How do spores wake up? Proteins involved in the first stages of spore germination
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1 General introduction

1.1 History of spore research

Research on bacterial spores started almost two centuries ago. In 1838, Ehrenberg reported the presence of refractile bodies in bacterial cells (Figure 1.1.1), which was the first description of bacterial spores. Posteriorly, in 1876, Ferdinand Cohn and Robert Koch reported that bacillus sp. are able to form spores that are resistant at 100 °C, which had an enormous impact on the science of bacteriology[2]. Between 1940 and 1970, several studies about spore resistance, sporulation and germination appeared. More recently, and related to the first sequenced Gram-positive bacterial genome, new genetics methods were applied to study the Bacillus subtilis 168 strain as a model organism (see below)[3].

Bacterial spore research is of high scientific and technological importance, because they are the most stress-tolerant cells known on our planet. They are metabolically dormant, environmentally resistant, and capable of surviving extreme temperatures, desiccation and high ionizing radiation[4]. The longevity of bacterial spores has been described as thousands to millions of years and these dormant cells can be found in every type of environment on earth[5].

Bacilli and Clostridia are the main spore-forming organisms although many Streptomyces species produce spores with some similarity to bacillus spores, but they are less resistant to harsh conditions[6]. It has been described that Clostridium botulinum, Clostridium perfringens and Bacillus cereus are involved in food-borne illnesses, and other spore formers such as Bacillus subtilis are known to cause food spoilage. Clostridium

Figure 1.1.1. Bacillus subtilis spores observed by phase-contrast microscopy. The arrowheads in panel A point to phase-bright spores (refractile bodies), and in panel B the arrowheads point to phase-dark spores. 1 μm bars. Picture modified from Real G et al[1].
difficile is the leading cause of hospital-acquired infections, and *Bacillus anthracis* has been studied extensively because of its potential in biological warfare\[7\]. For all these reasons understanding spore formation, spore germination and the mechanism(s) of spore killing is still of eminent importance these days.

### 1.2 *Bacillus subtilis* as model organism

*B. subtilis* is the model organism in most sporulation studies, because it is regarded as safe and genetically accessible; *B. subtilis* cells are naturally competent and readily transformed by foreign DNA\[8\]. Furthermore, given that the large majority of genes expressed during sporulation are not essential for growth, sporulation mutants are easily obtained\[2,9\]. *B. subtilis* was the first Gram-positive bacterium to be sequenced\[3\] and is up to date the best understood Bacillus species. *B. subtilis* sporulation has served as a model for prokaryotic cell differentiation\[4\].

### 1.3 Taxonomy and Phylogeny of *Bacillus subtilis*

*B. subtilis* taxonomy\[5,10\]:

- **Phylum**: *Firmicutes*
- **Class**: *Bacilli*
- **Order**: *Bacillales*
- **Family**: *Bacillaceae*
- **Genus**: *Bacillus*

The genus *Bacillus* includes Gram-positive and rod-shaped bacteria, occurring singly, in pairs and in chains. They are generally aerobic endospore formers\[6,10\]. A 16S rRNA maximum likelihood phylogeny of the selected strains from class *Bacilli* is depicted in Figure 1.4.1. This class can be divided into a number of orders, some with sporulating others with non-sporulating genera (orders or genera not shown)\[7,11\].

### 1.4 The life cycle of *B. subtilis*

The life cycle of *B. subtilis* is composed of two growth phases: vegetative cell growth (Stage 0 to I) and sporulation (Stage II to VII). The former has features present in other bacteria; each cell produces two identical daughter cells after each round of division (Figure 1.5.1). However, as nutrients become depleted from the environment and cells enter stationary phase, some of the cells in the population initiate the process of sporulation (see below) to increase their survival under harsh conditions\[8,12\]. Successful sporulation ultimately leads to the release of a mature dormant spore into the environment (Figure 1.5.1)\[13\]. Spores will remain dormant until they encounter specific nutrient stimuli, which cause rapid germination and loss of resistance properties. Eventually, the germinated spore will outgrow to become a normal vegetative cell (Figure 1.5.1)\[14,15\].
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1.5 Sporulation

Sporulation is a developmental process, in which the cells sense nutrient limitation and form a dormant spore. For some recent reviews about sporulation see [11,12,17–19]. During sporulation the cells suffer a morphologic differentiation that is initiated with asymmetric cell division. A division septum is formed near an extreme pole of the cell, creating a sporangium composed of two compartments: a smaller cell (the forespore) and a larger cell (the mother cell) [19,20]. Following completion of engulfment, the septum begins to curve and, eventually, the smaller forespore becomes totally contained within the mother cell in a process that resembles phagocytosis in higher cells. The forespore is fully engulfed and pitches off a free cell in the mother cell cytoplasm, so that the inner cell will become the spore and the outer mother cell nurtures the spore. Finally, the spore is released through cell lysis (Figure 1.5.1) [19,20]. Seven different morphological stages during sporulation are recognized through which metabolically active vegetative cells enter a stage of dormancy [12,17,21], as depicted in Figure 1.5.1.

1.5.1 Activation of histidine-sensor kinases and changes in gene expression

Sporulation is triggered by the activation of histidine sensor kinases (including KinA, KinB and KinC), which shuttle phosphate through an extended phosphorelay, resulting in phosphorylation of the master regulator of sporulation, the transcription factor Spo0A. Phosphorylated Spo0A controls a large regulon of genes, including those involved in asymmetric cell division and those involved in activation of the sporulation-specific sigma factors [20]. These changes in gene expression induce morphological differentiation in both the pre-divisional sporangium and later in the two compartments (Figure 1.5.1) [20].

1.5.2 Gene regulation of sporulation

Sporulation is controlled by five transcription factors: \( \sigma^H \), \( \sigma^F \), \( \sigma^E \), \( \sigma^G \) and \( \sigma^K \) (Figure 4). These transcription factors belong to a family of regulatory proteins in bacteria known as RNA polymerase sigma factors that direct the polymerase to particular promoter sequences.
Figure 1.5.1. Life cycle of *B. subtilis*. The vegetative and the sporulation cycle. For simplicity stage I has been omitted. Sporulation begins when a sporangium divides asymmetrically to produce two compartments (stage II): the mother cell and the forespore, which are separated by a septum. Next, the mother cell engulfs the forespore (Stage III), and following membrane fission at the opposite pole of the sporangium, a double-membrane bound forespore is formed. Coat assembly begins just after the initiation of engulfment and continues throughout sporulation. The peptidoglycan cortex between the inner and outer forespore membranes is assembled during late sporulation (Stage IV and V). In the final step, the mother cell lyses to release a mature spore into the environment. Modified from McKenney et al\cite{20}.

\[\text{Vegetative cycle}\]
Figure 1.5.2. Gene regulation during sporulation. (a) Spo0A and $\sigma^H$ are activated in the pre-divisional cell, which leads to asymmetric division and (b) early compartmentalized gene expression with $\sigma^F$ becoming active in the pre-spore and $\sigma^E$ in the mother cell. (c) A series of proteins produced in the mother cell degrade the asymmetric septum and trigger migration of the membrane around the pre-spore, which is called engulfment, (d) When the membranes fuse at the pole of the cell, the pre-spore is released as a protoplast in the mother cell, and a second round of compartmentalized gene expression occurs, with $\sigma^G$ becoming active in the prespore and $\sigma^K$ in the mother cell. These late factors activate transcription of the genes that build the structural components of the spore that provide its resistance qualities. (e) By lysis of the mother cell, the spore is released into the environment. When the dormant spore encounters an appropriate environmental stimulus, it initiates the process of germination that can result in the re-initiation of vegetative growth if sufficient nutrients are present. Modified from Higgins et al and Piggot and Hilbert [17,19].

on the chromosome[22] (Figure 1.5.2). Spo0A (mentioned before) and also $\sigma^H$ are activated in the pre-divisional cell, which leads to asymmetric division. After asymmetric division takes place, transcription factor $\sigma^F$ is activated. When $\sigma^F$ becomes activated in the forespore compartment, a sigma factor called $\sigma^E$ gets activated in the mother cell compartment.

After engulfment takes place, $\sigma^F$ gets replaced on the forespore by a transcription factor called $\sigma^G$. Lastly, in the mother cell, the final transcription factor $\sigma^K$ is activated[17]. The $\sigma^K$-regulated genes include spore coat proteins that are connected to the outer part of the spore, whereas in the spore, the ger operons encoding for germination receptors, are transcribed by a $\sigma^G$-regulated RNA polymerase[23]. In addition, in the last step of sporulation, the decrease of the core water content within the spore promotes the mother cell to lyse as a consequence of three autolysins referred to as programmed (mother) cell death[24], with the concomitant release of the spore into the environment.
1.6 Structure of bacterial spores

The bacterial spore is composed of a number of compartments that are termed (i) exosporium, (ii) coat, (iii) outer membrane, (iv) cortex, (v) germ cell wall, (vi) inner membrane, and (vii) central core (Figure 1.6.1). (i) The exosporium is the most external layer but is not found in all species of *Bacillus*. In *B. cereus* and its close relatives *B. anthracis* and *B. thuringensis*, the exosporium consists of a basal layer adorned with hair-like projections.

Recent structural studies have documented the crystalline bi-dimensional architecture of the basal layer\[25\]. It is composed of glycoproteins and may provide resistance to chemical and enzymatic treatments, providing the spore with the ability to adhere to surfaces\[26,27\].

The exosporium is separated by a gap called interspace\[20\]. (ii) In *B. subtilis* the coat is formed by up to 70 different proteins. The function of the coat has not been completely addressed, however, it is implicated in spore resistance to chemicals and predation by protozoa\[28\].

Recently, it has been shown that the coat is formed by three layers: the inner coat, the outer coat and the crust (Figure 1.6.1). (iii) The outer membrane is an essential structure during sporulation and may play a role as permeability barrier\[26,28\]. (iv) The cortex is essential for the formation of a dormant spore. It is composed of peptidoglycan, especially of muramic acid-\(\delta\)-lactam, which is important for the cortex lytic enzymes and recognition during spore germination. During spore germination, the cortex is degraded by cortex lytic enzymes in order to allow the expansion of the core and further outgrowth\[28,29\]. (v) The germ cell wall is also composed of peptidoglycan. During the final stage of spore germination the germ cell wall expands and forms the cell wall of the outgrowing spore. (vi) The inner membrane is a strong permeability barrier, playing a major role in resistance to a wide range of chemicals. It contains proteins that are important in germination such as the nutrient germinant receptors and the SpoVA proteins. The lipid molecules in the inner membrane of the spore are largely immobile\[30\]. The viscosity of the bilayer is much higher when compared to that of the cell membrane of a growing cell or fully germinated spore\[31\]. The phospholipids and fatty acids found in the inner membrane of the spore are almost identical to those of the growing cell, indicating that the high viscosity is caused by a tight packing of the lipids as a result of the dehydration. Note, the phase transition temperature of dioleoyl lipids is well below \(-10\) °C and increases to higher than \(60\) °C when membranes are dehydrated\[32,33\]. A similar

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**Figure 1.6.1.** Schematic representation of typical structures in *Bacillus subtilis* spores. The multiple layers of the spore serve to protect the genome and proteins, which are housed in the partially dehydrated central core. The inner forespore membrane and the outer forespore membrane are represented in gray. The core is protected by the cortex (green) and the spore coat, which consists of four layers: the basement layer (blue), inner coat (orange), outer coat (purple) and crust (red). The concentric rings of the basement layer and inner coat reflect the lamellar appearance of the inner coat in electron micrographs. Modified from McKenney et al\[20\].
The central core of the spore has a low water content (25-50% of wet weight) and is rich in pyridine-2,6-dicarboxylic acid (DPA, ~10% of total spore dry weight). The core plays an important role in spore resistance, containing most of the spore enzymes, DNA, ribosomes and tRNAs. Furthermore, the core is the reservoir of small acid-soluble spore proteins, which is crucial for spore resistance. The internal pH of the core is another important feature, ranging from 6.3 to 6.5 in dormant spores and 7.5 to 7.8 in germinated spores or growing cells. The low pH may contribute to the spore metabolic dormancy, however, the low water content in the core is likely the major reason for zero metabolic activity in the spore. Although proteins appear to be immobile in the core, Kaieda et al have suggested that most of the water molecules in the spore are highly dynamic.

1.7 Spore Germination

The spore can be resistant and dormant for a long period of time, but under appropriate environment conditions, the spore looses its resistance and becomes a growing and dividing cell. This process is called spore germination, which is an irreversible process that takes place in several stages (Figure 1.7.1). The germination process is triggered by the exposure to nutrients called ‘germinants’. The exact molecular mechanisms associated with the event are not well known, although it is associated with a major change in the inner membrane permeability and the inner membrane structure. Monovalent cations, including H\(^+\), K\(^+\), and Na\(^+\) are released during this stage, followed by the release of Ca-DPA (Stage I). The release of most Ca-DPA takes only a few minutes for individual spores and is most likely via channels composed of SpoVA proteins (seven in B. subtilis spores, see below). Ca-DPA release marks the end of stage I, while the spore still is in a metabolically inactive phase. In stage II, the cortex lytic enzymes become activated and specifically hydrolyze the peptidoglycan of the cortex by targeting muramic acid & lactam. Spores of Bacillus species generally contain two major cortex lytic enzymes, CwlJ and SleB, either of which alone is sufficient to allow the completion of spore germination. As the cortex is hydrolyzed, the spore core becomes further hydrated and expands, leading to further loss of resistance and dormancy (Figure 1.7.1). The re-start of the metabolic activity signifies the end of stage II of germination and the beginning of...
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outgrowth.

Upon completion of stage II of germination, the core water content has risen to 80% of wet weight, equal to that in vegetative cells. This increased core water content allows metabolism in the core to begin, followed by macromolecular synthesis, ultimately converting the germinated spore into a growing cell\[30\]. In addition during outgrowth, acid-soluble spore protein that were bound to the spore DNA during dormancy are degraded by the germination protease, making the DNA available for transcription. This process results in the synthesis of proteins and further metabolic activity. As metabolism and macromolecular synthesis proceed, the spore escapes the coat to become a growing cell again (Figure 1.7.1)\[41–43\].

1.8 Germinant receptors in Bacilli

Bacillus spores are equipped with a specific set of germinant receptors that continuously monitor the environment for proper outgrowth conditions. Germinants, including amino acids, sugars and purine nucleosides, are able to initiate germination when present in appropriate concentration and mixture in close proximity of the spore\[14,28,44,45\]. How these germinants transverse the outermost layers of the spore is not clear. However, there is evidence that GerP proteins are responsible for allowing nutrients and small molecules to permeate the outer layers (particularly the coat) of spores\[28,46–48\]. After the nutrients traverse the outermost layers of the spore, they interact with specific germinant receptors that reside in the spore’s inner membrane\[14,28,49,50\].

B. subtilis expresses three major families of germinant receptors: GerA, GerB and GerK. The genes belonging to GerA represent the first germination operon described and GerA is the most studied receptor, mediating L-Alanine or L-Valine-triggered germination\[51\]. Furthermore, GerB and GerK are both involved in a germination response to a mixture of L-asparagine, D-glucose, D-fructose and potassium ions (AGFK response)\[52\].

The GerA receptors comprise three genes: gerAA, gerAB and gerAC\[53\]. Disruption of any of these cistrons abolishes germination\[54–56\]. Recently, a putative fourth protein component of germinant receptors has been described as ‘D subunit’. It has been suggested that D subunit plays a role in modulating rates of germinant receptors-dependent spore germination, however the mechanism has not been fully understood\[29,40,57\].

GerAA is an integral membrane protein with 4 to 6 transmembrane (TM) segments with a large N-terminal hydrophilic domain and a small hydrophilic C-terminal domain\[29,58,59\]. GerAB is predicted to be an integral membrane protein with 10 to 12 TM segments flanked by short hydrophilic termin\[58,59\]. GerAC is predicted to contain a pre-lipoprotein signal sequence, suggesting that the C-subunit is anchored to the outer surface of the membrane via an N-terminally attached lipid moiety\[40,60,61\]. Mutational analysis indicates that lipidation of GerAC is essential for its role in germination\[60,62\]. The crystal structure of B. subtilis GerBC (homolog of GerAC) has been solved, but its function is still matter of debate\[63\]. Interestingly, all GerA receptors are expressed and localized in the inner membrane of the spore. Mutations of highly conserved residues in GerAA, GerAB and GerAC have been shown to affect germinant receptors function\[29,56,62,64,65\].

There is molecular-genetic and bioinformatic evidence that germinants bind specifically to germinant receptors in the inner membrane of the spore\[43,56,62,64,66,67\]. However, there are no studies showing that purified germinant receptors bind specific germinants, which would be definitive proof that these proteins are indeed serving as receptors\[28\].
1.9 GerD protein

Recent studies suggest that GerD, a lipoprotein of approx. 180-residues, is essential for germinant receptors-dependent germination\[29\]. It is located in the outer surface of the inner membrane of the spore\[28\]. Its function is unknown, however, deletion of the gerD gene decrease the rate of germinant receptors-dependent of germination. Spores, which lack GerD, germinate normally with non-nutrient germinants. It is also known that germinant receptors and GerD colocalize to a single cluster\[68\].

1.10 SpoVA proteins

In \textit{B. subtilis} the SpoVA proteins are encoded by a heptacistronic operon that comprises \textit{spoVAa}, \textit{spoVAb}, \textit{spoVAc}, \textit{spoVAd}, \textit{spoVAEa}, \textit{spoVAEb} and \textit{spoVAF}. From these protein, SpoVAA, SpoVAB, SpoVAC, SpoVAEb and SpoVAF are predicted to be membrane proteins, with two to five membrane spanning regions and most likely present in the inner membrane of the spore\[12,68–70\]. Mutations in any of the first six cistrons of the \textit{spoVA} operon eliminate Ca-DPA uptake during sporulation\[69,71,72\].

Previous studies suggested that proteins encoded by the \textit{spoVA} operon are involved in Ca-DPA release\[73,74\]. This release of Ca-DPA is triggered not only by nutrients but also by agents such as the cationic surfactant dodecylamine, which bypasses the germinant receptors\[74,75\]. There is strong evidence that one or more SpoVA proteins are involved in Ca-DPA release: (i) a temperature-sensitive \textit{spoVA} mutant is defective in Ca-DPA release in spore germination. (ii) overexpression of the \textit{spoVA} operon in spores increases the rate of Ca-DPA release in germination. (iii) loss of the SpoVAEa and SpoVAF proteins from \textit{B. subtilis} spores has an effect on the rate of DPA release during spore germination\[28,76\].

Furthermore, recent studies showed SpoVAC to share features with channel like proteins, that would allow the release of Ca-DPA during germination of the spore\[38\], this thesis chapter 3.

1.11 Non-nutrient germination

Germination of spores can be activated by non-nutrient germinants and regardless of the presence of any of the germinant receptors. The best-studied non-nutrient germinants are Ca-DPA\[77–79\] and cationic surfactants, in particular dodecylamine\[75,80\]. Addition of exogenous Ca-DPA appears to directly activate CwlJ, one of the cortex lytic enzymes, inducing the degradation of the cortex\[78,79\].

Dodecylamine induces quick loss of refractivity of the spore, release of DPA and loss of heat resistance. Mutants lacking all germinant receptors or the cortex lytic enzymes CwlJ and SleB germinated well with dodecylamine, indicating bypassing of the germinant receptors and the cortex lytic enzymes\[15,75\]. The molecular mechanism of this effect is not well understood, but it is possible that dodecylamine modifies the tension (lateral pressure profile) of the membrane with the concomitant activation of an associated protein channel\[74\]. There is strong evidence that SpoVA proteins are involved in Ca-DPA release triggered by dodecylamine. Notably, SpoVAC channel activity (see above) can also be gated by surfactants including dodecylamine\[38\].
Additional non-nutrient germinants include N-acetyl glucosamine and N-acetyl muramic (muropeptide) derived from the breakdown of peptidoglycans from growing cells. The muropeptide appears to trigger germination through activation of a spore protein kinase (PrkC), which phosphorylates serine/threonine residues, and whose kinase activity is required to trigger spore germination in response to muropeptides[81].

Bacillus spores also germinate when exposed to specific physical conditions like abrasion and high pressure[4,82–84]. High pressure is used in the industry to kill microbial contaminants in order to preserve certain food products. Pressures ranging from 50 to 350 Mpa and moderate temperature (20 to 50°C) induce spore germination by activation of one or more of the germinant receptors, while higher pressure (500 to 1000 MPa) initiates germination, probably by the release of Ca-DPA through SpoVA channels[29,43,83,85]. Abrasion causes mechanical damage to the spore, which in turn seems to activate CwlJ and SleB and leads to the degradation of the cortex[82] followed by ion fluxes and hydration of the core.

1.12 The role of mechanosensitive channels in germination of bacillus spores

B. subtilis is a ubiquitous soil bacterium[9]. The solute and moisture content of the soil environment frequently fluctuates due to desiccation or to rain fall. B. subtilis must adjust the levels of intracellular solute and water to avoid swelling or shrinkage due to excessive or minimal soil moisture. In situations with low soil moisture, the bacterium accumulates high levels of solutes (e.g. glycine betaine) to prevent dehydration of the cytoplasm[86]. The cell takes up water when soil water content increases, with an accompanying increase in cell turgor pressure to levels that can cause cell lysis. In this situation, the bacterium rapidly releases accumulated solutes, probably via one or more mechanosensitive channels. Mechanosensitive channels work as safety valves allowing cells to extrude ions and solutes upon exposure to an osmotic downshift (Figure 1.12.1)[87]. These channels were first detected in Escherichia coli spheroplast by using patch clamp[87] (Figure 1.12.2).

This electrophysiology method measures the ion currents through individual channel proteins. The best studied mechanosensitive channels are: (i) the mechanosensitive channel of large conductance (MscL), (ii) the mechanosensitive channel of Small conductance (MscS), (iii) the mechanosensitive channel depending of potassium in the medium (MscK) and (iv) the small mechanosensitive channel of minimum conductance MscM from E.coli[88]. These channels respond to changes in membrane tension by releasing the internal pressure of the cell under hypoosmotic conditions[88] It is known that during the spore germination in bacillus, Ca-DPA is excreted in the first seconds of germination of individual spores[75]. Given the dramatic osmolyte fluxes in sporulation and germination, it is not unreasonable to imagine that mechanosensitive channels are involved in this process[89]. However, all the known mechanosensitive channels expressed in vegetative cells of B. subtilis are not involved in germination and dodecylamine-triggered release of Ca-DPA[90]. We describe in chapter 3 of this thesis that SpoVAC reconstituted in liposomes works as a mechanosensitive channel, releasing a fluorescent dye triggered by ionic surfactants[38]. These findings are in line with previous studies that support that SpoVA proteins are working as a channel during spore germination.
Figure 1.12.1. Physiological function of mechanosensitive channels in bacteria. a) Low osmolarity cells in osmotic balance. b) Cells shrink due to water loss. c) Turgor regain. d) Mechanosensitive channels-assisted solute release. e) Normal growth. f) Channels fail to gate, which causes cell lysis. Taken from Booth et al[87].

Figure 1.12.2. Patch clamp set up. A) The tip of an electrode-containing glass pipette is brought in contact with the cell. A mild suction is then applied to form a very tight seal and to pull away the piece of membrane enclosed by the pipette tip. The bath solution is usually the same as for the pipette solution. The electrode, which is connected to specialized circuitry, can measure currents passing across ion channel of the cell. A second electrode in the bath solution serves as reference or ground electrode. The arrows represent the negative pressure applied to the membrane of the cell. B) A typical trace of MscL from giant unilamellar vesicles. Currents are depicted as pA and negative pressure as mmHg.
1.13 Outline of this thesis

Chapter I of this thesis summarizes the current view of sporulation and germination of bacillus spores. We describe crucial events during life cycle of *Bacillus subtilis*, together with insights in structure of the spore. In addition, we give new information about germinant receptors and SpoVA proteins that are required during the initial events in germination and describe the function(s) of mechanosensitive channels. Chapter 2 focuses on the biochemical characterization of the ABC subunits of GerA receptor proteins from *Bacillus subtilis*.

Chapter 3 elucidates the mechanism of one of the SpoVA proteins: SpoVAC. We present strong evidence that SpoVAC acts as mechanosensitive channel, presumably facilitating the release of Ca-DPA and other low molecular compound during germination in vivo.

In Chapter 4, we present some final conclusions and future perspective of this study.