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Chapter 3

Time-resolved proteomics of germinating spores of *Bacillus cereus*

Xiaowei Gao^{1,2}, Bhagyashree N. Swarge^{1,2}, Winfried Roseboom², Henk L. Dekker², Stanley Brul¹ and Gertjan Kramer²

¹ Molecular Biology & Microbial Food Safety, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands; x.gao@uva.nl (X.G.); b.n.swarge@uva.nl (B.N.S.); S.Brul@uva.nl (S.B.)

² Mass Spectrometry of Biomolecules, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands; w.roseboom@uva.nl (W.R.); h.l.dekker@uva.nl (H.L.D.); G.Kramer@uva.nl (G.K.)

Abstract

Bacillus cereus is a spore-forming human pathogen, which is a burden to the food chain. Dormant spores are highly resistant to harsh environmental conditions, but lose resistance after germination. In this study, we investigate the *B. cereus* spore proteome upon germination and outgrowth of the spores in order to get new insights into the molecular mechanisms involved. We used mass spectrometry combined with co-expression network analysis and obtained a unique global proteome view on germination and outgrowth processes of *B. cereus* spore by monitoring 2211 protein changeovers. We are the first to examine germination and outgrowth models of *B. cereus* spores experimentally by studying the dynamics of germinant receptors, other proteins involved in germination and coat and exosporium proteins. Further, by co-expression analysis, germination proteome data was clustered into eight modules (termed black, blue, brown, green, red, turquoise, grey and yellow), whose associated functions and expression profiles were investigated. Germination related proteins are clustered into module blue and brown, the abundances of which decreased after finishing germination. In modules brown and blue we identified 124 proteins that may be vital during germination. These proteins will be very interesting to study in future genetic studies into their function in spore revival in *B. cereus*.

3.1. Introduction

Germination is an orderly series of physiological and morphogenetic processes of spores initiated by activation of germinant receptors by small nutrients. Through this process spores transit from a dormant state to an active vegetative cell that in the case of *Bacillus cereus* may produce toxins. The stress resistant spores are formed when cells of Bacilli and Clostridia lack nutrients. The resistance of dormant spores to high temperature, uv, desiccation and various other environmental stresses is a result of their molecular structure. The structure of *B. cereus* spores, from inside to outside, consists of the spore core, inner membrane, the cortex, the outer membrane, coat and exosporium. Different from the most well studied spore forming organism *B. subtilis*, the outmost layer of *B. cereus* spores is the exosporium, a layer which is absent in *B. subtilis*. Moreover, the highly dehydrated core characterized by high levels of CaDPA and saturation of DNA with small acid soluble proteins (SASPs) is crucial to environmental stress resistance. The low permeable inner membrane and proteinaceous coat layers also contribute to the resistance properties of spores [1].

The tentative model of spore germination of Bacilli goes through two main stages (Figure 3.1a). In stage I, germination is triggered by stimulated germinant receptors. The water content in the spore core is raised gradually at the expense of monovalent cations and Ca-DPA release, transitioning the spore from phase bright to phase dark. Then spores enter into germination stage II. Two cortex-lytic enzymes CwlJ and SleB are activated to depolymerize the cortex, which allows spore expansion and rise of water content to the level found in vegetative cells. The cortex lytic enzyme CwlJ is activated by released DPA, while SleB is activated in an as yet unknown manner. The cortex hydrolysis pathway in *Bacillus* could be YpeB-SleB-CwlJ-GerQ, where YpeB is required for SleB localization and function and GerQ for CwlJ [2]. Full hydration of the spore core is required for metabolism, macromolecular biosynthesis and spore outgrowth. After outgrowth, spores eventually turn into actively growing vegetative cells [1]. Previously, the dynamics of protein and mRNA expression during germination was studied in *B. subtilis* [3]. Although the overall model for the germination process is thought to describe the process in *B. cereus*, this has never been studied experimentally. Specific high-quality data on *B. cereus* is still lacking and so differences and similarities remain to be studied in detail [1]. Of note, *B. cereus* is evolutionary speaking quite distinct from *B. subtilis* [4].

To get new insights into dynamic changes in the spore proteome during *B. cereus* spore germination and outgrowth, we conducted high temporal

resolution mass spectrometry analysis using label-free quantification. Our data show that the levels of germination associated proteins and morphological changes during germination and outgrowth of *B. cereus* spores were consistent with the current germination model of *Bacillus*. Network analysis provided a global view of protein level dynamic changes from 8 clustered modules. Furthermore, proteins that may have functions in germination were predicted by network analysis. From an applied point of view, our study reveals physiological processes during the awakening of dormant *B. cereus* spores and their return to vegetative life.

3.2. Results

3.2.1. Morphological and proteomic studies on spores during germination and outgrowth

To verify whether the model of germination is applicable to *B. cereus*, a series of samples were collected during spore germination and outgrowth. Firstly, the morphological changes of those samples were observed under the microscope and are shown in Figure 3.1b. It can be observed that spores were phase-bright before and after heat activation (PHA and AHA). After addition of germinants and transfer to germination medium (0 min), spores started germination immediately, transiting from phase bright to phase dark. The whole population of spores finished germination within 10 min. After 10 min, coming into the outgrowth stage, spores swelled gradually and grew out. After completion of germination and outgrowth, spores have revived back to the actively dividing vegetative stage and cell division was observed at 180 min.

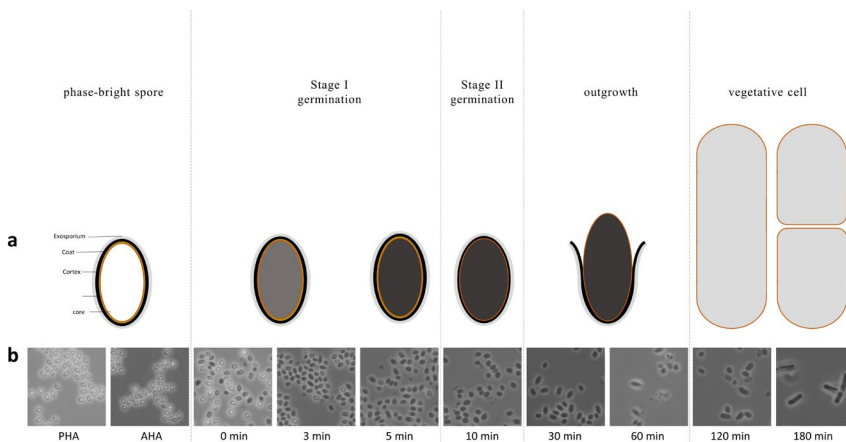


Figure 3.1. Scheme of spore germination and outgrowth (a). Morphological changes take

place during spore germination and outgrowth acquired by microscopy analysis (b). Spore samples were harvested at different time points throughout germination and outgrowth.

Next, we evaluated the spore germination and outgrowth of *B. cereus* at the protein level. For the proteomic study, in total 2211 proteins were identified and quantified. The reproducibility of samples was assessed by calculating Pearson correlation coefficients among three biological replicates at each time point. The pair-wise comparison of all the time points displayed high precision correlations over 0.8 (Figure 3.2A). The replicates collected between AHA to 60 min were relatively correlated. Moreover, the replicates collected during vegetative growth phase (120 -240 min) possessed high correlations. To investigate the proteome differences between samples, log₂ transformed LFQ intensity was used for principal component analysis (PCA) and hierarchical cluster analysis. As shown in Figure 3.2B, the first principal component clearly differentiated most samples along the time axis. It was noteworthy that sample PHA (Pre-heat activation) was distinctly separated from AHA (after heat activation) in the second principal component, indicating significant proteome changes induced during heat activation. This difference was also observed in the hierarchical cluster analysis (Figure 3.2C).

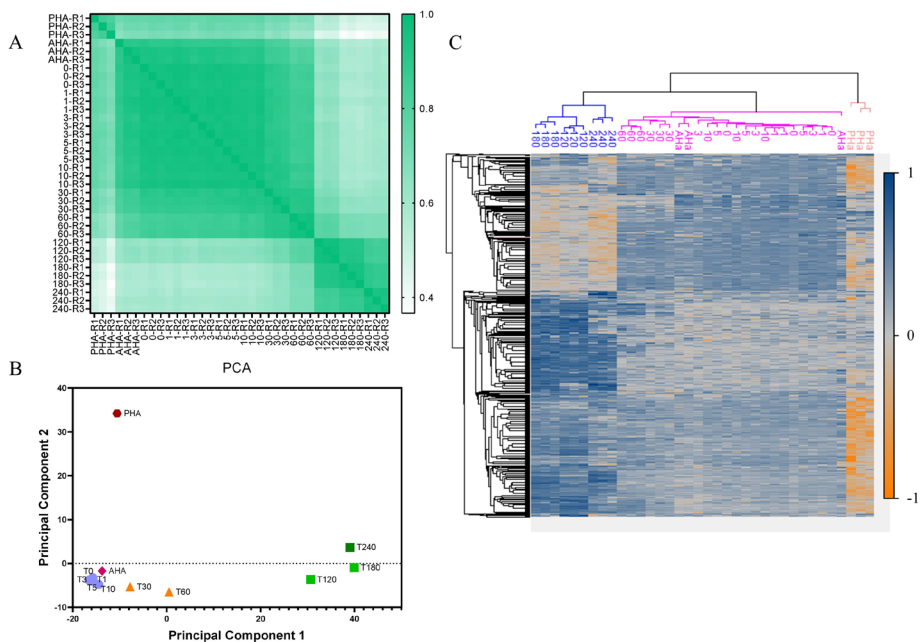


Figure 3.2. Comparison of the proteome at different time points during germination and outgrowth. (A) Pairwise Pearson correlations between all replicates. (B) PCA score plot of germination at different time points. (C) Cluster heatmap of proteome-

wide analysis. The color represents z-score transformed protein amounts of LFQ intensity. Color blue corresponds to an increased expression level; color grey to average level; color orange to a decreased level. columns and rows represent replicates at different time points and proteins, respectively. Time points are as follows: Pre-Heat activation (PHA), After Heat activation (AHA), 0 min (T0, addition of germinants), 1 min (T1), 3 min (T3), 5 min (T5), 10 min (T10), 30 min (T30), 60 min (T60), 120 min (T120), 180 min (T180), 240 min (T240).

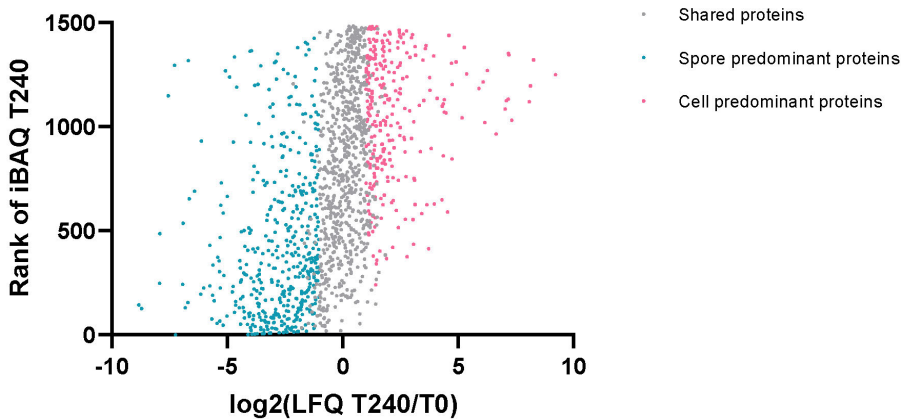


Figure 3.3. Scatter plot of proteome comparison of spore and vegetative cell. The Y-axis was the protein rank according to iBAQ intensity at T240 (vegetative cells). Proteins with higher iBAQ intensities at T240 ranked lower. The X-axis is the log₂ transformed LFQ intensity ratio of T240 and T0. The predominant proteins were determined by the limma algorithm.

The samples at each time point were compared with T0 and the significantly differentially expressed proteins (Supplementary Table S1) were calculated using the limma algorithm. According to the microscopy, spores eventually grow into vegetative cells and regained the ability of dividing from T180. As shown in Figure 3.3, comparison of the proteome at T240 and T0 revealed the differences between spores and vegetative cells. We found 457 proteins were spore predominant and 284 proteins were vegetative cell predominant proteins. 743 proteins were shared between spores and vegetative cells. Spore predominant proteins include spore specific proteins, such as spore coat protein and germination associated proteins. Vegetative cell predominant proteins are mainly involved in metabolism including energy, amino acid, lipid, nucleotide metabolism, and cell signal transduction and transportation. The abundances of 5 proteins were significantly increased at T10, when germination was just finished, 4 of which were associated with transcription. The levels of 130 proteins were significantly changed at T60, when spores finished outgrowth.

3.2.2. Dynamic changes of spore specific proteins

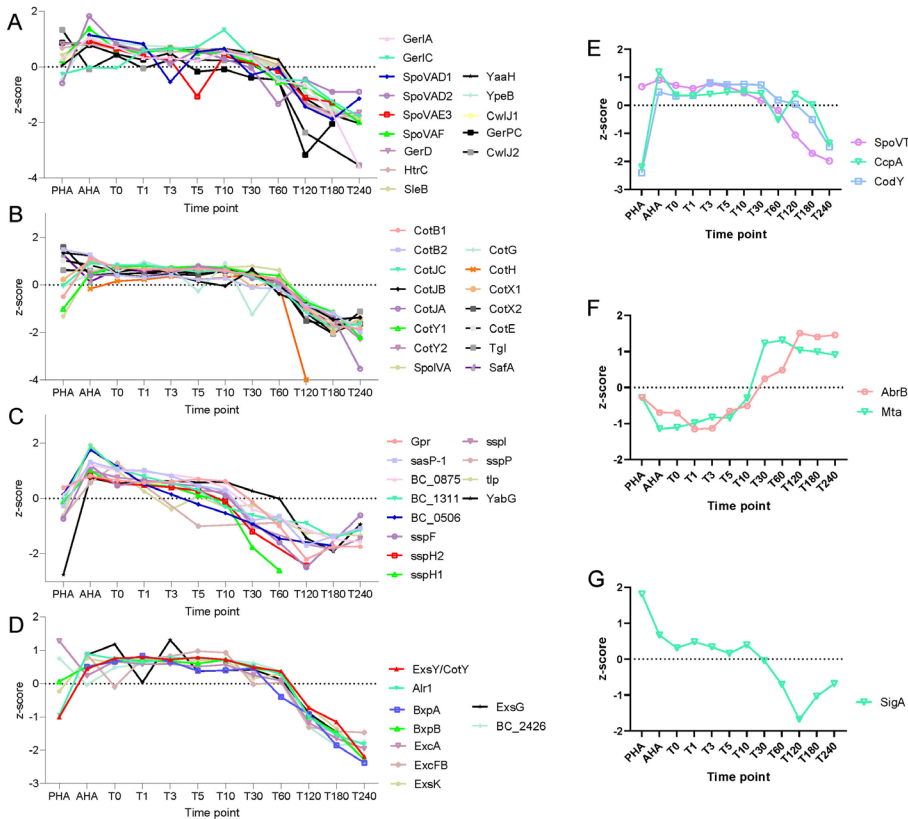


Figure 3.4. Dynamic changes of spore specific proteins. Z-score profiles of identified spore proteins across all the time points are shown. (A-D) Identified germination pathway proteins, coat proteins, SASP proteins and exosporium proteins. (E-G) The abundance variations of conserved regulators at different time points. The Y-axis displays z-scores transformed protein LFQ signal intensity and the X-axis shows time points.

According to the current model, germination requires the involvement of germinant receptors and channel proteins for signal transduction and degradation of SASPs, cortex and coat proteins. SASPs will be degraded during germination allowing DNA transcription and providing a pool of amino acids for initiation of metabolism. Spore coat proteins shed in outgrowth facilitating spore expansion. The dynamic changes of the spore specific proteins were quantified and are shown in Figure 3.4. The levels of all spore specific protein dropped during spore outgrowth (t=60-120 min), which were consistent with studies in *B. subtilis* [3] and *B. anthracis* [30] spore germination and outgrowth. Between T0 to T30, the intensities of proteins present in the germination pathway (Figure 3.4A) and coat layer

(Figure 3.4B) remained constant. Proteins characteristic to be part of the germination pathway (Figure 3.4A) declined gradually in quantity from 30 min onward, while coat proteins decreased from 60 min (Figure 3.4B). The GerIA level declined to an extremely low level at T240, but the intensity of GerIC dropped to a similar level with GerD, SpoVAD, SpoVAF. There are two paralogues of CwlJ. The level of CwlJ1 decreased to an extremely low level at T240, but the other one CwlJ2 remained relatively higher at 240 min. The intensity of CotH declined faster than other coat proteins during outgrowth (60-120 min). The abundances of SASPs gradually decreased from the onset of germination showing the biggest decline in outgrowth (10 min-30 min, Figure 3.4C). One noteworthy protein was a gamma-type SASP (BC0506) which saw a continued rapid decline from 0 min. The abundances of exosporium proteins showed decreases from T60, when spores were in the late stage of outgrowth.

By ortholog mapping to *B. subtilis*, highly conserved global regulators were quantified in *B. cereus*, including SigA, AbrB, CodY, CcpA and SpoVT [7]. Compared with the intensity at 0 min, the protein level of Mta significantly increased at 10 min just at the end of germination when the spore core was partly rehydrated (Figure 3.4F). The AbrB abundance raised dramatically between 10-30 min. The abundances of CcpA, SpoVT and CodY were trending downward from 30 min overall, even though there were oscillations of CcpA between 60 to 180 minutes (Figure 3.4E). The abundance of SigA continued to decrease until 120 min when it started to rise again (Figure 3.4G).

3.2.3. Co-expression Network construction

Proteomic data were further analyzed and visualized using weighted gene co-expression network analysis (WGCNA). WGCNA was developed in the field of systems biology to describe correlations among genes across microarray samples. The application of WGCNA analysis on proteomic datasets can be achieved by transforming proteomic datasets into a format similar to microarray and RNA-seq datasets after data normalization and imputation [5]. Proteins in a co-expressed module indicate close regulatory relationship, which can be used to infer unknown protein functions by their association with a cluster of well-characterized proteins [6].

In this study, we constructed a signed weighted co-expression network based on proteomics data to unravel protein dynamic patterns during spore germination and outgrowth and identify uncharacterized proteins behaving similar to well-known germination proteins inferred to have important functions during germination. Germination proteomics data were imputed

to avoid issues caused by missing values before network construction. Since it was observed that the heat activation caused tremendous impacts on the dormant spore proteome, the PHA and AHA samples were eliminated from network construction to remove the influence caused by heat activation on the module construction. To improve co-expression network analysis, a high-quality dataset of 1175 proteins identified in at least two replicates at each time point (except PHA and AHA) was used for further analysis. In total, 8 modules (clusters) were identified (Supplementary Table S2): “black” (41), “blue” (133), “brown” (131), “green” (87), “grey” (454), “red” (53), “turquoise” (153), “yellow” (123). Proteins that did not show any similarity to other modules were clustered into module “grey”. The protein correlation in the network was shown in a heatmap (Supplementary Figure S3). The correlation of each module was represented using an Eigenprotein, which is defined as the first principal component in one module and can be considered as a representative of the protein profiles in a module [8], and is shown in Supplementary Figure S2. To understand the relations between modules and spore germination and outgrowth processes, modules were correlated with time points, which is depicted in Supplementary Figure S4. All modules had weak coefficient of correlations with germination and early outgrowth processes (0-60 min). Module “blue” had a strong negative correlation with T240. Module “brown” was significantly negatively correlated with T180.

The changing trends of modules are shown in Figure 3.5. Proteins in module “brown” exhibited a quick decrease in intensity from 60 to 120 min and then remained constant. The protein level dynamics of module “blue” decreased gradually from 60 min. The abundances of proteins in module “green” slightly increased from 10 min when spores just finish germination and peaked at 120 min. The amounts of proteins in module “red” and “black” decreased from 60 min and showed an increase at 240 min. The protein level in module “turquoise” and “yellow” increased dramatically at 120 min. In module “turquoise”, the protein level kept the upward trend but module “yellow” stabilized from 120 min. To check whether modules were clustered with biological relevance, function enrichment analysis was performed for modules (Supplementary Table S3). Enrichment scores (FDR) suggested that modules were enriched with biological meaning.

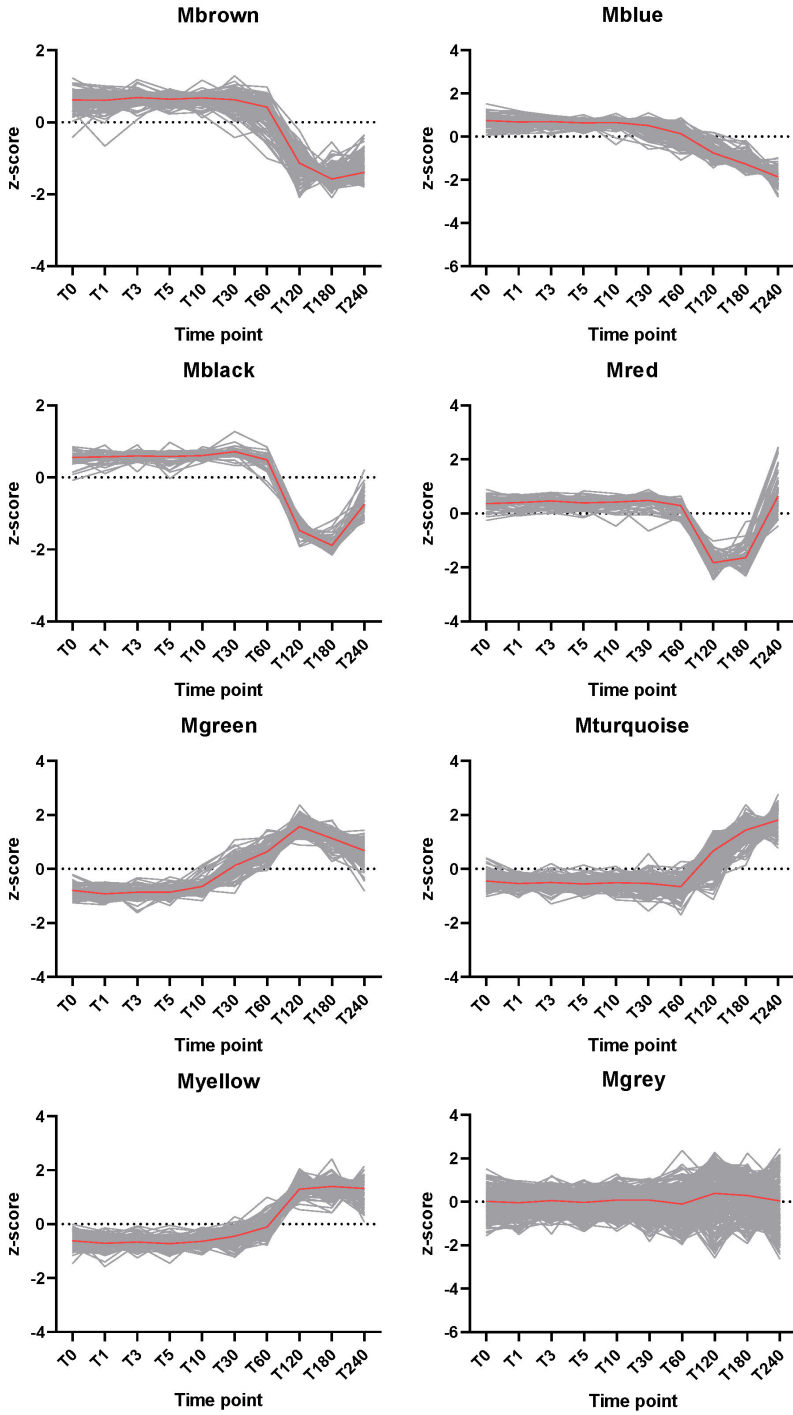


Figure 3.5. The trends in z-score transformed protein level of modules across time points. The grey lines indicate protein level changes. The red lines represent the median z-scores for each module.

We examined each module and found that proteins which are known to play important roles in germination and outgrowth were clustered into module “brown” and “blue” (Supplementary Table S4). As stated above, module “blue” was significantly negatively correlated with T240 and module “brown” was significantly negatively correlated with T180. To further screen for potential key proteins that account in module “brown” and “blue”, we calculated the correlations between proteins and T180 and T240 (gene significance (GS)), the correlations between proteins and module “brown” and “blue” (module membership (MM)). Proteins with high connectivity within a module (weight ≥ 0.3), correlations with T180 in module “brown” or with T240 in module “blue” ($GS \leq -0.5$) and correlations with modules (MM ≥ 0.8) were considered to be potential key proteins. In module “brown”, 59 proteins were sorted out to be potential key proteins and 65 in module “blue”. The top regulons among these key proteins were SigE, SigA, SigK and SigG.

3.3. Discussion

Under natural conditions, spores germinate, albeit heterogeneously, upon encountering nutrient molecules in the environment such as certain amino acids e.g., inosine, sugars and nucleosides. Heat activation is a sub-lethal short thermal treatment that enhances and synchronizes spore germination. As a tool to facilitate subsequent biochemical analysis of germinating and outgrowing spores, heat activation procedures are widely used in spore germination and outgrowth studies in a laboratory setting [9,10]. A previous study in *B. cereus* reported that L-Alanine did not induce significant germination without heat activation [11]. The temperature and heating time used in this study for activating *B. cereus* spores was adopted from previous study [32] to get activation effects on spores rather than causing detectable damages. However, we observed significant effects of heat activation on the dynamics of the proteome of *B. cereus* spores. The reasons for the differences in proteome with or without prior heat activation are still unknown. Such differences may have to do with changes in the cellular biophysical state [33] influencing protein structure and extractability. A reasonable assumption is that sub-lethal heat may change the structure of spores, i.e., their inner membrane or coat, which affects protein extractability and thus the spore proteome measured. Biophysical effects of spore heat activation, pertaining to the spore core state, were observed previously [12].

One aim of this study is directed towards a global view on the germination and outgrowth processes on the level of the proteome in *B. cereus* spores. The observations were consistent with the current germination model in *Bacillus* species. Although general morphological changes of *B. cereus* spores in germination and outgrowth followed similar steps to *B. subtilis* spores [3], *B. cereus* spores germinate much faster. In germination (T0-T10), the water content in the spore core increased gradually. The abundances of most proteins remain constant, which was different from the proteomic study during *B. subtilis* spore germination where obvious protein decreases of a number of proteins were detected upon germination initiation [3]. Nevertheless, the activities of protein synthesis in early germination were observed. The abundances of global regulators AbrB and Mta increased during the early phases of germination (T3-T10) and significant abundance changes were detected at 10 min. One SASP decreased during the whole germination and outgrowth phases. AbrB, as a key transition state regulator steering vegetative growth, has been studied in depth in *B. subtilis* [14]. The specific function of AbrB in *B. cereus* spore germination is unknown. Mta is a multidrug-efflux activator [15] and it may contribute to the resistances of the germinating spore to environmental killing factors. During early outgrowth (T10-T60), proteins in the germination signal transition pathway, such as cortex lytic enzyme CwlJ and channel protein SpoVA, started to degrade. Protein levels in module “green” were slightly increased, which are involved in translation, cell wall synthesis. During later outgrowth (T60-T180), the abundances of proteins in module “black” and “red” significantly increased, suggesting increased activity in protein synthesis and energy metabolism. The coat proteins were rapidly degraded during this period to make space for spore outgrowth. The noticeable degradation of CotH suggested that CotH may be a fundamental protein for the coat layer and degradation of it may be the key step in coat shedding. It is reported that the coat protein CotH is located in the inner coat as a morphogenetic factor mediating the assembly of at least 9 coat proteins [26,27,28]. In *B. subtilis* CotH is reported to play an assisting role in germination [13].

The main sigma factor, SigA, is active mostly for transcription of housekeeping genes in the exponential growth phase [16], the abundance of which decreased after spores were fully hydrated (T10-T120). Even though regulators are conserved, roles they play in the two organisms often are different. For example, SpoVT dramatically affects the completion of sporulation and deletion of *spoVT* causes premature germination of pre-spores in *B. cereus*, but is not essential in sporulation of *B. subtilis* [17]. Conversely, SpoVT is related to germination heterogeneity in *B. subtilis* [17].

Label-free quantification is widely applied in proteomics, it is widely applicable on varied sample types and allows for the comparison of large numbers of samples. At the same time label free quantification is economic and simple without extra steps of introducing stable isotopes in sample preparation. However, the label-free method cannot monitor new protein synthesis until abundance changes are detected. Further study on the onset of protein synthesis can be monitored by using a pulsed labeling method, such as dynamic SILAC (stable isotopic labeling of amino acids in cell culture). The SILAC incorporation experiments in *B. subtilis* reviving spores showed this method is sensitive to detect the onset of synthesis of low abundant proteins during spore germination [3].

Protein network analysis provides valuable data to study proteins with similar profiles based on a “guilt-by-association” approach [18]. The basis of this network analysis allows for studying protein behavior patterns, as well as the identification of novel protein functional interrelationships and correlations to germination and outgrowth stages. However, to reduce the influences caused by data quality, only proteins that were identified in every time points were used to generate the co-expression network. The proteins that were completely degraded or newly synthesized would not be analyzed using WGCNA in this way. The potential key proteins analysis based on network connections (weight ≥ 0.3) and GS and MM, lead to 59 proteins in module “brown” and 65 proteins in module “blue” with high possibilities to have putative roles in germination and outgrowth. It is worth noting that 17 proteins were uncharacterized proteins. The length of all uncharacterized proteins is longer than 100 amino acids, indicating none of them are likely to be SASPs. It will be very interesting to study the functions of these 17 proteins in spore germination and outgrowth in *B. cereus* spores in the future.

In summary, we successfully applied WGCNA on spore germination and outgrowth proteomic data. The analysis showed that there are a number of modules that clearly correlate with protein functionality during *B. cereus* spore germination and outgrowth. Future studies using genetic approaches where mutants in the identified genes with novel putative roles in germination and outgrowth will be interrogated are needed. Such approaches should provide more definitive functional data, allowing us to draw conclusions on the identification of functional protein modules that emerge during spore germination of the food borne toxigenic organism *B. cereus*. Such protein modules may be cause or consequence and hence follow or steer germination and outgrowth.

3.4. Materials and Methods

3.4.1. Culture Conditions

The strain *B. cereus* ATCC 14579 was used in this study. The details of spore culture and harvest were described previously [10, 19]. In brief, a single colony was inoculated in tryptic soy broth (TSB) and grown aerobically at 30 °C overnight. Then vegetative cells were collected by spinning down and cultured in a chemically defined growth and sporulation (CDGS) medium for 96 h to accomplish sporulation. Spores were harvested by centrifugation and washing with cold Milli-Q water for at least four times.

Fresh spores were heat activated at 70 °C for 15 min before germination. Around $OD_{600}=2$ (final concentration) spores were germinated in 100 ml YE medium at 30 °C with germinants. The final concentrations of germinants were 10 mM L-Alanine and 0.1 mM Inosine. The germination of *B. cereus* spores was a fast process which takes around 5-10 min. Samples were taken before and after heat activation and subsequently during germination and outgrowth as the various phases of spore revival (Figure 3.1) emerged. Overall it took spores over 2 h to outgrow into vegetative cells. Interval times were longer between later sampling time points. Summarizing, one ml of sample was taken at PHA (Pre-heat activation), AHA (After heat activation), 0 min, 3 min, 5 min, 10 min, 30 min, 60 min, 120 min, 180 min and 240 min. Samples were quenched with a final concentration of 20% cold methanol and kept in liquid nitrogen for short storage. Three biological replicates were obtained and stored at -80 °C. The YE medium [19] was composed of 0.00005% $FeSO_4 \cdot 7H_2O$, 0.0005% $CuSO_4 \cdot 5H_2O$, 0.0005% $ZnSO_4 \cdot 7H_2O$, 0.005% $MnSO_4 \cdot H_2O$, 0.02% $MgSO_4$, 0.008% $CaCl_2 \cdot 2H_2O$, 0.05% K_2HPO_4 , 0.2% $(NH_4)_2SO_4$, 0.2% yeast extract, 0.1% glucose. Stocks of all minerals were prepared individually and mixed before use.

3.4.2. Sample Preparation and LC-MS/MS Analysis

All samples were processed using the “one-pot” method as described previously [20]. The resulting peptides were resuspended in 0.1% formic acid and around 200 ng were injected into the Bruker tims-ToF. Mass spectrometry analysis of all samples was performed with tims-TOF pro (Bruker) coupled with an Ultimate 3000 RSLCnano UHPLC system (Thermo Scientific, Germeringen, Germany). Peptides were injected onto a 75 $\mu m \times$ 250 mm analytical column (C18, 1.6 μm particle size, Aurora, Ionopticks, Australia) and separated by a multi-step gradient of mixtures of solvent A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile): 55% solvent B for 55 min, 21% solvent B for 21 min, 31% solvent B for 12 min,

42.5% solvent B for 3408min and 99% solvent B for 7 min. The parameters of mass spectrometer were set to a spray voltage of 1.5 kV, a source gas flow of 3 L/min of pure nitrogen, a dry temperature setting of 180 °C, PASEF mode of acquisition, a scan range between 100-1700 m/z and a tims range of 0.6 - 1.6 V.s/cm². Fragmentation spectra was generated with 2 Th at 700 m/z and 3 Th at 800 m/z quad isolation width and the collision energy from 20 to 59 eV. A total of 10 PASEF MS/MS scans scheduled with a total cycle time of 1.16 s.

3.4.3. Data Analysis

Acquired LC-MS/MS data were processed with Maxquant (ver.1.6.14.0) [21] software and searched against *B. cereus* ATCC 14579 database downloaded from Uniprot (downloaded in September 2019). Trypsin/P was set as digestion enzyme. Maximum two missing cleavage were allowed. The carbamidomethylation (C) was set as fixed modification and oxidation (M) as variable modification. The option "Match between runs" was selected with a matching time window of 0.2 min and a matching ion mobility window of 0.05 indices. Both iBAQ and LFQ were used for label free quantification. Proteins identified in at least two replicates were considered to be valid. Then data was normalized and imputed in Perseus (version 1.6.15.0) [22] using LFQ intensity, as well as the analysis of PCA and cluster heatmap. The function enrichment was performed on DAVID (database for annotation, visualization and integrated discovery). For orthology analysis, OrthologueDB [24] and OMA browser [25] were used. Significantly differentially expressed proteins that were quantified in at least 24 of 36 samples were analyzed using R package limma [31]. Log₂ transformed LFQ abundances were fitted to a linear model and the standard errors are smoothed by empirical Bayes. Protein with a $|\log_2(\text{fold change})| \geq 1$ and adjusted p-value ≤ 0.05 were considered to be significantly expressed. The R package WGCNA was applied for network analysis [5,8]. Network was constructed based on a signed correlation. The parameter soft-threshold power β was carefully chosen to lead to an approximate ($R^2 \geq 0.9$) scale-free topology network. The WGCNA modules and eigenproteins (the first principal component of the modules) were identified and representative for the whole modules. Further, modules were correlated to the time points (traits) and those with p-value < 0.05 were considered significant.

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