Proteome dynamics during sporulation and heat resistance in Bacillus subtilis spores

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Chapter 1

General introduction

Bacterial endospores (commonly referred to as spores) are the metabolically dormant forms of the spore formers, discovered by Cohn and Koch in the late 19th century[1,2]. Their inhabitable environment includes, but is not limited to, soil, water and the gastrointestinal tract of animals and humans[3,4]. Species from the genera *Bacillus* and *Clostridium* can form spores when the environment is unfavorable, and the spores can survive under harsh environmental conditions, such as, heat, desiccation, radiation and chemical insult[1,5,6]. Due to their extreme resistance properties, contamination of bacterial spore formers of some species has become one of the challenges in industrial processing and their long-term persistence in and on food and medical products[7,8]. What's more, upon ingestion of the spores of some species, spore germination and outgrowth into vegetative cells can cause vomiting or diarrhea due to the action of toxins produced by growing cells[9–11]. In addition, spores of *Bacillus anthracis* have long been considered a potential bioterrorism agent[12]. On the bright side, spores' extreme resistance increases interest in using spores as a delivery vehicle for vaccines as well as growing probiotic bacteria[13–15]. Among the Bacillales, the model organism *Bacillus subtilis* is the most studied gram-positive bacterium and its complete genome sequence was reported in 1997[16]. *B. subtilis* is non-pathogenic, and cells are rod-shaped. The formed spores are phase bright, oval, 1.07 ± 0.09 μm long and 0.48 ± 0.03 μm in diameter[17]. In this chapter, general information about spore structure, resistance, germination, and formation (sporulation) will be introduced. Notably, general knowledge about bacterial spores is mostly based on studies of *B. subtilis*, unless noted otherwise.

1. Spore structure and resistance

The basic substructures of spores were first described by Koch[18]. Research on the resistance of bacterial spores to different conditions have identified a number of factors determining the resistance properties[19]. The spore core is surrounded by multiple layers (**Figure 1**) and is where DNA is saturated by the protective α/β-type small, acid-soluble proteins (SASPs). The core has a low level of water, 28—57% of wet weight with ⩾80% in a growing cell[20,21], and a high level of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) chelated with various divalent cations, mostly Ca^{2+}. The unique features of the

spore core contribute to spore resistance to desiccation, dry and wet heat, UV and γ-radiation and genotoxic chemicals. The spore also has enzymes to repair DNA damage caused by environmental stresses, when spores germinate and grow out[22–24]. Outside the core, there are two membranes, the inner membrane (IM) surrounds the spore core. Its low permeability to small molecules, even water, protects the spore from DNA-damage caused by biocidal chemicals[25]. Also, the IM is considered to play an important role in spore germination. The roles of the outer membrane in spores is to tether spore coat layers through SpoVM and SpoIVA, and may be involved in spore UV resistance as it contains pigments that can absorb UV[26,27]. The peptidoglycan (PG) cortex and the thinner germ cell wall are located between the two forespore membranes, with the germ cell wall outside the IM and will become the cell wall after spore germination. Proper formation of the cortex layers is necessary for dehydration of the spore core and essential for spore resistance properties[28,29]. Multiprotein protective layers, called the coat, are outside the outer membrane including the basement layer, inner coat, outer coat and crust (**Figure 1A, B**). In some species, such as the pathogenic bacteria *B. anthracis, B. thuringensis* and *B. cereus*, an additional layer called exosporium is loosely attached the outside of spores **(Figure 1C)**. Both coats and exosporium contribute to the spore resistance to extreme physical and chemical stresses and ingestion by bacteriovores[30–32]. Besides, the presence of exosporium provides a hydrophobic surface that may be involved in spores' adhesion to the host and other organisms[33,34]. The coat is mainly made of proteins. More than 70 proteins have been identified in the coat layers[30]. About 30% of the coat proteins are insoluble and highly cross-linked, contributing to the spore resistance[35–37]. Among the coat proteins, SpoIVA, SafA, CotE and CotX/Y/Z are morphogenetic proteins for the various coat layers (**Figure 2**). SpoIVA surrounds the outer membrane, anchoring the coat layers to the spore[27]. Mutation of *spoIVA*, as well as *spoVM* and *spoVID*, will result in the improper formation of coat layers[27,38,39]. Different from *spoIVA* mutation, either *spoVM* or *spoVID* mutants can still localize coat proteins to the spore surface but fail to surround the spore. SafA and CotE are necessary proteins for the formation of the inner and outer coats, separately[40,41], and the crust is absent when *cotX/Y/Z* are deleted individually or in combination[42,43].

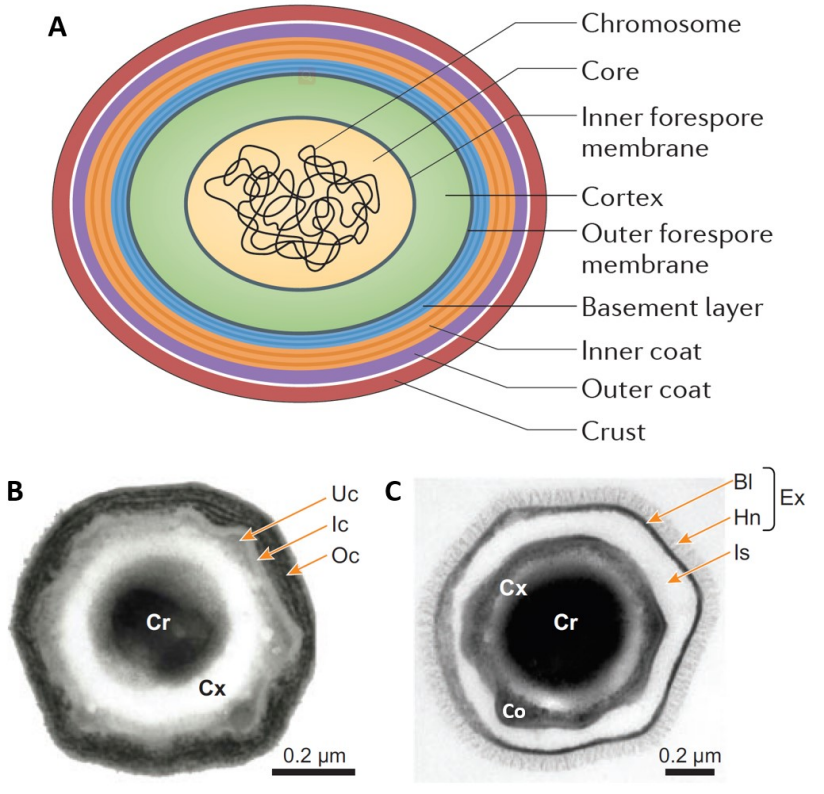

Figure 1. Cartoon of a typical *B. subtilis* spore (A), and electron micrographs of cross-sections of spores of *B. subtilis* (B) and *B. anthracis* (C). The following spore compartments or structures are normally recognized in electron micrographs of spores of *B. subtilis*: the spore core (Cr), the cortex PG layer (Cx), the undercoat region (Uc), the inner (Ic) and the outer (Oc) coat layers. In addition to these spore structures, the spore in panel **C** shows an exosporium (Ex) formed by a basal layer (Bl) and a hair-like glycoprotein nap (Hn). The exosporium is separated from the electrodense coat (Co) by a region called the interspace (Is). Adapted from[30,44].

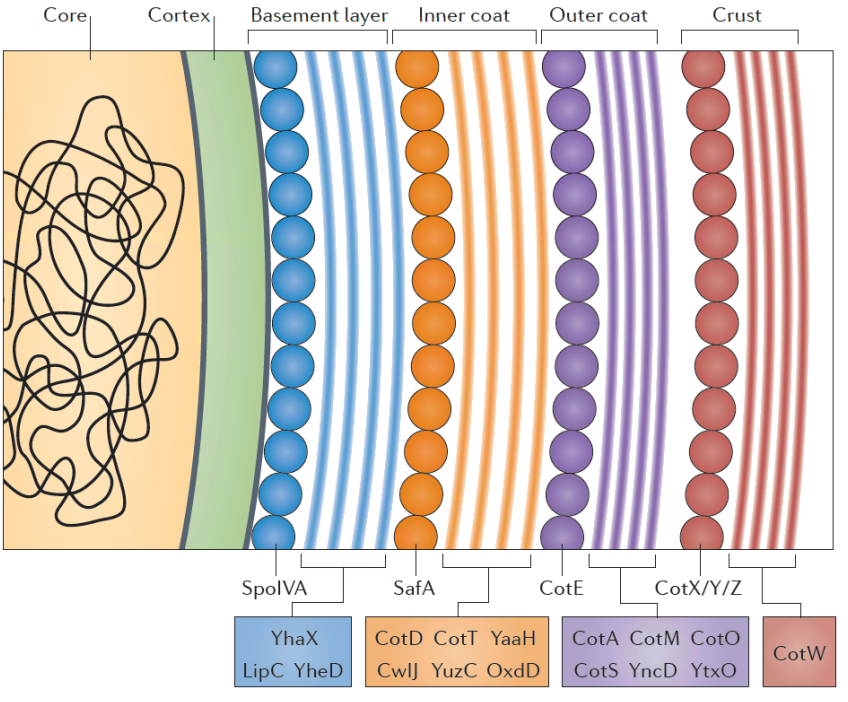

Figure 2. Morphogenetic proteins and representative coat proteins of *B. subtilis* spores. Polymerization of morphogenetic proteins, SpoIVA, SafA, CotE and CotX/Y/Z, creates the necessary binding sites for each of the individual coat proteins that make up each layer. Adapted from[44].

2. Spore germination and outgrowth

Spores can remain resistant and metabolically dormant until nutrients become available again. Ions and nutrients, including sugars and amino acids, will traverse the coat and cortex layers to reach the germinant receptors (GRs) located in the IM to trigger spore germination followed by outgrowth[45]. Spore's IM contains a set of GR proteins, with different GRs involved in sensing different nutrient molecules[45,46]. In contrast, in spores of *C. difficile*, a coat protein, CspC, senses bile salts which trigger germination[47]. Additionally, spore germination can often be triggered by cationic surfactants, high hydrostatic pressure and exogenous CaDPA[45,48,49]. Upon commitment to germinate following germinant sensing, CaDPA is released through an IM channel composed of SpoVA proteins[45,46,50]. In *Bacillus* spores, CaDPA release triggers the hydrolysis of the

cortex layer by CwlJ located in spore coat and/or cortex and SleB from the outer surface of the IM[51–53]. Following cortex hydrolysis, the spore core takes up water and remodels the IM and the germ cell wall PG. During this period the brightness of the spore in phase-contrast microscopy decreases drastically (**Figure 3)**, and spore core proteins and lipids in the IM become mobile[45]. Due to core rehydration, release of CaDPA and degradation of SASPs, most resistance properties of spores are lost. During this period, metabolism within the spore gradually becomes active, and finally, germinated spores enter the process of outgrowth[46,54]. Spores slowly increase their volume until the cell bursts out of the coat[55]. Germination of spores in a population is heterogenous[56]. Multiple factors contribute to this heterogeneity, including the amounts of GRs, sporulation conditions, etc.[57–59].

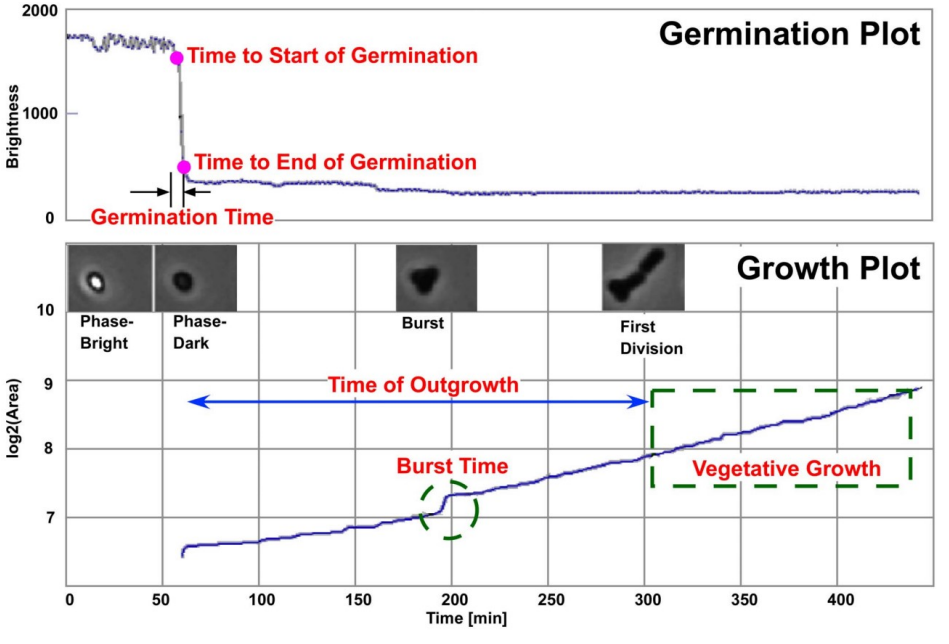

Figure 3. Plots of spore germination and outgrowth of a *Bacillus subtilis* spore analyzed with SporeTracker. Above: Phase-bright to phase-dark transition, marked with a small circle at 90% (start of germination) and 10% (end of germination) of the entire (pixel) intensity drop range (brightness). Below: various snapshots at different stages of germination and outgrowth. The burst of the cell out of the spore coat is accompanied by a relative short and significant increase in volume (marked by the green circle). Adapted

from[55].

3. Sporulation cycle

Vegetative *B. subtilis* can initiate a process to form spores, called sporulation, under unfavorable conditions, for instance nutrient limitation. Several morphological changes take place during sporulation (**Figure 4**). The process starts with an asymmetric division close to one pole of the cell, either the old pole or the newly formed pole in cell replication[60]. A smaller compartment, the forespore, and a larger compartment, the mother cell, are generated. The forespore is then engulfed by the mother cell, giving a sporangium with the engulfed forespore which is the developing spore (**Figure 5a**). Assembly of different spore layers, core dehydration and CaDPA uptake are completed late in sporulation. At the end of sporulation, the spore is released from the sporangium by the lysis of the mother cell. The generated spores may remain dormant and resistant for hundreds, maybe thousands of years[61,62], and are capable of terminating dormancy by quickly germinating and resuming vegetative growth, as described above.

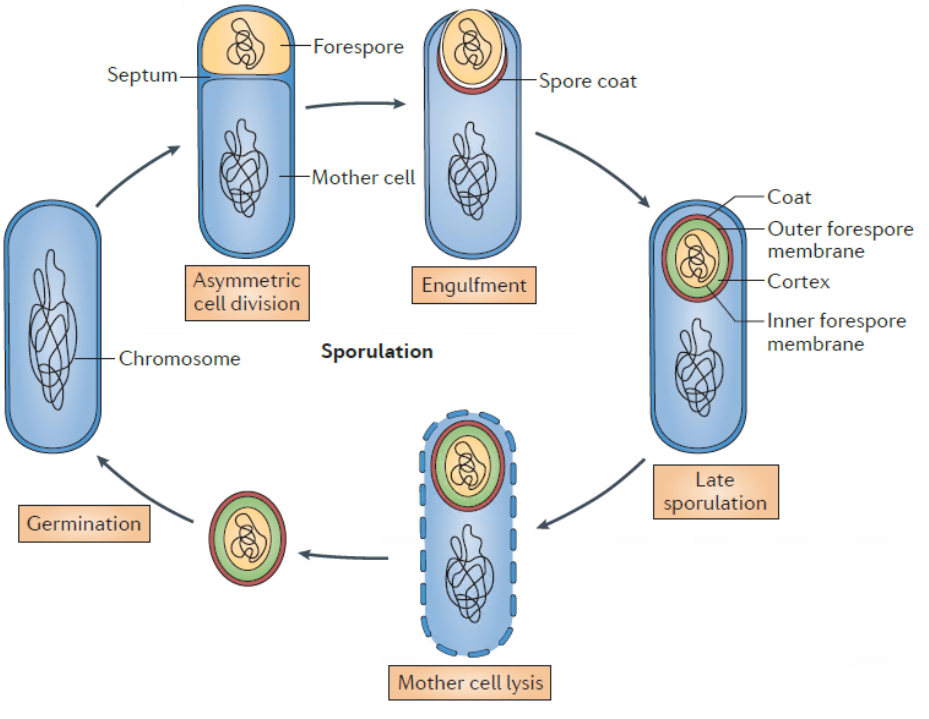

Figure 4. the sporulation and germination cycle in *Bacillus subtilis*, adapted from[44].

3.1. Mother cell line of sporulation

Coat and cortex assembly is mainly accomplished in the mother cell compartment[30,63,64]. Upon completion of asymmetric division, the morphogenetic protein SpoIVA is expressed in the mother cell, and localized near the mother cell side of the septum[65,66]. Following the engulfment stage, SpoIVA surrounds the forespore and localizes outside of the outer membrane, with help of SpoVM[27,66,67]. Concomitantly, SpoVID, SafA and CotE are assembled directly or indirectly associated with SpoIVA. CotE is ~75 nm away from the outer membrane[65]. The region delimited by SpoIVA and CotE forms a scaffold of the coat layers, called the matrix or precoat[65,68]. Subsequently, other structural and functional coat proteins are recruited and assembled into the precoat forming the inner and outer coat layers and the crust. However, the precise composition of the precoat, as well as the mechanism of coat assembly is not completely known[44,69,70]. After mother cell lysis, a final step of coat formation takes place, as some proteins are cross-linked into high-molecular-mass forms, mediated by coat proteins such as Tgl and YabG[71,72]. For the formation of cortex, the PG precursors and related proteins are synthesized in the mother cell, but the assembly mechanism is by no means completely understood[29,64].

3.2. Forespore line of sporulation

The pH of the spore core is ~6.5, which is lower than that of vegetative cells[73]. In sporulation, the pH in the forespore decreases at ~ the 4th hour of sporulation, while the pH in the mother cell remains constant, and similar to that in the vegetative cell[74,75]. The low pH in forespores may be involved in regulating activities of forespore enzymes[75]. The inner PG layer, the germ cell wall, is just outside the inner forespore membrane, and this PG's structure is more similar to the vegetative cell wall PG than cortex PG. In contrast to the synthesis of cortex PG in the mother cell, the germ cell wall PG is synthesized in the forespore, and prior to the synthesis of cortex PG[76,77]. The dehydration of the core is achieved in late sporulation coordinately with CaDPA uptake from the mother cell. How the water level in the core is decreased, and how the

permeability of the inner membrane decreases are not fully clear, but CaDPA uptake is via an IM SpoVA channel[78,79]. Expression of the SpoVA channel proteins, GRs and SASPs are carried out within the forespore[45,79,80].

4. Transcriptional regulatory system of sporulation

Sporulation initiation is governed by the master sporulation transcriptional regulator SpoA[81,82]. A large group of genes is controlled by Spo0A, including those involved in asymmetric division and early sporulation[82,83]. One important protein in formation of the asymmetric septum is FtsZ[84,85]. It assembles at polar positions on two sides of the cell and forms two FrsZ rings. One of the two rings develops into an asymmetric septum. Upon completion of asymmetric division, forespore and mother cell compartment-specific sigma factors direct the sporulation program, including two forespore sigma factors, SigF and SigG, and two mother cell sigma factors, SigE and SigK (**Figure 5b**). SigF is first activated after asymmetric division, and controls about 50 genes[80,86]. Its precursor, SpoIIAC, is expressed under control of Spo0A and expressed before asymmetric division. Activation of SpoIIAC involves SpoIIE, SpoIIAA and SpoIIAB, as well as genetic asymmetry between the forespore and mother cell[87,88]. Similar to SigF, the SigE precursor is also under the control of Spo0A and expressed prior to asymmetric division, but is only activated shortly after the completion of asymmetric division and directs expression of genes in the mother cell, including those for engulfment[89,90]. Like SigE and SigF, inactive SigG and SigK are expressed in forespore and mother cell, respectively, before the completion of engulfment and becomes active after engulfment.

Intercellular signaling between forespore and mother cell are essential for the subsequent activation of SigE, SigG and SigK (**Figure 5c**). Among the SigF controlled proteins, SpoIIR is expressed within the forespore and then secreted into the intermembrane space of the asymmetric septum, where mother cell protein SpoIIGA is activated by association with SpoIIR[91]. Then the activated SpoIIGA converts the SigE precursor into an active form[92], and *sigG* transcription is controlled by SigF and SigG itself. The activation mechanism of SigG is not fully clear, but involves the feeding tube connecting forespore and mother cell, and contains forespore protein SpoIIQ, SpoIIAA, mother cell SpoIIIA proteins and the

completion of engulfment[93–96]. In recent, a study shows that a hairpin structure in the *sigG* mRNA has effects on the expression of SigG and further affects proper SigG activation[97]. Expression of the SigK precursor is dependent on both SigE and SpoIIID[98]. SpoIIID is one of the SigE regulated proteins which includes around 270 genes[86,99]. SigK directs expression of around 150 genes, including another mother cell-specific transcription factor, GerE[86,99]. SpoIIID and GerE can act as either a repressor or an activator to regulate gene transcription in the mother cell. Activation of SigK is dependent on a protease SpoIVFB, which is located at the intermembrane space between the forespore and mother cell[100]. The protease activity of SpoIVFB is held inactive by forming a complex with SpoIVFA and BofA. The SigG controlled forespore protein SpoIVB is capable of reacting with SpoIVFA to convert inactive SpoIVFB into an active form[101].

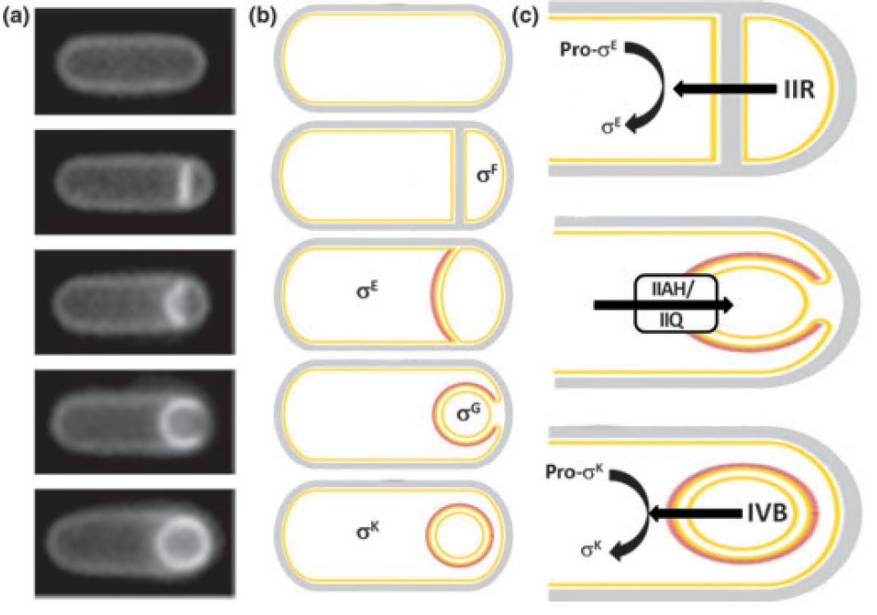

Figure 5. Transcriptional regulators during sporulation. (a) Asymmetric division and engulfment. (b) Mother cell and forespore-specific sigma factors. (c) Intercellular signaling cascade between mother cell and forespore. Adapted from[69].

5. Phosphorelay heterogeneity

In a population of vegetative growing cells, sporulation takes place

heterogeneously. This could be a bet-hedging strategy to allow the population to confirm the necessity of sporulation before entering this time and energy consuming process[60]. Initiation of sporulation is governed by the DNA-binding protein Spo0A. It is not a sigma factor but a regulator showing distinct regulatory behaviors between the high and low phosphorylated forms (Spo0A~P)[82,102] (**Figure 6A**). Around 500 genes are influenced by Spo0A~P in the early stages of sporulation and ~120 genes are directly affected by the low and high levels of Spo0A~P. Spo0A is constantly expressed during vegetative growth and stays at a low level of protein abundance and a low phosphorylated state. Progressive increases in Spo0A~P to a high level then result in the initiation of sporulation[103]. The sporulation program has been introduced in detail (see above). Phosphorylation of Spo0A is governed by the phosphorelay, a two component system[104] (**Figure 6B**). The phosphorelay comprises multiple components besides Spo0A, including histidine kinases (KinA-E), and two phosphotransferases Spo0F and Spo0B. The histidine kinases respond to environmental signals driven by starvation and phosphorylate themselves. Then the phosphoryl groups are transferred to Spo0A via phosphotransferases Spo0F and Spo0B. Increased Spo0A~P will activate a number of feedback loops to re-enforce the expression of KinA, Spo0F and Spo0A[69,105]. Finally, the high Spo0A~P triggers sporulation and then asymmetric division. One of the mechanisms making sporulation heterogenous is the highly dynamic expression of the phosphorelay genes between individual cells within the population[106]. Artificial induction of KinA-C can result in a more homogenous sporulation than wild-type sporulation[103,106].

Another source of sporulation heterogeneity is related to the high sensitivity or bi-stability of the decision to sporulate[107]. This is notable, in particular, concerning the need for high Spo0A~P threshold levels to trigger sporulation. Consequently, a minor change in Spo0A~P level at around the threshold will determine the fate of cell differentiation—vegetative division or asymmetric division, even though there are still discussions on the origin of this high sensitivity[107]. Fluctuation of the phosphate flux through the relay alters the phosphorylation rate of Spo0A and therefore affects the timing to reach the threshold of sporulation[108]. Additionally, phosphorylation of Spo0A is

coordinated with the completion of DNA replication (**Figure 6A**) and always happens after DNA replication in each cell replication cycle[109,110]. This is due to the unique chromosomal arrangement of *kinA* and *spo0F*. On the genome of *B. subtilis*, *kinA* is close to the terminus of DNA replication and *spo0F* is close to the origin. A transient high amount of *spo0F* is generated during DNA replication which represses the Spo0A phosphorylation via a negative-feedback loop, and releases the inhibition after the completion of DNA replication[109]. In this way, pulsed phosphorylation of Spo0A is formed in a continuous replicating cell under starvation[111]. Moreover, the pulsed Spo0A phosphorylation is indispensable for the successful entering of sporulation[109]. Along with the cell population growth, cells will gradually slow their growth rate and take more time to complete a cell cycle. The prolonged replication time lengthens the period between DNA replication events and further allows sporulating cell accumulation of Spo0A~P. Finally, the growth rate is slow enough to allow cells to accumulate Spo0A~P to the threshold needed for sporulation[112]. Cell growth rates in a population may differ between individuals and generations needed to trigger sporulation could also be highly dynamic or heterogenous[112].

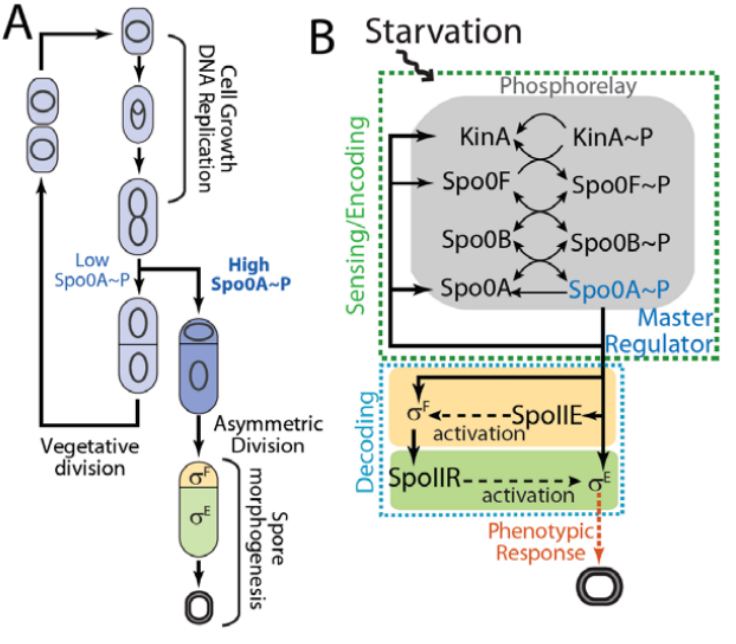

Figure 6. A high-level of Spo0A~P triggers the initiation of sporulation. A.

Schematic overview of the cell fate decision between vegetative division and sporulation made by *B. subtilis* cells during starvation. B. Diagram of the sporulation network that senses starvation and controls the cell-fate decision. Adapted from[107].

6. Outline of the thesis

Bacillus subtilis is the most well-studied spore former, however a lot remains unknown about the relationship between spore protein composition and spore high heat resistance. To that end we set out on a proteomic characterization of spores, first in the wild-type aiming at a detailed time-resolved analysis of spore protein biogenesis in sporulation. To do so, heterogeneity in sporulation induction was addressed and spore formation was artificially induced in a homogeneous manner. The resulting spore protein co-expression network was characterized. Next, spore proteomics analysis was performed on spores obtained from a foodborne isolate characterized by the formation of high heat resistant spores. Finally, the thesis describes the application of a novel protein extraction method that allows one to use SDS in proteome extraction protocols facilitating membrane protein analysis. This will be of use in more detailed time resolved germination protein analyses in the future aimed at resolving spore protein biogenesis as due to its compatibility with SDS the method is more versatile than the ONE-POT extraction protocol used in chapters 2-4. Given these overall aims, **Chapter 1** details background knowledge on the heterogeneous sporulation initiation, sporulation, spore structure, spore resistance and germination. To make sporulation initiation as homogeneous as possible, a *kinA*-inducible strain is used and the consequence of artificially homogenizing sporulation initiation on the spore proteome, structure, resistance, and germination is evaluated in **Chapter 2**. With induced expression of *kinA*, sporulation is synchronized to result in 70% spore formation of the population within 8 hours. During this homogeneous sporulation, the dynamic changeover of the proteome is investigated in **Chapter 3**. Comprehensive protein co-expression network analysis reveals the emergence of four modules of protein expression patterns during sporulation. In **Chapter 4**, *Bacillus subtilis* A163, a strain producing highly resistant spores, is investigated with respect to its spore proteome. The spore proteome comparison with a lab

strain probes for protein-based cues that might be the basis of the observed high thermal resistance of A163 spores. **Chapter 5** addresses the successful application of the single-pot, solid phase-enhanced sample-preparation (SP3) on spore proteome determination, indicating that SP3 is an alternative method in the study of spore proteomics. Overall, **Chapter 6** summarizes and discusses the results focusing on the implications of the data for spore thermal stress resistance and future perspectives derived from the results described in this thesis.

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