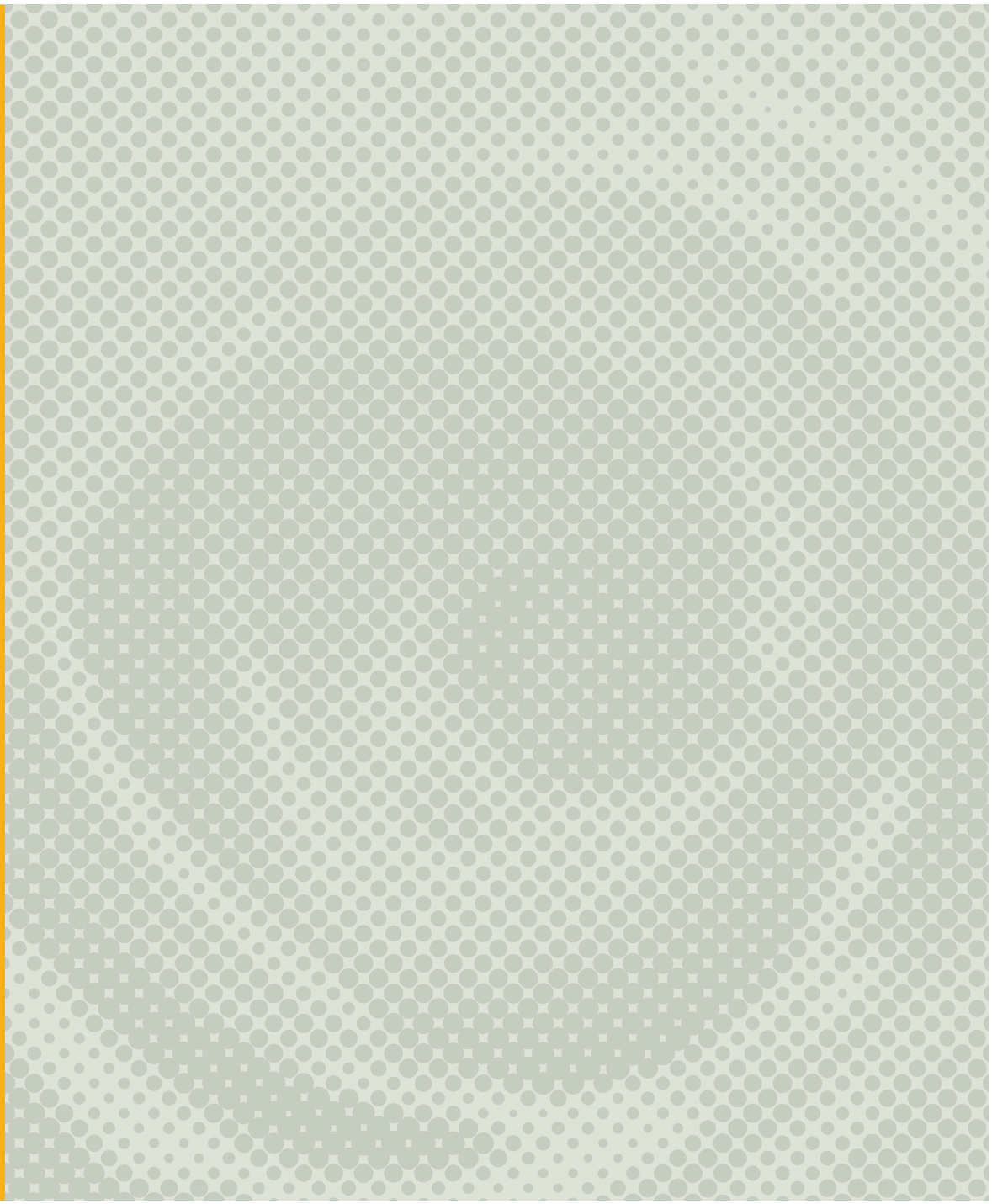
DIVERSITY IN GERMINATION RESPONSE AMONG *BACILLUS LICHENIFORMIS* STRAINS

KRISTIN CECILIA SAUE ROMUNDSET



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SAMMENDRAG

Flere studier har blitt utført for å beskrive germineringsegenskaper i forskjellige *Bacillus licheniformis*-stammer. Stammene har påviste forskjeller i operonet som koder for germineringsreseptoren GerA, som er forbundet med L-alanin-indusert germinering.

For å studere funksjonen til GerA-reseptoren ble en disrupsjons-mutant, der deler av *gerAA* i *gerA*-operonet er fjernet, konstruert fra en av stammene som er inkludert i undersøkelsene, MW3. MW3 ligner typestammen, men to type I restriksjons-modifikasjons-systemer er fjernet. Disrupsjonsmutanten ble benyttet til å konstruere to komplementeringsmutanter transformert med *gerA* fra MW3 og en annen stamme (NVH-1032). Ytterligere tre stammer (NVH-B357, NVH-800 og NVH-1112) ble inkludert senere, og tre nye komplementeringsmutanter med *gerA* fra disse stammene ble konstruert.

For å kartlegge uttrykk av *gerA* i komplementeringsmutanten sammenlignet med villtypen ble en real-time PCR-analyse gjennomført for *gerAA*, *sigG* og *rpoB*. Analysen ga relativ ekspresjon av *gerA* og *sigG*, som kontrollerer ekspresjon av reseptoren sammenlignet med et «house-keeping»-gen, *rpoB*, som uttrykkes relativt stabilt. Til å begynne med skal MW3 og dens komplementeringsmutant analyseres, og på bakgrunn av disse resultatene vil eventuelt flere stammer inkluderes i analysen.

Germineringsanalysene viste klare forskjeller mellom de forskjellige stammene, som muligens kunne relateres til forskjeller i *gerA*-sekvensene. Disrupsjonsmutanten germinerte i liten eller ingen grad. En analyse med kalsium og dipikolinsyre som germinant ble utviklet for å omgå reseptormediert germinering. Foreløpige resultater viser uensartet germinering for de forskjellige stammene. Analyseresultatene viser at disrupsjonsmutanten kan germinere med disse germinantene til tross for at GerA mangler.

På dette tidspunktet presenteres kun ett resultat fra real-time-PCR-analysen. Dette viser uttrykk av *gerA* gjennom sporulering av MW3. Det ser ut som tendensen er at *gerA*-uttrykkes omtrent ti ganger høyere i komplementeringsmutanten sammenlignet med villtypen. Videre analyser er nødvendige før det er mulig å konkludere med noe.

ABSTRACT

Several studies have been conducted to describe germination properties in different strains of *Bacillus licheniformis*. The strains showed differences in the operon encoding the germination receptor GerA which is associated with L-alanine induced germination.

As a means to study the function of the GerA receptor, a disruption mutant lacking part of *gerAA* in the *gerA* operon had previously been constructed from one of the strains included in the studies, MW3, which resembles the type strain and lacks two type I restriction modification system loci. This disruption mutant had been used to construct two complementation mutants with *gerA* from the strain described and another strain (NVH-1032) transformed on a low-copy plasmid. Three additional strains (NVH-B357, NVH-800 and NVH-1112) were included in the ongoing studies. The *gerA* operon was extracted from each of these strains and used to construct three new complementation mutants.

In order to determine whether the complementation mutants expressed the same amount of the GerA receptor as the wild types, a real-time PCR assay was conducted analysing *gerAA*, *sigG* and *rpoB*. This analysis showed the relative expression of *gerA* and *sigG*, which controls the expression of the receptor compared to a house-keeping gene, *rpoB*, with show a relatively stable expression. Initially MW3 and its complementation mutant will be studied, and based on these results more strains may be included as well.

The germination assays showed clear differences between the strains, which could possibly be related to differences between the *gerA* sequences. The disruption mutant showed little or no germination. A germination assay using fairly high concentrations of calcium and dipicolinic acid was developed in order to bypass receptor mediated germination. Preliminary results from this show a heterogeneous response to the germinants. The results show that the disruption mutant is able to germinate with these germinants even though it lacks the GerA receptor.

In this thesis, only one result from the real-time PCR assay, showing the expression of *gerA* during sporulation of MW3, is presented. Preliminary analyses have shown that the expression of *gerA* in the complementation mutant is about ten times higher than the wild type, but further analyses will have to be conducted before a conclusion can be reached.

CONTENTS

1 INTRODUCTION

Endospore (spore) forming bacteria are able to form dormant spores for long-time survival. This is accomplished during an alternate cell cycle with asymmetrical cell division and the formation of a spore (endospore) inside the original cell (mother cell), which lyses when the sporulation process is completed. The ability to form dormant spores is a trait found in many members of the genera of *Bacillus* spp. and *Clostridium* spp. and their relatives (Paredes-Sabja *et al* 2010; Setlow 2007; Granum 2007).

Bacterial spores have the ability to survive environmental extremes like desiccation, heat, pH, radiation, disinfection and other chemical treatments that would normally inhibit, injure or kill vegetative cells (Setlow 2007; Paredes-Sabja *et al* 2010; Granum 2007; Moir 2005). Many sporeformers are associated with food poisoning and/or spoilage. Studies of their pathogenic potential and their properties and behaviour in food are therefore of interest when attempting to prevent illness and food spoilage (Logan 2012; Mafart *et al* 2010; Carlin 2010).

In the industrialized parts of the World a public demand for more "natural", unprocessed foods with less additives and lower salt content is increasing. In addition to this, consumers also want food with fast and simple preparation, for example ready-to-eat meals or various cook-chill products (Gould 2000; Mañas, Pagán 2004; Kapperud 2007). Both of these demands come in addition to the public requirements for food safety – the easy-to-prepare, "natural" foods should not pose a threat (Mafart *et al* 2010; Gould 2000; Hornstra *et al* 2009). That is, microorganisms have to be removed from or controlled in food products. In total these demands are challenging the food industry in regard to microorganisms present in foods (Kapperud 2007).

Most microorganisms in foods are not harmful to man or animals, whereas some may cause disease. Some microorganisms are relatively easy to prevent from growing or to kill so that they present little or no risk for the consumers. However, not all microorganisms are easily controlled and might cause problems (Gould 2000). The fact that different species and strains are present in foods complicate matters, as a method developed to prevent problems caused by some species are not effective against others (Eijlander, Abee, Kuipers 2010; Carlin 2010). Endospores are the most difficult microorganisms to kill during food production, since many will not even be killed by boiling (Eijlander, Abee, Kuipers 2010; Granum 2007). When heat-treating food, the result may be that the vegetative cells are killed, whereas the endospores remain viable.

Thereafter the spores may germinate and form new vegetative cells (Granum 2007; Carlin 2010). If temperature and other growth conditions are favourable, they will grow rapidly to a large population without competition from other species.

Many sporeforming species and strains are found in soil, plants or on grain, and some live in or in contact with insects and animals (Nicholson 2002; Logan 2011; Carlin 2010). The possible routes into the food processing chain are therefore numerous, and are not always possible to avoid, as illustrated in Figure 1 (Carlin 2010). As more foods circulate the market from different parts of the world, microorganisms "travel", and countries are faced with the possibility of new foodborne diseases previously not relevant for these areas (Kapperud 2007). Introduction of new plants and animals may also introduce new microorganisms to the environment. Therefore studying strains that mainly cause food spoilage – and thereby economic challenges – may be of importance also when it comes to prevent foodborne diseases, as new strains able to cause disease may be introduced.

Many studies of endospore function and how to control or kill them have been performed, and with a growing understanding new questions and problems continuously arise (Mafart *et al* 2010). It might also be of interest to the food industry to know how to prevent vegetative cells from forming spores within the industry production plants.

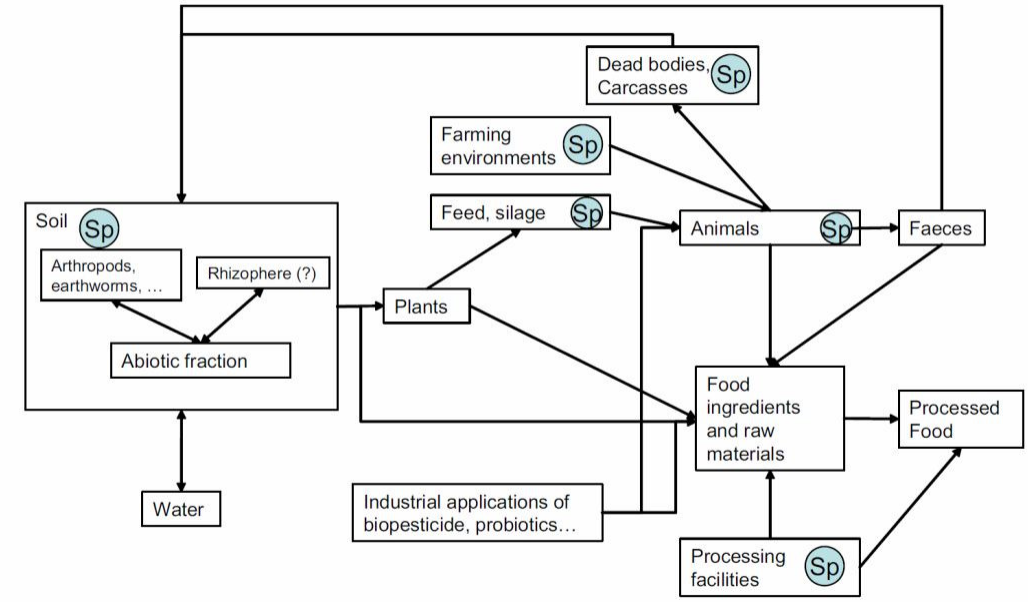

Figure 1: The various entry-ways of microorganisms in food production. Sp marks where endospores may enter the foods. (Carlin 2010)

Removal of spores is done either by autoclavation or by inducing spore germination, so the vegetative cells or germinated spores can be killed by temperatures well below 100 ºC (Mafart *et al* 2010; Eijlander, Abee, Kuipers 2010; Gould 2000). Studies on spore germination can therefore be of great interest. These studies include germination properties and conditions favouring germination, and studies on the molecular and the genetic level. To gain knowledge that can also be of practical use, several species and strains need to be studied under different conditions. Ultimately *in vitro* studies made with different strains in the laboratory will lead to a broader understanding, that later can be adapted to industrial food processing. (Mafart *et al* 2010)

There are still many unknown factors concerning spore germination. Most details of germination are based on results from studies of *Bacillus subtilis*, although detailed knowledge of other sporeformers is increasing. *Clostridia*, *Bacillus cereus* and *Bacillus anthracis* also have been studied to great detail because of their ability to cause disease. (Ross, Abel-Santos 2010; Paredes-Sabja, Setlow, Sarker 2010)

1.1 AIMS OF STUDIES

In this thesis, various analyses aimed to describe the germination properties in *Bacillus licheniformis* are presented. This work is part of an ongoing project aiming to describe germination properties, both phenotypic and genetic, in *B. licheniformis,* a bacterium previously not described in great detail. *B. licheniformis* is known as a frequent food contaminant able to cause food spoilage and in some cases foodborne illness (Salkinoja-Salonen *et al* 1999**)**. It is closely related to *B. subtilis*, and an ortholog of the germination receptor GerA is predicted to exist in *B. licheniformis* (Løvdal *et al* 2012; Paredes-Sabja, Setlow, Sarker 2010).

As part of the ongoing project, a disruption mutant lacking part of the *gerA* operon had previously been constructed. From this disruption mutant, two complementation mutants with *gerA* from two different strains introduced on plasmids had been constructed. (Løvdal *et al* 2012; Klufterud 2011)

The aims of the studies presented in this thesis were as follows:

1. Investigate the germination phenotype of the disruption mutant compared to the strain used in constructing this.

2. Ascertain whether the phenotype seen in the disruption mutant was due only to the disruption in *gerA*, by comparing germination properties in the strain used in constructing the disruption mutant and the complementation mutant harbouring *gerA* from this strain.
3. Examine the germination properties of another wild type strain and the complementation mutant with *gerA* from this new wild type strain.
4. Investigate non-nutrient induced germination in the wild type strains and the disruption mutant to determine whether the disruption mutant was capable of germination without the GerA receptor.
5. Construct three new complementation mutants with *gerA* from three strains with known genetic differences in the *gerA* operon and begin germination studies on these new strains.
6. Compare the expression of *gerA* in one wild type and the complementation mutant with *gerA* from this by means of real-time PCR.

1.2 THE ENDOSPORE

The endospore consists of an inner core surrounded by an inner spore membrane and a peptidoglycan layer named the germ cell wall. Outside the germ cell wall lies another, thicker peptidoglycan layer, named the spore cortex, and then the outer spore membrane. On the outside of this membrane lies the spore coat and for some species the exosporium. The various layers in the spore are illustrated in Figure 2. (Setlow 2003; Paredes-Sabja, Setlow, Sarker 2010)

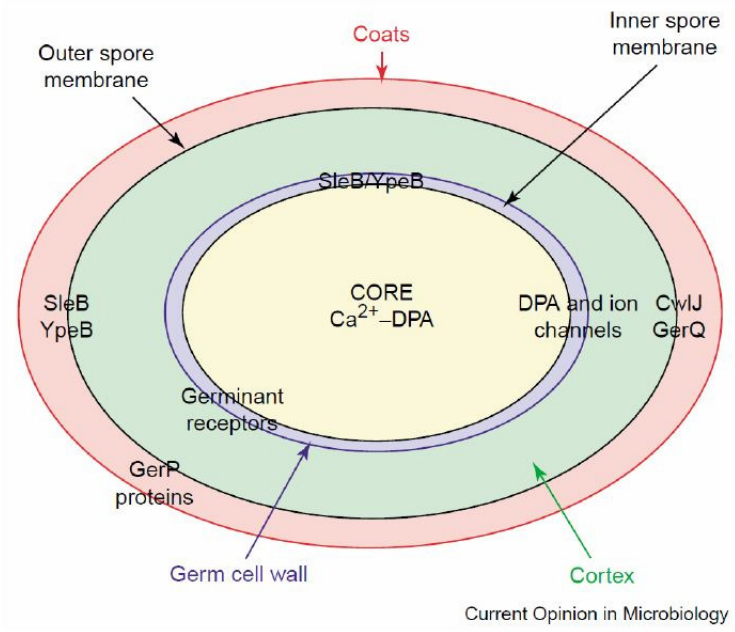

Figure 2: A schematic illustration of a bacterial spore. The exosporium is not shown, and the various structures and layers are not proportionally drawn. (Setlow 2003)

The core contains the spore DNA, RNA and most of the spore's enzymes. The DNA is saturated with small acid soluble proteins (SASP) and ~10 % of the core dry weight is a 1:1 chelate of calcium and pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA). Water provides 25–50 % wet weight. (Setlow 2003)

The inner spore membrane has a low permeability, and consists of a compressed, relatively immobile lipid layer (Setlow 2003). The spore's germination receptors and the cortex lytic enzyme SleB (also found in the spore coat) are found in the inner spore membrane (Hudson *et al* 2001; Setlow 2003). The germ cell wall consists of peptidoglycan with a structure similar to that of the cell wall in vegetative cells (Setlow 2003). It is believed that this peptidoglycan layer forms the cell wall in the vegetative cell emerging from the spore during germination and outgrowth. (Setlow 2003)

The cortex is a thicker peptidoglycan layer with structure similarities to the cell wall of growing cells, however with three structural traits specific for the spore cortex (Popham *et al* 1996): The cortex peptidoglycan has a lower degree of crosslinking due to a lower amount (~25 %) of substitution of the N-acetylmuramic acid (NAM) residues with short peptides. In addition ~25 % of the NAM residues carry a single L-alanine residue and ~50 % of the muramic acid residues are converted to muramic-δ-lactam, both of which are found in the cortex, but not in the germ cell wall. Muramic-δ-lactam has

been suggested to be the target of cortex-lytic enzymes during germination and outgrowth, as mutant strains without this structure have been shown to be unable to complete the germination process (Popham *et al*, 1996). The outer membrane has a higher permeability than the inner membrane. The spore coat consists of more than 50 spore specific proteins. The cortex lytic enzymes SleB and CwlJ are likely both located in the spore coat (Moir 2005).

1.2.1 Spore properties and ability to survive

The dormant spore can survive for long periods (up to thousands to millions of years) (Nicholson 2002) without nutrients, and express high resistance against environmental extremes (e.g desiccation, heat, radiation, toxic chemicals and extreme pH) that would kill the vegetative cell (Dworkin, Shah 2010; Nicholson *et al* 2007).

Dormancy, a period with no detectable metabolic activity, enables the spore to survive for long periods and still be able to come to life when the conditions are right (Dworkin, Shah 2010). There have been reports on spores found in Egyptian tombs or extracted from million years old amber have been successfully germinated when introduced in appropriate media (Nicholson 2002). The dehydration of the core and the spore contributes largely to the resistance against heat and desiccation (Sanchez-Salas *et al* 2011). The spore cortex is believed to contribute to this dehydrated state, and thereby to heat resistance (Nicholson *et al* 2000).

The spore coat has some resistance against chemicals and has low permeability to peptidoglycan lytic enzymes (Nicholson *et al* 2000). Other contributing factors to chemical resistance are dehydration, low permeability of the spore core to hydrophilic chemicals and protection of the spore DNA by SASPs (Setlow 2007).

Resistance against UV radiation is conducted by a DNA repair apparatus, where damages to the DNA are repaired during germination and outgrowth, and changed photo chemistry, likely by means of SASPs protecting the DNA (Setlow, Setlow 1996). Dehydration likely plays a role in the resistance against γ radiation. The low permeability of the inner membrane likely protects the core against external factors that might damage the DNA. (Nicholson *et al* 2000)

Spores react differently to hydrostatic pressure than vegetative cells, and have a much higher sustainability against pressure that kills vegetative cells. Spores may germinate under high pressure, and be killed by the pressure as a result of lowered resistance. (Paidhungat *et al* 2002; Gould 2000)

The spore properties mentioned make endospores difficult to kill or inactivate, and provide valuable competitive attributes for the sporulating species compared with non-sporeformers.

1.2.2 Sporulation

Sporulation describes the process where the vegetative cell ends the normal cell cycle and initiates an alternative route of the cycle, ending with one spore instead of two identical vegetative cells. Sporulation may be considered a last resort in preservation of the cell's DNA. When nutrients are depleted, cells in a culture enter the stationary phase, where the growth rate slows down, and during the following death phase, replicative growth is no longer supported. With scarce nutrition, sporeformers have the choice of sporulation. The cells are in a transition state, with gene expression of functions needed to explore other nutrient groups and competitive functions like antibiotics against other species. The cells may also express competence, in order to acquire new DNA that may be of help in scavenging the environment for nutrients. This is believed to be the state most *Bacilli* are expressing in the wild. The cell will undergo sporulation, an energy-costly process where more than 100 sporulation specific genes are expressed in *B. subtilis,* if it cannot find nutrients to support growth. Genes encoding spore specific proteins and receptors needed to undergo germination (see 1.3) are expressed during sporulation. (Phillips, Strauch 2002; Hornstra *et al* 2009)

It has been demonstrated that cells in the same population sporulate at different times, where each cell has its own decision point (Phillips, Strauch 2002). This decision point may be linked to the level of phosphorylation on the sporulation gene Spo0A. It is believed that the decision to induce sporulation is made generations before sporulation is initiated (Hornstra *et al* 2009).

The sporulation process is divided in seven stages, as shown in Figure 3, with stage 0 being the vegetative cell. Stage I describes the formation of an axial chromatin filament consisting of two chromosome copies in the cell centre. This filament divides and in stage II about 30 % of the DNA from one chromosome is enclosed by a forespore septum. This septum, formed by invagination of the membrane near one cell pole, creates two distinct cell compartments, each expressing their own set of genes during sporulation. During further sporulation the spore will acquire the rest of the chromosome as well. Stage III follows with the completion of the spore membrane as the mother cell fully engulfs the forespore, giving this two membranes. In the transition between stage III and

IV, small acid soluble proteins (SASP) are synthesized, binding to the DNA. The spore develops UV and chemical resistance at this stage, and water content and pH in the spore are lowered. In stage IV two peptidoglycan layers, which become the germ cell wall and the spore cortex, are formed between the membranes. The exosporium is developed at this stage in species carrying this structure (e.g. *B. cereus*). Sporulation continues with stage V and formation of the spore coat outside the outer membrane. Dehydration and chemical resistance development continues, along with resistance against γ-radiation. Between stage V and VI dipicolinic acid is produced in the mother cell and transported into the spore in 1:1 chelates with divalent cations, completing the dehydration of the spore. During stage VI the spore matures, developing greater resistance and reaches dormancy. The spore appears as phase bright inside the mother cell. Sporulation ends with stage VII, where the mother cell lyses, releasing the completed spore. (Løvdal 2011; Archunan 2004; Nicholson, Setlow 1990; Phillips, Strauch 2002)

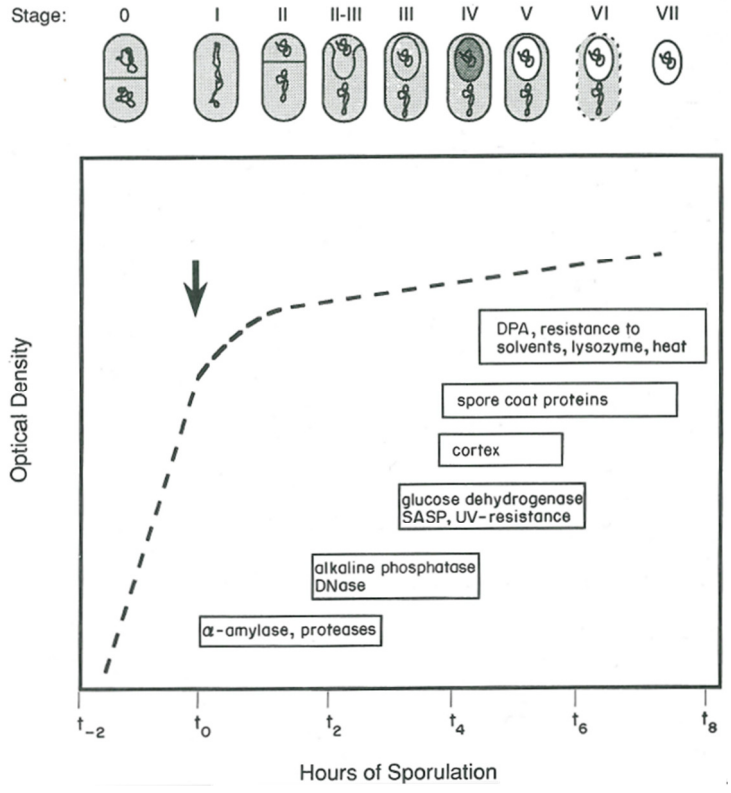

Figure 3: Idealized growth and sporulation curve in *B. subtilis*. Sporulation in this system is defined as the end of exponential growth and is marked by the arrow. The sporulation stages are shown as schematic drawings and the occurence of various molecular structures are outlined in the boxes below the curve. (Nicholson, Setlow 1990)

Several factors like pH (Baril *et al* 2011), temperature (Planchon *et al* 2010; Gounina-Allouane, Broussolle, Carlin 2007; Baril *et al* 2011; Garcia, van der Voort, Abee 2010), ions present (Riemann, Ordal 1963) and sporulation medium may influence sporulation and the properties of the finished spore (Eijlander, Abee, Kuipers 2010; Carlin 2010; Hornstra *et al* 2009). In addition stochastic variations within a sporulating population result in differences between individual spores in the same spore population (Maughan, Nicholson 2004; Dworkin, Shah 2010). Knowledge of how different factors are affecting the resulting spores during sporulation is important, as these properties need to be taken into consideration when studying germination or methods to minimize spores as a problem in the food industry (Eijlander, Abee, Kuipers 2010; Carlin 2010). The finished spores are also influenced by factors of the storage environment and will change properties as it ages (Sanchez.Salas *et al* 2011; Løvdal, Granum, Rosnes 2011; Hornstra *et al* 2009). The different factors that may influence the spore through sporulation, dormancy and germination are illustrated in Figure 4. As a result, great variations are found both between spores from different species and strains, but also within the same strain and even in the same spore population.

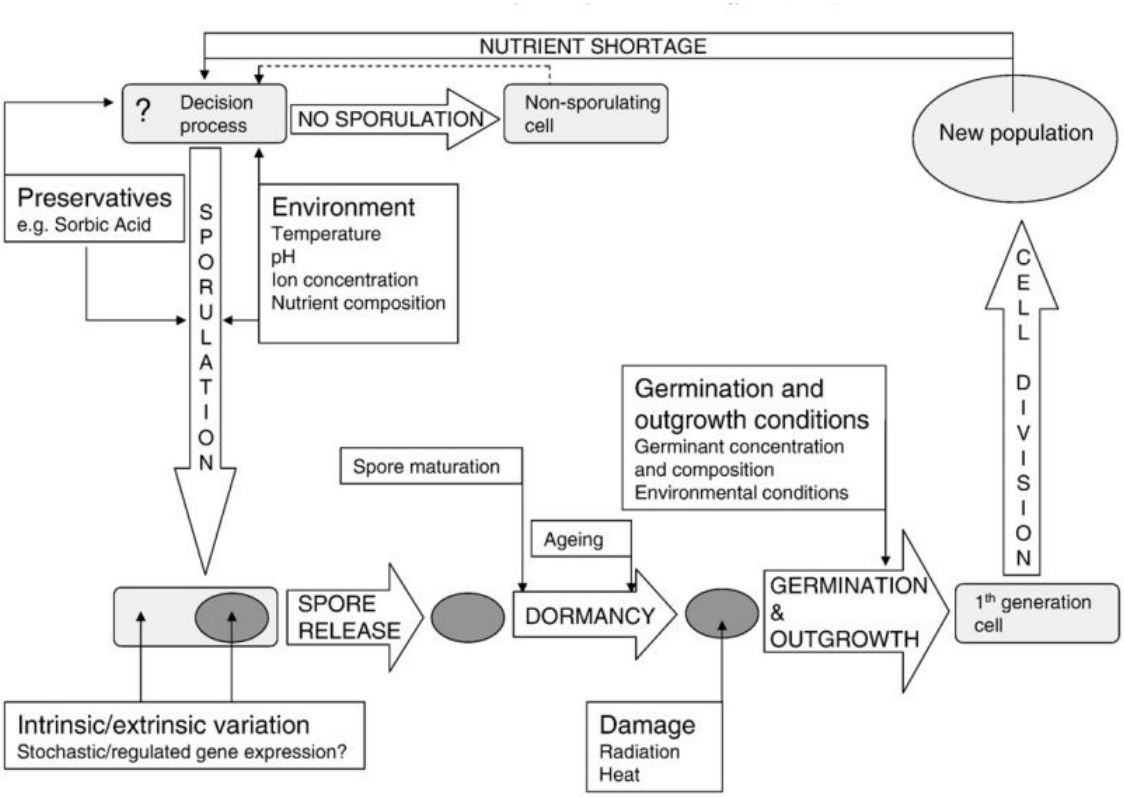

Figure 4: An outline of the various factors influencing spore properties during sporulation, dormancy and germination. (Hornstra, Beek et al 2009)

1.3 GERMINATION OF ENDOSPORES

The dormant spore has the ability to sense its environment, and when the environment turns favourable for vegetative cell growth, the spore can quickly undergo germination and outgrowth to a new vegetative cell. Several germination pathways are described, both with and without the use of relatively specific germination receptors. It is believed that receptor initiated germination is the main germination pathway in nature. All details of spore germination are not yet known, and especially the activation step of germination is still unclear. There are differences in germination properties and germination receptors, both between species and within the same species. (Paredes-Sabja, Setlow, Sarker 2010)

Germination can be a spontaneous process, seen for example in aged spores. However, it is believed that this also is germinant dependent in some way. With receptor mediated germination, the germinants need to penetrate the outer spore layers to access the inner membrane where the germination receptors are located (Moir 2005; Paredes-Sabja, Setlow, Sarker 2010).

In order to investigate germinant specification of the receptors present, a spore population can be exposed to different media or single substances such as amino acids and sugars in different concentrations and combinations, to see whether a germination response is initiated. During such experiments it is important to be aware of other factors that could affect the result as described in 1.2.2. Sporulation environment, storage conditions of the spores, spore age and germination environment all play a role in the germination properties of a spore (Eijlander, Abee, Kuipers 2010).

The importance of ionic environment both during sporulation and germination was described by Fleming and Ordal (1964). They demonstrated that germiniation of *B. subtilis* in L-alanine could be highly dependent on ionic concentrations in the germination and sporulation environment. Divalent cations were least active in supporting germination, while phosphate showed a synergistic response in the presence of germinants. Higher concentrations of NaCl in the sporulation environment resulted in spores with lower germination response. Addition of calcium reversed this effect. Germination with L-alanine and different buffers showed that a phosphate buffer gave better germination than a tris buffer.

It has also been demonstrated that some substances are able to inhibit the germination response. D-alanine is directly inhibiting germination with L-alanine, and some species also express the enzyme alanine racemase, which transforms L-alanine to D-

alanine (Eijlander, Abee, Kuipers 2010). It is believed that this is a spore precaution against early germination in environments that cannot sustain bacterial growth, or to prevent germination of the forespore during sporulation. When L-alanine reaches a threshold, the spores are not able to transform all the molecules into D-alanine, and germination starts.

1.3.1 Activation

Activation is a process that may occur or be induced before germination. The result of activation may be a more homogenous germination response within a spore population. Activated spores retain most of their properties, and dependent on the activation agent, the activation process may be reversed. Little is known about the mechanisms at work during activation of endospores. When looking at a spore population, different ways of activation can synchronize the germination response and make it faster, when presenting the spores to germinants. Heat-activation, the use of sub-lethal heat treatment, is most commonly used. This is also the most studied activation step and is described as being the most efficient activation procedure. Other activation procedures include low pH or reducing chemical agents. Prolonged storage of spores, including at low temperatures, such as in a refrigerator, will also activate the spores, in the process known as ageing. (Keynan, Evenchik 1969; Berg, Sandine 1970)

It is possible to reverse activation of spores. Studies of heat activated spores showed that storage of the activated spores at low temperatures resulted in fewer germinating spores when plating the spores on media with germinants. This deactivation has not been shown in activation by ageing. (Collado *et al* 2002; Keynan, Evenchik 1969)

van der Voort *et al* (2010) have demonstrated the diversity in germination properties in *B. cereus* strains, and that at least some strains need to be activated before they germinate, whereas others do not depend on activation in order to germinate.

1.3.2 Germination and outgrowth

The germination process deprives the spores of their ability to sustain environmental extremes and is the transition between metabolically dormant spores and vegetative growing cells. Germination is divided in two stages followed by outgrowth to a vegetative cell, as shown in Figure 5. (Moir, Corfe, Behravan 2002; Setlow 2003)

Stage I is characterized by release of monovalent cations, mainly K+ and H+ from the core, followed by loss of divalent cations and DPA and hydration of the spore core.

During this process the spore loses some of its resistance. Stage II follows with hydrolysis of the spore cortex and further core hydration. The germ cell wall expands, i.e. the spore expands in size, and the spore loses more of its resistance and its dormancy. (Setlow 2003)

The outgrowth follows germination, with metabolic activity, degradation of small acid soluble spore proteins (SASP), synthesis of ATP and escape from the spore coat. After completing the outgrowth to a vegetative cell, normal cell cycle continues as long as nutrients are provided. (Moir 2005)

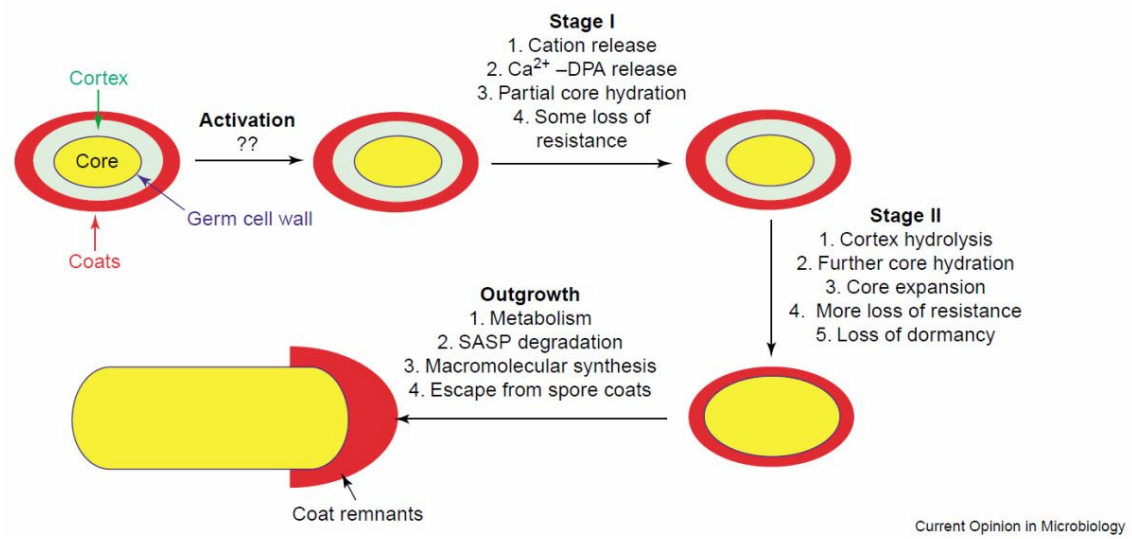

Figure 5. A schematic illustration of germination and outgrowth from a dormant spore to a vegetative cell. (Setlow 2003)

1.3.3 Germination receptors and nutrient germinants

Several germination receptors are described in the *Bacillus* genus, responding to nutrient germinants (Ross, Abel-Santos 2010). Nutrient germinants are small molecules of amino acids or sugars that can induce germination by interaction with one or more germination receptors. The germinants themselves are not metabolized during this process (Moir, Corfe, Behravan 2002). Germination receptors are often present in relatively low numbers (~25) in a spore (Eijlander, Abee, Kuipers 2010; Hudson *et al* 2001).

Some germination receptors respond to a single germinant, while some need a co-germinant to initiate germination (Ross, Abel-Santos 2010; Atluri *et al* 2005). In addition there are receptors that do not function alone, but can initiate germination only together

with other receptors (Ross, Abel-Santos 2010; Atluri *et al* 2005). The germination response is dependent on the germination environment, accessible nutrients and the spore's germination apparatus, including the number of different germination receptors present in the spore.

Receptor mediated germination involves interaction between a germination receptor and a germinant (Ross, Abel-Santos 2010). As described in 1.3, the germinant has to pass through the outer spore layers in order to reach the receptor. The receptor binds the germinant, leading to the cascade of steps known as germination of the spore (Ross, Abel-Santos 2010).

The first described step is commitment, where germination will continue regardless of the access to germinants. In a spore population some spores germinate even if the germinant is removed or the interaction between germinant and receptor is blocked. Heat-activation, germinant concentration and number of receptor proteins present have all been shown to increase the number of committed cells. (Yi, Setlow 2010)

B. subtilis, the type species, is one of the most thoroughly studied species, and the first germination receptors were described in *B. subtilis* spores (Moir 2005). Most details of how germination receptors work are based on studies of the GerA receptor in *B. subtilis*, which is the best studied germination receptor. This receptor has given name to the GerA receptor family, consisting of GerA homologues. The GerA receptor has been described as responding to L-alanine without the help of other germinants or receptors. However, the germination reaction to L-alanine can be enhanced by help of other proteins, like GerD in *B. subtilis* (Mongkolthanaruk, Robinson, Moir 2009), and various ions (Fleming, Ordal 1964). In the sporulating bacteria (e.g. *Bacilli, Clostridia*) there are several homologues to this receptor, responding to various germinants. Most sporulating bacteria have a variation of the GerA receptor, responding to L-alanine, either alone or in combination with other nutrients or receptors (Ross, Abel-Santos 2010).

The GerA receptor is encoded by a three-cistronic operon, *gerA*, which is transcribed by σ^G, and consists of three proteins (Ross, Abel-Santos 2010). These are predicted to be two integral proteins with transmembral segments (GerAA and GerAB) and one lipoprotein (GerAC) which is transferred through the forespore membrane and affixed on this, as shown in Figure 6 (Ross, Abel-Santos 2010). In order for the GerA receptor to function, all three subunits must be present (Paidhungat, Setlow 2000).

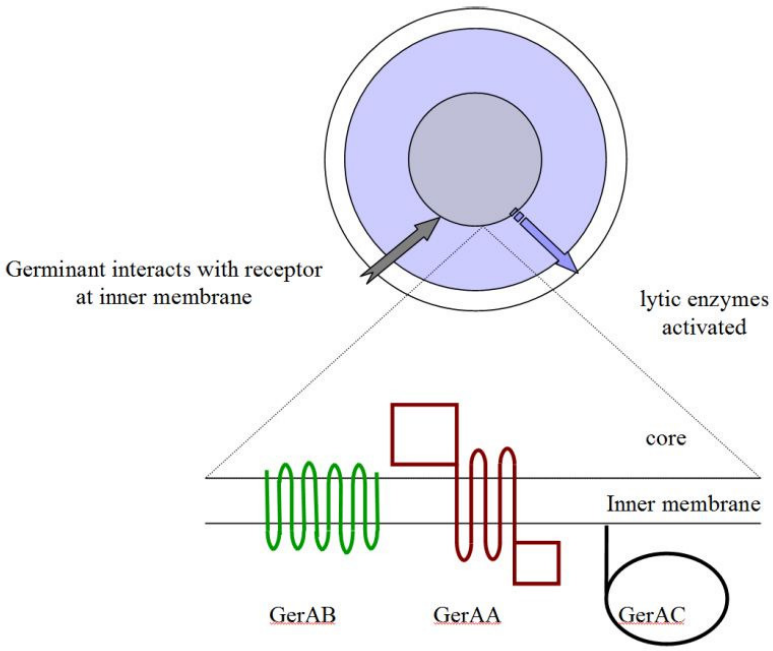

Figure 6. The localization of the three subunits of the GerA receptor in *B. subtilis*. (Corfe, Moir, Behravan 2002)

In *B. subtilis*, two other receptors in the GerA family have been described (Paidhungat, Setlow 2000). The GerB receptor works together with another receptor, GerK, to enable germination with a mix of asparagine, glucose, fructose and potassium ions (AGFK). Two additional GerA homologues (*yndDEF* and *yfkQRT*) with hitherto unknown function are predicted (Paidhungat, Setlow 2000).

The expression of germination receptors, i.e. the number of receptors present in a spore, is believed to affect the germination response. Overexpression may enhance the germination rate (Cabrera-Martinez *et al* 2003). However, overexpression of some germination receptors is possible only to some extent, as it can lead to premature germination during sporulation, where the spores lyse before they are completely developed, as has been demonstrated in overexpression of the *gerA* operon (Cabrera-Martinez *et al* 2003). It is a possibility that the low expression of germination receptors is a means to protect the spores against germinating prematurely in environments incapable of supporting vegetative growth (Ross, Abel-Santos 2010). Mutations in one or more genes encoding a receptor also affect germination (Paidhungat, Setlow 2000). A mutation may affect binding between receptor and germinant, or interaction between the different receptor proteins, it may be related to signal transmission, or it can lead to defect proteins.

1.3.4 Non-nutrient germination

Spores can also germinate without involving germination receptors, e.g. by help of various non-nutrient substances, such as CaDPA (de Vries 2004; Paidhungat, Ragkousi, Setlow 2001) or dodecylamine, or by outer stimuli, e.g. high pressure (Moir 2005; Setlow 2003). An overview of various ways of inducing germination in spores is outlined in Figure 7. Non-nutrient germination is likely not the main germination route in nature, but some germination inducing substances, e.g. CaDPA, and structures of peptidoglycan may be of some importance. CaDPA is released from germinating spores, and might trigger germination in neighbouring spores. It has also been shown that spores respond to muropeptides derived from breakdown of peptidoglycan from growing cells of the same or a related species to the spore. It is a possibility that this may trigger germination in nature as well as in the laboratory. Non-nutrient germination can bypass the germination receptor induced pathways in various manners, or may induce receptor mediated germination without nutrients present. High pressure may activate germination by means of germination receptors up to 100 MPa, while higher pressure does not activate said receptors (Moir 2005).

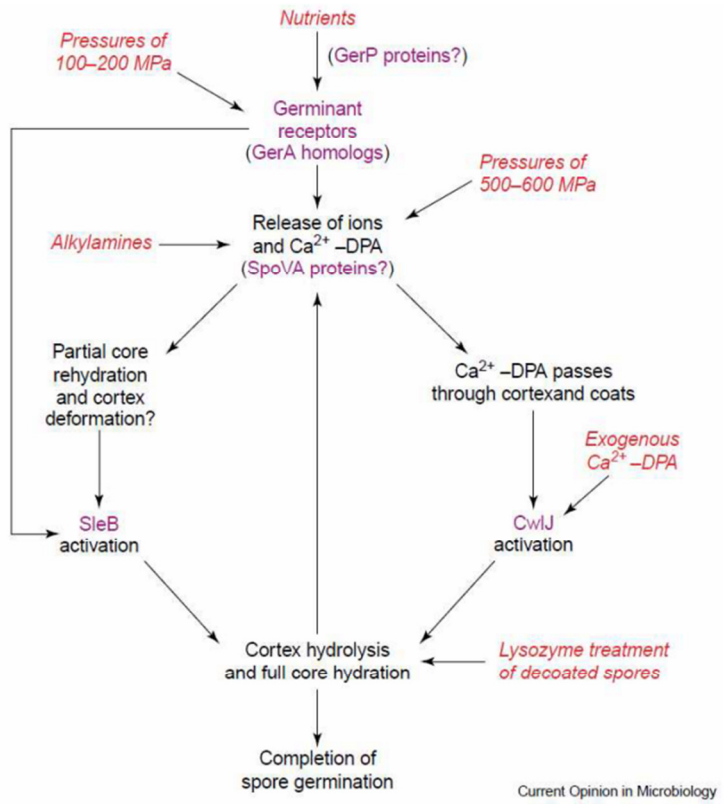

Figure 7. Model of nutrient and non-nutrient germination in *B. subtilis*. (Setlow 2003)

It is possible to germinate spores by exposing them to relatively high amounts of dipicolinic acid (DPA) and divalent ions, mainly calcium. CaDPA has been shown to activate the cortex lytic enzyme CwlJ directly, which then degrades the spore cortex (Paidhungat, Ragkousi, Setlow 2001; Ragkousi *et al* 2003). Endogenous DPA is released and the spore is hydrated, ultimately losing dormancy and resistance, growing out to a vegetative cell. Spores lacking CwlJ are still able to germinate in presence of nutrient germinants, due to the cortex lytic enzyme SleB (Ragkousi *et al* 2003). This enzyme does however not hydrolyse the cortex germination when only CaDPA is present. Riemann and Ordal (1961) investigated whether other metal ions could induce germination with DPA, and if DPA could be substituted with other pyridine dicarboxylic acids and induce germination in various *Bacillus* species. Their results showed that the effect on spores exposed to Ca^{2+} and DPA was specific for these substances, and that neither could be substituted and still induce germination of the spores.

1.3.5 Detecting germination

Germination is a process that can be measured and detected in various ways. As spores germinate, their light scattering properties are changed, and they release their stored CaDPA from the core and become less resistant to heat. All of these characteristics may be used to detect germination. (Løvdal 2011)

During germination the spore goes from phase bright to phase dark as seen in a phase contrast microscope (Figure 8), as refractivity light scatter is changed (Løvdal 2011). This translates to an OD_{600} loss of up to 60 % of the original OD_{600} for a spore population (Løvdal 2011). OD_{600} is the optical density, or light absorbance, at 600 nm, and describes transmission of light at this wave length through a solution or object. Usual methods for detecting germination include visual control with the help of a phase contrast microscope and various absorbance measurement assays (Løvdal 2011). In addition, measurement of DPA released from the spores can be used to determine germination. This is possible by measuring A_{270} in the supernatant fluid from the germinating spores (Vepachedu, Setlow 2004).

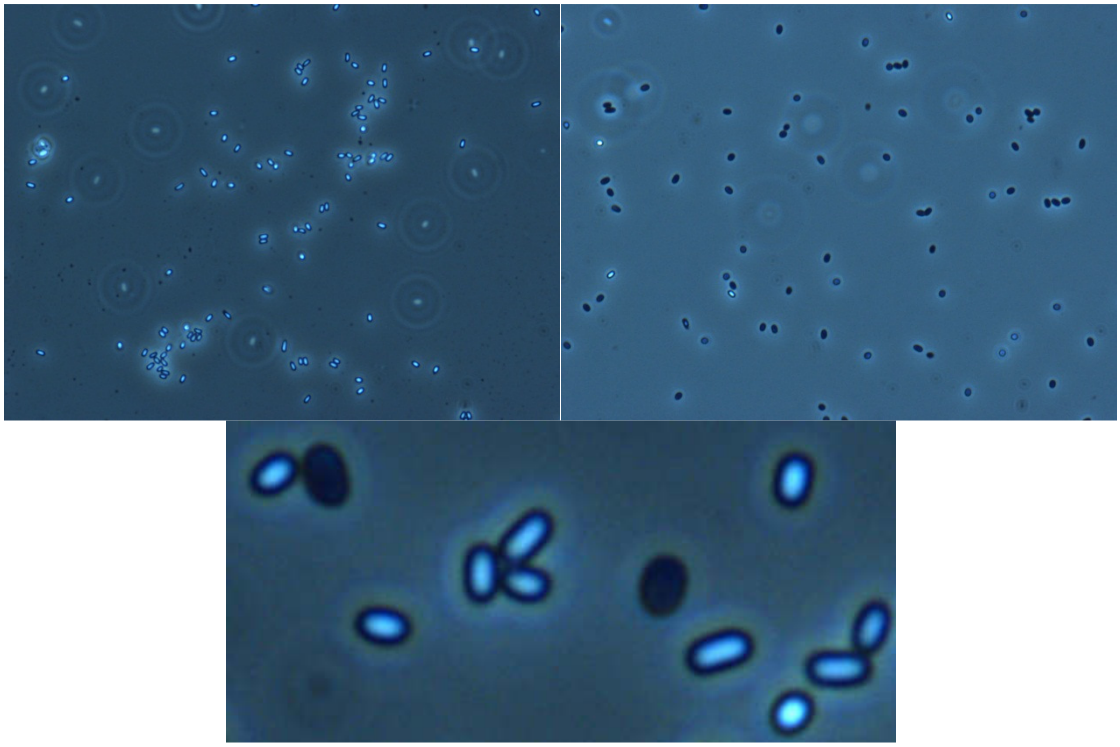

Figure 8. Phase contrast images (100 x) showing germination in *B. licheniformis*. Top left: un-germinated (phase bright) spores. Top right: Germinated (phase dark) spores. Bottom: Detailed image of two germinated and some ungerminated spores. (Pictures taken using Olympus BX51 with ColorView Illu camera and Cell^B software)

Measurement of heat-resistance is another possibility when detecting germination. Here both germinated and un-germinated (negative control) spores are heat-treated at temperatures which inactivate germinated but not un-germinated spores, before plating dilutions on appropriate solid media and counting the growing colonies after incubation. Detecting germination by measuring OD_{600} loss or loss of heat resistance are both useful when studying germination in spore populations. (Løvdal 2011)

Studying germination in populations by use of OD_{600} is associated with some disadvantages. In a population, the spores are not identical, and will respond differently to germinants and germinant environments. In a population only a germination tendency can be studied in the population as a whole, with a probability of each spore germinating within a given time. In populations with low germination, OD_{600} measurements may not even show the response. Spores can also clot together or adhere to the surfaces in the testing container where OD_{600} is measured, thereby complicating measurements and interpretation of these. When germination exceeds 90 % it is also difficult to show differences between populations in the assay. In other words, OD_{600} is a good tool for observing germination in populations which show a relatively good germination response.

When comparing different strains, the response should differ at least 10–20 % for this method of observation to be effective. Phase contrast microscopy can be used as a means of control when studying germination by measuring OD_{600} in a population. However, phase contrast microscopy as single method of observation is a time consuming process where quality control of the results are more difficult to ensure. (Løvdal 2011)

Some techniques to study germination in single spores are developed as well. These include Raman spectroscopy combined with automatically phase-contrast/differential interference contrast microscopy (Zhang, Setlow, Li 2009), and flow cytometry, where single spores are passed before one or more laser beams with measurement of scatter and fluorescence when fluorescent markers are used (Ormerod 2008). Flow cytometry as a means to detect germination would give the benefit of increased accuracy when determining the proportion of germinated cells in a population. However, monitoring germination through time could be a challenge using flow cytometry, as the spores would have to be passed before the laser beam and detected multiple times during germination. Raman spectroscopy is used to analyse scattering spectra, which changes as the spore germinates, and can be a technique with potentially high sensitivity (Zhang, Setlow, Li 2009).

1.4 THE *BACILLUS* SPECIES

The genus *Bacillus* is one of the most diverse genera based on 16S rRNA classification, and species of the genus inhabit a variety of environmental niches. *Bacillus* spp. are members of the phylum *Firmicutes* and can be classified into groups based on different criteria. One such classification is the division of members into two groups; the cereus and the subtilis group. The cells in the cereus group have a spore diameter larger than 1 µm and have non-swollen sporangia with cylindrical or oval spores. They are mainly mesophilic and neutrophilic, with some psychrotolerant strains. The cells in the subtilis group are smaller, less than 1 µm in diameter and the sporangia are non-swollen with cylindrical or oval spores. The cells are mesophilic and neutrophilic. Examples of species belonging to the cereus group consists are *B. anthracis*, *B. cereus*, and *B. weihenstephanensis*, whereas *B. licheniformis*, *Bacillus B. pumilus* and *B. subtilis* belong to the subtilis group. (Løvdal 2011)

In terms of foodborne illnesses, *B. cereus* is isolated as the reason in most cases where *Bacilli* cause the disease. *B. cereus* may produce two types of toxins. The heat-

stable emetic toxin (cereulide) causes intoxication after ingesting food that contains the toxin, and nausea and vomiting as typical symptoms. The various enterotoxins are produced during vegetative growth of bacteria in the intestine, and symptoms are abdominal pain, diarrhoea and sometimes nausea. (Logan 2012; Granum 2007)

1.4.1 *Bacillus licheniformis*

B. licheniformis belongs to the subtilis group and orthologs to approximately 80 % of the predicted coding sequences in *B. licheniformis* are found in *B. subtilis*, among them the germination receptor operon *gerA* (Rey *et al* 2004). *B. subtilis* has been studied in great detail, and because of their close relation, results from various studies of *B. subtilis* are of great interest when studying *B. licheniformis* as well. The GerD protein, which has been demonstrated to be part of the germination apparatus in *B. subtilis*, is predicted to exist in *B. licheniformis* as well (Paredes-Sabja, Setlow, Sarker 2010). Its possible function has not yet been described in the latter species.

B. licheniformis is a gram-positive, motile rod-shaped bacterium, able to form endospores. It is facultative anaerobic (Rey *et al* 2004) and thermophilic or thermotolerant. Some strains have been shown to grow at temperatures above 60 ºC, but usually the species is described to be growing between 15 and 55 ºC (Løvdal 2011; Sakai, Yamanami 2006).

The species is native to soil, and has also been isolated from tissue in healthy plants (Logan 2012). Some strains are believed to promote plant growth and have a role in plant protection. *B. licheniformis* is of some importance to the industry, as it has been used in the production of various enzymes, biochemicals and antibiotics (Rey *et al* 2004; Waschkau et al 2007).

Although not reported as cause in many outbreaks of foodborne illnesses, it has been isolated as causative agent in some cases, including meat products, pastry and vegetables (Salkinoja-Salonen *et al* 1999). The strains causing disease produce a toxin named lichenysin A, which may cause diarrhoea, vomit and abdominal pain (Salkinoja-Salonen *et al* 1999). In relation to foods, *B. licheniformis* is known as a spoilage organism. It causes ropiness in bread, and has been detected in various cases of food spoilage in dairy and meat products, in addition to canned foods (Logan 2012).

In *B. licheniformis* the germinant receptor operons *gerA* and *gerK* are found. Both are tricistronic operons, with A, B and C cistrons. The *gerA* operon thus consists of *gerAA*, *gerAB* and *gerAC*. Previously it has been shown that *B. licheniformis* responds to

nutrient germinants like L-alanine, L-cysteine, L-valine and casein hydrolysate, which consists of amino acids from hydrolysed casein (Martin, Harper 1964; Klufterud 2011; Løvdal et al 2012; Martin, Harper 1963).

Transformation of the species has been reported to be relatively difficult. This is likely due to restriction systems destroying foreign DNA. The *B. licheniformis* strain DSM 13 (which is isogenic to the type strain, ATCC 14580) has genes (*hsdR1* and *hsdR2*) encoding two type I restriction modification systems, destroying foreign DNA with restriction endonucleases (Waschkau et al 2007). It is not known whether all strains of the species carry these genes, but likely these or similar systems are present in a number of *B. licheniformis* strains. Because of these endonucleases the species is not naturally competent.

1.5 ENDOSPORES IN FOOD

As spores have properties that ensure higher resistance to a number of environmental strains than vegetative cells, and an apparatus that converts the spore to a vegetative growing cell in a short time span, spores have been and still remain a problem in the food industry. Sporeforming bacteria have numerous entry points to the food chain (Figure 1), and processing and storage which ensure minimal problems related to vegetative cells are not always enough to inactivate the spores or prevent bacterial growth after germination. (Eijlander, Abee, Kuipers 2010; Hornstra *et al* 2009)

A public demand for fresher, more natural foods – less processed, and without additives – may make spores more challenging today, as consumers at the same time request easy solutions for foods prepared at home, without lowering the food safety criteria (Mañas, Pagán 2004; Gould 2000). The increased production and consumption of refrigerated processed food with extended durability (REPFED) and other cook-chill products may present an increased risk of foodborne illnesses (Løvdal *et al* 2011; Gould 2000). REPFED can be described as refrigerated, cooked foods intended for extended refrigerated shelf life and are ready-to-eat or prepared with little or no additional heat treatment (Løvdal *et al* 2011). These products are typically prepared at temperatures between 65 and 95 ºC (Løvdal *et al* 2011). As temperature treatment remains one of the most used means of microorganism control in food processing, and the temperatures in REPFEDs are lower than the temperatures normally needed to inactivate spores, which

have been shown to survive 100 ºC, it is likely that more sporeformers will survive this form of food preservation (Løvdal *et al* 2011; Gould 2000).

Since endospores can be activated when exposed to nonlethal temperatures (Eijlander, Abee, Kuipers 2010), temperature treatment used in production of REPFEDs could in fact activate the spores, rather than inactivate them. As the vegetative cell population is eradicated or minimalized, the spores would have little or no competition when germinated to vegetative cells.

Higher, but not lethal temperatures could also delay spore germination (Eijlander, Abee, Kuipers 2010). This would be a problem when using double heat-treatment as means to inactivate or kill microorganisms in food, for example by canning foods or preparation of the aforementioned REPFEDs. In this process a primary heat treatment with a lower, and for spores non-lethal temperature starts the process, followed by a cooling period to ensure germination. The foods are then exposed to a secondary heat treatment with higher temperature, lethal for vegetative cells and germinated spores. Rather than activate germination, non-lethal temperatures may delay germination in some spores. If the start temperature delays germination, or spores germinate slowly, this could present a problem, as these un-germinated spores would not be inactivated by the second heat-treatment. Later, these spores may germinate and grow to a relatively large bacteria population, dependent on growth conditions in the food product. (Løvdal *et al* 2011b)

The composition of the food matrix also plays a role, as some substances could protect the spores or slow down germination of the spores. This is dependent on factors such as germinants present and water content. (Mañas, Pagán 2004; Gould 2000)

Additional techniques can be used in food preservation, either alone or in combination, to ensure food safety. The use of several factors combined to eliminate microorganisms is known as hurdle technology. Temperature during manufacturing and storage, acidity, water content, oxygen content, preservatives and even the addition of competing micro flora to the food product are described as hurdles. Each hurdle represents an obstacle the microorganisms present have to overcome in order to create a problem in the food. (Gould 2000)

In addition to this, there is variability between species, strains and spores formed in different environments and between spores of different age. This makes it difficult to develop a processing program that ensures that all spores are inactivated, or that a minimal number of spores remain in the food. This number should be as low as possible,

preferably none, to ensure little chance of growth during the storage time of the food, provided that the food is stored at the appropriate temperature. (Mafart *et al* 2010)

Many sporeformers may present a health risk in addition to their ability to cause food spoilage, as they either can cause infections or produce toxins harmful to humans or animals. Some toxin-producers, e.g. *B. cereus* do not present a risk when kept below 12 ºC as the emetic toxin is not produced at these temperatures. In contrast, non-proteolytic psychrotroph *C. botulinum* is able to produce toxins down to 3 ºC. A species related to *B. cereus*, *B. weihenstephanensis*, grows at temperatures down to 4 ºC, and has been shown to produce emetic toxin at 8 ºC. (Granum 2007; Gould 2000)

Some concerns have been raised regarding the use of *Bacillus* spp. as added probiotics in foods. These could potentially promote antibiotic resistance in strains associated with illnesses, or serve as opportunistic pathogens in patients with lowered immunological resistance. Alternatively, the probiotics may be contaminated with spores from different strains or species. The use of sporeformers as probiotic is currently more common in parts of Asia, and species used in these products include strains of *B. cereus*, *B. subtilis*, and *B. licheniformis*. (Logan 2012)

There may be a chance of sporeformers to develop biofilms or induce sporulation inside the production plant as well as being contaminants from the outside, or spores may attach to surfaces (Logan 2012; Carlin 2010).

B. licheniformis has been associated with foodborne illnesses to some degrees, but is usually considered a food spoilage bacterium. There is evidence that *B. licheniformis* spores are present in a greater number than spores of *B. cereus* in milk, but it is believed that the reason this bacterium do not cause problems to the degrees of *B. cereus*, is because *B. cereus* grows faster after germination, suppressing growth of *B. licheniformis*. (Logan 2012)

1.6 TRANSFORMATION OF *B. LICHENIFORMIS*

Most *Bacilli* are naturally competent, and are shown to express competence genes when nutrients are scarce, in order to try to achieve the ability to use alternative nutrients. When this fails, sporulation is likely initiated. These functions could also be initiated in separate cells of a population – some scavenge for food while others sporulate – thus ensuring survival of the species. (Phillips, Strauch 2002)

As described in 1.4.1, *B. licheniformis* is not naturally competent. Readily transformable strains of *B. licheniformis* DSM 13 were constructed and described by Waschkau *et al* (2007). They created three deletion mutant strains. One was a Δ*hsdR1* mutant and another a Δ*hsdR2* mutant. The third strain, MW3, was a double mutant, lacking both genes (Δ*hsdR1*Δ*hsdR2*). The construction of these mutants may be of great importance for the industry, as transformable *B. licheniformis* may be used in the production of even more substances.

The MW3 strain has later been used to construct a Δ*gerAA*spec+ mutant (Løvdal *et al* 2012; Klufterud 2011). This mutant was proved unable to germinate with L-alanine or casein hydrolysate, but germination could be induced by CaDPA. By use of a low-copy plasmid, complementation mutants expressing the GerA receptor from different strains can be constructed. This way it is possible to study the germination properties of the GerA receptor from multiple strains.

In order to successfully transform bacteria, chemically competent or electrocompetent cells are useful. Some competent bacteria species and strains can be obtained commercially, and for others protocols need to be developed in order to make the cells competent and useful in gene manipulation. This can be achieved by growing the cells in culture and treating them chemically or solve the cell pellet in chemicals protecting the cells during electroporation. Electroporation is a process where electrocompetent cells are transformed using an electro-magnetic field believed to momentarily increase the permeability of the plasma membrane so that plasmids can pass through more easily. (Weaver 1995)

1.7 EXPRESSION OF GERMINATION RECEPTORS

As described in 1.1, the germination properties of several wild type strains and complementation mutants with the *gerA* operon from these wild type strains are studied in this thesis (see 2.1). It would be useful to know whether the germination receptors are expressed differently in a complementation mutant compared to the wild type. An increased or decreased expression of the germination receptor potentially has great influence on a strain's germination properties (see 1.3.3). As the complementation is done by transforming the cells with a low-copy plasmid, it is likely that the expression will vary between the wild type and the complemented mutant.

The germination receptors are shown to be expressed during sporulation, and are mostly controlled by σ^G (Feavers *et al* 1990; Corfe *et al* 1994; Wang *et al* 2006; Igarashi, Setlow 2006). To study the expression of the germination receptor, it would thus be useful to monitor the expression of σ^G as well. σ^G is transcribed before asymmetric septation, and remains inactive, once translated, until completion of engulfment of the forespore (Evans, Feucht, Errington 2004). In addition a gene with relatively stable expression during sporulation (e. g. a house-keeping gene) should be included as a point of reference. The genes in question will then be related to the expression of the reference gene to give the relative expression of the genes studied during sporulation. In this instance *rpoB*, which encodes the β-subunit of RNA polymerase and should have a relatively stable expression during sporulation, was chosen as a reference gene.

The environment has been shown to affect the genetic expression, including *ger* operons, during sporulation (Hornstra *et al* 2006). When comparing the expression of specific genes in two strains during sporulation, this should thus be done under the same conditions. Considering this, the samples for investigation of *gerA* expression should be collected using the same sporulation conditions that were applied when preparing spores for germination analyses.

2 MATERIALS AND METHODS

The studies described in this thesis started with analysis of germination properties in five different strains of *B. licheniformis*: two wild type strains (one of them MW3, a Δ*hsdR1*Δ*hsdR2* mutant), a MW3_Δ*gerAA*spec+ mutant which had been modified from the Δ*hsdR1*Δ*hsdR2* mutant, and two complementation mutants where the *gerA* operons from the wild type strains had been introduced in the MW3_Δ*gerAA*spec+ mutant (Table 1).

Three new wild type strains were included later in the studies, in order to investigate further strain specific germination properties. The *gerA* operons from these were further used to construct three additional complementation mutants from the MW3_Δ*gerAA*spec+ mutant.

In order to investigate the relative expression of *gerA* in one wild type strain and the corresponding complementation mutant, a real-time PCR analysis was commenced. Samples were collected from the wild type strain during sporulation, and after RNA

extraction, the relative expression of the genes studied was analysed and estimated from analysing cDNA.

2.1 STRAINS

Several strains of *Bacillus licheniformis* were sporulated and studied in various germination assays (Table 1). In some experiments *B. subtilis* strain NVH-B252 or *B. cereus strain* NVH-1129 were used as positive controls.

The *B. licheniformis* strain NVH-1289, originally named MW3 is a Δh*sdR1*Δh*sdR2* of *B. licheniformis* DSM 13, an isogenic strain to the type strain ATCC 14580 (Waschkau *et al* 2007). As described in 1.6, *Hsdr1* and *hsdR2* are both loci in type I restriction modification systems found in *B. licheniformis* DSM 13, and are thought to destroy foreign DNA. The deletion of this restriction modification system in MW3 is suspected to be the reason why MW3, unlike DSM 13, is described as being readily transformable (Waschkau *et al* 2007). Therefore, MW3 offers the possibility of construction of and phenotypic studies of genetic modifications in *B. licheniformis*.

NVH-1289 had previously in our laboratory been used to create a mutant strain where part of *gerAA* in the *gerA* operon was substituted with a spectinomycin cassette. This had been accomplished by means of a modified pMAD shuttle vector, which harbours a resistance gene against spectinomycin, and a β-galactosidase gene. This allowed for antibiotic selection and blue-white screening on plates containing spectinomycin and X-Gal. The MW3_Δ*gerAA*spec+ mutant (NVH-1307) should have no expression of the *gerA* operon, and lacks a functional GerA receptor. (Løvdal *et al* 2012; Klufterud 2011)

To ensure that the observed phenotype was due to the introduced disruption in *gerAA*, a complementation mutant with the *gerA* operon from NVH-1289 on a free replication plasmid had been constructed (Løvdal *et al* 2012; Klufterud 2011). The low copy number plasmid pHT315 (Arantes, Lereclu 1991, Figure 9) was used for this purpose, as the low copy-number hopefully would ensure that the expression of *gerA* was kept at levels that allowed successful/complete sporulation. As described in 1.3.3, too high expression of a germination receptor may induce premature germination during sporulation. The plasmid pHT315 contains several restriction sites for different endonucleases, among them *SalI* and *XbaI*, which were used to insert the operon in the plasmid. In addition to this, the plasmid contains resistance genes against ampicillin and

erythromycin (Figure 9). These resistance properties were used in selection of transformed cells (Løvdal *et al* 2012; Klufterud 2011).

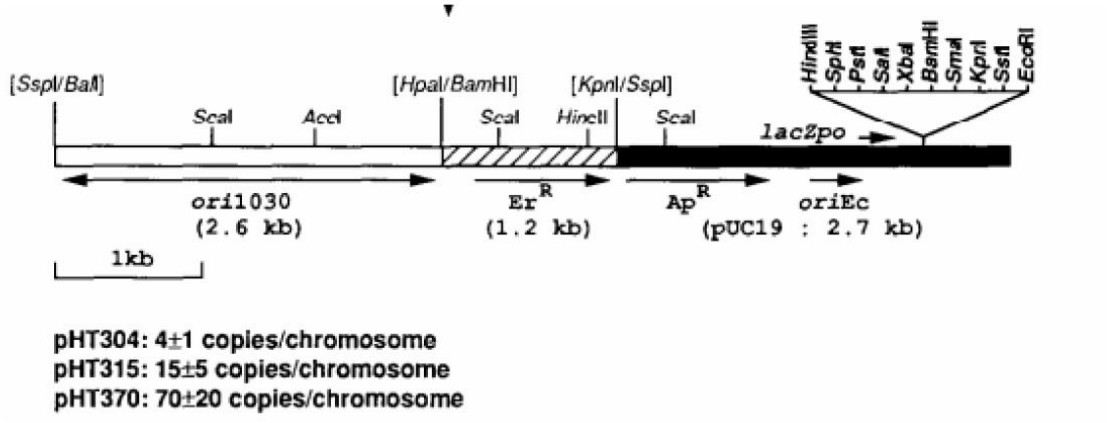

Figure 9. Schematic illustration of pHT315, the plasmid used in construction of complementation mutants. *gerA* is inserted between the restriction sites for *SalI* and *XbaI*. (Arantes, Lereclu 1991)

During the work presented in this thesis, three new complementation mutants of MW3_Δ*gerAA*spec+ were constructed, using the *gerA* operons from *B. licheniformis* strains that showed different strain history and genetic differences in *gerA*. By doing this, the various GerA receptors could be studied outside the strains from which they originated, without influence from the remainder of their germination apparatus. The GerA receptors (phenotype) would however be influenced by the germination apparatus present in the disruption mutant. In total the MW3_Δ*gerAA*spec+ mutant, five wild type strains (including NVH-1289) and two complementation mutants (Table 1) were studied, and three new complementation mutants were constructed for further studies of *gerA* in *B. licheniformis*.

The wild-type strains most extensively studied were NVH-1289 and NVH-1032. NVH-1032 originated from a canned meat product, where it was not killed by a double heat-treatment. This "problem" strain apparently failed to achieve complete germination during the cooling period between first and second heat treatment, and afterwards surviving spores gave rise to bacterial growth with gas production.

Differences are found between the sequences encoding the *gerA* operon in NVH-1032 and NVH-1289 (see Figure 9) (Henie-Madslien, unpublished results). Two strains with some similarities in genetic sequence to NVH-1032, NVH-800 and NVH-1112 were also included for future germination studies. NVH-800 originated from fresh dates and

NVH-1112 originated from a cattle abortion. The sequence of the *gerA* operon from these three strains differ from the sequence of the *gerA* operon in strain NVH-1289. In addition to these strains, a wild-type in which the *gerA* sequence resembles that of NVH-1289 was included. This strain, NVH-B357, originated from a water sample taken at a drinking water reservoir. An overview of the studied strains and their origin is given in Table 1 and Figure 10.

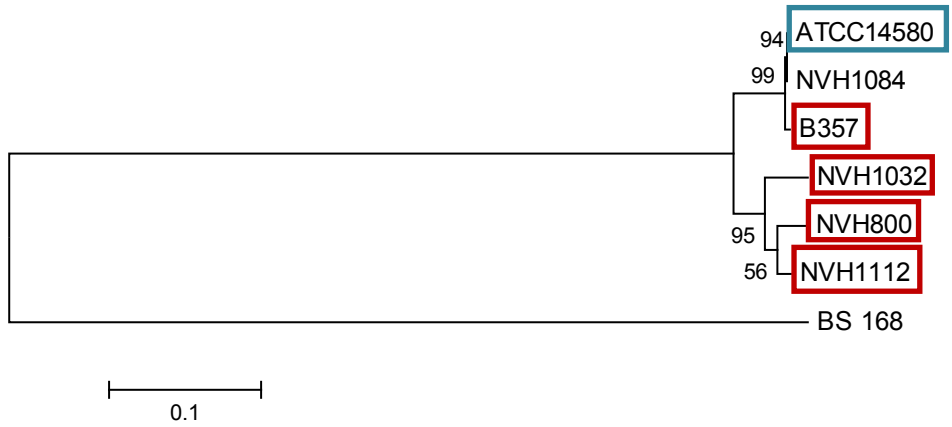

Figure 10. Neighbour-Joining phylogenetic tree created from the sequence alignment of *gerA* operon (start *gerAA*, stop *gerAC*) in six different strains of *B. licheniformis* and in *B. subtilis* 168 (the type strain). Strain ATCC14580 (marked in blue) is isogenic to DSM 13/MW3. The other wild types included in this study are marked with red. (Henie-Madslien, unpublished results)

Table 1.
The origins of the strains that were studied during the work presented in this thesis.

Strain	Description	Source
NVH-1289	*B. licheniformis* MW3	Prof. Dr. F. Meinhardt, Westfälische Wilhelms-Universität Münster (Waschkau *et al* 2007)
NVH-1032	*B. licheniformis*	Isolated from a canned meat-product
NVH-B357	*B. licheniformis*	Isolated from a drinking water reservoir
NVH-800	*B. licheniformis*	Isolated from fresh dates
NVH-1112	*B. licheniformis*	Isolated from a cattle abortion
NVH-1307	*B. licheniformis* MW3Δ*gerAA*spec+	Løvdal *et al* 2012

NVH-1311	*B. licheniformis* MW3Δ*gerAA*spec+ pHT315_*gerA*MW3	Løvdal *et al* 2012
NVH-1309	*B. licheniformis* MW3Δ*gerAA*spec+ pHT315_*gerA*1032	Klufterud 2011
NVH-1320	*B. licheniformis* MW3Δ*gerAA*spec+ pHT315_*gerA*B357	Constructed during the work presented in this thesis
NVH-1322	*B. licheniformis* MW3Δ*gerAA*spec+ pHT315_*gerA*800	Constructed during the work presented in this thesis
NVH-1321	*B. licheniformis* MW3Δ*gerAA*spec+ pHT315_*gerA*1112	Constructed during the work presented in this thesis
NVH-B252	*B. subtilis*	Isolated from tap water (From *et al* 2005)
NVH-1129	*B. cereus* ATCC 14579	*B. cereus* type strain, isolated from air (Ivanova et al. 2003)

2.2 CHEMICALS AND EQUIPMENT

An overview of the media used in these experiments is presented here. Complete lists of primers, chemicals, suppliers and other equipment are presented in Appendix 1 and 2.

Luria Bertani (LB) medium: 9 g tryptone (Oxoid), 4,5 g yeast extract (Oxoid), 9 g NaCl (Merck) to 900 ml distilled water.

LB-agar: 9 g tryptone (Oxoid), 4,5 g yeast extract (Oxoid), 9 g NaCl (Merck) to 900 ml distilled water, 1,5 % (w/v) agar bacteriological No. 1 (Oxoid).

Bacto-MS sporulation medium (van der Voort *et al* 2010): The sporulation medium consists of Difco Nutrient Broth (8 g/l, BD) with an addition of several salts to enhance sporulation. The salts used were $FeSO_4$ (1 µM, Merck), $CuCl_2$ (2,5 µM, Sigma), $ZnCl_2$ (12,5 µM, Sigma), $MnSO_4$ (66 µM, Merck), $MgCl_2$ (1 mM, J. T. Baker Chemicals B. V.), $(NH_4)_2SO_4$ (5mM, Merck), Na_2MoO_4 (2,5 µM, Riedel-de Häen), $CoCl_2$ (2,5 µM, Sigma) and $Ca(NO_3)_2$ (1 mM, Merck). The salts were kept refrigerated in watery solutions. With the exception of $FeSO_4$, $MnSO_4$ and $Ca(NO_3)_2$, the salts were added to the medium before autoclaving. $FeSO_4$, $MnSO_4$ and $Ca(NO_3)_2$ were sterile filtered (0,2 µm, Minisart Sartorius) and added after autoclaving and cooling the medium. Before autoclaving the medium pH was adjusted to ~7,6 (Metrohm). After autoclaving and addition of the sterile filtered salts, the pH was ~7,4. The sporulation medium was used within a week of making the medium.

PCR with purified DNA as template, used for amplification of *gerA* (Finnzyme Hot-start phusion polymerase kit)**:** 10 µl 5xPCR buffer, 1 µl dNTP (10 mM), 2,5 µl Forward primer 1 (10 µM), 2,5 µl Reverse primer 2 (10 µM), 0,5 µl Phusion Hot start II, 33,5 µl autoclaved MilliQ water, 0,5 µl template.

PCR with lysed cells as template, used in screening for correctly transformed cells: 5 µl 10x PCR buffer (Dynazyme), 1 µl dNTP (Dynazyme/Finnzyme), 1 µl Forward primer (Sigma), 1 µl Reverse primer (Sigma), 0,5 µl Taq polymerase (Dynazyme), 39,5 µl autoclaved Milli-Q water, 2 µl template. When including purified DNA as a positive control, 0,5 µl template was used, and the rest (1,5 µl) was replaced with autoclaved MilliQ water or LB (when analysing *E. coli* (2.5.4)).

2.3 SPORULATION

Spores of all the different strains (Table 1) were produced in order to assess the relationship between the genetic differences in the *gerA* operons and the strains' germination phenotypes.

The sporulation medium used, Bacto-MS, was a modified version of MSM (van der Voort *et al* 2010) that lack maltose (Løvdal *et al* 2012). Pre-culture and sporulation was done by incubation in Minitron (HF-Infors) or Sanyo Orbital Incubator (Sanyo). The bacteria were sporulated at 50 ºC as this temperature had previously been shown to yield stable spores for *B. licheniformis* (Løvdal *et al* 2012; Baril *et al* 2011; Klufterud 2011).

To obtain single colonies for pre-cultures, bacteria from cultures kept in Microbank tubes (Pro-Lab) at -80 ºC were spread on LB agar and grown overnight with appropriate antibiotics at 37 ºC. The wild type strains (NVH-1289, NVH-1032, NVH-800, NVH-B357 and NVH-1112) were grown without antibiotics, the MW3_Δ*gerAA*spec+ mutant (NVH-1307) was grown with 250 µg/ml spectinomycin (Sigma) and the complemented mutants were grown with 250 µg/ml spectinomycin (Sigma) and 1 µg/ml erythromycin (Sigma). A single colony was suspended in 50 ml LB medium in a 100 ml Erlenmeyer flask using the same antibiotic concentrations as on the agar plates. This pre-culture was grown at 50 ºC and 225–230 rpm for ~5 hours before transferring 20 µl of the pre-culture to the sporulation medium. Only the complementation mutants were grown with the presence of antibiotics in the sporulation cultures (1 g/ml erythromycin, Sigma). The sporulation culture, 100 ml Bacto-MS, was grown in a 500 ml Erlenmeyer flask at 50 ºC and 225–230 rpm overnight and for as long

as it took the cells to finish sporulation. In an alternative assay the pre-cultures were grown to OD_{600} 0,3–0,5 instead of for 5 hours before transfer to the sporulation culture.

The sporulation process was monitored by phase contrast microscopy (Olympus BX51) and sporulation was stopped by harvesting the spores when the spore content had reached 90 % or more and there were few vegetative cells left in the culture. If any germinated spores were observed during sporulation, sporulation was stopped immediately. The spores were harvested by transferring the culture to centrifugation flasks and centrifuged by 2806–6555 x *g* for 10 minutes (Sorvall RC-5B Refrigerated Superspeed centrifuge, GSA or SS34 rotor). The supernatant was removed and the spores were resuspended in cold autoclaved MilliQ water and washed ~10 times over the next days. Phase contrast microscopy (Olympus BX51) was used to determine the vegetative cell content in the washed spore solutions. The purified spore cultures were stored at 4 ºC in 10 ml water suspension in 45 ml sterile tubes (Falcon, BD).

To remove cell debris and vegetative cells during the washing process it was investigated whether a solution consisting of one part Ludox AS-40 colloidal silica, 40 wt. % suspension in water (Sigma-Aldrich) and five parts 50 mM tris (Trizma base, Sigma), 10 mM KCl (Merck) pH 7,4 could be used to create a density gradient during centrifugation, separating the spores from cell debris and vegetative cells, as described by Zhu *et al* (1989). For this purpose 1 ml spore stock was centrifuged at 16 100 x *g* for 3 minutes and the spore pellet was resuspended in 100 µl autoclaved MilliQ water. The spores were then transferred to the top of 1 ml of sterile filtered (0,2 m, Minisart Sartorius) Ludox solution in an Eppendorf tube and centrifuged at 16 100 x *g* (Eppendorf 5415D) at 4 ºC for 10 minutes. The bottom layer in the tube would then consist mostly of spores and these were extracted and washed at least three times in 1 ml autoclaved MiliQ water before using the spores in germination assays. Phase contrast microscopy was as before used to determine the vegetative cell content in the spore solutions. The spores were stored at 4 ºC in Eppendorf tubes.

2.4 GERMINATION STUDIES

The conducted germination studies mainly focused on nutrient germinants, with the exception of CaDPA which was used to induce non-nutrient germination. The studies compare the different strains of *B. licheniformis*, including the MW3_Δ*gerAA*spec+ mutant and the complementation mutants. Germination without receptor involvement was

included to investigate whether the MW3_Δ*gerAA*spec+ mutant would germinate, thus assessing whether the mutation had in any way damaged the ability to germinate without receptor interaction.

OD_{600} measurements were used to detect germination, with phase contrast microscopy as an additional control before and after germination. Some studies were done with only visual control using a phase contrast microscope.

2.4.1 Germination with L-alanine

An aliquot (usually ~300 µl) of the spore culture used in the assay was centrifuged at 16 100 x *g* (Eppendorf centrifuge 5415D) at 4 ºC for 3 minutes. The supernatant was removed to ensure little interference in the analysis caused by soluble molecules derived from spores or cells during storage. The spores were resuspended in 1 ml autoclaved MilliQ water and heat activated at 65 ºC for 20 minutes in water bath. The spores were centrifuged again and resuspended in the buffer used in the germination assay, either tris or K-phosphate (see below).

Before germination OD_{600} was adjusted to ~2 (Shimadzu UV-160A), which ensured that OD_{600} would be between 0,6 and 0,8 at the start of the germination assay after addition of germinant and negative control. Germination was done in a Tecan Infinite M200 microplate reader at 30 ºC using 96-well plates (Falcon Microtest 96, BD). Magellan 7.1 (Tecan) was used to program the microplate reader. 100 µl spore sample was added to each test well and all samples were analysed in parallels for germinant and negative control. 100 µl of the germination buffer was used as blanks. Addition of germinant (100 ml) and negative control (100 ml) was done by using the microplate injector and the germination was monitored by measuring OD_{600} every 30 seconds for 3 hours. Before and after germination the samples were checked in phase contrast microscope. The results were analysed using Microsoft Excel 2007.

Germination buffers, blanks, germinants and negative controls were sterile filtered (0,2 µm, Minisart Sartorius) before use. Two different buffers were used in the assay, and the results were compared in order to identify the most suitable buffer for germination with L-alanine.

Tris assay: The spore pellet was resuspended in germination buffer consisting of 50 mM tris (Sigma), 10 mM KCl (Merck) pH 7,4. L-alanine (Sigma) was solved in 50 mM tris, 10 mM KCl pH 7,4. 50 mM tris, 10 mM KCl pH 7,4 was used as negative

control. Final concentrations in the assay were 100 mM L-alanine and 50 mM tris, 10 mM KCl.

Phosphate assay: The spore pellet was resuspended in germination buffer consisting of 100 mM K_2HPO_4 (Merck)/KH_2PO_4 (Merck) pH 7,2. L-alanine (Sigma) was solved in distilled water. Distilled water was used as negative control. Final concentrations in the assay were 100 mM L-alanine and 50 mM K_2HPO_4/KH_2PO_4.

2.4.2 Germination with Casein hydrolysate

Casein hydrolysate (also named casamino acids) is a mixture of amino acids derived from acid hydrolysis of the milk protein casein (Løvdal *et al* 2012). It contains all the amino acids present in casein except from tryptophan, which is degraded during the hydrolysis.

The germination assay was performed by the same procedure as the L-alanine germination assay with tris buffer. The final germinant concentration was 1 % (w/v) casein hydrolysate (Merck) in 50 mM tris, 10 mM KCl, pH 7,4. Heat-activation, germination time, germinant addition and detection of germination was done as described in 2.4.1.

2.4.3 Germination with growth media

A couple of small pilot studies were conducted in order to see whether the MW3_Δ*gerAA*spec+ mutant would germinate in either Brain heart infusion (BHI, BD) or Luria Bertani (LB) medium (see 2.2). Spores were washed once in autoclaved MilliQ water before resuspension in autoclaved MilliQ water and addition of 2x BHI or 2x LB in water to a final 1x concentration of the media during the germination assay. The spores were germinated by incubation at 30 ºC and 150 rpm (Minitron, HT Infors) for three hours or overnight with phase contrast microscopy as method for detecting germination. These pilot studies were done without parallels and replicates. When the germination was done overnight 0,1 µg/µl chloramphenicol (Sigma) was added to the samples to prevent outgrowth. A similar pilot was later conducted on strains which showed poor germination with L-alanine as germinant, now using BHI as germinant.

2.4.4 Germination with calcium and dipicolinic acid (DPA)

The original assay was done by washing 300 µl spore stock in autoclaved MilliQ water, resuspending them in germination buffer (125 mM tris base (Sigma), 50 mM pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA, Aldrich), pH 7–8) and adjusting OD_{600} to 1–

1,5 (Shimadzu UV-160A). Addition of 25 µl 0,5 or 1,0 M $CaCl_2$ (Riedel-de Häen) in distilled water to 225 µl sample was done just before assay start in Tecan Infinite M200 microplate reader. The germination was done at 30 ºC for 3–21 hours with OD_{600} measurements every 5 minutes. The final concentrations were 45 mM DPA, 50–100 mM $CaCl_2$ and 112 mM tris. These are germinant concentrations that have previously been demonstrated sufficient to induce germination in *B. cereus* spores (Keynan, Halvorson 1962). *B. cereus* NVH-1129 (ATCC 14579) and *B. subtilis* NVH-B252 were both used as positive controls.

During further analysis, this protocol was modified in order to discover the proper germination environment for *B. licheniformis*. Germination response was analysed using several temperatures, DPA/$CaCl_2$/tris base concentrations and germination times. Final DPA concentrations in the assays varied between 20 and 80 mM with $CaCl_2$ in excess. Final tris base concentrations were 100–200 mM. The temperatures varied between 21 and 30 ºC in different assays, and the germination times were between 3 and 23 hours. The germination was done in Tecan Infinite M200 microplate reader, incubator (Minitron, HT Infors) with 150 rpm, or on roller mixer (Sarstedt) at room temperature. As calcium crystals precipitated during the assay, visual control in phase contrast microscope was used instead of OD_{600} measurement to detect germination.

When the germination was done overnight, 0,1 µg/µl chloramphenicol (Sigma) was added to the samples to prevent outgrowth. To prevent precipitation of calcium crystals it was investigated whether addition of 0,4 % gelatine (Oxoid) in the germination buffer could be effective. Keynan and Halvorson (1962) had previously shown that this gelatine concentration could prevent precipitation of calcium when germinating *B. cereus* spores. They also demonstrated that 20 ºC was the optimal temperature for CaDPA induced germination.

2.5 CONSTRUCTING NEW COMPLEMENTATION MUTANTS

To extend the knowledge regarding the function and the variability of the *gerA* operon in *B. licheniformis*, three new complementation mutants with *gerA* operons from different strains were constructed. This was done by the method described by Løvdal *et al* (2012) by transforming the Δ*gerAA*spec+ mutant with plasmids (pHT315 (Arantes, Lereclu 1991), Figure 9) containing the sequences from the chosen strains.

2.5.1 PCR amplification of the different *gerA* operons

DNA had been extracted using the following protocol (performed by Elisabeth Henie-Madslien, FFI): Bacteria from freeze cultures were grown on sheep blood agar at 30 °C overnight. A single colony was then used as inoculate in 10 ml LB. The bacterial culture was grown overnight at 30 °C and centrifuged at 3000 x *g* for 10 min. The supernatant was discarded and the pellet was resuspended in 1 ml enzymatic lysis buffer (20 mM Tris·Cl, pH 8.0, 20 mM Tris·Cl, pH 8.0, 1.2% Triton® X-100, 20 mg/ml lysozyme). Further DNA extraction was performed according to the protocol provided by DNeasy Blood and Tissue Kit (Qiagen, USA). The final DNA concentration was approximately 10 ng/µl (Nanodrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, USA).

The primers pHT315gerAwt_SalI F1 (Sigma) and pHT315gerAwt_XbaI R1 (Sigma) (see Table 7, Appendix 1) and the Hot-start phusion polymerase kit from Finnzymes were used in the PCR assay to amplify the *gerA* genes from the three strains (see Table 7, Appendix 2) with 0,5 µl template. The *gerA* operons were amplified in Mastercycler ep*gradient* S (Eppendorf), using the following PCR amplification protocol:

1. 98 ºC for 30 seconds
2. 98 ºC for 10 seconds
3. 58 ºC for 30 seconds
4. 72 ºC for 2 minutes
5. 72 ºC for 10 minutes
6. 4 ºC ∞

Step 2–4 were repeated 30 times.

2.5.2 Amplification of pHT315 used as complementation plasmid

The vector used for complementation was pHT315 (Arantes, Lereclu 1991, Figure 9). This plasmid is about 6500 bp and harbours resistance genes towards ampicillin and erythromycin. These resistance properties were used to select transformed clones of *E. coli* (ampicillin) and *B. licheniformis* (erythromycin).

E. coli One Shot TOP10 Electrocomp (Invitrogen) electrocompetent cells harbouring pHT315 were grown on LB agar (see 2.2) with 100 µg/ml ampicillin (Sigma) overnight. A single colony was suspended in 10 ml LB medium with 100 µg/ml ampicillin and grown overnight at 37 ºC and 230 rpm. 100 µl from the pre-culture was transferred to 100 ml LB medium with 100 µg/ml ampicillin and grown overnight at 37 ºC and 230 rpm.

The plasmids were extracted using Plasmid Midi kit (Qiagen). Extraction was done following the kit protocol, centrifuging in GSA and SS34 rotors in Sorvall RC-5B Refrigerated Superspeed centrifuge. The plasmids were kept refrigerated in elution buffer until use.

2.5.3 Inserting the operon in the complementation plasmid

The restriction enzymes *XbaI* (BioLabs) with NEB buffer 4 (BioLabs) and Bovine Serum Albumine (BioLabs) and *SalI* (with buffer included, Sigma) were used to cut pHT315 and the PCR products. The restriction reactions were done separately, both being done in a 37 ºC waterbath for 3 hours. Each 100 µl reaction consisted of 40–45 µl plasmid or PCR product, 5–10 µl of the enzyme and 10 µl 10x buffer. In the *XbaI* restriction reactions 10 µl 10x Bovine Serum Abumine (BioLabs) were added. The remaining volume consisted of water. After each reaction, the DNA was purified using Qiaquick PCR purification kit (Qiagen). The plasmid was further purified by 0,7 % agarose gel (SeaKem) electrophoresis and use of the Qiaquick gel extraction kit (Qiagen).

The PCR products were inserted in the plasmid using T4 ligase (Invitrogen) in a 10 µl reaction with 1 µl T4 Ligase and 2 µl 5x ligation buffer (Invitrogen) at 16 ºC over night in PTC-100 Peltier Thermal Cycler (MJ Research). Based on visualized products from the restriction reactions in a 0,7 % agarose gel (SeaKem), two ligation reactions were performed to construct each complementation plasmid. One ligation reaction consisted of 3 µl pHT315 and 4 µl PCR product, and another consisted of 2 µl pHT315 and 5 µl PCR product.

2.5.4 Transformation of *E. coli* One Shot TOP10 with complementation plasmids

In order to amplify the ligated complementation plasmids, they were transformed into *E. coli* One Shot TOP10 Electrocomp (Invitrogen) electrocompetent cells by electroporation. Cells with verified correct transformation were further grown in culture, and the amplified plasmid was then extracted.

Just before transformation 40 µl cells thawed on ice were mixed carefully with 2 µl pHT315*gerA* and incubated on ice for one minute. The mix was then transferred to chilled 0,2 cm gap width electroporation cuvettes (BioRad) and transformed using the electroporation apparatus MicroPulser (BioRad), with the program Ec2 (2,5 kV). After transformation the cells were recovered in 0,5 ml SOC medium (Invitrogen) 90–120

minutes before they were plated on LB agar with 100 µg/ml ampicillin (Sigma) and incubated overnight at 37 ºC.

The following day some of the colonies growing on the agar plates were harvested and stored in 10 µl LB medium at 4 ºC. 2 µl of this material was used in PCR reactions to verify whether the plasmids contained the *gerA* insert. The primers used were the same that were used to amplify gerA; pHT315gerAwt_SalI F1 (Sigma) and pHT315gerAwt_XbaI R1 (Sigma). PCR reactions were done in either PTC-100 Peltier Thermal Cycler (MJ Research) or MasterCycler ep*gradient* S (Eppendorf), using the following protocol:

1. 94 ºC for 3 minutes
2. 94 ºC for 1 minute
3. 55 ºC for 1 minute
4. 72 ºC for 2 minutes 40 seconds
5. 72 ºC for 10 minutes
6. 4 ºC ∞

Step 2–4 were performed 30 times.

The transformed *E. coli* containing the complementation plasmids were further grown and the plasmids were harvested using Plasmid Midiprep (Qiagen) as described in chapter 2.5.2. The changes made to this protocol were that 5 µl of the LB material with positive PCR results were used to inoculate 20 ml LB with 100 µg/ml ampicillin which was grown overnight and used to inoculate the 100 ml culture. For long time storage of *E. coli* cells with verified complementation plasmids, 700 µl of the overnight culture was mixed with 300 µl sterile filtered (0,40 µm, Sartorius) 50 % glycerol (Merck) and stored at -80 °C.

2.5.5 Transformation of *B. licheniformis* MW3_Δ*gerAA*spec+

In order to transform *B. licheniformis* NVH-1307 (MW3_Δ*gerAA*spec+) with the new complementation plasmids, electrocompetent cells were prepared using a modified protocol from Xiaomin Hu (Løvdal *et al* 2012).

~10 µl of freeze cultures (-80 ºC) was used as inoculate in 25 ml BHI with 250 µg/ml spectinomycin in a 100 ml Erlenmeyer flask. This pre-culture was grown at 37 ºC and 150 rpm overnight. 1 ml of the pre-culture was then transferred to 200 ml pre-tempered (37 ºC) BHI with 250 µg/ml spectinomycin in a 1 litre Erlenmeyer flask and grown at 37 ºC and 150 rpm. The culture was grown to OD_{600} 0,9–1,0. The culture was

harvested in a Sorvall RC-5B Refrigerated Superspeed centrifuge with GSA-rotor at 2074 x *g* and room temperature for 15 minutes. The supernatants were removed and the cell pellets were washed twice in 200 ml autoclaved MilliQ water and centrifuged at 6555 x *g* and room temperature for 15 minutes. The pellets were resuspended in 10 ml sterile filtered PEG6000 solution (40 g PEG6000 (Merck) in 100 ml distilled water). The cells were centrifuged for 15 minutes at 2560 x *g* and room temperature and the cell pellets were resuspended in 1,5–2 ml of the PEG6000-solution. The now electrocompetent cells were transferred to Eppendorf tubes, 100 µl in each tube, and stored at -80 ºC. ~ 10 µl of the pre-culture was spread on LB-agar with 250 µg/ml spectinomycin for purity control of the culture.

MicroPulser (BioRad) was used to transform the electrocompetent *B. licheniformis* NVH-1307 with a manual program: 1,4 kV for 4 ms. Just before transformation 100 µl cells thawed on ice were mixed carefully with 2 µl of the plasmid and incubated on ice for 1 minute before transforming them to chilled 0,2 cm gap width electroporation cuvettes (BioRad). After transformation the cells were mixed with 1,5 ml pre-tempered (37 ºC) LB medium and incubated ~4 hours at 37 ºC and 150 rpm before spreading these solutions on LB agar with 250 µg/ml spectinomycin and 1 µg/ml erythromycin ad incubating overnight at 37 ºC. 250 µl was spread on each agar plate.

Several of the growing colonies were transferred to 10 µl LB each and stored at 4 ºC. 5 µl of this was used as inoculate in 10–20 ml LB with 250 µg/ml spectinomycin and 1 µg/ml erythromycin. This culture was grown to turbidity and the cells were harvested in Eppendorf tubes at 16 100 x *g* for 3 minutes. Slow-growing cultures were grown overnight and used as inoculate in a new culture that was grown and harvested as described above. The cells were suspended in 50–150 µl autoclaved MilliQ water and lysed either by boiling and cooling them 3 times repeatedly for a minute each, or by freeze-thawing them on dry-ice mixed with ethanol (Kemetyl) for 3 minutes and 37 ºC water bath repeated 3–4 times. 2 µl of the lysed cells were used as template in a PCR reaction to verify *gerA* in the plasmid, using the same primers and protocol as described in 2.5.4. Some of the 10 µl LB storage cultures were used directly as template without growing cultures and lysing the cells. For long-time storage of the transformed cells at -80 °C, 700 µl bacteria culture was mixed with 300 µl sterile filtered (0,40 µm, Sartorius) 50 % glycerol (Merck).

Confirmed correctly transformed mutants were stored in Microbank tubes (Pro-Lab) at -80 ºC.

2.5.6 Verifying correctly transformed *B. licheniformis* and insert direction

As an extra precaution, additional PCR reactions were done using lysed cell material from the strains where *gerA* was confirmed inserted. Here, the primers (Table 7, Appendix 1) used in amplification of *gerA* and primers from each end of the pUC19 region in pHT315 (Figure 10) were used in different combinations. The primer pairs pHT315gerAwt_SalI F1 and pUC19F, and the primer pairs pHT315gerAwt_XbaI R1 and pUC19R should give amplifications of correctly inserted *gerA* operons. The reactions were done in MasterCycler ep*gradient* S (Eppendorf), using the following PCR-protocol:

1. 94 ºC for 3 minutes
2. 94 ºC for 1 minute
3. 52 ºC for 1 minute
4. 72 ºC for 2 minutes 40 seconds
5. 72 º for 10 minutes
6. 4 ºC ∞

Step 2–4 were performed 30 times.

Germination analyses promoted some uncertainty as to whether the correct plasmids were inserted in the new complementation mutants. To investigate whether the correct complementation plasmids had been inserted, lysed cell cultures from the strains which should be complementation mutants with *gerA* from NVH-B357 and NVH-800 respectively were used as template. Genomic DNA from both strains was used as control. The primers used, A1F and A1R, were specific to the NVH-B357 strain. The PCR protocol was as follows:

1. 94 ºC for 3 minutes
2. 94 ºC for 1 minute
3. 52 ºC for 1 minute
4. 72 ºC for 1 minute
5. 72 º for 10 minutes
6. 4 ºC ∞

Step 2–4 were performed 30 times.

2.6 GERA EXPRESSION DURING SPORULATION

As described in 1.7, the genes encoding germination receptors are expressed during sporulation. Together with the expression of *gerAA*, the expression of *sigG* and the house-keeping gene *rpoB* were analysed in strain NVH-1289. The assay gives a relative expression of the two genes in question (*gerAA* and *sigG*) compared to the expression of *rpoB* during sporulation by detecting the relative expression of each gene in a total of 1,25 ng (50 pg/µl) cDNA.

2.6.1 Samples collected during sporulation

The samples for real-time PCR analysis were collected during sporulation. In order to get a true picture of the *gerA* expression during sporulation of the spores analysed in the germination assays, the sporulation protocol used was the same as described in 2.3. The NVH-1289 strain was grown on LB agar at 37 ºC for 10–12 hours, before transferring a single colony to liquid LB medium. The pre-culture was grown to OD_{600} 0,3–0,5 before transfer to the sporulation medium, Bacto-MS. The sporulation culture was grown for 5 hours before the first sample was collected.

During sporulation the OD_{600} was measured and the culture was studied in phase contrast microscope to see how far the sporulation had progressed during sampling. Pictures were taken each hour (Olympus ColorView Illu camera with Cell^B Soft Imaging System, Olympus). To collect samples for real-time PCR analysis 600 µl sample was mixed with 600 µl cold methanol (-20 ºC, VWR) in Eppendorf tubes and stored at -80 ºC before RNA extraction. Two samples were taken each hour 5–20 hours after starting the sporulation culture. At the same times OD_{600} was measured and pictures taken for use as reference when repeating the sporulation experiment for real-time PCR sampling.

2.6.2 RNA extraction and reverse transcription

RNA was extracted from the sporulation samples using Tri-reagent solution (Invitrogen). The samples were thawed on ice and centrifuged for 5 minutes at 15 700 x *g* and 4 ºC (Eppendorf centrifuge 5415D). The cell pellets were suspended in 1 ml Tri-reagent and transferred to a tube with silica beads (Lysing Matrix B, MP Biomedicals) and homogenized for 3x1 minute with cooling on ice between the homogenizing steps (Mini-Beadbeater, BioSpec Products). After homogenizing, the sample was incubated at room temperature for 5 minutes. 100 µl 1-bromo-3-chloropropane (Sigma) was added to the

samples and the samples were mixed before incubating for 8 minutes at room temperature. The samples were centrifuged at 12 000 x *g* and 4 ºC (Eppendorf centrifuge 5415D) for 15 minutes and the colourless upper layer was transferred to a new Eppendorf tube. 500 µl propane-2-ol (VWR) was added to the samples and the samples were mixed before incubating them at room temperature for 5 minutes and centrifuging the samples at 12 000 x *g* and 4 ºC for 10 minutes. The supernatants were removed and the pellet was washed in 1 ml 70 % ethanol (Kemetyl) and centrifuged at 12 000 x *g* and 4 ºC for 5 minutes. The supernatant was removed and the pellet was allowed to dry ~5 minutes before solving the RNA in 90 µl nuclease-free water (Ambion or Applied Biosystems). If the samples were not directly treated with DNase, they were stored at -80 ºC.

The RNA was DNase treated using Turbo DNA-free DNase kit (Ambion). The sample (90 µl) was mixed with 10 µl 10x DNase buffer and 2 µl Turbo DNase and incubated at 37 ºC for 15 minutes. Another 1–2 µl Turbo DNase was added to each sample, and the samples were incubated at 37 ºC for 15 minutes. 10 µl DNase Inactivation agent was added to each sample and the samples were incubated at room temperature for 2 minutes and vortexed occasionally. The samples were centrifuged for 90 seconds at 15 700 x *g* and room temperature (Eppendorf centrifuge). 70–85 µl of the supernatant was transferred to a new Eppendorf tube and mixed with 9 µl 3 M Sodium Acetate (May & Baker Ltd.) pH 5,2 and 200 cold (-20 ºC) µl absolute ethanol (Kemetyl). The samples were centrifuged at 16 100 x *g* and 4 ºC for 15 minutes (Eppendorf centrifuge) and the supernatants were removed. The samples were washed once in 70 % ethanol and centrifuged at 16 100 x *g* and room temperature for 2 minutes. The supernatants were removed and the pellets were allowed to dry ~5 minutes at room temperature before dissolving the RNA in 10–20 µl nuclease-free water. RNA concentration and purity of the samples were measured using NanoDrop 1000 (Thermo Scientific). The samples were stored at -80 ºC.

cDNA was prepared using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) as follows: 500 ng RNA in 10 µl in nuclease-free water was mixed with 10 µl mastermix for reverse transcription reaction. 10 µl mastermix: 4,2 µl nuclease-free water, 2,0 µl 10x RT buffer, 0,8 µl 25x (100 mM) dNTP mix, 2,0 µl 10x RT random primers and 1,0 MultiScribe Reverse Transcriptase. The reverse transcription was done in PTC-100 Peltier Thermal Cycler (MJ Reasearch) using the following protocol:

1. 25 °C i 10 min
2. 37 °C i 120 min

3. 85 °C i 5 min

4. 4 °C ∞

After the reverse transcription reaction the cDNA samples were stored at -20 ºC.

For samples taken early and late during sporulation 1200 µl culture (mixed with 1200 µl methanol) was used to prepare the RNA. Otherwise 600 µl culture (mixed with 600 µl methanol) was used.

2.6.3 Real time PCR

The primers used for the analyses were RTgerAf, RTgerAr, sigGf, sigGr, rpoBf and rpoBr (Table 7, Appendix 1). These were constructed based on the genetic sequence for *B. licheniformis* DSM 13 using the program PrimerExpress (version 3.0, Applied Biosystems). pDRAW32 (AcaClone) was used to retrieve reverse complementary sequences.

To construct standard curves for the primers used in the study RNA isolated from the 13 hours sporulation culture were used. The standard curves were made using genomic DNA in the sample before DNase-treatment. The sample was diluted in autoclaved MilliQ water as follows: 1:50, 1:250, 1:1250, 1:6250 and 1:31250. 5 µl of each dilution were used as template in one Real-time-PCR reaction with the following reagents: 12,5 µl Express SYBR green ER SuperMix with premixed Rox (Invitrogen), 5,5 µl MilliQ water (not autoclaved), 1 µl forward primer (5 µM) and 1 µl reverse primer (5 µM). Each dilution was analysed in triplicates for each primer pair. In addition to the sample dilutions one negative control (water) was included for each gene.

The StepOne real-time PCR machine (Applied Biosystems) was used to conduct the analyses. Linearity and amplification efficiency for the assay were calculated based on the serial dilutions for each gene. The reaction efficiency was calculated using the slopes of the standard curves and the equation E = 10-1/s (s being the slope). Calculations were done by use of StepOne Software (Applied Biosystems).

All cDNA samples were diluted 1:100 with nuclease-free water before analysis. 5 µl cDNA were used in each reaction and the analyses were done in triplicates for each gene. Two negative controls (water) were included for each gene, and two additional DNase controls (DNase treated RNA diluted 1:100 in nuclease-free water) from each sample were included with the RTgerA primers and analysed in duplicates. The threshold value was set to 0,1 for all genes.

Relative Expression Software Tool (REST, Qiagen) was used to get the relative expression of *sigG* and *gerA* in relation to *rpoB*. The sample collected 5 hours (OD_{600}=0,204) after starting the sporulation culture was used as point zero, as this sample was collected early during exponential growth, before the start of sporulation.

3 RESULTS

Comparisons of sporulation and germination times show a tendency that wild-type strains which sporulate slowly also germinate slower / to a lesser extent. In addition, some differences between young and aged spores and between spore batches were observed.

3.1 SPORULATION

In general, the use of pre-cultures grown to OD_{600} 0,3–0,5 gave more synchronous sporulation for all strains compared to the sporulation cultures where the pre-cultures were grown for 5 hours. The strains NVH-1289 (MW3) and NVH-B357 showed faster sporulation than the other wild types (NVH-1032, NVH-800 and NVH-1112). NVH-1112 in particular sporulated slowly. NVH-1307 (MW3_ΔgerAAspec+) sporulated within the same time range as NVH-1289. The complementation mutant strains, which were sporulated with erythromycin in the medium, sporulated somewhat slower than NVH-1289 and NVH-1307. Table 2 shows the approximate sporulation times in Bacto-MS for each strain.

Centrifuging spore stock through the Ludox solution removed almost all vegetative cells and cell debris without losing many spores in the process.

Table 2. The sporulation times in Bacto-MS observed in the studied strains.

Strain	Sporulation time (days)
NVH-1289	1–2
NVH-1032	2
NVH-800	2–3
NVH-B357	2
NVH-1112	2–3
NVH-1307	2
NVH-1309	2
NVH-1311	2
NVH-1320	2

3.2 GERMINATION ASSAYS

Generally NVH-1289, NVH-1311 and NVH-B357 showed best germination in assays with nutrient germinants. NVH-1032, NVH-1309, NVH-800 and NVH-1112 had a poorer germination response. NVH-1307 did not germinate to any extent.

Regarding non-nutrient induced germination by CaDPA, NVH-1289 and NVH-1307 showed the best results, whereas the other strains showed some variations of lower germination responses.

Spores purified using Ludox expressed some minor differences during germination in casein hydrolysate, compared to spores purified in water. These differences were not apparent in L-alanine induced germination.

3.2.1 L-alanine

L-alanine was used as germinant in analyses of multiple batches of spores from the wild type strains NVH-1289 and NVH-1032, the Δ*gerAA*spec+ mutant and the complementation mutants NVH-1311 and NVH-1309. Each batch was analysed at least two times. In addition, one batch from each of the newly included strains (NVH-B357, NVH-800 and NVH-1112) and the corresponding complementation mutants were included and analysed with one repetition.

The tendencies using 100 mM L-alanine as germinant were fast and almost complete germination of the strains NVH-1289 and NVH-1311 (complementation mutant with *gerA* from NVH-1289) within three hours. NVH-1032 and NVH-1309 (complementation mutant with *gerA* from NVH-1032) germinated slower, and ≤ 50 % of the spores would germinate within three hours. The *gerA* disruption mutant, NVH-1307 showed little or no germination (< 10 % in three hours). NVH-B357 behaved similar to NVH-1289, with ~80 % of the spores germinating in three hours. NVH-800 showed 5–10 % germination and NVH-1112 less than 5 % germination in three hours. There were some complications with the new complementation mutants (see 3.3). Figure 11 and 12 and Table 3, show germination measured as fall in OD_{600} for the different strains.

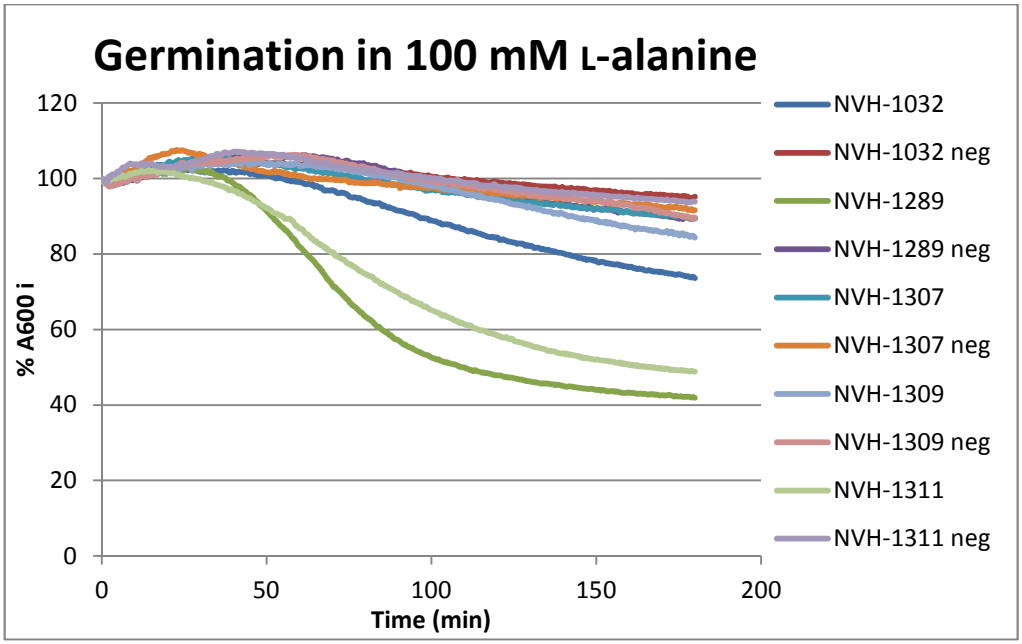

Figure 11. Germination with 100 mM L-alanine in 50 mM K-phosphate buffer pH 7.2. This shows representative results for strains NVH-1289, NVH-1032, NVH-1307, NVH-1309 and NVH-1311. Samples labeled "neg" are the negative controls. NVH-1307 is the gerA disruption mutant, NVH-1309 is the complementation mutant with gerA from NVH-1032, and NVH-1311 is the complementation mutant with *gerA* from NVH-1289.

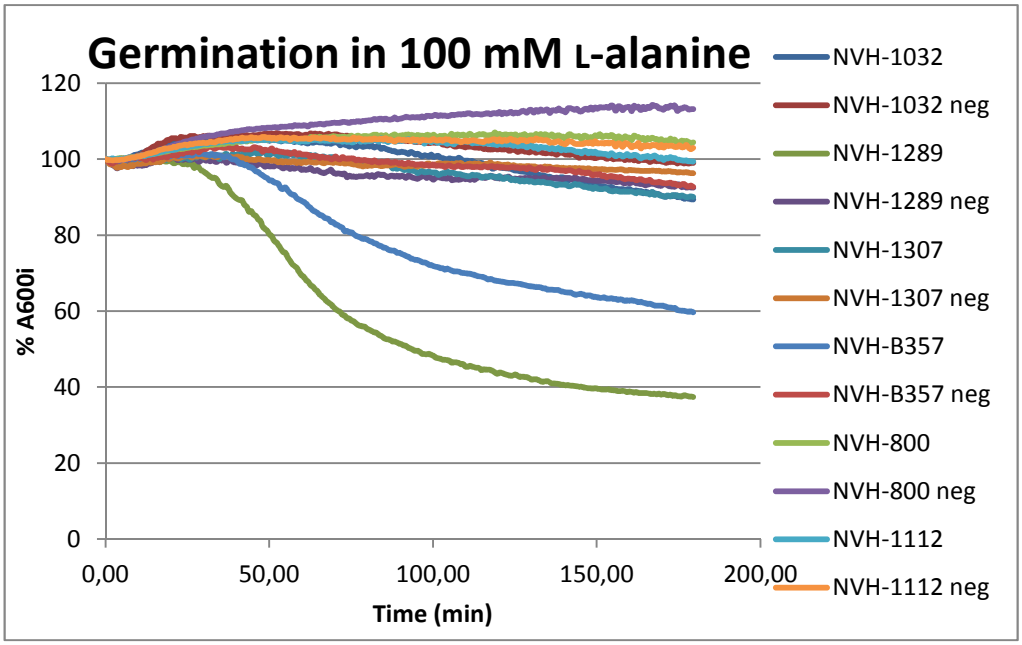

Figure 12. Germination with 100 mM L-alanine in 50 mM K-phosphate buffer pH 7.2. This shows preliminary results for the strains NVH-B357, NVH-800 and NVH-1112 in relation to the strains NVH-1032, NVH-1289 and NVH-1307. Samples labeled "neg" are the negative controls.

Some differences between spore batches and between new spores compared with older spores were seen. Older spores tended to germinate more rapidly than new spores when analysing spores from the same spore batch. In general, germination in phosphate buffer provided better germination and clearer differences between NVH-1289 and NVH-1032 than the tris buffer. No great differences were detected between spores washed only in water and spores purified through a Ludox gradient. Supplementary results from germination with L-alanine in tris buffer can be found in Appendix 3 (Table 9 and Figure 18), and a comparison of the germination properties of spores purified using water or Ludox can be studied in Figure 20 and Table 10, Appendix 4.

Table 3. Detected fall in OD_{600} (% A600i) after three hours for all strains germinated with 100 mM L-alanine in 50 mM K-phosphate buffer pH 7.2. The spores are divided according to their age at the time of germination, without showing spore batches individually.

Strain	Age of spore batch (months)	% A600i after germination (3 hours)	% A600i negative control
NVH-1289	4–5	39–51	90–99
	2–3	42	88–89
	0,5	37–41	85–98
NVH-1311	4–5	46–55	83–99
	1–2	49–50	91–94
	0,5	42–45	92–95
NVH-1307	10	87–93	94–98
	4	89–100	90–102
	1–2	90–97	89–92
	0,5	90–101	93–96
NVH-1032	4–5	49–79	97–107
	1–2	74–78	94–95
	0,5	89–90	96–99
NVH-1309	3	79–85	89–101
	5	56–60	88–94
	1–2	82–84	84–90
	0,5	76–82	90–98
NVH-B357	0,5	58–60	92–93
NVH-800	0,5	103–104	112–113
NVH-1112	0,5	97–99	101–103

3.2.2 Casein hydrolysate

The strains NVH-1289, NVH-1311 and NVH-1307 were analysed using multiple batches of spores with at least one repetition of the analyses done on each batch using casein hydrolysate as germinant. Germination of the strains NVH-1032, NVH-B357, NVH-800 and NVH-1112 were each analysed two times using the same batch.

The same differences in germination rates between strains, as described using L-alanine as germinant, were seen using 1 % casein hydrolysate as germinant. However, no strain was fully germinated after three hours. This may be due to a lower concentration of germinants in casein hydrolysate. NVH-1289 showed best germination, with ~50 % phase dark spores as seen in phase contrast microscope after three hours. NVH-1311 showed similar results to those of NVH-1289, whereas NVH-1307 had no detectable germination. NVH-B357 germinated ~30 %, and NVH-800, NVH-1032 and NVH-1112 all showed 10–15 % germination. These results can be viewed in Figure 13 and 14 and Table 4.

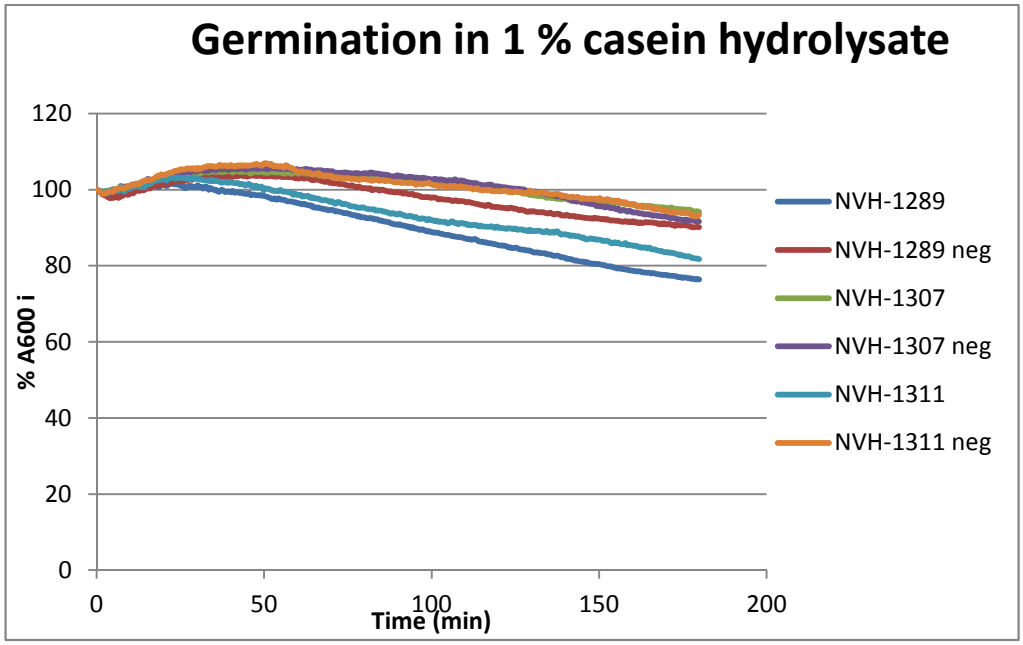

Figure 13. Germination with 1 % casein hydrolysate, 50 mM tris pH 7.4, 10 mM KCl. This shows representative results for strains NVH-1289, NVH-1307and NVH-1311.

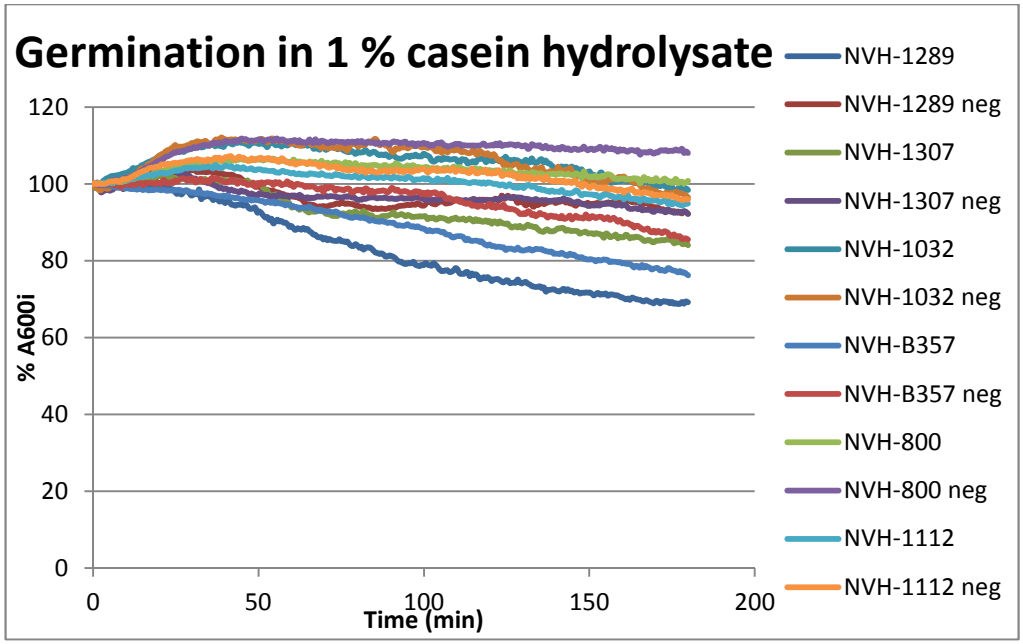

Figure 14. Germination with 1 % casein hydrolysate, 50 mM tris pH 7.4, 10 mM KCl. This shows representative results for the strains NVH-B357, NVH-800, NVH-1032 and NVH-1112 in relation to the strains NVH-1289 and NVH-1307.

Table 4. Detected fall in OD_{600} (%A600i) after three hours for all strains germinated with 1 % casein hydrolysate in 50 mM tris, 10 mM KCl buffer pH 7.4. The spores are divided according to their age at the time of germination, without showing spore batches individually.

Strain	Age of spore batch (months)	%A600i after germination (3 hours)	% A600i negative control
NVH-1289	2–3	70–76	91–95
	7	76	90
	1	69–81	92–95
NVH-1311	1–2	78–92	87–99
	3	79	85
	7	82	93
NVH-1307	2–3	93–100	90–100
	7	94	92
	1	84–99	92–97
NVH-B357	1	76–93	86–101
NVH-800	1	96–101	103–108
NVH-1112	1	90–95	96–99
NVH-1032	1–2	91–98	86–97

Germination by casein hydrolysate in tris buffer showed some differences between spores washed in water and spores purified using the Ludox gradient. In general spores purified using Ludox showed somewhat better germination in three hours compared to spores (from the same batch) washed in water only. These results are shown in Appendix 4 (Figure 19, Table 11).

3.2.3 Calcium and dipicolinic acid (DPA)

The CaDPA studies started with the use of 45 mM DPA and 50–100 mM Calcium as germinant either at 30 ºC or at room temperature. At these conditions *B. cereus* NVH-1129 showed complete germination, and the *B. subtilis* NVH-B252 and the *B. licheniformis* strains tested showed very little or no germination. With concentrations of 20 mM DPA and 30 mM DPA, *B. cereus* NVH-1129 germinated 40–50 % in three hours at room temperature, whereas none of the other strains showed signs of germination. At any tested concentrations using ≥ 45 mM DPA with Calcium in excess, the *B. cereus* strain would show a complete germination within 3 hours.

At 60 mM DPA and 100 mM Calcium *B. subtilis* NVH-B252 germinated almost completely within three hours, and the *B. licheniformis* strains showed 40–70 % germination with apparent variations between repetitions. The spores germinated better at room temperature than at 30 ºC.

Finally the concentrations of 80 mM DPA and 100 mM calcium at room temperature showed complete germination for *B. subtilis* and 70–100 % germination in *B. licheniformis* NVH-1289 and NVH-1307, which were analysed using three different spore batches from each strain. At the same conditions NVH-B357 germinated ~95 %, NVH-1032 germinated ~80 %, NVH-800 germinated 40-–0 % and NVH-1112 germinated ~10 % when these were tested. Only one spore batch from each of the last four strains was tested. These results are presented in Table 5.

Table 5. Germination detected by use of phase contrast microscopy for spores germinated with CaDPA.

Germinant concentrations		Percent germinated spores after 3 hours on roller mixer at room temperature			
[DPA]	[Ca2+]	NVH-1129	NVH-B252	NVH-1289	NVH-1307
80	100	100	~ 100	70–100	70–100
45	100	100	50–60	10–15	5–10
20	100	40–50	0	0	0
[DPA]	[Ca2+]	NVH-1032	NVH-B357	NVH-800	NVH-1112
80	100	80–90	95	40–50	~ 10

3.2.4 Germination in LB and BHI

It was investigated whether NVH-1307 or any of the strains with slow/little germination in L-alanine or casein hydrolysate (NVH-800, NVH-1032 and NVH-1112) would germinate in BHI. NVH-1289 was used as a positive control. NVH-1289 germinated ~40 %, whereas NVH-1307 did not germinate in BHI. None of the other strains showed germination beyond 10–20 % in BHI. In LB medium the results were similar for NVH-1289 and NVH-1307, the only strains tested.

3.3 NEW COMPLEMENTED MUTANTS

After transforming NVH-1307 with complementation plasmids containing the *gerA* operon from NVH-B357, NVH-800 or NVH-1112 and producing spore stocks from these new strains, analyses of germination in 100 mM L-alanine were done. The results showed similarities in the germination properties of the new complementation mutant strains which should have contained *gerA* from NVH-B357 and NVH-800 respectively (see Figure 15). These similarities raised suspicions regarding the identity of the complementation mutant strains, and a PCR with primers specific to NVH-B357 was used to investigate this further. The results from this PCR confirmed that both of the strains indeed had been transformed with the complementation plasmid containing *gerA* from NVH-B357. New complementation mutants with *gerA* from NVH-800 and NVH-1112 were constructed, as there was some uncertainty of the identity of NVH-1321 (complemented with *gerA* from NVH-1112) as well. No further analyses were conducted on the new complementation mutants.

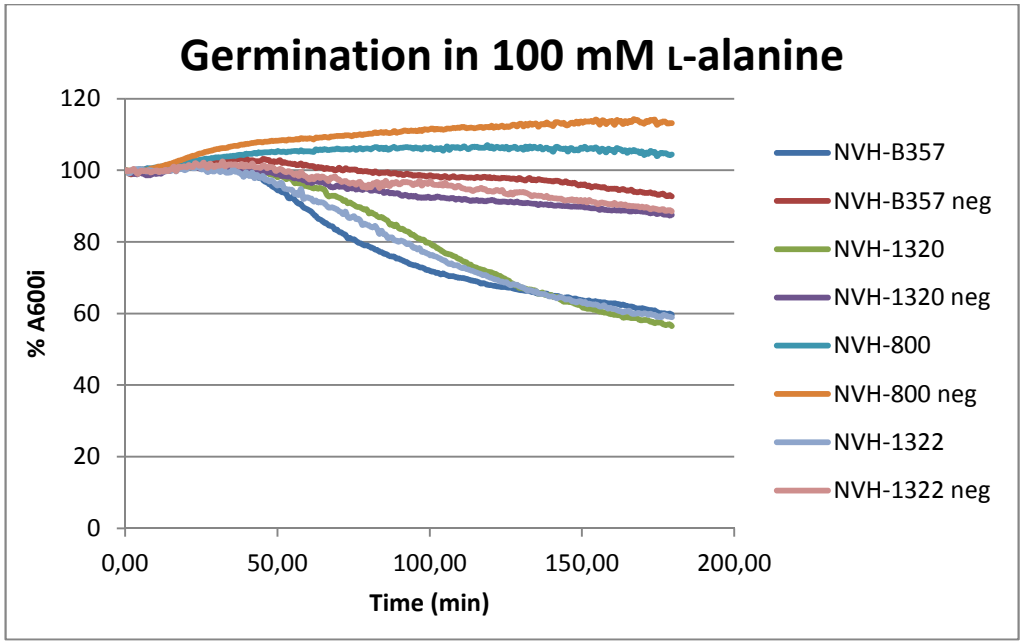

Figure 15. Germination with 100 mM L-alanine in 50 mM K-phosphate buffer pH 7,2. This shows the complementation mutants NVH-1320 (gerAB357) and NVH-1322 (gerA800) compared to the wild types. Notice the similar OD_{600} curves for NVH-B357, NVH-1320 and NVH-1322.

3.4 REAL-TIME PCR

The reaction efficiencies for the primer pairs are shown in Table 6. Figure 21 (Appendix 5) shows the standard curves, and melt curves for the standard curves and the experiments can be viewed in Figure 22 and 23 (Appendix 5) respectively.

Table 6. Calculated reaction efficiency for each primer pair used in the real-time PCR analysis.

Primer pair	Reaction efficiency (%)
RTgerAf/RTgerAr	95,359
sigGf/sigGr	98,064
rpoBf/rpoBr	101,128

Real-time PCR showed a peak for the expression of *gerA* in NVH-1289 sometime before or about 8 hours after starting the sporulation culture. The highest peak detected happened around 13 hours after starting the sporulation culture, with a smaller peak a couple of hours later. At 20 hours the expression was also growing. This is illustrated in Figure 16.

The expression of *sigG* decreased somewhat towards 10 hours, and then increased rapidly and peaked at 15 hours. After this, the expression decreased until 19 hours, and

showed some increase between 19 and 20 hours. The expression of rpoB was fairly stable throughout the analysis.

The growth curve (OD_{600}) showed transmission from exponential growth to stationary phase around 10 hours after starting the sporulation culture. The first phase-bright spores appeared at 8 hours and the first free spores appeared 13 hours after starting the sporulation culture. After 16 hours phase-bright spores dominated and at 19 hours free spores were dominating the culture. Sampling stopped at 20 hours, when more than 65 % of the culture consisted of free, phase-bright spores and less than 20 % were vegetative cells with or without enclosed forespores. At this time some of the free spores had begun to germinate spontaneously. Pictures taken during sporulation are shown in Figure 17.

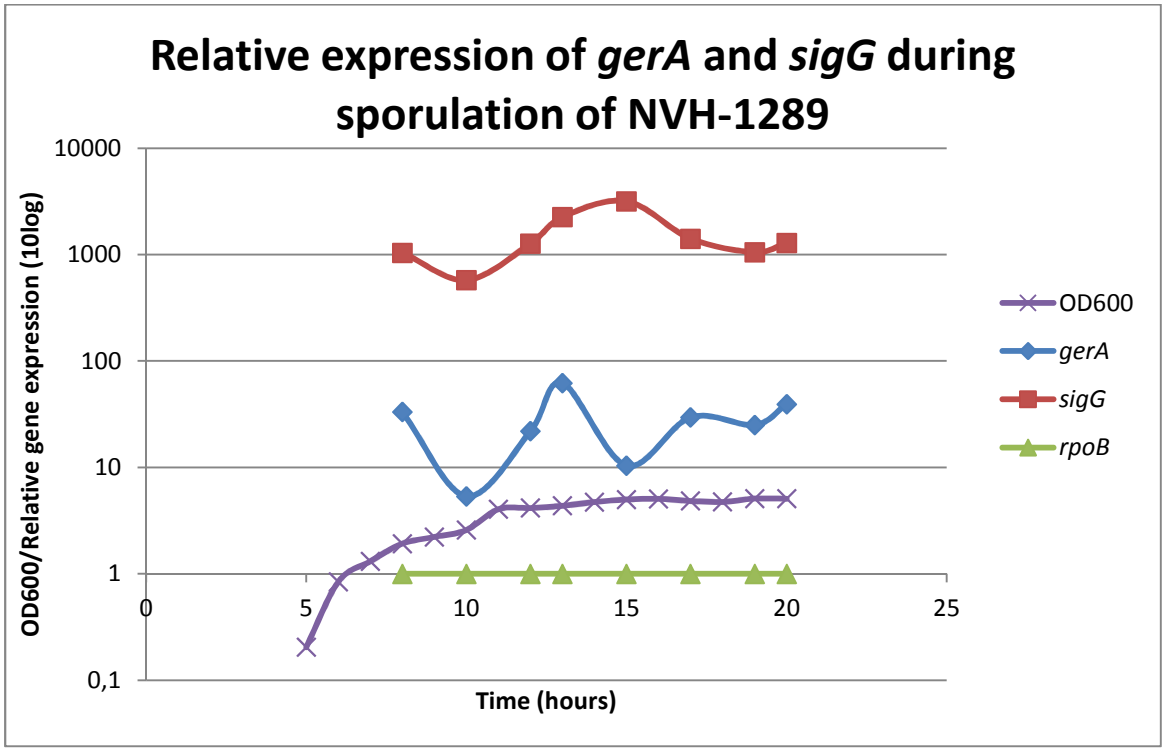

Figure 16. Relative expression of *gerA* and *sigG* throughout sporulation of NVH-1289 using *rpoB* as reference gene in NVH-1289 sporulated in Bacto-MS. The growth/sporulation curve (OD_{600}) is indicated.

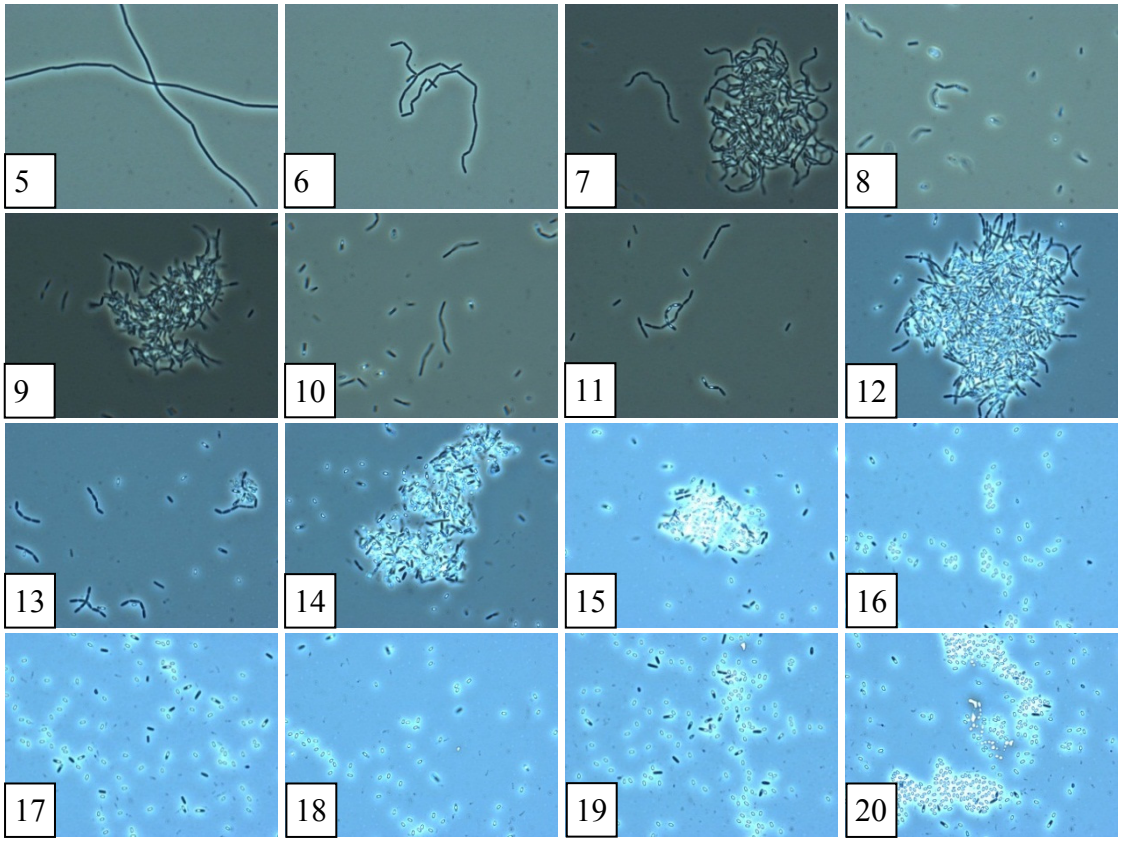

Figure 17. Phase contrast images of vegetative cells (dark) contra spores (bright) in the sporulation culture (NVH-1289) 5–20 hours after transfer to the sporulation medium (Bacto-MS). The numbers indicate hours after transfer to the sporulation medium. (Pictures taken using Olympus BX51 with ColorView Illu camera and Cell^B software)

4 DISCUSSION

Based on the results from the germination assays with nutrient germinants, it seems that the GerA receptor in *B. licheniformis* indeed is involved in germination with L-alanine, and possibly other amino acids present in casein hydrolysate. In addition, results from germination with CaDPA showed that the MW3_Δ*gerAA*spec+ mutant is capable of germination, and that it seems unlikely that the germination apparatus in this strain is damaged beyond the disruption in *gerAA*. For the different *B. licheniformis* strains tested (NVH-1289, NVH-1307, NVH-1032, NVH-B357, NVH-800 and NVH-1112), it seems that *B. licheniformis* needs higher concentrations of CaDPA than the strains used as positive controls (*B. cereus* NVH-1129 and *B. subtilis* NVH-B252) in order to germinate efficiently. To the author's knowledge, this has not been reported earlier, and for now there are no explanations for these observations.

4.1 SPORULATION

Sporulation of *B. licheniformis* was done at a relatively high temperature (50 ºC). Sporulation of some of the strains studied had previously been done at 37 ºC (Klufterud 2011). As increasing the temperature in some cases seemed to yield more stable spore cultures, with less spontaneous germination (Klufterud 2011), this sporulation temperature was maintained. In addition, the sporulation time was decreased by days and spore germination was enhanced. (Løvdal *et al* 2012; Klufterud 2011).

Interpreting the results from the germination studies, it is important to remember that germination properties are influenced by the sporulation conditions, which have been demonstrated to have great impact on spore properties (Fleming, Ordal 1964; Baril *et al* 2011; Garcia, van der Voort, Abee 2010; Planchon *et al* 2010; Eijlander, Abee, Kuipers 2011; Carlin 2010). This includes the media used, ions and salts present, the temperature and the sporulation time. Baril *et al* (2011) have demonstrated that heat-resistance in *B. licheniformis* increases as a function of the sporulation temperature. For their strain tested (AD978) a maximum heat resistance was detected in spores sporulated at 45 ºC. It could be worthwhile to investigate correlations between sporulation conditions and spore properties in order to optimize the sporulation process in the examined strains. In addition, spores have been shown to mature for weeks after sporulation (Sanchez-Salas *et al* 2011), and exposing spores to different environments during this maturation time could influence germination properties (Løvdal, Granum, Rosnes 2011).

All the strains studied showed great heterogeneity during sporulation. Some cells sporulated early, and there was a tendency of "nest" formation in the sporulation cultures (see Figure 17; 7, 9, 12 and 14 hours after transfer to the sporulation medium). These "nests" consisted of vegetative and sporulating cells, where some cells sporulated quicker than others. In some strains (e.g. NVH-1289) there was a tendency of spontaneous germination of the spores formed early during sporulation, and these could germinate before all sporulating cells had finished sporulation. Some cells never showed any signs of sporulation. A challenge with sporulation to construct spores for laboratory use is that the sporulation environment cannot be kept stable in all senses: during sporulation in liquid media, the nutrients are gradually depleted, and the pH is affected. Spores made early will have been exposed to different environments than spores made late in a spore population. (Sanchez-Salas *et al* 2011; Hornstra *et al* 2009) When sporulating bacteria on solid media, sporulation will occur in micro-colonies, where each colony consists of a

different spore population (Hornstra *et al* 2009). Thus spores made under relatively controlled conditions are still to be seen as a heterogeneous group of spores. In addition to the differences between spores within one population or spore batch, there will be differences between spore batches as well, contributing to the diversity seen in for example germination analyses.

As described by Zhu *et al* (1989), Ludox proved to be a good tool for purifying the spores from the vegetative cells after they were removed from the sporulation medium. By this technique the spores were separated from vegetative cells and cell debris without losing too much spore material. Germination analyses done on the same spore batch with spores washed only in water and spores centrifuged through the Ludox gradient showed similar results, which indicate that this treatment do not largely interfere with the germination properties that were tested. However, some differences were seen, and further analyses, using several spore batches from different strains, are required before a definitive conclusion can be reached.

4.2 GERMINATION STUDIES

The germination assays performed in this study are studies on populations of spores and not on single spores or cells. By measuring the change in OD_{600} after addition of the germinant, the OD_{600} gives an overall illustration of the germination rate in the populations. Slow or no change in OD_{600}/germination within a given time interval after addition of the germinant may be interpreted as either poor or slow germination in the specific spore population at the given conditions. Clear differences between the strains' abilities to germinate with L-alanine and casein hydrolysate were detected. Some clear differences were observed during germination in calcium and dipicolinic acid as well. The strain NVH-1112 appeared to be the slowest to germinate, and further analyses are needed to determine if the germination rates in this strain can be increased by using higher concentrations of the germinants, or by changing the sporulation and/or germination conditions. The assay with casein hydrolysate showed the most promising results in term of germination in this strain as seen in phase contrast microscope. The *gerAA* disruption mutants complemented with either *gerA* from NVH-1289, NVH-1032 or NVH-B357 showed a similar germination pattern as the wild types NVH-1289, NVH-1032 and NVH-B357. No results from germination of the new NVH-1307 mutants

complemented with *gerA* from NVH-800 and NVH-1112 are currently available, but it is likely that this will be the case for these mutants as well. This remains to be investigated.

The *gerA* sequence from the plasmid used in NVH-1322 has been proved to be identical to *gerA* from NVH-800 (Henie-Madslien, unpublished results). The *gerA* on the other plasmids are currently only partly sequenced, but preliminary results show identical sequences as the strains from which *gerA* was extracted. Probably the sequences will be correct, as was the case for both NVH-1309 and NVH-1311, which were constructed using the same procedure.

The slow germination in NVH-1032 ("problem" strain isolated from a canned meat product) compared to NVH-1289 (laboratory strain) is supported by the finding that this strain was in fact not killed by the double heat treatment employed during the canning process as a result of slow germination of the spores. Since the "problem" strain, NVH-1032, was isolated from a canned meat product where it probably germinated with little or no air present, it would be interesting to study this strain's properties during anaerobic germination. The other strains in this study should be included in this as well, thus giving some information on anaerobic germination of *B. licheniformis* as a species. A small pilot study comparing aerobic and anaerobic germination indicated no great differences in germination properties with L-alanine as germinant for the wild type strains (Henie-Madslien, unpublished results).

The preliminary results from the on-going studies including the new *B. licheniformis* strains indicate great diversity in germination properties, and that mutations in the *gerA* operon may cause large differences in germination capability with L-alanine and possibly other amino acids. Results from germination with 80 mM DPA and 100 mM Ca^{2+} also show great strain-specific variations. A comparison of the sequences for the genes encoding CwlJ in the tested strains could possibly shed some light on the reason for these differences. As described in 1.3.4, CaDPA has been shown to activate the cortex lytic enzyme CwlJ, and thereby induce germination in spores (Paidhungat, Ragkousi, Setlow 2001; Ragkousi *et al* 2003). Including *cwlJ* from the tested *B. cereus* and *B. subtilis* strains in the analysis may give some indications on whether sequence differences in *cwlJ* influence germination rates with CaDPA as germinant.

The reasons for the apparent differences in the germination abilities among the studied strains could have different explanations. One possible reason is that the sequence differences found between the *gerA* operons in the studied strains may prevent germination if these mutations in some way cause dysfunctional receptors. Another

possibility is that these sequence differences make germination a slower process as a consequence of changes in the receptor, e.g. by poorer interaction between germinant and receptor. Further analyses are required to ascertain whether the GerA receptors of the strains showing slow or no germination function properly. This work has been initiated with the construction of complementation mutants harbouring *gerA* from the different strains.

Another possibility that may explain the observed differences is that the receptors in some strains could need co-germinants or other environmental factors during sporulation or during germination to initiate germination of the spores. Atluri *et al* (2005) have shown that the cooperation between receptors in *B. subtilis* may enhance germination in various ways, including stimulation of L-alanine induced germination by adding glucose and K^+ to spores with functional GerA and GerK receptors. This will, however, need further testing. The relevance of other genetic factors can also not be excluded. In *B. subtilis* the need of a functional GerD protein for efficient germination with L-alanine has been demonstrated (Mongkolthanaruk, Robinson, Moir 2009). This protein is predicted to exist in *B. licheniformis* ATCC 14580/DSM 13 as well (Complete proteome *B. licheniformis* strain DSM 13 / ATCC 14580), and it could be useful to explore the presence of this protein in the studied strains. If GerD indeed should be responsible for some of the germination differences between the strains, it is likely that these differences could be eliminated in the complementation mutants, as the protein is predicted to exist in the strain used to construct these (MW3, which has been used to construct the studied mutant strains was constructed from strain DSM 13). The existing results indicate that differences between the GerA receptors between NVH-1289 and NVH-1032 are responsible for at least some of the observed germination variability between the strains.

As mentioned earlier, it should be emphasised that all results are dependent on the environmental conditions during sporulation, preparation and storage of the spores and during germination. As described in the results, some experiments were performed with only one spore batch. These germination experiments should be repeated with at least one more spore batch in order to verify the results presented here. In addition to differences between spore batches and within the same spore batch, the time the experiments were conducted after sporulation was of some importance. Storage for a month could seemingly contribute greatly to enhance germination in a spore batch. Differences between batches or storage-dependent differences have been observed in all germination

assays with L-alanine, casein hydrolysate and CaDPA as germinants. However, it remains uncertain whether the mentioned factors are responsible for some of the observed differences.

It is also important to remember that all strains are studied in pure cultures, with only one strain present in each sample. Mostly the germinant concentrations tested and the population density are different and/or higher than they would be outside the laboratory. How a certain strain behaves alone, with a high population density and high amounts of germinants present, will only give an indication of how this strain behaves in the wild (Nicholson 2002). However, germination studies are an important tool for understanding differences between species and strains, and to investigate why certain strains behave differently during for example heat treatment of processed food, as the aforementioned "problem" strain, NVH-1032. Based on results from monocultures, assays to study the interactions between more strains and species could be developed, including assays for studying germination in food products.

Microscopy as means to study germination is highly subjective, and may probably give higher deviation between runs and batches than the photometric measurements. Still, it is sometimes the only possibility to study a specific germinant, e.g. the Ca-DPA assay because of precipitation of calcium crystals. Microscopy might also serve as a control for the photometric measurements. Some strains, e. g. NVH-800, show a germination curve, as measured by OD_{600}, which increase greatly before decreasing compared to the measurements shortly after addition of germinant (figures 12 and 14). However, in the microscope, evidence of germination for these samples was clear, but could not be detected from the OD_{600}-curve. The reason for this increase is not known, but flocculation of the spores is a possible explanation. For strains with poor germination, microscopy provides valuable information.

4.3 REAL-TIME PCR

The real-time PCR results from strain NVH-1289 show multiple peaks for the expression of *gerA* during germination. Studies done by Igarashi and Setlow (2006) have shown the tendency of two clear peaks for the expression of germination receptors (GerA, GerB and GerK) in *B. subtilis* during sporulation, with the highest peak fairly late in the sporulation progress. Corfe *et al* (1994) have also shown that there is some variation in the expression of a germination receptor (GerB) during the early stages of sporulation. The tendency of

more than one peak that was detected in these experiments could be coincidental, as only one sporulation culture has been analysed. However, it could also be explained by the sporulation process of this *B. licheniformis* strain, which in Bacto-MS is not synchronized (this applies to the other studied strains as well). Some cells in the culture sporulate early and some late, which would result in that the expression during sporulation may vary throughout the process. To verify the results, further sporulation experiments and real-time PCR will have to be repeated. As the goal of studying the expression of *gerA* during sporulation is to compare the expression in the wild type with the expression in the complementation mutant carrying the same gene, OD_{600}-values and the phase contrast microscopic images should be used to make sure the cultures are in roughly the same phase of sporulation when the samples are prepared. In this study, the 5 hours-culture (OD_{600} ~0,2) was used as point zero, as this time point is early in the exponential growth phase, well before the stationary phase and therefore before the sporulation is initiated. Samples taken before this would have a poor RNA yield, and would probably be of little use in the assay.

Preliminary results from real-time PCR studies of NVH-1311 (MW3Δ*gerAA*spec+ pHT315*gerA*1289) indicate tenfold expression of *gerA* in this strain compared to NVH-1289 (Henie-Madslien, unpublished results). This is to be expected, as the pHT315 plasmid has been shown to have ~15 copies in *B. thuringiensis* (Arantes, Lereclu 1991; Mahillon *et al* 1989).

The real-time-assay is based on detection of signals from the SYBRgreen dye, which binds unspecific to dsDNA. There is a possibility that more specific results could be obtained using a specific TaqMan Probe, which is more expensive. However, based on the melting curves and the reaction efficiency, the presented results should prove sufficient regarding specificity of the assay.

An alternative to real-time PCR to visualize the amount of the GerA receptor in the spores is use of membrane extraction, SDS-page and western blotting with antibodies against the GerA protein in the spores. This would include uniform spore cultures (with approximately the same number of spores, preferably based on plate count after inactivating vegetative cells in the cultures). To perform this experiment, a specific antibody against part of the GerA receptor would be needed. Instead of comparing the expression of the receptor encoding gene during sporulation, one would get a visual picture of the receptor protein present in the spore. If the concentrations of the receptor protein are fairly similar between the studied strains, western blotting would perhaps have

a too low sensitivity. In this case, another method using immunologic reactions, such as ELISA, could be of use. Probably the spores would have to be de-coated or fragmented in order for this to work, as the protein is localized on the inner membrane.

4.4 FUTURE PROSPECTS AND STUDIES

Future work with germination in *B. licheniformis* will include finishing the germination studies with L-alanine, casein hydrolysate and CaDPA in the additional strains, including the new complementation mutants. The real-time PCR analyses should be repeated on at least three separate spore batches for the strains NVH-1289 and NVH-1311. It would probably be useful to repeat these analyses in another wild type and the corresponding complementation mutant (e.g. NVH-1032 and NVH-1309), as this would give a clearer picture of whether the tendency of ten-fold expression in the complementation mutants compared to the wild type is universal for this gene. In addition, the Δ*gerAA*spec+ mutant (NVH-1307) could be included, in order to verify that this strain in fact has no transcription of the *gerA* operon.

To examine germination differences between the studied strains further, and possibly describe the germination apparatus in *B. licheniformis* more extensively, a Δ*gerA*Δ*gerK* double mutant could be constructed. This mutant would lack both of the predicted germination receptors (GerA and GerK) in *B. licheniformis*, and using this in germination studies with complementation mutants would eliminate the possibility of interactions between these germination receptors. This double mutant could then be used to study each germination receptor in *B. licheniformis* more extensively, and hopefully reveal more about the relationship between genotype and germination phenotype.

Further germination analyses in this species could target the cortex lytic enzymes CwlJ and SleB and see whether both of these are functional in the different strains studied. The differences between the strains tested with CaDPA as germinant indicate some differences in CwlJ, which is associated with CaDPA induced germination in *B. subtilis* (Paidhungat, Ragkousi, Setlow 2001; Ragkousi *et al* 2003). It would also be of interest to study whether the predicted protein GerD has the same function in *B. licheniformis* as in *B. subtilis*, namely enhancing L-alanine induced germination which is associated with the GerA receptor (Mongkolthanaruk, Robinson, Moir 2009; Paredes-Sabja 2010).

The type I restriction modification system genes *hsdR1* and *hsdR2* are so far detected in *B. licheniformis* DSM 13/ATCC 14580 (Waschkau *et al* 2007). It is likely that some system destroying foreign DNA is present in many *B. licheniformis* strains, but it remains uncertain whether naturally competent strains that may not harbour these or similar genes exist. It would be of interest to see whether any of the strains in this study could in fact be naturally transformable (e.g. lacking functional HsdR1 and HsdR2), and using these in further germination studies.

Increasing the amount of germinants used in the conducted assays would be of interest, to see whether this could have any effect on the strains which are difficult to germinate. As both L-cysteine and L-valine have been showed to induce germination in *B. licheniformis* (Martin, Harper 1963; Klufterud 2011), these, and possibly other amino acids present in casein hyrolysate, could be investigated as possible germinants, either alone or in various combinations. Different growth media, perhaps with the addition of sugars or ions not already present, could be investigated as germinants. Adding different ions or sugars to either L-alanine or casein hydrolysate could also be investigated as stimulants enhancing germination in *B. licheniformis*.

If possible, higher concentrations of DPA with calcium ions in excess should be tested as germinants. In addition, the strains which germinate relatively easy in 80 mM DPA and 100 mM Ca^{2+} could also be studied in company with germinating *B. cereus* spores. It might be a possibility that released CaDPA from germinating *B. cereus* spores could initiate germination in *B. licheniformis*. To eliminate receptor mediated germination, the MW3_Δ*gerAA*spec+ mutant, NVH-1307, would probably be the best choice in such experiments. Supernatant from *B. cereus* germinated with nutrient germinants could be used to try to induce germination in *B. licheniformis*. A small pilot was conducted without indication that CaDPA from *B. cereus* could germinate *B. licheniformis* (results not shown), but it is possible that higher amounts of *B. cereus* would increase the CaDPA concentration enough to induce a response in *B. licheniformis.*

The impact of sporulation conditions regarding germination are of interest. Comparing germination properties of spores made at different temperatures could be of value in view of industrial methods to eliminate spores. This should include tests for heat resistance and resistance against other factors, as well as germination studies. Based on this, a storage analysis could also be considered. This could involve analysing spores from different strains or batches at given intervals after sporulation. Alternatively a

double heat-treatment assay could be developed, where for example the long-time effects of heat activation could be studied.

It has been shown that heat-activation is a reversible process (Collado *et al* 2002), and from an industrial view it could be of interest to know the impact this has on *B. licheniformis*. In cook-chill products such as REPFEDs (see 1.5), it is valuable to know whether the non-lethal (to spores) temperatures applied could in fact activate spores, inducing better germination, and how long this effect is apparent. This could also be used to optimize a double heat-treatment process in regards to inactivation of *B. licheniformis* as a possible food pathogen or spoilage bacterium. Inclusion of other sporeformers known to cause problems in food products could be of interest, but so far no assays are developed for this purpose. Other methods known to inactivate spores, e.g. high pressure (Gould 2000), either alone or in combinations with for example heat treatment, could also be interesting to investigate.

Another possibility of extending the germination studies is to include known toxin-producing strains of *B. licheniformis* as well, as this could give valuable information of strains associated with foodborne diseases in addition to be important for the food production industry.

A factor believed to affect the spore properties in the studies conducted is the presence of antibiotics during growth and sporulation of the mutant strains (e.g. NVH-1307, NVH-1311 and the other complementation mutants). To abolish the need to maintain selective pressure to prevent plasmid curing would be valuable for future studies. Complementation mutants where *gerA* is deleted without replacing it with an antibiotic resistance cassette, and the deleted gene is replaced with *gerA* from another strain directly into the chromosome, would give a truer picture of the *gerA*-linked germination properties, as this mutant would likely harbour only one copy of the *gerA* operon.

Finally, it could be of great value to conduct studies based on single spores, rather than spore populations. This would include new equipment, either for flow cytometry or raman spectroscopy, which is expensive. However, to learn more about the spore properties in *B. licheniformis* it could prove effective.

4.5 FINAL REMARKS

Based on the results from various germination assays conducted with the wild-type NVH-1289, the MW3_Δ*gerAA*spec+ mutant NVH-1307 and the complementation mutant NVH-1311, it can be concluded that the GerA receptor induces germination with L-alanine, and possibly other amino acids present in casein hydrolysate. The analyses with CaDPA as germinant show that other parts of the germination system in the MW3_Δ*gerAA*spec+ mutant are not affected by the disruption in the *gerAA* locus.

The "problem" strain NVH-1032 showed slower germination (compared to NVH-1289) in the experiments conducted under the sporulation and germination conditions used in this study. Other strains with sequence variations in the *gerA* operon, compared to the type strain, show similar characteristics: NVH-800 and NVH-1112 show slow or no germination. However, NVH-B357, which resembles the type strain, germinates effectively.

It can be argued that the L-alanine concentrations used are much higher than those found in foods, and that germination studies based on lower concentrations may prove valuable as well with respect to the food industry.

The transformable *B. licheniformis* strain MW3 has been of great value to uncover properties of the GerA receptor in *B. licheniformis*, and probably it can be valuable in studies of other parts of the germination systems in the species as well.

The same germination analysis performed weeks apart on the same spore batch generally has shown better or faster germination of the older spores. It may be assumed that spore contaminations from outside the food production plant are a heterogeneous mix of spores from different species, strains, environments and ages. Spores coming into the food production from the outside may be older than spores made inside the production facility. Ageing of spores has been shown to activate spores, enhancing germination in a spore culture, and these results also support this. Thus it may be assumed that spores made inside the production facility could be more resistant to various inactivation methods. Whether this is the case remains to be investigated, but if so, it would be wise to avoid promoting spore formation in a food production plant.

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APPENDIX 1–5

Appendix 1: List of primers

Table 7. The Table shows sequences for the primers used in this thesis.

Primer name	Sequence
Sequencing analyses	
ASF	AAAGAAGCCTTGGAGAAGTGA
AOR	CGCTTTGCCCTGGATATAGA
4AF	CAATCCGCTAGGCCAGAC
6AF	GCGGACTGAGCCTGAATATG
8AF	CGCTCAGGATCCGTCTAAAG
A9F	CAGATCGAAGCGCTGAATTT
A1R	GCG TTA ATC GCG TCC TTT T
A5F	TCGGATTAGCACGTATGAAGC
A6F	GGG TGG AGC TGA TGA TGT TT
A7R	TGCAGATGCTGCGAGAATAC
A8F	CGCTCAGGATCCGTCTAAAG
pHT315F	TTTATGCTTCCGGCTCGTAT
pHT315R	GGAGAAAATACCGCATCAGG
A1R-8	GGCAGCGCAAAAATAACATT
A2R	CGATCCGTCCATTAAACAGC
A2F	GGAACCCGTTACAGCAAAAA
A6R	TCCAAGGTAGTCGCTGAAGG
A7F	GGATTTGGGATACCGCTCTT
A3F	TTCCGCAGATCCAGAATAC
Separating gerAB357 from gerA800	
A1F	TCAATCTCCTCCGGTTTCC
A1R	GCGTTAATCGCGTCCTTTT
Amplification of gerA from NVH-B357, NVH-800 and NVH-1112	
pHT315gerAwt_SalI F1	CAATCTGTCGACGTTTCCCCGTAAGCCTGATT
pHT315gerAwt_XbaI R1	GTGAGGTCTAGACCGATCGTGAAGAAAAGCAT
Checking correct insert and direction	
pHT315gerAwt_SalI F1	CAATCTGTCGACGTTTCCCCGTAAGCCTGATT
pHT315gerAwt_XbaI R1	GTGAGGTCTAGACCGATCGTGAAGAAAAGCAT
1383 pUC19 F	GTTTTCCCAGTCACGACGTT
1384 pUC19 R	TGTGGAATTGTGAGCGGATA
Real-time PCR	
RTgerAf	CGCTCGGCAAGAGCAAGT
RTgerAr	CCGCTTTGCC CTGGATATAG
sigGf	GCAGATATTTGCGCGATAACAA

sigGr	TGTACGCGATATCCCTGAGTGA
rpoBf	AGCTCCGCTGCGGGTTA
rpoBr	TCCTTTACTTCGCCGGTTTCT

Appendix 2: List of chemicals and equipment

Table 8. The Table shows chemicals and equipment used in the work on this thesis.

Assay	Reagent/equipment	Manufacturer
CaDPA germination	$CaCl_2$*$2H_2O$	Riedel-de Haën, Seelze, Germany
	DPA - pyridin-2,6-dicarboxylic acid	Aldrich, St. Louis, MO
	Tris (Trizma-base)	Sigma, St. Louis, MO
	Gelatine	Oxoid, Basingstoke, UK
	Chloramphenicol	Sigma
L-alanine germination	L-alanine	Sigma
	Tris (Trizma-base)	Sigma
	KCl	Merck, Whitehouse Station, NJ
	Fosfatbuffer - KH2PO4	Merck
	Fosfatbuffer - K2HPO4	Merck
Casamino acids germination	Casein-hydrolysate	Merck
	Tris (Trizma-base)	Sigma
Sporulation medium	Difco nutrient broth	Becton, Dickinson and Company, Franklin Lakes, NJ
	$FeSO_4x7H_2O$	Merck
	$CuCl_2x2H_2O$	Sigma
	$ZnCl_2$	Sigma
	$MnSO_4x4H_2O$	Merck
	$MgCl_2x\ 6H_2O$	J. T. Baker Chemicals B. V., Center Valley, PA
	$(NH_4)_2SO_4$	Merck
	$Na_2MoO_4x2H_2O$	Riedel-de Haën
	$CoCl_2x6H_2O$	Sigma
	$Ca(NO_3)_2x4H_2O$	Merck
	Erythromycin	Sigma
	Spectinomycin	Sigma
LB medium/agar	Tryptone	Oxoid
	Yeast extract	Oxoid
	NaCl	Merck
	Agar bacteriological (agar no. 1)	Oxoid
PCR	Taq Polymerase	DynaZyme, Vantaa,

		Finland
	dNTP	DynaZyme/Finnzyme, Vaanta, Finland
	Primers	Sigma
Gel-electrophoresis	SeaKem LE Agarose	Lonza, Basel, Switzerland
	Loading buffer	Sigma
	1 kB and 100 bp ladders	Merck
	EtBr	Sigma
Gel-UV-camera	Gel-logic 200	Kodak, Rochester, NY
Electric supply electrophoresis		BioRad, Hercules, CA
TAE buffer gel electrophoresis	Tris (Trizma base)	Fermentas, Vilnius, Lithauania
	Acetic acid	Fermentas
	EDTA	Sigma
BHI germination	Bacto Brain Heart Infusion	Becton, Dickinson and Company
Plasmid extraction	Ampicillin	Sigma
	Qiagen Plasmid Midi Kit	Qiagen, Venlo, The Netherlands
PCR product purification	Qiaquick PCR purification kit	Qiagen
Plasmid purification	QIAquick PCR gel extraction kit	Qiagen
Exonucleases	XbaI	BioLabs, Ipswich, MA
	NEB buffer 4 for *XbaI*	BioLabs
	SalI with supplied buffer	Sigma
	Bovine serum albumine	BioLabs
Ligation	T4 Ligase	Invitrogen, Carlsbad, CA
	Ligation buffer	Invitrogen
PCR *gerA* amplification	Hot start phusion kit	Finnzyme
	Primers	Sigma
Real-time PCR	Primers	Sigma
	Express SYBRgreen ER SuperMix with premixed ROX	Invitrogen
	High capacity cDNA Reverse transcription kit	Applied Biosystems, Foster City, CA
	Turbo DNA-free Dnase kit	Ambion, Carlsbad, CA
Real-time PCR machine and software	StepOne	Applied Biosystems
RNA extraction	Trireagent	Sigma
	Bcp	Sigma
	Propan-2-ol	VWR, West Chester, PA
	EtOH	Kemetyl, Stockholm, Sweden / Vestby, Norway
	Na-acetat	May & Baker Ltd., M&B, Dagenham, UK

	MetOH	VWR
	Lysing Matrix B (silica-kuler i rør)	MP Biomedicals, Solon, OH
Transformation	One Shot *E. coli* TOP10 Competent Cells	Invitrogen
	Micro-pulser	BioRad
	0,2 µm width electroporation cuvettes	BioRad
Elektrokompetent *B. licheniformis*	BHI	Becton, Dickinson and Company
	Spectinomycin	Sigma
	PEG6000	Merck
Sequencing	Primers	Sigma
	Sequencing company	Source BioScience, Nottingham, UK
Minisart sterile filters 0,2 and 0,45 µm		Sartorius, Goettingen, Germany
Ludox AS-40 colloidal silica, 40 wt. % suspension in water		Sigma-Aldrich
Roller mixer		Sarstedt, Nümbrecht, Germany
Incubator	Minitron	HT Infors, Bottmingen/Basel, Switzerland
	Sanyo orbital incubator	Sanyo Biomedical, San Diego, CA
Phase contrast microscope	Olympus BX51	Olympus, Shinjuku, Tokyo, Japan
Phase contrast microscope camera	ColorView Illu	Olympus
Software phase contrast microscope camera	Cell^B Soft Imaging System	Olympus
Spectro-photometer	Shimadzu UV-160 A	Shimadzu, Kyoto, Japan
PCR machines	PTC-100 Peltier Thermal Cycler	MJ Research, St. Bruno, Canada
	Mastercycler ep*gradient* S	Eppendorf, Hamburg, Germany
pH-meter		Metrohm, Herisau, Switzerland
Waterbath		Techne, Burlington, NJ
Freeze cultures	Microbank tubes	Prolab, Toronto, Canada
	Glycerol	Merck
Homogenizer	Mini-Beadbeter	BioSpec Products, Bartlesville, OK
Centrifuges	Centrifuge 5451D	Eppendorf
	Sorvall RC-5B Refrigerated Superspeed centrifuge	Sorvall, Buckinghamshire,

		England
Microplate reader	Tecan Infinite M200	Tecan, Männedorf, Switzerland
Software microplate reader	Magellan 7.1	Tecan

Appendix 3: Germination with L-alanine in tris buffer

Table 9. The Table shows results from germination with 100 mM L-alanine in tris buffer. The age at the time of germination are shown.

Strain	Age (months)	% A600i after germination (3 hours)	% A600i negative control
NVH-1289	3–4	56–59	94–98
NVH-1311	3–4	55–60	91–102
NVH-1307	3–4	96–100	93–102
NVH-1032	3–4	58–72	100–102
NVH-1309	2–3	82–87	98–100

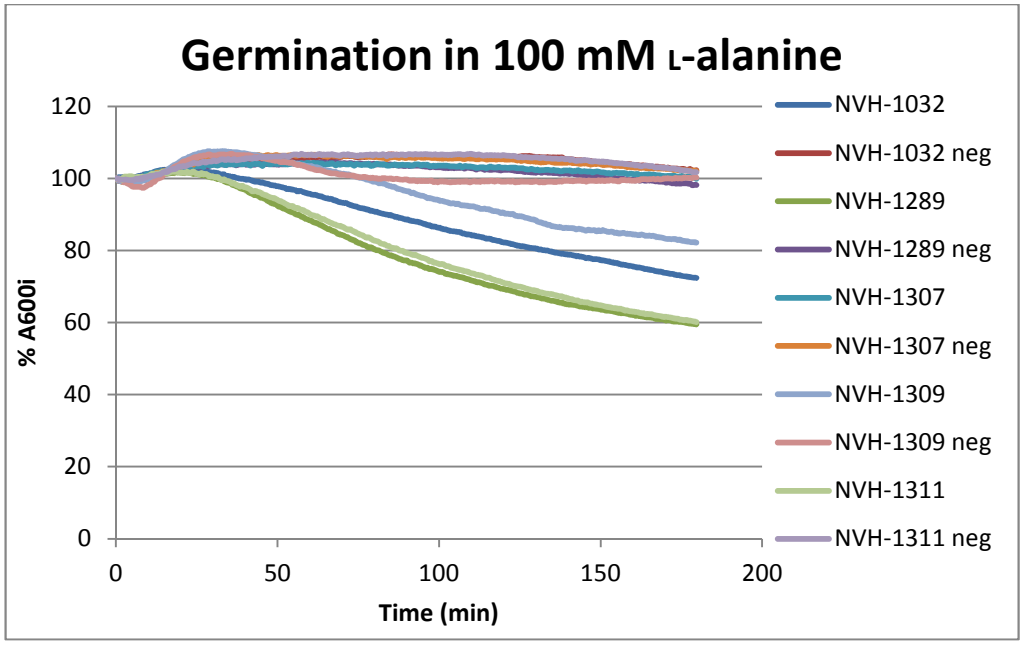

Figure 18. Germination in 100 mM L-alanine, 50 mM tris pH 7,4, 10 mM KCl,

Appendix 4: The effect of Ludox on germination

Table 10. Differences between spores purified in water and Ludox. Results from germination with 100 mM L-alanine in K-phosphate buffer.

Strain	Age of spore batch(months)	% A600i after germination (3 hours)		% A600i negative control	
		Ludox	Water	Ludox	Water
NVH-1289	0,5	37–41	40	92–98	85–89
NVH-1307	0,5	90	96–101	93–96	93–96
NVH-1309	0,5	76–78	82	90–92	93–98

Table 11. Differences between spores purified in water and Ludox. Results from germination with 1 % casein hydrolysate in tris buffer.

Strain	Age of spore batch (months)	% A600i after germination (3 hours		% A600i negative control	
		Ludox	Water	Ludox	Water
NVH-1289	1	69–76	77–81	92–95	92–93
NVH-1307	1	84–87	96–99	92–94	94–97
NVH-B357	1	76	88–93	86–87	96–101

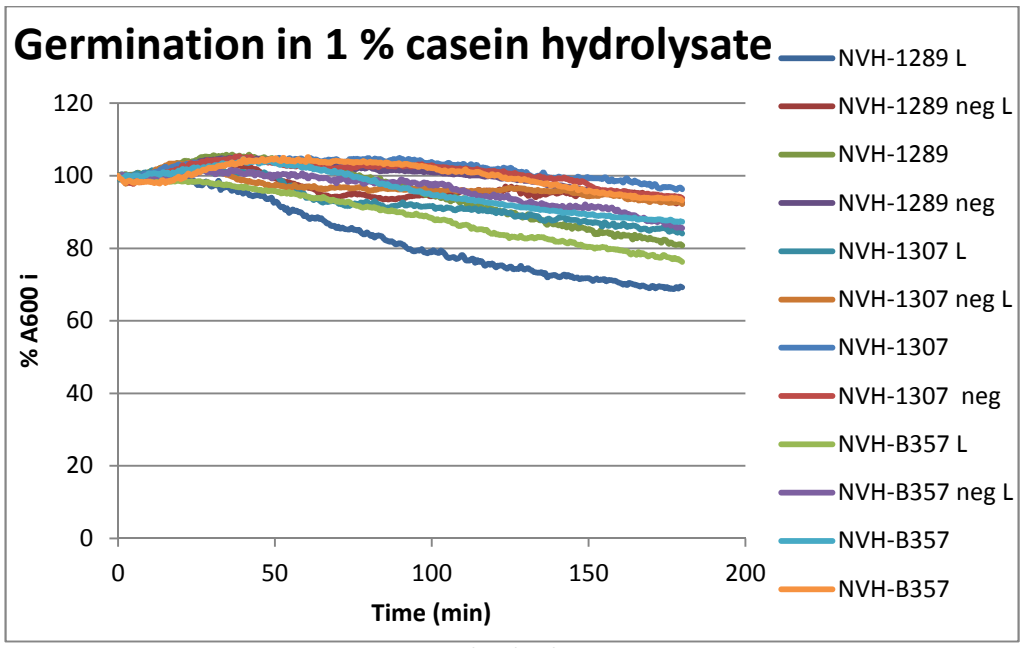

Figure 19. Germination in 1 % casein hydrolysate, 50 mM tris pH 7,4, 10 mM KCl. Differences in germination efficiency between spores purified in water and spores purified by use of Ludox gradient centrifugation. Samples marked with an L are purified by means of Ludox.

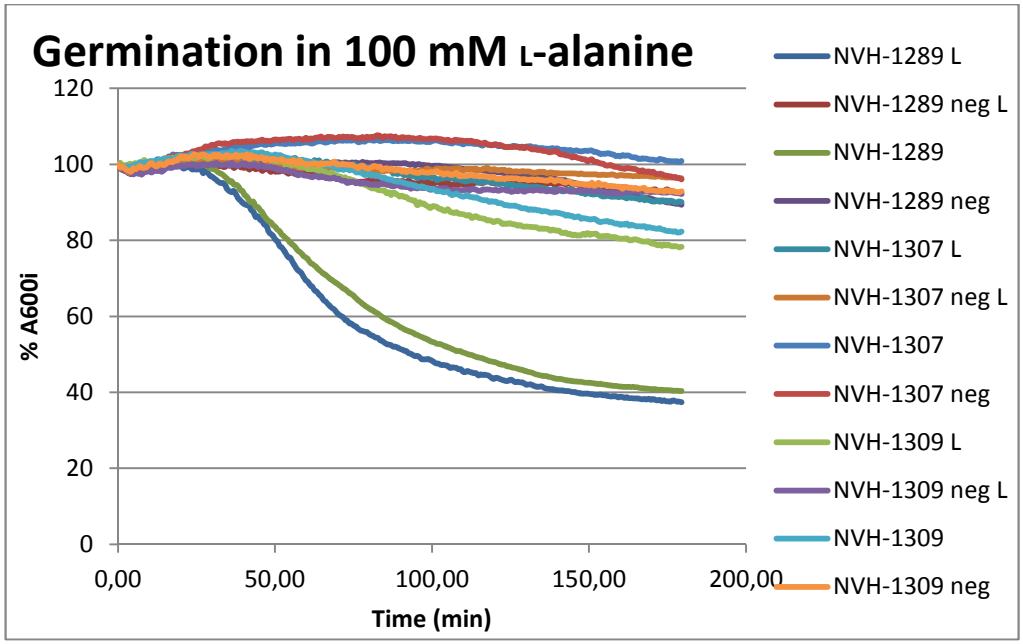

Figure 20. Germination in 100 mM L-alanine in 50 mM K-phosphate buffer 7,2. Differences in germination efficiency between spores purified in water and spores purified by use of Ludox gradient centrifugation. Samples marked with an L are purified by means of Ludox.

Appendix 5: Real time PCR

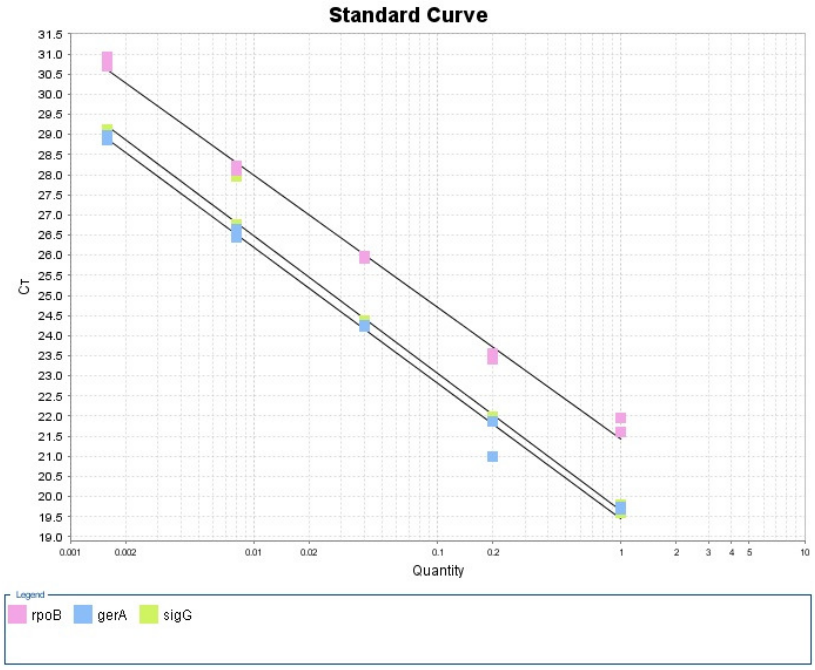

Figure 21. The Figure shows the standard curves for *gerA*, *sigG* and *rpoB*.

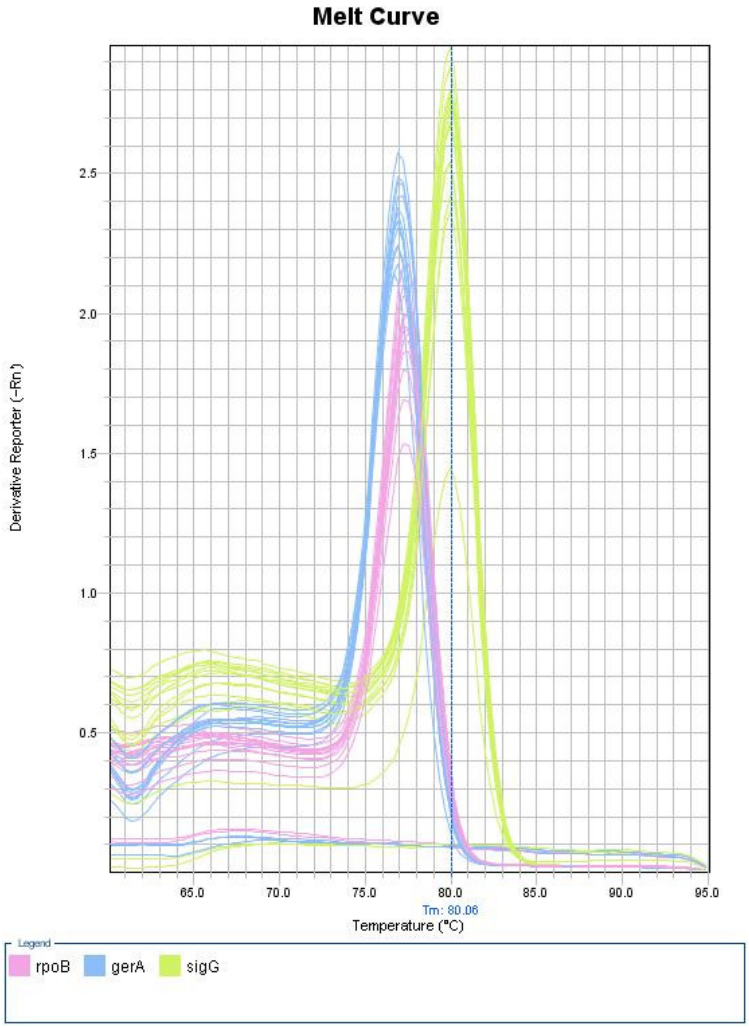

Figure 22. The Figure shows melt curves from the standard curves.

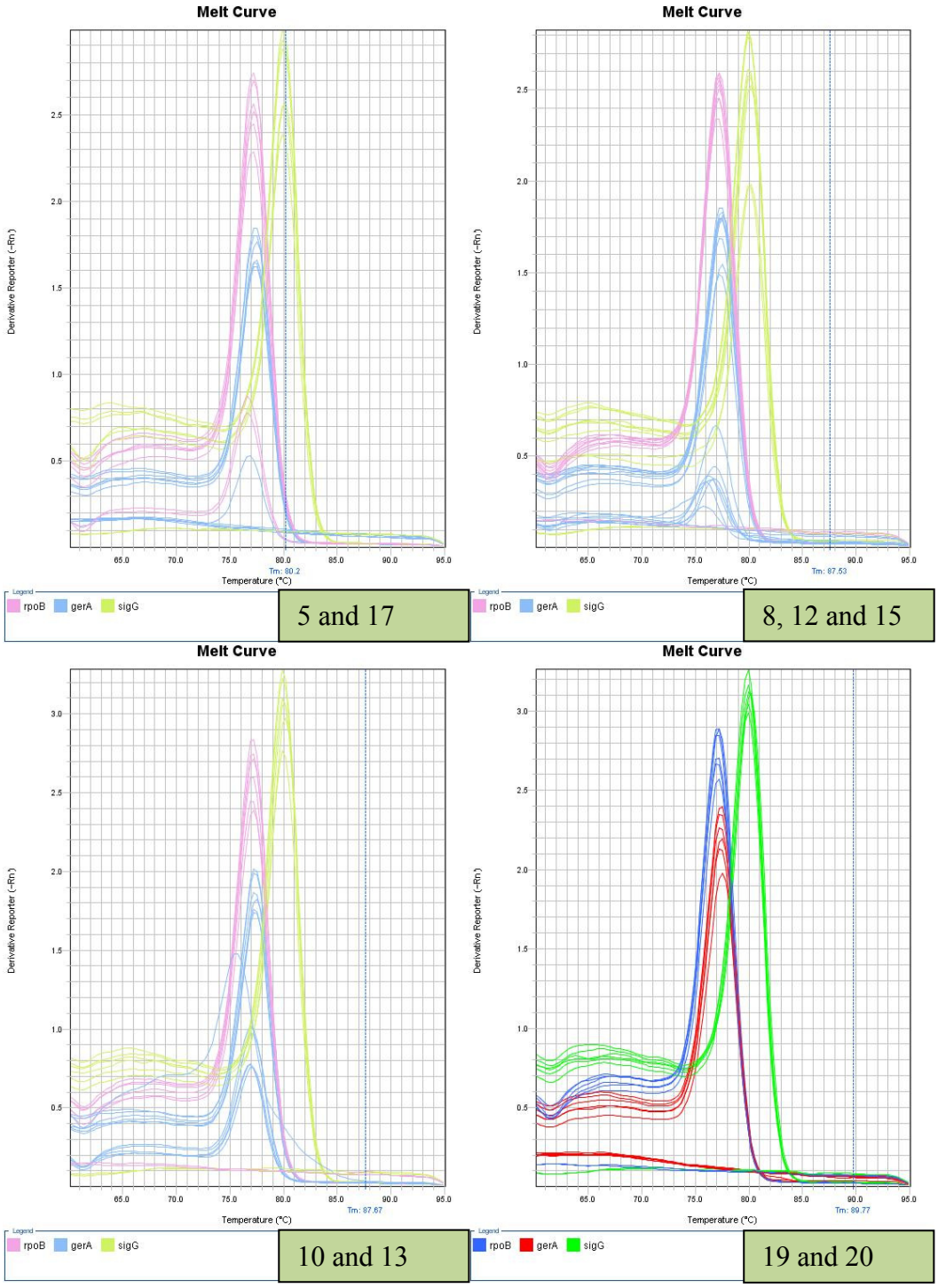

Figure 23. The figures show melting curves from the real-time PCR experiments. Time points for extraction from the sporulation culture are indicated in the green boxes.