Chapter 1

General introduction and outline of the thesis
1.1 The Bacillus Genus

The Bacillus genus consists of Gram-positive, rod-shaped bacteria. Its bacteria are ubiquitously present and inhabit water, soil and live on as well as inside plants and animals (Jensen et al., 2003; Nicholson, 2002; Tam et al., 2006; Vilain et al., 2006). Among all Bacillus species, B. subtilis is the most studied. It is the model organism for Gram-positive bacteria. The genome of B. subtilis was one of the first bacterial genomes to be sequenced. The organism exhibits natural competence (incorporates exogenous DNA thus allowing for genetic modification) (Kunst et al., 1997; Moszer, 1998). Bacillus forms endospores that enables it to survive under unfavorable environmental conditions (Gould, 2006; Setlow, 2006; Setlow, 2003). The position of the endospore differs among different bacterial species. For example bacteria having terminal, sub terminal, centrally placed endospores are Clostridium tetani, Bacillus subtilis and Bacillus cereus respectively. Bacterial spores are phase bright, oval, dormant, metabolically inactive, stress resistant structures formed when the cells are exposed to unfavorable conditions. The process through which they are formed is called sporulation.

1.2 Sporulation cycle

The process of spore formation has been studied in detail by using B. subtilis as model organism. Fig. 1.1 shows the sporulation cycle in detail. It is divided into seven different stages. The vegetative cells are considered to be in Stage 0. These undergo an unequal cell division to form a small forespore (also known as the prespore), which develops into the spore, and large mother cell, which is necessary to sustain spore formation after which it ultimately lyses (programmed cell death). The next phase, Stage I, is the condensation stage, where the two genomes of the vegetative cell (post-asymmetric cell division) fuse to form a single axial filament of chromatin. At Stage II a septum is formed by membrane invagination that takes place at one pole of the mother cell. Soon after the division, distinct programs of gene expression are initiated in the mother cell and forespore, which are directed by sporulation-specific RNA polymerase σ factors, σF in the forespore and σE in the mother cell. At Stage III, a protoplast is formed within the mother cell. After division, the mother cell engulfs the forespore, resulting in the double membrane enveloped spore structure. On completion of the engulfment, σG becomes active in the forespore and σK in the mother cell (Fig. 1.2). Next, Stage IV is the stage where the deposition of the primordial germ cell wall and cortex between the membranes of the spore protoplast occurs (Errington, 2003). In Stage V the spore coat deposition around the cortex takes place and at Stage VI the "maturation" of the spore occurs, upon which the spore develops its characteristic
1.3. Endospore structure and its resistance

Resistance properties. Finally during Stage VII, the mother cell lyses and releases the completed spore (Piggot et al., 2004; Stephens 1998). After the release, the spore undergoes further maturation with a final increase in (thermal) stress resistance (Abhyankar et al., 2014).

![Figure 1.1: The sporulation cycle of spore-forming bacteria.](adapted from Errington, 2003)

1.3. Endospore structure and its resistance

The environmental resistance properties of spores are generally considered to be due to their well-assembled structure. The spore is made up of different layers i.e. exosporium, coat, cortex and core (Fig. 1.3). The exosporium is the outer layer that lies on top of the spore coat (Driks, 1999; Henriques et al., 2007). It is composed of a low-density network of glycosylated proteins. The network is loosely attached to the outer coat. The exact function of the exosporium is, however, not known (Driks, 1999; Henriques et al., 2007; Waller et al., 2004).

![Figure 1.2: Gene regulation during sporulation.](adapted from Piggot and Hilbert, 2004)
This layer is optional and present in for example *B. cereus* but not in *B. subtilis*. The spore coat is present around the outer membrane. It is made-up of proteins and works like a sieve that excludes large molecules like lysozyme. The coat contains enzymes that are involved in spore germination. Furthermore it allows interaction with the surroundings of the spore (Driks, 1999; Henriques et al., 2007). Underneath the spore coat lays the cortex, which consists of peptidoglycan. Next follows the inner membrane which encloses the spore core (Takamatsu et al., 2002). The inner membrane is made up of closely packed lipids that display far less lateral mobility than lipids in the plasma membrane of vegetative cells (Cowan et al., 2003). It is selectively permeable for small and large molecules (Setlow et al., 1993; Cortezzo et al., 2004). It also contains germination receptor proteins, which are important for the regulation of the start of the germination and outgrowth process of spores.

The core is present beneath the cortex. It contains the chromosomal DNA, which is saturated with $\alpha/\beta$-type small acid-soluble spore proteins (SASPs)(5-10%) (Raju et al., 2006). In dormant spores the SASPs provides the spore’s resistance to heat and UV light but during germination and outgrowth, the SASPs get degraded and generate amino acids which are subsequently used for protein synthesis (Mason, et al., 1986; Setlow, 1987; Setlow, 1987). The core has a low water content amounting to about 25-50% wet weight (Beaman et al., 1986). Under optimal non-stress conditions the pH in the core has been reported to be around 6.0-6.5 and hence to be slightly more acidic than vegetative cells (Magill, et al., 1994; Setlow and Setlow, 1993; Van Beilen et al., 2013). The acidic nature of the core is due to a large amount of pyridine-2,6-dicarboxylic acid or dipicolinic

![Spore anatomy](image)
acid (DPA) present in the core which is synthesized in the mother cell (Setlow, 2007). This DPA chelates with divalent calcium ions in a 1:1 ratio. The chelation in spores is merely in the solid phase as the DPA concentration is above its level of water solubility (Ablett et al., 1999; Leuschner et al., 2001, 2000). The compound stabilizes the DNA and protects it from heat and UV radiation by enhancing the amount of spore photoproduct (SP) production (Setlow and Setlow, 1993; Setlow, 2006).

### 1.4 Spore germination and outgrowth

Spore germination is a process by which the dormant and stress resistant spore wakes up and initiates vegetative cell growth. All of the components necessary to facilitate germination are pre-packaged into the spore during sporulation. Figure 1.4 shows a schematic representation of different layers and elements that comprise the germination receptor system of *Bacillus* spores. Germination starts by interaction of appropriate germinants such as amino acids and sugars with germinant receptor proteins (Corfe et al., 1994b; Sammons et al., 1981; Zuberi et al., 1987). The germinant receptor proteins are present at very low level (24-40 per spore) in inner membrane of *B. subtilis* spores. The germinants receptors are encoded by tricistronic operons, which are composed of three subunits proteins (A, B, and C, Fig. 4) (Paidhungat et al., 1999; Moir et al., 2002) and are under the control of a σG promoter (Corfe et al., 1994a; Kemp et al., 1991). The “A” subunit is composed of two parts, first part has five or six predicted membrane spanning domains facing toward the core and second part has a large hydrophilic N-terminal domain facing to the cortex. The “B” and “C” subunits include ten predicted transmembrane domains and predicted lipoprotein signal sequence facing toward the cortex respectively. Thus the “B” subunit is know as an integral membrane protein and “C” subunit is known as membrane anchored (Moir et al., 2002; Zuberi et al., 1987). Although the exact roles of each of these proteins are not well comprehended, it is likely that their mutual interaction(s) (Hudson et al., 2001; Igarashi et al., 2005; Paidhungat et al., 2001) is (are) required for proper functioning of the receptor complex as a whole.

To start the germination process, germinants must reach receptors in the inner membrane of the spore by crossing the exosporium, coat, and cortex. The process is not well understood. It is known from *B. cereus* and *B. subtilis* studies that the germinant entry may be facilitated by GerP proteins (Behravan et al., 2000). The germination process is divided into two different stages: stage I, early germination and stage II, the actual germination stage (Fig. 1.5). In the early germination the germinant receptors, located in the inner membrane of the spore, sense the pres-
ence of germinant in their environment. This event commits spores to germinate. Ones germination is committed, it becomes irreversible and continues even after removal of the germinants (Stewart et al., 1981; Yi et al., 2010). After this, the release of monovalent cations such as $H^+$, $Na^+$ and $K^+$ as well as divalent $Ca^{2+}$ of dipicolinic acid (DPA) from the spore core occurs. Simultaneously the pH of the core rises from 6.5 to 7.7 (Setlow et al., 2008; van Beilen et al., 2013).

**Figure 1.4:** Schematic diagram of spore layers and elements that comprise of the germination receptor system of textitBacillus spores (modified from Moir et al., 2002)

Due to partial rehydration and release of DPA, the heat resistance property of the spores is reduced. In stage II, the degradation of cortex peptidoglycan by two lytic enzymes called SleB and CwlJ takes place (Wuytack et al., 1998; Paidhungat et al., 1999, 2001, 2002; Setlow, 2003). This allows further uptake of water, due to which the core and inner membrane expand, dormancy breaks and hence enzyme activity recommences (Christie, 2012). At this stage the spores are completely germinated and become phase dark. On the one hand the germination process is complex but on the other hand it is extremely efficient and rapid as 95% of spores germinate within minutes in rich media. The spore can germinate in any
of the three ways. One, the nutrient mediated germination where the germinants come in contact with the germination receptors and hence activate them. Second, the germination is independent of nutrient or germinant receptors. The dormant spores sense peptidoglycan fragments that are released in the environment by other bacteria in an asyet unknown way and thus stimulate germination (Shah et al., 2008). The third is the non-nutrient route where germination occurs with the help of surfactants, pressure or exogenous Ca-DPA. The exogenous Ca-DPA allow spores to germinate by activating the lytic enzyme CwlJ, which in turn degrades the peptidoglycan of the cortex.

Germination is an irreversible process in which physical and chemical properties of spores changes, such as release of Ca-DPA, loss of heat resistance, and decrease in absorbance (Stewart et al., 1981; Yi et al., 2010b). Spore germination can be measured indirectly by correlating the mentioned properties of germinating spores (Stewart et al., 1981; Yi et al., 2010b). The time-resolved analysis of the mentioned properties of the spores is a frequently used to inspect germination (Paidhungat et al., 2002). Other method could be used to analyze the germination mutants where genes involved in germination are mutated thus allowing us to study different germination components of the spore with respect to spore germination.

The germination process is followed by the outgrowth stage. This outgrowth stage is also divided into two stages. In the first stage of outgrowth, the conversion of 3-phosphoglycerate results in the generation of ATP. After ATP production the second stage of outgrowth starts. In this stage the spore starts using extracellular nutrients and producing macromolecules needed to reconstitute biochemical pathways, nutrient uptake, and the replication process. After approximately 30 min chromosomal replication is initiated. Due to the action of germination-specific lytic enzymes (GSLEs) full rehydration of the spore occurs and activation of other enzymes and more ATP production begins. During this process the degradation of SASPs in the spore core and transcription of core DNA resume (Setlow, 2007). In the outgrowth phase the RNA, protein, and DNA synthesis results in formation of metabolically active vegetative cells.

The SASPs in the spore core responsible for maintaining DNA stability are degraded, hence allowing transcription to resume (Setlow, 2007). The initiation of RNA, protein, and DNA synthesis during outgrowth results in a newly formed, metabolically active vegetative cell.
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1.5 *Bacillus subtilis* spores in the Food Industry

Food industries are concerned with spore forming bacteria such as *Bacillus* spp. The organisms cause immense problems to the food industry because they are resistant to many preservation processes that are commonly used in industry. This allows spores and thus their parent vegetative cells to survive commonly applied food processing and conservation methods. Thus food industries must invest huge amounts in food quality control to reduce spore-related problems and ensure a microbiologically safe supply of high quality food products to consumers. Currently, the food industry aims for less intense, more fit for purpose preservation methods. These should fulfill consumer’s demands of healthy nutritious and natural food products at the required microbiological stability levels.

It is known that dry and wet heat induce damage via different mechanisms. On the one hand dry heat primarily causes DNA damage (Setlow, 2006), on
1.6 Heterogeneity in bacterial (spore) physiology during germination and outgrowth

The other hand, wet heat causes protein denaturation in a rather nonspecific way (Coleman et al., 2007). Similar to these two thermal treatments often preservation strategies have different molecular bases. This characteristic has been exploited in the application of what has been coined as ‘the hurdle preservation concept’. This preservation technology generally consists of the combination of more than one antimicrobial strategy such as the use of low concentrations of salt or acidification with specific weak organic acids often along with the application of a mild heat regime. The most naturally occurring and common weak organic acid used in food industries are sorbic acid, propionic acid, lactic acid, and acetic acid (Beales, 2004; Theron et al., 2007). The antimicrobial activity of these acids in aqueous solution is pH dependent, with the maximum effect occurring at low pH, where the undissociated state of the acid is favoured (Piper et al., 2001). The undissociated acid molecules are lipophilic and will penetrate plasma membranes, thus entering the cells. Inside the cell the acid encounters an internal pH, which is nearly neutral. As the pKa values of many weak acid preserving acids are between 4.2 and 4.9, inside the cells a rapid dissociation of acid molecules into charged protons and anions takes place. These charged compounds cannot subsequently diffuse back across the plasma membrane. As a result, intracellular acidification of the cell cytosol, resulting from the accumulation of protons, takes place and inhibits key metabolic activities involved in glycolysis (Krebs, et al., 1983) thereby perturbing the ATP yield of cellular metabolism (Van Beilen et al., 2014).

1.6 Heterogeneity in bacterial (spore) physiology during germination and outgrowth

Conventional microbial culturing methods allow us to measure heterogeneity at the population level, but the analyses average out the single cell effects. An example of such data is the continuous measurement of the change in optical density (OD) during the germination and outgrowth phase of bacterial spores. Hence different methods have been developed to study the heterogeneity at single cell level. These methods include the use of Anopore filters, flow cytometry, laser tweezers Raman microscopy, as well as differential interference contrast microscopy etc. (Chen et al., 2006; den Besten et al., 2007, 2010; Smelt et al., 2008; Wang et al., 2011).

Heterogeneity is not only evident in the germination phase but also in different phases of spore outgrowth as well as the growth of vegetative cells (Pandey et al., 2013; Stringer et al., 2011). Some spores are often called super dormant spores, as they remain dormant when exposed to germinant or, in alternative, they start germinating extremely slowly. Hence, potentially, such spores may ‘come back to
life long after inactivation (preservation) treatments have been applied (Ghosh et al., 2010, 2009; Chen et al., 2014). The generally agreed view is that the cause of germination heterogeneity may be found in the number of receptors and accessory germination proteins present in (super) dormant spores and the number of cations available during sporulation (Ghosh et al., 2012; Chen et al., 2014). It is believed that outgrowth heterogeneity may well be influenced by stresses incurred during spore formation, for example the temperature at the time of sporulation (Melly et al., 2002). In addition stress in the spore’s environment, for example a thermal stress treatment, may influence the heterogeneity observed at this stage of transition of a dormant spore to a vegetative cell (Smelt et al., 2008). Figure 1.6 exemplifies this by schematically indicating the effect of a thermal treatment on a spore population.

Figure 1.6: Effect of heat treatment on spore, which generate subpopulation of sub-lethally damage spores.

When a dormant spore population is treated with heat the single spores may subsequently be divided into three subpopulations i.e. viable, damaged and dead spores. The viable spores can germinate as fast as non-treated spores. The damaged spore tends to germinate slower than the viable spore as most likely the germination machinery is itself damaged. In addition outgrowth may be affected as time may be needed to repair damage in the machinery that regulates DNA duplication and general cellular metabolism (Smelt et al., 2008; Stringer et al., 2011; Pandey et al., 2013). Clearly irreversibly damaged spores often do not even germinate (Coleman et al., 2007). A higher level of stress tends to correlate with an increased heterogeneity in spore behavior (e.g. Smelt et al., 2008).

In fact a heat treatment may have various effects on spores. It may, dependent on its intensity, trigger activation, germination as well as destruction of dormant
1.6. Heterogeneity in bacterial (spore) physiology during germination and outgrowth

Spores through a complex multistage process. In order to minimize or eliminate food spoilage caused by the bacterial spore formers, it is very important to detect and estimate the amount of viable spores in the food. Effective elimination of spores is only possible once spore detection and inactivation systems are available. To develop such systems it is necessary to understand the molecular mechanisms behind the processes of germination and outgrowth as well as the stress resistance properties of spores. Not much is known about the mechanisms that operate during the germination and outgrowth phase. Keijser et al. (2007) have shown in a population based transcriptome study prominent functional modules in spore germination and outgrowth. Interestingly some of the modules contain stress response genes which are transiently expressed during outgrowth even in the absence of the cognate stress. In this way spores may anticipate adverse environmental conditions. The vegetative cell (metabolically active form) is much easier to kill than the dormant spore. Therefore, it would be advantageous to deliberately drive spores in food into their vegetative form in order to facilitate their inactivation with food preservation treatments. In practice this should be done by triggering spores with germinants or physical treatments that allow their ‘rapid return to life’ through the process of germination and outgrowth. The timing of this process is difficult to predict accurately as spores are generally seen to germinate at different times and at different rates. There have been few reports on spore germination at the single spore level. A study on the timing of germination and outgrowth at the level of individual cells has shown that spore outgrowth occurs up to 150 hrs. with or without a prior thermal treatment (Smelt et al., 2008). For this study wild type thermally treated spores were sorted individually in 96 wells of micro titer plates and tested for (out) growth under product-relevant conditions. The method gave clear insight in the heterogeneity of the timing of outgrowth, but did not allow for the monitoring of the individual successive developmental processes of spore germination, outgrowth to vegetative cells and subsequent cell divisions. These complex cell processes have been traditionally studied at the population level. The studies have shown that single cell heterogeneity is a widespread phenomenon in biology and have directed the development of the single cell approaches. In this regard flow cytometry, florescence in situ hybridization, microscopy etc. was deployed allowing measurement of cell properties at individual level. Stringer et.al used phase-contrast microscopy to determine the relation between different stages in germination and subsequent outgrowth of spores from the anaerobically growing non-proteolytic Clostridium botulinum strain (Stringer et al., 2011). Their conclusion was that the distribution of the times to germination as well as outgrowth and subsequent growth, showed considerable variability. All stages contribute to the overall variability in the observed lag time with the time to germination and outgrowth being
most affected by a thermal stress. The time to germination, spore emergence, cell maturation and cell doubling were not correlated. In a relatively recent study Veening et al. presented a method to mount Bacilli spp. on a microscope slide and study their sporulation. The authors reported qualitative details on the growth of vegetative cells (De Jong et al., 2011). However, no quantitative validation of the growth performance of vegetative cells growth under the condition for live imaging versus those prevailing in well aerated shake flask was performed. Huang et al. (2007) showed that microfluidic Raman tweezers can be used of a variety of Bacillus species and strains to measure levels of Ca-DPA in individual spores in populations. 

Phase-contrast microscopy allows us to observe at the single spore level the process of germination by assessing the transition of spores from phase bright to phase dark, their outgrowth by measuring the time interval between the phase dark formation and the first cell division as well as vegetative cell divisions for every individual cell of a population (Pandey et al., 2013; 2014). By using such high-resolution single-cell analysis techniques in combination with time-lapse microscopy, quantitative image analysis and observation of the activity of fluorescent reporter proteins is facilitated. This has enabled us and other researchers to make a time resolved study of the expression and activity of various proteins in individual cells and even bacterial spores (amongst others this thesis). In fact we can analyze individual spores, study their molecular physiology and thus the mechanistic basis of the observed heterogeneity in spore behavior during germination and outgrowth. In this way we provide a framework for future contemporary studies aimed at further detailing the mechanistic basis of food preservation and spoilage models targeting bacterial spores.

1.7 Thesis outline

In summary, bacterial spores are a major concern of the food industry. They are omnipresent and hence occur frequently in food ingredients and raw materials. Spores are highly resistant and thus escape the mild food processing regimes, which allows the spores to survive in the final food products and in some cases induces germination. This germinated spore then grows out and forms a vegetative cell, which, of this occurs in food products, readily leads to food-spoilage. The industries invest a huge amount in food quality control to reduce spore growth-related problems and to fulfill the consumer’s demand of mild processed foods as being natural and healthy. A thorough understanding of germination and outgrowth and heterogeneity is required. Such information might enable the design of processes that efficiently eliminate viable spores from foods. This would lead to

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an extended shelf life of food products, a reduction of spoilage and better product quality as well as restricted spore-mediated outbreaks. Moreover studying the heterogeneity in germination and outgrowth under stress conditions may improve the success of a hurdle preservation concept. Chapter 1 provides basic knowledge about bacterial spore structures, sporulation, germination and outgrowth, as well as the challenges for the food industry. Finally, the chapter briefly indicates techniques that can be used to study heterogeneity either in germination and/or outgrowth. Chapter 2 describes the in-house developed data analysis tool “SporeTracker”. The tool was developed to allow for efficient accurate and automated data processing from germination to outgrowth as well as doubling of vegetative cells. Chapter 3 describes a novel closed air-containing chamber developed for live imaging. The efficiency of the chamber was checked by monitoring the growth and division of B. subtilis vegetative cells, which was comparable to those obtained in well-aerated shake flask cultures. Finally the chamber was used to analyze Bacillus subtilis spore germination, outgrowth, as well as subsequent vegetative growth. The influence of a heat stress of 85°C for 10 min on germination, outgrowth, and subsequent vegetative growth was investigated in detail. In Chapter 4, the effect of tea compounds: gallic acid, gallo catechin gallate, Teavigo (>90% epigallocatechin gallate), and theaflavin 3,3’-digallate on germination and outgrowth of Bacillus subtilis spores was quantitatively analyzed at single cell resolution. The tested compounds had a significant effect on most stages of germination and outgrowth. Gallic acid most strongly reduced the ability to grow out. Additionally, all compounds, in particular theaflavin 3,3’-digallate, clearly affected the growth of emerging vegetative cells.

Chapter 5 describes the effect of sorbic acid, heat and combination of both on germination and outgrowth of Bacillus subtilis spores, which was quantified at, single cell resolution. Chapter 6 describes the Intracellular pH response to weak acid stress in individual Bacillus subtilis vegetative cells by making use of IpHluorin live-imaging. An improved version of the genetically encoded ratiometric, IpHluorin is used. This IpHluorin version, with an approximate 40% increase in cell specific fluorescence intensity, was expressed from the native B. subtilis promoter that is specifically active during vegetative growth on glucose (PptsG). Dual wavelength excitation ratio imaging was set up and allowed us to resolve the population data at single cell level. The weak organic acid food preservatives sorbic acid and acetic acid caused concentration-dependent intracellular acidification. In the presence of sorbic acid a decrease in the pHi and increase in generation time of growing cells were observed. Similar effects were observed when cells were stressed with acetic acid but at a higher concentration (25 mM). This shows that sorbic acid (KS) lowers the pHi more effectively than acetic acid (KAc). Cells with a lower pHi all showed a lower growth rate. Finally the Discussion, con-
clusion remark and future perspectives are described in Chapter 7 followed by summary of this research.

1.8 References


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