Proteome dynamics during sporulation and heat resistance in Bacillus subtilis spores

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Chapter 6

General discussion

To form a metabolically dormant and stress resistant spore, a vast number of genes become active and their gene-products direct assembly of the spore during the process of sporulation. Sporulation is a heterogeneous event in a population, well understandable from an evolutionary and metabolic viewpoint. For experimental analysis using various 'omics' tools, heterogeneity in a population is, however, an important hurdle. In order to cope with this, we used a *kinA* inducible strain for time-resolved proteomic analysis. *KinA* is the starting point of the phosphorelay system that initiates the sporulation process culminating in Spo0A phosphorylation. We report on our studies in **Chapter 2**. Compared with wild-type spores, the spores obtained from *kinA*-induced cells showed a thicker coat and cortex layers and some enhanced thermal resistance. The spore proteome and germination behavior were also different from wild-type spores. These results corroborate the intricate link between spore formation and spore stress resistance mechanisms and alerted us to take this into account in our studies. To further unravel mechanisms central to the formation of *Bacillus* spores, we performed a detailed time-resolved proteomic analysis on synchronized sporulating cultures in **Chapter 3**. Through protein co-expression analysis, four co-expressed modules (i.e., modules brown, green, blue, and yellow) were revealed. Among them, modules brown and green are associated with sporulation, module blue is associated with ribosomal and metabolic proteins and module yellow is co-expressed with all other three modules. Remarkably, the levels of some of the coat proteins, for instance the morphogenetic coat proteins, decreased late in sporulation. At present it is unclear why these coat proteins needed to decrease, and no earlier study has reported such degradation of coat proteins in sporulation. A logical assumption is that these coat proteins could have played roles in guiding or helping assembly of other coat proteins, after which they became "surplus" and were degraded. Our study highlights the dynamics of protein expression during sporulation at high temporal resolution and illustrates its highly dynamic nature. In **Chapter 4**, we investigated the proteome of a high heat resistant strain, *Bacillus subtilis* A163. We found no SpoVM homologs present in *Bacillus subtilis* A163. Quantitative analysis revealed both high and low abundance of spore and cellular proteins in *Bacillus subtilis* A163 compared to those in a laboratory wild-type strain. These findings might be some of the basis of the high thermal

resistance of the *B. subtilis* A163 spores. In order to facilitate future time resolved proteomic analyses we described a novel sample preparation method in particular aimed at a higher recovery of membrane proteins. In **Chapter 5**, we successfully applied the single-pot, solid phase-enhanced sample-preparation (SP3) on a proteomic study of *B. subtilis* spores and cells. With SP3, more membrane proteins were qualitatively and quantitively identified.

In this concluding chapter, we discuss our results focusing on the implications of the data for spore thermal stress resistance and future perspectives derived from the results described in this thesis.

1. Mass spectrometry-based spore proteomics

Proteomics was defined as "the use of quantitative protein-level measurements of gene expression to characterize biological processes and decipher the mechanisms of gene expression control"[1]. The insolubility of many spore proteins makes the study of the spore proteome quite challenging. With the development of proteomics technologies, mass spectrometry-driven proteomics has opened new avenues to resolve the spore proteomes, including soluble and insoluble fractions[2–4]. To quantitively study the spore proteome, two quantitative proteomics strategies were employed in this thesis—^{15}N metabolic labelling (**Chapter 2 and 3**) and label-free quantification (**Chapter 4 and 5**). Quantitative data utilizing metabolic labelling are acquired by mixing a "heavy" sample grown in one condition with a "light" sample grown in another condition. After LC-MS analysis, relative protein abundance from two conditions can be calculated through isotopic peptide ratios (**Figure 1a**). Stable isotope-based labeling methods are the gold standard for quantification. However, ^{15}N metabolic labeling is limited to strains that can be easily grown in the laboratory. Label-free quantification is the simplest and most economical approach and is applicable to any kind of material (**Figure 1b**). With the latest development of accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction (MaxLFQ), label-free quantification with MaxLFQ has already achieved quantification accuracies similar to that of SILAC (stable isotope labeling by amino acids in cell culture)[5].

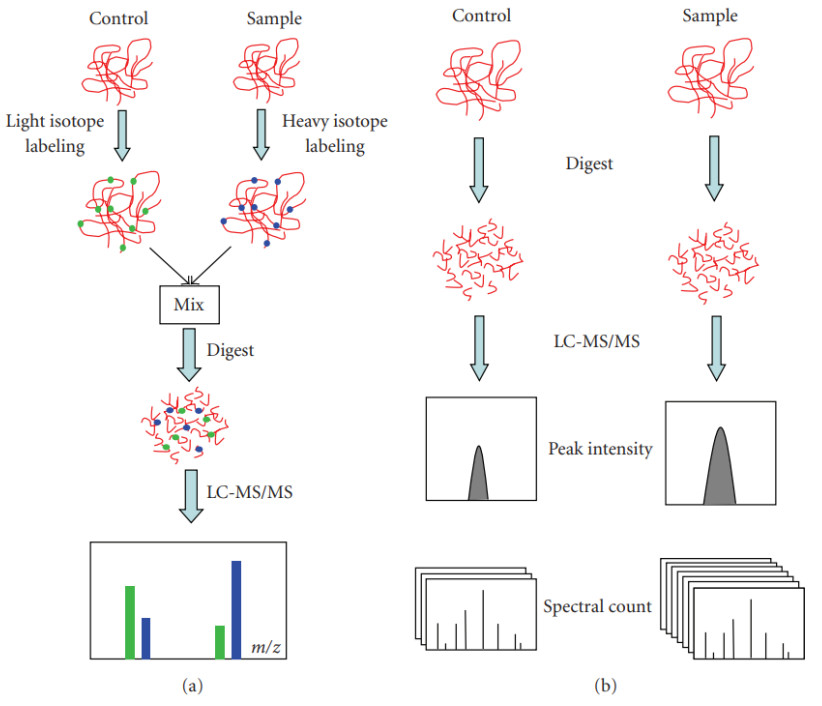

Figure 1. General approaches for quantitative proteomics. (a) Shotgun isotope labeling method. After labeling by light and heavy stable isotopes, the control and sample are combined and analyzed by LC-MS/MS. The quantification is calculated based on the intensity ratio of isotope-labeled peptide pairs. (b) Label-free quantitative proteomics. Control and sample are subject to individual LC-MS/MS analysis. Quantification is based on the comparison of peak intensity of the same peptide or the spectral count of the same protein. Adapted from[6].

2. Sporulation and spore resistance

To facilitate proteomic research on sporulation, a *kinA*-inducible strain was used. **Chapter 2** has addressed the influence of KinA induction on the spores produced. In doing this work we noticed that induction of sporulation via KinA in a nutrient rich-liquid medium (Luria Bertani broth i.e., LB) results in less heat resistant spores (**Figure 2**, unpublished data). This is in accordance with previous findings that culture conditions affect spores' thermal resistance, not only the temperatures and medium states (liquid or solid)[7–10], but also the richness of the medium. Still, many questions remain unanswered regarding the way in which

sporulating cells sense medium environments and respond accordingly. In vegetative cells, SigB dependent and independent-general stress proteins are induced by heat shock, salt and ethanol stress, and glucose or phosphate starvation[11]. The thermal resistance of the vegetative cells of *Clostridium perfringens* was increased two- to threefold when a sublethal heat shock was applied[12]. During sporulation, heat shock also increases spore heat resistance significantly[13,14]. A heat shock stimulates expression of heat shock proteins, but the heat shock induced proteins disappeared later in sporulation[14]. However, how the heat shock proteins influence the sporulation regulatory system is unknown. The time resolved proteomics shown in **Chapter 3** are designed to monitor the changeover of the spore proteome during sporulation. In the results from this experiment, not only were sporulation proteins identified, but also proteins of the general stress response regulon (SigB regulon) and stringent response, as well the proteins involved in metabolism and protein synthesis. With these data a protein co-expression network of sporulation was revealed. This will become the basis for future studies of sporulation regulatory mechanisms under different conditions, such as sporulation temperature and richness of the medium, and between different strains producing spores with high and low resistance.

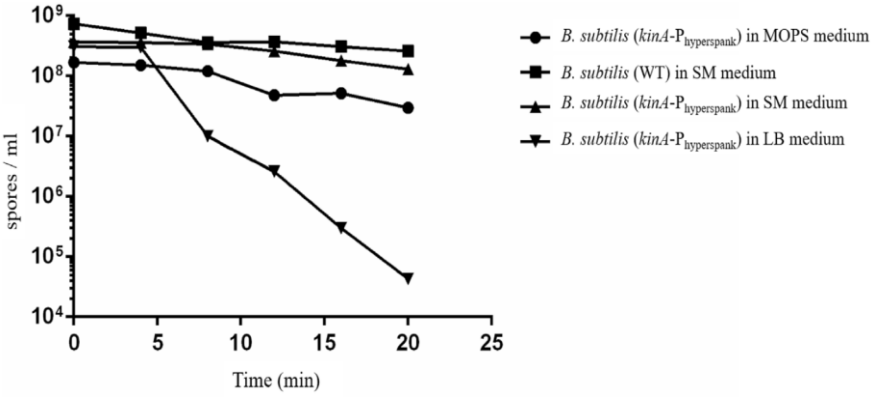

Figure 2. Wet heat resistance at 85°C of spores obtained from of *B. subtilis* (*kinA*-$P_{hyperspank}$) sporulated in different media. The wild type (WT) strain PY79 sporulated in SM (Sterlini and Mandelstam) medium is used as a control. The *kinA*-$P_{hyperspank}$ strain 1887 was obtained from[15]. Spore preparation,

purification and assessment of heat killing were carried out as described in[3,16,17].

The spore proteome of high heat resistant spores from *B. subtilis* A163 was extensively studied for the first time in **Chapter 4**. However, some germinant receptors (GRs) and several proteins of the SpoVA channel and *spoVA*^{2mob} operon were not identified. In **Chapter 5**, we described the application of the SP3 protein clean-up to increase the sensitivity of the proteomic analysis of bacterial spores. Since the SP3 protein clean-up showed advantages in the identification of membrane proteins, and GRs and SpoVA proteins are located in the spore inner membrane (IM)[18–20], SP3 could be promising for the identification of low abundance membrane proteins. Furthermore, a liquid handling robot for automated processing (autoSP3) of sample lysates in a 96-well format embedded with SP3 is capable of handling multiple samples at the same time[21], which should greatly facilitate time resolved analysis of spore protein synthesis upon sporulation of different strains under relevant environmental conditions.

In **chapter 2 and 4**, we described the varied spore proteomes in spores exhibiting very different thermal resistance. Abhyankar et al. has also reported the varied spore proteomes of spores prepared in a solid and a liquid medium, and spores prepared in the solid medium showed elevated thermal resistance[8]. Among the differentially presented proteins of spores in the three conditions (**Table 1**), protein YckD and proteins from the *opp* operon were always in a low abundance in the high thermal resistance spores, and coat proteins from the *cotJ* operon were in high abundance. This observation confirms the notion that variation in the spore thermal resistance involves remodulation of the spore molecular composition. However, how *B. subtilis* remodulates the spore proteome in different conditions is unknown, and how remodulation of the spore proteome leads to increased spore thermal resistance is also unknown.

Table 1. Differentially presented proteins in spores of different strains and sporulation conditions of *B. subtilis*[a]

UniProt IDs	Proteins	Abundance in spores of	Up- or down-regulation in	Up- or down-regulation in spores

		B. subtilis A163	spores of *kinA*-induced *B. subtilis* PY79	of *B. subtilis* PY79 sporulated in a solid medium
O34563	GlnH	Low	Downregulation	NA
P54466	YqfA	Low	NA	Downregulation
P24141	OppA	Low	Downregulation	Downregulation
P24139	OppC	Low	Downregulation	NA
P24136	OppD	Low	Downregulation	NA
P24137	OppF	Low	Downregulation	NA
O32076	YuaG	Low	Downregulation	NA
P42089	CgeA	Low	Downregulation	NA
P42402	YckD	Low	Downregulation	Downregulation
P37558	YabP	Low	NA	Downregulation
P39801	CotG	NA	Downregulation	Downregulation
P96619	YdcC	NA	Downregulation	Downregulation
O06010	YraD	Low	Upregulation	NA
Q45535	CotH	High	NA	Downregulation
P46915	CotSA	High	NA	Downregulation
O31802	CotU	NA	Upregulation	Downregulation
P07790	CotC	NA	Upregulation	Downregulation
Q45536	CotJA	NA	Upregulation	Upregulation
Q45537	CotJB	NA	Upregulation	Upregulation
Q45538	CotJC	High	Upregulation	Upregulation
Q7WY67	GerT	High	Upregulation	NA
O34413	YtcC	High	Upregulation	NA
P39668	YyxA	High	NA	Upregulation

a, adapted from chapter 2 and 4, and[8]. NA, not identified or not differentially presented. High or low abundance (upregulation or downregulation) of proteins indicates protein amounts in the high thermal resistance spores are at least twofold or less than half of the protein amounts in the spores of low thermal resistance, respectively. Proteins differentially present in at least two conditions are shown.

3. Future perspectives

Resistant spores are formed in sporulation. We have shown in Chapter 2 that *kinA* induction alters spores' proteome. A question could be asked—does

induction of other kinases also cause the same effect on spores? In the phosphorelay system controlling sporulation, there are in total five kinases with KinA being the most active[22,23]. Sporulation is decreased to almost zero in a strain mutated both in *kinA* and *kinB*[24]. In an attempt to artificially induce sporulation, *kinA*, *kinB* or *kinC* can trigger sporulation, but *kinD* or *kinE* cannot (**Figure 3**). Since these different kinases are thought to play roles in response to different environmental signals, it may be worthwhile to investigate the effect of induction of sporulation by different kinases on the spore proteome and properties.

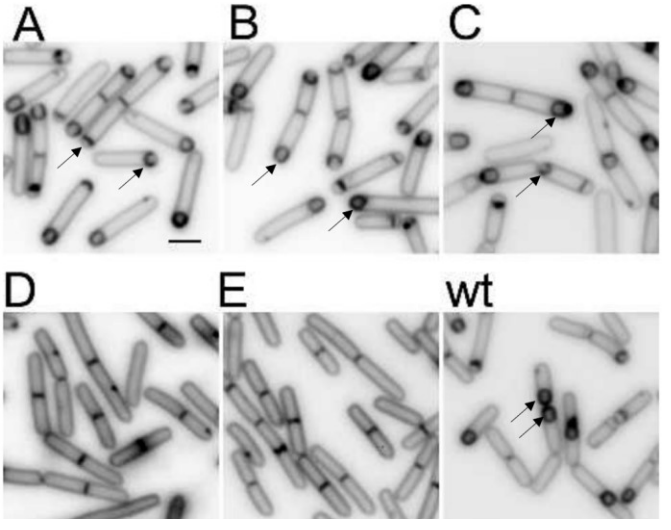

Figure 3. Induced synthesis of KinA, KinB, or KinC, but not of KinD or KinE, triggers sporulation. Cells of strain MF1887 (A, *Phy-spank*-*kinA*), MF1888 (B, *Phy-spank*-*kinB*), MF1889 (C, *Phy-spank*-*kinC*), MF2147 (D, *Phy-spank*-*kinD*), MF2148 (E, *Phy-spank*-*kinE*), and PY79 (wt) were induced to sporulate in CH medium (a rich medium for which casein hydrolysate is the sole carbon source) followed by IPTG addition or in SM medium [PY79 (wt)]. Cells were treated with the vital membrane stain FM4-64 at hour 3 post-induction and observed by fluorescence microscopy. Forespores were visualized within cells in A, B, C, and wt (pointed by arrows). Bar, 2 µm. Adapted from[25].

As indicated above, the spores' thermal stress varies and is highly dependent on sporulation conditions. However, how the sporulation regulatory system responds to varying conditions is not known. One possible technical

direction to investigate sporulation regulatory mechanisms is the time resolved proteomic analysis of sporulation of the cells sporulated under different conditions, such as different temperatures or in nutrient poor and rich medium, as well as under other stresses. For the high heat resistant spores from *B. subtilis* strain A163, a combination of purification of the spore IM and then processing the spore IM fraction using SP3 could improve the identification of important IM spore proteins. More research is also needed with respect to the encasement of spore coat layers in *B. subtilis* A163, as SpoVM is crucial to proper formation of the coat in *B. subtilis* PY79[26,27], but no homologs of SpoVM were found in *B. subtilis* A163 (**Chapter 4**).

In conclusion, the details regarding the proteome dynamics of sporulation and the high resistance of spores from strain *B. subtilis* A163, as well as the successful application of the SP3 protein clean-up in the study of the bacterial spore proteome, as described in this thesis, have provided novel insights in the spore proteome and sporulation, and offer opportunities for future studies to further understand the molecular physiology of high thermal resistance of bacterial spores.

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