (red) hybrid into the S-layer, validating the use of fluorescent reporters in measuring SLH protein assembly.

Fig. 2.

Purified, recombinant Sap_{SLH}-mCherry, but not mCherry, binds to the cell walls of *B. anthracis* strains harboring *csaB*. (a) Coomassie-stained SDS-PAGE of hexahistidyl-purified mCherry and a translational hybrid between the Sap SLH domains (amino acids 30 to 210 of the *sap* gene) and mCherry. Abundant, soluble forms of both proteins were purified from *E. coli* and, at equal concentrations, exhibited equivalent fluorescent yields with identical peak excitation (585 nm) and emission (608 nm) spectra. (b) Fluorescent micrographs of *B. anthracis* Sterne, *csaB*, *csaB* pLM5, and *csaB* pcsaB cells incubated with 1 μ M

mCherry or Saps^{SLH}–mCherry. Staining of the cell wall depends upon the presence of the SLH domain and the *csaB* gene, validating the use of this as an *in vitro* model of S-layer assembly.

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Fig. 3. Crude, soluble peptidoglycan and HF-PS inhibit Sap_{SLH}-mCherry binding to the *B. anthracis* cell wall. (a) Fluorescence intensities in the reaction sediment of Sap_{SLH}-mCherry (1 μM) incubated with equal numbers of *csaB* cells (open squares) or with Sterne cells (filled squares) and the indicated concentration of GlcNAc (normalization control), or with Sap_{SLH}-mCherry (1 μM) incubated with Sterne cell suspensions containing the indicated concentrations of soluble peptidoglycan from Sterne (filled triangles) or *csaB* cell walls (open triangles). Only soluble peptidoglycan fragments from Sterne cell walls can inhibit the

binding of Sap_{SLH}-mCherry to the cell wall. (b) Fluorescence intensities in the reaction sediment of Sap_{SLH}-mCherry (1 μ M) incubated with equal numbers of *csaB* cells (open squares) or with Sterne cells (filled squares) and the indicated concentration of D-glucose (normalization control), or with Sap_{SLH}-mCherry (1 μ M) incubated with Sterne cell suspensions containing the indicated concentrations of HF-PS from Sterne (filled triangles) or *csaB* cell walls (open triangles). Only HF-PS from Sterne cell walls can inhibit the binding of Sap_{SLH}-mCherry to the cell wall. (c) Fluorescence micrographs of cells from representative reactions plotted in (a) validate the measurements in the upper panel. The scale bar represents 5 μ m. (d) Fluorescence micrographs of cells from representative reactions plotted in (b) validate the measurements in the upper panel.

Fig. 4.

rpHPLC purification of the HF-PS from Sterne and *csaB* strains reveals a single peak containing the SLH ligand. (a) *rpHPLC* chromatographs (A_{206}) of HF-PS extracts from *B. anthracis* Sterne (black trace) and *csaB* (gray trace) both contain a single, predominant peak. (b) All fractions from the purifications in (a) were dried, dissolved in neutral buffer, and incubated with Sap_{SLH}-mCherry (1 μ M) and Sterne cell walls. Fluorescence intensity in the sediment is plotted. The large, predominant peak from the Sterne strain was inhibitory in this reaction and was subjected to further analysis.

Fig. 5. MALDI-TOF mass spectra of *rHPLC*-purified HF-PS from *Sterne* and $\Delta csaB$. (a) MALDI-TOF spectra were collected from *rHPLC*-purified HF-PS. Both *Sterne* and $\Delta csaB$ spectra contain the same series of ions, which is identical with those ions described in the identification of the SCWP structure. Ions in bold represent multimers of the basic Hex₃HexNAc₃ oligosaccharide unit of the SCWP. These spectra identify the SLH ligand polysaccharide as the SCWP of *B. anthracis*. (b) A more detailed view of the mass spectra from (a), displaying ions from $m/z=1200$ to $m/z=1800$. A series of ions (bold) were discovered in the *Sterne* HF-PS that were absent from $\Delta csaB$ HF-PS and differed in mass ratio from ions present in both by $m/z=70$, the mass ratio difference expected from a ketal pyruvate modification.

Fig. 6. High-resolution FTMS of the HF-PS from Sterne. (a) Full mass spectrum of HF-extracted, SLH ligand polysaccharide from *B. anthracis* Sterne. The monoisotopic masses and structures of 115 species are given in Table 3. Ion **a** is the most abundant and represents the repeating SCWP hexasaccharide (right inset structure); ions **b–i** arise from structural perturbation of (a). Notably, many less abundant ions have structures composed of **a–i** and **z**, which comprise two N-acetylated amino hexose sugars and a ketal pyruvate (right inset structure). Ions whose m/z ratios are italicized are doubly charged ($z=2$). (b) Isolated parent ion $m/z=1114.4152$. (c) Collision-induced disassociation (CID) tandem MS of $m/z=1114.4152$ ion generates 15 major daughter ions. (d) Structure of $m/z=1114.4152$ parent ion and CID events that yield the daughter ions labeled in (b).

Fig. 7. nSIM measurement of pyruvylated forms of the SCWP. Four independent nSIM experiments were performed to accurately measure the mass of four pyruvylated forms of the SCWP and, therefore, to confirm our structural model. Monoisotopic signals are displayed in bold; peaks with m/z values increasing by 0.5 amu represent isotopic variants of the same compound.

Fig. 8.

Treatment of *B. anthracis* with tunicamycin inhibits Saps_{SLH}-mCherry binding to the cell wall and TagO from *B. anthracis*. (a) Fluorescence micrographs of untreated *B. anthracis* cells (upper panels) or cells treated with 1 $\mu\text{g mL}^{-1}$ tunicamycin and then stained with vancomycin-BODIPY (green) or with Saps_{SLH}-mCherry. *B. anthracis* cells treated with tunicamycin form aberrant, rounded shapes and appear to have increased, delocalized peptidoglycan synthesis and multiple division septa (white arrows on green micrographs). Treated cells also bound dramatically less Saps_{SLH}-mCherry, requiring much higher

exposure times (indicated in the upper right of the micrographs) to visualize any Sap_{SLH}-mCherry binding. Sap_{SLH}-mCherry labeling occurred only at a few poles (white arrows on red micrographs), presumably those that existed prior to tunicamycin treatment. (b) PAGE analysis of WTA extracts from *S. aureus* RN4220 and its variants. RN4220 produces abundant WTA, which requires *tagO* for synthesis. WTA synthesis can be complemented in trans with a plasmid encoding the RN4220 *tagO* gene (*ptagO^{S.aureus}*) or with a plasmid encoding the *tagO* gene from *B. anthracis* Sterne (*ptagO^{B. anthracis}*). *S. aureus* RN4220 *tagO ptagO^{B. anthracis}* grown in the presence of the indicated concentrations of tunicamycin showed a dose-dependent abrogation of WTA synthesis, indicating that tunicamycin inhibits TagO from *B. anthracis*. The arrow indicates the position of the bromophenol blue dye front. Lipoteichoic acid synthesis was unaffected regardless of plasmid concentration or growth condition, demonstrating tunicamycin's specificity for TagO inhibition.

Ions present in MALDI-TOF spectra from HF-PS extracted from both Sterne and *csaB*

Table 1

Observed m/z (Sterne PS) a	Observed m/z (<i>csaB</i> PS) b	Proposed structure	Theoretical m/z (monoisotopic) c	Sterne m/z e-a	<i>csaB</i> m/z e-b
1118.3309	1118.3593	[GlcNAc ₂ ManNAc ₁ Gal ₃ -H ₂ O+Na] ⁺	1118.3843	0.0534	0.0250
1136.3836	1136.3666	[GlcNAc ₂ ManNAc ₁ Gal ₃ GlcNAc ₂ ManNAc ₁ Gal ₃ +Na] ⁺	1136.3948	0.0112	0.0282
1152.3512	1152.3607	[GlcNAc ₂ ManNAc ₁ Gal ₃ +O+Na] ⁺	1152.3897	0.0385	0.0290
1178.3952	1178.3367	[GlcNAc ₂ ManNAc ₁ GalOAcGal ₂ +Na] ⁺	1178.4053	0.0101	0.0686
1321.3619	1321.4404	[GlcNAc ₃ ManNAc ₁ Gal ₃ -H ₂ O+Na] ⁺	1321.4633	0.1014	0.0229
1339.4456	1339.4075	[GlcNAc ₃ ManNAc ₁ Gal ₃ +Na] ⁺	1339.4738	0.0282	0.0663
1362.4947	1362.4520	[GlcNAc ₃ ManNAc ₂ Gal ₂ -H ₂ O+Na] ⁺	1362.4898	-0.0049	0.0378
1380.5203	1380.4245	[GlcNAc ₃ ManNAc ₂ Gal ₂ +Na] ⁺	1380.5003	-0.0200	0.0758
1403.4946	1403.4623	[GlcNAc ₃ ManNAc ₂ Gal ₂ +2Na] ⁺	1403.4901	-0.0045	0.0278
1421.5325	1421.4854	[GlcNAc ₄ ManNAc ₂ Gal ₁ +Na] ⁺	1421.5268	-0.0057	0.0414
1483.4787	1483.4963	[GlcNManNGlcNAc ₃ ManNAc ₁ Gal ₂ -O+Na] ⁺	1483.5634	0.0847	0.0671
1499.5223	1499.5162	[GlcNManNGlcNAc ₃ ManNAc ₁ Gal ₂ +Na] ⁺	1499.5583	0.0360	0.0421
1524.5672	1524.5162	[GlcNAc ₃ ManNAc ₁ Gal ₄ +2Na] ⁺	1524.5161	-0.0511	-0.0001
1542.6977	1542.5310	[GlcNAc ₃ ManNAc ₂ Gal ₃ +Na] ⁺	1542.5528	-0.1449	0.0218
1565.5451	1565.5510	[GlcNAc ₃ ManNAc ₂ Gal ₃ +2Na] ⁺	1565.5426	-0.0025	-0.0084
1583.6014	1583.5364	[GlcNAc ₄ ManNAc ₂ Gal ₂ +Na] ⁺	1583.5793	-0.0221	0.0429
1625.6340	1625.5233	[GlcNAc ₄ ManNAc ₂ GalOAcGal+Na] ⁺	1625.5898	-0.0442	0.0665
1661.6583	1661.5688	[GlcNManNGlcNAc ₃ ManNAc ₁ Gal ₃ +Na] ⁺	1661.6108	-0.0475	0.0420
1686.5755	1686.5566	[GlcNAc ₃ ManNAc ₁ Gal ₅ +2Na] ⁺	1686.5686	-0.0069	0.0120
1703.7262	1703.6128	[GlcNGlcNAc ₃ ManNAc ₂ Gal ₃ +Na] ⁺	1703.6213	-0.1049	0.0085
1727.6852	1727.6150	[GlcNAc ₃ ManNAc ₂ Gal ₄ +2Na] ⁺	1727.5951	-0.0901	-0.0199
1745.7051	1745.5923	[GlcNAc ₄ ManNAc ₂ Gal ₃ +Na] ⁺	1745.6318	-0.0733	0.0395
1848.6754	1848.6306	[GlcNAc ₃ ManNAc ₁ Gal ₆ +2Na] ⁺	1848.6211	-0.0543	-0.0095
1865.7980	1865.6704	[GlcNGlcNAc ₃ ManNAc ₂ Gal ₄ +2Na] ⁺	1865.6738	-0.1242	0.0034
1889.6858	1889.6458	[GlcNAc ₃ ManNAc ₂ Gal ₅ +2Na] ⁺	1889.6476	-0.0382	0.0018

Observed m/z (Sterne PS) a	Observed m/z (<i>csaB</i> PS) b	Proposed structure	Theoretical m/z (monoisotopic) c	Sterne m/z c-a	<i>csaB</i> m/z c-b
1907.7384	1907.6608	[GlcNAc ₄ ManNAc ₂ Gal ₆ +Na] ⁺	1907.6843	-0.0541	0.0235
1931.7430	1931.6667	[GlcNGlcNAc ₂ ManNAc ₂ Gal ₆ +2Na] ⁺	1931.6581	-0.0849	-0.0086
2009.7289	2009.7438	[GlcNGlcNAc ₂ ManNAc ₂ Gal ₅ +2Na] ⁺	2009.6896	-0.0393	-0.0542
2027.6312	2027.7323	[GlcNGlcNAc ₃ ManNAc ₂ Gal ₅ +Na] ⁺	2027.7263	0.0951	-0.0060
2051.7870	2051.7031	[GlcNAc ₃ ManNAc ₂ Gal ₅ +2Na] ⁺	2051.7001	-0.0869	-0.0030
2069.8491	2069.7141	[GlcNAc ₄ ManNAc ₂ Gal ₅ +Na] ⁺	2069.7368	-0.1123	0.0227
2093.8138	2093.7082	[GlcNAc ₃ ManNAc ₂ GalOAcGal ₅ +2Na] ⁺	2093.7106	-0.1032	0.0024
2111.8258	2111.7463	[GlcNAc ₄ ManNAc ₂ GalOAcGal ₆ +Na] ⁺	2111.7473	-0.0785	0.0010
2189.8865	2189.7595	[GlcNGlcNAc ₃ ManNAc ₂ Gal ₆ +Na] ⁺	2189.7788	-0.1077	0.0193
2213.8951	2213.7842	[GlcNAc ₄ ManNAc ₂ Gal ₆ -H ₂ O+Na] ⁺	2213.7788	-0.1163	-0.0054
2231.8272	2231.7766	[GlcNAc ₄ ManNAc ₂ Gal ₆ +Na] ⁺	2231.7893	-0.0379	0.0127
2254.8276	2254.7766	[GlcNAc ₄ ManNAc ₂ Gal ₆ +2Na] ⁺	2254.7791	-0.0485	0.0025
2274.8674	2274.7607	[GlcNAc ₃ ManNAc ₂ GalOAcGal ₅ +Na] ⁺	2274.7838	-0.0836	0.0231
2294.8876	2294.7683	[GlcNAc ₃ ManNAc ₂ GalOAcGal ₄ +H] ⁺	2294.8123	-0.0753	0.0440
2312.9262	2312.8281	[GlcNGlcNAc ₃ ManNAc ₃ Gal ₃ +Na] ⁺	2312.8583	-0.0679	0.0302
2354.9798	2354.8459	[GlcNAc ₆ ManNAc ₃ Gal ₃ +Na] ⁺	2354.8688	-0.1110	0.0229
2432.9039	2432.8970	[GlcN ₂ GlcNAc ₄ ManNAc ₃ Gal ₄ +Na] ⁺	2432.9003	-0.0036	0.0033
2456.9717	2456.8613	[GlcNGlcNAc ₄ ManNAc ₃ Gal ₅ +2Na] ⁺	2456.8741	-0.0976	0.0128
2499.1209	2498.8989	[GlcNAc ₃ ManNAc ₃ Gal ₅ +Na] ⁺	2498.8846	-0.2363	-0.0143
2517.0349	2516.9009	[GlcNAc ₃ ManNAc ₃ Gal ₄ +Na] ⁺	2516.9213	-0.1136	0.0204
2577.0763	2576.8870	[GlcN ₂ GlcNAc ₂ ManNAc ₂ Gal ₅ +2Na] ⁺	2576.9161	-0.1602	0.0291
2595.0364	2594.9209	[GlcN ₂ GlcNAc ₄ ManNAc ₂ Gal ₅ +Na] ⁺	2594.9528	-0.0836	0.0319
2620.1232	2619.8814	[GlcNAc ₅ ManNAc ₂ Gal ₇ +2Na] ⁺	2619.9106	-0.2126	0.0292
2637.0117	2636.9578	[GlcNGlcNAc ₃ ManNAc ₃ Gal ₅ +Na] ⁺	2636.9633	-0.0484	0.0055
2660.9568	2660.9664	[GlcNAc ₅ ManNAc ₃ Gal ₆ +2Na] ⁺	2660.9371	-0.0197	-0.0293
2800.2063	2800.0208	[GlcNAc ₅ ManNAc ₃ Gal ₇ +Na] ⁺	2799.9998	-0.2065	-0.0210
2823.1167	2823.0181	[GlcNAc ₅ ManNAc ₃ Gal ₇ +2Na] ⁺	2822.9896	-0.1271	-0.0285

Observed m/z (Sterne PS) a	Observed m/z (<i>csaB</i> PS) b	Proposed structure	Theoretical m/z (monoisotopic) c	Sterne m/z c-a	<i>csaB</i> m/z c-b
2841.2496	2841.0042	[GlcNAc ₆ ManNAc ₃ Gal ₆ +Na] ⁺	2841.0263	-0.2233	0.0221
2862.1650	2862.1125	[GlcNAc ₃ ManNAc ₃ GalOAc ₂ Gal ₅ +H] ⁺	2862.0388	-0.1262	-0.0737
2964.3171	2964.0210	[GlcNAc ₄ ManNAc ₂ GalOAc ₂ Gal ₆ +Na] ⁺	2964.0203	-0.2968	-0.0007
2987.2883	2987.0659	[GlcNAc ₅ ManNAc ₂ GalOAc ₂ Gal ₇ -H ₂ O+Na] ⁺	2987.0363	-0.2520	-0.0296
3003.1480	3003.0693	[GlcNAc ₆ ManNAc ₃ Gal ₇ +Na] ⁺	3003.0788	-0.0692	0.0095
3045.2293	3045.0647	[GlcNAc ₆ ManNAc ₃ GalOAcGal ₆ +Na] ⁺	3045.0893	-0.1400	0.0246
3165.3916	3165.1147	[GlcNAc ₆ ManNAc ₃ Gal ₈ +Na] ⁺	3165.1313	-0.2603	0.0166
3309.3999	3309.1826	[GlcNAc ₆ ManNAc ₃ Gal ₉ -H ₂ O+Na] ⁺	3309.1733	-0.2266	-0.0093
3327.3966	3327.2034	[GlcNAc ₆ ManNAc ₃ Gal ₉ +Na] ⁺	3327.1838	-0.2128	-0.0196
3367.4485	3367.2002	[GlcNGlcNAc ₆ ManNAc ₄ Gal ₇ +Na] ⁺	3367.2263	-0.2222	0.0261
3732.6504	3732.3103	[GlcNGlcNAc ₇ ManNAc ₄ Gal ₈ +Na] ⁺	3732.3578	-0.2926	0.0475
4098.6257	4098.4512	[GlcNAc ₈ ManNAc ₄ Gal ₁₀ +Na] ⁺	4098.4733	-0.1524	0.0221
4221.7969	4221.5103	[GlcNAc ₁₀ ManNAc ₅ Gal ₇ +Na] ⁺	4221.5528	-0.2441	0.0425
4260.5003	4260.5073	[GlcNAc ₈ ManNAc ₄ Gal ₁₁ +Na] ⁺	4260.5258	0.0255	0.0185
4422.4986	4422.6673	[GlcNAc ₈ ManNAc ₄ Gal ₁₂ +Na] ⁺	4422.5783	0.0797	-0.0890

Table 2

Ions present only in MALDI-TOF spectra of HF-PS extracted from Sterne and predicted to contain a ketal pyruvate

Observed m/z (Sterne PS) a	Proposed structure	Theoretical m/z (monoisotopic) b	Sterne m/z b-a
1247.3360	$[\text{GlcNAc}_3\text{ManNAc}_1\text{Gal}_2\text{kPyr}+\text{Na}]^+$	1247.4267	0.0907
1451.6573	$[\text{GlcNAc}_3\text{ManNAc}_1\text{GalOAcGal}_2\text{kPyr}+\text{Na}]^+$	1451.4897	-0.1676
1409.5652	$[\text{GlcNAc}_3\text{ManNAc}_1\text{Gal}_3\text{kPyr}+\text{Na}]^+$	1409.4792	-0.0860
1612.6528	$[\text{GlcNAc}_3\text{ManNAc}_2\text{Gal}_3\text{kPyr}+\text{Na}]^+$	1612.5582	-0.0946
1653.5932	$[\text{GlcNAc}_4\text{ManNAc}_2\text{Gal}_2\text{kPyr}+\text{Na}]^+$	1653.5847	-0.0085

Table 3 Summary of monoisotopic ions present in FTMS of HF-PS extracted from *B. anthracis* Sterne

Observed (monoisotopic) m/z a	Proposed structure	Theoretical (monoisotopic) m/z b	Dm/z b-a
495.1823	[GlcNAc-ManNAc-kPyr+H] ⁺	495.1817	-0.0006
537.1930	[GlcNAc-ManOAcNAc-kPyr+H] ⁺	537.1922	-0.0008
586.2456	[GlcN-GlcNAc-ManNAc+H] ⁺	586.2448	-0.0008
628.2561	[GlcNAc ₂ -ManNAc+H] ⁺	628.2553	-0.0008
693.7611	[GlcN-GlcNAc ₂ -ManNAc ₂ -Gal ₂ -kPyr+2H] ²⁺	693.7605	-0.0006
706.2882	[GlcNAc ₂ -ManNAc-Gal+H] ⁺	706.2868	-0.0014
714.7668	[GlcNAc ₃ -ManNAc ₂ -Gal ₂ -kPyr+2H] ²⁺	714.7658	-0.0010
730.2882	[GlcN-GlcNAc-ManNAc-Gal-H ₂ O+H] ⁺	730.2868	-0.0014
735.7722	[GlcNAc ₃ -ManNAc ₂ -GalOAc-Gal-kPyr+2H] ²⁺	735.7710	-0.0012
739.2940	[GlcN ₂ -GlcNAc ₂ -ManNAc ₂ -Gal ₂ +2H] ²⁺	739.2921	-0.0019
748.2988	[GlcN-GlcNAc-ManNAc-Gal+H] ⁺	748.2973	-0.0015
756.7789	[GlcNAc ₃ -ManNAc ₂ -GalOAc ₂ -kPyr+2H] ²⁺	756.7763	-0.0026
760.2993	[GlcN-GlcNAc ₃ -ManNAc ₂ -Gal ₂ +2H] ²⁺	760.2973	-0.0020
772.2995	[GlcNAc ₂ -ManNAc-Gal-H ₂ O+H] ⁺	772.2973	-0.0022
774.7882	[GlcN-GlcNAc ₃ -ManNAc ₁ -Gal ₃ -kPyr+2H] ²⁺	774.7868	-0.0014
790.3094	[GlcNAc ₂ -ManNAc-Gal+H] ⁺	790.3078	-0.0016
795.7933	[GlcNAc ₃ -ManNAc ₂ -Gal ₃ -kPyr+2H] ²⁺	795.7920	-0.0013
816.7988	[GlcNAc ₃ -ManNAc ₂ -GalOAc-Gal ₂ -kPyr+2H] ²⁺	816.7973	-0.0015
820.3196	[GlcN ₂ -GlcNAc ₂ -ManNAc ₂ -Gal ₃ +2H] ²⁺	820.3183	-0.0013
829.3262	[GlcN ₂ -GlcNAc ₂ -ManNAc ₂ -Gal ₃ +H ₂ O+2H] ²⁺	829.3236	-0.0026
832.3202	[GlcNAc ₂ -ManNAc ₁ -GalOAc+H] ⁺	832.3183	-0.0019
837.8029	[GlcNAc ₃ -ManNAc ₂ -GalOAc ₂ -Gal-kPyr+2H] ²⁺	837.8025	-0.0004
841.3248	[GlcN-GlcNAc ₃ -ManNAc ₂ -Gal ₃ +2H] ²⁺	841.3236	-0.0012
850.3314	[GlcN-GlcNAc ₃ -ManNAc ₂ -Gal ₃ +H ₂ O+2H] ²⁺	850.3288	-0.0026
862.3310	[GlcNAc ₄ -ManNAc ₂ -Gal ₃ +2H] ²⁺	862.3288	-0.0022
876.8190	[GlcNAc ₃ -ManNAc ₂ -Gal ₄ -kPyr+2H] ²⁺	876.8183	-0.0007
892.3413	[GlcN-GlcNAc-ManNAc-Gal ₂ -H ₂ O+H] ²⁺	892.3393	-0.0020
897.8234	[GlcNAc ₃ -ManNAc ₂ -GalOAc-Gal ₃ -kPyr+2H] ²⁺	897.8235	0.0001
901.3466	[GlcN ₂ -GlcNAc ₂ -ManNAc ₂ -Gal ₄ -kPyr+2H] ²⁺	901.3446	-0.0020
910.3517	[GlcN-GlcNAc-ManNAc-Gal ₂ +H] ⁺	910.3498	-0.0019
922.3520	[GlcN-GlcNAc ₃ -ManNAc ₂ -Gal ₄ +2H] ²⁺	922.3498	-0.0022
931.3559	[GlcN-GlcNAc ₃ -ManNAc ₂ -Gal ₄ +H ₂ O+2H] ²⁺	931.3551	-0.0009
934.3518	[GlcNAc ₂ -ManNAc-Gal ₂ -H ₂ O+H] ⁺	934.3498	-0.0020
943.3567	[GlcNAc ₄ -ManNAc ₂ -Gal ₄ +2H] ²⁺	943.3551	-0.0017

Observed (monoisotopic) m/z a	Proposed structure	Theoretical (monoisotopic) m/z b	$\Delta m/z$ b-a
952.3620	$[\text{GlcNAc}_2\text{-ManNAc-Gal}_2\text{+H}]^+$	952.3603	-0.0017
964.3620	$[\text{GlcNAc}_4\text{-ManNAc}_2\text{-GalOAc-Gal}_3\text{+2H}]^{2+}$	964.3603	-0.0017
973.3656	$[\text{GlcN-GlcNAc}_3\text{-ManNAc}_2\text{-Gal}_5\text{-C}_2\text{H}_4\text{O}_2\text{+2H}]^{2+}$	973.3656	0.0000
976.3637	$[\text{GlcNAc}_2\text{-ManNAc-GalOAc-Gal-H}_2\text{O+H}]^+$	976.3603	-0.0034
982.3722	$[\text{GlcN-GlcNAc}_3\text{-ManNAc}_2\text{-Gal}_4\text{+2H}]^{2+}$	982.3708	-0.0014
991.3777	$[\text{GlcN-GlcNAc}_3\text{-ManNAc}_2\text{-Gal}_4\text{+H}_2\text{O+2H}]^{2+}$	991.3761	-0.0017
994.3725	$[\text{GlcNAc}_2\text{-ManNAc-GalOAc-Gal+H}]^+$	994.3708	-0.0017
1003.3776	$[\text{GlcN-GlcNAc}_2\text{-ManNAc}_2\text{-Gal}_5\text{+2H}]^{2+}$	1003.3761	-0.0016
1012.3820	$[\text{GlcN-GlcNAc}_2\text{-ManNAc}_2\text{-Gal}_5\text{+H}_2\text{O+2H}]^{2+}$	1012.3813	-0.0007
1015.3763	$[\text{GlcNAc}_4\text{-ManNAc}_2\text{-Gal}_5\text{-H}_2\text{O+2H}]^{2+}$	1015.3761	-0.0003
1024.3831	$[\text{GlcNAc}_4\text{-ManNAc}_2\text{-Gal}_5\text{+2H}]^{2+}$	1024.3813	-0.0018
1032.8964	$[\text{GlcN-GlcNAc}_4\text{-ManNAc}_2\text{-Gal}_4\text{+H}_2\text{O+2H}]^{2+}$	1032.8946	-0.0019
1036.3817	$[\text{GlcNAc}_2\text{-ManNAc-GalOAc}_2\text{+H}]^+$	1036.3813	-0.0004
1045.3883	$[\text{GlcN-GlcNAc}_4\text{-ManNAc}_2\text{-Gal}_4\text{+H}_2\text{O+2H}]^{2+}$	1045.3866	-0.0018
1054.3920	$[\text{GlcN}_2\text{-GlcNAc}_2\text{-ManNAc}_2\text{-Gal}_6\text{-H}_2\text{O+2H}]^{2+}$	1054.3918	-0.0002
1063.3987	$[\text{GlcN}_2\text{-GlcNAc}_2\text{-ManNAc}_2\text{-Gal}_6\text{+2H}]^{2+}$	1063.3971	-0.0016
1066.3956	$[\text{GlcNAc}_4\text{-ManNAc}_2\text{-GalOAc}_2\text{-Gal}_3\text{+2H}]^{2+}$	1066.3918	-0.0038
1072.4044	$[\text{GlcN-GlcNAc-ManNAc-Gal}_2\text{+H}]^+$	1072.4023	-0.0021
1084.4040	$[\text{GlcN}_2\text{-GlcNAc}_2\text{-ManNAc}_2\text{-Gal}_6\text{+2H}]^{2+}$	1084.4023	-0.0017
1092.9188	$[\text{GlcN}_2\text{-GlcNAc}_3\text{-ManNAc}_2\text{-Gal}_7\text{+H}_2\text{O+2H}]^{2+}$	1092.9156	-0.0032
1096.4043	$[\text{GlcNAc}_3\text{-ManNAc-Gal}_3\text{-H}_2\text{O+H}]^+$	1096.4023	-0.0020
1100.4114	$[\text{GlcNAc}_5\text{-ManNAc}_3\text{-Gal}_3\text{-kPyr+2H}]^{2+}$	1100.4105	-0.0009
1105.4093	$[\text{GlcNAc}_4\text{-ManNAc}_2\text{-Gal}_6\text{+2H}]^{2+}$	1105.4076	-0.0018
1114.4148	$[\text{GlcNAc}_2\text{-ManNAc}_1\text{-Gal}_3\text{+H}]^+$	1114.4128	-0.0020
1121.4158	$[\text{GlcNAc}_5\text{-ManNAc}_3\text{-GalOAc-Gal}_2\text{-kPyr + 2H}]^{2+}$	1121.4158	-0.0001
1126.4155	$[\text{GlcNAc}_4\text{-ManNAc}_2\text{-GalOAc-Gal}_5\text{+2H}]^{2+}$	1126.4128	-0.0027
1131.4420	$[\text{GlcN-GlcNAc}_2\text{-ManNAc-Gal}_2\text{+H}_2\text{O+H}]^+$	1131.4393	-0.0027
1134.9285	$[\text{GlcNAc}_5\text{-ManNAc}_2\text{-Gal}_5\text{+H}_2\text{O+2H}]^{2+}$	1134.9261	-0.0024
1138.4152	$[\text{GlcNAc}_2\text{-ManNAc}_1\text{-GalOAc-Gal}_2\text{-H}_2\text{O+H}]^+$	1138.4128	-0.0024
1147.4144	$[\text{GlcNAc}_4\text{-ManNAc}_2\text{-GalOAc}_2\text{-Gal}_4\text{+2H}]^{2+}$	1147.4181	0.0037
1156.4252	$[\text{GlcNAc}_2\text{-ManNAc}_1\text{-GalOAc-Gal}_2\text{+H}]^+$	1156.4233	-0.0019
1165.4297	$[\text{GlcN-GlcNAc}_3\text{-ManNAc}_2\text{-Gal}_5\text{+2H}]^{2+}$	1165.4286	-0.0011
1169.4402	$[\text{GlcN-GlcNAc}_4\text{-ManNAc}_3\text{-Gal}_4\text{-kPyr+H}_2\text{O+2H}]^{2+}$	1169.4368	-0.0034
1174.4331	$[\text{GlcN-GlcNAc}_3\text{-ManNAc}_2\text{-Gal}_7\text{+H}_2\text{O+2H}]^{2+}$	1174.4338	0.0007
1181.4389	$[\text{GlcNAc}_5\text{-ManNAc}_3\text{-Gal}_4\text{-kPyr+2H}]^{2+}$	1181.4368	-0.0022
1186.4353	$[\text{GlcNAc}_4\text{-ManNAc}_2\text{-Gal}_7\text{+2H}]^{2+}$	1186.4338	-0.0015

Observed (monoisotopic) m/z a	Proposed structure	Theoretical (monoisotopic) m/z b	$\Delta m/z$ b-a
1190.4426	[GlcNAc₅-ManNAc₃-Gal₄-kPyr+H₂O + 2H]²⁺	1190.4420	-0.0006
1194.9508	[GlcN-GlcNAc ₄ -ManNAc ₂ -Gal ₆ +H ₂ O+2H] ²⁺	1194.9471	-0.0038
1198.4353	[GlcNAc ₂ -ManNAc ₁ -GalOAc ₂ -Gal+H] ⁺	1198.4338	-0.0015
1202.4457	[GlcNAc₅-ManNAc₃-GalOAc-Gal₃-kPyr+2H]²⁺	1202.4420	-0.0037
1211.4495	[GlcNAc₅-ManNAc₃-GalOAc-Gal₃-kPyr+H₂O+2H]²⁺	1211.4473	-0.0023
1214.9717	[GlcN ₂ -GlcNAc ₄ -ManNAc ₃ -Gal ₄ +H ₂ O+2H] ²⁺	1214.9683	-0.0034
1234.4578	[GlcN-GlcNAc-ManNAc ₁ -Gal ₄ +H] ⁺	1234.4548	-0.0030
1241.4622	[GlcN-GlcNAc₄-ManNAc₃-Gal₅-kPyr+2H]²⁺	1241.4578	-0.0044
1250.4643	[GlcN-GlcNAc₄-ManNAc₃-Gal₅-kPyr+H₂O+2H]²⁺	1250.4630	-0.0013
1262.4645	[GlcNAc₅-ManNAc₃-Gal₅-kPyr+2H]²⁺	1262.4630	-0.0015
1266.4742	[GlcNAc₃-ManNAc₂-Gal₁-kPyr+H]⁺	1266.4712	-0.0030
1271.4719	[GlcNAc₅-ManNAc₃-Gal₅-kPyr+H₂O+2H]²⁺	1271.4683	-0.0037
1276.4675	[GlcNAc ₂ -ManNAc ₁ -Gal ₄ +H] ⁺	1276.4653	-0.0022
1283.4701	[GlcNAc₅-ManNAc₃-GalOAc-Gal₄-kPyr+2H]²⁺	1283.4683	-0.0019
1292.4777	[GlcNAc₅-ManNAc₃-GalOAc-Gal₄-kPyr+H₂O+2H]²⁺	1292.4735	-0.0042
1308.4826	[GlcNAc ₅ -ManNAc ₃ -Gal ₆ +2H] ²⁺	1308.4866	0.0040
1304.4719	[GlcNAc₅-ManNAc₃-GalOAc₂-Gal₃-kPyr+2H]²⁺	1304.4735	0.0016
1313.4803	[GlcNAc₅-ManNAc₃-GalOAc₂-Gal₃-kPyr+H₂O+2H]²⁺	1313.4788	-0.0015
1322.4853	[GlcN₂-GlcNAc₃-ManNAc₃-Gal₆-kPyr+2H]²⁺	1322.4840	-0.0013
1329.0025	[GlcNAc ₆ -ManNAc ₃ -Gal ₅ +2H] ²⁺	1328.9998	-0.0027
1331.4937	[GlcN₂-GlcNAc₃-ManNAc₃-Gal₆-kPyr+H₂O+2H]²⁺	1331.4893	-0.0044
1343.4923	[GlcNAc₅-ManNAc₃-Gal₆-kPyr+2H]²⁺	1343.4893	-0.0030
1352.5004	[GlcNAc₅-ManNAc₃-Gal₆-kPyr+H₂O+2H]²⁺	1352.4945	-0.0059
1364.4977	[GlcNAc₅-ManNAc₃-GalOAc-Gal₅-kPyr+2H]²⁺	1364.4945	-0.0032
1373.0083	[GlcNAc₆-ManNAc₃-Gal₅-kPyr+H₂O+2H]²⁺	1373.0078	-0.0005
1377.0226	[GlcN ₂ -GlcNAc ₄ -ManNAc ₃ -Gal ₆ +H ₂ O+2H] ²⁺	1377.0208	-0.0018
1385.4979	[GlcNAc₅-ManNAc₃-GalOAc₂-Gal₄-kPyr+2H]²⁺	1385.4998	0.0018
1389.0226	[GlcN-GlcNAc ₅ -ManNAc ₃ -Gal ₆ +2H] ²⁺	1389.0208	-0.0018
1398.0293	[GlcN-GlcNAc ₅ -ManNAc ₃ -Gal ₆ +H ₂ O+2H] ²⁺	1398.0261	-0.0032
1410.0305	[GlcNAc ₆ -ManNAc ₃ -Gal ₆ +2H] ²⁺	1410.0261	-0.0045
1419.0344	[GlcNAc ₆ -ManNAc ₃ -Gal ₆ +H ₂ O+2H] ²⁺	1419.0313	-0.0031
1428.5271	[GlcNAc₃-ManNAc₂-Gal₂-kPyr+H]⁺	1428.5237	-0.0034
1470.0510	[GlcN-GlcNAc ₅ -ManNAc ₃ -Gal ₇ +2H] ²⁺	1470.0471	-0.0040
1479.0597	[GlcN-GlcNAc ₅ -ManNAc ₃ -Gal ₇ +H ₂ O+2H] ²⁺	1479.0523	-0.0074
1491.0571	[GlcNAc ₆ -ManNAc ₃ -Gal ₇ +2H] ²⁺	1491.0523	-0.0048
1500.0636	[GlcNAc ₆ -ManNAc ₃ -Gal ₇ +H ₂ O+2H] ²⁺	1500.0576	-0.0060

Observed (monoisotopic) <i>m/z</i> a	Proposed structure	Theoretical (monoisotopic) <i>m/z</i> b	<i>Dm/z</i> b–a
1512.5538	[GlcNAc₃-ManNAc₂-GalOAc₂-kPyr+H]⁺	1512.5447	–0.0091
1551.0746	[GlcN-GlcNAc ₅ -ManNAc ₃ -Gal ₈ +2H] ²⁺	1551.0733	–0.0013
1560.0836	[GlcN-GlcNAc ₅ -ManNAc ₃ -Gal ₈ +H ₂ O+2H] ²⁺	1560.0786	–0.0050
1572.0835	[GlcNAc ₆ -ManNAc ₃ -Gal ₈ +2H] ²⁺	1572.0786	–0.0050
1581.0867	[GlcNAc ₆ -ManNAc ₃ -Gal ₈ +H ₂ O+2H] ²⁺	1581.0838	–0.0029
1590.5824	[GlcN ₂ -GlcNAc ₃ -ManNAc ₃ -Gal ₁₀ +2H] ²⁺	1590.5811	–0.0014
1632.5934	[GlcNAc ₅ -ManNAc ₃ -Gal ₁₀ +2H] ²⁺	1632.5916	–0.0018
1653.1103	[GlcNAc ₆ -ManNAc ₃ -Gal ₉ +2H] ²⁺	1653.1048	–0.0055
1662.1193	[GlcNAc ₆ -ManNAc ₃ -Gal ₉ +H ₂ O+2H] ²⁺	1662.1101	–0.0093

Ions in bold contain a ketal pyruvate.