The proteome of spore surface layers in food spoiling bacteria

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Proteomic characterization of spore coat protein mutants of *Bacillus subtilis*

Wishwas Abhyankar, Linli Zheng, Stanley Brul, Leo J. de Koning, Chris G. de Koster

**Abstract** Spore coat proteins play an important role in maintaining spore structure as well as the resistive capacity of the spores. Spore morphogenetic proteins are responsible for layering the proteinaceous layers during spore morphogenesis. Previous studies have analyzed the dependence of certain coat proteins on the well-known morphogenetic proteins such as SpoIVA, CotE, CotH etc. Yet many coat proteins remain to be studied for regulation of their assembly and their dependence on the morphogenetic or other coat proteins. With the aim to map the dependence of coat protein deposition on CotA, CotE, Tgl, we studied protein profiles from the ΔcotA, ΔcotE and Δtgl spores. We identified a subset of coat proteins dependent and/or affected by the absence of the candidate protein genes cotE and tgl in these spores. ΔcotA spores did not show an effect on the overall protein composition of spore coat. The thermal stress resistance tests of ΔcotE and Δtgl mutant spores compared to the wild-type spores showed that Δtgl spores are more resistant to thermal stress than ΔcotE spores. Δtgl spores are compared to the equally thermal resistant spores from control wild-type cells, under our test conditions.
Chapter 4

Introduction

Spore coat, crust and exosporium are the outermost layers of bacterial endospores. Spores are formed by bacteria belonging to the genera *Bacillus* and *Clostridium* in response to environmental stress conditions. Spore formation is a highly controlled processed where each layer of spore is deposited progressively leading to further maturation of the spores. The process starts with an initial asymmetric division of the bacterial cell, followed by separation of the larger mother cell and the smaller pre-spore and finally ending up into the lysis of the mother cell. Chapter 1 discusses the details of the sporulation process as well as the details of the different spore layers. The coat acts as a shield protecting the spores from degradative enzymes, chemicals and reactive oxygen species [1]. The coat is also important for germination as it houses the spore cortex lytic enzymes (SCLEs) required for degrading the cortex during initial stages of germination [2, 3]. The coat is also capable of accommodating changes in spore volume that occur during spore formation and germination [4, 5]. The coat layer is unique to bacterial spores, as is the majority of the coat proteins from *B. subtilis*. This uniqueness is confirmed by the fact that these proteins do not have homologues except among the *Bacilli* and *Clostridia*.

The molecular mechanisms behind coat and exosporium assembly are only partially understood with at least 70 proteins known to form the coat. With the advents of new methods such as protein-GFP tagging with fluorescence microscopy, Atomic Force Microscopy (AFM), mass spectrometry, access to the as yet unknown coat and exosporium proteins has become possible. In such an effort, as discussed in Chapter 2 and Chapter 3 we identified 21 putative novel coat proteins from *B. subtilis* 168 using our gel-free technique. Given that only a small subset of coat proteins play essential roles in the determination of coat morphology [6, 7] it is essential to know the function of these proteins. Moreover, the identification of novel proteins could be important to uncover putative mediators of spore resistance and novel compounds important to the spore structure. A group of spore coat proteins listed in Table 1 controls the assembly of the coat proteins in a supra-molecular structure. These proteins are called morphogenetic proteins [6]. Although their functions in coat assembly have been characterized to some extent, the complete understanding of their role in establishing the protein interaction network in the coat layers has not been achieved. Essentially, the coat morphogenetic proteins only control the assembly of proteins but do not affect the gene expression. The coat-associated transglutaminase (*tgl*) [8] and superoxide dismutase (*sod*) [9] also have been shown to play a role in modulating coat layer by introduction of glutamyl-lysine and di-tyrosine cross-links respectively. As an extension to these findings, we attempted to map the inter-protein dependences in the coat proteins from *B. subtilis*. Also, several reports about the role of laccases in protein cross-linking are available. Though the spore coat laccase CotA in *Bacillus subtilis* is known to be involved in a melanin-like pigment production [10] (during sporulation) and spore-resistance against UV radiation [11], we hypothesize its participation in protein cross-linking in our study. We discuss the findings of the proteomic characterization of ΔcotE, ΔcotA and Δtgl mutants of *B. subtilis* in this chapter.
Table 1. The known morphogenetic proteins in the spore coat of *Bacillus subtilis*.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpoIVA</td>
<td>Attaches the coat to the forespore membranes and is suggested to be required for formation of the cortex[12]</td>
</tr>
<tr>
<td>SpoVID</td>
<td>Required for attachment of the coat to the forespore[12]</td>
</tr>
<tr>
<td>SafA</td>
<td>The <em>safA</em> mutants are defective in germination and possess a defective coat that fails to protect the cortex from lysozyme[13]</td>
</tr>
<tr>
<td>CotE</td>
<td>Layered between the inner and outer coat, directs assembly of a subset of coat proteins including most if not all of the outer coat proteins[14]</td>
</tr>
<tr>
<td>CotH</td>
<td>Directs assembly of CotG[14, 15]</td>
</tr>
<tr>
<td>CotO</td>
<td>Directs assembly of a subset of coat proteins that overlaps with the CotH-controlled proteins[16]</td>
</tr>
</tbody>
</table>

Results and Discussion

Protein CotE affects the assembly of the outer coat proteins.

It is well known from the prior research that protein CotE plays a pivotal role in assembly of the outer coat[14]. A possible protein interaction network was predicted by Kim and co-workers[17] based on their study performed using cell biological (GFP-tagging) and protein biochemical (SDS-PAGE) methods. Some of their findings were confirmed in the current study. As seen in Table 2, peptide identifications of highly abundant coat proteins CotA, CotB, CotZ and YqfA were significantly affected by the absence of CotE. Interestingly only 1 to 2 peptides were identified from the inner coat morphogenetic protein SpoIVA in the ΔcotE spores as opposed to the wild-type. Little and Driks[14] showed that the C-terminal region of CotE directs deposition of CotA, CotB, CotG, CotSA, CotS, CotR and YaaH (Figure 1). The C-terminus of CotE does not contain any tryptic cleavage sites and therefore no tryptic peptides would be identified from this region. On the contrary, this region is rich in glutamic acid and aspartic acid residues (Figure 1). These residues might be good candidates for isopeptide linkages or the salt bridge interactions discussed in Chapter 7. In agreement with Kim and colleagues[17] as well as Little & Driks[14], outer coat proteins CotSA, CotS, CotI and inner coat protein CotH were not identified from the ΔcotE spores indicating dependence of these proteins on CotE for their assembly in the outer coat. It is noteworthy, that CotS and CotSA have been identified previously to depend either on CotH or on CotE for their assembly. Other findings of the current study are shown in Table 2 where a selected group of proteins is represented. The complete list of identified proteins can be found in Appendix Table I. On the contrary to the above mentioned observations, certain coat proteins were indicative of easy extraction from the coat as predicted from the higher number of peptide MS/MS spectra identified and the MASCOT scores (Table 1). Of these proteins SafA has been predicted to depend on the presence of CotE for its extractability[14]. Although less likely, an up regulation of these proteins in an effort to compensate for the loss of most of the other coat proteins might take place. Though proteins CotG, CotR, YaaH all are dependent on CotE for their proper coat deposition,
they were identified as their dependence is specific to the C-terminal end of CotE. In fact, in our ΔcotE spores, the N-terminal of CotE has been replaced by the chloramphenicol resistance marker while the C-terminus is intact [18]. Despite the aforementioned mutation the oligomerization sequence is still present in the cotE gene of our mutant and yet it appears that CotE fails to form multimers indicating importance of the N-terminus of CotE in overall protein biochemistry. Thus, it is also intriguing that though not completely structured and produced at low levels, CotE can still control the deposition of these proteins. Previously researchers have tested the resistance behaviour of ΔcotE spores towards thermal stress and lysozyme. These researchers found that ΔcotE spores are, in spite of a loose coat structure, resistant to thermal stress but are sensitive towards lysozyme [18]. Overall decrease in the protein identification due to absence of functional CotE is shown in Figure 2. As seen in ΔcotE spores, from the insoluble protein fraction, proteins with molecular mass of 20kDa and proteins with molecular masses in the range of 40-50 kDa are missed in identification. Little & Driks[14], in their study on soluble coat protein fraction, also observed smear bands in the region of 30 kDa and 43 kDa indicative of multiple protein species which they could not identify using a MALDI-TOF-MS peptide mass fingerprinting approach. In our study, there were a few putative novel proteins identified yet further studies are needed to confirm their dependence on CotE. Intriguingly, in the absence of an electron dense outer coat as observed under electron microscope (EM), ΔcotE spores are also seen to be affected in their germination rates with spores germinating slower in presence of L-alanine and faster in presence of dodecylamine compared to the wild-type [19]. The proteins and the signal mechanisms that are affected by the absence of outer coat still remain to be a mystery. Future studies, including gene knock-out studies as well as protein-protein cross-linking studies, will aid in solving the inter-protein dependence to a deeper extent.

Transglutaminase (Tgl) mediated cross-links may only affect assembly of a fraction of spore coat proteins.

It is known from previous studies that deletion of one protein from the spore coat does not alter the structure of the coat extensively unless the deleted gene encodes a morphogenetic protein as seen in the cotE mutants above. One such important protein that renders certain coat proteins insoluble is the enzyme transglutaminase encoded by the gene tgl in B. subtilis. Transglutaminases are known to catalyze covalent bond formation between a free amine group (such as from lysine) and an acyl group (such as from glutamine) at the end of peptide side chain. Such glutamyl-lysine bonds are resistant to proteolytic digestion. A previous study by Ragkousi and Setlow [20] showed that Tgl-mediated cross-linking is involved in the assembly of the spore coat protein GerQ. Though the presence of glutamine-lysine cross-links has been predicted in the spore coat, no other proteins except GerQ have been identified yet to be cross-linked by Tgl-mediated cross-linking. In the current study, we also analyzed the protein composition of spore coats from Δtgl mutants of B. subtilis. It appeared that only a subset of proteins was
Spore coat protein mutants of *B. subtilis*

Figure 1. Structural regions of CotE involved in assembly and deposition of spore coat proteins. The N-terminal region (a.a. 1 to 101) underlined in black is required for deposition of certain coat proteins. The region shown in bold and orange lettering (a.a. 58 to 75) is responsible for oligomerization of CotE. The region shown in bold and blue lettering is required for targeting CotE to the spore. Deposition of protein CotA, CotR and YaaH is dependent on the C-terminal region highlighted in grey (a.a. 156 to 158), of proteins CotG, CotB is dependent on region highlighted in green (a.a. 159 to 169) and of proteins CotS and CotSA on the region highlighted in yellow (a.a. 176 to 178). The C-terminal region rich in aspartic acid and glutamic acid is underlined in red colour. The regions are demarcated based on the study by Little and Driks[14].

Figure 2. Comparison of protein identification in wild type (WT) and ΔcotE spores of *B. subtilis* PY79. Results of three independent biological replicates (R1, R2 and R3) are shown in terms of frequency distributions of number of proteins identified with respective molecular masses. These results are consistent with previous findings[14].
Table 2. Identification of spore coat proteins in ΔcotE spores of B. subtilis. The wild type strain used was B. subtilis PY79.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>Mass (Da)</th>
<th>Wild Type</th>
<th>ΔcotE Replicate1</th>
<th>ΔcotE Replicate2</th>
<th>ΔcotE Replicate3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MASCOT Score</td>
<td># peptide MS/MS spectra</td>
<td>MASCOT Score</td>
<td># peptide MS/MS spectra</td>
</tr>
<tr>
<td>CotA</td>
<td>Spore coat protein A</td>
<td>58690</td>
<td>1947</td>
<td>27</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>CotB</td>
<td>Spore coat protein B</td>
<td>42946</td>
<td>1771</td>
<td>266</td>
<td>8</td>
<td>87</td>
</tr>
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<td>Spore coat protein E</td>
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<td>1400</td>
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<td>38</td>
<td>38</td>
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<tr>
<td>CotSA</td>
<td>Spore coat protein SA</td>
<td>43056</td>
<td>1045</td>
<td>27</td>
<td>27</td>
<td>27</td>
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<tr>
<td>CoZ</td>
<td>Spore coat protein Z</td>
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<td>1005</td>
<td>15</td>
<td>219</td>
<td>4</td>
</tr>
<tr>
<td>YdcC</td>
<td>Sporulation protein</td>
<td>38170</td>
<td>654</td>
<td>4</td>
<td>38</td>
<td>1</td>
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<tr>
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<td>Spore coat protein I</td>
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<td>488</td>
<td>12</td>
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<td>AkeL</td>
<td>Calcium-transporting ATPase</td>
<td>97516</td>
<td>449</td>
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<td>1</td>
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<td>YqfA</td>
<td>UPF0365 protein</td>
<td>35760</td>
<td>435</td>
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<td>DacF</td>
<td>D-alanyl-D-alanine carboxypeptidase</td>
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<td>402</td>
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<td>SpoVA</td>
<td>Stage IV sporulation protein A</td>
<td>55177</td>
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<tr>
<td>SodA</td>
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<td>CoH</td>
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<td>CotN</td>
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<td>4</td>
<td>4</td>
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<tr>
<td>CotQ</td>
<td>Uncharacterized FAD-linked oxidoreductase</td>
<td>50167</td>
<td>562</td>
<td>15</td>
<td>546</td>
<td>19</td>
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<tr>
<td>CotR</td>
<td>Putative sporulation hydrolase</td>
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<td>619</td>
<td>15</td>
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<tr>
<td>CoC</td>
<td>Spore coat protein C</td>
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<td>366</td>
<td>13</td>
<td>170</td>
<td>9</td>
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<tr>
<td>SieB</td>
<td>Spore cortex-lytic enzyme</td>
<td>34151</td>
<td>315</td>
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<td>295</td>
<td>7</td>
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<td>YjkC</td>
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<td>YgkC</td>
<td>Uncharacterized FAD-linked oxidoreductase</td>
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<td>CotDC</td>
<td>Protein Cot/C</td>
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<td>YjH</td>
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<td>YodJ</td>
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<td>9245</td>
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<td>YxeE</td>
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<td>76</td>
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<td>13</td>
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<td>SafA</td>
<td>SpoIVD-associated factor A</td>
<td>43429</td>
<td>46</td>
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<td>688</td>
<td>21</td>
</tr>
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</table>
affected by the deletion of this protein. In fact a set of glutamine and/or lysine rich proteins was only identified from the ΔtgI mutant. These results point to fact that these selected group of proteins (Table 3) could be made accessible for digestion by the absence of TgI. It was observed previously that the protein GerQ could be completely extracted from the spore coat in the absence of TgI. We confirmed this observation by identifying all possible C-terminal peptides from this protein but the number of MS/MS spectra for peptides was significantly reduced when compared to the wild type. The N-terminal region of GerQ is rich in glutamine (Q) and does not have a suitable tryptic cleavage site and hence with the use of trypsin as the protease no peptide was identified from this region. Proteins CotM and CotX have been suggested as other targets for TgI-mediated cross-linking [8] however we did not identify the CotM protein in our analyses also from the wild type. We identified CotX in the wild type as well as in the tgI-mutant however the number of peptide MS/MS was slightly lower in mutants and hence no conclusion can be drawn (Appendix Table II). Interestingly, proteins CotN, GerE, YxeD, YuzC - all rich in glutamine and/or lysine, were identified in the wild type but not in the ΔtgI mutant in our study. It will be interesting to study the precise location of these proteins in spores as well the effect of deletion of these proteins on the thermal and chemical resistance properties of spores.

Deletion of cotA does not affect the spore coat protein composition

The cotA gene is expressed under the control of σK, with GerE being a transcriptional repressor. Protein CotA is a copper-dependent laccase. Laccases are multicopper oxidases known to take part in oxidative reactions [21]. CotA plays an important role in the biosynthesis of the brown pigment, which is thought to be a melanin-like product[10]. The ΔcotA spores also lack the brown pigment. The absence of brown pigment is generally diagnostic of a block in spore formation, however in our experiments we obtained intact phase-bright spores. It has been thought that, the production of pigment is somehow tightly coupled to sporulation [22]. The ΔcotA spores have been shown to be sensitive to UV-B as well as UV-A radiations. In the functional study of CotA, Hullo et al. [11] recognized the role of CotA in UV-resistance where the brown melanin-like pigment produced by CotA during sporulation is said to play a role. Though, in contrast to tyrosinases, most laccases are not known to oxidize tyrosines; it is still not known if CotA has any oxidase activity in B. subtilis. Therefore to assess a possible role of CotA in oxidative linking of proteins (mostly via di-tyrosine formation [21]) we tested the protein profile of ΔcotA spores. Our results did not show a significant effect on the spore coat protein composition and the protein identification profile of mutants was similar to that of the wild type. Hullo and co-researchers [11] have reported that CotA is a classical laccase without tyrosinase activity. Nevertheless proteins CotS, CotSA and CotQ were identified with reduced peptide MS/MS spectra pointing to lower protein abundance or extraction efficiency (Table 4). Incidentally, these three proteins are rich in tyrosine. It is not clear if CotA-mediated cross-linking plays any role in assembly of spore coat proteins and thus detailed studies are required. Contrary to these
Table 3. Identification of spore coat proteins in Δtgl spores of *B. subtilis*. The wild type strain used was tryptophan prototroph *B. subtilis* 168.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>Mass (Da)</th>
<th>Wild type</th>
<th>Δtgl Replicate 1</th>
<th>Δtgl Replicate 2</th>
<th>Δtgl Replicate 3</th>
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<tbody>
<tr>
<td></td>
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<td>MASCOT score</td>
<td># peptide MS/MS spectra</td>
<td>MASCOT score</td>
<td># peptide MS/MS spectra</td>
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<td>GerE</td>
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<td>GerQ</td>
<td>Spore coat protein</td>
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<td>Tgl</td>
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<td>Spore protein</td>
<td>11396</td>
<td>42</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Identified proteins affected by deletion of *tgl*

- GerE Spore germination protein
- GerQ Spore coat protein
- Tgl Protein-glutamine gamma-glutamyl transferase
- YahP Spore protein

Identified proteins possibly dependent on Tgl-mediated links

- YkD Uncharacterized protein
- YkuA Uncharacterized protein
- YciC Putative metal chaperone
- YhmM Uncharacterized protein
- YhcX UPF0012 hydrolase
- YkuG UPF0180 protein
- YtxJ Uncharacterized protein
- YneT Uncharacterized protein
- YusN Uncharacterized protein
- YznB Uncharacterized protein
- YyeD Uncharacterized protein
- YurZ Uncharacterized protein
- YurT Uncharacterized protein
- YuzM Uncharacterized protein
- YlbN Uncharacterized protein
- YktB UPF0637 protein

86
Table 4. Identification of spore coat proteins in ΔcotA spores of *B. subtilis*. The wild type strain used was *B. subtilis* PY79.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>Mass (Da)</th>
<th>Wild type</th>
<th>ΔcotA Replicate 1</th>
<th>ΔcotA Replicate 2</th>
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<td># peptide MS/MS spectra</td>
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<td>CotA</td>
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<td>CotSA</td>
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<td>562</td>
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<td>YkuD</td>
<td>Putative LD-transpeptidase</td>
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</tr>
<tr>
<td>CotW</td>
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<tr>
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<td>Ferredoxin</td>
<td>9092</td>
<td>22</td>
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observations, a group of proteins with very less or no tyrosine was identified in wild type but not in mutants. Since these proteins were identified with very few MS/MS spectra per protein it is still premature to derive any firm conclusion. The complete list of proteins identified from ΔcotA spores can be seen in Appendix Table III.

**Thermal resistance of ΔcotE, Δtgl spores**

Wet-heat resistance of spores is the most studied survival challenge of wild-type and mutant spores. Zheng and colleagues [18] studied the thermal resistance of ΔcotE spores and found that these spores did not show any deviation in terms of survival level when the behaviour was compared to the wild type spores. In our study, we tested the ΔcotE spores for their thermal resistance and observed that ΔcotE spores possessed lower resistivity to the thermal stress conditions used in our experiment (Figure 3). It is noteworthy that, these spores lack or contain lower levels of structural coat proteins such as CotA, CotB, SpoIVA etc. indicating the role of individual spore coat layers in spore heat resistance. Additionally, proteins CotB, CotI, CotS, CotSA appear to be involved in spore maturation (Chapter 5). Absence of these proteins in ΔcotE spores can thus also be linked to the lower thermal resistance of these spores. In a similar experiment, the Δtgl spores did not show any effect on their resistant nature towards thermal stress as compared to the wild type spores (Figure 3). Sanchez-Salas et al. [23] also showed that heat stress did not affect the survival of Δtgl spores. Impressively, in the absence of the transglutaminase (inducer of cross-links) the resistance levels of spores are not affected. We did not study the thermal resistance of ΔcotA spores but these spores have been reported to possess normal heat resistance that is comparable to the wild-type [21]. Hence we conclude that while outer coat spore proteins are important for proper spore assembly and wet-heat resistance, the formation of glutamine-lysine cross-links plays no role of major importance in spore thermal stress resistance. This observation also implies the presence of other types of cross-links, as discussed in Chapter 7, which might be involved in spore maturation which in turn is linked with spore wet-heat resistance. The role of small set of possibly CotA-dependent proteins in UV-resistance as well as effect of UV on the ΔcotE and Δtgl mutant spores remains a topic of future research.
Concluding remarks

Analysis of spore coat protein mutants in our study revealed a small group of coat proteins that were affected by the mutation in question. CotH, CotI, CotS, CotSA previously suggested to be directly or indirectly dependent on CotE for their assembly were not identified in our study confirming the original hypothesis. These proteins along with a few others are seen to participate in spore maturation. A loss of these proteins also appears to be linked to a lower heat resistance as seen in our study. Similar to SafA, we also suggest the extractability of certain coat proteins to depend on CotE as observed from our results. Absence of transglutaminase affects the oligomerization of GerQ but in addition we suggest that proteins GerE, YabP as well as other glutamine/lysine rich putative novel proteins are also affected by the absence of transglutaminase activity. CotA is seen to affect the pigmentation phenotype in mutant spores and only few putative coat proteins likely depend on CotA for their localization and interaction in spore coat. Heat resistance tests of Δtgl spores argue a presence of other types of cross-links in the spore coat.

Materials and Methods

Bacterial strain and sporulation conditions

_Bacillus subtilis_ 168 (Trp') lab-strain and _Bacillus subtilis_ PY79 wild-type were the background strains used in this study. The ΔcotE, ΔcotA (both with PY79 background) and Δtgl (B. subtilis PS832 (Trp') background) mutant strains were obtained from the Eichenberger lab (New York University, USA). Bacteria were pre-cultured and sporulated as described previously [24]. The pre-culturing of mutant strains was done in Luria Bertani (LB) broth medium (pH 7.5). For ΔcotE and ΔcotA mutant strains the pre-culture medium was supplemented with chloramphenicol (5 mg/L) and the Δtgl mutant strain was pre-cultured in medium supplemented with erythromycin (1 mg/L). For sporulation a defined minimal medium, buffered with 3-(N-morpholino) propanesulfonic acid (MOPS) to pH 7.4, was used[25]. Three independent biological replicates for both wild-type and mutants were analyzed. After 96 hours, the spores were harvested as described elsewhere[24].

Spore coat isolation and protein extraction

The harvested spores were subjected to spore coat isolation & protein extraction as described previously [24, 26]. The isolated coat material was freeze-dried overnight and immediately used for mass spectrometric analysis.

Measurements of thermal resistance

Thermal resistance of spores to wet heat was assessed using the previously used screw-cap tube method[25]. In short, a 1 ml (heat-activated (70°C, 30 min); OD 600 ~2) spore suspension in sterile milli-Q water, for each time point, was injected with a syringe into a preheated metal screw-cap tube containing 9.0 ml of sterile milli-Q water. The heat activation helped to kill all the remaining vegetative cells in the sample. The tube was heated by immersing it completely in a glycerol bath (85°C for 10 min). After 10 min the tube was transferred to ice-water. Dilution series of spore suspension were prepared in sterile milli-Q water and 100 μl of sample was spread on Tryptic Soy agar plates. The number of colonies was counted after 24 hours of incubation at 37°C. The thermal resistance of spores was thus determined by the loss of their ability to germinate and form colonies (i.e., viability counts). As a control a same dilution series for
non-heat stressed spores were plated and the final colony counts from the stress samples were normalized with those from the control sample. The significance of the thermal resistance tests was tested by one-way ANOVA test.

Sample preparation for MS analysis

The freeze dried samples were reduced with 10 mM dithiothreitol in 100 mM NH$_4$HCO$_3$ (1 hour at 55°C) followed by a reaction with 55 mM iodoacetamide in 100 mM NH$_4$HCO$_3$ for 45 min at room temperature in the dark. The samples were directly digested for 18 hours at 37°C with trypsin (Trypsin gold Promega, Madison, WI) using a 1:60 (w/w) protease: protein ratio. The tryptic digests were desalted using Omix μC18 pipette tips (80 μg capacity, Varian, Palo Alto, CA) according to the manufacturer’s instructions.

LC-FT-ICR MS/MS analysis

LC-MS/MS data were acquired with a Bruker ApexUltra Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 7 T magnet and a nano-electrospray Apollo II DualSource™ coupled to an Ultimate 3000 (Dionex, Sunnyvale, CA, USA) HPLC system. Samples containing up to 160 ng of the tryptic peptide mixtures were injected as a 10 μl 0.1% TFA, 3% ACN aqueous solution and loaded onto a PepMap100 C18 (5-μm particle size, 100-Å pore size, 75-μm inner diameter x 250 mm length) analytical column (Thermo Scientific, Etten-Leur, The Netherlands) to the nano-electrospray source. Gradient profiles of up to 120 min were used from 0.1% formic acid / 3% CH$_3$CN / 97% H$_2$O to 0.1% formic acid / 50% CH$_3$CN / 50% H$_2$O 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 6x10$^{-6}$ mbar (measured at the ion gauge) and the fragment ions were detected in the ICR cell at a resolution of up to 60000. In the MS/MS duty cycle, 3 different precursor peptide ions were selected from each survey MS. The MS/MS duty cycle time for 1 survey MS and 3 MS/MS acquisitions was about 2 s. Instrument mass calibration was better than 1 ppm over a m/z range of 250 to 1500. Raw FT-MS/MS data were processed with the MASCOT DISTILLER program, version 2.4.3.1 (64bits), MDRO 2.4.3.0 (MATRIX science, London, UK), including the Search toolbox and the Quantification toolbox. Peak-picking for both MS and MS/MS spectra were optimized for the mass resolution of up to 60000. Peaks were fitted to a simulated isotope distribution with a correlation threshold of 0.7, with minimum signal to noise of 2. The processed data, from the three independent biological replicates, were searched with the MASCOT server program 2.3.02 (MATRIX science, London, UK) against a complete B. subtilis 168 ORF translation database (Uniprot 2011 update, downloaded from http://www.uniprot.org/uniprot). The database was complemented with its corresponding decoy data base for statistical analyses of peptide false discovery rate (FDR). Trypsin was used as enzyme and 1 missed cleavage was allowed. Carbamidomethylation of cysteine was used as a fixed modification. The peptide mass tolerance was set to 25 ppm and the peptide fragment mass tolerance was set to 0.025 Dalton. The threshold for MASCOT peptide identification score was set to 20. At this cut-off and based on the number of assigned decoy peptide sequences, a peptide false discovery rate (FDR) of ~2% for all analyses was obtained. For each sample > 200 proteins were identified of which 90 were known as well as candidate coat proteins (see Appendix Tables I,II and III) while the rest were remnants of cytosolic proteins from the mother cell.

References
Spore coat protein mutants of *B. subtilis*

