

Identification and role of interspace saccharide layer(s) on the structure and properties of *Bacillus cereus* spores

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Research Article

Keywords: *B. cereus*, saccharides, exosporium, interspace, spore

Posted Date: October 14th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11160566/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background

Spores of *Bacillus cereus sensu lato* are surrounded by an exosporium, the outermost spore layer, which contributes to spore protection and adhesion to surfaces. The exosporium is a balloon-like structure separated from the coat layer by the interspace. Some proteins may be located in this compartment and the presence of two distinct saccharide layers within the interspace of *B. anthracis* spores have been recently evidenced, although the exact composition and structure of these saccharides has not been described. Whether the interspace of *B. cereus* spores also contains some saccharides remains unknown, as well as their composition and their contribution to spore structure and properties.

Results

We demonstrated, by fluorescence microscopy, the presence of N-acetylglucosamine (GlcNAc) in the interspace of *B. cereus* ATCC 14579 spores. A bioinformatics approach identified five gene clusters, predicted to be involved in saccharide biosynthesis or assembly and potentially expressed during sporulation. Corresponding deletion mutants were constructed and chromatography analysis of the monosaccharide composition of the spore fraction, containing both interspace and exosporium, revealed significant differences in monosaccharide quantities between mutant and wild-type spores. When analyzed by electron microscopy, more exosporium-less spores were observed in mutant spores compared to wild-type and spores of two mutants exhibited multilayers external to spore coat, whereas another one displayed a thinner cortex associated with a thicker core. Some gene deletions also affected spore heat-resistance.

Conclusions

Our results provide evidence for the presence of a GlcNAc-containing saccharide in the interspace of *B. cereus* spores and indicate that interspace-associated saccharides contribute to spore structure and properties.

Background

Under unfavourable conditions, bacteria of the *Bacillaceae* and *Clostridiaceae* family produce dormant spores encased in a series of concentric shells, which play a role in their resistance properties, adhesion to biotic and abiotic surfaces and germination capacities. The overall structure is well conserved among species and is composed of a central core that contains the DNA, surrounded by the cortex, a layer of peptidoglycan and the coat. The most outerlayer differ among *Bacillus* species: in the model organism *Bacillus subtilis*, the inner and outer coats are covered by the crust, while spores of *Bacillus cereus sensu*

lato, exhibit a single layer coat separated by an interspace from the external balloon-like layer, named the exosporium (1).

The crust surface of *B. subtilis* spores is composed of six proteins, CgeA, CotV, CotW, CotX, CotY and CotZ, the three latest being considered as morphogenetic proteins, and of glycans (2). The glycoprotein CgeA acts as a linker between the polysaccharides layer and the rest of the spore (3). Crust polysaccharides and proteins play a role on spore surface properties (4). Analysis of the monosaccharides of the crust showed the presence of N-acetylglucosamine (GlcNAc), rhamnose (Rha), galactose (Gal), 3-O-methyl-rhamnose (3-O-Me-Rha), muramic lactam and quinovose (5, 6). Recent publications have described two new saccharides in the crust fraction, legionaminic acid, whose biosynthesis is mediated by enzymes encoded by the *sps* genes (7), and the quinovosan (8). The saccharide composition can be impacted by the composition of the sporulation medium or the presence of oxygen, consequently modifying spore adhesion properties (6).

The exosporium of *B. cereus* and *B. anthracis* spores is composed of about twenty proteins, including the morphogenetic proteins ExsY and CotY (9). ExsY is the main structural component of the exosporium and self-assembles to form a two-dimensional crystalline basal layer while CotY, in association with ExsY, forms the cap in *B. anthracis* forespores (10, 11). Previously, we demonstrated that the assembly of these proteins during *B. cereus* exosporium formation is a bidirectional process guided by the morphogenetic protein CotE, which positioned CotY to form one cap at the Mother Cell Proximal (MCP) and at the Mother Cell Distal (MCD) poles and then guides ExsY for the forespore encasement (12). In addition, CotE, together with the exosporium proteins CotO and ExsA, anchor the exosporium to the spore coat (13, 14). The exosporium is surrounded by an external nap of hair-like projections, composed of different glycosylated proteins, with BclA being the main one (15, 16). The analysis of the saccharide composition of *B. cereus* exosporium revealed the presence of GlcNAc, N-acetylgalactosamine (GalNAc), Rha and methyl forms of rhamnose [2,4-methyl-rhamnose (2,4-Me-Rha), 2-O-methyl-rhamnose (2-O-Me-Rha) and 3-O-Me-Rha], with some variations according to the strains (17). The glycosylation of BclA has been studied in *B. cereus* and in *B. anthracis* (18, 19). The protein BclA is structured in three regions, the N-terminal domain (NTD) which enables the fixation to the basal layer of exosporium, the intermediate collagen-like region (CLR) with short O-glycans, such as Rha, 3-O-Me-Rha and GalNAc, bearing anthrose corresponding to 2-O-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-didesoxy-D-glucose for *B. anthracis* or cereose (Cro) corresponding to 3-C-Methyl-6-deoxygulose for *B. cereus* (18, 20, 21). The last region, the C-terminal globular domain (CTD), is composed of high-molecular weight saccharides, whose carbohydrate composition and abundance depend on the species (21). Cereose is not exclusive to *B. cereus* spore glycoprotein BclA, as it was also detected in $\Delta bclA$ spores (22). This observation suggests that cereose may also be associated with other spore macromolecules, such as urea-resistant glycoproteins or SDS-resistant glycolipids, since it remained detectable after combined urea and SDS treatment. These treatments are known to release exosporium glycoproteins BclA and BclB. Spores of *B. cereus sensu lato* are also adorned of numerous appendages, referred as ENAs, anchored to the exosporium and involved in adhesion and biofilm formation (23). As glycosyltransferase genes are present in the locus encoding F-ENA genes, the authors suggested that the F-BclA domain could be

glycosylated. The exosporium contributes to spore adhesion by modulating the surface hydrophobicity and isoelectric point of spores (9, 18). The composition of the interspace region, localized between the coat and the exosporium, is still poorly described. Some proteins have been assigned to the interspace, such as the inosine hydrolase *IunH* of *B. anthracis* (24), this protein having a role in moderating nucleoside-induced germination in *B. thuringiensis* (25). The protein *ExsK* was also located by fluorescence microscopy beneath the exosporium in *B. anthracis* (26, 27) and additional proteins, such as *BclC* and *ExsFB* may also be located in the interspace (24, 28). In addition, using a series of lectins which binds different saccharides, the presence of saccharides has been reported in the interspace on *B. anthracis* spores (27). Taking into account the differential binding of lectins to selected coat and exosporium mutant spores, the authors proposed a model with two distinct interspace saccharide layers, consisting of a first internal layer made, at least of GlcNAc and formed at the engulfment stage and of a second layer of GalNAc and/or Gal observed during the formation of the exosporium cap. The layers of saccharides seem to play a role in spore assembly, as the formation of the cap and the exosporium appears to depend on these inner saccharides (27). These authors also hypothesized that they maintain the contact between the forespore and the exosporium during sporulation and potentially plays a role during germination as nutritional reserve.

Based on these new data, we hypothesized that the interspace of *B. cereus* may also contain some saccharides. It has been shown that the glycosylation of *BclA* differs between *B. anthracis* and *B. cereus* (21) and the saccharide composition of the interspace may also be different.

The aims of this work were i) to evidence the presence of saccharides in the interspace of *B. cereus* spores, ii) to identify among candidate genes those involved in their biosynthesis or assembly and iii) to reveal their contribution to spore structure and properties. To this end, we generated deletion mutants of selected genes predicted to encode enzymes involved in the biosynthesis or assembly of spore saccharide during sporulation. We determined the monosaccharide composition of the mutant spores by GC-MS and RP-HPLC-FL and compared it with that of the wild-type (WT). We then examined the structure and resistance properties of the mutant spores. We showed that deletion of genes involved in saccharides assembly alters the saccharide quantity of the spore supernatant fraction, impacts the outer spore layers and modifies some spore properties.

Methods

Strains and gene designation

All strains are described in Table 1. For clarity and to improve the flow of the text, genes classified as having an unknown function were referred to by their locus_tag. The locus_tag annotations corresponding to genes used in this study were based on the *B. cereus* ATCC 14579 genome sequence available under GenBank accession number AE016877.1. Because locus_tags in this genome sequence are designated either as "BCNumber" or "BC_Number", the corresponding gene names were standardized by omitting the underscore and formatting in italics. For example, the gene annotated with the locus_tag

BC1204 or BC_1204 was referred to as “BC1204”. The same nomenclature was used for genes derived from the *B.anthraxis* genome sequence available under GenBank accession number AE017225.1. For example, the locus tags “BAS1128” and “BAS_RS1128” were both designated as *BAS1128* for the corresponding gene.

Table 1
Strains and plasmids used in this study.

Strain and plasmid used	Genotype	Reference
<i>Bacillus cereus</i> ATCC 14579 W/O	<i>Bacillus cereus</i> ATCC 14579 without pClin15 plasmid	Laboratory collection
<i>B. cereus</i> Δ 1204–1206	<i>Bacillus cereus</i> ATCC 14579 W/O strain deleted of BC1204, BC1205 and BC1206 genes	This study
<i>B. cereus</i> Δ 1943–1945	<i>Bacillus cereus</i> ATCC 14579 W/O strain deleted of BC1943, BC1944 and BC1945 genes	This study
<i>B. cereus</i> Δ 3427	<i>Bacillus cereus</i> ATCC 14579 W/O strain deleted of BC3427 gene	This study
<i>B. cereus</i> Δ 3514–3517	<i>Bacillus cereus</i> ATCC 14579 W/O strain deleted of BC3514, BC3515, BC3516 and BC3517 genes	This study
<i>B. cereus</i> Δ 4863–4867	<i>Bacillus cereus</i> ATCC 14579 W/O strain deleted of BC4863, BC4863, BC4864, BC4865, BC4866 and BC4867 genes	This study
<i>B. cereus</i> ATCC 14579 Δ exsA	<i>Bacillus cereus</i> ATCC 14579 strain deleted of gene encoding for protein ExsA (SafA)	(50)
<i>B. cereus</i> ATCC 14579 W/O Δ cotE	<i>Bacillus cereus</i> ATCC 14579 W/O strain deleted of gene encoding for protein CotE	(29)
<i>B. cereus</i> ATCC 14579 Δ bclA	<i>Bacillus cereus</i> ATCC 14579 strain deleted of gene encoding for protein BclA	(18)
<i>B. cereus</i> ATCC 14579 Δ exsY	<i>Bacillus cereus</i> ATCC 14579 strain deleted of gene encoding for protein ExsY	Gift of Christine Faille
<i>E. coli</i> SCS110	<i>rpsL thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44 (lac-proAB)</i>	Laboratory collection
<i>E. coli</i> DH5 α	<i>fhuA2 lacU169 phoA glnV44 80 = lacZM15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 L</i>	Laboratory collection
pLM4	Spec ^r cloning vector	(51)
pLMGG1	Kan ^r cloning vector, derived to pLM4 plasmid	S. Chamot, personal communication

Standardized growth of strains

Bacillus cereus ATCC 14579 (WT) (29) and isogenic mutants were grown in triplicate at 30°C in LB (Luria-Bertani broth) medium at 200 rpm overnight. The precultures were diluted to 1/100 until reaching an OD_{600nm} equal to 0.8. The bacterial cultures were then inoculated in microplates with OD_{600nm} equal to 0.05. The growth monitoring was realized at 20°C and 37°C in LB with the reading of OD_{600nm} (TECAN, Nano Quant Infinite) every 15 min during 24h under agitation.

Spore production

B. cereus spores were produced on Fortified Nutrient Agar (FNA) plates (30) at 20°C, as previously described (29). When the majority of spores was refringent and free under a phase-contrast microscope with 1,000X magnification, spores were and washed four times with cold water by centrifugation at 4°C, twice at 4,550 g then 2,320 g and finally 1,485 g. Spore pellets were resuspended in 1 ml of cold water and stored at 4°C until use. Initial concentration of spore suspensions was determined by serial dilution, plated on to LB plates and incubated 24h at 30°C. Three independent batches of spores were produced for each strain. For RP-HPLC-FL analyses, spores were submitted to two additional washes at 1,500g during 15 min at 4°C.

Sporulation capacity

Precultures were grown in triplicate in LB media at 30°C at 200 rpm during 16 h and diluted to 1/100 until reaching an OD_{600nm} equal to 1.0. The cultures were centrifuged during 10 min at 6,000g at 12°C. The bacteria pellets were resuspended in Sporulation Mineral Buffer (SMB) (31) and incubated during 72 h at 20°C. Sporulation was evaluated by taking a 1 mL-sample aliquot at 72 h, from which 500µL of the suspension were used for total viable count (vegetative cells and spores) and 500µL were heated 10 min at 70°C for spore counts. An enumeration was performed after spreading each aliquot- fraction on LB media and after incubation at 30°C during 24 h.

Selection of candidate genes based on their putative functions

A search of homology with *Bacillus anthracis* proteins involved in saccharides biosynthesis or assembly was performed with the Protein Blast (BLASTP) tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and with *Bacillus subtilis* genes using the Subtiwiki website (<https://subtiwiki.uni-goettingen.de/v5/welcome>) to obtain a first list of candidate genes. This list was completed with the CAZy database (Carbohydrate Active enZYme) (<http://www.cazy.org/b139.html>), used to select genes implicated in saccharide biosynthesis in the genome of *B. cereus* ATCC 14579. The research was continued by focusing on proteins identified in literature with biochemical appropriate activities such as saccharide biosynthesis, transfer and degradation.

Among these proteins, we only selected those which corresponding genes were likely expressed during sporulation, by searching the presence of sporulation sigma factor consensus boxes with FIMO Tool

(<https://meme-suite.org/meme/tools/fimo>) on the 200 bp region upstream of each gene and 500 bp for gene *BC1943*. Different sigma factors consensus binding sites were researched: σ^G G[ACT]ATANNNNNNNNNNNNNNNNNNNNN[AC]A[AT]A[AC]TA (32), σ^K [AC] [AT]C[AC]NNNNNNNNNNNNNNNNNNNNCATANNNT[AGT] (33), σ^F G[CT]ATANNNNNNNNNNNNNNNNNNNNGGNNANA[ACT]T[AG] (32), σ^H [AG]NAGGA[AT][AT] [AT]NNNNNNNNNNNN[AG]NNGAAT[AT][AT] and [AG]NAGGA[AT][AT] [AT]NNNNNNNNNNNN[AG]NNGAAT[AT][AT] (34), and σ^E [AGT][CT][AC]T[AG][AT] [AT]NNNNNNNNNNNNNNNNNNNCATA[ACT]A[AT]T (35). Transcriptomic data on *B. anthracis* obtained during sporulation were also used (36) to check potential expression of the candidate genes during sporulation, via homologs in *B. anthracis*.

Finally, the NCBI database (National Center for Biotechnology Information) was used to check the synteny of saccharide biosynthesis genes in *B. cereus* ATCC 14579 genome AE016877.1 (<https://www.ncbi.nlm.nih.gov/nuccore/AE016877.1?report=graph>). Analysis of genes conservation within *B. cereus sensu lato* was performed with the optimized 'Highly similar sequences BLASTN algorithm (megablast program) (<https://blast.ncbi.nlm.nih.gov/>), and by selecting the 'refseq_genomes database' and each appropriate known taxid in this bacterial group (Table S1).

Construction of mutant strains

Regions of 800 bp located upstream and downstream of *BC1204-1206*, *BC1943-1945*, *BC3427*, *BC3514-3517* and *BC4863-4867* genes were amplified by PCR (Table 2) and cloned in the *BsaI* sites of the thermosensitive plasmid pLMGG, which derives from pLM4, using the Golden Gate assembly method (37)(Chamot et al, personal communication) Plasmids were then transformed in *E. coli* DH5 α , sequenced and finally transferred in *E. coli* SCS110 (*dam*⁻/*dcm*⁻) before introduction in *B. cereus* by electroporation, as already described (38). For the double crossing-over, bacteria were first grown on LB-kanamycin 150 μ g/mL at 42°C and then on LB at 30°C. The deletions were verified by PCR with external primers (Table 2) and by sequencing the amplicons.

Table 2

Oligonucleotides used in this study. Uppercase nucleotides indicate the non-complementary region, whereas lowercase nucleotides indicate the complementary region involved in primer hybridization.

Name	Sequence
up 1204_fwd	ggctacggtctcgttgcTAAGAATGCTTGTCTTAATTGATAG
up 1204_rev	ggctacggtctcaaaatACTACTCTGCAAAATTAAAAGTG
down 1206_fwd	ggctacggtctcaATTTGTGCCTCCTGCAAAATG
down 1206_rev	ggctacggtctcatgccATAATTATTCAAATGGATTAAATCCCGATG
ext_1204_F	GACACTTGCGCCTAGAAAGC
ext_1206_R	CTTTCGGTTTTGCATCTAC
down_1206_F (800 bp)	CCAGCGGAGTTAAATGC
ext_1206_R (1400 bp)	CAACGGGCTATTGTCTCCTCC
up 1943_fwd	ggctacggtctccttgcATAGCGTCACAACTTTTATC
up 1943_rev	ggctacggtctcacattctttttgTTAACTCTCTCACCTCTTC
down 1945_fwd	ggctacggtctcaAATGAATGCTTTTTATTTTATCCC
down 1945_rev	ggctacggtctcttgccTTTCGTTATATCCGACGATTTG
ext_1943-F	GAGTGATCCGACGTATGAATTG
ext_1945_R	CCGTCTGCATAAGCTCGGAG
up 3427_fwd	ggctacggtctctttgcATCTTTTCTCTCCTCAATC
up 3427_rev	ggctacggtctcttccatttAATATTCCCCCTTATTTTATAAGG
down 3427_fwd	ggctacggtctctTGGAGAAAATCAGAAGAGG
down 3427_rev	ggctacggtctcgtgccAATACTGTTCAATTGCTTCG
ext-3427-F	GTAGCTCTATGGATAGGTACTCTTAG
ext-3427-R	CTCTGTAAGCGACACTTGC
3514-up_fwd	ggctacggtctccttgcATATCTAATATTGTTGAACAAAATTGG
3514-up_rev	ggctacggtctcttAGATTTATTTCTTCCTCTC
3517-down_fwd	ggctacggtctcttctACTACTCCCACTTCCCCATAC
3517- down_rev	ggctacggtctcgtgccTGTAATAAGAAGACAATAAAGAAAACAAAAAAG
ext-3514-F	GGGGAAATCGAGATGAAGCGGTTG
ext-3517-R	CGTTCTATCAGGTGAACTTAGAACTGTG

Name	Sequence
up_4867_fwd	ggctacgggtctcggtgcAAACTTGACGATAATGTGATC
up_4867_rev	ggctacgggtctcccaatatagcaacctctTATGCTCACCTCACTTAG
down 4863_fwd	ggctacgggtctccATTGGGAGATATATTATCGATTTC
down 4863_rev	ggctacgggtctcctgccATTGATTCTCTCGTCAATC
ext_4867-F	GAGACGTAGCGACATCTTC
ext_4863_R	GCATTTTCGAAATACCAGATGG

Lectin assay

Pellets of WT and mutant spores were fixed in a paraformaldehyde solution 4% overnight at 4°C. Paraformaldehyde was removed by washing three times with phosphate buffer saline (PBS) by centrifugation at 10,000 g at room temperature for 3 min. Next, the pellets of fixed spores were individually labelled with the Wheat Germ Agglutinin lectin (WGA) coupled to Alexa fluor 594 (Invitrogen, 10 µg/ml), which binds GlcNAc. The lectin was diluted in Tris-cation buffer (1 mM Tris-HCl pH 7.5 containing 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂, and 0.1% [v/v] Tween 20) (27). Incubation was done in dark tubes during 1 h at room temperature on shaking tray. Spores were washed three times with PBS, by centrifugation at 10,000 g at room temperature for 3 min and were suspended in 10 to 30 µL of PBS before being observed by fluorescence microscopy (Olympus BX 50, Rungis, France). A minimum of 100 spores were counted for each strain, except for WT spores treated with the French Press as described below, for which at least 21 spores were counted.

Measurement of glycogen level in spores

Spores were produced on FNA plates supplemented with 0.5% glucose and purified as described above in the spore production section. To measure the glycogen in spores, $2 \cdot 10^9$ spores suspended in sterile water were lysed by bead beating using a FastPrep II instrument (MP Biomedicals) for 10 cycles of 45 s at speed 6.5. Cell debris were removed by centrifugation at $7,000 \times g$ for 10 min. Glycogen levels in the resulting supernatant were quantified using the Glycogen Assay Kit (MAK465, Sigma-Aldrich), according to the manufacturer's instructions.

Exosporium and interspace extraction, and saccharide composition analysis

Spore suspensions were mechanically treated with French press (SLM instruments, Urbana, IL) to remove the exosporium by two successive passages at 14,000 psi. After that, two successive centrifugations (6,800 g, 15 min, 4°C) were applied to separate the supernatant and the pellet fractions.

The two pellets corresponding to spores and insoluble exosporium fragments were combined and the efficiency of the French press treatment was evaluated on mutant and WT spores by Transmission Electron Microscopy (TEM) with negative staining, as described below. The supernatant fraction, containing interspace content and soluble exosporium fragments was stored at -20°C before lyophilization prior to analysis. The monosaccharide composition of the supernatant fraction was established by Gas chromatography–mass spectrometry (GC-MS) as alditol acetate derivatives, as previously described (21) on the GC-MS Glycoconjugate Analysis Platform, PAGés (Lille, France). Release, derivation and separation of the supernatant monosaccharides by RP-HPLC-FL were performed, as already described (6). Briefly, after acid hydrolysis, monosaccharides were coupled to anthranilic acid and analysed by HPLC coupled with a fluorescence detector. The detection of monosaccharides was realized by measure of fluorescence with a Waters 2,475 fluorimeter ($\lambda_{exc} = 360$ nm, $\lambda_{em} = 425$ nm). For quantification of monosaccharides a standard containing GlcN, GalN, Gal, Glc, and Rha was used. The amount of each monosaccharide (pmol) was estimated by measuring the area of the peak and by reporting this area to a standard curve of the corresponding monosaccharide. For a better comparison with WT results, values were normalized by the OD_{600nm} unit of the spore preparations before French Press treatment (pmol/OD_{600nm} unit).

Spore properties

To test spore germination, spore suspensions were adjusted at an OD_{600nm} between 1.4 and 1.5, activated 10 min at 70°C and sonicated (Sonorex Bandelin Electronics, 3 min, 50 KHz). Spores were transferred into 200 µL of sterile 10 mM Tris-HCl / 10 mM NaCl, pH 7.4 buffer with inosine 1 mM, or without as a control, or into 200 µL of LB medium. Spores were incubated at 37°C during 30 min with agitation for inosine germination assay and during 4 h for LB germination assay, in microplate reader (BioTek Synergy H1, Agilent instrument). The OD_{600nm} was taken every 10 min. The percentage of germination was calculated with this formula $(1 - (OD_{600nm} T1 \text{ inosine} - (OD_{600nm} T1 \text{ control} - OD_{600nm} T0 \text{ control}))) / (OD_{600nm} T0 \text{ inosine}) * 100$.

The spore heat-resistance was determined in capillarity tubes by incubation at 90°C during 30 min of 0.1 mL of spore suspension (10^7 CFU·mL⁻¹), which have been prior activated 10 min at 70°C and sonicated during 3 min at 50 kHz. The reduction of concentration of viable spore was determined by enumeration (spores/mL) on LB media at t = 0 and every 5–10 min, and expressed as logarithmic reduction by reference to the number of spores/mL at t₀, i.e., $\log_{10} (N/N_0) = f(t)$, where t=time in min, N=spores/mL at time t, N₀ = spores/mL at t = 0 min.

To test spore hydrophobicity, a suspension of 1.9 mL of spore (at initial OD_{600nm} equal 1) was sonicated during 3 min at 50 kHz. Hexadecane preheated to 37°C (100 µL) was added to the spore suspension, the mix was vortexed during 1 min at maximum speed, then left to settle during 15 min at room temperature. The final OD_{600nm} was measured in the aqueous phase. The percentage of spores transferred from the aqueous phase to the solvent phase was calculated with this formula: $100 - ((\text{final OD}_{600nm} / \text{initial}$

OD_{600nm}) *100). All experiments to determine spore properties of WT and mutant spores were performed with three independent spore batches.

Electron microscopy analyses

Spore analysis by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) were realized on SAVE and μ A pole of PROPHYLE microscopy platforms. Spores were fixed with 2.5% glutaraldehyde (v/v) in a 0.2 M sodium cacodylate buffer (pH 7.4) during 1.5 h at room temperature and overnight à 4°C. For SEM observation, spores were then washed three times in 0.2 M sodium cacodylate and subjected to 20 successive dehydration baths containing increasing concentrations of ethanol (50% to 100%) before critical-point drying under CO₂. Samples were mounted on aluminum SEM sample stubs (15 mm diameter x 6mm M4, Micro to Nano, Haarlem, Netherlands) with conductive double-sided adhesive tape (Agar Scientific, Oxford Instruments SAS, Gometz-la-Ville, France) and sputter coated with Gold-Palladium (Polaron SC7640, Milexia, Verrières-le-buisson, France) for 220 s at 10 mA. Samples were visualized by field emission gun scanning electron microscopy (SEM FEG). They were viewed as secondary electron (2 kV) with a Hitachi S4500 instrument (Milexia, Verrières-le-buisson, France). Around eighteen images were taken and twenty-five spores were observed for each strain. To further investigate the ultrastructure of the spores, observations were also performed by TEM, either after negative staining to the examine the overall aspect of the exosporium of after embedding and ultrathin sectioning for cytological analysis, For negative staining, a drop of fixed spores was filled on the grid and incubated with ammonium molybdate solution 1% (p/v). Around twenty pictures were taken for each strain. For thin sections, fixed spores were washed three times in 0.2 M sodium cacodylate by 60 min-centrifugations at 3,500g, post-fixed 1h with 1% osmium tetroxide, and gradually dehydrated in ethanol (30% to 100%) and gradually substituted in a mix of propylene oxyde-epon and embedded in Epon (Delta Microscopie, Labège, France). Thin sections (80 nm) were collected onto 200-mesh copper grids, and counterstained with lead citrate to allow TEM visualization with a Hitachi HT7800 instrument (Hitachi High-Technologies). About forty images were taken for each strain with longitudinal and cross-section images, with at least 100 spores per strain counted. The presence of multilayers external to spore coat and the structure of coat and exosporium were observed. The core radius and cortex thickness were measured on cross-section images, three times for each spore (n = 14 spores for Δ 3427 and n = 11 for WT spores). When core measures showed high variability, only the minimal measure was kept as the core radius if the spore is spherical (39). Image analysis and measures of layers were realized with ImageJ software (Version 1.54p).

Statistical analysis

Statistical tests and figures were performed using GraphPad Prism software (Version 10.6.1(862) and Version 11.0.2(92)). Prior to statistical analysis, normality of the data was assessed using the Shapiro-Wilk test. For data with a normal distribution, a one-way analysis of variance (ANOVA) or two-way ANOVA, was performed. For non-normally distributed data, a non-parametric Kruskal Wallis and Dunn's T3 multiple comparison test analysis was performed. To compare the core radius and cortex thickness between WT and Δ 3427 spores, a Welch t-test was performed.

Results

The interspace of *Bacillus cereus* spores contains saccharides

We hypothesized that the interspace of *B. cereus* spores contains saccharides, as described for *B. anthracis* (27). To evidence their presence, we used WGA lectin, conjugated to Alexa Fluor 594, which preferentially recognizes terminal GlcNAc but can also recognize other terminal N-acetylated residues (40). If such residues are present in the interspace of *B. cereus* spores, WGA lectin should bind the surface of *B. cereus* $\Delta cotE$ spores, because the loss of CotE causes exosporium detachment and thereby exposes the underlying interspace (29) but not to WT spores. Our fluorescence microscopy experiments confirmed this hypothesis, as WGA lectin bound $\Delta cotE$ spores (65%) but not WT spores (2%) (Fig. 1). WGA lectin also bound $\Delta exsA$ spores (46%), exhibiting a defect in exosporium assembly (14). However, WGA lectin did not bind $\Delta exsY$ spores (0%), which lacks an exosporium except in the cap region (41). In addition, we tested the WGA lectin on WT spores submitted to a French Press (FP) treatment which removed, at least partially, the exosporium, with 20% of spores without an exosporium and 42% with a damaged exosporium (Fig. S1). We observed that 14% of these spores were stained by WGA lectin (Fig. 1). Therefore, WGA binding does not occur when the exosporium is present and correctly attached to the spore. When the exosporium of WT spores is removed or damaged by French press treatment or only partially attached in $\Delta cotE$ and $\Delta exsA$ spores (14, 29), WGA bound spores, revealing the presence of saccharides containing GlcNAc or other N-acetylated residues. This GlcNAc is probably located beneath the exosporium, within the interspace or associated with or bound to the inner side of the exosporium, as no WGA binding was observed in $\Delta exsY$ spores.

Selection of candidate genes potentially involved in saccharide assembly during sporulation of *B. cereus*

Using lectin, we evidenced, at least the presence of GlcNAc or other N-acetylated residues in the interspace of *B. cereus* spores. Thus, we looked for genes potentially involved in the synthesis of saccharides in *B. cereus* ATCC 14579 genome. First, we found fifty genes in *B. cereus* on the CAZy database and approximately one hundred and fifty genes in *B. anthracis* and *B. subtilis* based on the literature and the SubtiWiki website, whose annotations suggest biochemically appropriate activities. We finally obtained a list of two hundred genes potentially involved in saccharide synthesis/assembly in *B. cereus*.

Among those two hundred genes, we excluded genes with essential functions, genes with already described function, for example genes involved during vegetative growth, as well as genes with a low percentage of homology with genes found in *B. anthracis* or *B. subtilis*. Next, we searched for the presence of sporulation sigma factor binding sites in the promoter region of the resulting genes, in order to screen those expressed during sporulation. For those genes, we checked the timing of expression or of the synthesis of the encoding proteins during sporulation in *B. anthracis* and *B. cereus*, respectively (36) (Lablaine et al, personal communication); Finally, we checked their predictive gene function, such as

saccharide biosynthesis, assembly or degradation, and obtained a list of genes that responded to all criteria. Among those genes, some were organized in cluster.

We examined genetic conservation of at least one gene from each selected cluster across the different major phylogenetic groups of *B. cereus sensu lato* (42) by screening homolog genes in the different NCBI taxids. Among the tested genes, some were of interest due to their conservation feature (Table S1). *BC1206* was well conserved in the major groups II to VI, but its homolog was nearly absent from subgroup III-8a (*B. anthracis*) and from the most distant groups I and VII; it was partially conserved in subgroup II-2 (*B. mobilis*) and VI-like M sub-group (*B. proteolyticus* and *B. nitratedreducens*). In contrast, *BC1204* was present only in group IV and VI and was partially conserved (45%) in the subgroup III-8b (*B. cereus anthracis*-like). *BC1943* was well conserved in groups II to V. A similar conservation pattern was observed for *BC1944*, except that it was absent from groups II-2 and V. *BC3427* was conserved in groups II-1, III, IV, V and the VI-like M sub-group whereas *BC3514* appeared to be more specific to groups I and IV, which includes *B. cereus sensu stricto*, *B. thuringiensis* and *B. pseudomycoides* strains. Finally, *BC4867* (*glgB*) was highly conserved across all the phylogenetic groups, whereas *BC4863* (*glgP*), encoding a glycogen phosphorylase responsible for breaking down glycogen to glucose-1-phosphate, appeared to be absent from group VII (*B. cytotoxicus*) (Table S1).

Taking into account all these selective steps, we finally selected five genes or groups of genes which may be organized in operons, *BC1204-1207*, *BC1943-1945*, *BC3427*, *BC3514-3517* and *BC4863-4867*, coding for proteins potentially involved in saccharide synthesis and we identified either the presence of the sporulation sigma factor boxes σ^H , $\sigma^{E/H}$ or $\sigma^{H/G}$ in the promoter regions of these genes (Fig. 2).

We looked at the similarity of the five selected genes with known genes from *B. anthracis* and *B. subtilis*. The *BC1204-1206* genes are located at the beginning of *BC1204-BC1222* genes cluster. This cluster contains the following known genes: the *bclA* gene (*BC1207*), encoding the major glycoprotein component of the exosporium nap layer, as well as several genes involved in BclA glycosylation, the spore coat protein gene *cotO*, *bxpB* encoding an exosporium basal layer protein and the exosporium protein genes *exsY* and *cotY*. *BC1204* gene encodes a glycosyltransferase containing an SpsA domain (Spore Coat Polysaccharide Biosynthesis Protein) referred to the SpsA protein of *B. subtilis* involved in the spore coat synthesis, and that shares 66.3% identity (80.1% similarity) with BAS1129 from *B. anthracis* genome (GeneBank Accession AE017225.1). *BC1205* gene encodes a methyltransferase sharing 79.2% identity (88.2% similarity) with BAS1128, while *BC1206* gene encodes a macrocin O-methyltransferase sharing 87.9% identity (94% similarity) with BAS1127. These three genes form an operon (43). The proteins BC1204 and BC1206 were previously detected in a proteomic analysis performed during a *B. cereus* sporulation kinetics experiment (Lablaine et al, personal communication), and the transcripts of these three genes were detected during wave 4 of the five distinct transcriptional waves observed as *B. anthracis* progressed from germination to sporulation, a late sporulation stage associated with the expression of genes involved in spore coat and exosporium assembly (36). The exact role of the proteins encoded by these three genes has not yet been characterized and they may be involved in the biosynthesis of interspace or exosporium saccharides.

The proteins encoded by the *BC1943-BC1945* genes showed a high sequence similarity to three proteins encoded in the operon *BAS3322-BAS3318* and involved in anthrose biosynthesis in *B. anthracis*, anthrose being the terminal sugar of the pentasaccharide O-linked to BclA (20, 44). The glycosyltransferase encoded by *BC1943* shared 84.2% identity (96.2% similarity) with its *B. anthracis* homolog, whereas the aminotransferase encoded by *BC1944* shared 81.1% identity (92.8% similarity), and the O-acetyltransferase encoded by *BC1945* shared 82.4% identity (94.7% similarity). The transcripts of these three genes were also detected during the late sporulation stage (wave 4) of *B. anthracis* (36). Notably, anthrose is absent in *B. cereus*. In addition, the *BC1943-1945* gene cluster is not involved in cereose biosynthesis; instead cereose is synthesized by the *BC3358-BC3361* operon (22).

The gene *BC3427* encoded a glycosyltransferase containing a SpsA domain. The transcript of this gene was also detected during the late sporulation stage (wave 4) of *B. anthracis* (36).

BC3514-BC3517 genes encoded a predicted Glucose-1-phosphate cytidyltransferase (*BC5314*) bearing a SpsA domain showing 51.2% identity and 69.7% similarity with YfnH from *B. subtilis*, a glycosyltransferase bearing a SpsA domain (*BC3515*) showing only 12.5% identity and 22.2% similarity with YfnF from *B. subtilis*, a predicted CDP-abequose synthase (*BC3516*) and a predicted CDP-glucose 4,6-dehydratase (*BC3517*) showing only 29.1% identity and 46% similarity with YfnG from *B. subtilis*. *B. subtilis yfnHGFED* are σ^K -controlled genes involved in synthesis of quinovosan, a polysaccharide composed of D-quinovose residues found in a non-covalently bound form within the crust (Evangelaire, 2025). Quinovose has been reported in *B. subtilis* spores but not in *B. cereus* spores (5, 45). Three of these proteins *BC3514*, *BC3515* and *BC3517* were detected in a proteomic analysis performed during a *B. cereus* sporulation kinetics experiment (Lablaine et al, personal communication).

Finally, *BC4863-4867* (*glgBCDAP*) operon encoded proteins homologous to proteins implicated in glycogen metabolism in *Clostridioides difficile* (39) and in glycogen synthesis during sporulation of *B. subtilis* (46). These genes are σ^E -controlled genes in *B. subtilis* and their transcripts were detected during the early sporulation stage (wave 3) of *B. anthracis*, corresponding to the onset of sporulation and early differentiation events, such as asymmetric septation and forespore formation (36). $\sigma^{E/H}$ binding sites are present in the promoter region of *BC4867*. Recently, the *BC4863-4867* gene cluster was shown to be under the control of σ^H (47).

Deleting genes putatively involved in saccharide assembly does not alter growth properties and sporulation but impacts the outer layers of *B. cereus* spores

To determine the role of these selected genes, one or several genes of each of the five loci were inactivated by homologous recombination (see Material and Methods) (Fig. 2), and the phenotypes of resulting mutants were analyzed. These mutants will be referred as InterSpace Saccharide (ISS) mutants in this study. The five ISS mutant cells displayed similar growth to that of the WT in LB medium at both 37°C and 20°C (Fig. S2A-B). The sporulation capacity of the ISS mutant strains in LB-SMB medium at 20°C was also compared with that of the WT and no significant difference was observed, with 7.8 log₁₀

spores/ml obtained after 72h for all strains (Fig. S2C). The glycogen content was measured in WT and $\Delta 4863-4867$ spores produced on FNA plates supplemented with 0.5% of glucose. WT spores contained a significant amount of glycogen, whereas glycogen was below the limit of detection in $\Delta 4863-4867$ spores (Fig. S2D), supporting the predicted role of the *BC4863-4867* gene cluster in glycogen metabolism. Interestingly, glycogen was not detected on WT spores produced on sporulation medium without addition of glucose (data not shown).

We then investigated by electron microscopy whether the deletions made in the five selected loci have an impact on the spore structure. The surface morphology of the ISS mutant spores was first observed by SEM and compared to that of WT spores. The appearance of the exosporium of these five mutant strains (Fig. S3B-F) exhibited wrinkled patterns, similar to that of the WT (Fig. S3A), suggesting that the absence of the saccharides encoded by the deleted genes does not have a major impact on surface morphology of the exosporium.

Next, we observed the spore structure of the ISS mutant by TEM. Images after negative staining revealed two types of spores for all strains, spores with an intact exosporium and others without exosporium (Fig. 3A-B). Number of exosporium-less spores was higher for all ISS mutant spores than that of the WT (with $n \geq 99$), particularly for $\Delta 3514-3517$ spores with 19.3% of spores without exosporium, approximately twice the proportion observed for WT spores (7.9%) (Fig. 3C).

We then observed thin sections of ISS mutant spores by TEM to evidence potential modifications in their structure compared to WT spores. Interestingly, while the overall structure of $\Delta 1204-1206$, $\Delta 1943-1945$ and $\Delta 3427$ spores were similar to that of the WT (Fig. 4A), $\Delta 3514-3517$ and $\Delta 4863-4867$ spores exhibited additional multilayers external to spore coat (Fig. 4B-C), for 44% and 42% of spores, respectively vs 16% for WT spores (Fig. 4D). TEM images of $\Delta 3427$ spores also revealed that the core of these mutant spores seemed bigger than the WT ones, while the cortex was smaller (Fig. 5B-A, respectively). To confirm this observation, we measured the radius of the core and the thickness of the cortex for WT and $\Delta 3427$ spores ($n = 11$ and $n = 14$, respectively) and showed that the core radius of $\Delta 3427$ spores was significantly higher than that of the WT, with a mean value of 274.2 ± 25.3 nm and 225.9 ± 28.1 nm respectively ($p < 0.0001$) (Fig. 5C), and the cortex was significantly reduced in $\Delta 3427$ spores, with a mean value of 68.9 ± 21.1 nm, compared to the WT spores with a mean value of 97.9 ± 24.2 nm ($p < 0.0001$) (Fig. 5D).

Some spore properties are altered by the deletion of saccharide assembly genes

Considering that more spores of the five ISS mutant strains were exosporium-less than those of the WT strain (Fig. 3), we wondered if this could impact spore hydrophobicity. We used the BATH assay, a partitioning method based on the affinity of hydrophobic spores to hexadecane. The mean hydrophobicity of all spores was between 50 and 60% and no significative difference was observed between the five mutant and the WT spores (Fig. S4).

We also tested the resistance of ISS mutant and WT spores to a heat-treatment at 90°C at different time points. Spores of the $\Delta 4863-4867$ mutant were significantly less resistant than WT spores after 2 min at 90°C (with 2.2 log and 0.5 log reduction, respectively; $p < 0.05$), after 5 min (with 5.5 log reduction and 2.7 log, respectively; $p < 0.001$) and at 10 min (with, 7.6 log and 5.6 log, respectively; $p < 0.05$) (Fig. 6). In contrast, spores of $\Delta 1943-1945$ were significantly more resistant than WT spores after 5 min at 90°C (with 0.8 log and 2.7 log reduction, respectively; $p < 0.05$) and after 10 min (with 3.8 log and 5.6 log reduction, respectively; $p < 0.05$). Similarly, spores of $\Delta 3427$ were significantly more heat-resistant than the WT spores after 10 min at 90°C (with 2.9 log and 5.6 log reduction, respectively; $p < 0.001$). $\Delta 3514-3517$ and $\Delta 1204-1206$ spores were as heat-resistant as WT spores.

Finally, we tested the germination capacity of the WT and five ISS mutant spores at 37°C during 30 min in buffer in presence of 1mM inosine. Spore germination was efficient for all tested spores and no significant difference was observed between the ISS mutant and the WT spores (Fig. S5A). Moreover, germination and outgrowth of WT and mutant spores in LB during 4 h were similar (Fig. S5B).

Saccharide quantities are modified in the supernatant fraction of ISS mutant spores

To determine the impact of these gene deletions on the interspace saccharide composition, we investigated the monosaccharide composition of the supernatant fraction obtained after French press treatment of spores, which contains the interspace content and exosporium soluble fragments. We first determined monosaccharide composition of WT spores by GC-MS analysis (Fig. 7). A wide range of monosaccharides was identified, according to their specific retention times in comparison with standards, and according to the electronic impact-MS fragmentation patterns of reduced per-acetylated monosaccharide derivatives, rhamnose (Rha) and rhamnose derivatives (2,4-O-Me-Rha, 2-O-Me-Rha, 3-O-Me-Rha), fucose (Fuc), glucose (Glc), galactose (Gal), GlcN/GlcNAc and GalN/GalNAc, as previously described for the exosporium of this strain (17). The presence of rhamnose and its derivatives confirmed the presence of exosporium fragments in the analyzed fraction, as these monosaccharides are specifically associated with the exosporium (17).

The relative amounts of these monosaccharides in the supernatant fractions of WT and of Δ exsY spores, lacking an exosporium except in the cap region (41) were then evaluated by reverse-phase high-performance liquid chromatography coupled with fluorescence detection (RP-HPLC-FL). The RP-HPLC-FL chromatogram displayed 8 major peaks for the WT strain (Fig. S6). Co-injection with standards allowed the identification of five peaks corresponding to GlcN/GlcNAc (retention time ~ 11.3 min), GalN/GalNAc (~ 12 min), Gal (~ 15.2 min), Glc (~ 16 min), and Rha (~ 19.9 min). A co-elution with fucose (Fuc) was also performed allowing the identification of the peak eluting at 20.7 min as fucose. All of these monosaccharides, except the fucose, were also detected in Δ exsY, although their relative abundances differed (Fig. 8). The supernatant fraction of Δ exsY contained approximately threefold less GlcN/GlcNAc and 1.7-fold less Rha, but approximately threefold more Gal than that of the WT. Similarly, monosaccharide levels were determined in the supernatant fractions of the five ISS mutant strains

(Fig. 9). The supernatant fraction of all five ISS mutants contained two- to fourfold more Gal than that of the WT spores, they also contained 1.7 to 2.3-fold less Rha, with the exception of the $\Delta 4863-4867$ spores, which contain a level of Rha similar to that of the WT. In addition, the supernatant fractions of the $\Delta 1204-1206$ and $\Delta 1943-1945$ spores contained approximately two-fold more Glc than that of the WT. Together, these changes in monosaccharide composition indicates that the deleted gene clusters affect the relative abundance of saccharides present in the spore supernatant fraction and likely contribute to the composition of the interspace associated saccharides.

Discussion

In this study, we aim to evidence the presence of saccharides in the interspace of *B. cereus* spores and to reveal their impact on spore structure and properties. We showed that WGA lectin, which recognizes GlcNAc residues or other N-acetylated residues, did not bind intact spores but bound to $\Delta cotE$ and $\Delta exsA$ spores, both exhibiting exosporium defects, as well as to WT spores from which the exosporium had been mechanically removed. These observations suggest that N-acetylated-containing saccharides are located in the interspace, between the spore coat and the exosporium. These results were consistent with those reported by Lehmann *et al.* who also observed WGA binding $\Delta cotE$ spores of *B. anthracis* (27). No WGA binding was detected on $\Delta exsY$ spores, which lack an exosporium, suggesting that GlcNAc or other N-acetylated residues are localized within the interspace and/or on the inner surface of the exosporium. Using a bioinformatics approach, we selected gene clusters potentially involved in the synthesis or assembly of the interspace saccharides (ISS). Five ISS mutants were constructed and none exhibited defect at the surface of the exosporium. Consistent with this observation, WGA lectin did not bind any of the ISS mutant spores (data not shown) and spore hydrophobicity was not affected, suggesting that deletion of these genes does not globally alter the outer exosporium saccharide composition.

We then determined the monosaccharide composition of the supernatant fraction of WT spores after French press treatment, which contains both interspace and exosporium fragments. This fraction contains GlcNAc/GlcN, GalNAc/GalN, Gal, Glc, Fuc, Rha, 2,4-O-Me-Rha, 2-O-Me-Rha, and 3-O-Me-Rha, all of which have been identified previously as monosaccharides of total *B. cereus* spores (48). The HPLC results, combined with WGA-binding phenotype, suggest that the amino sugars detected in the interspace-exosporium fraction may occur in their N-acetylated forms, GlcNAc and GalNAc rather than exclusively as GlcN and GalN. The major exosporium glycoprotein, BclA, is glycosylated with GalNAc/GalN, Rha, 2,4-O-Me-Rha, 2-O-Me-Rha, and 3-O-Me-Rha in *B. cereus* spores (18). In addition, the saccharide composition of the exosporium has been reported to include GlcN/GlcNAc, GalN /GalNAc, Rha, 2,4-O-Me-Rha, 2-O-Me-Rha, and 3-O-Me-Rha and also Gal, that may be, at least in part, due to agar contamination (17). In our study, the analysis of the WT spore supernatant fraction suggests that Glc, Fuc and Gal may be specific components of the interspace.

We then quantified the identified monosaccharides of the supernatant fractions from WT and mutant spores. Analysis of the $\Delta exsY$ fraction revealed lower levels of Rha and GlcN/GlcNAc and higher levels of

Gal than in the WT and no Fuc was detected. Δ exsY spores retain BclA in the cap region (49); however, it is not known whether BclA is present at the same level as in WT spores. Therefore, the decrease in Rha observed in the Δ exsY mutant could either reflect a reduced amount of BclA or indicate that Rha may be less efficiently recovered from Δ exsY spores. The level of GalN/GalNAc was similar in WT and Δ exsY samples. Because GalN/GalNAc is abundant on BclA, either the amount of BclA is not affected in Δ exsY spores, or if reduced, the same level of GalN/GalNAc suggests that GalN/GalNAc may be more efficiently recovered from Δ exsY spores. Overall, except in Δ 4863–4867 spores, the GalN/GalNAc quantity in the ISS mutants was similar to that of the WT. These results are consistent with GalN/GalNAc being primarily associated with BclA. The quantity of Rha was decreased in all mutant spores vs WT spores, except in Δ 4863–4867 spores. Together with the reduction observed in the Δ exsY, this finding suggests that a proportion of Rha is associated with saccharides located within the interspace. The decrease of Rha in the ISS mutant spores further indicates that the biosynthesis and/or assembly of these Rha-containing saccharides is impaired. The concomitant increase in Gal and/or Glc in Δ 1204–1206, Δ 1943–1945, Δ 3427 and Δ 3514–3517 spores may reflect remodelling of saccharide composition rather than a simple loss of material. The deletion of these genes could impair the incorporation of Rha into saccharide, leading to the relative accumulation of saccharide precursors or incomplete saccharide structures enriched in Gal or Glc, and/or in the rerouting of metabolic fluxes towards alternative saccharide biosynthetic pathways. Consistent with this hypothesis, the predicted functions of the genes within these clusters suggest that their products are involved in saccharide assembly. In fact, the BC3514–BC3517 gene cluster is predicted to encode the biosynthesis and transfer of a CDP-activated deoxyhexose. BC3514 and BC3517 proteins are likely involved in the synthesis of a CDP-deoxyhexose precursor, whereas BC3516 probably catalyzes its final conversion and BC3515 mediates its incorporation into a saccharide. The altered monosaccharide composition observed in Δ 3514–3517 (decrease of GlcN/GlcNAc and Rha, increase of Gal) suggests that disruption of this pathway affects saccharide assembly rather than the synthesis of a monosaccharide itself. The decrease in Rha together with the increase in Gal and Glc is consistent with the accumulation of incomplete saccharide intermediates caused by impaired incorporation of the deoxyhexose. Deletion of this cluster resulted in 44% of spores exhibiting multilayers external to the spore coat, suggesting that the saccharide assembled by BC3514–3517 proteins contributes to the proper assembly of the outermost spore layers, as also suggested by (27). A similar phenotype has been reported in *B. anthracis* spores overproducing ExsY (49). It is therefore tempting to speculate that ExsY accumulates in Δ 3514–3517 spores, resulting in the formation of these multilayers external to the spore coat. The predicted functions of BC1943–BC1945 proteins suggest a role in saccharide assembly and maturation. BC1943 is predicted to be a glycosyltransferase, BC1944 belongs to the StrS family of polysaccharide biosynthesis proteins, and BC1945 likely catalyzes O-acetylation of galactose-containing saccharides. The altered monosaccharide composition observed in Δ 1943–1945 fraction (decrease of GlcN/GlcNAc and Rha, increase of Gal and Glc) suggests that disruption of this pathway could affect interspace saccharide biosynthesis or maturation, leading to the accumulation of incomplete saccharide intermediates enriched in Gal and Glc and reduced in Rha.

The predicted functions of BC1204-BC1206 suggest a role in saccharide assembly and modification. *BC1204* is predicted to encode a glycosyltransferase, whereas *BC1205* and *BC1206* encode putative O-methyltransferases. Together, these enzymes may participate in the maturation of a saccharide through the addition and subsequent methylation of sugar residues. Although the proteins BC1204 and BC1206 share 66.3% and 87.9% sequence identity with *B. anthracis* proteins BAS1129 and BAS1127 respectively, the corresponding genes are not conserved in *B. anthracis* strains. Interestingly, 2-O-Me-Rha has not been reported in spores or BclA saccharides of *B. anthracis* (21, 48). Since the *BC1204-1206* operon is located immediately upstream of *bclA* (*BC1207*), it may participate in the biosynthesis and/or assembly of BclA saccharides. One possibility is that it contributes to the incorporation of the 2-O-Me-Rha into *B. cereus* BclA saccharides. Alternatively, *BC1204*, which encodes a putative glycosyltransferase, could be involved in the addition of cereose to the BclA saccharide. Indeed, the cereose biosynthetic locus (*BC5538-BC3361*) does not encode a glycosyltransferase (22). Consistent with these hypothesis, deletion of this operon did not affect spore structure and heat-resistance. Disruption of the *BC1204-1206* cluster could impair saccharide maturation, either by affecting interspace saccharide biosynthesis or BclA glycosylation, leading to the accumulation of incomplete saccharide intermediates enriched in Gal and Glc and reduced in Rha.

Deletion of *BC3427*, which encodes a glycosyltransferase, affected spore structure, resulting in a thinner cortex and a thicker core. These structural changes were associated with increased resistance after 10 min of heat-treatment at 90°C compared with the WT spores. Although the mechanism remains unclear, the increased core thickness may reflect changes in core/cortex organization contributing to enhanced heat-resistance.

The gene clusters *BC4863-BC4867* encodes a complete glycogen metabolism pathway. The absence of detectable glycogen in $\Delta 4863-4867$ spores, formed on a sporulation medium supplemented with glucose, confirms the predicted function of this operon. Although disruption of this pathway altered the monosaccharide composition of the spore supernatant fraction, Rha levels remained similar to those of the WT, suggesting that glycogen metabolism does not directly contribute to Rha incorporation into interspace saccharide. Deletion of the glycogen synthesis pathway might have been expected to result in glucose accumulation. However, the fractions analyzed were obtained from spores produced on FNA medium without glucose supplementation, and under these conditions, no glycogen accumulation was detected in WT spores (data not shown). This suggests that glycogen synthesis may not be activated in the absence of an exogenous carbon source, as described for *B. subtilis* (46). However, even under these conditions, these genes are expressed, as their transcripts were detected during early sporulation (wave 3) of *B. anthracis* grown in sporulation medium without glucose (36). Similarly, the corresponding gene are expressed in *B. subtilis* during sporulation, even though glycogen is not detected under these conditions (46). In our study, even when spores were produced in the absence of an exogenous carbon source, the monosaccharide composition of the supernatant fraction from $\Delta 4863-4867$ spores differed from that of the WT spores. Together, these observations suggest that, even in the absence of detectable glycogen accumulation, the *BC4863-4867* operon influences the composition of interspace-associated saccharides. The increase level of Gal in this mutant may reflect changes in carbon flux or precursor

availability. Deletion of the *BC4863-4867* operon did not affect sporulation rate, however, it resulted in a reduced spore heat-resistance. Increased sensitivity to bleach, NaOH and hydrogen peroxyde was reported for *glg* mutant spores in *C. difficile* (39). The structure of $\Delta 4863-4867$ spores was similar to that of the WT except that 42% of spores exhibit a double layer external to spore coat (only 16% of the WT spores showed this phenotype), while the spores of the corresponding *glg* mutant of *C. difficile* exhibit a thinner core with a thicker cortex than WT spores (39). The authors speculated that glycogen is stored in the spore core, however, they did not mention whether the spores used for this analysis were produced in medium supplemented with carbon sources. In our study, the spores observed by TEM were produced on medium without exogenous carbon sources, condition under which no glycogen accumulation was detected and thus unlikely to affect spore structure.

Conclusion

Overall, our results provide evidence for the presence of a GlcNAc and/or other N-acetylated residues containing saccharide within the interspace of *B. cereus* spores, and partially elucidate its genetic determinants as well as their impact on spore structure and properties. In addition to GlcNAc, several other monosaccharides were identified in the supernatant spore fraction, supporting the evidence of spore-associated saccharide. Further work will be required to determine its structure and to elucidate the biosynthetic pathway responsible for its assembly. The identification of a new saccharide also raises several important questions regarding its organization within the spore. In particular, it remains to be determined whether it is present as a free glycan or covalently anchored to another spore component, and if anchored, whether it is associated with the outer surface of the coat or the inner face of the exosporium. Finally, the contribution of the interspace saccharide in the assembly and organization of the outer spore layers during sporulation remains to be established.

Declarations

Acknowledgments/funding

The authors thank Hugo Mathey for technical assistance. ER PhD work is funded by a fellowship from the French Ministry for research and higher education (Ministère de la recherche et de l'enseignement supérieur). This work was supported by grants from INRAE MICA division, SFR Tersys, and Avignon Université.

Ethics, Consent to Participate, and Consent to Publish declarations

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

Use of AI-assisted technologies

During the preparation of this manuscript, the authors used ChatGPT to language-edit the manuscript. After using this tool, the authors reviewed and edited the output and take full responsibility for the content of the manuscript.

Author contributions

ER- Design the study, experiments, data analysis, writing, review & editing; MHG- Experiments, data analysis, review & editing; CL- Experiments, data analysis, review & editing; TD- Experiments, data analysis, review & editing; IB- Experiments, data analysis, review & editing; TM- Experiments, data analysis, review & editing; FK- Experiments, data analysis, review & editing; MC- Experiments, data analysis, review & editing; CG- Experiments, data analysis, review & editing; VB- Funding acquisition, design and experimental conceptualization, data analysis, supervision, writing, review & editing; AC- Funding acquisition, design and experimental conceptualization, data analysis, supervision, writing, review & editing.

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Figures

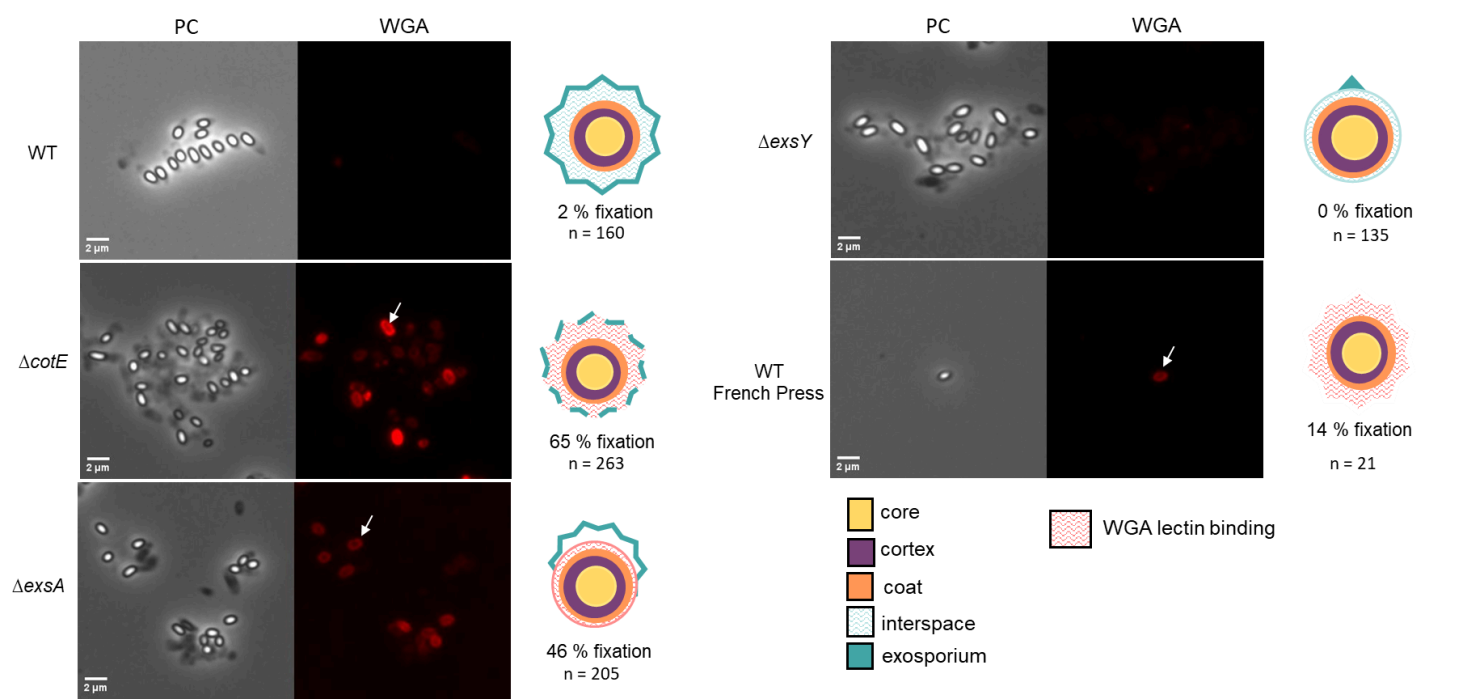


Figure 1

B. cereus spores stained with WGA lectin. WT, $\Delta cotE$, $\Delta exsA$, $\Delta exsY$ spores and French Press treated-WT spores were fixed with 4% paraformaldehyde and stained with WGA lectin conjugated to Alexa Fluor 594. Phase contrast (PC) and fluorescence microscopy images (WGA) were acquired (white arrows indicate the WGA lectin signal). Schematic representations of the spore structure and the corresponding WGA lectin binding pattern are shown for each spore type, with the % of lectin fixation (n, spores counted)

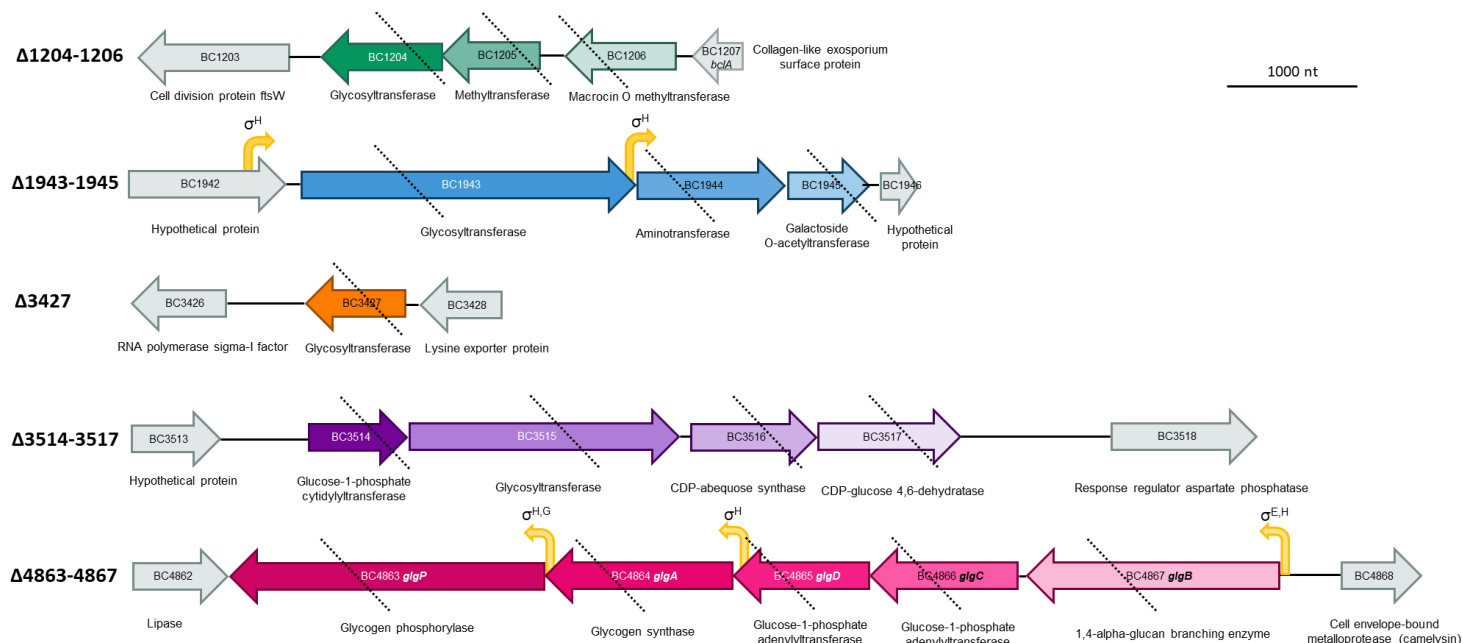


Figure 2

Selected genes potentially involved in saccharide synthesis/assembly during sporulation of *B. cereus* ATCC 14579, represented through their locus-tag in genome ATCC 14579 (GenBank accession number AE016877.1). The genes crossed out with dotted lines are the genes deleted in the corresponding mutant strains. Yellow arrows indicate the predicted sigma factor boxes downstream. Genes putatively forming a monocistronic unit are designed with the same color.

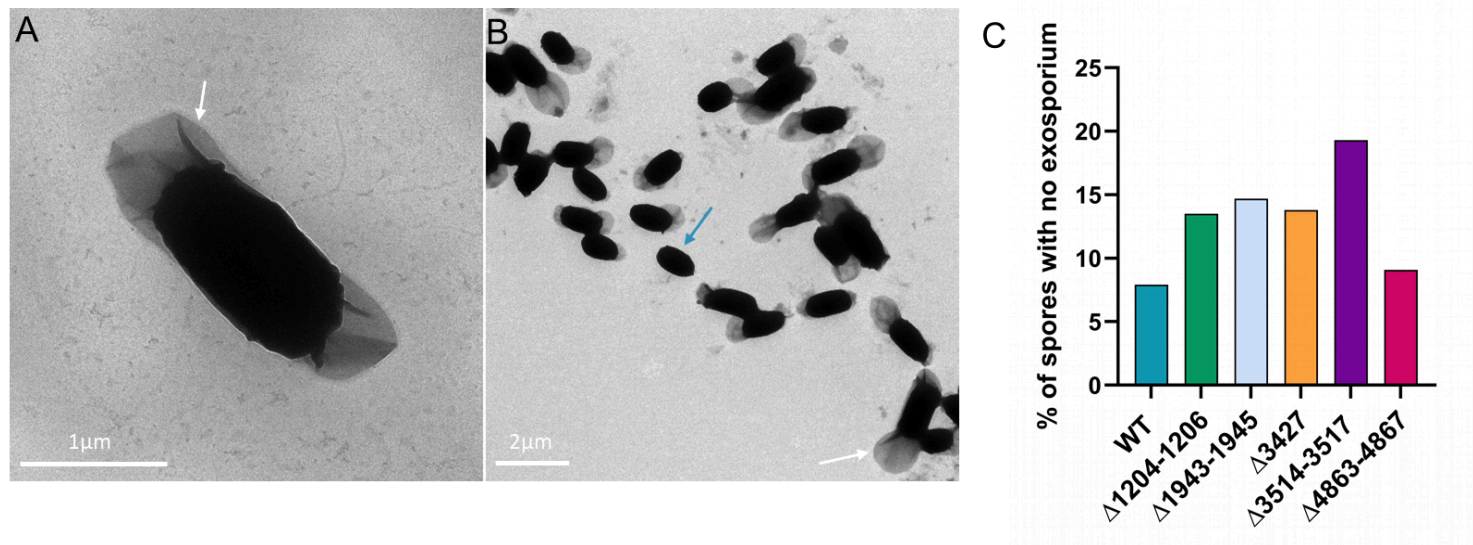


Figure 3

B. cereus spores observed by transmission electron microscopy after negative staining. (A) WT spore with a normal exosporium (white arrow) and (B) without exosporium (blue arrow). (C) Counts of exosporium-less spores for WT, Δ1204-1207, Δ1943-1945, Δ3427, Δ3514-3517 and Δ4863-4867 strains; n ≥ 99 spores for each strain.

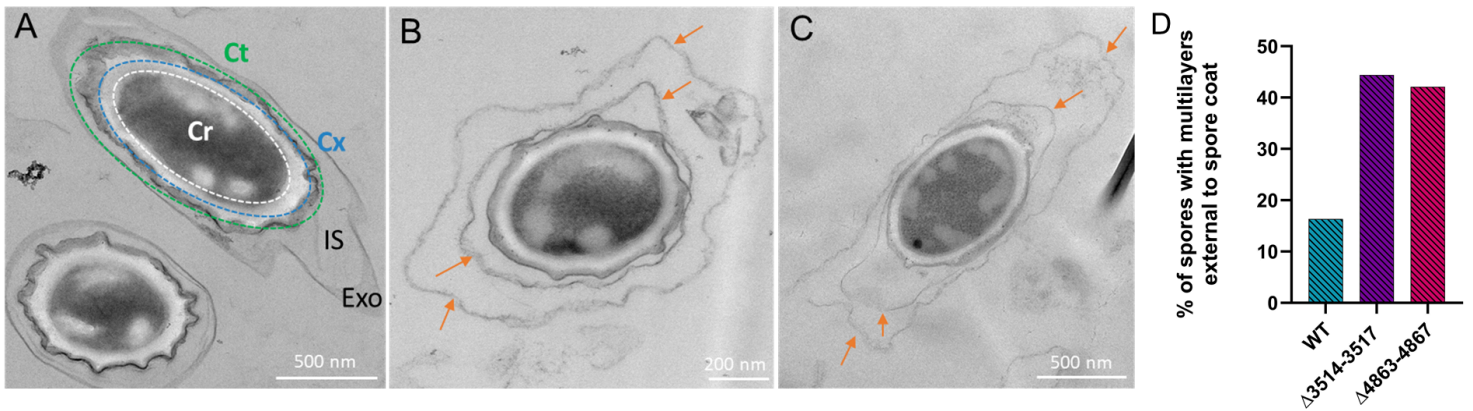


Figure 4

Ultrastructure of WT, $\Delta 3514-3517$ and $\Delta 4863-4867$ spores observed by transmission electron microscopy. Thin sections of (A) WT spores (exo, exosporium; IS, interspace; Ct, coat; Cx, cortex and Cr, core); (B) $\Delta 3514-3517$ spores; (C) $\Delta 4863-4867$ spores with multilayers external to spore coat (orange arrow); (D) Percentage of spores with multilayers external to spore coat ($n \geq 100$ spores for each strain).

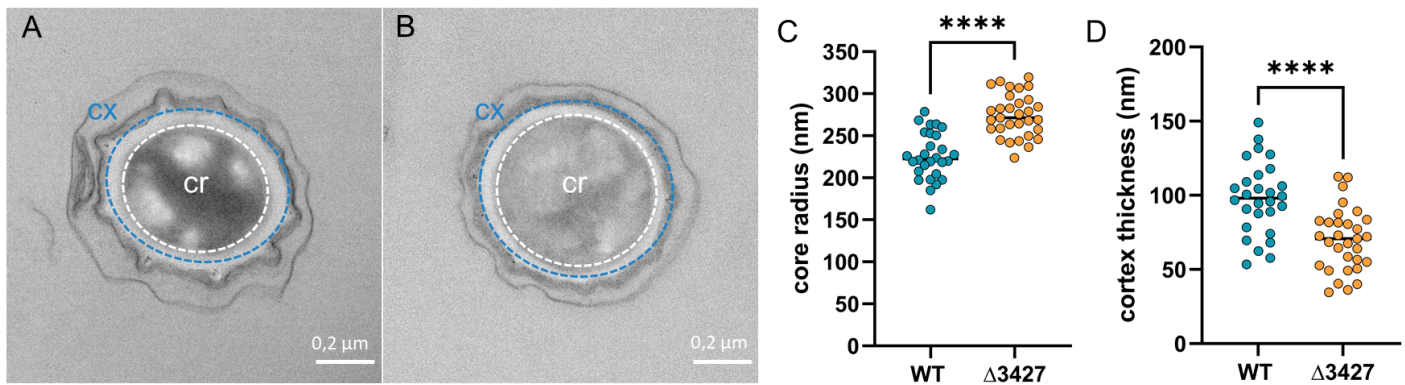


Figure 5

Thin section of spores observed by transmission electron microscopy. (A) WT and (B) $\Delta 3427$ spore structure; white dashes define the periphery of core (Cr) and blue dashes define the periphery of cortex (Cx). (C) Measures of radius of the core and (D) thickness of cortex of WT and $\Delta 3427$ spores ($n=14$ and 31 measures for WT; $n=11$ and 28 measures for $\Delta 3427$). A Welch' t-test was applied with p value < 0.0001 .

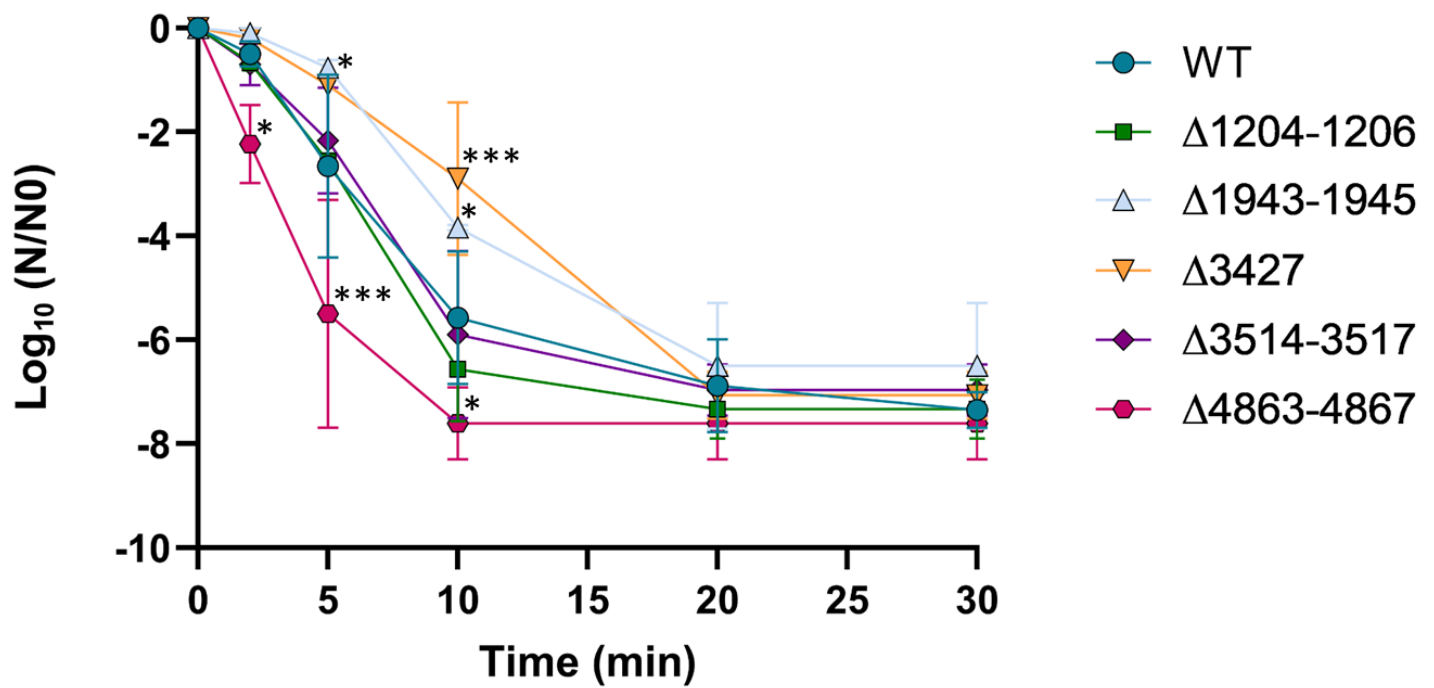


Figure 6

Heat-resistance of WT and ISS mutant spores, expressed as logarithmic reduction of viable spores (spores/ml). Spores of WT, $\Delta 1204-1207$, $\Delta 1943-1945$, $\Delta 3427$, $\Delta 3514-3517$ and $\Delta 4863-4867$ were submitted to heat-treatment for 30 min at 90°C. Data represent the mean of three independent spore batches $\pm$ SD; p-values of the two-way ANOVA statistical test vs WT, * $p < 0.05$ and *** $p < 0.001$

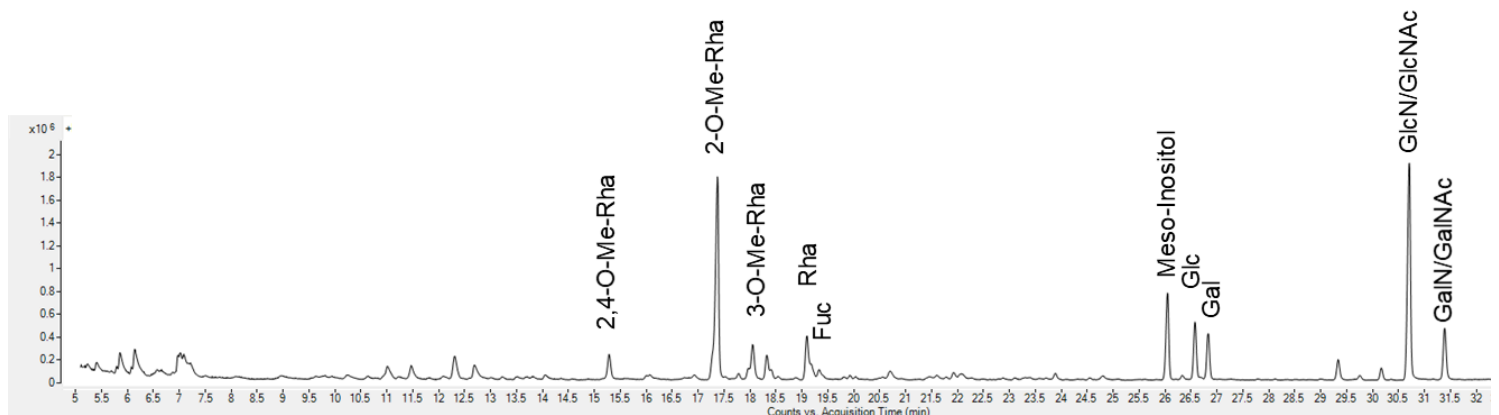


Figure 7

GC-MS chromatogram from alditol derivative analysis of the supernatant fraction of WT spores. The identified saccharides are annotated above their respective peaks. The experiment was conducted solely on one independent spore batch. Meso-Inositol is the internal standard that was used.

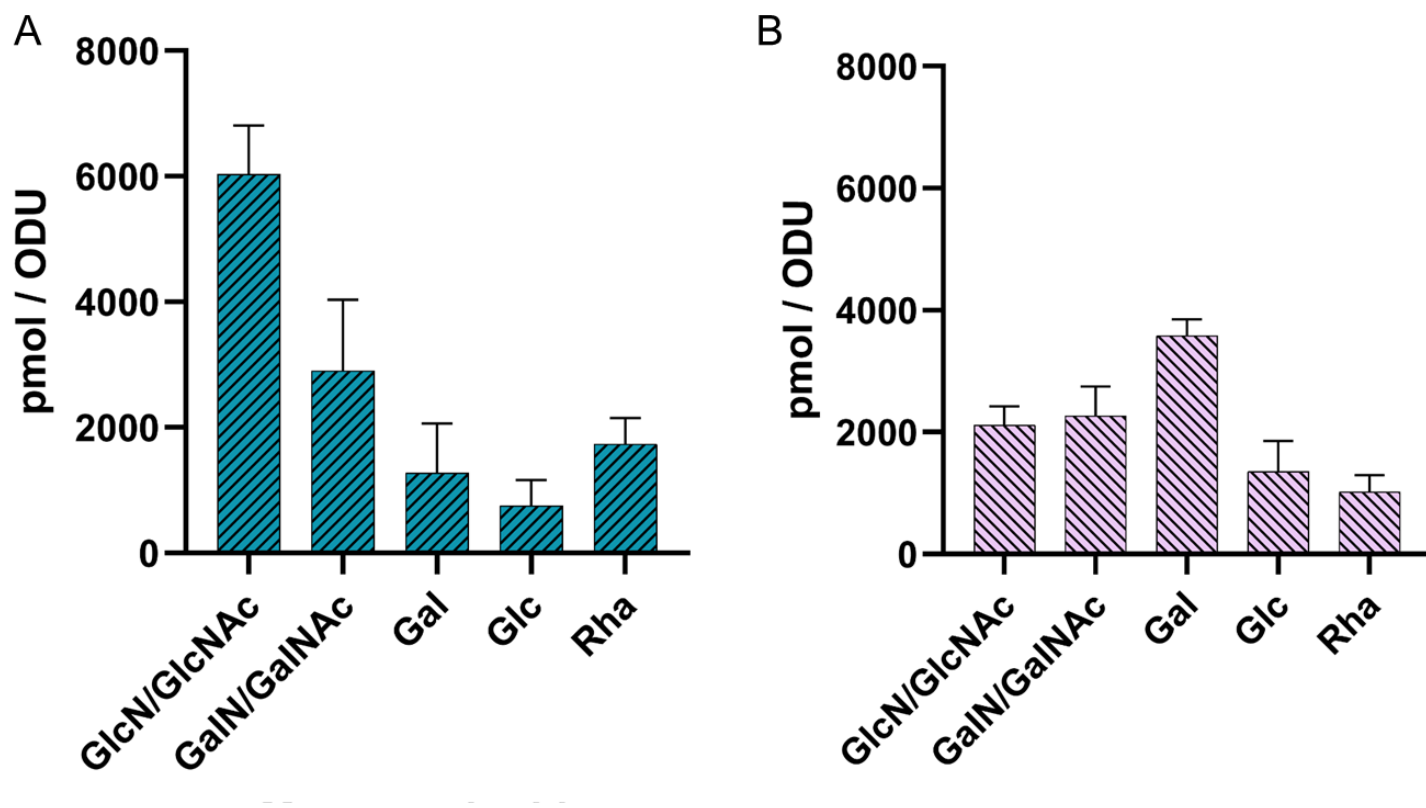


Figure 8

Monosaccharide composition of supernatant fractions from (A) WT and (B) $\Delta exsY$ spores. The relative amounts of GlcN/GlcNAc, GalN/GalNAc, Gal, Glc and Rha were determined by RP-HPLC-FL. The results were normalized to the OD_{600nm} of the spore preparations. Data represent the mean $\pm$ SD of four independent spore batches.

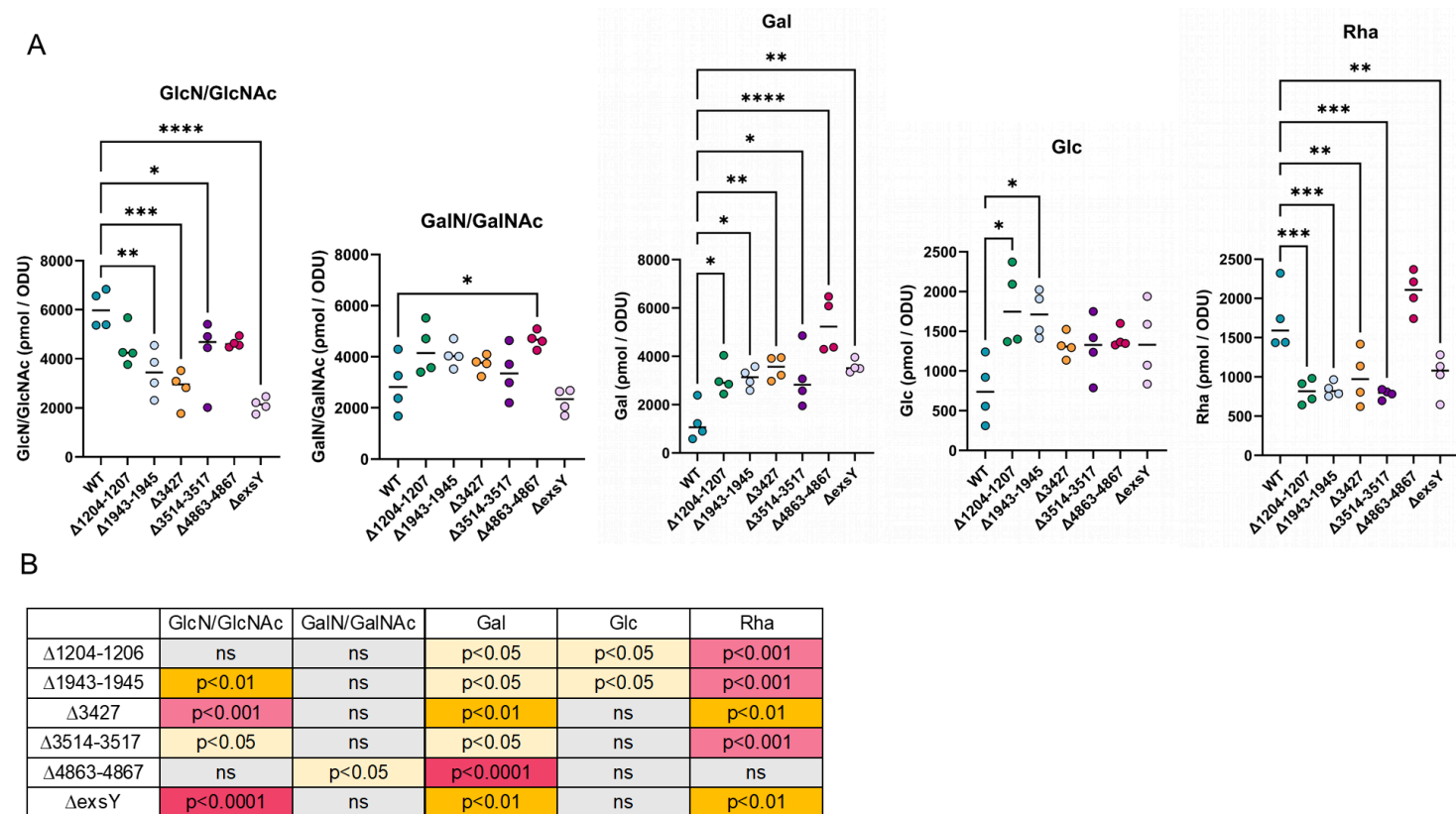


Figure 9

Quantification of monosaccharides in the supernatant fraction of *B. cereus* WT and ISS mutant spores. A. The relative amounts of GlcN/GlcNAc, GalN/GalNAc, Gal, Glc and Rha were determined by RP-HPLC-FL. The results were normalized to the OD_{600nm} of the spore preparations. Data represent the mean ± SD of four independent spore preparations. Asterisks indicate statistically significant differences compared to the WT spores (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001). B. Statistical comparison of saccharide levels in each mutant relative to WT spores.

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