Letter

# Identification of Native Cross-Links in *Bacillus subtilis* Spore Coat Proteins

Rick Ursem,[⊥] Bhagyashree Swarge,[⊥] Wishwas R. Abhyankar,[⊥] Hansuk Buncherd, Leo J. de Koning, Peter Setlow, Stanley Brul,* and Gertjan Kramer

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**ABSTRACT:** The resistance properties of the bacterial spores are partially due to spore surface proteins, ∼30% of which are said to form an insoluble protein fraction. Previous research has also identified a group of spore coat proteins affected by spore maturation, which exhibit an increased level of interprotein cross-linking. However, the proteins and the types of cross-links involved, previously proposed based on indirect evidence, have yet to be confirmed experimentally. To obtain more insight into the structural basis of the proteinaceous component of the spore coat, we attempted to identify coat cross-links and the proteins involved using new peptide fractionation and bioinformatic methods. Young (day 1) and matured (day 5) *Bacillus subtilis* spores of wild-type and transglutaminase mutant strains were digested with formic acid and trypsin, and cross-linked peptides were enriched using strong cation exchange chromatography. The enriched cross-linked peptide fractions were subjected to Fourier-transform ion cyclotron resonance tandem mass spectrometry, and the high-quality fragmentation data obtained were analyzed using two specialized software tools, pLink2 and XiSearch, to identify cross-links. This analysis identified specific disulfide bonds between coat proteins CotE−CotE and CotJA−CotJC, obtained evidence of disulfide bonds in the spore crust proteins CotX, CotY, and CotZ, and identified dityrosine and ε-(γ)-glutamyl-lysine cross-linked coat proteins. The findings in this Letter are the first direct biochemical data on protein cross-linking in the spore coat and the first direct evidence of the cross-linked building blocks of the highly ordered and resistant structure called the spore coat.
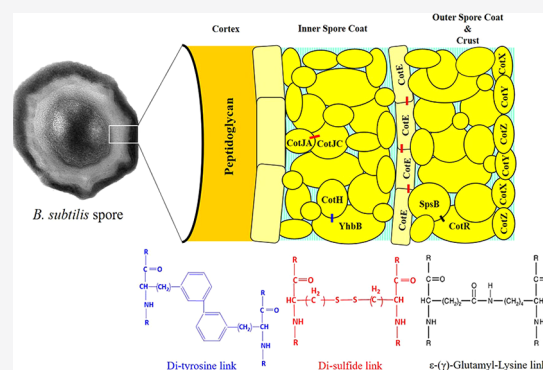
## 1. INTRODUCTION

The resistance properties of *Bacillus subtilis* spores are partially due to the assembly of the coat during sporulation in addition to the spore core levels of Ca$^{2+}$-dipicolinic acid (DPA), small acid-soluble DNA-binding proteins, and water.[1] The morphologically complex coat is a proteinaceous layer with ∼25% of total spore proteins and has posed a significant challenge in the extraction of coat proteins, many of which are insoluble.[1] It has been reported that at least 30% of coat proteins are characterized by interprotein cross-linking.[1,2] Three types of cross-links are suggested to be present in the spore coat: dityrosine, ε-(γ)-glutamyl-lysine, and disulfide cross-links. However, although some spore coat proteins are rich in lysine, glutamine, tyrosine, and cysteine, estimating the proportions of proteins incorporating the cross-links is still a challenge. Dityrosine cross-links are likely formed by an unknown peroxidase, which is thought to be associated with multimerization of the outer coat protein CotG, and in the presence of the hydrogen-peroxide-producing enzyme SodA.[3] There are several tyrosine-rich proteins in the outer spore coat, of which proteins CotC and CotU have been shown to assemble into high mass forms.[4] The ε-(γ)-glutamyl-lysine links are formed

by the enzyme transglutaminase (Tgl).[5] Proteins GerQ and SafA of the inner coat are among the known substrates of Tgl,[6,7] but it is not known which proteins they are linked to. Finally, there are also disulfide links connecting the cysteine-rich outermost crust proteins CotX, CotY, and CotZ, which are encoded in the *cotVWXYZ* gene cluster.[8] For the proposed cross-linked protein partners,[4,9−11] the exact locations and the nature of these interprotein interactions remain unknown. There are also many other coat proteins that have been predicted to form cross-links,[2] and spore maturation based on such interprotein cross-linking has been suggested to be important in spores' thermal resistance and germination behavior.[2] By finding a way to directly identify the cross-linked proteins, the type of linkage, and the regions responsible for the cross-link formation, valuable knowledge will be gained
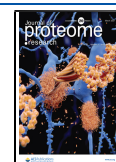
on the assembly of the proteins in the spore coat and on cross-linking-mediated spore resistance. To identify cross-linked proteins, mass spectrometric analysis of cross-linked peptides can be performed, and recent improvements in the accuracy of mass spectrometers and better database search capabilities have facilitated this type of research.[12] During tandem mass spectrometry, cross-linked peptides fragment similarly to linear peptides, and the fragments can be used to identify the two peptides involved in a cross-link.[13] This allows for the identification of the cross-linked proteins and the exact location and the type of cross-link. This technique is often used in combination with chemical cross-linking. Nevertheless, the cross-linked peptides will be at low abundance in any sample and therefore need to be enriched prior to identification.[14] In this study, we have enriched cross-linked peptides by strong cation exchange (SCX) chromatography based on the notion that cross-linked peptides will, on average, have a higher positive charge than linear peptides and thus will elute in later fractions in this mode of chromatography.[14] Subsequently, the bulkier cross-linked peptides can be further purified by reversed-phase chromatography on a $C_8$ column to improve their chromatographic behavior, followed by their detection on a highly accurate Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer. Identification of cross-linked peptides was then achieved by database searching using two specialized cross-link specific database search engines, XiSearch[15] and pLink2.[16] Doing so allowed the identification of native cross-links between the coat proteins CotE−CotE and CotJA−CotJC through disulfide bridging. Evidence was also found of dityrosine cross-links between coat proteins SpsB−CotJB and CotH−YhbB and an $\varepsilon$-($\gamma$)-glutamyl-lysine cross-link between coat proteins SpsB−CotR. This systematic study of native cross-links in the *B. subtilis* spore coat shows that despite the difficulty and complexity of such analysis, the identification of such cross-links is possible. Importantly, this work gives the first direct evidence of interprotein cross-linking in intact *B. subtilis* spores.

## 2. MATERIALS AND METHODS

### 2.1. Sporulation and Spore Harvesting

The *B. subtilis* wild-type 168 strain PY79 (WT) and an isogenic transglutaminase (*tgl*; Erm$^{R+}$(PY79 background)) deletion mutant were used in this study. MOPS-buffered minimal medium,[17,18] was used for growth and sporulation and, unless noted otherwise, contained 40 mM $NH_4Cl$ and 40 mM glucose. A single colony was grown in this MOPS medium and incubated at 37 °C at 200 rpm. After 16 h of incubation, the cells were inspected by phase-contrast microscopy to ensure that the overnight culture was free of spores. This culture was diluted into a fresh MOPS-buffered medium to a final $OD_{600}$ of 0.1 and incubated at 37 °C at 200 rpm until the $OD_{600}$ reached 0.6. After an additional 90 min, the culture was diluted five-fold into the MOPS-buffered medium but without glucose to trigger sporulation. The phase-bright spores were harvested 1 and 5 days after inoculation, as previously described.[2,19] For the young (1 day) spore preparations, the cells and spores were separated with a Histodenz gradient, as previously described.[2] The mature (5 day) spores were washed seven times with cold, sterile Milli-Q (MQ) water. During the second washing step, Tween 20 was added to 0.01% to lyse the remaining vegetative cells. This was sufficient to obtain spore preparations with >99% free phase-bright spores, as observed by phase-contrast

microscopy. Harvested spores were frozen in liquid nitrogen to halt biological processes such as cross-linking and freeze-dried, the dry weight of the spores was determined, and the dry spores were stored at −80 °C.

### 2.2. Sequential Digestion

Spore digestion was performed in three steps (Supplementary Figure 1): (1) initial formic acid (FA) digestion, (2) reduction−alkylation, and (3) trypsin digestion.

**2.2.1. Formic Acid Digestion.** *2.2.1.1. Identification of Disulfide Bonds.* 250 $\mu$L of 2% FA (Biosolve BV, Valkenswaard, The Netherlands) was added to 4 mg dry weight of spores to digest proteins[20] in a 2 h incubation at 108 °C. During this incubation, the spores were manually shaken every 30 min to ensure efficient digestion. The solubilized proteins and peptides were separated from the spore pellet by centrifuging for 10 min at 13 200 rpm in a tabletop Centrifuge 5415 D (Eppendorf, Germany). The supernatant was transferred to a separate Lo-Bind tube (Eppendorf, Germany), and 100 $\mu$L of ULC/MS-grade water (Biosolve BV) was added to the spore pellet to wash it, followed by centrifugation to harvest as much loose peptide material as possible in the supernatant. This supernatant was added to the previously collected supernatant in the Lo-Bind tube. The pellets and supernatant samples were freeze-dried overnight. Freeze-dried samples were further suspended in 100 $\mu$L of 100 mM $NH_4HCO_3$. To examine disulfide bonds present in proteins, the spore pellet and the supernatant samples were split in half and processed with and without dithiothreitol (DTT) and subsequent alkylation (see later), where samples with the DTT treatment served as controls for the identification of disulfide bonds. The samples that were not subjected to reduction were only alkylated to block any free cysteines from forming disulfide links, whereas DTT-reduced samples were alkylated to block further disulfide bond formation.

*2.2.1.2. Identification of Dityrosine and $\varepsilon$-($\gamma$)-Glutamyl-lysine Cross-Links.* 250 $\mu$L of 2% FA (Biosolve BV) was added to 2 mg dry weight of spores of WT and *tgl* strains. The remaining protocol for FA digestion was as previously described.

**2.2.2. Reduction and Alkylation of Spore Pellets and Supernatants.** For the control pellets (disulfide cross-link identification) and test pellets (dityrosine and $\varepsilon$-($\gamma$)-glutamyl-lysine cross-link identification), the reduction and alkylation protocol published previously[17] was followed. Additionally, after the alkylation step, 60 mM thiourea was added, and the samples were incubated for an additional 20 min at room temperature in the dark to block overalkylation of samples.[18] The samples were centrifuged, the supernatants were discarded, the pellets were washed twice with 100 $\mu$L of 100 mM $NH_4HCO_3$, and the pH was measured to be ~8.

For the control supernatants (disulfide cross-link identification) and test supernatants (dityrosine and $\varepsilon$-($\gamma$)-glutamyl-lysine cross-link identification), the freeze-dried samples were dissolved in 100 $\mu$L of 100 mM $NH_4CO_3$. For reduction, DTT was added to 5 mM to the tubes that were incubated for 1 h at 55 °C; then, iodoacetamide (IAA) was added to 15 mM for alkylation, with incubation at room temperature for 45 min. Finally, thiourea was added to 20 mM, the samples were incubated at room temperature for 20 min, and the pH was measured as ~8 prior to further steps.

**2.2.3. Trypsin Digestion.** For the samples described above, 3 $\mu$g of trypsin (1:60 protease/protein ratio; Sigma-

Aldrich, The Netherlands) was added to the pellet samples, and 2 $\mu$g of trypsin (1:200 protease/protein ratio) was added to the supernatant samples; then, all samples were incubated for 18 h at 37 °C in a rotary shaker. After incubation, the pellet samples were centrifuged and washed three times with 100 $\mu$L of ULC-MS-grade water. Each time the supernatants were collected in single Lo-Bind tubes per sample. To quench the trypsin reaction, the pH of all supernatants was adjusted to 3 with 10% trifluoroacetic acid (TFA). Samples were then freeze-dried and stored at −80 °C until use.

### 2.3. SCX Cross-Linked Peptide Enrichment

Freeze-dried samples were reconstituted in 215 $\mu$L of buffer A (10 mM ammonium formate, 25% acetonitrile (ACN), pH 3.0), and 200 $\mu$L of sample was loaded onto a polysulfethyl aspartamide column (2.1 mm ID, 10 cm length) (PolyLC, Columbia, MD). An isocratic flow with 100% buffer A for 5 min was followed by a gradient of 0−1% buffer B (500 mM ammonium formate, 25% ACN, pH 3.0) in the next 5 min and then from 1 to 50% between 10.25 and 19.25 min, followed by an increase from 50 to 100% between 19.25 and 23.25 min. This SCX chromatography program lasted 42.5 min with a 400 $\mu$L/min flow rate. Peaks were fractionated based on absorbance measurements at 280 nm. All peptide fractions were freeze-dried.

### 2.4. FT-ICR Analysis

Freeze-dried SCX fractions were dissolved in 100 $\mu$L of 0.1% TFA. Fractions were pooled based on peak appearance from the SCX, resulting in four fractions per sample. An 80 $\mu$g capacity $C_{18}$ ZipTip (Agilent Technologies, Santa Clara, CA) was used for desalting the samples, as per the manufacturer's instructions. ZipTipped fractions were freeze-dried to remove ACN and TFA and stored at −80 °C until further use. Before FT-ICR MS/MS measurements, to prevent peptide aggregation issues, 30 $\mu$L of TFA was added to the freeze-dried samples, and the samples were sonicated for 10 min before the TFA was evaporated by blowing argon over the liquid. Then, 20 $\mu$L of ULC-MS-grade water was added, and the peptide concentration was estimated by measuring the absorbance at 215 nm on a DeNovix DS-11+ spectrophotometer (DeNovix, Wilmington, DE). Approximately 300 ng of peptides was used for each analysis with 1 $\mu$L of 75 fM [Glu1]-fibrinopeptide B as an internal standard. Data were acquired with a Bruker Apex Ultra FT-ICR mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 7 T magnet and a nanoelectrospray Apollo II dual source coupled to an Ultimate 3000 ultra-performance liquid chromatography (UPLC) system (Dionex, Sunnyvale, CA).

Low charge (<3$^+$) SCX fractions were loaded onto a PepMap100 $C_{18}$ (5 $\mu$m particle size, 100 Å pore size, 300 $\mu$m inner diameter × 5 mm length) precolumn. Following injection, the peptides were eluted at 30 °C via an Acclaim PepMap 100 $C_{18}$ (5 $\mu$m particle size, 100 Å pore size, 75 $\mu$m inner diameter × 500 mm length) analytical column (Thermo Fisher Scientific, Etten-Leur, The Netherlands) to the nanoelectrospray source. Gradient profiles of up to 100 min were used from 0.1% FA, 3% ACN to 0.1% FA, 50% ACN at a flow rate of 300 nL/min. Data-dependent Q-selected peptide ions were fragmented in the hexapole collision cell at an argon pressure of 6 × 10$^{−6}$ mbar (measured at the ion gauge), and the fragment ions were detected in the ion cyclotron resonance (ICR) cell at a resolution of up to 60 000. Each MS/MS data set was mass calibrated internally or externally depending on

the experiment based on the [Glu1]-fibrinopeptide-B peptide fragment ion masses.

The highest charged SCX fraction of each sample was loaded onto a PepMap $C_8$ (5 $\mu$m particle size, 0.1 mm inner diameter × 5 mm length) trap column. Following injection, the peptides were chromatographed on an Acclaim PepMap 100 $C_8$ (3 $\mu$m particle size, 100 Å pore size, 75 $\mu$m inner diameter × 150 mm length) analytical column (Thermo Fisher Scientific). The peptides were eluted from the column by applying a gradient from 0.1% FA−3% ACN to 0.1% FA−72% ACN (flow rate 300 nL/min) in 60 min. The MS/MS settings and buffers used for the $C_8$ column remained identical. The entire protocol is summarized in the flowchart shown in Supplementary Figure 1.

### 2.5. Linear and Cross-Linked Peptide Identification

**2.5.1. Linear Peptide Identification.** Linear peptides were identified with Mascot server version 2.6.2 (MATRIX science, London, U.K.) using the protein database of *B. subtilis* 168. Trypsin + FA was manually added as the enzyme. Maximum missed cleavages were set to 9. The precursor and fragment mass error tolerance was set to 50 ppm. Carbamidomethyl was added as a fixed modification on cysteine, and oxidation of methionine and deamidation of asparagine and glutamine were added as variable modifications.

**2.5.2. Identification of Cross-Linked Peptides.** Identification of cross-links was performed with pLink2[16] and XiSearch.[15] The in-house *B. subtilis* 168 spore coat protein database used for this search was prepared based on previous literature and contained 128 proteins, including all of the known and predicted spore coat proteins as well as the most abundant spore proteins (Supplementary File 1). For both of the cross-link search programs, the precursor mass error tolerance was set to 10 ppm, and the fragment mass error tolerance was set to 15 ppm. Disulfide and dityrosine cross-links were defined as cross-links between two cysteines and two tyrosines, respectively, with a cross-linker mass of −2.01565 (−H$_2$), whereas the $\varepsilon$-($\gamma$)-glutamyl-lysine cross-links were defined as cross-links between glutamine and lysine with the cross-linker mass of −17.026549105 (−NH$_3$). This mass was based on the chemical processes underlying cross-linking. The false discovery rate (FDR) was set to 5%. Trypsin + FA digestion of proteins was added manually as a combination of enzymes. The full XiSearch configurations used for the searches are also available as configuration files (Supplementary File 2). For pLink2, each of the cross-link types was searched separately. For samples where disulfide bonds were not reduced, no modifications were specified. For all other samples, alkylation of cysteines was set as a fixed modification. The number of missed cleavages was set to 5, which was the maximum number possible. Full pLink2 configurations used are available as Supplementary File 3.

**2.5.3. Verification of Cross-Links.** Candidate cross-links identified by pLink2 and XiSearch were manually verified. Candidates were examined for the presence of the ion fragments belonging to the proposed cross-link. Fragments resulting from the MS fragmentation of a single peptide and of both peptides in the cross-link were considered. Measured masses were compared with the theoretical fragments of the candidate cross-links. The mass error tolerance was set to 15 ppm, and theoretical fragments with one water loss or one ammonia loss were also included for the verification. Theoretical fragment masses were automatically generated

**Table 1. Cross-Linked Peptides Identified in the *B. subtilis* Spore Coat[a]**

| type of cross-link | proteins involved in the cross-link[b] | peptides identified as cross-linked | tool used for cross-link identification | PSM score[c] | no. of replicas[d] | age of spores (days) | strain | fraction |
|---|---|---|---|---|---|---|---|---|
| S–S | CotE(C$_{22}$)–CotE (C$_{22}$) | FTQ**C**TNTISPEK KFTQ**C**TNTISPEK | XiSearch | 11.53 | 1 | 1, 5 | WT | pellets + supernatant |
| | CotE (C$_{22}$)–CotE (C$_{113}$) | FTQ**C**TNTISPEK–VLQQPN**C**LEVTISPNGNK | XiSearch | 9.06 | 1 | 1 | WT | pellets + supernatant |
| | CotJA (C$_{23}$)–CotJC (C$_{17}$) | P**C**PPIGK–VST**C**NPTLAK | XiSearch | 6.63 | 1 | 1, 5 | WT | pellets + supernatant |
| Y–Y | SpsB (Y$_{156}$)–CotJB (Y$_9$) | GDSKQYLETACSLLAP**Y**D–VDAE**Y**YR | pLink2 | $1.3 \times 10^{-1}$ | 1 | 1, 5 | WT | pellets + supernatant |
| | | | | | 2 | 1, 5 | *tgl* | pellets + supernatant |
| | CotH (Y$_{269}$)–YhbB (Y$_{278}$) | IHGERMAAD**Y**VR–GNMPLVNAQT**Y**D | pLink2 | $3.34 \times 10^{-1}$ | 2 | 1, 5 | WT | pellets + supernatant |
| | | | | | 2 | 1, 5 | *tgl* | pellets + supernatant |
| Q–K | SpsB (K$_{139}$)–CotR (Q$_{165}$) | REIADSC**Q**KGD–SSGAPAT**Q**RAYQNYVD | pLink2 | $1.15 \times 10^{-1}$ | 1 | 1, 5 | WT | pellets + supernatant |

[a]Amino acid residues in bold are involved in cross-link formation. [b]Residue positions in the proteins involved in cross-link formation are given in parentheses. [c]Peptide spectrum match (PSM) score for pLink2[16] and XiSearch.[15] [d]In total, two biological replicates were performed. The number is indicative of the number of replicas in which the cross-links were identified.

and then compared with those generated with a custom-made program in Microsoft Excel. Additionally, the spectrum quality, MASCOT identifications of linear peptides, and alternative explanations for the spectrum, such as modifications of linear peptides, were assessed. This was done for each mass spectrum identified as a cross-link to prevent regular peptides from being misidentified as cross-linked peptides.

## 3. RESULTS AND DISCUSSION

### 3.1. SCX Fractionation of Cross-Linked Peptides

SCX has effectively separated peptides based on charge, and Supplementary Figure 2 shows a typical elution profile of sequentially digested spore peptides. As depicted in Supplementary Table 1, fractions 1−3 are enriched with a few singly and mostly doubly or triply charged peptides. Although fraction 3 is seen to have enriched a minor percentage of 4$^+$ peptides, the majority of the 4$^+$ to 7$^+$ peptides are enriched in fraction 4. This fraction is expected to have the majority of the cross-linked peptides.

### 3.2. Identification of CotE−CotE and CotJA−CotJC Disulfide Cross-Links

Our study attempts to identify the native disulfide bonds in the spore coat fraction of *B. subtilis* by avoiding reducing environments during the extraction and purification of spore coat proteins (Table 1). Although the spore crust proteins CotX, CotY, and CotZ are said to form an insoluble protein fraction via disulfide cross-linking, our current study failed to identify these cross-links for two main reasons: (a) If these proteins are indeed cross-linked via disulfide bonds, then it is plausible that each of the cysteines from these proteins is involved in cross-link formation. This is suggested by the observation made in Figure 1, where no peptides were identified/extracted from these proteins in the areas containing cysteine(s). This observation makes it difficult to analyze such cross-links because if the peptides are released from these protein parts, then they might be cross-linked to multiple other peptides, an assumption that is constant in this study for all types of cross-links. (b) Alternatively, perhaps the disulfide linked peptides can still contain additional free alkylated cysteines (not involved in disulfide bond formation) that are

not included in the analyses. Nevertheless, our analyses confidently identify the links between CotE dimers and CotJA−CotJC proteins. In the high-charge SCX fractions from FA + trypsin-digested 1 and 5 day old PY79 spores, we identified two disulfide cross-links in the CotE homodimers. These cross-links were identified in both the pellet and the supernatant fractions of these spores and involve cysteines at positions 22 and 113 in the protein. Thus the C$_{22}$−C$_{22}$ and C$_{22}$−C$_{113}$ cross-links identified in this study provide two possible means of stabilizing the loosely formed CotE oligomers. The MS/MS fragmentation spectra of these cross-links are shown in Figure 2A,B. However, the C$_{22}$−C$_{113}$ cross-link was not identified in the day 5 samples, suggesting that it might be initially formed to stabilize the CotE oligodimers and later becomes unextractable owing to spore coat maturation. With regards to the spore's inner coat assembly, the current analyses confirm for the first time the idea that CotJA and CotJC assembly is dependent on each other. The disulfide cross-links observed in these proteins involve cysteines C$_{23}$ in CotJA and C$_{17}$ in CotJC. These cross-links have also been identified from the high-charge SCX fractions of pellets and supernatants of both 1 and 5 day old PY79 spores. Figure 2C displays the MS/MS fragmentation spectrum of this cross-link.

### 3.3. Identification of Dityrosine and ε-(γ)-Glutamyl-lysine Cross-Links in Spore Layers

The dityrosine and ε-(γ)-glutamyl-lysine cross-links discussed as follows have been identified only with the pLink2 tool. In this study, dityrosine cross-links in SpsB (Y$_{156}$)−CotJB (Y$_9$) and CotH (Y$_{269}$)−YhbB (Y$_{278}$) have been observed in highly charged peptide fractions obtained from pellets and supernatants of both *tgl* and WT spores harvested after day 1 and day 5. It is reported that CotE helps in the assembly of SpsB[21] and that the latter protein is involved in spore coat polysaccharide synthesis.[22] The identification of a cross-link between SpsB and CotJB found in the inner coat layer indicates a plausible step in inner coat maturation. On the contrary, YhbB, an inner coat protein, is suggested to be in the insoluble fraction.[23] In addition, CotH control of YhbB assembly is said to be temperature-dependent, as YhbB− GFP rings in the spore coat region are clearly visible only at 25
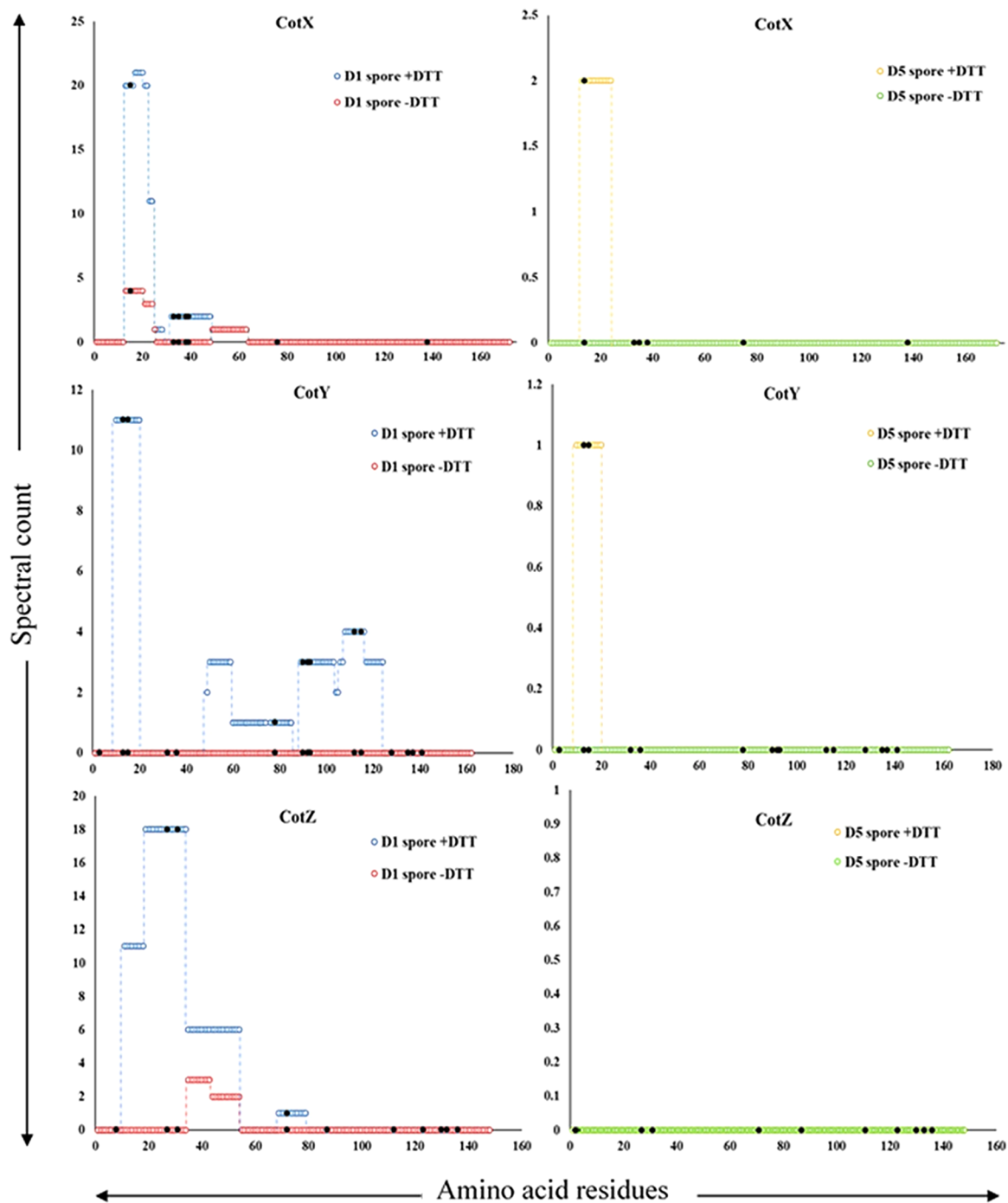
**Figure 1.** Spectral counts of CotX, CotY, and CotZ peptides in 1 and 5 day spores at the amino acid residue level with and without reduction of disulfide bonds by DTT. The number of peptide sequence matches for each residue is shown on the *Y* axis for day 1 (left panel) and day 5 WT (right panel) spores for CotX, CotY, and CotZ. The *X* axis shows the number of amino acid residues per protein. The cysteine amino acid positions in the proteins are marked as black circles. The amino acid residues are identified from the $C_{18}$ column as linear peptides. As mentioned in the text, the cysteine-rich regions in CotX, CotY, and CotZ were extracted poorly (i.e., low spectral count), if at all, without the reduction treatment.

°C.[23] Consequently, the identification of a CotH–YhbB cross-link suggests that further studies of these proteins' assembly may be warranted. Supplementary Tables 2 and 3 show the fragmentation patterns of SpsB–CotJB and CotH–YhbB dityrosine cross-links, respectively. Coat protein GerQ is suggested to be cross-linked via ε-(γ)-glutamyl-lysine cross-links,[7] but we have not been able to identify any cross-links in this protein. This could be because trypsin and FA digestion of GerQ could generate either very small or very large peptides,

making their identification via MS/MS difficult. In addition, as previously mentioned, if the peptides are released from the insoluble coat proteins, then they might be cross-linked to multiple other peptides about which no definitive analysis can yet be made. In addition, it is difficult to simulate such multiple cross-links in MS/MS analysis; therefore, it is difficult to identify such cross-linked proteins. Nevertheless, we have identified a ε-(γ)-glutamyl-lysine cross-link between SpsB ($K_{139}$) and CotR ($Q_{165}$) in wild-type spores, and as expected,
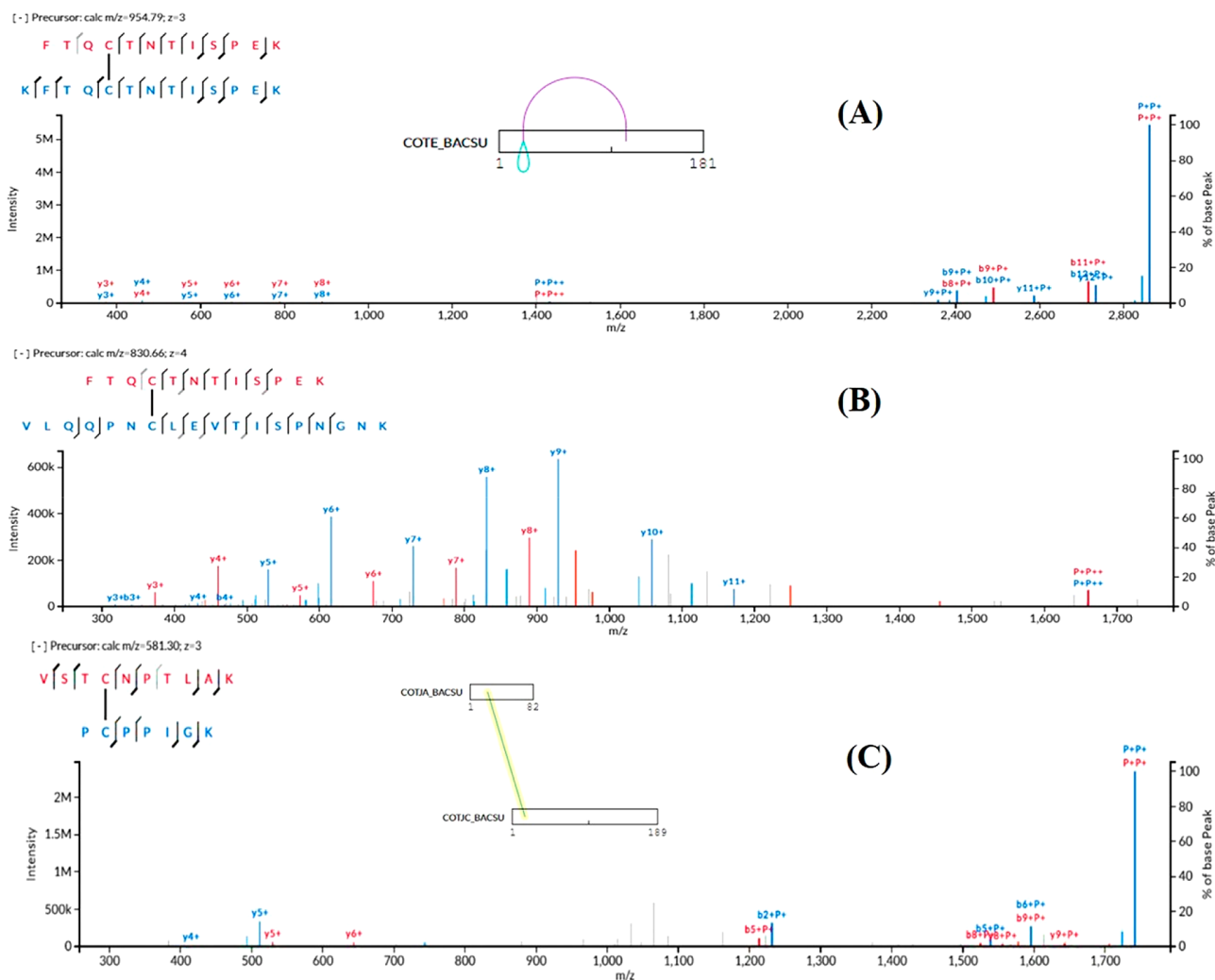
**Figure 2.** Fragmentation of the disulfide-bonded peptides in (A) CotE ($C_{22}-C_{22}$), (B) CotE ($C_{22}-C_{113}$), and (C) CotJA ($C_{23}$) and CotJC ($C_{17}$). The fragment mass is shown as M + H. Solid black lines in the sequence show b and y ions. Gray lines in the sequence show b and y ions with a loss of $H_2O$ or $NH_3$. P+, monoisotopic peak of the parent ion of the respective peptide; BACSU, *Bacillus subtilis*. The inset figures in panels A and C show the positions of the identified link in respective proteins. The inset figure in panel A can be extended to the fragmentation spectrum in panel B. These fragmentation spectra were obtained from XiView.[25]

it is absent in *tgl* spores. Both of these proteins are involved in spore coat formation.[24] Supplementary Table 4 shows the fragmentation pattern of the SpsB−CotR cross-link.

## 4. CONCLUSIONS

The current study has for the first time identified cross-linked proteins directly from the spore coat at the residue level. These cross-links involve coat proteins CotE, CotJA, CotJB, CotJC, CotH. CotR, SpsB, and YhbB, and the actual cross-linking positions within these proteins have been identified. In addition, further evidence is provided for the presence of disulfide bonds in the cysteine-rich spore crust proteins CotX, CotY, and CotZ. Thus mass spectrometry in combination with SCX and the use of a $C_8$ column for peptide fractionation is a promising tool for future research into spore coat protein assembly and coat protein interactions.

## ■ ASSOCIATED CONTENT

### ⓈⒾ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.1c00025.

Supplementary Figure 1. Workflow for the detection of disulfide, dityrosine, and $\varepsilon$-($\gamma$)-glutamyl-lysine cross-links in WT and $\Delta tgl$ *B. subtilis* spores. Supplementary Figure 2. SCX elution profile of day 1 *B. subtilis* spore pellet with reduction of disulfide bonds by DTT (PDF)

Supplementary Table 1. Charge distribution of identified peptides across SCX fractions. Supplementary Table 2. Fragmentation pattern of the SpsB−CotJB dityrosine link. Supplementary Table 3. Fragmentation pattern of the CotH−YhbB dityrosine link. Supplementary Table 4. Fragmentation pattern of the SpsB−CotR $\varepsilon$-($\gamma$)-glutamyl-lysine link (PDF)

Supplementary File 1. *B. subtilis* Spore Coat database used for cross-link identification (TXT)

Supplementary File 2. XiSearch configuration used for disulfide link identification (TXT)

Supplementary File 3. pLink configuration used for dityrosine and $\varepsilon$-($\gamma$)-glutamyl-lysine link identification (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

**Stanley Brul** − *Department of Molecular Biology and Microbial Food Safety, University of Amsterdam, Amsterdam 1098 XH, The Netherlands;* ⊙ orcid.org/0000-0001-5706-8768; Email: s.brul@uva.nl

### Authors

**Rick Ursem** − *Department of Molecular Biology and Microbial Food Safety and Department of Mass Spectrometry of Biomolecules, University of Amsterdam, Amsterdam 1098 XH, The Netherlands*

**Bhagyashree Swarge** − *Department of Molecular Biology and Microbial Food Safety and Department of Mass Spectrometry of Biomolecules, University of Amsterdam, Amsterdam 1098 XH, The Netherlands;* ⊙ orcid.org/0000-0003-1086-8295

**Wishwas R. Abhyankar** − *Department of Molecular Biology and Microbial Food Safety and Department of Mass Spectrometry of Biomolecules, University of Amsterdam, Amsterdam 1098 XH, The Netherlands;* ⊙ orcid.org/0000-0002-9644-7856

**Hansuk Buncherd** − *Faculty of Medical Technology, Prince of Songkla University, Songkhla 90110, Thailand*

**Leo J. de Koning** − *Department of Mass Spectrometry of Biomolecules, University of Amsterdam, Amsterdam 1098 XH, The Netherlands*

**Peter Setlow** − *Department of Molecular Biology and Biophysics, UConn Health, Farmington, Connecticut 06030-3305, United States*

**Gertjan Kramer** − *Department of Mass Spectrometry of Biomolecules, University of Amsterdam, Amsterdam 1098 XH, The Netherlands*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acs.jproteome.1c00025

### Author Contributions

[⊥]R.U., B.S., and W.R.A. contributed equally.

### Notes

The authors declare no competing financial interest.
The raw proteomics data have been deposited to the PRIDE repository with the data set identification number PXD023492.

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