The role of *gerK* operon in germination of *Bacillus licheniformis* spores

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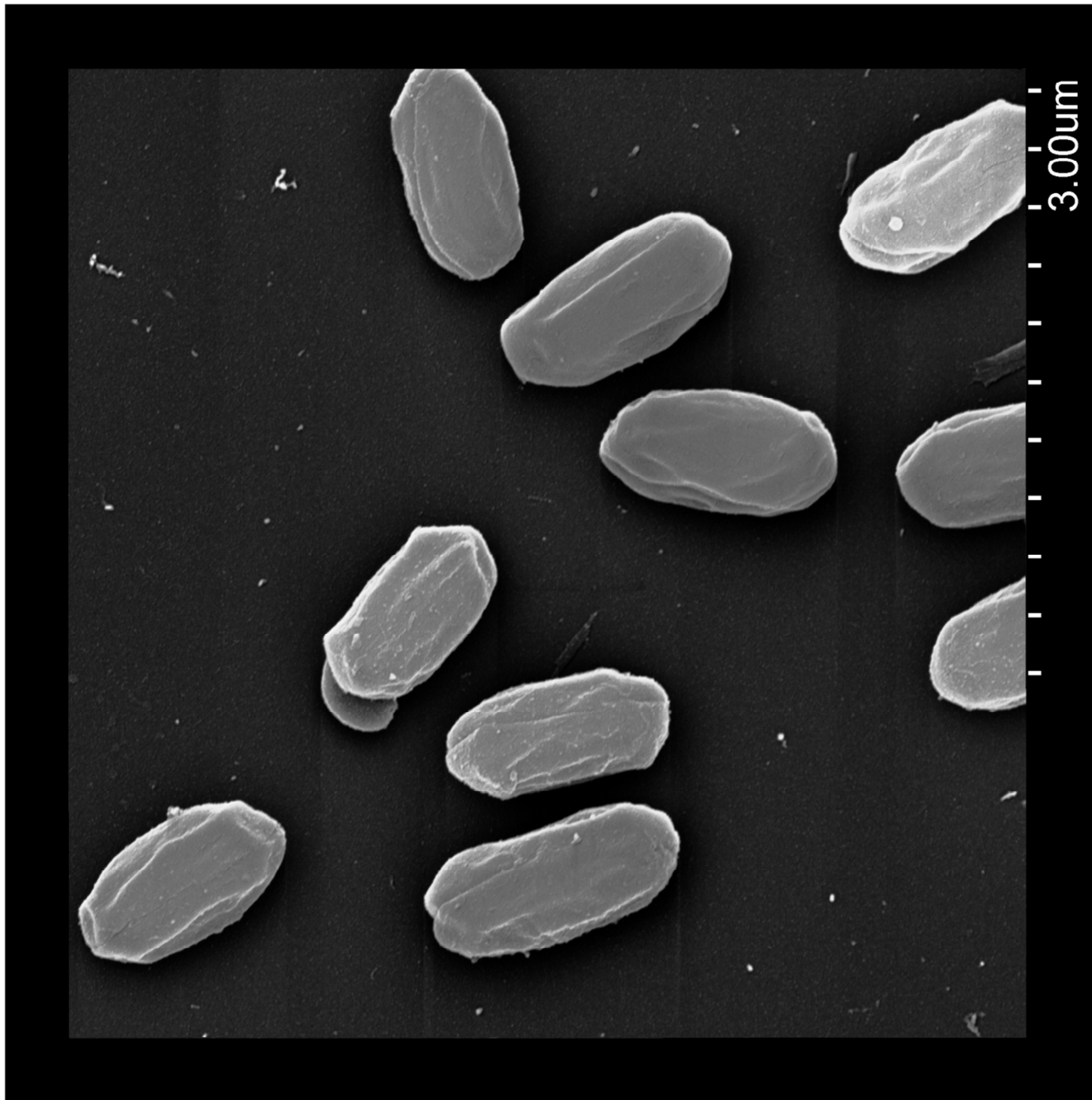

Photo of *Bacillus licheniformis* MW3 spores by E.H. Madslien, FFI and A. Hoenen, EM lab IBV, UiO, 2013.

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Abstract

Sporeforming bacteria are one of the main challenges in food preservation. The consumer demand for refrigerated foods of extended durability (REPFED) increases the need for effective ways of reducing the threat of food borne illness stemming from sporeforming bacteria. Studying germination receptors and their role in germination and outgrowth of spores might uncover the Achilles heel of spores.

This study is part of an ongoing project at the Norwegian School of Veterinary Science to study germination receptors in *Bacillus licheniformis*. Previous work done by the same research group has demonstrated that the *gerA* operon in *B. licheniformis* is involved in L-alanine induced germination. This thesis builds on this work and focuses on the *gerK* operon.

In order to study the function of the GerK receptor, a deletion mutant was made using the transformable *B. licheniformis* MW3 strain. *B. licheniformis* MW3 lacks two type I restriction modification enzymes present in the type strain. Two mutants were created, a single *gerK* deletion in *B. licheniformis* MW3 and a double deletion mutant, *gerA* and *gerK,* created by deletion of *gerK* in the *gerA* mutant.

No involvement of *gerK* could be found in germination with L-alanine, L-cysteine, L-isoleucine or casamino acid, but further studies are needed to rule out a possible cooperation between GerA and GerK receptors in relation to germination with L-alanine, glucose and K^+.

After creating the *gerK* deletion mutants, a set of *CwlJ* deletion mutants were created. CwlJ is an enzyme which is involved in depolymerization of cortex peptidoglycan during germination. Due to timeframe restriction these were not included in the germination study for this thesis, but will be studied by the research group.

Sammendrag

Sporedannende bakterier er en av hovedutfordringene innen matkonservering. Forbrukernes ønske om kjølevarer med lang holdbarhet øker behovet for effektive måter å redusere faren for matbåren sykdom utløst av sporedannende bakterier. Ved å studere germineringsreseptorer og deres rolle for germinering og utvekst av sporer, så er det kanskje mulig å oppdage sporenes akilleshæl.

Denne studien er en del av et prosjekt ved Norges Veterinærhøgskole for å kartlegge germineringsreseptorer i *Bacillus licheniformis.* Tidligere arbeid utført av den samme forskningsgruppen har vist at *gerA*-operonet er involvert i L-alanin-indusert germinering. Denne masteroppgaven bygger på dette arbeidet og fokuserer på *gerK*-operonet.

For å kunne studere funksjonen til GerK-reseptoren, ble det laget delesjonsmutanter ved hjelp av den transfomerbare *B. licheniformis* MW3 stammen. *B. licheniformis* MW3 mangler to type I restriksjonsmodifikasjonssystemer som er tilstede i typestammen. Det ble laget to mutanter, en enkel *gerK*-delesjonsmutant dannet fra *B. licheniformis* MW3 og en dobbeldelesjonsmutant, *gerA* og *gerK,* dannet ved å fjerne *gerK* i *gerA*-mutanten.

Det ble ikke påvist noen *gerK*-funksjon i forbindelse med germinering i L-alanin, L-cystein, L-isoleucin eller hydrolysert kasein, men videre studier må utføres for å utelukke et eventuelt samarbeid mellom reseptorene GerA og GerK, med tanke på germinering med L-alanin, glukose og K$^+$.

Etter *gerK*-mutantene ble det laget ett sett *CwlJ*-mutanter. *CwlJ* er et enzym som er involvert i depolymerisering av sporebarken ved germinering. Grunnet tidsrestriksjoner ble ikke disse studert i denne masteroppgaven, men mutantene vil bli studert av forskningsgruppen.

In memory of my father, David W. Kidd,

who taught me to never take shortcuts.

Acknowledgements

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Last, but not least, I wish to thank my family for their support. Thank you; Bengt Olav and Ingebjørg, for your keen eye when it comes to punctuation and grammar.

Content

Introduction

Humans and disease-causing microorganisms have been at war since the dawn of the human era. Even though microorganisms were not observed and described until Antonio van Leeuwenhoek made his own microscope in 1675 (Porter 1976), there had been a working knowledge on how to avoid illness. Alexander the Great was taught by Aristotle to boil all water before drinking it to avoid disease. One of the earliest descriptions is by Marcus Terentius Varro in the 1st century AD. He wrote *"Precautions must also be taken in the neighbourhood of swamps, (…) because there are bred certain minute creatures which cannot be seen by the eyes, which float in the air and enter the body through the mouth and nose and there cause serious diseases"* (Hooper 1934).

Through the discovery by Nicolas Appert in 1810 (Jay et al. 2005), the canning food industry was born. John Tyndall invented the process of tyndallisation (double heat treatment) in 1876 (Strick 2009), when he discovered that a single heating would not kill all microorganisms in his boiled broth, which had been contaminated with spores from hay (Tyndall 1877). At the same time Ferdinand Julius Cohn observed and described the bacterial spore of *Bacillus subtilis* and explained why boiled infusions could resume microbial growth (Strick 2009). In the early 1920s Charles Olin Ball devised the formula for thermal death time and reduced the problem of spoiled and toxic canned foods (Herndon 1971).

Centralised, industrialised food production has proved to be an effective way of spreading pathogens. A few generations ago contaminated foods had a limited distribution and only affected a small geographic area. With modern logistics a contaminated product can affect people across the whole of Europe (Kapperud 2007), and even the world.

Consumers of today want quick and simple meal solutions, but demand that it be "natural" with fewer additives and low in sodium. Ready to eat and cook-chill products pose complex problems for the food industry. To be able to produce a safe product they need to identify the types of microorganisms that might be present and how to remove them completely or control their growth (Marth 1998). Most microorganisms present in foods are not pathogenic, but to the industry spoiled goods cut in to the profits. Of all the microorganisms, endospores are the most difficult to kill during food production since many can survive heat treatment and cleaning agents (Heyndrickx 2011).

Even though over 130 years have passed since Cohn described the spore, there are many elements of spore science that still remain abstruse. Ball's equation assumes log linear inactivation of spores, but published curves of spore heat survivors show non log-linear behaviour with shoulders and tails (Gould 2006).

This thesis is part of an ongoing study to describe germination properties in *Bacillus licheniformis*, led by Professor Per Einar Granum at the Norwegian School of Veterinary Science (NVH). *B. licheniformis* is a spore former that frequently contaminates food and causes spoilage and sometimes food poisoning (Logan 2012). *B. licheniformis* is closely related to *B. subtilis*. The study of spores has so far centred on *B. subtilis* because of the ease in which this organism can be manipulated and the early availability of its complete genome sequence. (Leggett et al. 2012)

Previous work done by Irene S. Løvdal et al. has successfully shown that *B. licheniformis* germination with L-alanine functions via the GerA receptor (Løvdal et al. 2012). The work in this thesis builds upon this discovery and by eliminating other germination receptors or germination specific enzymes; their functions can be mapped by loss of germination in the spore.

The aim of the work in this thesis was to create *gerK* and *CwlJ* deletion mutants based on *B. licheniformis* MW3 (wild type) and *gerA* deletion mutant, and map their response to different germinants

Biological classification

Evolutionary relationships were of importance to the early microbiologists and they adopted the Linnean classification system used by botanists and zoologists. While bacteria have simple morphologies, animals and plants have complex morphological detail that forms a firm basis for their phylogenetic classification. Phylogenetic classification of bacteria was inherently flawed and the subject became discredited, the only remnant was the bacterial taxonomy based on Linnaean classification (Sapp 2005). Until 1977, bacteria were classified in a determinative manner based on characteristics of growth and biochemistry (Woese 1987). At that time there were only two basic forms of life, eukaryotes and prokaryotes, those who had a nucleus and those that did not (Sapp 2005).

The phylogenetic tree of life, as set forth by Carl Woese in 1977, divides life into three main domains, Bacteria, Archaea and Eucarya. The Eucarya domain contains the divisions of Animalia, Fungi and Plantae. The domain of Archaea (named *Archaeabacteria* in 1977 but changed to Archaea by Woese in 1990, to remove the notion that they were just another group of bacteria) contains single celled microorganisms. This group was previously thought to be bacteria, but through 16S rRNA gene sequencing it was discovered that Archaea were more phylogenetically related to eukaryotes (Woese et al. 1990). None of the Archaea are spore producers but some of the Eucarya species are.

Sporogenesis in Eucarya is for the most part a reproductive pathway, although a few can switch from reproduction to formation of dormant spores when needed. Examples of these dormant spores are *chlamydospores*, *teliospores* and *zygospores* produced mainly by fungi (Gould 2009).

Following Carl Woese's publication, Bacterial Evolution (Woese 1987), the domain Bacteria had six branches. One of them was the Gram positive bacteria. The Gram positive bacteria were split into four phyla based on high G+C content (*Actinobacteria*), low G+C content (*Firmicutes*), photosynthetic species (*Heliobacterium*) and species with Gram negative walls.

To this day there is no official classification of bacteria, but there is an official nomenclature from class down to subspecies. To keep up with the ever increasing and ever changing prokaryotic nomenclature, an online database was established in 1998. The database "*List of Prokaryotic names with Standing in Nomenclature*" (LPSN) bases itself on the criteria set forth in the *International Code of Nomenclature of Bacteria* (1990 Revision). Today (August 14th 2013) the domain of Bacteria is listed with 30 phyla, and even though phyla are not controlled by any nomenclature rules, most are derived from the plural genus of the main bacteria in its grouping. The exceptions are Firmicutes, Cyanobacteria, and Proteobacteria.

Firmicutes derive their name from the latin words, firmus, meaning strong, and cutis, meaning skin and referring to the cell wall. According to the database LPSN, this phylum contains six bacterial classes: Bacilli, Clostridia, Erysipelotrichia, Mollicutes, Negativicutes, and Thermolithobacteria. Although there is some strife over the placement of Mollicutes, as some assign the class to the phylum Tenericutes (Schleifer 2001).

When bacteria were classed by strain characteristics, the endospore forming bacteria were grouped in a few genera, the most important in relation to food poisoning were genus *Bacillus* and genus *Clostridium*. Today, the endospore forming bacteria are spread across a range of genera under the phylum Firmicutes. A brief description of *Clostridium* is included in this thesis for its relevance to the subject of food safety in the food industry.

The class Clostridia harbours the family of Clostridiaceae, in which we find the genus *Clostridium*. The definition of this genus used to be non-sulfate-reducing, anaerobic endospore forming bacteria, but it has also been changed by discoveries done by use of 16S rRNA analysis. The genus *Clostridium* has become more defined and are all phylogenetically related to the type species *Clostridium butyricum* (Wiegel et al. 2006). Several of the clostridia cause disease but not all are food borne. The food borne illnesses are caused by *Clostridium botulinum* and *Clostridium perfringens* (Granum 2007a; Granum

2007b). Botulism is the most serious type of food poisoning, since the toxin is extremely potent and causes paralysis with respiratory failure, resulting in death (Granum 2007a).

The family of *Bacillaceae* lies under the class of *Bacilli*, order *Bacillales* and contains the genus *Bacillus* together with 49 other genera. The *Bacillus* genus was previously thought of as the genus of the aerobic endospore forming bacteria, but since the 1990s the genus has been through several taxonomic rearrangements. The aerobic endospore forming bacteria are now found within a number of families, often together with non sporeformers, although the main *Bacillus* species: *B. subtilis, Bacillus anthracis, Bacillus cereus, B. licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus sphaericus* and *Bacillus thuringiensis* remain (Logan & De Vos 2009). The genus *Bacillus* itself contains 268 species and *B. subtilis* is its type species (LPSN, August 14th 2013).

Bacillus species

The genus *Bacillus* was established by Cohn in 1872 and included *B. anthracis* and *B. subtilis*. In 1920 the genus was defined as Gram positive, aerobic sporeformers. This held for 75 years, until *Bacillus infernus* (Boone et al. 1995), an asporogenus strict anaerobe, was proposed included in the genus *Bacillus.* Since then, other *Bacillus* species that divert from the original definition have been included because of 16s rRNA gene sequence analysis (Logan 2009). The *Bacillus* genus is the most diverse genus in the family of *Bacillaceae*, but it harbours no characteristics that will easily separate it from the other genera in the family and its species inhabit a variety of environmental niches (Fritze 2004).

The endospore forming *Bacillus* species have been subdivided into *B. cereus* and *B. subtilis* groups based on strain characteristics, in order to distinguish certain strains for scientific purposes*.* Bacteria of the *B. cereus* group have a cell diameter larger than 1 μm. They have non-swollen sporangia and are mainly mesophilic and neutrophilic, but some strains are psychrotolerant (Logan & De Vos 2009). The *B. cereus* group also have protoplasmic inclusions of poly-β-hydroxybutyrate, which the *B. subtilis* group lacks (Drobniewski 1993). The *B. cereus* group comprises of 7 species: *B. cereus, B. anthracis, B. weihenstephanensis, B. thuringiensis, Bacillus mycoides, Bacillus pseudomycoides* and *Bacillus cytotoxicus* (Guinebretiere et al. 2012). The *B. subtilis* group have a cell diameter of less than 1 μm and are all considered to be mesophilic and neutrophilic. A selection of species from this group is *B. subtilis*, *B. pumilus* and *B. licheniformis* (Fritze 2002). All these strains have useful applications, for example fermentative processes in food and production of enzymes, although they can cause problems in some instances (Schallmey et al. 2004). Both groups have ellipsoidal to cylindrical spores (Logan & De Vos 2009).

B. cereus is an opportunistic pathogen and most often the culprit when bacilli are involved in foodborne disease. *B. cereus* was described as disease-causing as early as 1950 by Steinar Hauge, after an outbreak caused by custard sauce. Just to be certain, he isolated the offending strain, grew a solution to 4 X 10^6 CFU/ml and drank 200 ml, then took notes as the illness progressed (Hauge 1955). *B. cereus* foodborne disease can have two types of symptoms depending on which toxin has been produced, diarrheal and emetic type (Stenfors Arnesen et al. 2008). Unlucky victims might experience both at the same time, due to both toxin and bacteria being present. The emetic toxin is a heat stabile cereulide that binds to the cell wall of the duodenum and causes vomiting by stimulation of the vagus nerve (Stenfors Arnesen et al. 2008). For the diarrheal type of illness to occur, *B. cereus* needs to establish itself in the gut and produce enterotoxin. Different types of enterotoxins have been described (Stenfors Arnesen et al. 2008). *B. cereus* can grow from 10 °C to 46 °C, although psychrophilic strains have been found causing problems in milk products (Stenfors Arnesen et al. 2008). Emetic toxin production peaks at 12-15 °C and is undetectable during growth over 37 °C according to one study on *B. cereus* (Finlay et al. 2000).

Much rarer than *B. cereus* incidents, *B. subtilis* group bacteria: *B. subtilis*, *B. pumilus* and *B. licheniformis,* have all been associated with food poisoning. Small cyclic lipopeptides have been isolated from the offending strains. The lipopeptides are suspected of being emetic like toxins but this has not yet been verified (Granum & Baird-Parker 2000; From et al. 2005; From et al. 2007). These bacilli grow in the temperature range of 10-40°C (Stenfors Arnesen et al. 2008), and can produce toxins at 10-30 °C (From et al. 2005).

Bacillus licheniformis

B. licheniformis belongs to the *B. subtilis* group of the *Bacillus* species and is a motile Gram positive endospore forming bacterium present in soil and plants (Logan 2012). *B. licheniformis* is a facultative anaerobe, which will enable it to grow where other aerobic *Bacilli* cannot (Rey et al. 2004).

Closely related to the type species *B. subtilis*, *B. licheniformis* has not previously been described in great detail owing to the greater focus on the type species *B. subtilis*. The restriction modification enzymes present in *B. licheniformis* and lack of completed genome sequence have also delayed the characterisation process (Raleigh & Brooks 1998; Veith et al. 2004; Rey et al. 2004). The industry products produced by *B. licheniformis* are many: proteases, α-amylase, penicillinase, pentosanase, cycloglucosyltransferase, β-mannanase and several pectinolytic enzymes (Schallmey et al. 2004). Together with the other *Bacillus* species they are preferred hosts for a large range of products (Schallmey et al. 2004). *B. subtilis* and *B. licheniformis* are approximately 84.6 % identical at the nucleotide level (Rey et al. 2004). They show considerable organizational similarity which makes

results from studies of *B. subtilis* interesting when studying *B. licheniformis* (Veith et al. 2004; Rey et al. 2004).

B. licheniformis produces surfactants called lichenysin (A, B, C, D, G) and surfactant BL86. Lichenysins are potent, heat stable, anionic cyclic lipoheptapeptide biosurfactants (Nerurkar 2010). They are structurally similar to cereulide produced by *B.* cereus, but they have lipid tails and induce toxicity in a different manner. Lichenysin A produces ion channels in the host membrane rather than affecting mitochondria in the way cereulide does (Logan 2012), but these toxins are generally poorly characterised (Løvdal 2011). Foodborne disease by *B. licheniformis* has occurred with substances such as ice cream, desserts, meat pies and sandwiches, but the dose needed to induce sickness is unknown (Logan 2012).

Transformable *Bacillus licheniformis* MW3

Through the work of Waschkau et al. (2008), a transformable *B. licheniformis* has been achieved. Previously, genetic engineering of *B. licheniformis* has been hampered by its type 1 restriction modification system (Waschkau et al. 2008). Three types of restriction modification systems are known (Raleigh & Brooks 1998). The genomic DNA is protected by specific modifications, such as methylation of adenine or cytosine residues, while foreign DNA is cut by restriction endonucleases. The type 1 restriction modification system was knocked out and both single and double knock out of Δhs*dR1* and Δhs*dR2* were produced. Single knock out mutants (Δhs*dR1* or Δhs*dR2*) were modifiable with plasmids sourced from *Bacilli* but only the double knock out mutant (Δhs*dR1* and Δhs*dR2, B. licheniformis* MW3) was transformable with plasmids from *Escherichia coli* (Waschkau et al. 2008). This breakthrough has aided the process of describing *B. licheniformis* characteristics by simplifying the mutational process.

At the Norwegian School of Veterinarian Science, *B. licheniformis* MW3 has previously been used to create a Δ*gerAA*spec+ mutant and this disruption mutant was complemented with a plasmid-borne *gerA* copy (Løvdal et al. 2012). That study (Løvdal et al. 2012) concluded that the *gerA* gene plays a fundamental role in germination with L-alanine and casein hydrolysate.

The endospore

Bacterial sporeformers are characterised by their ability to form endospores. Sporeforming bacteria are usually found in soil and Hong et al. (2009) determined that the concentration in soil is around 10^6 spores/g and 10^4 spores/g in human faeces (Postollec et al. 2012).

Spore capsules are a vessel for survival for bacteria in unfavourable environmental conditions. By entering into dormancy the spore can function as a seed bank for when the environmental conditions have improved (Jones & Lennon 2010). Spores isolated from insects embedded in amber have been viable even after 25-40 million years in dormant state (Gould 2006).

The spore structure, as shown in Figure 1, gives the bacteria resistance against desiccation, radiation, extreme pH and chemicals poisonous to the vegetative cell (Setlow 2006).

The outermost layer of the spore is the exosporium, although some *bacilli* have this structure, (*B. cereus, B. anthrasis* and *B.thuringiensis*) *B. subtilis* does not (Todd et al. 2003; Terry et al. 2011; Leggett et al. 2012). The exosporium does not seem to protect the spore significantly from biocides (Leggett et al. 2012), although it may be important for the spore's interaction with target organisms in relation to pathogenicity (Setlow 2006). This lack of exosporium in *B. subtilis* has reduced the amount of information available regarding its composition in exosporium coated spores (Terry et al. 2011; Leggett et al. 2012).
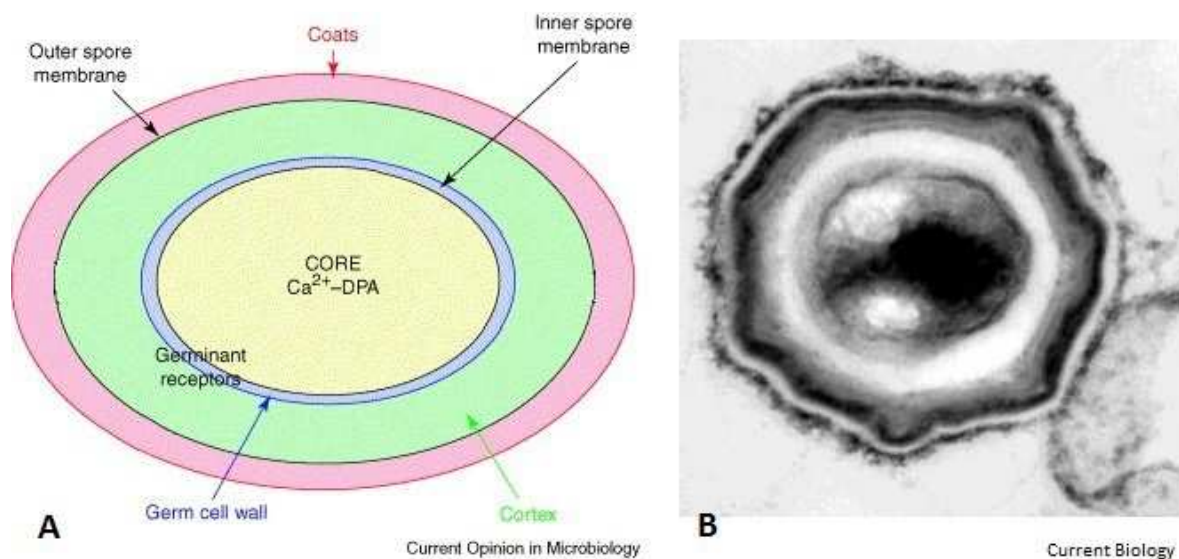
Figure 1: A Generic illustration of spore structure. Illustration is not drawn to scale, nor is exosporium included (Edited from Setlow 2003). **B** Thin-section transmission electron microscope photo of *B. subtilis* stained with Ruthenium red (McKenney et al. 2010). **A and B** are reprinted with permission from Elsevier.

Sporulation

When nutrients are depleted or the environment turns unfavourable, the sporulation signal is triggered. Sporulation starts with a phosphorelay process, which results in phosphorylation of the master transcription regulator, Spo0A (Fujita & Losick 2003). The signals result in an asymmetrical

division of the cells cytoplasm, as shown in Figure 2, as well as the transcription of *spoIIA*, *spoIIE* and *spoIIG* loci, which encode key developmental regulators (Piggot & Hilbert 2004; Zhao et al. 2002). The smallest part is called the forespore and the remainder the mother cell (Figure 2). In these two compartments different gene expressions are initiated. The gene expressions are regulated by sporulation specific RNA polymerase σ factors, σ^F in the forespore and σ^E in the mother cell (Lewis et al. 1994). To create a double membrane, the mother cell engulfs the forespore in a process similar to phagocytosis. After engulfment the forespore σ factor changes to σ^G and the mother cell to σ^K (Phillips & Strauch 2002). This leads to further differentiation in the two compartments. The forespore now has a double membrane in which the cortex can be assembled.

Traag et al. (2013) have recently identified a sporulation gene *ylyA* that encodes a novel RNA polymerase-binding protein, which influences sporulation sigma factor σ^G in *B. subtilis*. When acting on σ^G, *ylyA* influences the level of germination receptors and a protein channel that releases DPA. A deletion of *ylyA* resulted in a deficit in the spore germination efficiency (Traag et al. 2013).

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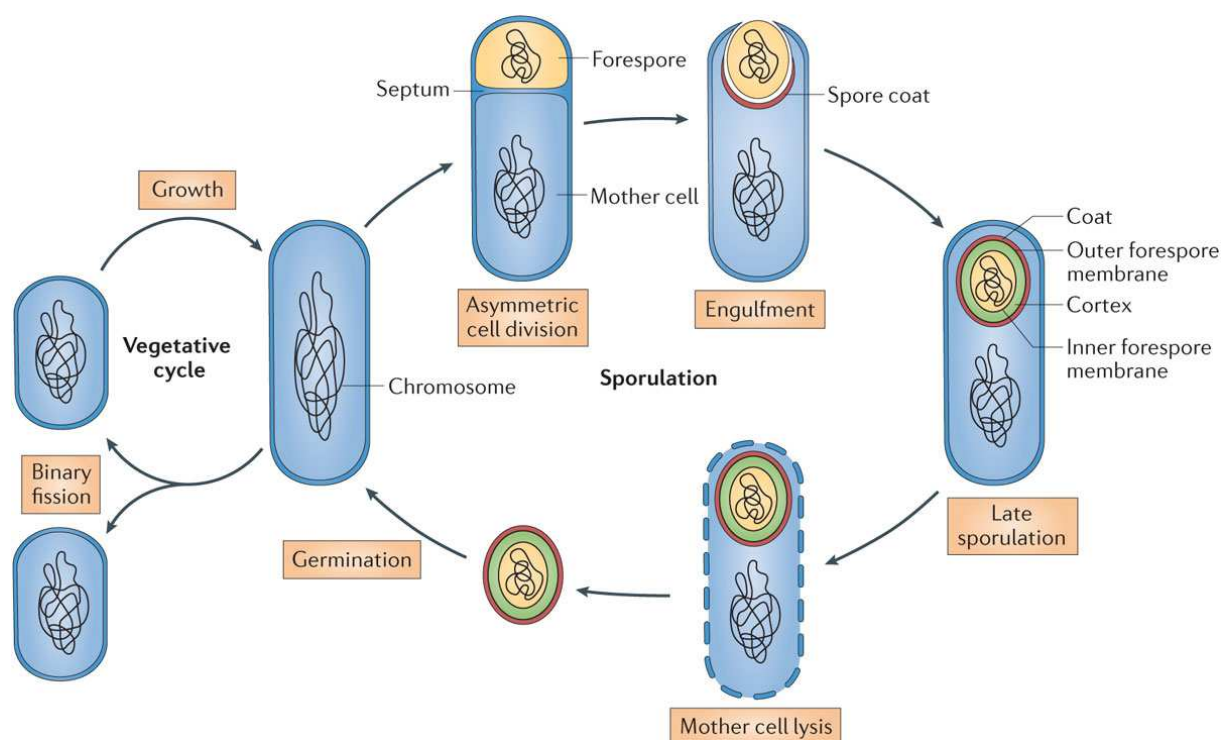
Figure 2: Illustration of the steps in sporulation and germination of *B. subtilis* (McKenney et al. 2013). This figure is reprinted with permission from Nature Publishing Group.

The cortex is a thick peptidoglycan layer between the inner and outer spore membrane (Figure 1). It is similar to the peptidoglycan layer of a vegetative cell but contains some modifications. Spore cortex

completely lacks teichoic acids from the N-acetylmuramic acid (NAM) residues (Leggett et al. 2012). The germ cell wall lies between the cortex and the inner spore membrane and also contains peptidoglycan. The difference between these two layers of peptidoglycan is that 50% of the spore cortex NAM is cyclized to form spore specific muramic-δ-lactam, while muramic-δ-lactam is absent in the germ cell wall NAM (Leggett et al. 2012). The muramic-δ-lactam functions as a marker for the selective degradation of spore cortex, as the cortex lytic enzymes can recognise this key substrate (Atrih et al. 1998). During germination, the spore cortex is degraded but the germ cell wall remains and forms the cell wall of the new vegetative cell after germination (Leggett et al. 2012).

Building of the spore coat also commences after engulfment. The spore coat is a proteinaceous layer either as the outmost layer or within the exosporium. In *B. subtilis* three layers have been observed in the spore coat, by thin-section electron microscope (Figure 1 B). The recently identified outer layer called crust (McKenney et al. 2010), a coarsely layered outer coat and a lamellar inner coat (McKenney et al. 2013). So far 70 coat proteins have been identified in *B. subtilis* (Leggett et al. 2012).

The inner membrane lies within the germ cell wall. The lipid composition is similar to that of the vegetative cell plasma membrane but they have very different protein composition (Leggett et al. 2012). The protein composition of the inner membrane consists of germinant receptor structures and SpoVA proteins that are not found in the vegetative plasma membrane (Setlow 2003).

In the centre of the spore is the spore core, it contains the spores DNA, RNA, ribosomes and enzymes. The water content in the spore core is extremely low which restricts macromolecular movement and enzymatic activity (Algie & Watt 1984). The core contains pyridine-2,6-dicarboxylic acid, also called dipicolinic acid (DPA). The DPA is produced in the mother cell of the sporulating bacteria and is absorbed by the forespore (Setlow 2006). Another feature of the core is small acid soluble spore proteins (SASP) of α/β-type (Mason & Setlow 1986). SASP is synthesised in the developing spore prior to DPA uptake (Pedreza-Reyes et al. 2012). SASP has been found to protect the DNA from UV damage, as well as supply amino acids for protein synthesis (Mason & Setlow 1986).

When the spore is complete the mother cell lyses and releases the spore into the environment. A study (Segev et al RNA dynamics 2012) has shown that there is a maturation period of several days after sporulation of *B. subtilis*. In this period the amount of RNA in the cell is influenced by the temperature in their environment. Spores stored at a high temperature (50 °C) degrade more of their RNA than spores stored at a low temperature (4 °C). The same study showed that spores stored at an intermediate temperature (37 °C) were slower to germinate than high- and low temperature stored spores. This delayed germination is attributed to the RNA status of the cell with neither enough intact RNA nor enough degraded RNA available for synthesis.

Spore properties

The spore's layers and chemical composition defines its total resistance. As shown in Figure 1 the spore is comprised of multiple layers around a core. The dehydrated condition of the spore core is what is thought to give it most of its resistance properties. The spore core conditions protect the DNA from damage from heat and radiation (Setlow 2006).

The coat protects the spore from bacteriovores since the structure serves as a barrier for large molecules and makes it indigestible. If lysozyme, a peptidoglycanlytic enzyme, had access to the cortex it would be able to degrade it (Leggett et al. 2012). Smaller molecules must be able to penetrate to reach the germinant receptors in the inner membrane. The coat also protects against a range of harmful chemicals such as hydrogen peroxide, peroxynitrite, chlorine dioxide and hypochlorite, but no single coat component has been identified as essential for protection (Leggett et al. 2012). Removal of the spore coats and the outer membrane does not affect the spore's resistance to heat, radiation and some chemicals (Setlow 2006).

In a study by Imae and Strominger (1976), a mutant of *B. sphaericus* was used to test the resistance properties connected to the spore cortex. The amount of cortex present in *B. sphaericus* could be controlled by changing the amount of meso-diaminopimelic acid available in the growth medium. The study revealed that a critical mass of cortex is needed for resistance against xylene, octanol and heat, but due to the complexity of spore development they were unable to specifically show that the cortex was the main contributor to resistance (Imae & Strominger 1976).

The inner spore membrane has a major role in controlling permeability to the spore cortex and this may give the spore resistance to some chemicals. The inner membrane increases twofold within minutes of germination and therefore is thought to be compressed in the dormant spore (Setlow 2006).

The core composition seems to be the most important feature of the spores dormancy and ability to survive. To be able to successfully germinate, the spore needs to protect its DNA from accumulating more damage than it can fix. Many of the spore's resistance properties are connected to protecting its DNA from damage. It has been determined that the low water content of the core is the most important factor in determining resistance to wet heat (Beaman & Gerhardt 1986), while SASP protects the DNA (Pedreza-Reyes et al. 2012). SASP coats the DNA and alters the structure making the DNA thread form a toroidal shape (Englander et al. 2004; Lee et al. 2008). This doughnut like shape stabilises the DNA and protects it from damage caused by heat, chemicals, and UV radiation (Setlow

2007; Lee et al. 2008). Upon germination the spore also harbours multiple DNA repair systems to take care of DNA damage that has accumulated while in dormancy (Setlow 2007).

In food

For decades microbial spoilage has been a problem for the producers of products with long shelf lives. Problems with unwanted gas production in cheese, ropy bread, unstable canned food, spoiled juice and soft drinks are well known to them, but problems with gas production in packed meats has also surfaced (Postollec et al. 2012). A new niche is Refrigerated processed foods of extended durability (REPFED). REPFEDs include a range of minimally processed foods such as meat-, seafood-, egg- and vegetable salad as well as fresh pasta, sauces, soups and ready to eat meals (Marth 1998). Sous vide foods cooked inside hermetically sealed plastic under vacuum are also considered to be REPFED since the heat treatment they receive is less than required for commercial sterility (Marth 1998).

In a study by Postollec et al. (2012) 90 food samples were tested for presence of sporeforming bacteria. The 90 samples were equally divided into three categories; egg-based products, milk and dairy products, canned foods and food ingredients. *Bacillus* genera were detected in 28, 26 and 19 samples of the categories mentioned above, although no direct link could be established between detection and spoilage.

Sporeforming bacteria and their outgrowth are related to high economic losses for the industry through spoilage and foodborne disease. The majority of foodborne illness is a result of inadequate cooling and/or reheating, causing spores to germinate, multiply and, if possible, produce toxins (Logan 2012). These foodborne incidents are more common as sporadic cases in private homes, restaurants and institutions where such temperature and storage malpractice can occur (Andersson et al. 1995). Food poisoning incidents caused by *Bacillus* species are suspected to be underreported since most have quite mild symptoms (Granum & Lund 1997).

The emergence of highly heat resistant endospores (HRS), that are able to survive commercial sterilising and ultrahigh temperature (UHT) processing, may be an indicator that food production plants lead to species adaptation (Postollec et al. 2012). In this manmade environment, free of competitive micro flora, such adapted species could cause great problems. As heat still is the most efficient way of destroying spores, this development is foreboding.

When the process of dormancy and germination is fully understood, spores might be enticed, by a mild physical or chemical treatment, to start germination and become vulnerable. This would enable

milder heat treatments in food processing, reducing spoilage and foodborne pathogen risk but also avoiding unwanted organoleptic and nutritional changes caused by high heat (Berg & Sandine 1970).

Germination of endospores

A dormant spore is not metabolically active, yet it is still aware of its environment and can respond when presented with favourable conditions. The spore does not metabolise available nutrients from the environment to start germination (Moir 2006); it contains all elements needed to germinate, much like an egg contains everything it needs to make a chicken.

The process of germination can be divided into three stages, as shown in Figure 3 (Setlow 2003). A pre-germination event is called activation, where cells are made more sensitive to germination signals, but unlike germination, activation can be reversed (Berg & Sandine 1970). "Ageing" also makes the spore more prone to germinate but this is not a reversible process (Keynan et al. 1964).

When a spore enters the germination stage, either by reacting to a germinant or undergoing a physical treatment, it will irreversibly lose its spore properties and become sensitive to heat, radiation, desiccation, and chemicals (Foster & Johnstone 1990). The spore's refractility will be lost, the spore core will become hydrated and it will finally emerge from the spore coats as a vegetative cell during outgrowth (Moir 2006; Setlow 2003).

Spore germination can be investigated by exposing spore populations to controlled substances: single amino acids, sugars, and ribosides; and controlled environment: pH, temperature, media, ions, and concentration. Combined with genetic mutations and improved techniques of measuring germination response the spores germination system can be mapped (See Figures 3, 4 and 5).
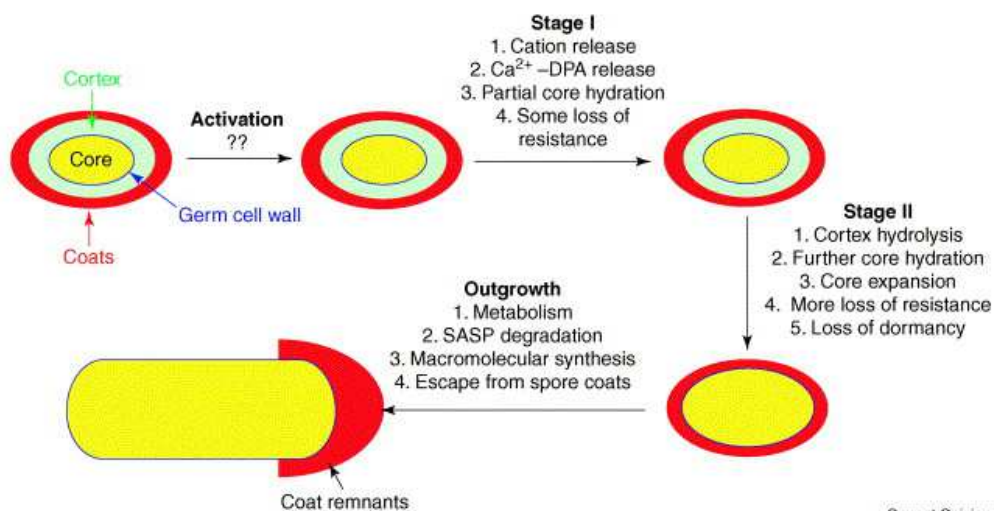
Activation

Activation seems to prime spores for germination and heat activation is widely used in laboratory conditions (Løvdal et al. 2013; Keynan et al. 1964). Other chemical and physical treatments can also be used to induce activation, such as pH, reducing agents, ionizing radiaton, high pressure, and various chemicals (D-cycloserine, D-carbamyl-D-serine, dimethylformamide, and urea) (Berg & Sandine 1970). The specific mechanism remains unknown (Indest et al. 2009).

A study (van der Voort et al. 2010) shows that there is diversity in the germination response to heat activation by different strains of *B. cereus*, where some strains need heat activation to germinate efficiently while others germinate without. Ghosh et al. (2009) reported that superdormant spores of *B. cereus, B. megaterium* and *B. subtilis* have a higher heat activation requirement, 8-15°C above

population optimum. Activation can also be induced at moderately elevated temperatures (<40°C) but the incubation period increases as the temperature decreases (Løvdal et al. 2013). At one point it will cross over into the ageing segment, where the activation can no longer be reversed (Keynan et al. 1964). These moderate temperature increases can occur in soil, decomposing organic matter and the gut of animals or humans (Løvdal et al. 2013; Keynan et al. 1964). Keynan et al. (1964) note that spores stored for three years were still responsive to heat activation but lost the ability to reverse the process and proposed that ageing can be viewed as the loss of activation reversal.

Germination and outgrowth

When a spore germinates, it loses its dormant properties of resistance and goes through a metamorphosis to once again become a vegetative cell. The process is described in Figure 3 and can be divided into two stages before outgrowth (Setlow 2003).

Figure 3: Model of events in spore germination and outgrowth. Activation precedes germination but the specific mechanism is unknown. Germination is separated into two stages followed by outgrowth (Setlow 2003). This figure is reprinted with permission from Elsevier.

In the first stage after the spore has received a signal to germinate, it releases monovalent cations (H^+, K^+) in order to raise the pH in the core from ~6.4 to 7.7 (Swerdlow et al. 1981; Setlow 2003). The neutralisation of the environment within the spore core is essential for spore metabolism at a later stage. The spore also releases its depot of DPA and its chelate Ca^{2+}, which is replaced by water, hydrating the spore (Dring & Gould 1971; Foster & Johnstone 1990; Setlow 2003).

In the second stage of germination, the peptidoglycan of the spore cortex is hydrolysed, a very crucial step that leads to total rehydration of the spore core and makes enzyme action possible (Moir 2006). The spore enzyme recognises the spore cortex peptidoglycan by its special muramic-δ-lactam and thus avoids breaking down the germ cell wall (Atrih et al. 1998). The spore core continues to take in

water and swells, expanding within the germ cell wall (Setlow 2003). After finishing these two stages the spore has lost all its resistance properties and will begin the final preparations to break out of the spore coats and function as a vegetative cell.

When the spore core is hydrated enough to allow enzymatic action, the spore regains metabolism and starts degrading SASP; but it also needs to repair damage to the DNA before macromolecular synthesis begins and outgrowth can be completed (Setlow 2007; Setlow 2003). In growing cells, UV radiation gives rise to cyclobutan-type pyrimidine dimers and 6-4-photoproducts which form between adjacent pyrimidine residues in the DNA (Slieman & Nicholson 2000; Nicholson et al. 1991). The binding of SASP to the DNA in the spore promotes thymidyl-thymidine photoproduct (spore photoproduct, SP) as a favoured DNA lesion (Nicholson et al. 1991). This damage can be repaired before outgrowth by at least three repair mechanisms, one which is specific for SP damage (Moeller et al. 2008). The SP specific repair pathway requires spore photoproduct lyase, an enzyme that uses a radical mechanism to split the thymidine dimer. The SP lesion can also be repaired by recombination or excision, via the RecA repair pathway (Setlow 2006). The spore has a single chromosome; therefore repair via homologous recombination is impossible while in the process of germination. The latest theory is that the tight packing caused by SASP, keep the ends of broken double strands in close proximity and thus allowing error free repair by non-homologous end joining (Englander et al. 2004; Frenkiel-Krispin et al. 2004).

To complete outgrowth, the germinated cell needs to escape the confinement of the spore coats. According to an analysis of spore structure during germination and outgrowth, the spore coat cracks along one or both sides and the bacteria can elongate to its preferred size (Leuschner et al. 2000).

Nutrient and non-nutrient germination

It is believed that spores in nature generally germinate in response to nutrients, via receptor dependent germination. Receptors can sense single amino acids, sugars and purine nucleosides and these nutrients are called germinants. Not only single nutrients can cause germination, but a mix of asparagine, glucose, fructose and K^+ (AGFK) can trigger germination in *B. subtilis* spores (Moir et al. 2002) even though the individual constituents cannot. In Figure 4 the entry points and pathways of germination in *B. subtilis* are displayed (Setlow 2003). Germination is an irreversible process which the spore must complete even though the environment might have become unfavourable again (Keynan et al. 1964). The spore is said to have become committed when it germinates after the stimulant has been removed. The level of committed spores in a population can be raised by heat activation, increased germinant concentration and increased amount of germinant receptors by mutation (Yi & Setlow 2010).
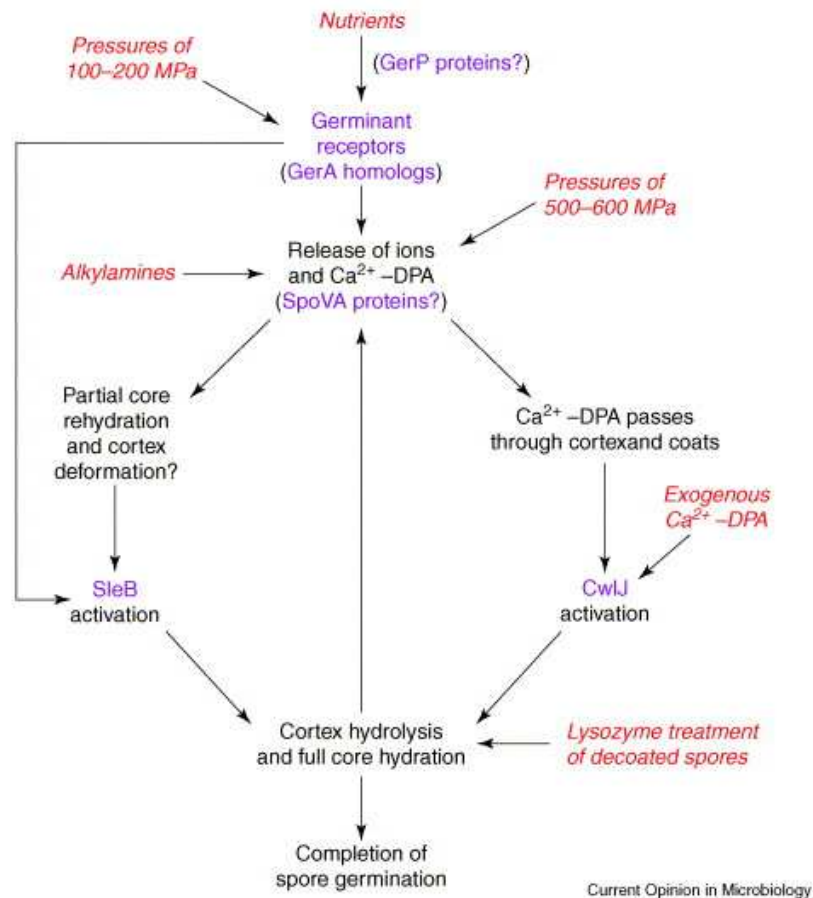
Figure 4: Map of germination response to nutrient and non-nutrient stimulation in *B. subtilis* (Setlow 2003). This figure is reprinted with permission from Elsevier.

The non-nutrient germinants are different chemical and physical stimuli, including lysozyme, salts, high pressure, dodecylamine, DPA and its chelate Ca^{2+}. These stimulants use several components of the nutrient germination pathway (Figure 4) (Moir 2006; Setlow 2003). Lysosyme, when spores are decoated, and Ca^{2+} chelated DPA can germinate spores that lack germinant receptors (Paidhungat & Setlow 2000; Setlow 2003).

In *B. subtilis* five tricistronic operons have been discovered to be involved in germination, *gerA, gerB, gerK, yndDEF,* and *yfkQRT* (Paidhungat & Setlow 2000). Of these GerA, and when acting cooperatively, GerB and GerK have been confirmed as functional germination receptors (Moir et al. 2002; Atluri et al. 2006). Mutant spores of *B. subtilis* show slow spontaneous germination when all known germination receptors have been removed, the mechanism behind this germination is unknown (Paidhungat & Setlow 2000). In Figure 5 the assembly of the three proteins of *B. subtilis* GerA germination receptor, and their predicted structure is shown (Moir et al. 2002). These are the products of the *gerA* locus and reside in the inner membrane of the cell.

17
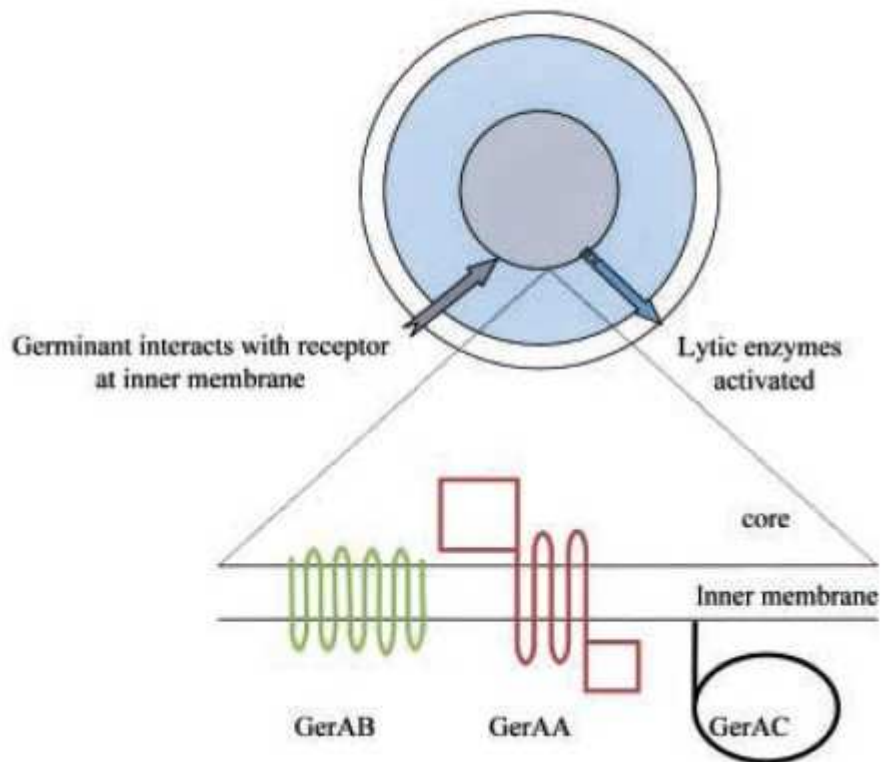
Figure 5: Location and predicted structure of germination receptor GerA in *B. subtilis.*This is the predicted organisation of the components of the germination receptor complex in the inner membrane of a *B. subtilis* spore (Moir et al. 2002). This figure is reprinted with permission from Springer.

In *B. licheniformis,* orthologus proteins similar to those encoded by *gerA, gerK* and *yndDEF* have been found (Ross & Abel-Santos 2010)*,* but none similar to *gerB* (Løvdal and Madslien, unpublished results). A *B. licheniformis gerA* disruption mutant was created by Irene Løvdal. The *gerAA* gene was exchanged with a spectromycin cassette and the *B. licheniformis* lost its ability to germinate in response to L-alanine (Løvdal et al. 2012).

Detection of germination

Germination of bacterial spores can be detected through different properties, e. g. light scattering (refractility), DPA release and heat resistance (Hashimoto et al. 1969). Refractility, and the loss of it, can easily be viewed by phase contrast microscopy (Powell 1957). As shown in Figure 6, the spores go from phase bright (left) to phase dark (right).
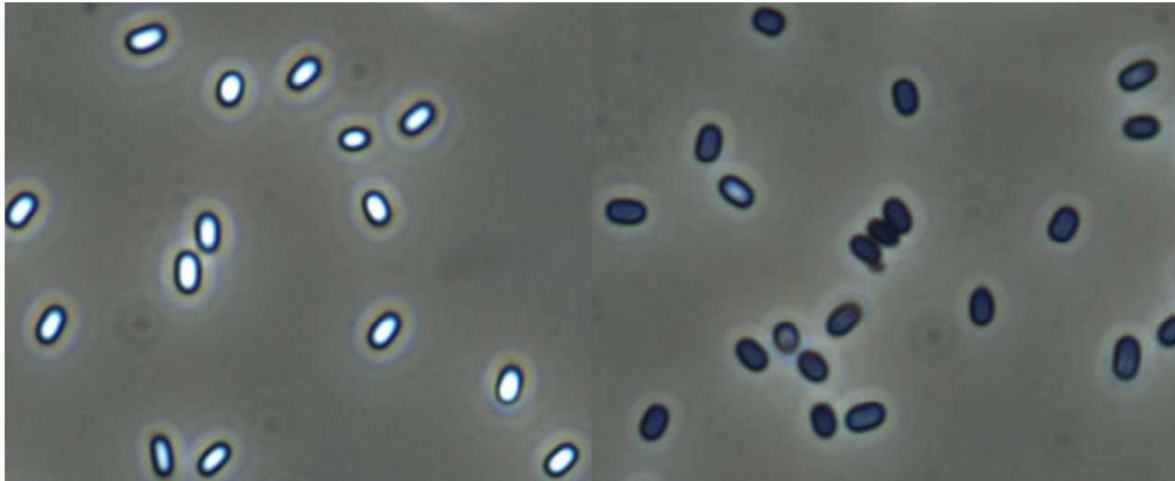
Figure 6: Phase contrast images (100x) of dormant spores (left) and germinated spores (right). Picture was taken using Olympus BX51 with ColorView Illu camera and Cell[B] software, by S. Kidd. (These photos have been cropped).

The spores' refractive changes can be measured in a spore population by measuring the change in absorbance (A) at 600 nm (A_{600nm}) (Powell 1950). Absorbance is light, set to a specific wavelength, which has been transmitted through a sample.

$$\% \ loss \ of \ A \ at \ time \ t \ (t_1) = \left[1 - \left(\frac{A \ at \ t_1}{A \ at \ t_0}\right)\right] \times 100$$

When plotted against time, the maximum germination rate is obtained from the maximum slope of the graph (Nicholson & Setlow 1990). A plate reader taking multiple absorbance readings over time, makes an efficient method for observing effects from mutations on germination with multiple strains (Hornstra et al. 2005; Løvdal 2011). The loss of refractility for a germinated *B. licheniformis* spore is equivalent to a drop of around 60% from the original refractility of the spore (Løvdal et al. 2012). Phase contrast microscopy is still used to check the germination behaviour and correlate the percentage of germinated spores to the refractility loss as a control. The method has some weaknesses, as it is not sensitive enough to measure neither low germination (10-15 % of spore population), nor when germination exceeds 90%. The measurements can also be influenced by adherence of spores to each other or the surface of the sample container. When comparing different

strains the germination response should differ at least 10-20% for this method to be effective (Løvdal 2011).

Another equation, made by McCormick in 1964, could predict the time-course of any spore germination curve as long as three constants *k, c* and *a* (function of temperature, germinant and heat) were known (McCormick 1965). The frequency distribution of his equation was verified by Vary and Halvorsen (1965). McCormick's hope was that a model for breaking the dormant state would emerge if a few more variables could be incorporated into his equation. While germination by loss in refractility is measured directly on the spore, DPA release is measured in the supernatant fluid sampled from the germinating spores at A_{270nm} (Vepachedu & Setlow 2004).

A third possibility in population studies on spore germination lies in the heat resistance of ungerminated spores. A germinated sample and negative control sample of ungerminated spores are heat treated at a temperature lethal to germinated spores before plating on solid medium at appropriate dilutions. After incubation, the colonies that have grown can be counted. This method takes into account the viability of the spore population; whereas a change in spore refractility does not confirm that the spore is viable (Powell 1957).

Methods and materials

To study the function of the germination receptor GerK in *B. licheniformis* deletion mutants were made. Two cistrons of the tricistronic *gerK* operon, *gerKA* and *gerKC,* referred to as Δ*gerKA-KC,* were deleted, see Figure 7. Since this work is part of a project to characterise germination properties in *B. licheniformis,* deletion mutants lacking spore cortex lytic enzyme *CwlJ* were also created. Only the germination properties of *gerK* mutants were explored for this master thesis.

The process of markerless gene replacement is displayed in Figure 7 (Janes & Stibitz 2006). A DNA fragment, containing an upstream and a downstream part of the gene to be deleted, was created by PCR and cloned into the pMAD-*I-SceI* shuttle vector (Appendix V, p. 67). The the pMAD-*I-SceI* vector contained an *I-Scei* restriction site that is not found in *B. licheniformis* DNA. The first crossover incorporates the pMAD-*I-SceI* vector into the genome. A second plasmid (pBKJ223, appendix V, p. 67), transformed into the *B. licheniformis* recombinants, carries a gene encoding a restriction enzyme that cuts the *I-SceI* site in the pMAD-*I-SceI* vector. The cut creates a double strand break which is repaired by homologous recombination either splicing out the target sequence or regenerating the wild type sequence. If the target sequence is deleted, it is replaced with ATGTGA (5'-3') in Δ*gerKA-KC*

and ATGTAA (5'-3') in Δ*CwlJ.* This is done to keep the reading frame intact, as not to cause frame shift mutations and interfere with genes downstream from the excision site.

When the deletion was confirmed, the strains were sporulated and a series of germination assays were done. The germination assays compares germination response of *B. licheniformis* MW3 (type strain), *B. licheniformis* MW3Δ*gerA* (Løvdal et al. 2012), *B. licheniformis* MW3Δ*gerA* Δ*gerKA-KC* (constructed for this thesis) and *B. licheniformis* MW3 Δ*gerKA-KC* (constructed for this thesis).
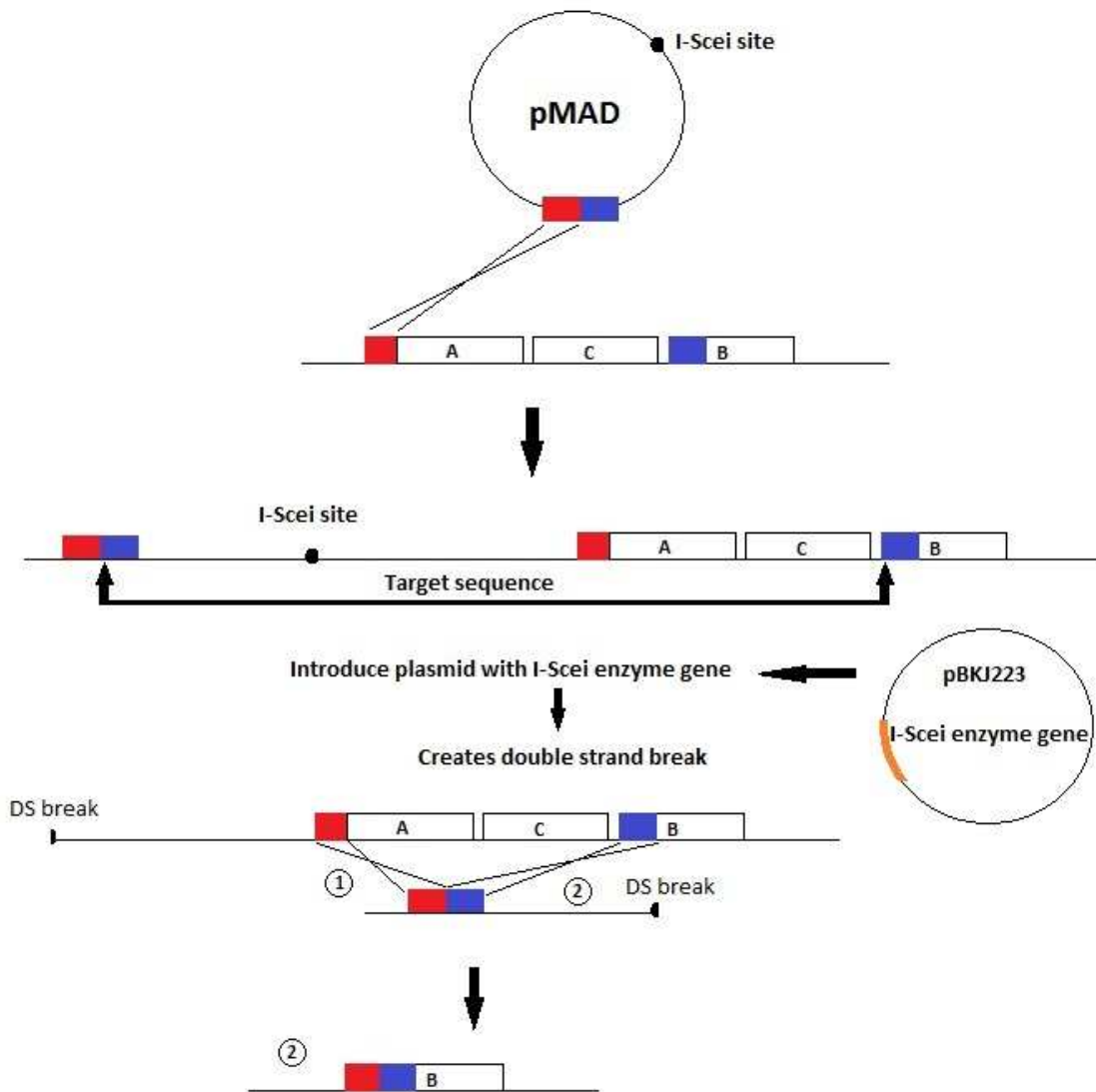
Figure 7: Schematic representation of the procedure in markerless gene replacement (Janes & Stibitz 2006). The figure shows gene replacement in the tricistronic operon of *gerK* (*gerKA, gerKC and gerKB*). The red and blue boxes represent DNA sequences located upstream and downstream of the excision site and their homologues in the pMAD-*I-SceI* vector insert. Integration of pMAD-*I-SceI* vector into the genome can take place in either red or blue area, in this figure the red crossover is displayed. Only homologue recombination of the blue areas (shown as ② in figure) in the second step will result in excision of the target sequence. Homologue recombination of the red areas will result in reversion to wild type gene expression. Target sequence was replaced with a start and a stop codon.

Strains

Table 1: Bacterial strains and plasmids used in the construction of the mutants in this thesis

Strain	Description	Reference
Escherichia coli TOP10	Oneshot TOP10 chemically competent *E. coli* for cloning	Invitrogen
B. licheniformis MW3	*Bacillus licheniformis* DSM13 Δhs*dR1* Δhs*dR2*	(Waschkau et al. 2008)
B. licheniformis MW3Δ*gerA*	*B. licheniformis* MW3Δ*gerA*	(Løvdal et al. 2012)
Plasmid	**Description**	**Reference**
pCR 2.1 TOPO	*E. coli* PCR product cloning vector	Invitrogen (Appendix V, p. 66)
pMAD-*I-SceI*	*E. coli/B. licheniformis* shuttle vector with *I-sceI* site	(Arnaud et al. 2004)/Dr. Anette Fagerlund, University of Oslo, Norway (Appendix V, p. 67)
pBKJ223	Plasmid for producing *I-SceI* enzyme	(Janes & Stibitz 2006)

Chemicals and equipment

For detailed lists of primers, chemicals, equipment and suppliers, please see Appendix I and II, p. 53 and 55.

Growth and sporulation media

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: Luria-bertani medium with 1,5 % (w/v) agar bacteriological No. 1 (Oxoid).

Bacto^{TM} Brain heart infusion (BHI) medium: 37 g/L (BD) in distilled water.

BHI agar: BHI medium with 1,5 % (w/v) agar bacteriological No. 1 (Oxoid)

All media were autoclaved at 121 °C for 15 minutes. BHI and LB was used propagate bacteria.

Bacto MS sporulation medium (van der Voort et al. 2010): The sporulation medium consists of Difco^{TM} Nutrient Broth (8 g/L, BD) with an addition of several salts to enhance sporulation. The salts used were 1 µM $FeSO_4$ (Merck), 2,5 µM $CuCl_2$ (Sigma), 12,5 µM $ZnCl_2$ (Sigma), 66 µM $MnSO_4$ (Merck), 1 mM $MgCl_2$ (,J. T. Baker Chemicals B. V.), 5 mM $(NH_4)_2SO_4$ (Merck), 2,5 µM Na_2MoO_4 (Riedel-de Häen), 2,5 µM $CoCl_2$ (Sigma) and 1 mM $Ca(NO_3)_2$ (Merck). The salts were kept refrigerated in stock solutions and were added to the medium before autoclaving, with the exception of $FeSO_4$, $MnSO_4$ and $Ca(NO_3)_2$ which were added after autoclaving. $FeSO_4$, $MnSO_4$ and $Ca(NO_3)_2$ were sterile filtered

(0,2 μm, Minisart Sartorius) and added after the medium had cooled. The Bacto-MS sporulation medium was made fresh for each sporulation.

Bacto MS was used to enhance the sporulation of *B. licheniformis.*

S. O. C medium (supplied in pEXP5-TOPO$^®$ TA expression kit from Invitrogen)**:** 2 % tryptone, 0,5 % yeast extract, 10 mM NaCl, 2,5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose.

S. O. C. medium was used to recover cells after transformation.

Construction of deletion mutants

Combining sequence and ligation independent cloning (SLIC) (Li & Elledge 2007) and markerless gene replacement (Janes & Stibitz 2006), it was possible to replace the target sequence of *gerK* and *CwlJ* with ATGTGA and ATGTAA (5'-3'), respectively. The deletion mutant strains were all based on the transformable *B. licheniformis* MW3 strain. *B. licheniformis* MW3 is a mutated strain of *B. licheniformis* DSM 13 (Type strain), lacking the restriction enzyme genes *hsdR1* and *hsdR2* (Δ*hsdR1* Δ*hsdR2*).

Dr. Toril Lindbäck designed the primers used to create the *gerKA-KC* insert and assisted in creating the primers used for the *CwlJ* insert. All primers are listed in Appendix I, p. 53. Kristin O'Sullivan produced the Δ*CwlJ* insert and ligated it into pCR 2.1 TOPO vector, before handing the project over to me.

DNA extraction

Genomic DNA was extracted following the protocol from "A versatile quick-prep of genomic DNA from Gram-positive bacteria" by Pospiech & Neumann.

SET buffer (Pospiech & Neumann 1995): 75 mM NaCl (Merck), 25 mM EDTA, pH 8.0 (Sigma) and 20 mM Tris, pH 7,5 (Sigma).

B. licheniformis MW3 was cultured in LB to saturation before 3 ml was pelleted by centrifugation. The pellet was resuspended in 495 μl SET with 50 μl of 100 μg/ml lysozyme and incubated at 37 °C for 1 hour. 50 μl of 10 % SDS and 5μl of 25 mg/ml Proteinase K were added after 1 hour. The solution was gently mixed and incubated at 55 °C for another 2 hours.

After incubation 200 μl 5.0M NaCl and 700 μl chloroform:isoamyl alcohol, at a ratio of 24:1, was added to the solution and incubated at room temperature for half an hour, with frequent inversions to separate out the DNA. After half an hour the solution was centrifuged for 25 minutes at 15.700 x *g* in an Eppendorf table centrifuge. The aqueous phase was siphoned off and mixed with an equal volume of isopropanol in which the DNA precipitated, followed by centrifugation at 15.700 x *g* for 10

minutes. The precipitate was washed with 70 % ethanol and let dry in room temperature. The DNA was then resuspended in 100 µl autoclaved distilled water and frozen for later use.

Fusion PCR

Primer pairs A-B and C-D (see appendix I, p. 54), where B and C primer sequence overlapped in opposite directions, were used to amplify upstream and downstream regions of the targeted gene. 0,5 µl *B. licheniformis* MW3 DNA, dilution 1:5, was used as template.

PCR products of A-B and C-D primers were produced separately by Phusion high-fidelity DNA (Finnzymes) polymerase and cleaned by Qiaquick PCR purification kit (Qiagen), see appendix III, p. 60. The two PCR products were mixed together and a new round of Phusion polymerase PCR was run to assemble the fragments.

The following PCR amplification protocol was used:
1.   98 °C for 30 seconds
2.   98 °C for 10 seconds
3.   52 °C for 20 seconds
4.   72 °C for 20 seconds
5.   72 °C for 5 minutes
6.   4 °C ∞
Steps 2 through 4 were repeated 30 times.

(A cleaner PCR product was achieved by raising the temperature in step 3 to 58°C)

The PCR product was then incubated at 72°C with Taq polymerase (Finnzymes) for 15 minutes to produce sticky ends to allow cloning in TOPO vector.

Cloning

The assembled PCR fragment with sticky ends was cloned into pCR 2.1 TOPO vector (Invitrogen, Appendix V, p. 66) and transformed into chemically competent *E. coli* One Shot TOP10 (Invitrogen) (pEXP5-TOPO$^{®}$ TA expression kit).

Ligation reaction:
2 µl PCR fragment
0.5 µl salt solution (1,2 M NaCl, 0,06 M $MgCl_2$)
0.5 µl TOPO vector
Incubated at room temperature for 5 minutes.

A vial of chemically competent *E. coli* One Shot TOP10 was thawed on ice and 2 µl of the ligation mix was added. The chemically competent *E. coli* was gently stirred with the pipette tip and left to incubate on ice for 30 minutes.

After ice-incubation the cells were heat shocked in a water bath at 42 °C for 30 seconds before being transferred back to the ice. 500 µl S.O.C., heated to room temperature, was added to the vial, which was capped and incubated in a water bath at 37 °C for 1 hour.

Following the TOPO cloning transformation, the *E. coli* cells were spread on LB agar plates with 100 µg/ml ampicillin and incubated at 37°C. Single colonies were screened for the insert by using primers A and D and a positive colony was selected and incubated in LB with 100 µg/ml ampicillin in a minitron HT ingfors incubator, at 37 °C and 150 rpm overnight.

The pCR 2.1 TOPO vector with insert was isolated from *E. coli* by QIAprep Spin miniprep kit (Qiagen), see appendix III, p. 58.

EcoRI digestion of pCR 2.1 TOPO vector with insert:
40 µl Plasmid DNA
10 µl 10X buffer
5 µl *EcoRI* enzyme
45 µl sterile distilled water
Incubated at 37°C overnight.

The pCR 2.1 TOPO vector with insert was cut by *EcoRI* and run on a 1 % agarose electrophoresis gel (SeaKem LE Agarose). The smallest fragment (*gerK:* 1056bp, *CwlJ:* 1235bp) was excised from the gel and purified using QIAquick Gel extraction kit (Qiagen), see appendix III, p. 62.

The cloned fragment was digested from the pCR 2.1 TOPO vector by *EcoRI* and ligated into the thermo sensitive pMAD shuttle vector (Arnaud et al. 2004) with an added *I-SceI* site (Kind gift from Dr. Anette Fagerlund, University of Oslo, Norway)(Appendix V, p. 67). The pMAD-*I-SceI* vector had been prepared by K. O'Sullivan.

EcoRI digestion of pMAD-*I-SceI*:
30 µl DNA
10 µl 10X buffer
5 µl *EcoRI* enzyme
55 µl sterile distilled water
Incubate for 2 hours at 37 °C
10 µl phosphatase buffer and 3µl arctic phosphatase was added and incubated at 37°C for another 30 minutes. The enzyme was deactivated by incubation at 65 °C for 10 minutes.

Ligation of *EcoRI* fragment into *EcoRI* site in pMAD-*I-SceI*:
2 µl 5X Ligation buffer
1 µl T4 ligase
4 µl pMAD-*I-SceI* vector (*EcoRI* digested)
3 µl insert (*EcoRI* digested)

Incubated at room temperature for 1 hour.

6 µl of the reaction was transformed into chemically competent E. coli One Shot TOP10 (Invitrogen), as described on page 24.

Single colonies were checked for pMAD-*I-SceI* vector with correct insert by PCR by pMAD primers in combination with up F primers pertaining to the individual gene (Appendix I, p. 53). Insertion of pMAD into the chromosome was made possible by homologous recombination when the temperature was raised to 37 °C. pMAD cannot replicate at this temperature because of its temperature sensitive replicon. A positive colony was grown over night in BHI with 100 µg/ml ampicillin at 37 °C and shaking.

pMAD-*I-SceI* is a low copy vector and therefore pMAD-*I-SceI* with insert was isolated from 12 ml cell culture with QIAprep Spin miniprep kit (Qiagen)(Appendix III, p. 58). To verify that the correct insert was present, a small amount was treated with *EcoRI* and checked by gel electrophoresis. The pMAD-*I-SceI* vector with insert was sent to Source Bioscience (Nottingham) for DNA sequencing.

The remaining pMAD-*I-SceI* isolate was concentrated by a precipitation reaction:
180 µl plasmid
20 µl NaAc 3 M pH 5.2
550 µl ethanol 100 %
The mix was frozen at -20 °C for 1 hour and then centrifuged at 15.700 RCF for 15 minutes.

The supernatant was removed and 200 µl 70 % ethanol was added. This was centrifuged for 5 minutes at 15.700 x *g*.

The supernatant was removed and the alcohol dissipated. When dry, 30 µl autoclaved distilled water was added.

Electrocompetent *B. licheniformis* and electroporation

Electrocompetent *B. licheniformis* MW3 were produced using a modified protocol from Xiaomin Hu (Løvdal et al. 2012).

40 % Polyethylene glycol 6000 (PEG6000) (Merck)**:** 40 g PEG6000 dissolved in 100 ml sterile distilled water. This was made fresh for each electrocompetent treatment.

B. licheniformis MW3 freeze culture (-80 °C) was streaked on LB agar and incubated at 37 °C overnight, before material from a single colony was inoculated in 25 ml BHI in a 100 ml Erlenmeyer flask. The inoculate was incubated in a Minitron HT Ingfors incubator, at 37 °C and 150 rpm overnight. 1 ml of the overnight culture was added to 200 ml room temperature BHI and incubated at 37 °C for 4-5 hours. The culture was kept in the incubator until A_{600} reached 0,9-1,0 at which it was harvested in a Sorvall RC-5B refrigerated super speed centrifuge in two 200 ml centrifuge tubes. The centrifuge

temperature was set to 20 °C with GSA-rotor at 4500 rpm for 15 minutes. The supernatant was drained off and the pelleted material was washed in 100 ml autoclaved distilled water by manual shaking. The solution was centrifuged at 8000 rpm for another 15 minutes at 20 °C. The washing step was repeated. The washed pellet was then resuspended in 5 ml freshly made 40 % PEG6000 and centrifuged at 5000 rpm for 15 minutes. The spent PEG6000 was drained off and the pellet was resuspended in 750 µl 40 % PEG6000. The prepared cell solution was transferred to 1,5 ml Eppendorf tubes containing aliquots of 100 µl and stored at -80 °C.

When transforming, 4 µl plasmid was added to the 100 µl aliquot of prepared *B. licheniformis* and incubated for 1 minute on ice. 40 µl was transferred to a 0,2 cm electroporation cuvette and electroporated at 2,5 kV for 4 ms (Micropulser™, Biorad).

After electroporation of *B. licheniformis*, 500 µl S. O. C. was added and the electroporated cells were transferred to an Eppendorf tube and incubated at 30 °C for 4 hours. The electroporated cells were then spread on LB agar containing 1 µg/ml erythromycin and 40 µl X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and incubated at 37 °C for 48 hours.

The pMAD-*I-SceI* vector carries a constitutively expressed transcriptional fusion with the *bgaB* gene encoding a thermostabile β-galactosidase from *Bacillus stearothermophilus*, thus allowing for blue/white selection when grown on agar containing X-Gal (Arnaud et al. 2004). The pMAD-*I-SceI* vector also contains a temperature sensitive mutant replicon derived from pE194, (naturally occurring plasmid in *Staphylococcus aureus*) called pE194[TS]. Vectors carrying this replicon display an extremely tight replication block above 37 °C, but maintain wild type copy numbers at temperatures below 32 °C (Arnaud et al. 2004).

The *B. licheniformis* transformants were incubated at 37 °C, which is higher than the permissive temperature reported for pMAD (Arnaud et al. 2004). The higher temperature has been successfully used by the lab at NVH, which believe it might cause a more direct integration of the plasmid into the genome (Løvdal 2011).

Blue colonies were restreaked on LB agar containing 1 µg/ml erythromycin and colonies positive for the first homologous recombination were confirmed by PCR using primers, *gerK*: 695-1565 or *CwlJ*: 1359-695. The pMAD-*I-SceI* positive *B. licheniformis* recombinants were made electrocompetent by the above mentioned method.

Creating the deletion

pBKJ223 was isolated from *E. coli* by QIAprep Spin miniprep kit (Qiagen) and concentrated by precipitation reaction as detailed previously on page 26. The pBKJ223 encodes the restriction

enzyme, *I-SceI*. This enzyme recognizes an 18 bp sequence that has been introduced in the pMAD-*I-SceI* vector and is unique in the genome of *B. licheniformis*/ pMAD-*I-SceI*. The double stranded break produced by the enzyme cleaving the unique site within the chromosome, leads to repair of the break by homologous recombination of the homologue regions flanking the ends of the break (Janes & Stibitz 2006).

Electroporation was done as described previously, on page 26, and after initial incubation in S. O. C. at 37 °C the cells were spread on BHI agar with 10 µg/ml tetracycline and 40 µl X-Gal and incubated at 37 °C overnight.

White colonies, showing loss of pMAD-*I-SceI,* were checked by PCR using primer pairs, *gerK*: 1565-1555 or *CwlJ*: 1359-1333, and confirmed deletion mutants were cultivated for 5 hours in BHI medium at 37 °C and shaking. This was done without tetracycline to rid the mutants of the pBKJ223 plasmid. The mutants was then spread on BHI agar and incubated at 37 °C overnight.

Single colonies were patched on to BHI agar with and without tetracycline to screen for loss of pBKJ223. When proven tetracycline sensitive the mutant was propagated in BHI medium at 37 °C with shaking before being added to a Microbank freeze culture tube (Prolab Diagnostics) and frozen at -80 °C. Genomic DNA was isolated and PCR using primer pairs, *gerK*: 1565-1555 or *CwlJ*: 1359-1333, were done to verify the correct deletion. PCR product was purified with Qiaquick PCR purification kit (Qiagen) and sent to Source Bioscience (Nottingham) for DNA sequencing.

Sporulation

To run germination tests there was a need to produce large batches of spores. This was done by a protocol developed by Irene S. Løvdal (Løvdal et al. 2012).

Freeze cultures were streaked on LB agar (for *B. licheniformis* MW3 Δ*gerA* 100 µg/ml spectinomycin was used) and incubated at 37 °C overnight. 50 ml liquid LB medium in a 100 ml Erlenmeyer flask, with or without antibiotic, was inoculated with scrapings from a single colony and incubated at 37 °C and 225 rpm overnight. 20 µl pre-culture was transferred to 100 ml Bacto-MS in a 500 ml Erlenmeyer flask and incubated at the same temperature and shaking as the pre-culture. The sporulation process was checked by phase contrast microscopy every day until >70 % of the cells had sporulated and the spores could be harvested.

When the spores were ready to harvest (>70 % spores), 50ml of spore-laden bacto-MS was transferred to a 50ml falcon tube and centrifuged for 20 minutes at 3900 x *g* in an Allegra™ X-22R centrifuge at 4 °C. The pellet was washed with 10 ml distilled water and centrifuged at the same
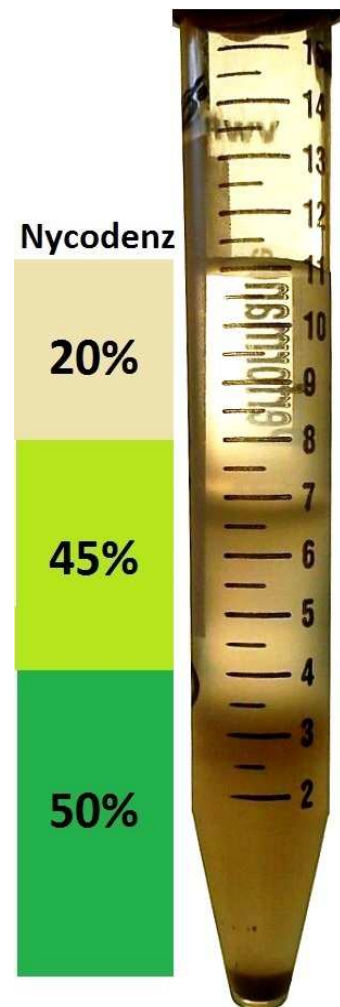
speed and temperature for 10 minutes. The pelleted material was resuspended in 3 ml 20 % Nycodenz (GE Healthcare) and mixed well by pipetting. All Nycodenz solutions were sterile filtrated through a 0,45 µM filter.
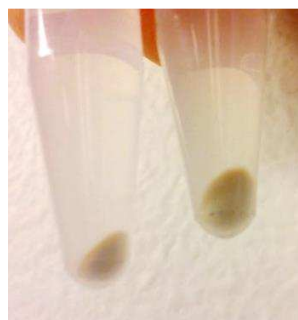
In a 15 ml falcon tube a gradient was created by layering 4 ml 50 % Nycodenz with 4 ml 45 % Nycodenz on top, as shown in Figure 8. The 3 ml 20 % Nycodenz spore solution was added as the top layer and the tube was centrifuged at 3900 x g at 4 °C for 80 minutes.

After centrifugation, the sporesolution had moved through the different Nycodenz layers leaving vegetative cells and detritus at the top while the spores were collected in a pellet at the bottom and an opaque layer of 2-3 ml (Figure 8). The top layer and part of the opaque layer was removed, approximately 5-6 ml in total. Then cold sterile distilled water was added to make a total volume of 15 ml, in which the spores were resuspended and centrifuged for 10 minutes at 3900rcf and 4 °C. This step was repeated 2 more times for a total of 3 washes.

Figure 8: Layers in Nycodenz after sentrifugation of spore solution

When a solid pellet was achieved it was dissolved in 2 ml sterile distilled water and transferred to two Eppendorf tubes. The Eppendorf tubes where then centrifuged at 4500 x g for 5 minutes at 4 °C in an Eppendorf table centrifuge. The supernatant was removed and fresh distilled water was added. The pellet was dissolved by pipetting and centrifuged a second time. The supernatant was removed and fresh distilled water was added a second time, the pellet was dissolved by pipetting again and then the spore solution was left to sedimentate at 4 °C for five days. After five days the spore solution was checked by phase contrast microscopy to confirm 99 % clean phase bright spores. The spore pellet of a single Eppendorf tube as shown in Figure 9 is enough to make 2 x 900 µl potassium phosphate spore solution for germination assay.

Figure 9: Clean phase bright spores ready for germination

Germination

The spore solution was centrifuged at 200 x g for 3 minutes and the pellet was resuspended in room temperature sterile distilled water before being heat activated for 20 minutes at 65 °C. The warmed spore solution was centrifuged at 4500 x g for 5 minutes at 4°C and resuspended in 900 µl 100 mM potassium phosphate buffer (pH 7,1) and the absorbance was adjusted to an initial A_{600} of 2,15 in a spectrophotometer (Shimadzu UV-VIS 160A, Shimadzu Europa GMBH). Aliquots of 100 µl of potassium phosphate spore solution were added to two rows of eight wells in a 96 well micro plate. The bottom row (negative control) had 100 µl of sterile distilled water added while the germinant row had 100 µl of germinant solution added. The germination response was then measured (A_{600}) every 2 minutes in a Tecan Infinite M200 micro plate reader with Magellan 7.1 software, incubating the germinating spores at 37 °C for 2 hours with shaking. The number of strains tested in different assays varied, but no more than two rows of eight wells were used since longer read times would break the two minute cycle. When the germination time was up (2 hours), the germinated spores and negative controls were checked in a phase contrast microscope and the percentage of phase dark spores was noted. L-cysteine was made fresh before germination, due to rapid oxidation to cystine and crystal formation.

A variation of this germination assay was used to test for inhibitory action on the part of amino acids. Aliquots of 100 µl of potassium phosphate spore solution were added to two rows of eight wells in a 96 well micro plate. 50 µl of inhibitor was added to one half of the wells while 50 µl of sterile distilled water was added to the other half. The plate was preincubated for 15 minutes before 50 µl of germinant was added to wells with and without inhibitor. 50 µl sterile distilled water was added to negative control wells before the plate was incubated and germination was measured for two hours as a regular germination assay.

Germination of CaDPA could not be measured by absorbance due to the precipitate that forms when DPA and $CaCl_2$ are mixed together. The germination was estimated by viewing the progress, in a microscope, every hour for three hours. Spore solution was centrifuged at 200 x g for 3 minutes at 4°C, before being resuspended in sterile distilled water and adjusted to an initial A_{600} of 2,15. The solution was split in two parts, where one half was heat activated at 65 °C for 20 minutes while the other was kept on ice. Both solutions where then centrifuged at 4500 x g for 5 minutes at 4°C and resuspended in 1000µl each of 100mM DPA in Tris buffer (pH 7,1). 200 µl triplicates were made of both heat activated and ice incubated and 50 µl 500mM $CaCl_2$ was added. The Eppendorf tubes were incubated at room temperature on a tilting mixer wrapped in alu-foil. All triplicates were checked in a phase contrast microscope every hour for three hours.

Results

Creation of deletion mutants

The MW3 wild type strain and the previously constructed MW3 Δ*gerA* mutant were used to create Δ*gerKA-KC* and Δ*CwlJ* mutants. The strains created during this work are presented in Table 2 on page 37. Figure 10 displays the upstream and downstream PCR products of *gerK* (left) and the fusion product (right), hereby referred to as *gerK* insert.
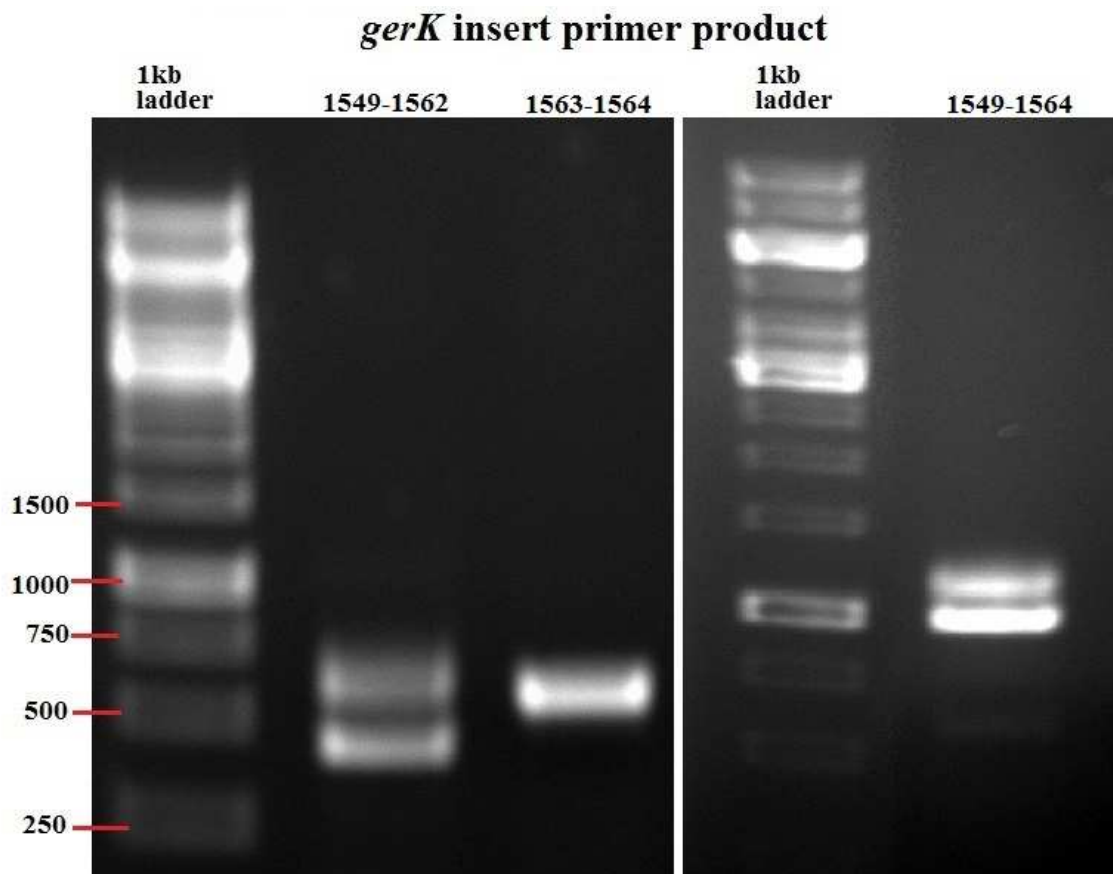

Figure 10: Left: Upstream (1549-1562) and downstream (1563-1564) PCR fragments. Upstream fragment had a primer specificity problem; upper band is the correct length, i. e. 541 and 515bp respectively. **Right:** Fusion PCR fragment, upper fragment is correct length, 1056bp. Dual bands are a run-on problem from the upstream fragment. (Fermentas generuler 1 kb, Appendix I, p. 54)

The unspecific binding of primer in the upstream fragment resulted in in two fusion products. This problem was carried over into the pCR TOPO 2.1 and transformed into *E. coli* TOP10 cells*.* Seven colonies were checked for incorporation of TOPO vector by PCR using primers 1549-1564 (Appendix I, p. 54) and four were positive but only one of the four had the correct 1056bp insert. The colony with the correct *gerK* insert was propagated and the vector was isolated. The TOPO vector with *gerK* insert was digested with *EcoRI* and the *gerK* insert was purified by gel electrophorese as shown in Figure 11 (left). pMAD was also cut with *EcoRI* and insert, *gerK* or *CwlJ*, was ligated into the *EcoRI* site of pMAD-*I-SceI*. The pMAD vectors with insert were propagated in *E. coli* TOP10 and positive colonies were verified by PCR and gelelectrophoresis, as shown in Figure 11 (right) and 12.
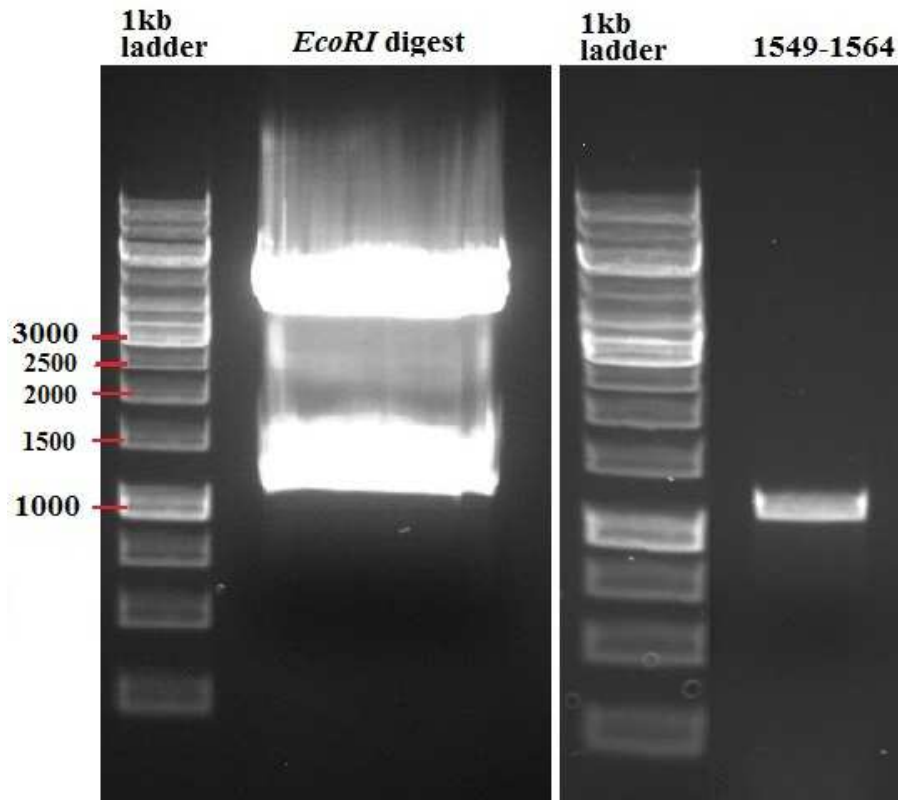
Figure 11: Left: Vector isolated from *E. coli* and digested over night by *EcoRI*. **Right:** PCR verification of *gerK* insert in pMAD. Fragment is 1056bp. (Fermentas generuler 1 kb, Appendix I, p. 54)
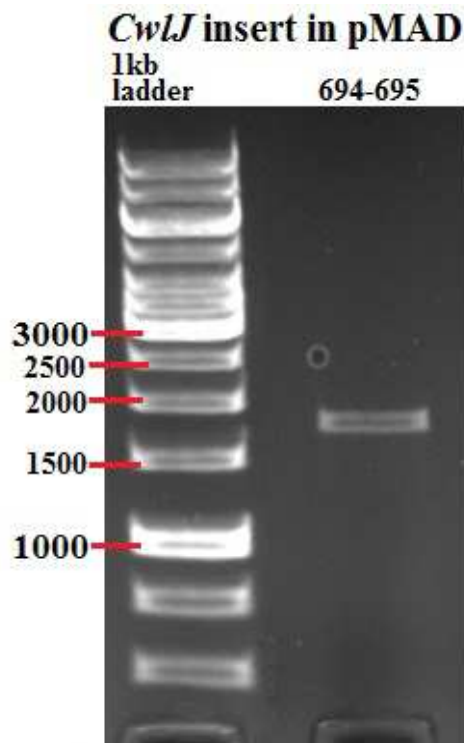

Figure 12: PCR verification of *CwlJ* insert in pMAD. Fragment is 1759bp. (Fermentas generuler 1 kb, Appendix I, p. 54)

After pMAD with insert had been confirmed in an *E.coli* TOP10 colony (all tested colonies were positive), the colony was propagated and the vector isolated. The isolated pMAD and insert was checked by PCR using primers 1565-695 for *gerK* and 694-695 for *CwlJ* (Appendix I, p. 54) and the PCR products were sent to Source Bioscience (Nottingham) for DNA sequencing.

The sequence from Source Bioscience (Nottingham) confirmed Δ*gerKA-KC* as error free. The insert for Δ*CwlJ* on the other hand, contained a base substitution in the downstream area of the deleted gene. This error was also found in the deletion mutant. A guanine base had been substituted for a thymine. The substitution occurred in the last base of the DNA codon and the substitution did not affect the amino acid sequence nor did it affect the reading frame. The deleted gene was replaced by ATGTGA (5'-3') in Δ*gerKA-KC* and ATGTAA (5'-3') in Δ*CwlJ*. The DNA sequences are included in appendix VI, p. 68.

Introducing pMAD-*I-SceI*-Δ*gerKA-KC*/ Δ*CwlJ* into *B. licheniformis*

The pMAD-*I-SceI* vector with insert was electroporated into the relevant *B. licheniformis* strain and the first crossover was induced by growing the transformants at 37 °C for 48 hours. The expected upstream crossover events for the two mutants are illustrated in Figure 13. The downstream crossover event is not illustrated because crossover direction is not important in this step only that the crossover has happened.
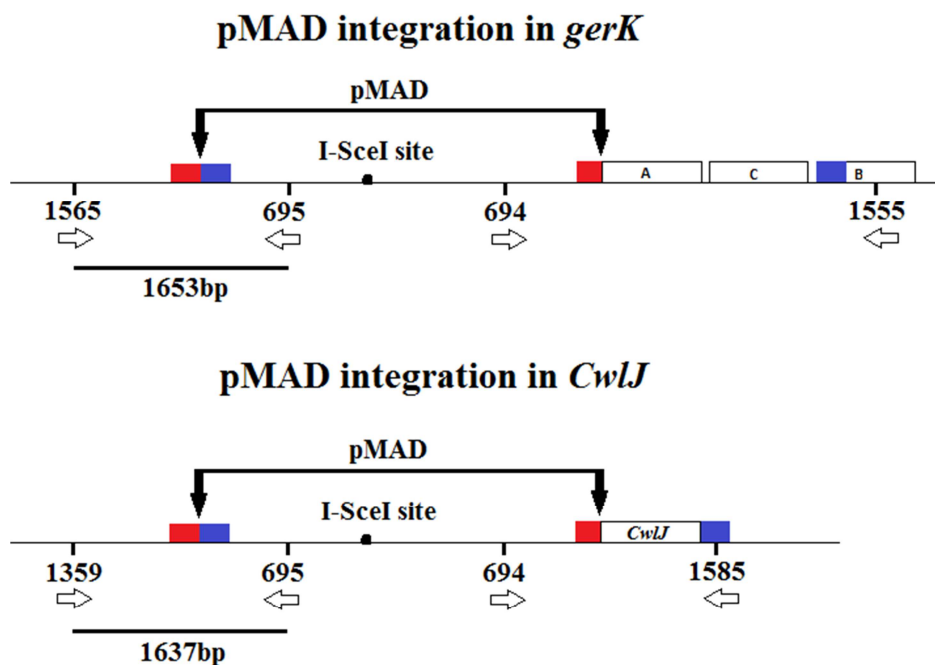

Figure 13: Schematic representation of pMAD-*I-SceI* integration into *B. licheniformis* genome. The red and blue boxes represent homologous areas cloned in pMAD, see Figure 7. pMAD-*I-SceI* integration was checked by PCR.

Colonies, which were blue after 48 hours, were picked and restreaked before being checked by PCR to verify and determine the location of the crossover. The results of the PCR are shown in Figures 14 and 15.
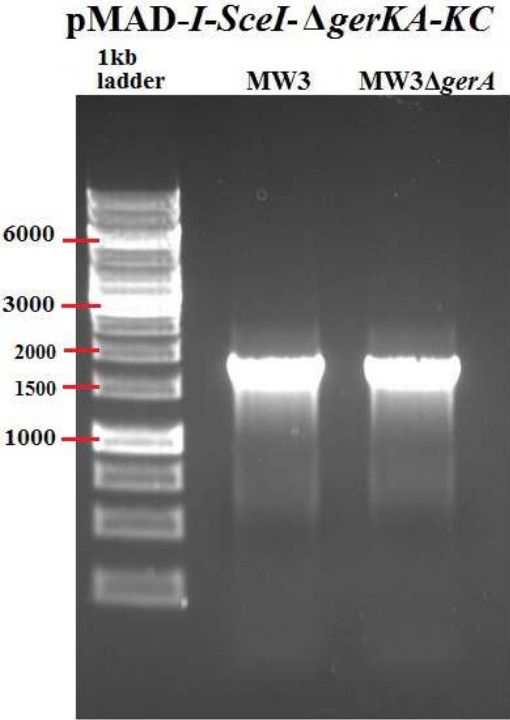
Figure 14: pMAD-*I-SceI-gerK* PCR using primers 695-1565. Fragment is 1653bp (Fermentas generuler 1 kb, Appendix I, p. 54)

The pMAD-*I-SceI-ΔgerKA-KC* insertion, as shown in Figure 13, crossover happened in the upstream homologue section and the fragment of 1653bp produced by primers 1565-695 (Figure 14), showed correct size.
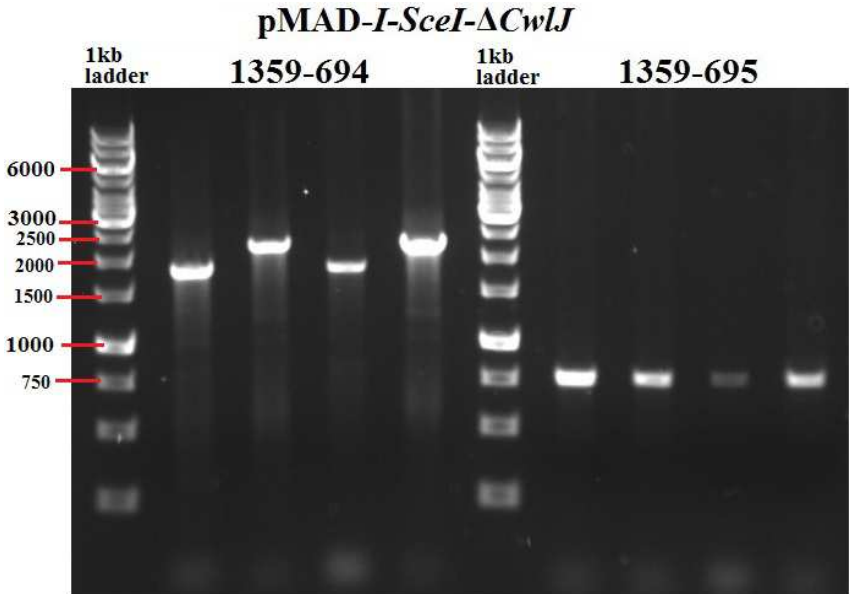

Figure 15: pMAD-*I-SceI-ΔCwlJ* PCR fragments from four colonies of MW3 transformants. Fragments 1359-694: 1750bp and 2250bp. Fragments 1359-695: 750bp (Fermentas generuler 1 kb, Appendix I, p. 54)

The pMAD-*I-SceI-ΔCwlJ* construct did not result in the expected crossover, which would yield a fragment of 1637bp when using primers 1359-695. The PCR fragments produced when using primers 1359-695 were roughly 750bp (Figure 15). The pMAD-*I-SceI-ΔCwlJ* transformants also yielded fragments varying from 1750bp to 2250bp when using primers 1359-694. Since the same forward primer (1359) was used in both primer sets, one of the pMAD primers (694 and 695, appendix V p. 67) should not have resulted in PCR product. PCR from the opposite side of the pMAD-*I-SceI-ΔCwlJ* insertion was not attempted due to lack of primer outside the downstream area. It was decided to move on to the second crossover, rather than order a new primer, since the site of the first crossover was of low importance.

Gene deletion

After electroporation with the second plasmid pBKJ223, colonies that remained white after 48 hours on X-gal were restreaked on BHI with 10 µg/ml tetracycline. The second crossover led to deletion of target sequence or reversion to wildtype. Verification by PCR of the deletion of correct size is shown in Figures 16 and 17. The five deletion mutants are presented in Table 2 on page 37.
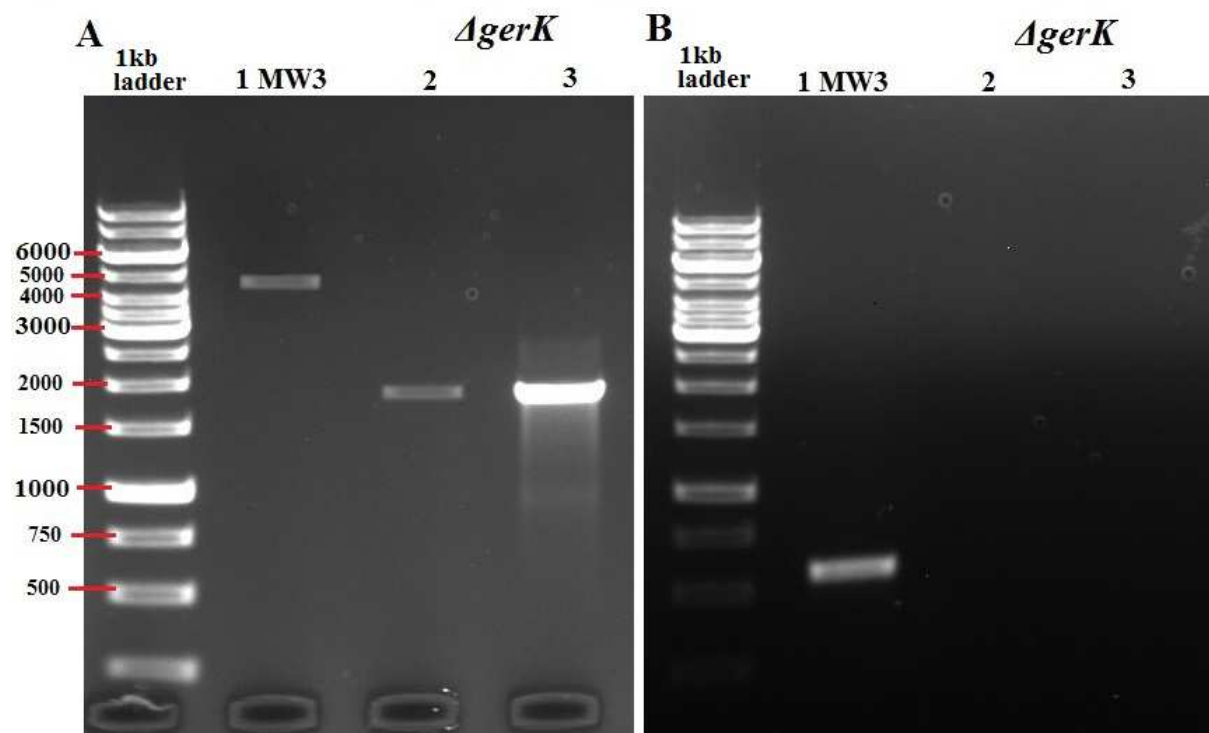
Figure 16: Confirmation of *gerK* gene deletion.   **A:** Primer pair 1565-1555, external to *gerK*. **B:** Primer pair 1566-1567, internal in *gerK*. **A and B:** Fermentas generuler 1 kb (Appendix I, p. 54) Lane 1: MW3 wild type strain, Lanes 2 and 3: Deletion mutants 1323 and 1324, respectively.

Figure 16 A shows the fragments made using primers 1565-1555 (Figure 13) in *B. licheniformis* MW3 wild type and the two deletion mutants (1323, 1324). The size of the *B. licheniformis* MW3 (wild type)

band is 4662bp and the size of the fragments from the *gerK* deletion mutants is 1747bp. A segment of 2915bp has been deleted from the *gerK* mutants.

Figure 16 B shows the single fragment, 538bp, produced by primers 1566-1567 (internal in the deleted fragment) in the *gerKA-KC* gene in *B. licheniformis* MW3. The two deletion mutants did not produce this PCR product.
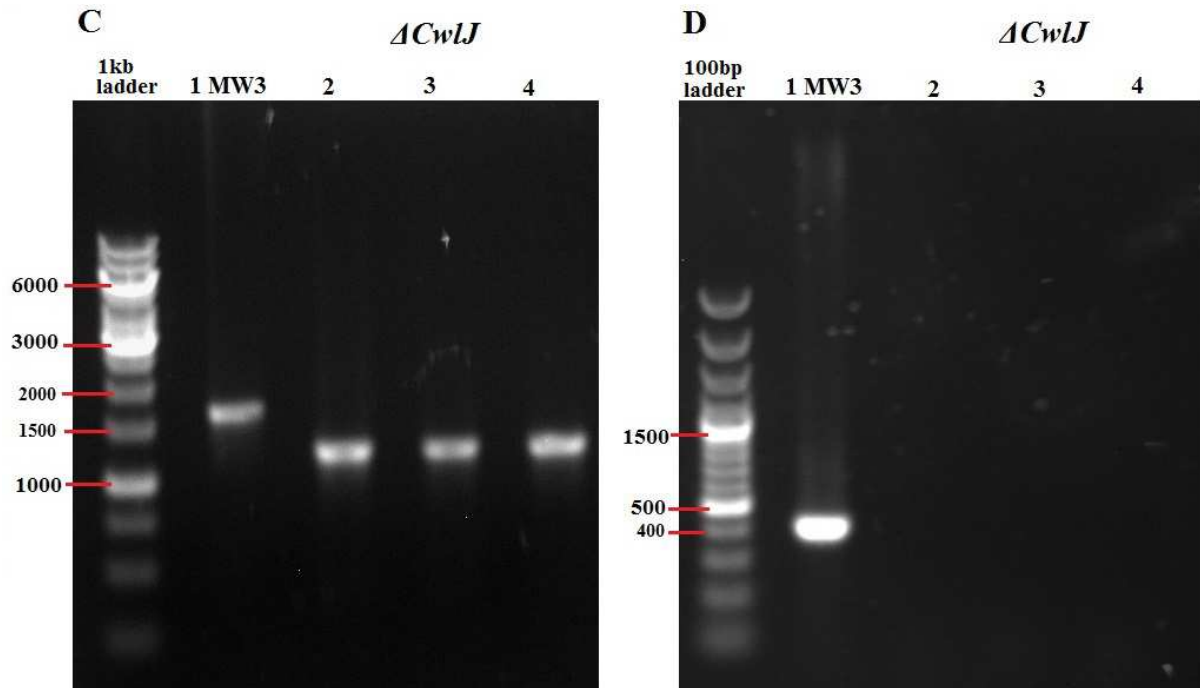

Figure 17: Confirmation of *CwlJ* gene deletion. **C:** Primer pair 1359-1585, external to *CwlJ*. **D:** Primer pair 1342-1341, internal in *CwlJ* .**C:** Fermentas generuler 1 kb. **D:** Fermentas generuler 100 bp Plus (Appendix I, p. 53) Lane 1: MW3 wild type, Lanes 2, 3 and 4: Deletion mutants 1327, 1332, 1333, respectively.

Figure 17 C shows the fragments made using primers 1359-1585 (Figure 13) in *B. licheniformis* MW3 and the three deletion mutants (1327, 1332, 1333). The size of the *B. licheniformis* MW3 (wild type) band is 1614bp and the size of the fragments from the *CwlJ* deletion mutants is 1155bp. A segment of 459bp has been deleted from the *CwlJ* mutants.

Figure 17 D shows the single fragment, 370bp, produced by primers 1342-1341 (internal in the deleted fragment) in the *CwlJ* in *B. licheniformis* MW3. The three deletion mutants did not produce this PCR product.

DNA sequencing of the PCR product shown in Figure 16 and 17 (A: lane 2, 3 and C: lane 2, 3, 4) verified that the deletion was correct and that five different deletion mutants (two of *gerK* and three of *CwlJ*) had been constructed. The five different mutants are displayed in Table 2.

Table 2: Deletion mutants constructed in this thesis

Strain #	Created from	Mutant description	Figure ref.
1323	*B. licheniformis* MW3Δ*gerAA*::spec	*B. licheniformis* MW3Δ*gerAA*::spec Δ*gerKA-KC*	Figure 16 A2/B2
1324	*B. licheniformis* MW3	*B. licheniformis* MW3Δ*gerKA-KC*	Figure 16 A3/B3
1327	*B. licheniformis* MW3Δ*gerAA*::spec Δ*gerKA-KC*	*B. licheniformis* MW3Δ*gerAA*::spec Δ*gerKA-KC* Δ*CwlJ*	Figure 17 C2/D2
1332	*B. licheniformis* MW3Δ*gerKA-KC*	*B. licheniformis* MW3Δ*gerKA-KC* Δ*CwlJ*	Figure 17 C3/D3
1333	*B. licheniformis* MW3	*B. licheniformis* MW3Δ*CwlJ*	Figure 17 C4/D4

Germination

Due to timeframe and problematic spore batches, only germination of the two *gerK* mutants, *B. licheniformis* MW3Δ*gerAA*::spec Δ*gerKA-KC* and *B. licheniformis* MW3Δ*gerKA-KC* were examined during the work described in this thesis. Several spore batches had to be discarded due to spore clumping which affected germination efficiency and absorbance reading.

To study the effect of gene deletion on germination phenotype the *gerK* mutants were germinated with L-alanine and L-cysteine together with the wild type MW3 and the *gerA* deletion mutant. All negative controls remained stable (<1% phase dark). All germination results presented here have been made with one week old spores stored at 4 °C, unless otherwise specified.

Figures 18 and 19 are an average of two individual germination assays with two different spore batches.

Table 3 shows the % of phase dark spores of the two spore batches, as viewed in a phase contrast microscope. The average of the two percentages can be correlated to the germination end points shown in Figures 18 and 19.

Table 3: % phase dark spores as observed in a microscope after 2 hours germination in assay.

Strain	L-alanine	L-cysteine	All negative controls
MW3	>95%/>95%	>95%/>95%	1%
Δ*gerA* (1307)	20%/5%	40%/20%	1%
Δ*gerA* Δ*gerK* (1323)	40%/40%	60%/50%	1%
Δ*gerK* (1324)	>95%/>95%	>95%/>95%	1%

L-alanine

Figure 18 shows the germination average, using two different spore batches of *B. licheniformis* MW3, *B. licheniformis* MW3Δ*gerAA*::spec, *B. licheniformis* MW3Δ*gerAA*::spec Δ*gerKA-KC* and *B. licheniformis* MW3Δ*gerKA-KC,* named MW3, Δ*gerA*, Δ*gerA*Δ*gerK* and Δ*gerK* respectively, using 100 mM L-alanine as germinant. The Δ*gerA* and Δ*gerA*Δ*gerK* mutants germinated poorly, while the Δ*gerK* mutant germinated as the MW3 wild type. Δ*gerK* mutant seems to germinate better than MW3 when being measured by absorbance, but this was not confirmed by phase contrast microscopy (Table 3).
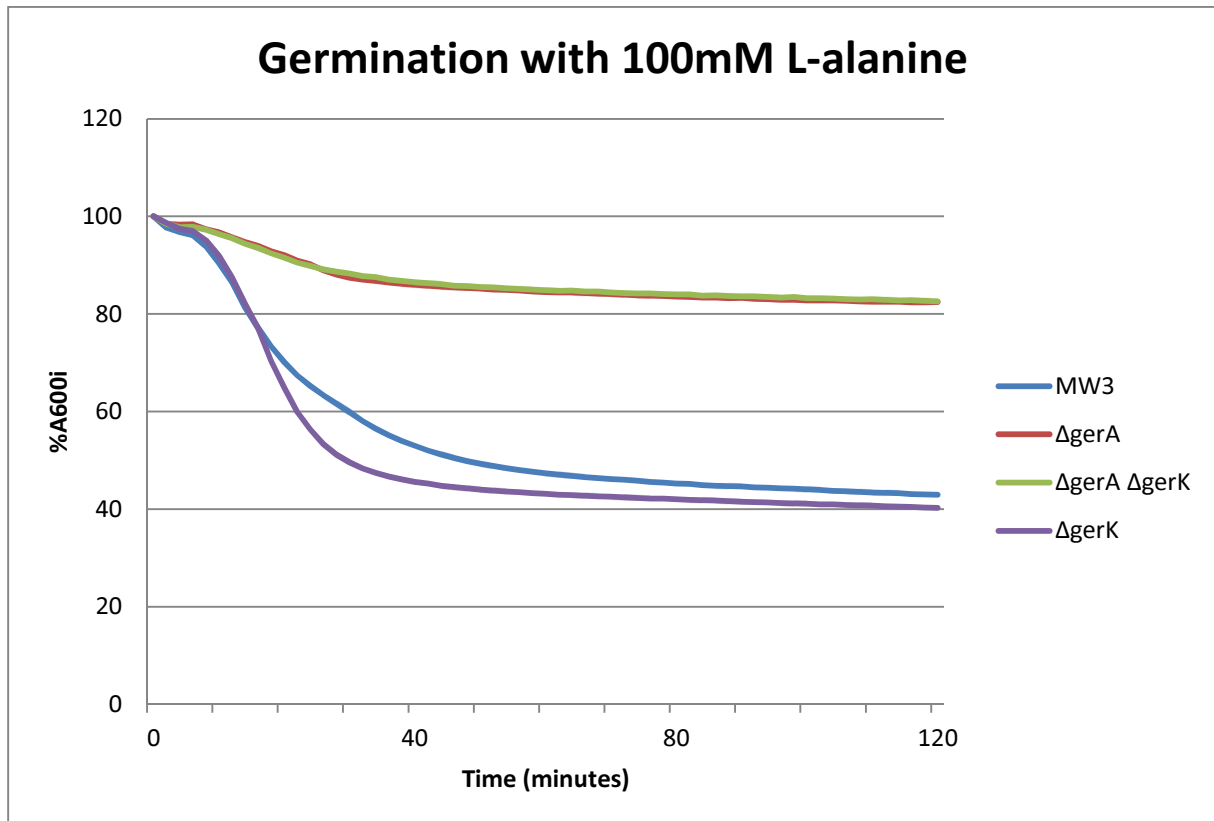
Figure 18: Germination with 100 mM L-alanine in 50 mM potassium phosphate buffer (pH 7,1). The germination curve is an average of two different spore batches.

L-cysteine

Figure 19 shows the germination average using two different spore batches of MW3, Δ*gerA*, Δ*gerA*Δ*gerK* and Δ*gerK* with 100 mM L-cysteine. The Δ*gerA* and Δ*gerA*Δ*gerK* mutants germinated poorly, while the Δ*gerK* deletion mutant germinated essentially as the MW3 wild type. As with L-alanine it seemed like the Δ*gerK* mutant germinated better than the MW3 wild type in L-cysteine but this was not confirmed by phase contrast microscopy (Table 3). The Δ*gerA*Δ*gerK* mutant seemed to germinate a little better than the Δ*gerA* mutant. Δ*gerA*Δ*gerK* germinates 10-30 % (Table 3) better than Δ*gerA*. It has been observed that the Δ*gerA* mutant, germinated slightly better in L-cysteine than it did in L-alanine (personal communication from E. Madslien).
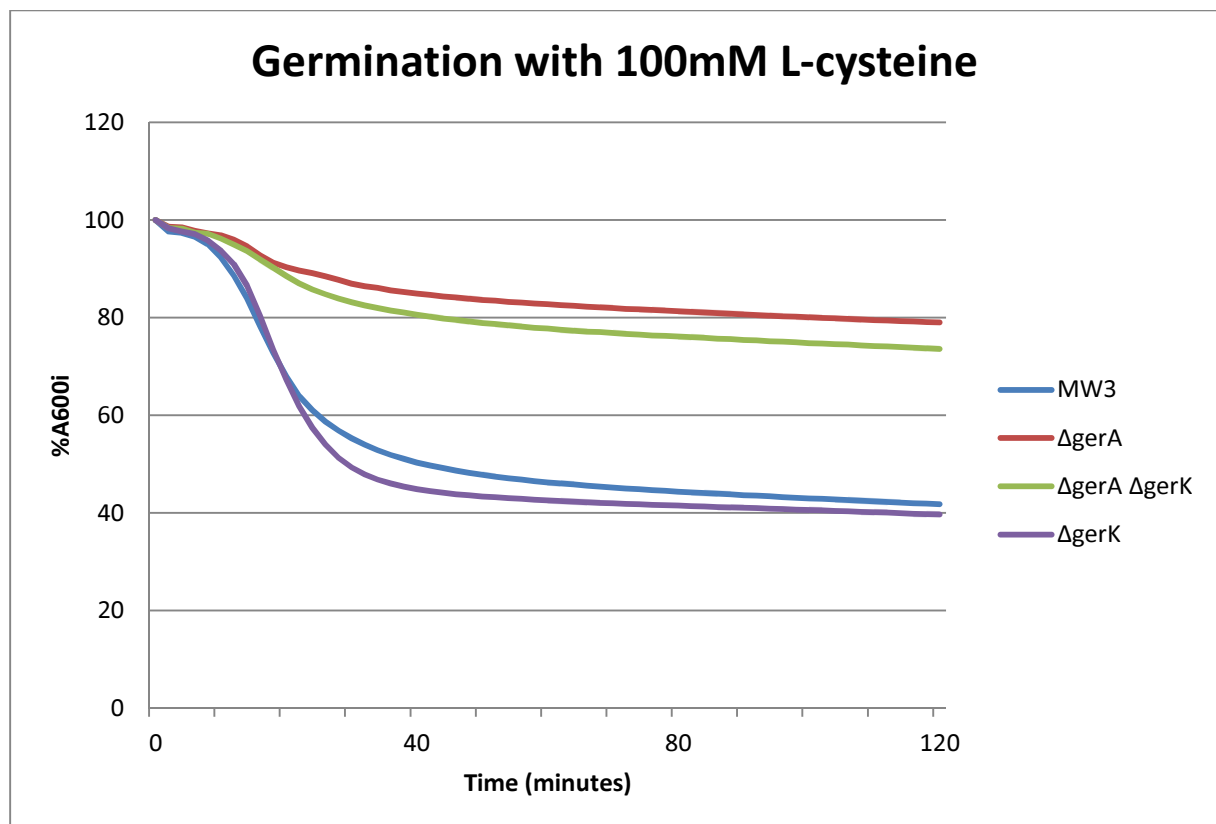
Figure 19: Germination with 100 mM L-cysteine in 50 mM potassium phosphate buffer (pH 7,1). The germination curve is an average of two different spore batches.

Since the *gerK* deletion mutant did not seem to be affected by its loss of *gerK* when germinated in L-alanine and L-cysteine and compared to the wild type, other germinants had to be tested. A preliminary test of germinants with *B. licheniformis* MW3 (wildtype) (Appendix VII, Figure A, p. 69) had shown that it does not respond to germination with a mix of AGFK (asparagine, glucose, fructose, and K$^+$), so casein hydrolysate was selected for further study.

Casein hydrolysate

To explore the germination properties of *gerK,* casein hydrolysate, referred to as casamino, and several amino acids were tested. Løvdal et al. (2012) lists the amino acids present in casein hydrolysate, as referred in Merck Microbiology manual 12th edition: (%w/w) alanine (2.00), arginine (2.20), aspartic acid (4.40), glutamic acid (12.50), glycine (1.20), histidine (1.80), isoleucine (2.40), leucine (3.40), lysine (5.60), methionine (1.20), phenylalanine (2.50), proline (6.10), serine (2.70), threonine (2.20), tyrosine (0.60), valine (3.90). Of these amino acids, eight were chosen, excluding alanine and valine, on the basis of immediate availability. The amino acids and their groupings are listed in Table 4.

Table 4: Germinants in casamino acid study of wild type spore (MW3) and Δ*gerK* mutant (1324)

Name	Amino acids in germinant	Note
Casamino		All naturally occurring amino acids in casein-hydrolysate
Amino	L-arginin, L-aspartic acid, L-glutamic acid, L-histidine , L-isoleucine, L-leucine, L-methionine, L-proline	Amino acid mix equivalent to casamino acid without L-alanine and L-Valine.
Group 1 (did not show germination, removed from assays)	L-arginin, L-aspartic acid, L-glutamic acid, L-histidine	Double % w/w as in casamino acid
Group 2	L-isoleucine, L-leucine, L-methionine, L-proline	Double % w/w as in casamino acid
L-Isoleucine	L-isoleucine	50 mM

Figure 20 shows a representative result of germination of *B. licheniformis* MW3 (wildtype) and the Δ*gerK* mutant using the germinants listed in Table 4. Due to negative results from germination with amino acids in Group 1, early in the testing process, they were exchanged for the single germinant isoleucine, which had shown to give some germination in MW3 (Appendix VII, Figures B and C, p. 70). Figure 20 shows that for all the germinants tested, there was no difference in germination response between the wild type strain and the *gerK* deletion mutant.
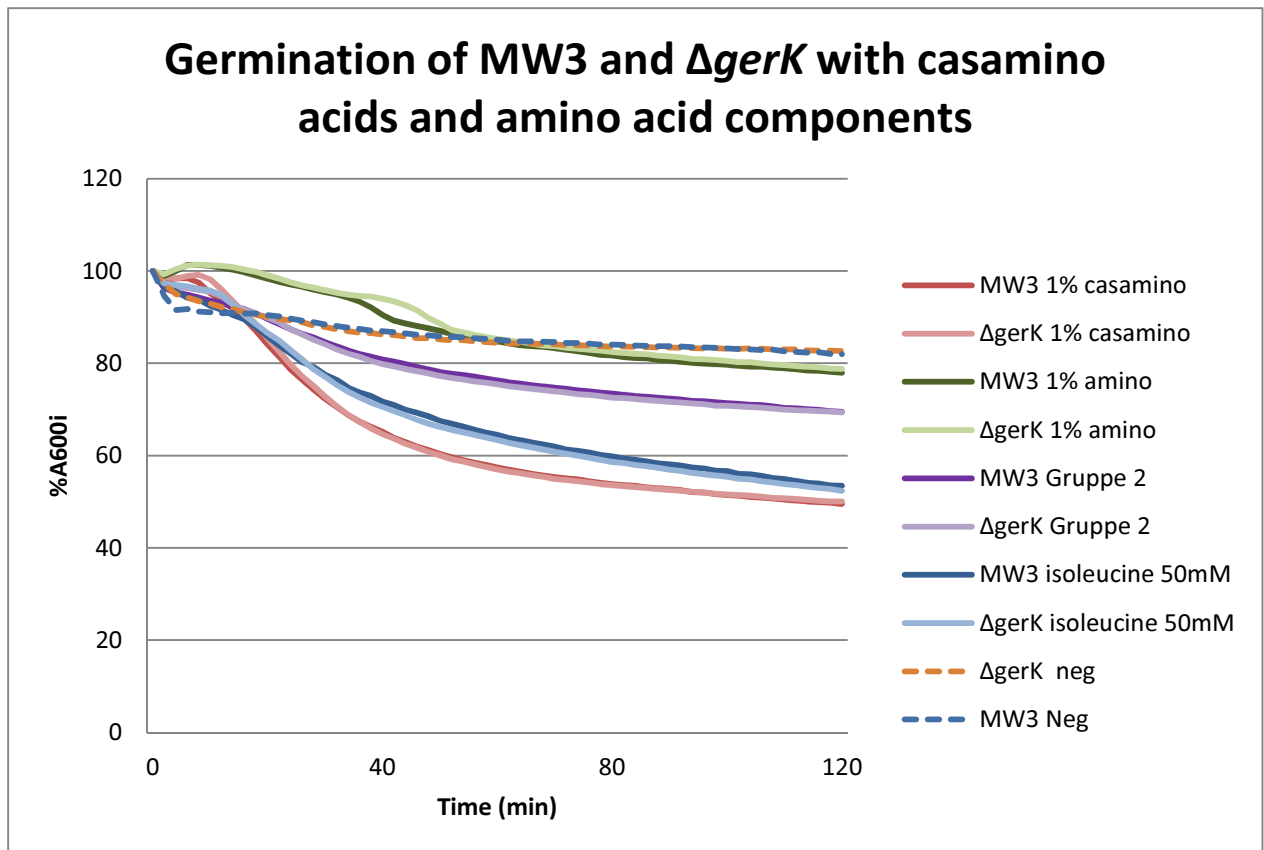
Figure 20: Germination with different amino acids in 50 mM potassium phosphate buffer (pH 7,1). The germination curve is a representative result for MW3 and Δ*gerK*.

Germination at elevated pH

Signs of an amino acid germination inhibitor in the Group 1 amino acids had previously been observed (Appendix VII, Figure D, p. 72). This led to a series of germination assays involving L-Arginine and various other germinants. During these experiments, a set of conflicting results arose, showing both inhibitor and germination effect (Appendix VII, Figures E and F, p. 71). Aqueous solutions of L-Arginine were shown to be strongly alkaline and the potassium phosphate buffer capacity was too low to counter the effect. The inhibitor effect was most probably a result of the pH sky rocketing to 10. Keeping the germinants in petri dishes prior to use, let the L-Arginine solution absorb carbon dioxide form the atmosphere and the pH when mixed with phosphate buffer only rose to 8-9.

Figure 21 shows the germination average of two different germination assays where a rise in pH to 8-9 was caused by arginine in addition to germinant compared to germinant alone in neutral pH 7.1. The germination was done on two different batches of *B. licheniformis* MW3 (wildtype). Due to germination tests, some of which are included in appendix VII, p. 69, the germinants have been adjusted to 25mM. This also reduced the risk of coming across solubility problems with the different amino acids.
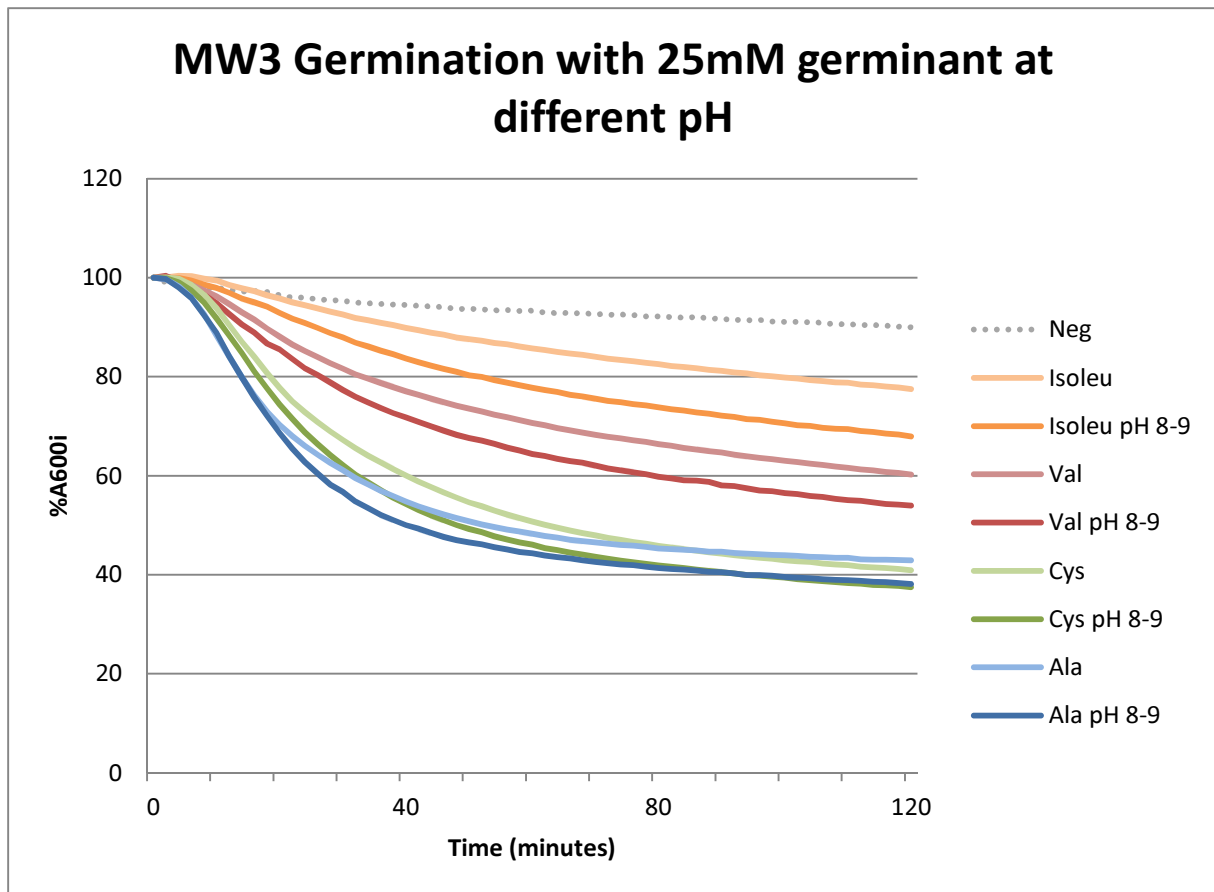
Figure 21: The high pH is induced by 25mM L-Arginine overloading the buffer (50 mM potassium phosphate pH 7,1) The germination curve is an average of two different spore batches.

For all the four germinants, a combination with L-Arginine and a pH between 8 and 9, resulted in an increased germination effect.

Calcium and dipicolinic acid (DPA)

A calcium and DPA germination assay was set up to check if the non nutrient germination pathway (Figure 4, p. 17) had been influenced by the *gerK* deletion. Due to the labour intensiveness of the assay, only a single germination test was done. There are no major differences between the four strains and they all germinate well with external DPA and calcium ions at pH 7.1. Table 5 shows the result of germination as detected in a phase contrast microscope hourly for three hours.

Table 5: Percentage of phase dark spores when germinated with CaDPA

Time (Hours)	Incubated in room temperature				Ice incubated				
	MW3	Δ*gerA*	Δ*gerA*Δ*gerK*	Δ*gerK*	MW3	Δ*gerA*	Δ*gerA*Δ*gerK*	Δ*gerK*	Neg ctrls
1	20 %	30 %	40 %	20 %	20 %	40 %	50 %	30 %	1 %
2	40 %	50 %	50 %	30 %	40 %	60 %	60 %	50 %	1 %
3	50 %	60 %	60 %	40 %	60 %	80 %	90 %	60 %	1 %

Discussion

Creation of deletion mutants

Creation of the deletion mutants via sequence and ligation independent cloning (SLIC) and markerless gene replacement was efficient and took only 16 days to produce a mutant. The method used was different to that used in creation of the *gerA* mutant, where a spectromycin resistance gene replaced the *gerA* gene. In this work the fusion insert was made as one whole sequence, rather than two separate PCR fragments (Klufterud 2011; Løvdal et al. 2012). Only one restriction site in pMAD was used, making the construction of pMAD with insert easier. Using the markerless gene replacement method, the deletion of the gene would not interfere with downstream transcription as an antibiotic cassette insert would do, since the deletion is inframe. The method could be repeated for as many gene deletions as necessary without the need for different antibiotic cassettes. The deletion mutant created by this method would not be influenced by the need to add antibiotics to the sporulation medium and thus eliminating a factor that could cause unwanted differences between mutant and wild type.

The *gerA* deletion mutant created by Løvdal was fitted with a spectromycin cassette insert in the *gerAA* area of the trisistronic gene. This cassette might be lost without antibiotic pressure, but the gene will not revert to wild type, since a part of the gene sequence is deleted.

The construction of the *gerK* deletion mutants (MW3 and MW3 Δ*gerA*) was a textbook case and the mutants were ready within 16 days. The generation of the *CwlJ* deletion mutants did not go as smoothly, with the insertion of pMAD unable to be verified and the second crossover in the wild type strain (*B. licheniformis* MW3, creating the single *CwlJ* deletion mutant) proving hard to induce. The insertion verification proved not to be an issue, as the next steps were successful, and the single *CwlJ* deletion mutant proceeded without a hitch when it was decided to start over with transformation of pMAD-*I-SceI-ΔCwlJ* into MW3.

Sporulation

The spores that were used in this work came from *B. licheniformis* sporulated at 37 °C, while 50 °C is the optimum sporulation temperature for *B. licheniformis* (Klufterud 2011). The decreased temperature means longer sporulation time, lower spore yield, lowered germination efficiency and a risk of more spontaneous germination (Klufterud 2011; Løvdal et al. 2012). However, the lower sporulation temperature used in this study was chosen since sporulation and germination in the gut of humans and animals is of importance. The lowered temperature leads to a more heterogeneous spore population because the sporulating bacteria are exposed to different environments in the

liquid sporulation medium as the nutrients are depleted and the pH is affected (Ramirez-Peralta et al. 2012). Since this work was about discovering differences in germination response connected to deletion of the *gerK* receptor gene, the lowered temperature was not considered to be an important factor.

Sporulation conditions have been demonstrated to have a strong influence on spore properties, including germination response (Fleming & Ordal 1964; Eijlander et al. 2011; Ramirez-Peralta et al. 2012). It is important to have this in mind when comparing results from germination studies, but this is less of a problem when probing for differences in deletion mutants from a single strain, than when comparing properties related to different strains of *B. licheniformis*. However, even small changes in the spore preparation protocol may influence on the germination properties of the spore batch.

E. Madslien (PhD student) assisted in harvesting of spore batches, since the criteria for harvesting is quite subjective and requires a trained eye (>70% spores, observed by phase contrast microscopy). As the mutants were sporulated without the need for antibiotic pressure, they behaved much like the wild type and were ready for harvesting after 48 hours in sporulation medium. Sporulation of *B. licheniformis* MW3 mutants in the presence of antibiotics was observed to prolong the sporulation to 72-96 hours (Mutants not presented or used in this work). The protocol, described in this thesis on page 28, produced roughly equal amounts of spores for the three mutants and the wild type used in the germination study.

The washing procedure utilised Nycodenz but whether the gradient influenced the germination properties compared to spores washed only in water, has not been explored in this work. The spores were washed using the same protocol each time but batch variation, related to the problem of spore clumping, was observed to be person dependent. This difference might be caused by variation in the measure of force used to dissolve spore pellet. This is one of the few factors that we are able to influence as other factors, e.g. micro environment during sporulation is undetectable.

Germination

Based on the results from germination assays with nutrient germinants, it seems that the GerK receptor is not involved in germination with L-alanine, L-cysteine, L-Isoleucine or some of the other amino acids present in casein hydrolysate. There are still nutrient germinants that need to be tested, before any final conclusion can be drawn. The *gerK* receptor might not be expressed in *B. licheniformis* and real time PCR to test this hypothesis is not included in this work. Even though a real time PCR shows expression of *gerK* it is no guarantee that the receptor works. Other possible methods to detect GerK receptor would be an immunoassay, such as ELISA (Enzyme-linked

immunosorbent assay) or SDS-PAGE (poly acrylamide gel electrophoresis) and immunoblotting with antibodies against the GerK receptor. This was not possible to do since we lack antibodies against GerK. The location of the receptor in the spore's inner membrane also complicates immunoassay detection as the spore would have to be decoated or fragmented in order for the assay to work.

The *gerK* deletion mutant behaved almost like the wild type strain *B. licheniformis* MW3 for all germination nutrients tested. The small difference between MW3 and the Δ*gerK* mutant, seen in Figure 18 and 19, may be caused by spore batch variation and might disappear if the experiment were repeated many times. Even though some variation in germination characteristics for parallel spore batches made with the same sporulation protocol was observed in this work, all successful germinations followed certain trends, as shown in Figures 18, 19, 20 and 21. Deviation from these germination trends were always a result of spore clumping, causing fluctuation in absorbance readings and skewed germination results. It was observed that spore clumps germinated less than single spores and this may be due to nutrients not being able to penetrate the clump. The cause of these spore clumps was not pinpointed beyond being related to the washing process, but the problem was aggravated when spores were added to the potassium phosphate buffer.

The weak germination in response to L-cysteine seen in the *gerA* deletion mutant (E. Madslien, unpublished results) did not cease when *gerK* was removed, rather L-cysteine germination seems to have increased slightly in the Δ*gerA*Δ*gerK* mutant. The small difference between Δ*gerA* and Δ*gerA*Δ*gerK* in germination with L-cysteine (Figure 19) persisted in all germination assays. This result was completely unexpected. One of the hypotheses connected to *gerK* initiating this work, was that GerK receptor caused the slight L-cysteine germination seen in the Δ*gerA* mutant (E. Madslien, unpublished results) and a deletion of *gerK* would abolish this. The results described in this study prove the hypothesis wrong.

The Δ*gerA* mutant does have a spectromycin cassette which the Δ*gerK* mutant lacks, but both the mutants have been sporulated under the same conditions given that the spectromycin cassette replaced part of the *gerAA* gene and wild type would not be reinstated even if spectromycin resistance was lost.

Paidhungat & Setlow (2000) constructed a *B. subtilis* quintuple mutant (deletion of *gerA, gerB, gerK, yndDEF* and *yfkQRT*) and tested its germination properties in four germination mediums. It remained ungerminated in all of them. This indicates that there are only these five germination receptors in *B. subtilis* and probably that there is other L-alanine responding germination receptors present in the *B. licheniformis* Δ*gerA*Δ*gerK* mutant. Ross & Abel-Santos (2010) have found a gene similar to the one encoding the *B. subtilis* YndDEF receptor present in *B. licheniformis*. It would be of great interest to

make a triple deletion mutant in *B. licheniformis*, lacking *gerA*, *gerK* and *yndD,* and see if all L-cysteine germination would be eradicated.

In *B. subtilis*, germination receptors GerB and GerK work together and induce germination with AGFK (Moir et al. 2002) but this knowledge cannot be used in comparison to *B. licheniformis*, as it lacks a GerB receptor homologue and does not germinate in response to AGFK. A study by Atluri et al. (2006) found that interaction with GerK, glucose, and K^+ somehow stimulates spore germination with L-alanine via GerA in *B. subtilis*. This might be the fate of GerK receptor, to function in cooperation with other Ger receptors in *B. licheniformis* MW3 under special circumstances.

In relation to the arginine induced pH studies, the effect of arginine on the potassium phosphate buffer was discovered at the last minute. Due to the nature of the discovery this experiment needs to be redone with basic and acidic phosphate buffer solutions to control the pH and eliminate any effect L-Arginine might induce. It has yet to be established if the germination effect is due to the increased pH or L-Arginine itself. The negative controls with arginine at first showed no signs of germination, but when tested again at the lower pH, a germination response seems to have been induced (Appendix VII, Figures E and F, p. 71).

Conclusion and future prospects

This thesis has resulted in an open ending. No involvement of *gerK* could be found in germination with L-alanine, L-cysteine, L-Isoleucine or casamino acid, but it is too early to draw a final conclusion. To further examine germination in *B. licheniformis* a triple mutant, Δ*gerA*Δ*gerK*Δ*yndD* should be constructed. This mutant would lack all germination receptors predicted in *B. licheniformis* so far.

A preliminary test was done with L-Valine on the three deletion mutants and the wild type of *B. licheniformis* studied in this work. Even though the result was compromised by spore clumping, spore germination was detected in wild type and Δ*gerK* mutant but not in Δ*gerA* and Δ*gerA*Δ*gerK* mutants. More germination assays need to be performed in order to eliminate statistical insignificant differences and test the remaining amino acids. The possibility of cooperation between GerA and GerK receptors in relation to germination with L-alanine, glucose, and K^+ is also a hypothesis worth testing.

All the *CwlJ* mutants created by this work are untested and the impact of the deletion has yet to be explored. As this work is part of a larger project, the *CwlJ* mutants will be studied at a later stage. The majority of germination receptor work is on *B. subtilis* and now followed by *B. cereus* and *C. perfringens*. Our understanding of the germination process benefits from a wide search, yielding small pieces of the puzzle that is spore dormancy.

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Appendix

Appendix I – Primer and ladders

Table 1: primers used in this thesis, replacement sequence highlighted in green and red, corresponding to start and stop codons, marking the beginning of primer overlap.

Number	Name	Sequence
	gerK	
1549	*gerK* A	ACGAGGTTATCGGCAATACG
1562	*gerK* B	TCTCTTTCATTCACATATTTTTCCTTGCGCAAGC
1563	*gerK* C	AAGGAAAAATATGTGAATGAAAGAGAGAGGAGG
1564	*gerK* D	TTTCCAAGAATGGGCAAAAG
1565	*gerK* up F	AAGCGTCGCAAGTCTTCATT
1555	*gerK* down R	CGTCTGGATCGTACTCAGCA
1566	*gerK* int F	CTTGAGACGATTGCAGGTGA
1567	*gerK* int R	GAATAAAAACGGCAGGGACA
	CwlJ	
1359	*CwlJ* up F	CGGTTAGATTGCCTTTCCAA
1585	*CwlJ* down R	AACGCCTTTCAATCATACCG
1570	*CwlJ* A	ACAGTCCCCCATCCATGATA
1571	*CwlJ* B	TTTTTGTTACATTTTTAGTCACCCCCAT
1572	*CwlJ* C	TGACTAAAAATGTAACAAAAACATCCCTTT
1573	*CwlJ* D	AAAACTTTCGGCAGCTTGAA
1342	*CwlJ* int F	TCTCACACTCTTCCGCTGTC
1341	*CwlJ* int R	GGCAGTCGTTCAGGCTTC
	pMAD	
694	pMAD F1	CCATCAGACGGTTCGATCTT
695	pMAD R1	GTCCCTGATGGTCGTCATCT
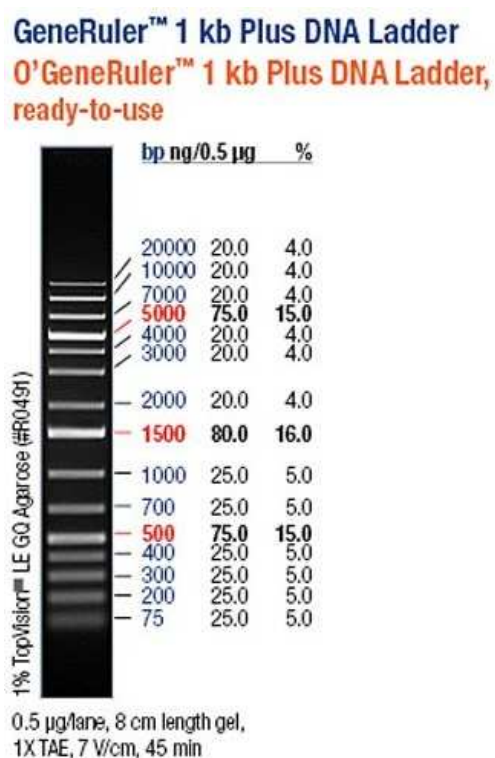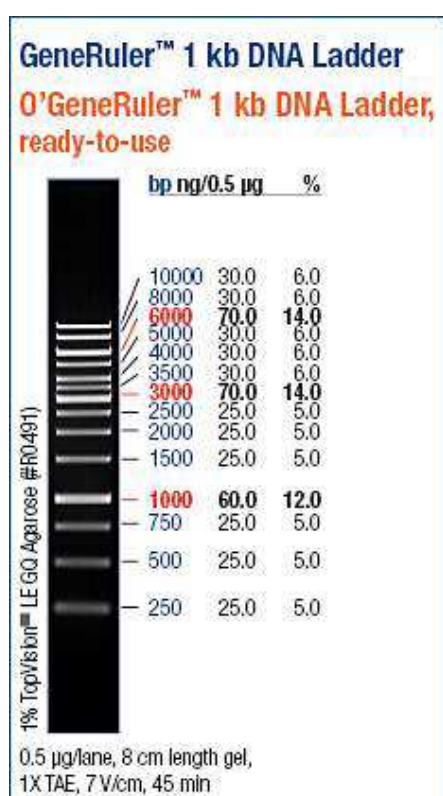
Appendix II – Chemicals, equipment and suppliers

Chemical/Equipment	Manufacturer	Notes
L-alanine	Sigma-Aldrich, St. Louis MO, USA	Germinant
L-Arginin	Sigma	Germinant
L-Asparagine	Sigma	Germinant
L-Aspartic acid	Sigma	Germinant
L-cysteine	Sigma	Germinant
L-Glutamic acid	Sigma	Germinant
L-Histidine	Sigma-Aldrich	Germinant
L-Isoleucine	Sigma	Germinant
L-Leucine	Sigma	Germinant
L-Methionine	Sigma	Germinant
L-Proline	Sigma	Germinant
L-Valine	Sigma-Aldrich	Germinant
KCL	Merck, Whitehouse station NJ, USA	Germinant
Glucose	Sigma	Germinant
Fructose	Sigma	Germinant
Casein-hydrolysate	Merck	Casamino acid germinant
Difco nutrient broth	Becton, Dickinson and Company, Franklin Lakes NJ, USA (BD)	Sporulation medium
$FeSO_4$	Merck	Sporulation medium
$CuCl_2$	Sigma	Sporulation medium
$ZnCl_2$	Sigma	Sporulation medium
$MnSO_4$	Merck	Sporulation medium
$MgCl_2$	J. T. Baker Chemicals B. V., Center Valley PA, USA	Sporulation medium
$(NH_4)_2SO_4$	Merck	Sporulation medium
Na_2MoO_4	Riedel-de Häen, Seelze, Germany	Sporulation medium
$Ca(NO_3)_2$	Merck	Sporulation medium
$CoCl_2$	Sigma-Aldrich	Sporulation medium
KH_2PO_4	Merck	Phosphate buffer
K_2HPO_4	Merck	Phosphate buffer
Ampicillin	Sigma	
Erythromycin	Sigma	
Spectinomycin	Sigma	
Tetracycline	Sigma	
Tryptone	Oxoid, Basingstoke, UK	Luria-Bertani (LB) medium
Yeast extract	Oxoid	Luria-Bertani (LB) medium
NaCl	Merck	Luria-Bertani (LB) medium/ SETbuffer
Agar bacteriological no.1	Oxoid	Agar
Bacto™ Brain heart infusion medium	BD	BHI
S. O. C. medium	Invitrogen, Carlsbad CA, USA	Suppied in pEXP5-TOPO TA expression kit

Taq polymerase	Dynazyme, Vantaa, Finland	PCR
Phusion high fidelity DNA plymerase	Finnzymes produced by BioLabs, Ipswich MA, USA	PCR
dNTP	Finnzymes, Vanta, Finland/Quanta Biosciences Gaithersburg MD, USA	PCR
Primers	Sigma	PCR
SeaKem LE Agarose	Lonza, Basel, Switzerland	Gel electrophoresis
Loading buffer	Sigma	Gel electrophoresis
1kB and 100bp ladder	Fermentas, Vilnius, Lithuanina	Gel electrophoresis
Ethidium Bromide	Sigma	Gel electrophoresis
Electric supply electrophoresis	Biorad, Hercules CA, USA	
Tris (Trizma base)	Sigma	TAE buffer/SET buffer
Acetic acid	Fermentas	TAE buffer
EDTA	Sigma	TAE buffer/SET buffer
SDS	Sigma	
EcoRI	BioLabs	
T4 ligase	Invitrogen	
Ligation buffer	Invitrogen	
Lysozyme	Sigma-Aldrich	
Proteinase K	Sigma-Aldrich	
Chloroform	VWR West Chester PA, USA	
Isoamylalcohol	Merck	
Ethanol	Kemetyl, Vestby, Norway	
QIAquick PCR purification kit	Qiagen, Venlo, The Nederlands	
QIAquickPCR gel extraction kit	Qiagen	
QIAprep spin miniprep kit	Qiagen	
pEXP5-TOPO TA expression kit	Invitrogen	
One Shot® TOP10 Chemically Competent *E. coli*	Invitrogen	
Sodium acetate (NaAc)	May & Baker Ltd. Dagenham, UK	
Polyethylene glycol 6000	Merck	
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)	Sigma-Aldrich	
Microbank	Prolab^{TM} Diagnostics, Toronto, Canada	Freeze culture tube
Nycodenz	GE Healthcare	
Minisart sterile filters, 0,2 µm, 045µm	Sartorius, Goettingen, Germany	
Falcon conical centrifuge tubes, 50 ml, 15 ml	BD	
Falcon 96 well microplate, flat bottom with lid	BD	
Mastercycler epgradient S	Eppendorf, Hamburg, Germany	PCR machine
Waterbath	Teche, Burlington NJ, USA	
pH-meter	Methrohm, Herisau, Switzerland	
Centrifuge 5451D	Eppendorf	
Sorvall RC-5B refrigerated	Sorvall, Buckinghamshire,	

superspeed centrifuge	England	
Allegra^{TM} X-22R centrifuge	Beckman Coulter, Brea CA, USA	
Minitron incubator	HT ingfors, Bottmingen, Switzerland	
Autoflow NU-5500	NuAire, plymouth MN, USA	
Dry block Heating system QBD2	Grant Instruments, Cambridge, UK	
Micropulser	Biorad	Electroporator
Electroporation cuvette 0,2cm	Biorad	
Tecan Infinite M200	Tecan, Männedorf, Switzerland	Microplate reader
Magellan 7.1	Tecan	Microplate reader software
Shimadzu UV-160 A	Shimadzu, Kyoto, Japan	Spectrophotometer
1,5ml disposable cuvettes	Brandtech, Essex CT, USA	Spectrophotometer cuvette
CaCl$_2$*2H$_2$O	Riedel-de Häen	
Pyridine-2,6-dicarboxylic acid (DPA)	Aldrich	

Appendix III – Qiagen kit protocols

QIAprep Spin miniprep kit

Quick-Start Protocol

QIAprep® Spin Miniprep Kit

The QIAprep Spin Miniprep Kit (cat. nos. 27104 and 27106) can be stored at room temperature (15–25°C) for up to 12 months.

For more information, please refer to the *QIAprep Miniprep Handbook*, which can be found at www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting

- **Optional:** Add LyseBlue® reagent to Buffer P1 at a ratio of 1 to 1000.
- Add the provided RNase A solution to Buffer P1, mix, and store at 2–8°C.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 13,000 rpm (~17,900 x g) in a conventional table-top microcentrifuge.
- Symbols: ● centrifuge processing; ▲ vacuum processing.

1. Pellet 1–5 ml bacterial overnight culture by centrifugation at >8000 rpm (6800 x g) for 3 min at room temperature (15–25°C).

2. Resuspend pelleted bacterial cells in 250 μl Buffer P1 and transfer to a microcentrifuge tube.

3. Add 250 μl Buffer P2 and mix thoroughly by inverting the tube 4–6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 min. If using LyseBlue reagent, the solution will turn blue.

4. Add 350 μl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. If using LyseBlue reagent, the solution will turn colorless.

5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.

For Material Safety Data Sheets, see www.qiagen.com/safety.

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6. Apply the supernatant from step 5 to the QIAprep spin column by decanting or pipetting. ● Centrifuge for 30–60 s and discard the flow-through, or ▲ apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source.

7. Recommended: Wash the QIAprep spin column by adding 500 μl Buffer PB. ● Centrifuge for 30–60 s and discard the flow-through, or ▲ apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source.

   Note: This step is only required when using endA$^+$ strains or other bacterial strains with high nuclease activity or carbohydrate content.

8. Wash the QIAprep spin column by adding 750 μl Buffer PE. ● Centrifuge for 30–60 s and discard the flow-through, or ▲ apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source. Transfer the QIAprep spin column to the collection tube.

9. Centrifuge for 1 min to remove residual wash buffer.

10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 μl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAprep spin column, let it stand for 1 min, and centrifuge for 1 min.

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QIAquick PCR Purification Kit Protocol
using a microcentrifuge

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting

■ Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

■ All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.

■ Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5.

■ Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.

■ If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.

    For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).

2. If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow.

    If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. Place a QIAquick spin column in a provided 2 ml collection tube.

4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.

5. Discard flow-through. Place the QIAquick column back into the same tube.

    Collection tubes are re-used to reduce plastic waste.

6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.

7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.

    IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

Quick-StartProtocol

QIAquick® Gel Extraction Kit

The QIAquick Gel Extraction Kit (cat. nos. 28704 and 28706) can be stored at room temperature (15–25°C) for up to 12 months.

For more information, please refer to the QIAquick Spin Handbook, March 2008, which can be found at: www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting

- The yellow color of Buffer QG indicates a pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Isopropanol (100%) and a heating block or water bath at 50°C are required.
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge.

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg ~ 100 µl). For >2% agarose gels, add 6 volumes Buffer QG.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.

5. Add 1 gel volume of isopropanol to the sample and mix.

Step 5 was not used in the work for this thesis

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6. Place a QIAquick spin column in ▲ a provided 2 ml collection tube or into ● a vacuum manifold.

7. To bind DNA, apply the sample to the QIAquick column and ▲ centrifuge for 1 min or ● apply vacuum to the manifold until all the samples have passed through the column. ▲ Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 μl, load and spin/apply vacuum again.

8. If the DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 0.5 ml Buffer QG to the QIAquick column and ▲ centrifuge for 1 min or ● apply vacuum. ▲ Discard flow-through and place the QIAquick column back into the same tube.

9. To wash, add 0.75 ml Buffer PE to QIAquick column and ▲ centrifuge for 1 min or ● apply vacuum. ▲ Discard flow-through and place the QIAquick column back into the same tube.

   Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, blunt-ended ligation), let the column stand 2–5 min after addition of Buffer PE.

10. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min at 17,900 x g (13,000 rpm) to remove residual wash buffer.

11. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

12. To elute DNA, add 50 μl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 μl Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.

13. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.
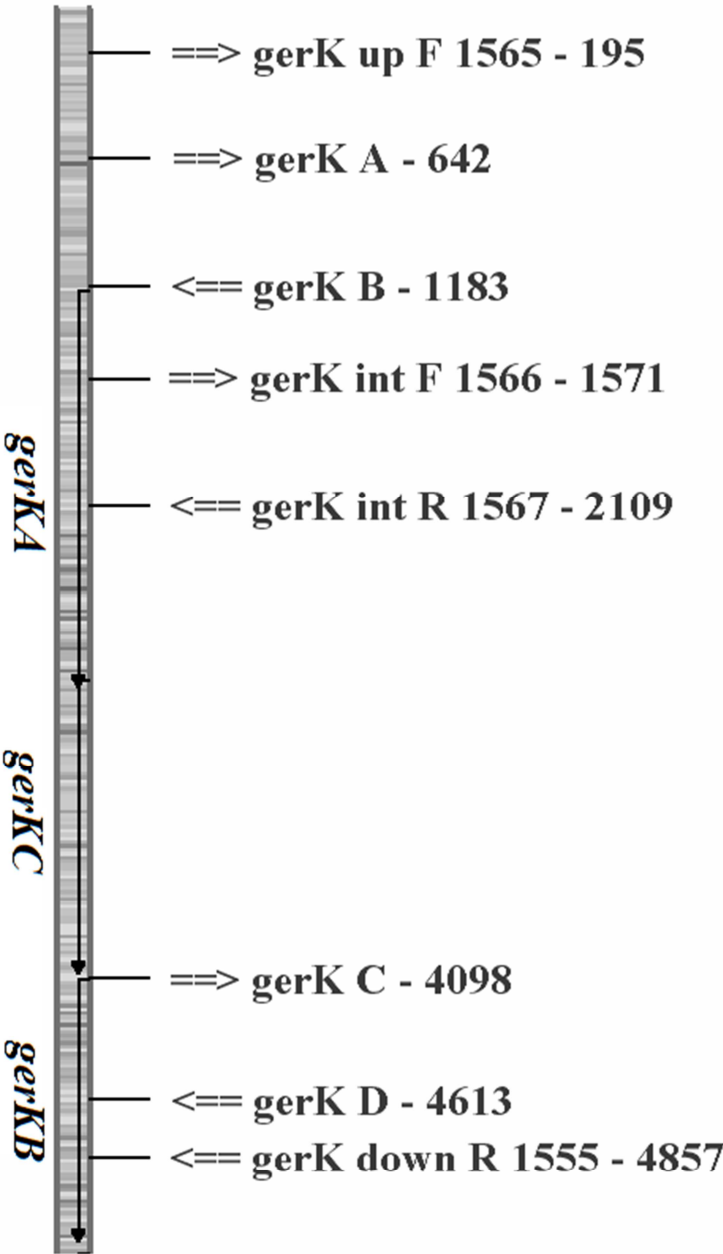
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Appendix IV – *B. licheniformis* MW3 genomic DNA segments and primer sites

DNA segments are shown as wild type, before gene deletion.

==> CwlJ up F 1359 - 1698

==> CwlJ A - 1950

<== CwlJ B - 2445
==> CwlJ int F 1342 - 2480

<== CwlJ int R 1341 - 2850
==> CwlJ C - 2904
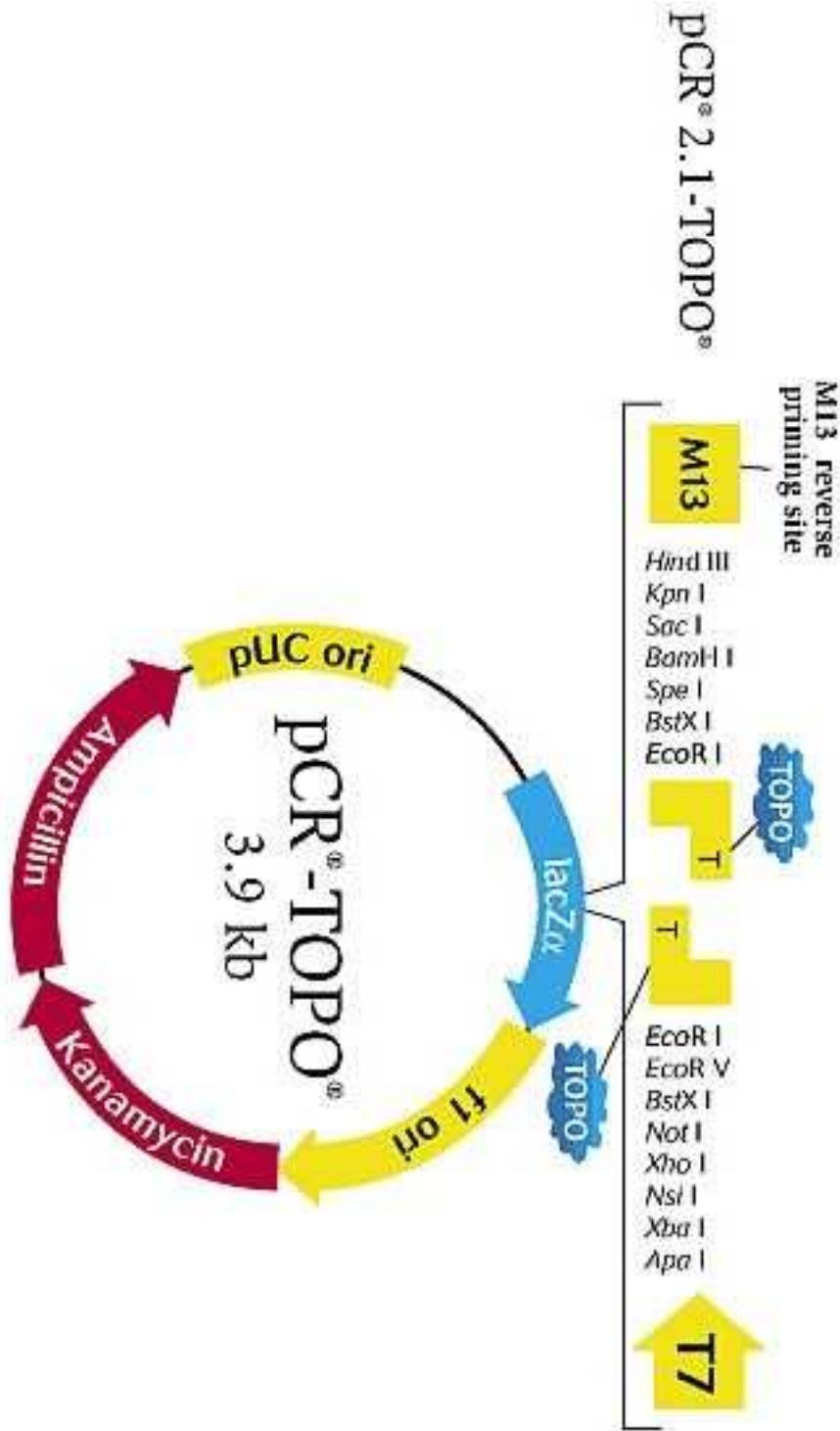
<== CwlJ down R 1585 - 3312

<== CwlJ D - 3644
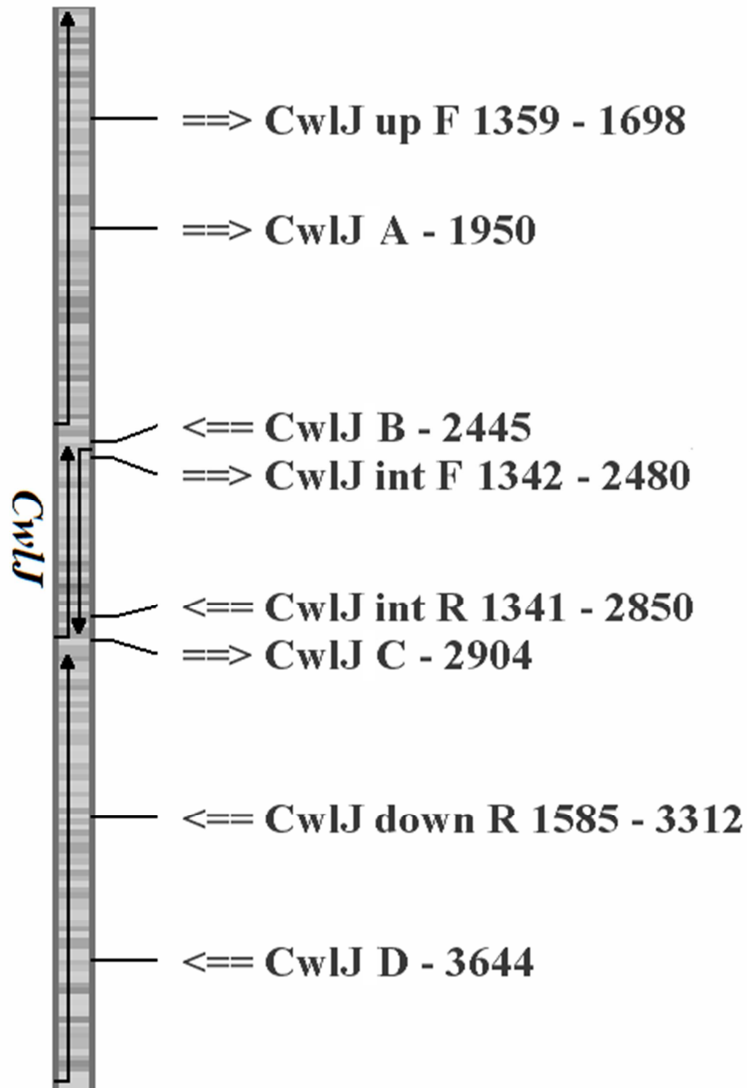
# Appendix V – Cloning vectors

pCR 2. 1 TOPO
Produced by Invitrogen

pMAD

The pMAD vector used in the construction of the deletion mutants in this thesis. The insert was placed in the EcoRI seat. The first crossover was made possible by the temperature sensitive G+ ori and the second crossover by the unique I-SceI site introduced in the pMAD vector by Dr. Anette Fagerlund, University of Oslo, Norway.
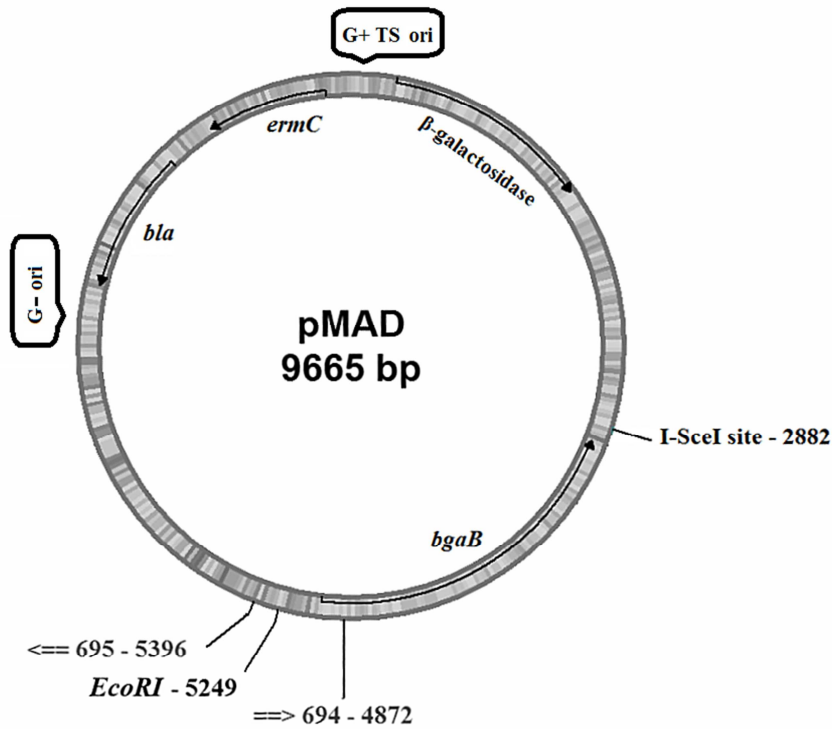
pBKJ223

As published in Routine markerless gene replacement in *Bacillus Anthracis* by Janes and Stibitz, Infect. Immun. 2006, 74(3)
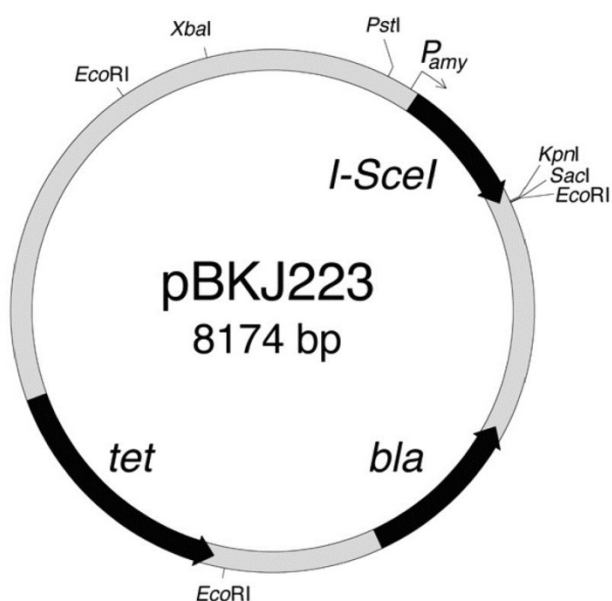
Appendix VI – Gene sequence from Source Bioscience

The highlighted areas in blue are the replacement sequences introduced by deletion of the target gene. The red highlight marks the base substitution that occurred in the Δ*CwlJ* mutant.

gerK

AAATTATATTGAAAACGACGTACCTGAACAAAGGTCCGGCACCGCTTTCAGCATCACGCAATCAGACAATGTAAC
ATGTTCCGGCAATCATATTGCAAATGTCGTTCTTGCTGTCAGGAGCACAGGGAACGACGTCCAAATCAAGCATAA
CGTCATTAAGCAATTTTCCAGAGGCATATGGATATCTCAAGGAAATGCTGTAATAGAAGGAAACTTTATTTCGCCG
AACGGCTTTCAAACGGTGCCGGAGTCATATGCTGTATCCGTCACAAACACAGCTTCGGCAGTTATTCAACAAAAC
ACCATCACACGCTTCAGAAACTTTCCAATATACTGCTCGACAAATCAGCATACAAAAATTATCGGAAACTATATTGA
AGATTCACCGCTGATCGTCACGGTGTTTCTCACCCGCGGAACACACGAGGTTATCGGCAATACGATAACGCTCAG
CAGGCCGTCCTTGCCGGCTGTCCTCATTTATCTTGACCAATCGTCGGACTCGGCGATCCTCAACAACACGCTTCGA
AGCAATACGGAAGCAAGAGTAACGGCCATTCAAACCAATACGTCAAGGCAAACGCTCATCATCGGCAATACGATT
GTCAAAGGAATGATCCATTCCCACCAAACAGATACAGTCAATGGAAATATCGAAGTTTAAGCTCCCTTTGGACAG
GGAGCCTTTTATGTCCTTTTTCTCTTTTTGCGAAAGCTTTCCGAGGCTTCGAGAAAAATGATAAACAAAAATAAAA
GGATGATGATCCACTCGCCCACTTCCAGGATGGCAAACGTCAGGGAGTTTACAATGGCTCTTTGCAAGGTCATGC
CTTGAAATACATCCAGCATGACGGCAAAAGCCTCTAAGGAAGCTAATAATAAAAGCCCGATTCCGATTAATTTCAA
CTTTGCACCACTCTCCGTTCCCCGTTATTTTTTCTCCTTTCATAAAGCACTATTCACCCGGGAATAGTATAAATGTGC
TTGCGCAAGGAAAAAT<mark style="background:cyan">ATGTGA</mark>ATGAAAGAGAGAGGAGGGCTTTAAATGGAGAAAGCAAAAATCAGTGCTGCA
CAGCTGTTCGTGCTTATTGTTCTGTTTGAACTCAGCAGCTCTCTGCTGATTTTTCCAGGACAATCTGCCGAACAGG
ACGCCTGGATCGCGATCTTGTTCGGAACAGTCGGAGGGCTGGCTTTGTTTATGATGCATCACTTTTTCTACCAGGT
GAATCCCGACGCCTCTCCCTATGAAACGCTGATGAATATTTTTGGAAAACCGGTTGGCTGGAGCATGACATTTATC
TACATTGTTTATTATTGTTACATTGCGGCAAGGGTTTTGCGCGATTTTGGAGAAATGCTGCTGACATCGGCTTATCC
GAACACGCCGATTATCTTCGCTAATGGATTGTTGATTGCAGTTTGTATTTTTACTGTGAGGAAGGGAATCGAGGTC
CTTGCCCGTTCAGGCGAGCTGTTGTTTGGCATCATGTTTTGGCTTGCTGTCACAGGCCTAATCCTGATAGTTTCGT
CAGGCTTGATCAAACCGAAAGAGCTTTTGCCCATTCTTGGAAACGGACCCGGTCCGATGTTTCATGCCGTGTTCA
CGCAAACGCTGTATTTTCCGTTTGGAGAAATTATCGCCTTTACATTGATCCTGCCGTATTTAAAGGAAAAGAAACT
AGCGAAAAAGCCGGGTTGTTGGGAATTGCCATCAGCGGCCTCATCCTGGCTTTTAATGTCGCATTGAACATCAG
CGTGCTGGCGTCGATCTCAATATGCGGTCGCGCTTCCCATT

CwlJ

GTTAGATTGCCTTTCCAAAAAAAGTTTTTAGCGCCGGGAATATGGCCGGCCCGATGATCGAGAGGCTCGGTTTCC
CCGGTGTATCTTGAAGGATCGCGCGCATCGATCAGAATGGAGCCTTCTCTGCCGATTCGCTTCAGTACTTCTTTGG
CATCGAGCAGTTCGTTTTCTCTGAGCGCCGGCTTAAATTCGCGCTCGCTAGGACGAGGATCCTCGGCGTTCACCT
CGTAGCCCGCTTTGATCCAGTTGGACAGTCCCCCATCCATGATATAGACGCTGTCATGCCCGAGATAGTGAAGCTG
CCACCAAAAGCGCGCTGCAAACATGCCGCCGCTGTCATCATAAACAACGACATCTGTTTGCCGGTCAATGCCGAT
TTTTCCAAGCTTCGCGGCCAGAAGGTCAGGGTCTTGAAGCGGGTGTCTGCCGCCGTGCTCTTCCGGGTTGCGCG
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GTATACCCATTCAGGGCTTTTTAATACTGACAATTCACGATCCCTCCGTTCCCCTTTGTTTTTTCTCGGGTTCTGATA
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AAAGGGATGTTTTTCGTTCAAAATAATTCTGCCAGTCTTTCGTAAGACTGCTTCTTATCTTCAAACCGGTAGACGTT

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CGATCACCATACGGCTGCGGTTTTGGGCAAGCTTCTTTTTATCGGCAGCTGTGTAAGCGGCCGATTTCGCCTCTTC
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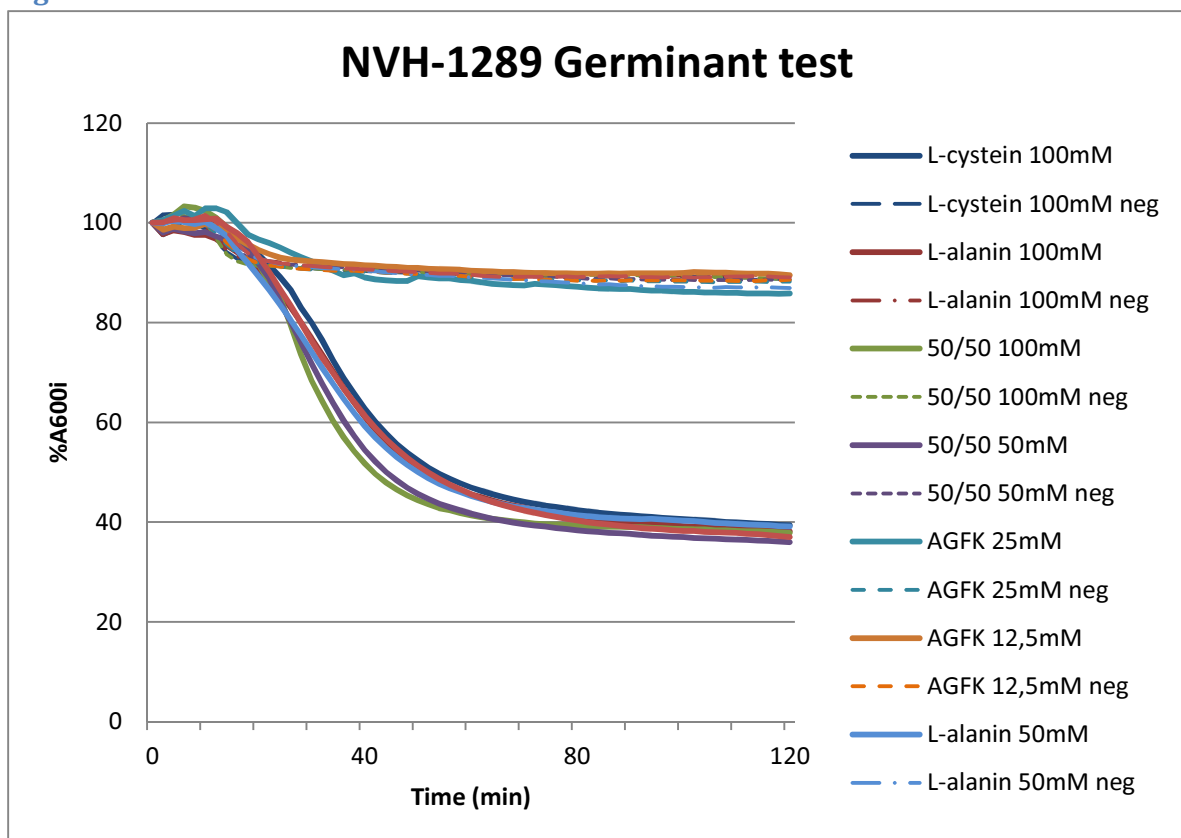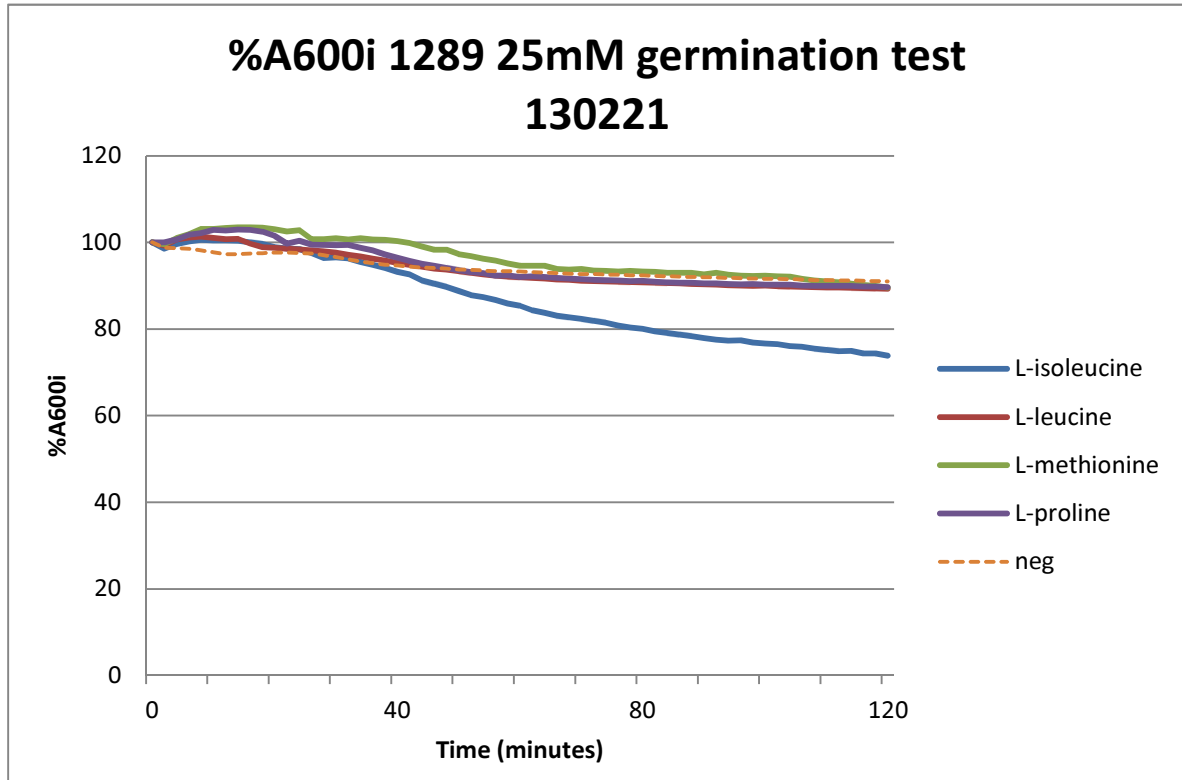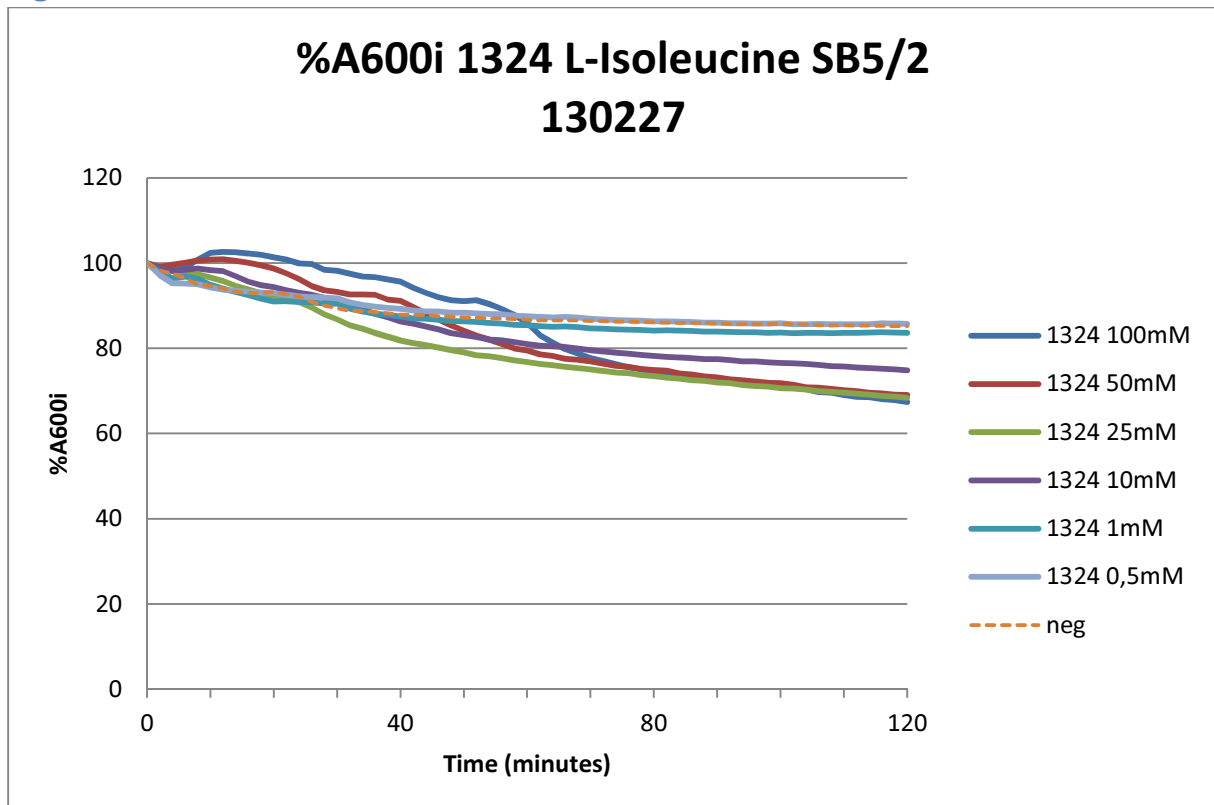## Appendix VII – Germination test curves

Figure A

Figure B

%A600i 1289 25mM germination test 130221

Figure C

%A600i 1324 L-Isoleucine SB5/2 130227

Figure D

%A600i 1324 inhibited germination with 25mM
L-isoleucine SB5/2
130320

Legend: Arginine/Isoleucine, Glutamic acid/Isoleucine, Histidine/Isoleucine, MQ/Isoleucine, neg

Figure E

%A600i 1289 25mm germinant SB17/4
130422

Legend: Neg, Arginine, Arg/Isoleu, Isoleu, Arg/Ala, Ala, Arg/Cys, Cys

%A600i 1289 25mm germinant SB17/4 130421