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Chapter 4

Changes in the spore proteome of *Bacillus cereus* in response to plasmid based genetic tools.

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Abstract

Fluorescent fusion proteins were expressed in *Bacillus cereus* to visualize the germinosome by introducing a plasmid that carries fluorescent fusion proteins of germinant receptor GerR or scaffold protein GerD. The effects of plasmid insertion and recombinant protein expression on the spore proteome were investigated. Proteomic analysis showed that overexpression of the target proteins has negligible effects on the spore proteome. However, plasmid-bearing spores displayed dramatic abundance changes of spore proteins involved in signaling and metabolism. Our findings suggested that introduction of plasmid alone alters spore protein composition dramatically with 916 proteins significantly down-regulated and 379 proteins significantly up-regulated among 3551 identified proteins. This shows that empty vector controls are more apt to compare changes to than WT when using plasmid based genetic tools. Furthermore, researchers should keep in mind that molecular cloning techniques can alter more than their intended targets in a biological system, and interpret results with this in mind.

4.1. Introduction

B. cereus is a Gram-positive, facultative anaerobic, spore-forming human pathogen, well known as a causative agent for food spoilage and food-borne illnesses: diarrhoeal and emetic disease [1]. The removal of this organism from the food chain is difficult due to its ability to form spores. When the environment is not favorable, *B. cereus* vegetative cells divide asymmetrically and release spores. This developmental process is called sporulation [2]. Spores are dormant and highly resistant to extreme conditions, such as high temperature, radiation, desiccation and so on, making them hard to kill. The resistant properties are mostly the results of the composition of spore core and to a lesser extent of the multi-layered structures that encase the core [3]. The spore core, the innermost layer, encloses DNA, RNA and most enzymes. DNA is protected by saturation with small, acid soluble spore proteins (SASPs) against heat and toxic chemicals [4]. The spore core contains a high level of a 1:1 chelate of dipicolinic acid (DPA) with Ca^{2+} , which also contributes to spore resistance [3]. The spore core is surrounded by the spore inner membrane, which is highly compressed and has a low permeability to small molecules [5, 6]. Outside the inner membrane is a germ cell wall layer, the peptidoglycan composition of which is identical with the growing cell wall. The spore cortex peptidoglycan layer, covering the germ wall, possesses modified peptidoglycans for enzyme recognition and cleavage. This layer is required for spore core dehydration and dormancy [7]. Above the cortex is the outer membrane and the proteinaceous coat layer, which protect the spore against chemicals and enzymes. In *B. cereus*, the outermost layer is the exosporium and participates in pathogenesis [2].

Even though, the spore is dormant, it keeps monitoring the environment and small nutrients can be sensed by the germinant receptors (GRs) located in the inner membrane. Once committed to germinate, spores release the deposit of CaDPA from the spore core, degrade the spore cortex and coat layer and grow out into vegetative cells, losing their extreme resistance properties [3]. The growing cell produces toxins resulting in food spoilage or poisoning. Hence, potential strategies for spore eradication are to trigger germination to kill spore easily or prevent germination to avoid toxin production [1]. To improve and develop new strategies for spore eradication, more detailed knowledge about spore germination processes is required, especially about the germinant receptors. However, germinant receptors in spores are expressed at low levels [8]. The germinant receptor GerR complex consists of three subunits: A, B and C encoded in a single operon [9]. Subunit A and B are made up of multiple transmembrane domains, making it more

technically challenging for protein extraction, solubilisation and purification. The GerD protein is critical for germination mediated by germinant receptors [10]. It is suggested to interact with the germinant receptors to form a germinosome in the early stage of germination [11, 12].

The green fluorescent protein (GFP) was first discovered and isolated in the early 1960s [13]. This discovery revolutionized cell biology research. Fluorescent proteins are widely used as fusion tags to monitor protein localization in living organism. Fluorescent chimeric proteins are also used as a common tool in the field of *in vitro* functional and structural studies by overexpression and purification of membrane proteins. Taking advantages of this technology, localization of GerR and the visualization of germinosomes in *B. cereus* were reported after genetically fusing the fluorescent proteins to the N-termini of GRs to act as a reporter, followed by microscopic detection in the whole spore [11, 12].

Although fluorescent proteins are a vital tool in molecular studies, their introduction into a biological system can have a variety of unexpected and undesired effects. Apart from the importance to validate whether a fusion protein localizes and functions similarly to its endogenous counterpart [14, 15], the introduction through genetic tools can potentially cause a variety of unintended effects in both pro- and eukaryotes [16-18]. Well known side-effects of recombinant protein production shown by global studies in *Escherichia coli*, are a significant plasmid metabolic burden altering gene expression of central metabolism as well as marked increases in expression levels of heat shock proteins [19, 20]. To visualize GRs and find how they associate in *B. cereus* spores, a variety of plasmids containing a single or combinations of GR genes fused to fluorescent proteins were introduced into wild type *B. cereus* [11, 12]. We were interested whether the introduction of plasmids bearing fusion proteins would affect the global protein content of the mature spore in any way. To study this, we used label-free quantitative proteomics to profile the protein content of mature spores from five strains transformed with different plasmids containing fusions of GRs to fluorescent proteins, an empty plasmid, a plasmid with GFP and a deletion of *cotE* [21]. We quantitatively compare the protein content to spores from a WT strain. In this manner we can make a distinction between effects of plasmid introduction, protein expression (GFP), and specific GR fusion proteins on the protein composition of mature spores.

4.2. Results

4.2.1. The influence of molecular cloning on spore proteome.

The strains used in this study are listed in Table 4.1. The deletion of *cotE* is constructed by allele exchange and verified by DNA sequencing [21]. The *cotE* null strain was used as a positive control for large scale changes to the spore proteome composition versus wild-type spores. Strain M-001 contained only an empty plasmid to check for transformation and plasmid maintenance effects. M-003 and M-006 contain a plasmid with the GerR promoter from the *B. cereus* genome controlling expression of either only the GerRB subunit fused to SGFP or the entire GerR operon (GerRA, GerRB and GerRC) of which GerRB is fused to SGFP. Strain M-005 carries a plasmid with the constitutive aminoglycoside phosphotransferase A30 promoter and SGFP to check the effect of constitutive protein expression. M-007 contained GerD fused to the red fluorescent protein mScarlet controlled by the GerD promoter. Strains M-008 and M-009 are two gene constructs with either GerRB-SGFP or the complete GerR operon (with GerRB-SGFP) under GerR promoter control combined with GerD-mScarlet controlled by the GerD promoter.

Table 4.1. Strains used in this study.

Strains	Relevant genotype	Source
<i>B. cereus</i> WT	<i>B. cereus</i> ATCC 14579 wild type	lab stock
<i>B. cereus</i> CotE	<i>B. cereus</i> Δ <i>cotE</i> knockout mutant Sp ^r	[21]
<i>B. cereus</i> 001	<i>B. cereus</i> carrying pHT315 Ery ^r	
<i>B. cereus</i> 003	<i>B. cereus</i> carrying pHT315-PgerR-gerRB-SGFP2 Ery ^r	
<i>B. cereus</i> 005	<i>B. cereus</i> carrying pHT315-PaphA3'-SGFP2 Ery ^r	[12]
<i>B. cereus</i> 006	<i>B. cereus</i> carrying pHT315-PgerR-gerR-SGFP2 Ery ^r	
<i>B. cereus</i> 007	<i>B. cereus</i> carrying pHT315-PgerD-gerD-mScarlet-I Ery ^r	
<i>B. cereus</i> F09 (008)	<i>B. cereus</i> carrying pHT315-PgerR-gerRB-SGFP2-I-PgerD-gerD-mScarlet-I Ery ^r	[11]
<i>B. cereus</i> F06 (009)	<i>B. cereus</i> carrying pHT315-PgerR-gerR-SGFP2-PgerD-gerD-mScarlet-I Ery ^r	

Sp^r, spectinomycin; Ery^r, Erythromycin.

The spore proteome of these different strains was investigated to estimate the effects caused by recombinant gene expression. In total, 3551 proteins were identified in the whole set of samples. As shown in Figure 4.1A, over 2600 proteins were quantified from the biological replicates of the different strains. The average CV (coefficient of variation) between replicates was below 0.05, showing a high quantitative reproducibility between replicates.

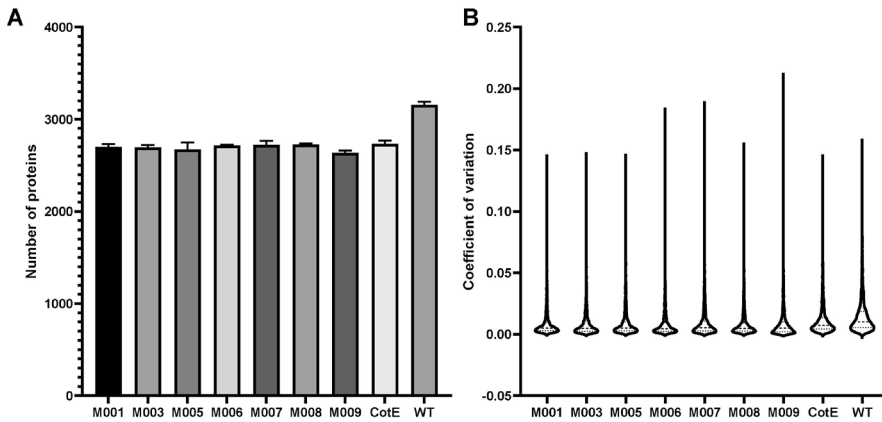


Figure 4.1. A: The number of identified proteins in each strain. B: Coefficient of variation between replicates of each strain.

To detect significantly altered content of spores a T-test was conducted on pairwise groups and the difference of expression is shown in a volcano plot (Figure 4.2). Proteins with a $\log_2(\text{fold change}) \geq |\pm 1|$ and $-\log(\text{p-value}) \leq 0.01$ are considered to be significantly differentially present in the spore. First, we compared both the CotE deletion strain and M001 (empty plasmid) to the WT spores. Compared with WT, the knockout of gene *cotE* resulted in 698 proteins significantly down-regulated and 502 proteins significantly up-regulated. Introduction of an empty plasmid caused 916 proteins significantly down-regulated and 379 proteins significantly up-regulated (Figure 4.2, Supplementary Table S1). The differentially expressed proteins in strain CotE and M001 compared to WT are quite evenly distributed over global KEGG pathways (Figure 4.3), only the KEGG term “Unclassified: signaling and cellular process” containing spore specific proteins had slightly more protein abundance changes found in the CotE deletion strain, This is a surprisingly large number, which shows that introduction of an antibiotic resistance bearing plasmid actually has quite a marked effect on the protein content of the mature spore, on par with a deletion mutant of a major spore coat constituent protein.

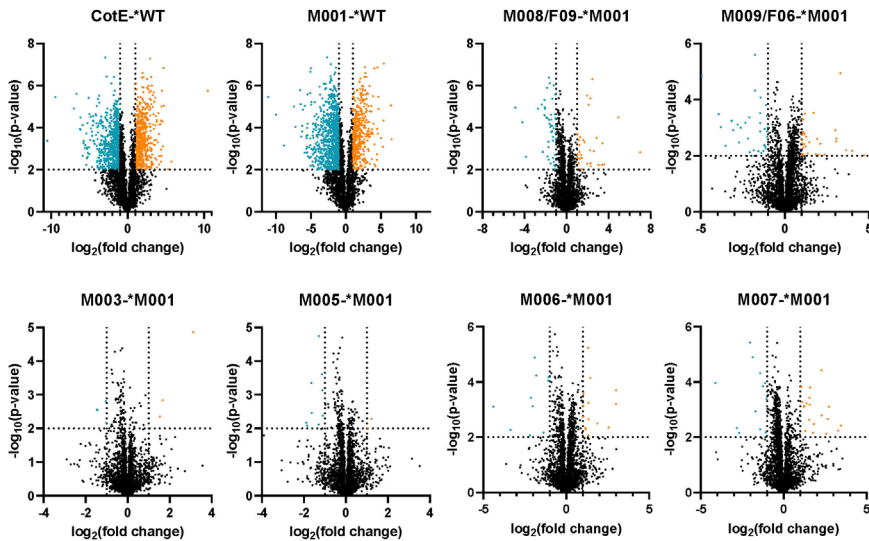


Figure 4.2. Volcano plots of t-test between different strains. * represents control group.

The introduction of the plasmid already induces so many alterations in protein content. As such, to be able to identify changes induced by specific GR fusion protein expression (strains M003, M006 and M007), combinations of two GR fusion proteins (strains M008 and M009) and constitutive expression of a fluorescent protein (M005) we compared these to the empty plasmid containing spores (M001). As is obvious this resulted in far fewer altered proteins (see below) and underscores the necessity of using a proper control (not WT) when doing global studies on strains altered through the introduction of a plasmid. When we look at additional effects from constitutive expression of SGFP there are only 9 proteins less abundant and 2 proteins more abundant compared to empty plasmid containing spores. Only one of which (Hemolysin BL binding component) is also downregulated in four out of five of the other strains bearing plasmids containing fusion proteins. None of the other proteins are found back in more than one or two of the other strains as being regulated in the same manner. Together with the relatively small number of additional altered proteins this suggests that the effect of expressing a recombinant protein has relatively minor additional effects on the protein composition compared to the introduction of the plasmid.

Likewise, the alteration in protein content from spores containing a single GR fusion protein, M003 (3 up, 4 down), M006 (14 up, 10 down), M007 (19 up, 13 down) is markedly smaller than plasmid introduction and only

slightly higher in 3 strains compared to constitutive expression of GFP. Among the proteins of altered abundance, in spores expressing the entire GerR operon (GerRB-SGFP, M006) and GerD-mScarlet (M007) there were 3 proteins related to the sporulation (Sporulation-specific protease YabG in M007) and germination pathways (GerA and GerC family proteins in M006). The two strains which had plasmids containing both GerRB-SGFP and GerD-mScarlet (M008) and the GerR operon with GerRB-SGFP and GerD-mScarlet (M009) there was a larger number of proteins that showed an altered abundance in M008 (30 up, 46 down) and M009 (31 up, 32 down) compared to the empty plasmid spores. Of these, 16 proteins correlated in abundance changes between the two strains compared to an empty plasmid (Supplemental Table S1), but relatively few proteins seemed directly related to sporulation or germination, suggesting that no large changes in the spore protein content related to these pathways is induced by the fusion protein expression.

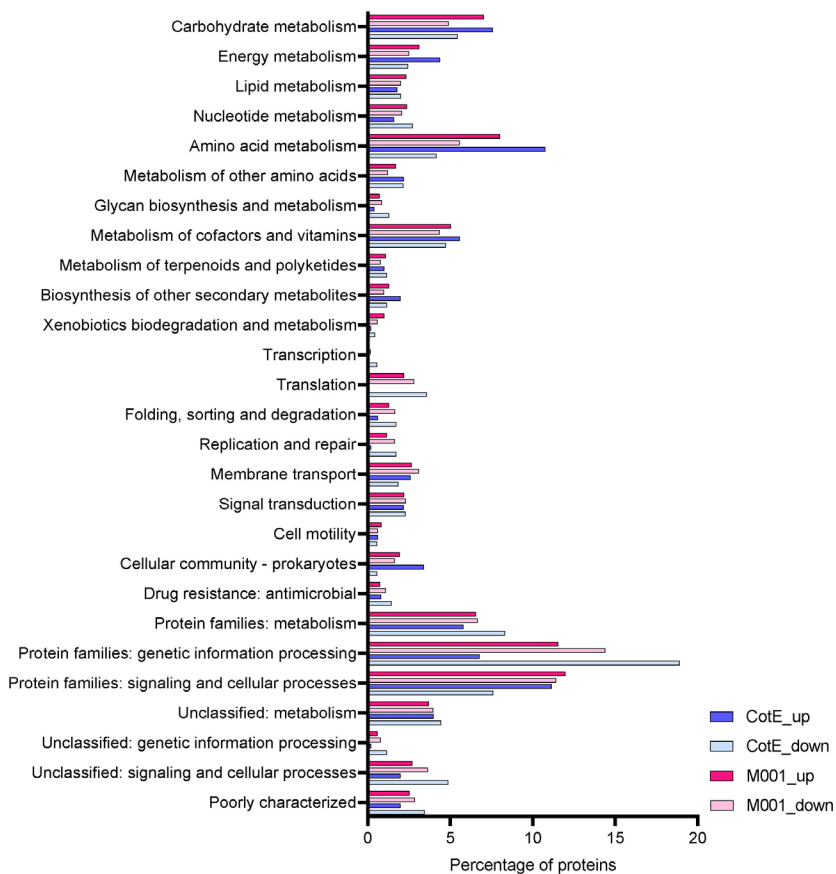


Figure 4.3. The KEGG functional enrichment of differentially expressed proteins in strain CotE and M001 compared with WT spores.

4.2.2. Examining relative levels of spore proteins in the different strains.

The strains were constructed to study signaling events relating to spore-revival, as such it is of particular interest to observe how genetic alterations influence the expression of spore proteins. As stated in the above the strains produce a fusion protein of either only GerRB (M003), the entire GerR operon but only GerRB fused to SGFP (M006) and GerD fused to mScarlet (M007). The other two strains are a combination of one of the first two with GerD fused to mScarlet on the same plasmid (M008 and M009). The relative abundances (z-scored normalized iBAQ values) of the GerR operon, GerD and CotE are shown in in Figure 4.4. In the CotE mutant strain three peptides were still detected due to the match between run algorithm of Maxquant, but these are likely due to carry-over between samples and not caused by expression of CotE in the mutant strain. When you compare the WT spore levels of the 4 proteins shown to the strains carrying an empty plasmid (M001) and the plasmid constitutively expressing SGFP (M005) there is a clear reduction, which does not seem to change much due to SGFP expression. Compared to M001 and M005 the expression of CotE in the other strains is relatively constant, while in the strains where only GerRB is expressed from the plasmid (M003 and M008 in combination with GerD) there is a small increase in its expression level while the other proteins of the GerR operon also remain unaffected when compared to strain M001. While when the entire Ger operon is expressed there is a marked increase in the levels of all 3 proteins (M006 and M009 vs M001). GerD expression from a plasmid (M007-009) increases its own expression level compared to strain M001, while its expression seems to not clearly influence the GerR operon (M007 and M008 vs M001).

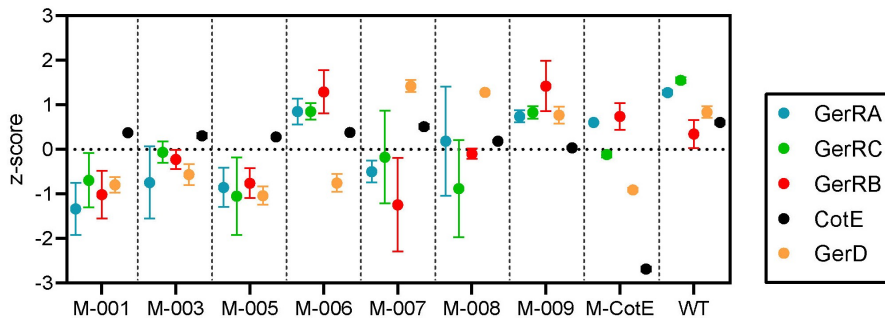


Figure 4.4. The abundances of three proteins encoded by the GerR operon (GerRA, GerRC and GerRB), GerD and CotE. The z-score of the iBAQ intensities are shown to compare relative expression levels between different strains of a given protein, please note that the quantitative data for GerRB is based on a single peptide only.

The influence of expressing the GerR operon or GerD from a plasmid on the abundance of proteins in spores involved in germination and sporulation are shown in Figure 4.5A. Based on the results from Figure 4.2, significantly altered proteins are marked with an asterisk. Among these, GerK, GerS, GerQ, GerI, GerB and GerL are germinant receptors that respond to different nutrients [9]. SpoVA is a channel protein involved in CaDPA release. The coat protein GerP is involved in transport of signals to germinant receptors [22]. Gpr is the enzyme that degrades SASPs during germination. The cortex lytic-enzymes SleB and CwlJ are involved in degradation of the cortex and proteins YpeB and GerQ (coat) are required for their stability [23]. Again, compared with WT most are markedly less abundant in empty plasmid containing spores (M001), as much or more than in a CotE deletion mutant. On the other hand, abundances of GerK, CwlJ and proteins in the coat layer (GerP, GerQ) were not significantly affected. The transcription of germinant receptors is regulated by sigma factor SigG [24] and repressor SpoVT [25]. Compared with WT, the abundances of these two transcription regulators were also reduced in strain M001. However as is clear from Figure 4.2, the additional expression of different proteins from the plasmid does not seem to induce further alteration in protein abundance in mature spores, GerLC being the only significantly downregulated protein found in M008 compared to M001.

We also looked at the influence of empty plasmid transformation and *cotE* deletion on the composition of the spore coat layer and SASPs in the spore core (see Figure 4.5B-D). The effects of *cotE* deletion on spores were studied previously [26]. Consistent with the previous study [26], the abundance of CotE-independent proteins (CotH, CotG, CotS, CotD) were significantly increased, while the abundances of CotE-dependent proteins, such as SafA, decreased. Most of coat proteins were significantly downregulated in M001 versus WT, except CotH, CotD, CotG, CotW, CotS2 and BC5056. Apart from SasP-1, the amounts of SASPs were significantly downregulated in strain M001. The SspE protein has been reported to be CotE dependent [26], but was not identified. The SASP protein SspO levels were significantly downregulated in the CotE deletion strain, suggesting its CotE dependence. Overall these data again underscore that WT strains are not apt controls when using plasmid based systems to study processes in spores.

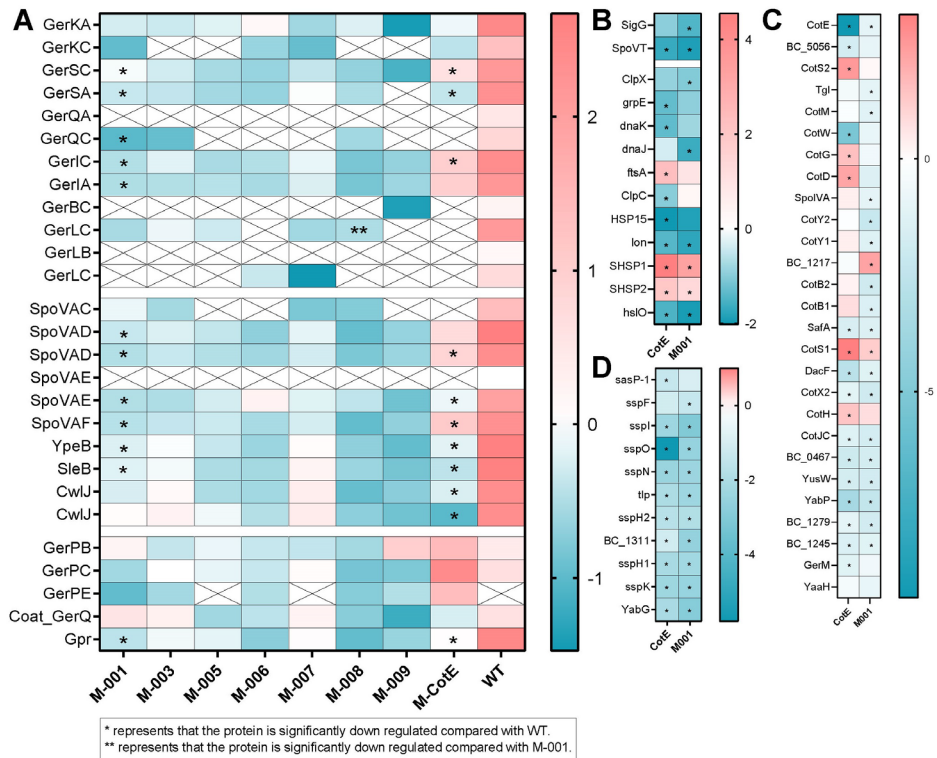


Figure 4.5. The heatmap of proteins associated with germination (A). Color represents z-score of LFQ values, a cross means not detected. The \log_2 (fold change) of SASPs (D), coat proteins (C), heat shock proteins (B) are shown in a colored heatmap. The red represents elevated protein abundance and blue represents depressed LFQ intensities compared with WT.

4.2.3. Plasmid induced physiological changes are apparent in the spore proteome

Prior research has reported on the plasmid metabolic burden altering gene expression of central metabolism as well as heat shock proteins [19, 20]. Dormant spores, having no metabolic activity themselves, do carry with them proteins to restart metabolism upon germination. Furthermore, the vegetative proteins found in spores can be thought to be a representation of the metabolic state their mother cell was in when they formed [27]. It has been demonstrated that spores have different properties in response to the different temperatures [28-30]. In the above (Figure 4.3), many of the proteins found altered in the spore proteome when comparing the spores from strain M001 carrying an empty plasmid to WT belonged to the central metabolic pathways. The quantitative information of Glycolysis, Tricarboxylic Acid (TCA) cycle and Pentose Phosphate (PP) pathway proteins found in spores are shown in Figure 4.6. Compared with WT the

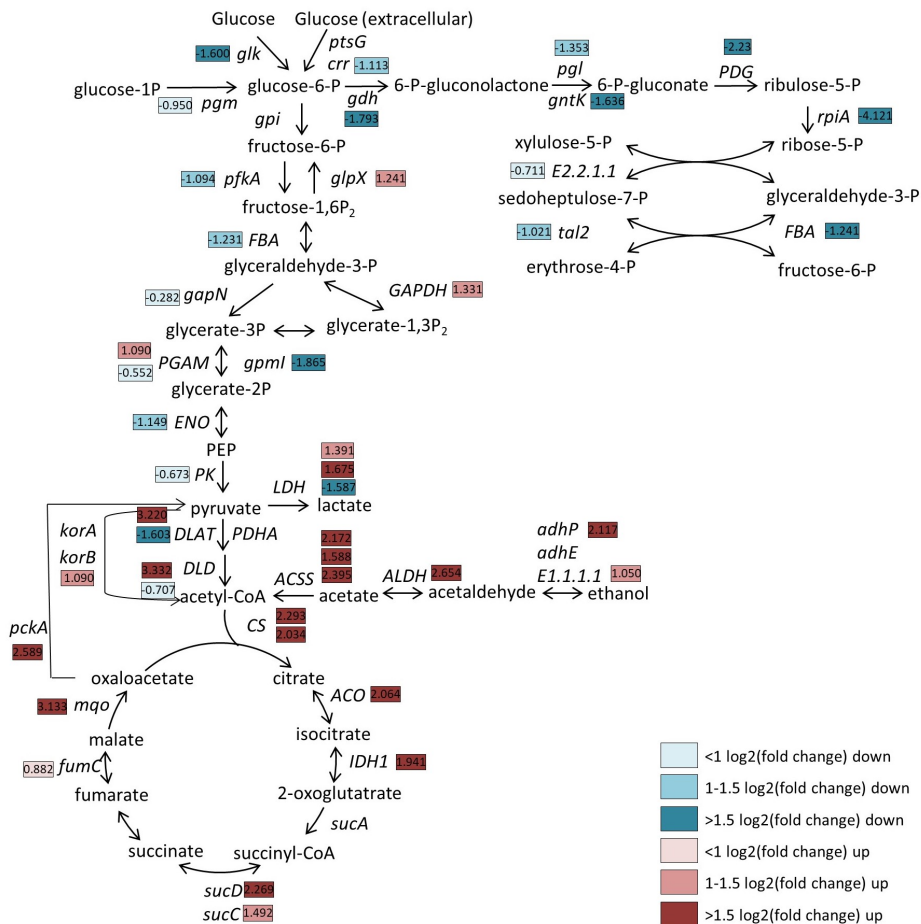


Figure 4.6. The enzyme levels ($\log_2(\text{fold change})$) in central metabolic pathway of *B. cereus* strain M001. The enzyme levels colored in blue are reduced, while in red are increased as compared to WT. The multiple boxes next to some enzymes correspond to isoforms of the same enzyme. The regulation of the relevant genes and the enzymes for which they are coding are presented in Supplementary Table S6. Abbreviations used: PEP, phosphoenolpyruvate.

introduction of an empty plasmid resulted in higher levels of TCA cycle proteins. The key enzyme CS (citrate synthase) in the cycle is significantly up-regulated as were the enzyme levels in the pathway transforming acetyl-CoA from acetate and ethanol. In contrast there was a reduction in proteins from the PP pathway found in spores. Glycolysis was mostly repressed, apart from GAPDH and PGAM, showing that alterations in central metabolism induced by plasmid transformation are apparent in the spore proteome.

As the central metabolism, the protein abundance of heat shock proteins and heat-induced proteins (DnaJ, DnaK, ClpC, ClpX, GrpE) [31] in the spore

proteome are shown in Figure 4.5B. Compared to WT, only two small heat shock proteins (SHSP1, SHSP2) showed an increased abundance, while ClpX, DnaJ, Lon and HslO were downregulated in strain M001. The small heat shock proteins were also found to be upregulated in strain CotE, but ClpX and DnaJ levels were not significantly affected. Compared to M001, the abundances of heat shock proteins in strain M003-M009 were not affected (Supplementary Table S2), showing again that plasmid introduction has a far larger effect compared to fusion protein expression.

4.3. Discussion

There are quite a few studies about the influences of environmental conditions during sporulation on the composition of spores. In previous studies on spore formation in *B. cereus*, damage of the exosporium was observed in spores formed at high temperature [32], and differences in coat protein composition were observed when sporulation occurred at 20 °C or 37 °C [21]. The concentrations of minerals accumulated in the spore core and composition of the cortex peptidoglycans are affected by the composition of sporulation medium [5, 33]. However, very little is known about the response of *B. cereus* to perturbations in the intracellular environment. We investigated the alteration of the spore proteome induced by plasmid introduction and expression of plasmid-encoded proteins. The experiments were conducted using *B. cereus* mutant strains constructed to localize and visualize the protein-protein interactions between the multi-subunit germinant receptor GerR with the scaffold protein GerD in *B. cereus*.

The abundances in the spore proteome of the proteins expressed on the different plasmids were investigated. Results showed that only overexpression of subunit GerRB of the GerR operon (consisting of GerRA, GerRC and GerRB) did only marginally increase the abundance of GerRB itself and did not affect the level of the other two subunits. In contrast, expression of all 3 subunits from the plasmid did show marked increases of their levels in the spore proteome. This result suggests that the three subunits of GerR could be assembled together during sporulation and excess free GerRB protein may be degraded. Studies on GerA in *B. subtilis*, a homologue of GerR, showed that overexpression of A or C subunits increased the germination rate via the GerA receptor [34, 35]. Similarly, overexpression of the whole GerA operon increased the germination rate [36]. Our results on GerR operon expression suggest that GerR assembly and function in *B. cereus* may differ from *B. subtilis*. Inferred from homology with *B. subtilis*, the GerR operon in *B. cereus* could be a member of the SigG regulon [24] and putative SigG binding sites are also found in up-stream

regions of operons encoding germinant receptors in *B.cereus* [37]. In *B. subtilis*, the SigG regulon controls other spore specific proteins, e.g. germinant receptors GerK, GerD and SASP proteins, in parallel [38]. In our dataset, overexpression of the GerR (M006 and M009) operon did not affect other possible members of the SigG regulon like GerD, other germinant receptors and proteins in the germination pathway, which was in line with a previous study in *B. subtilis* that genes under SigG regulon are expressed in parallel [38].

The largest effect we found on the spore proteome of mature spores was the introduction of an empty plasmid only containing the antibiotic resistance marker used for selection. The number of proteins significantly altered was slightly larger than a deletion mutant of CotE compared to WT spores. Protein content of the spore proteome altered by introduction of the plasmid, ranged from central carbon metabolism, small heat shock proteins and a lot of spore specific and germination receptor proteins. As spores are metabolically dormant these protein levels likely reflect the state of the mother cell during sporulation. Introduction of plasmids having effects on central carbon metabolism and heat shock proteins has been reported before in cellular expression systems such as *E. coli* [16, 19, 20, 39]. We however report their effects on the spore proteome for the first time.

When introduction of fluorescent fusion proteins to an empty vector is compared, the additional effects on the spore proteome composition were minor. Whereas introduction of an empty plasmid caused a differential abundance in 1293 (916 of which were downregulated) amongst which many spore coat proteins. This may explain the observation that autofluorescence attributed to coat proteins in *B. subtilis* [40] was reduced in M001 compared to WT spores [12]. A limitation of our current study is that we cannot differentiate between plasmid introduction and antibiotic resistance marker effects. When interpreting differences in the spore proteome of the CotE deletion strain compared to WT a possible confounding factor is the antibiotic resistance marker of the deletion strain. Overall, our results indicate that an empty plasmid (carrying the same selection marker) is a more apt comparison than a WT sample. Furthermore, researchers should keep in mind that molecular cloning can influence more than only its target genes. Consequently, results should be interpreted with care, as the empty plasmid transformed spores were markedly different in protein composition compared to WT spores.

4.4. Materials and Methods

4.4.1. Strain and Culture Conditions

The wild type strain and mutant strains used in this study was *B. cereus* ATCC 14579. The details of bacterial culture and spore harvest were described previously [12, 26]. In brief, a single colony was inoculated and grown aerobically at 30 °C overnight in tryptic soy broth (TSB) with 275 µg/mL spectinomycin or 10 µg/mL erythromycin (Sigma-Aldrich Chemie B. V. The Netherlands). Then cells were spin down and cultured in a chemically defined growth and sporulation (CDGS) medium for 96 h. Spores were harvested by centrifugation and washing with 0.1% Tween-80 and cold Milli-Q water for at least four times.

4.4.2. Sample Preparation and LC-MS/MS Analysis

Samples of OD₆₀₀=2 spores were processed using the “One-Pot” method [41]. To improve identification depth, each WT sample was also separated into 10 fractions using ZIC-HILIC-based peptide fractionation [41]. Around 200 ng peptides for each sample were dissolved in 0.1% FA (formic acid) for mass spectrometry analysis. All samples had three biological replicates. Mass spectrometry analysis of all samples was performed as described previously [42].

4.4.3. Data Analysis

Raw MS/MS data was searched in Maxquant (version: 1.6.14.0) [43] against *B. cereus* ATCC 14579 data base downloaded from Uniprot, to estimate false spectrum assignment rate a reverse version of the same database was also searched. Settings were default for timsDDA. The Trypsin/P was selected as digestion enzyme with a maximum of 2 missed cleavages. The Oxidation (M) was set as variable modifications and Carbamidomethyl (C) as fixed modifications. The “Match between runs” was selected with a matching time window of 0.2 minutes and a matching ion mobility window of 0.05 indices. For label-free quantification, both iBAQ and LFQ were enabled.

Data analysis was performed in Perseus (version: 1.6.15.0) [44]. If not mentioned specifically otherwise, LFQ intensity was used for data analysis. Data was filtered based on valid values with “min 2” “in at least one group”. The second replicate of Sample M009 was removed, since it was found to be an outlier. The functions of proteins were categorized according to KEGG pathway. The LFQ intensity was used for a volcano plot using a T-test for determining significant changes of protein levels. Strain CotE and M001 were compared with WT and other mutant strains were compared with M001. Data was performed with z-scoring of rows, which correspond to protein expression levels over the different samples in the series. In Figure 4.4, iBAQ intensities were normalized by subtracting the median of protein expression levels per sample and z-scoring of rows as mentioned above

before examining the abundances of germinant receptor subunits, GerD and CotE.

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