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The role of CwlJ and SleB in germination of *Bacillus licheniformis* spores

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Sammendrag

Sporer fra Gram-positive bakterier er et stort problem for helsesektoren og matindustrien. Sporene blir dannet inne i bakteriecellen når det er lite næringsstoffer i en prosess kalt sporulering. Sporene kan overleve svært tøffe omgivelser over en veldig lang periode. Når omgivelsene bedrer seg vil sporen germinere, dvs gjenoppta vegetativ vekst. *Bacillus licheniformis* er en slik sporedannende bakterie. Den forårsaker først og fremst forderving av mat, men kan gi matforgiftning. På grunn av problemer hos en matprodusent hvor *B.licheniformis* overlevde standard steriliseringmetoder, ble det satt i gang et prosjekt for å studere germinering i *B. licheniformis* ved Institutt for Mattrygghet og Infeksjonsbiologi, NMBU. En viktig fase i germinering er nedbrytning av et veldig tykt peptidoglykanlag i sporen, kalt cortex. Tidligere studier gjort på den nært beslektede *Bacillus subtilis* har vist at to enzymer er ansvarlige for nedbrytingen av cortex. Disse to enzymene er kalt CwlJ og SleB, og deres homologer i *B.licheniformis* vil bli studert i denne oppgaven.

Tre mutanter ble lagd for å undersøke CwlJs og SleBs rolle i *B. licheniformis*. Enkeltmutantene Δ*cwlJ* og Δ*sleB* ble lagd på et tidligere tidspunkt av forskningsgruppen, mens dobbelmutanten Δ*cwlJ*,Δ*sleB* ble konstruert fra Δ*cwlJ* i denne oppgaven . Det ble lagd sporer av alle mutantene og av villtypen. Tre forskjellige metoder ble brukt for å studere germinering i mutantene og villtype stammen. I to av metodene ble germinering indusert med L-alanine. I den tredje metoden ble germinering indusert ved eksternt tilsatt Ca^{2+}-Dipicolinic acid (CaDPA) som aktiverer germinering i *B.subtilis*.

Fullstendig germinering ble observert i begge enkeltmutantene (Δ*cwlJ* and Δ*sleB*) og i villtypen (MW3). Delvis germinering, men ingen utvekst ble observert i sporer av dobbeltmutanten (Δ*cwlJ*,Δ*sleB*). Disse observasjonene i samsvar med det man tidligere har sett i *B. subtilis*.

Abstract

Bacterial spores are a major issue for the health sector and the food industry. These spores are made inside a cell when nutrients become scares in a process called sporulation, and can survive very harsh conditions for a very long time. When conditions return to normal, they undergo germination where they return to vegetative growth.
Bacillus Licheniformis is one such spore forming bacterium. It causes food spoilage and may cause food poisoning. Because of reported problems with *B.licheniformis* in production of cooked ham where the bacteria survived sterilization methods that usually worked, a project studying germination in *B.licheniformis* was started at the Department of Food Safety and Infection Biology, NMBU.

An important part of the sporulation is the breakdown of a very thick modified peptidoglycan layer called the cortex. Studies done on the closely related species *Bacillus subtilis* have shown that there are two enzymes responsible for this breakdown of the cortex. These are CwlJ and SleB, and their homologues in *B. licheniformis* will be investigated in this thesis.
Three mutants were created to study the role of CwlJ and SleB. The single mutants Δ*cwlJ* and Δ*sleB* were made previously by the research group, but the double mutant Δ*cwlJ*,Δ*sleB*was made from Δ*cwlJ* in this thesis using a "markerless gene replacement" method.
Spores of the mutants and the wild type were made, and germination measured by three different assays. In two of the assays germination is induced by L-alanine. In the third assay, germination is induced by exogenous Ca^{2+}-Dipicolinic acid (CaDPA) which activates CwlJ in *B.subtilis.*

Successful germination was observed in both the single mutants (Δ*cwlJ* and Δ*sleB*) and the wild type (MW3), but only partly germination, and no out-growth was observed in the double mutant (Δ*cwlJ*,Δ*sleB*). These observations are in agreement to what is seen in *B.subtilis*.

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Table of content

1 Introduction

1.1 Spore forming bacteria and their implications

Spore forming bacteria are found among the gram positive phyla and vary a lot in their morphology and metabolic properties. The majority is found in soil and some are found in animals and plants. In soil there is an average of 10^6 spores/gram, and in human feces there is an average of 10^4 spores/gram (Hong*, et al.*, 2009). Of the spore forming bacterial species it is the genus Bacillus and Clostridium which is the best studied. This is because they are the ones that affect human health more than other spore forming bacteria. Both hospitals and the food industry have challenges in regards to *Bacillus* and *Clostridium* even though many can be harmless. A few are pathogenic though and perhaps most famous is *Bacillus Anthracis*. This bacterium is the culprit behind anthrax. A spore is the equivalent of a lifeboat in which the DNA of the bacterium can survive when the bacterial cell no longer can sustain itself. This lifeboat, or spore, can survive very harsh conditions for an extremely long time. Unlike a real lifeboat, the spore can become a new vegetative cell and continue normal cellular activity in a process called germination (Setlow, 2014).

Because spores can remain dormant for a long time, they can be used as a biological agent as they can be stored. In agriculture there has been a wide use of spores of *Bacillus thuringiensis* as a pesticide against a variety of insects such as moth larva. Spores are sprayed on the crops and subsequently eaten by insects. When entering the gut they start to grow and produce a toxin which kills the insect (Bravo*, et al.*, 2011). Since spores survives the stomach acid, they can be used as probiotics. Researchers are even testing if spores can be used in vaccination by applying them to the mucosal layers in the oral cavities, or in the gut. One goal could even be to use this as a vaccination against tuberculosis (Duc le*, et al.*, 2003, Reljic*, et al.*, 2013).

Another famous spore forming bacteria is *Clostridium botulinum* which produces the worlds' most potent toxin, the botulinum toxin. In medicine and the beauty business this is more

commonly known as botox. Spores of botulinum are present in the soil and can contaminate food and colonize the gut where it produces the botulinum toxin. Botulism results in temporal paralysis and can be lethal if untreated (Del Torre*, et al.*, 2004, Shukla & Sharma, 2005). *Bacillus cereus* is another spore forming bacteria that cause a milder and more common gastrointestinal disease through food. *B.cereus* infection causes diarrhea, and often nausea and vomiting. This is the classic food poisoning example and a great deal of the population will experience it once in their lifetime. The emetic toxin of *B.cereus* is produced when growing on food and can cause disease even though the bacteria dead. If this toxin is ingested in a very high amount, it can in worst case cause liver failure (Stenfors Arnesen*, et al.*, 2008). Other closely food borne related species of *B.cereus* that can cause food poisoning are *Bacillus Subtilis*, *Bacillus pumilus* and *Bacillus licheniformis (From, et al., 2007)*. *B. subtilis* is used as a model spore forming bacteria. Most of what know about sporulation and germination comes from the studies of this bacterium. Results from this thesis will therefore be compared with the results from similar experiments done on *B.subtilis.*

Contamination of food by spores is almost impossible to avoid. The problem is increased by the difference in growth condition the bacteria thrive in. So even though the conditions at a manufacturer such as temperature, is not optimal for growth, the bacteria can still survive as spores and contaminate the food.

1.2 *Bacillus licheniformis*

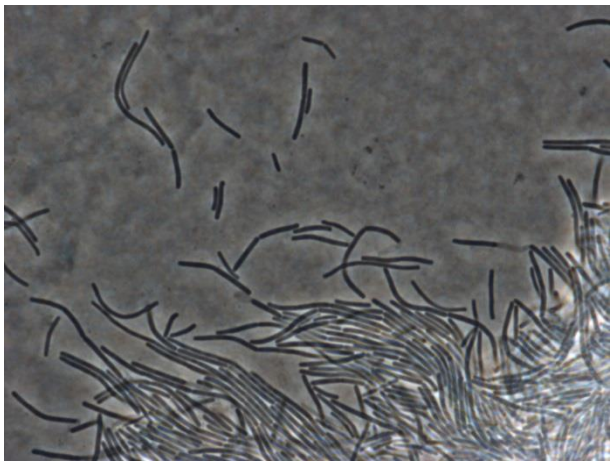


Figure 1: *Bacillus licheniformis*

B.licheniformis is a motile, Gram-positive, rod shaped, facultative anaerobe, endospore-forming bacterium (figure 1). It is commonly found in soil, and in feces of birds and mammals (Logan*, et al.*, 2009). *B.licheniformis* is closely related to *B.subtilis*. Both have a genome of 4.2 Mbp and on a nucleotide level they show 84.6 % similarity. However, *B.licheniformis* has over 100 more genes but fewer rRNA operons (7 in *B.licheniformis* and 10 in *B.subtilis*) (Veith*, et al.*, 2004). Optimum growth temperature of *B.licheniformis* is around 50 ˚C but it grows readily between 30 – 50 ˚C. The optimum temperature can vary between strains (Warth, 1978). Morphology of the colonies varies between strains and under different growth conditions. The most distinguishable appearance of B.licheniformis when grown on a petri dish are colonies with systems of what looks like fungal hyphae or lichens (figure 2). It adheres well to the plate and it possess β-hemolysis which makes clear haloes on blood agar (Logan*, et al.*, 2009). In addition it produces the antibiotic bacitracin (Ducluzeau*, et al.*, 1976). *B.licheniformis* and other *Bacillus* species are used in industry as producers of a variety of enzymes like proteases, penicillinase, α-amylase and many more (Schallmey*, et al.*, 2004).

Figure 2: A colony of *Bacillus licheniformis*

However, it is not wanted in the food industry. The bacterium spoils food and even though it is regarded as fairly safe to ingest, it may cause systemic infections and food poisoning (Santini*, et al.*, 1995). The symptoms are usually mild

gastrointestinal symptoms such as abdominal pain and diarrhea. Vomiting can also occur. The incubation time is 2-14 hours and last from 6-24 hours with an infective dose of $>10^7$ CFU g^{-1} (Granum, 2003). It has been speculated if it is responsible for many more cases of food poisoning then reported. This may be because of relatively mild symptoms and short duration of symptoms. People will thus be reluctant to seek medical attention and so it may be underreported.

However, there has been one death of an infant where *B.licheniformis* was isolated as the allegedly perpetrator (Salkinoja-Salonen*, et al.*, 1999). *B.licheniformis* produces a cytotoxin called lichenysin A. Cytotoxicity has been found at a concentration at 10 µg ml^{-1} (Madslien*, et al.*, 2013).

B.licheniformis is in theory a naturally competent bacterium. It has orthologous genes that are required for natural genetic competence in *B.subtilis* (Rachinger, *et al*., 2013). However, this competence is poor in many strains of *B.licheniformis*. It has been theorized that it is due to two genes, *hsd*RI and *hsd*R2, encoding a type 1 restriction enzyme (Schallmey*, et al.*, 2004). Waschkau and coworkers created a deletion mutant (Δ*hsdRI*,Δ*hsdR2*) which significantly increased the rate of transformation (Waschkau*, et al.*, 2008). This mutant was named MW3 and is used as a laboratory wild type strain in this study.

1.2.1 Background for this project and study aim

A manufacturer based in Norway wanted to produce canned meat. They applied a heat treatment procedure called Tyndallization (Tyndall, 1877). It was developed by John Tyndall in 1877 and involves activating spores by heating. A first heat treatment kills the vegetative microorganisms. As the heat drops, a temperature is reached where bacterial spores can germinate. After incubating for some time, the heat is once more raised and the germinated spores are killed (figure 3). This did not seem to work for the manufacturer, as the cans bloated

up because of gases produced by bacteria. They did of course never reach the marked.

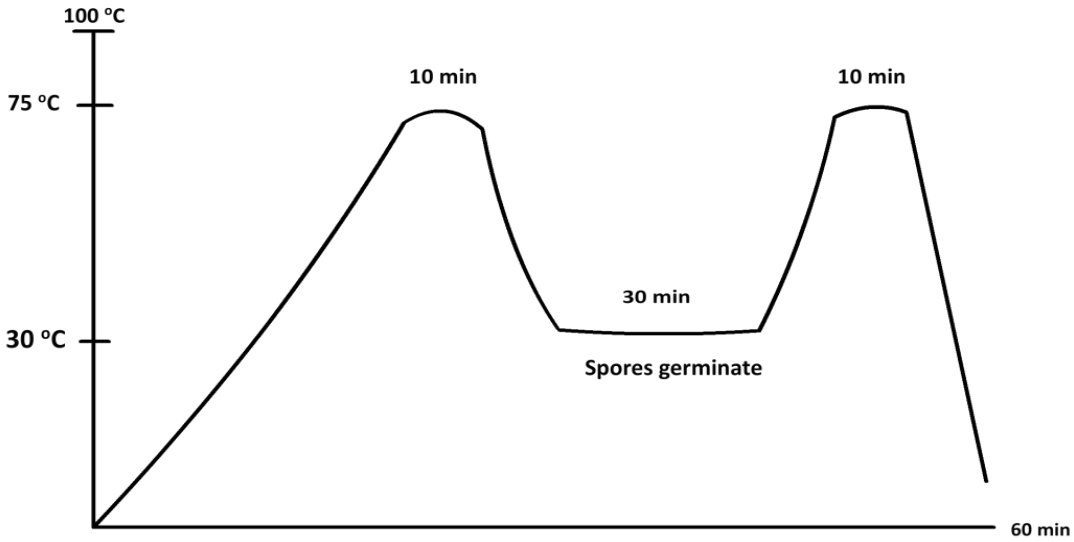


Figure 3: An example of Tyndallization. A product is heated to kill all vegetative cells. The product is cooled to a temperature where spores germinate. A second heating kills the germinated cells.

The isolated bacterium was a strain of *B.licheniformis*. Tests done at the "Department of Food Safety and Infection Biology" revealed that this strain was very slow to germinate (Lovdal*, et al.*, 2013). The solution was to wait longer before the second heat treatment. Given the difference in this germination behavior, it was seemingly necessary to map the germination mechanisms of *B.licheniformis* and get a better understanding of germination in general.

This thesis is part of this mapping project. The goal is to see if there are any differences in the cortex lytic enzymes CwlJ and SleB in *B.licheniformis* during germination compared to *B.subtilis*. This will be done by studying three mutants in which two single mutants, Δ*cwlJ* and Δ*sleB,* have already been constructed. A double mutant, Δ*cwlJ,*Δ*sleB,* will be constructed from one of these in this study. Spores of these three mutants plus the wild type MW3 will be made and used in three different germination assays. These assays will show implications each mutation have. In addition to the presentation of the results, the methods and results will be compared with *B.subtilis,* which has already been studied thoroughly.

1.3 The bacterial endospore

When nutrition becomes scares or the environment becomes unfavourable, cells can lyse, go into a low activity stasis or form spores. The latter is an option used by Gram-positive bacteria. Bacterial spores are extremely resilient to environmental extremes such as drought, heat, cold, and radiation (Nicholson*, et al.*, 2000, Setlow, 2006). They are known to last for thousands and even allegedly millions of years (Gest & Mandelstam, 1987, Cano & Borucki, 1995). The spore is made through an asymmetrical cell division which is then engulfed by the mother cell. Once the spore is engulfed it undergoes modifications that make it sustain the environmental challenges. The spore is built up by the core, inner membrane, core wall, cortex, outer membrane, spore coat, crust and exosporium (figure 4).

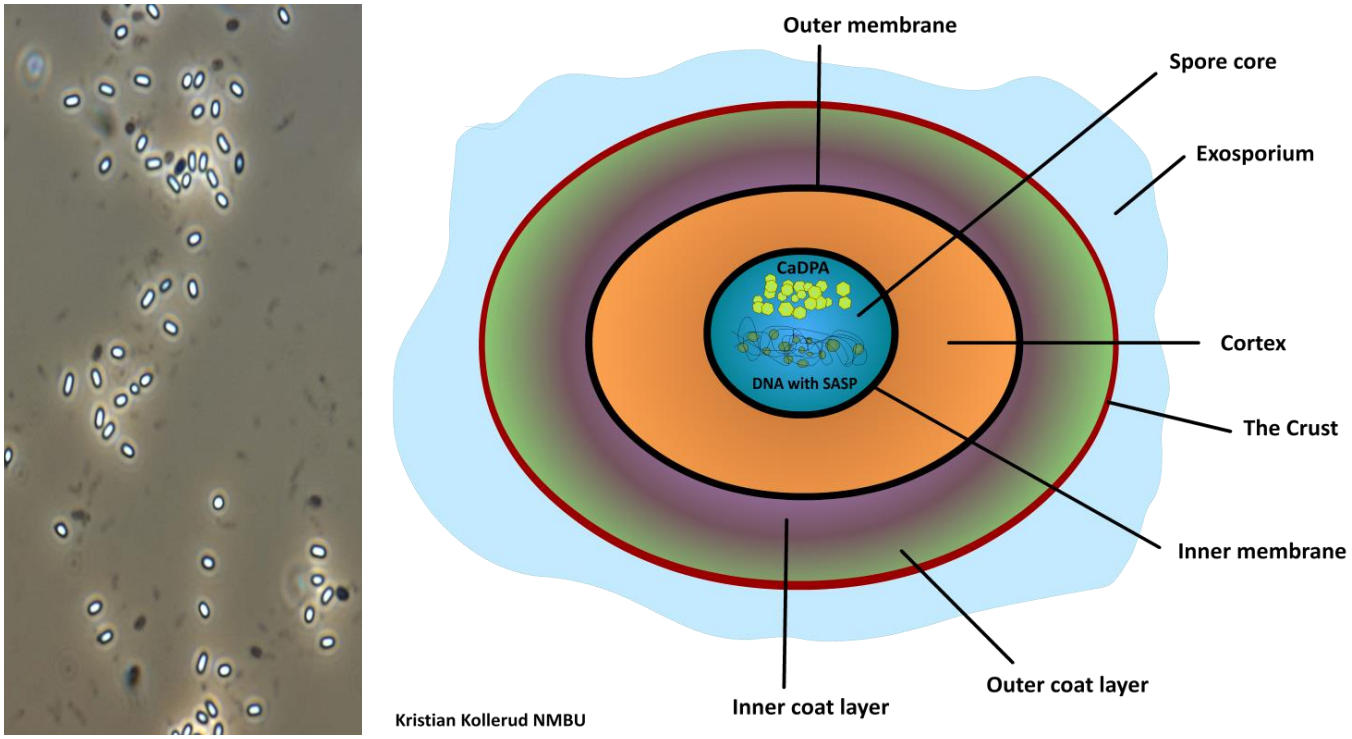


Figure 4: The spore. **Left**: Spores as seen in a phase microscope. The spores are phase bright due to the low water content. Dark spores are hydrated. **Right:** An illustration of the structure of the spore.

In the core you find DNA, small acid-soluble proteins (SASP), dipicolinic acid (DPA) and ions, mainly Ca^{2+}. DPA and Ca^{2+} make a polymer complex (figure 5) which reduces water availability within the spore. The amount can be as much as 25% of the spore dry weight. Under a microscope, the spores will look phase bright because of the dehydration and phase dark when they are hydrated. The low water content protects the DNA from heat damage (Setlow, 2006, Setlow, 2007) .

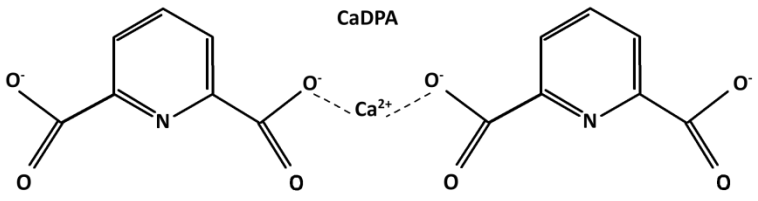


Figure 5: CaDPA polymer complex

The SASPs bind tightly to the DNA and change the molecular structure of DNA from B to A form. The A form is more compact then the B form. This makes it more resistant to formation of pyrimidine dimers caused by UV radiation (figure 6). In addition the SASPs contribute to protect the DNA from dry heat and dry freezing, and function as a carbon and nitrogen reserve for when the spore germinates (Setlow & Setlow, 1993, Fairhead*, et al.*, 1994, Pogliano*, et al.*, 1995, Setlow & Setlow, 1995, Setlow, 2006, Setlow, 2007).

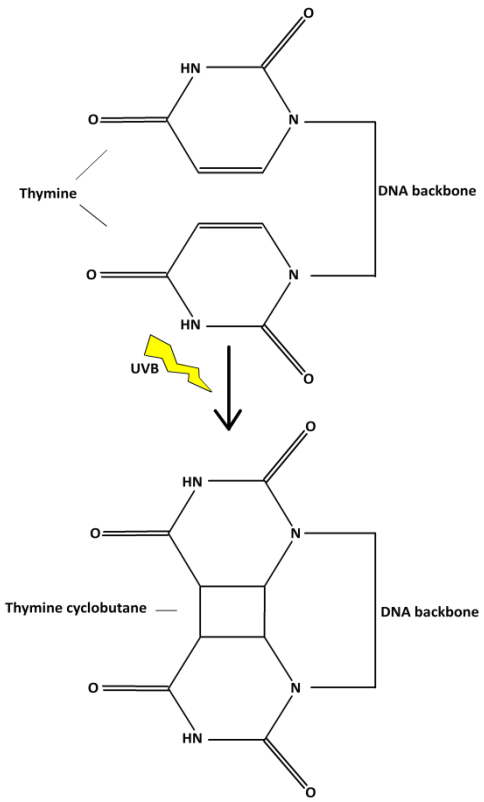


Figure 6: UVB damage. Thymine cyclobutane

Surrounding the core is the inner membrane, the core wall and the cortex. The core wall is a standard peptidoglycan layer but this is not the case for the cortex. This is a modified peptidoglycan layer with less peptide cross-links between the glycan strands, and a muramic-δ-lactam modified sugar. The muramic-δ-lactam functions as a specific marker for cortex lytic enzymes during germination (Popham, 2002, Leggett*, et al.*, 2012). The cortex is surrounded by the outer membrane and a

spore coat. The spore coat consists of a large number of proteins in different layers which protect the spore from various environmental factors. Spores lacking the spore coat can easily be rendered non-viable when exposed to salts, acids, enzymes and other chemicals that would otherwise not reach the cortex (Yi*, et al.*, 2011). It is possible that the spore coat is there to protect the spores from protozoan predator by surviving digestion (Klobutcher*, et al.*, 2006, Carroll*, et al.*, 2008). It also enables pathogenic spores to colonize the gut by surviving the acid barrier (Ceuppens*, et al.*, 2012). The number of spore coat layers can differ from species to species. In *B.subtilis* there is one basement layer, one inner coat and an outer coat layer (Imamura*, et al.*, 2010). Outside the outer layer there has recently been found to be a special protein layer called the crust. The crust is believed to be important in protecting the spore layers by creating a protein barrier (McKenney*, et al.*, 2010, McKenney*, et al.*, 2013). Outside the crust is the loose-sitting exosporium. This layer consists of a less dense layer of enzymes and protein homologues of the spore coat. The combination of these proteins varies from species to species and some do not even possess an exosporium (Redmond*, et al.*, 2004). In fact, the absence of an exosporium can even vary between strains within the same species (Hong*, et al.*, 2009).

1.4 Sporulation

The prosess in which a cell form a spore is called sporulation (figure 7).

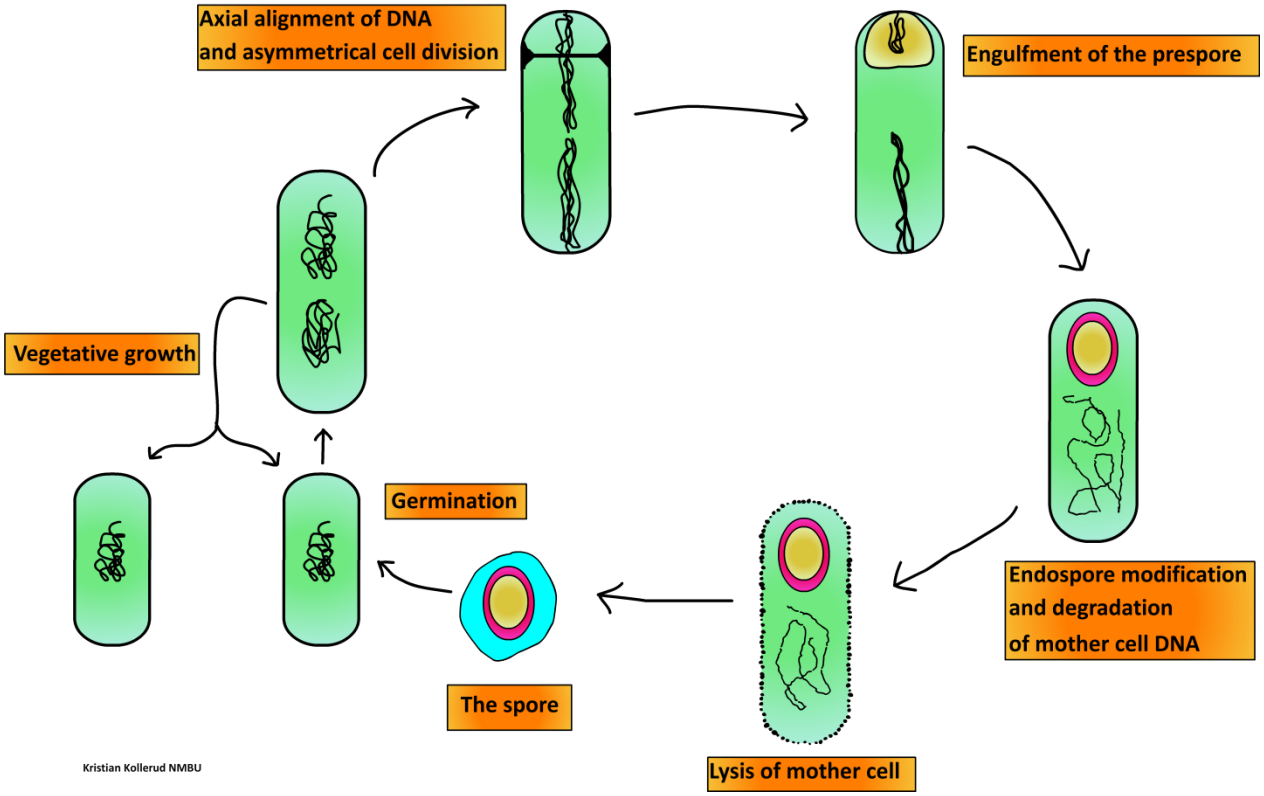


Figure 7: Sporulation. A cell replicates its genome and aligns it at the poles in an axial position. Asymmetrical division is initiated by assembly of the divisome and a septum is formed. Instead of cleavage of the septum, the newly formed compartment is engulfed and is now an endospore. It is now modified and the gnome of the mother cell is degraded. The spore breaks free as the mother cell lysis. When environmental conditions becomes favorable again, the spore germinates and return to vegetative growth

The sporulation can take up to 8-10 hours and is roughly divided into 6 stages/phases (Piggot & Coote, 1976, Piggot & Hilbert, 2004). The first stage is the initiation, which is regulated by signals deriving from cell density, nutrition, or cell cycle. The exact signals are not yet fully agreed upon (McKenney*, et al.*, 2013). These signals activate kinases which will drive a self-regulatory system consisting of Spo0F and Spo0B, and the transcription factor Spo0A. This system is called the phosphoreley (Figure 8) (Burbulys*, et al.*, 1991, de Jong*, et al.*, 2010). Dephosphorylation of Spo0F is done by Rap proteins. Rap is in turn inhibited by the uptake of

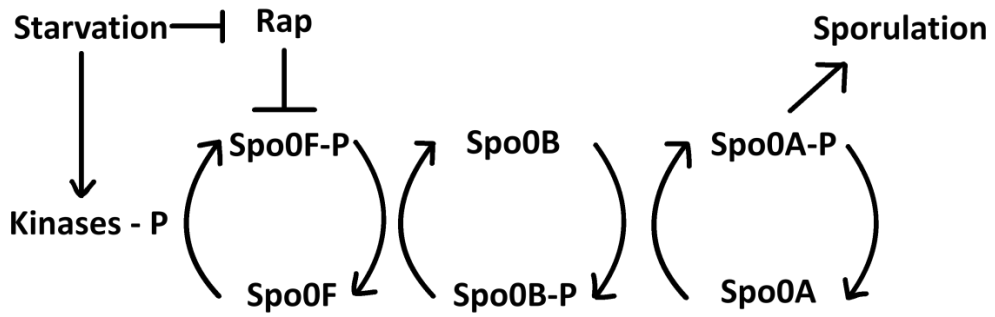


Figure 8: The phosphoreley

small polypeptides (signals for Gram-positive bacteria regarding quorum sensing) which increases with the cell density (Jiang*, et al.*, 1999). Nutrition activation is dependent on low GTP concentration, as high GTP levels activates CodY which in turn repress genes involved in the positive activation of the phosphoreley (Ratnayake-Lecamwasam*, et al.*, 2001).
Spo0A-P activates the transcription of *spoII*A, *spoII*E and *spoII*GA. This will in turn activate sigma factor σ^F and σ^E which in a cascade activates a total of over 500 genes (Hoch, 1993, Molle*, et al.*, 2003). Together with σ^H, Spo0A engages the cell into an asymmetric cell division (Sonenshein, 2000). The large compartment is called the mother cell, and the small compartment is called the prespore.

The chromosome is replicated, but RacA proteins will anchor the origins of the chromosomes to the end of the poles. This makes the chromosome lie in a stretched axial position (Ben-Yehuda*, et al.*, 2003, Hilbert & Piggot, 2004). Spo0A activated genes will also transcribe *fts*ZA (septum formation complex units). SpoIIE lead the FtsZA to the poles, and not to the center as one would see in a normal dividing cell (Ben-Yehuda & Losick, 2002). The alignment prevents the divisome from forming in the center of the cell and an asymmetrical septum is formed. SpoIIE also activates σ^F which is only expressed in the forming endospore (Arigoni*, et al.*, 1996). Even though the septum has fully formed, the chromosomes lie in the same axial positions. This means that the mother compartment have one whole chromosome plus two thirds of another.

The endospore only has only one third of a chromosome (figure 9) (Hilbert & Piggot, 2004).

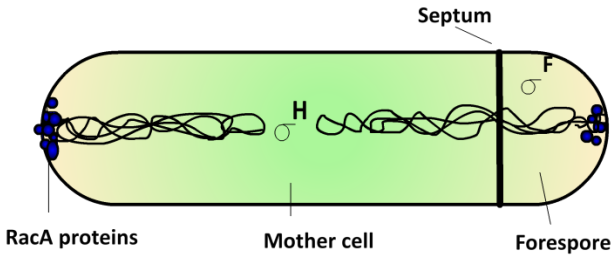


Figure 9: Axial chromosome alignment and septum formation. σ^H is active in the mother cell compartment while σ^F is active in the forespore

The septum is not cleaved as in normal cell division, but instead there is another septum formed as σ^E is activated in the mother compartment. This septum will engulf the prespore into and endospore now covered by two membranes. As this engulfment initiates, the peptidoglycan layer is partly degraded between the mother cell and the prespore. This happens from the middle and outwards and works as a septal thinner. The peptidoglycan layer between the mother compartment and the prespore is thick, because it is made as if the outcome would be two cells, ergo two cell walls (Illing & Errington, 1991, Londono-Vallejo*, et al.*, 1997, Abanes-De Mello*, et al.*, 2002). As the thinning of the peptidoglycan layer proceeds, the engulfment of the prespore begins (figure 10).

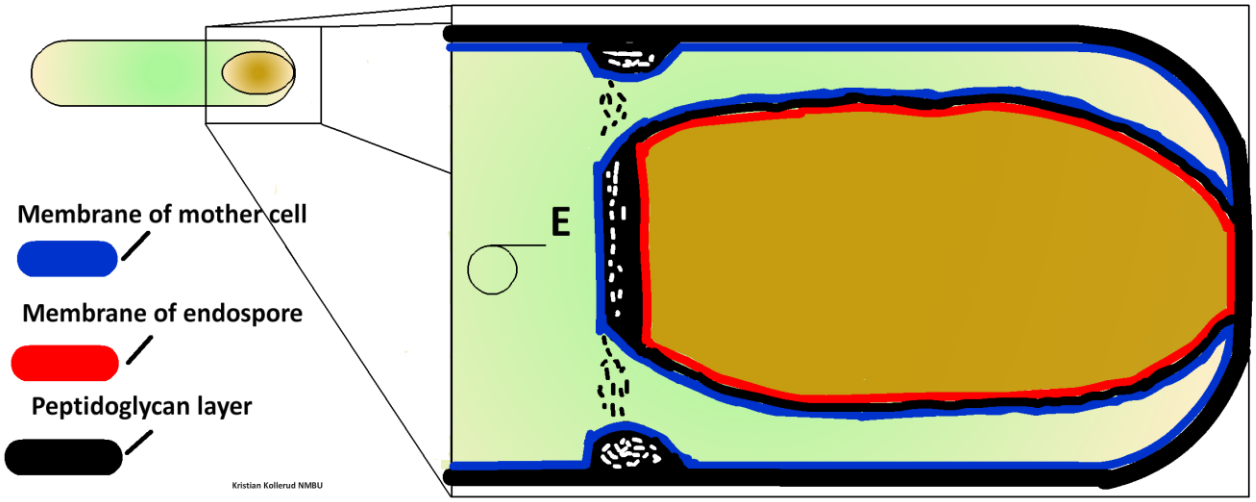


Figure 10: Engulfment of the prespore. The peptidoglycan layer of the septum begins to partly degrade (black dots and white dots). Two new septums are formed at each end of the old septum. They will move along the peripheral of the cell. The prespore is being packed in with the first membrane (red), then a peptidoglycan layer before a second membrane covers it (blue).

In addition, σ^E will transfer the remainder of the chromosome into the prespore. The end result is an endospore with its own chromosome, membrane, and two layers of peptidoglycan. One layer belongs to the mother cell, and one layer belongs to the newly formed endospore. On top of this is the membrane of the mother cell. The chromosome in the mother cell is now beginning to break down (Wu & Errington, 1994, Pogliano*, et al.*, 2002).

As the chromosome is transported into the endospore during the engulfment, new genetic material is available for the endospore. The genetic implications of this event are not yet understood (Hilbert & Piggot, 2004). s*po* genes are now transcribed which turn on σ^G. This sigma factor will modify the endospore so it can endure different types of environmental stress. It is also responsible for genes involved in germination such as germination receptors (Hilbert & Piggot, 2004). To protect the DNA from damage like UVB radiation and heat degeneration, the endospore synthesizes small acid-soluble proteins (SASP)(Pogliano*, et al.*, 1995, Setlow & Setlow, 1995). In addition, a very thick modified peptidoglycan layer with muramic-δ-lactam residues are formed called the cortex. The cortex is located outside the inner membrane and the germ cell wall before the outer membrane. Spo proteins are located in the outer membrane of the endospore which are cleaved and will activate σ^K in the mother cell compartment (Rudner & Losick, 2002, Dong & Cutting, 2003).The σ^K encode genes producing dipicolinic acid (DPA) and the transport system of DPA into the endospore. Ca^{2+} ions follow DPA to make the Ca^{2+}-DPA (CaDPA) complex and the core is dehydrated. The spore coat, which consists of several layers, is now assembled (McKenney*, et al.*, 2013).

The final step is the lysis of the mother cell. σ^K encodes the enzymes CwlC and CwlH whose activity is cell wall hydrolase of the peptidoglycan layer (Petersohn*, et al.*, 1999).

This is possibly why only Gram-positive bacteria would form spores. A Gram-negative bacterium would have 4 membranes, 2 peptidoglycan layers and 2 periplasms. The complexity of such a structure would give rise to so many obstacles that this has simply not evolved (that we know of).

1.5 Germination

When environmental factors become favorable again, the spore undergoes germination. This is the process in which the spore goes back into a vegetative state (Figure 11).

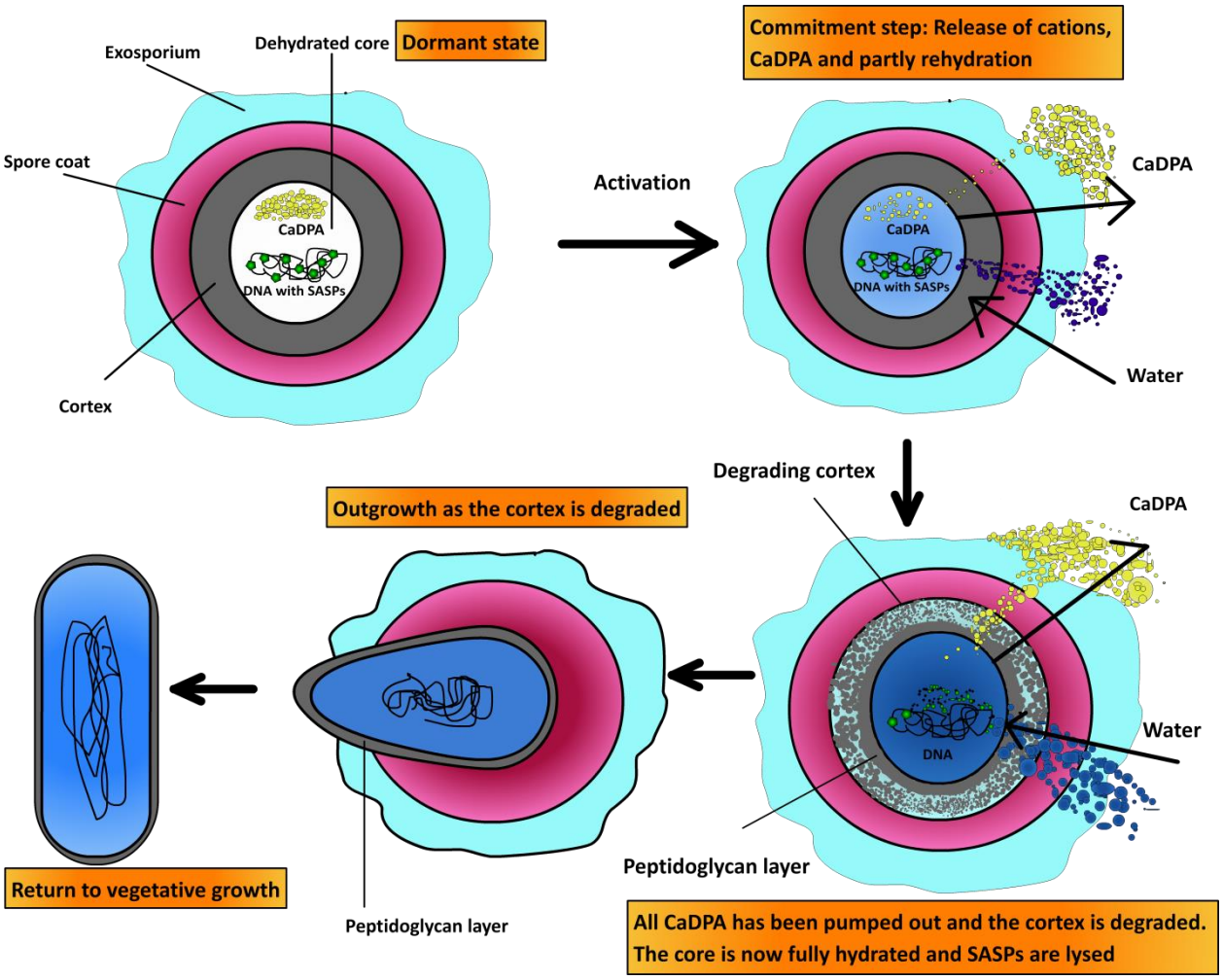


Figure 11: The germination process. A dormant spore exposed to a germinant commits to germination marked by an efflux of cations, CaDPA and some rehydration. The cortex will then start to lyse and the core and the rest of the CaDPA is released following a full hydration. The SASPs are degraded and the spore grows out of the cortex and return to vegetative growth.

Unlike sporulation which takes 8-10 hours, germination can take as little as 20 min from initiation. Activation of germination is related to *ger*A family receptors embedded in the membrane reacting to different nutrients mainly amino acids such as L-alanine (Ross & Abel-Santos, 2010, Lovdal*, et al.*, 2013, Setlow, 2014). However, the triggering nutrition, or germinant, varies a lot between species and subspecies and there are several ways to initiate

the germination (figure 12) (Paidhungat & Setlow, 2000). When a germinant reaches a species specific threshold, the spore irreversibly commits to germination even if the germinant is subsequently removed (Yi & Setlow, 2010). The commitment is characterized by increased permeability of the membrane resulting in an efflux of cations (H^+, Na^+, K^+), CaDPA and amino acids, leading to partial rehydration of the core (Setlow, 2003, Setlow, 2014). After initial commitment, the breakdown of the muramic-δ-lactam peptidoglycan layer begins where the cortex lytic enzymes SleB and CwlJ play redundant roles. This has been observed in *B.subtilis* (Ishikawa*, et al.*, 1998). CwlJ can be activated by exogenous CaDPA and thus function as a non-nutrient germinant (Setlow, 2003, Magge*, et al.*, 2008). As the cortex lyse the spore releases the remaining CaDPA and the spore becomes completely rehydrated. The final step is the degradation of the SASPs surrounding the DNA and the return of genetic and metabolic activity. Protease degradation of the SASPs yields amino acids to fuel initial synthesis of new proteins, but supplement nutrition is needed for further vegetative outgrowth. (Foster & Johnstone, 1990, Magge*, et al.*, 2008, Setlow, 2014).

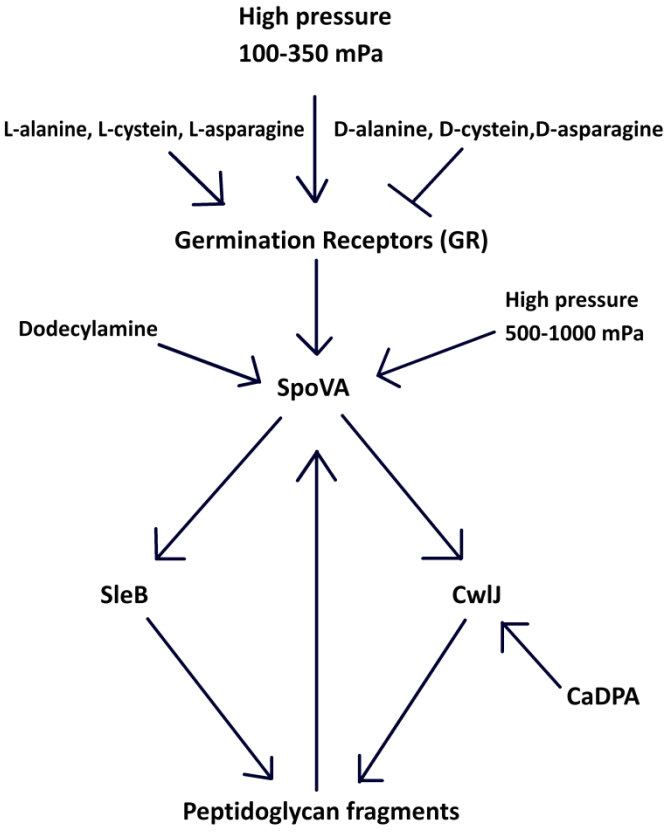


Figure 12: Possible germination flow chart in *B.subtilis*. The flow chart is currently being tested in *B.licheniformis.*

1.6 The cortex lytic enzymes

1.6.1 SleB

sleB is transcribed by the σ^G factor in the forespore compartment, and the protein is transported across the inner membrane in the cortex just next to the spore's coat where it lies dormant until germination is triggered (Moriyama*, et al.*, 1999). Being a lytic transglycosylase (amidase), it breaks down the muramic-δ-lactam into large pieces by cutting the peptide link. These pieces are further broken down by some other minor enzymes that are non-essential for germination (Boland*, et al.*, 2000, Atrih & Foster, 2001, Chirakkal*, et al.*, 2002). Its exact triggering mechanism is not yet clear, but its activation is post-initial to CaDPA-release from the core. However, the location of SleB in the outer part of the cortex do suggests that the signaling could come from the outside rather than the inside of the spore core (Yi & Setlow, 2010).

1.6.2 CwlJ

Unlike *sleB*, *cwlJ* is transcribed by the σ^E factor in the mother compartment, the site of spore coat synthesis (Ishikawa*, et al.*, 1998). The enzyme is therefore located in the spore's coat but, exactly how the enzyme can act on the cortex from the coat is not yet fully understood. Since CwlJ does not contain any transmembrane regions it has been suggested that it is located in the spore's inner coat, close to the cortex (Bagyan & Setlow, 2002). In *B.cereus* it has also been shown that the assembly and localization of CwlJ into the spore's coat is dependent on YwdL. Δ*ywdL* mutants do not show CwlJ in the spore's coat of *B.cereus* (Ragkousi*, et al.*, 2003, Terry*, et al.*, 2011). The exact catalytic specificity of CwlJ on the breakdown of muramic-δ-lactam is still unclear, but it has the same catalytic glutamate residue in the active site as SleB (Jing*, et al.*, 2012, Li*, et al.*, 2012). Even though both SleB and CwlJ show redundancy in enzymatic activity of the cortex, CwlJ is triggered by CaDPA. It is suggested that the enzyme is activated when the spore commits to germination and an efflux of K^+, H^+, Na^{2+} and CaDPA takes place (Bagyan & Setlow, 2002). Because of CwlJ location in the spore's coat, it is exposed to external signaling and it can thus be activated by exogenous CaDPA. (Zhang*, et al.*, 2012). It is therefore not unthinkable that this mechanism can work as a quorum sensing when germination is favorable.

2 Materials and Methods

2.1 Media, buffers, solutions, strains and primers

2.1.1 Media

The medium chosen in each experiment were meant to give the bacteria the best possible growth and sporulation condition. The media that were made was not stored more than two weeks at the most, with the exception of Bacto-MS and S.O.C. These were freshly made before each use. BHI was the preferred medium after transformation as it is very nutritious, but due to its dark colour BHI-agar was not suitable when screening for blue and white colonies. LB medium was thus the preferred medium for making agar plates. All media was autoclaved and cooled to room temperature before use.

Luria Bertini (LB) medium 1000 ml:

- 10 g Tryptone (Oxoid)
- 10 g NaCl (Merck)
- 5 g Yeast Extract (Oxoid)
- 1000 ml distilled H_2O

Bacto™ Brain- Heart infusion (BHI) medium 1000 ml:

- 37 g Bacto Brain- Heart infusion (BD)
- 1000 ml distilled H_2O

Bacto-MS sporulation medium (van der Voort*, et al.*, 2010):

- Difco™ Nutrient Broth (8 g/L, BD)
- 2.5 µM $CuCl_2$ (Sigma)
- 12.5 µM $ZnCl_2$ (Sigma)
- 1 mM $MgCl_2$ (Merck)

- 5 mM $(NH_4)_2SO_4$ (Merck)
- 2.5 µM Na_2MoO_4 (Sigma-Aldrich)
- 2.5 µM $CoCl_2$ (Sigma-Aldrich)

The solutions were kept refrigerated when stored as stocks over time. The following solutions could not handle autoclavation, and had to be sterile filtrated using a 0.2 µm filter (Minisart Sartorius) and added after autoclavation.

- 1 µM $FeSO_4$ (Merck)
- 66 µM $MnSO_4$ (Merck)
- 1 mM $Ca(NO_3)_2$ (Merck)

S.O.C Medium:

- 2 % Tryptone (Oxoid)
- 0.5 % yeast extract (Oxoid)
- 10 mM NaCl (Merck)
- 2.5 mM KCl (Merck)
- 10 mM $MgCl_2$ (J.T Backer)
- 10 mM $MgSO_4$ (Merck)
- 20 mM glucose (Sigma-Aldrich)

The medium should be made fresh, but was stored at - 20 ˚C in batches of 500 µl for later use in electroporation of cells.

Agar:

- 1.5 % (w/v) Bacteriological Agar No 1. (Oxoid), for both LB and BHI.

Solidify around 40 °C.

2.1.2 Buffers and solutions

SET (Salt EDTA Tris) (Pospiech & Neumann, 1995):

- 75 mM NaCl (Merck)

- 25 mM EDTA (Ethylenediaminetetraacetic acid) (pH 8.0) (Sigma-Aldrich)

- 20 mM Tris (pH 7.5) (Sigma-Aldrich)

2.1.3 List of strains and plasmids

Table 1: Bacterial strain used in germination assays, and for creation of the double mutant.

Strain	Genotype	Source
NVH-MW3	*B. licheniformis ATCC14580/DSM13ΔhsdR1ΔhsdR2*	(Waschkau, *et al.*, 2008)
NVH-1331	*B. licheniformis* MW3Δ*sleB*	Tina O'Sullivan
NVH-1333	*B. licheniformis* MW3Δ*cwlJ*	Shani Kidd
NVH-1341	*B. licheniformis* MW3Δ*sleB*Δ*cwlJ*	This project

Table 2: Genetic tools used for constructing the desired mutant

Plasmid	Description	Source
pCR 2.1 TOPO	PCR product cloning vector	Invitrogen
pMAD-I-*Sce*I	Shuttle vector with I-*Sce*I restriction site. *E. coli* was used as replication host.	(Arnaud, *et al.*, 2004)/Dr. Annette Fagerlund, University of Oslo.
pBKJ223	Plasmid producing I-*Sce*I restriction enzyme. *E. coli* was used as replication host.	(Janes & Stibitz, 2006)

Table 3: Primers used in this thesis. SleB-B and SleB-C have an overhang which is complimentary to each other. The ATG start codon in blue is marking the beginning of the overlap. The rest of the complimentary overlap is marked in red.

Primer name	Identification number	Sequence 5'-3'
SleB-A	1574	GAGGGCAGTTTTGTCGTTTC
SleB-B	1575	**TCGCTTTTA**CATGTTTTAAGCCTCCT
SleB-C	1576	TTAAAAC**ATG****TAAAAGCGA**GGTGCTA
SleB-D	1577	AAATGGTCGTACCCTTGCTG
SleB ext. fwd	1582	TTGACGCATCGTTTAAGCTG
SleB ext. rev	1583	AGCTATGACCTCGCGAAAAA
pMad fwd	694	CCATCAGACGGTTCGATCTT
pMad rev	695	GTCCCTGATGGTCGTCATCT
CwlJ fwd	1359	CGGTTAGATTGCCTTTCCAA
CwlJ rev	1585	AACGCCTTTCAATCATACCG
SleB int. fwd	1568	GGCCGAAAACAAAACGATTA
SleB int. rev	1569	ATTCGGGAACGTAGGGCTAT

2.2 Creation of deletion mutant Δ*cwlj* Δ*sleB*

2.2.0 Technique: Markerless gene replacement

This technique involves replacing a gene without the use of a cassette containing antibiotics. One vector and a plasmid are involved in the process. The pMAD is a temperature sensitive vector created by Arnaud and coworkers (Arnaud*, et al.*, 2004) (figure 13). This has then been modified with an insertion of an 18 bp restriction site called I-*Sce*I (a kind gift from Annette Fagerlund). It will not be copied above 37 ˚C, but keeps a wild type copy number below 32 ˚C. The vector is transformed into the target bacterium and integrated into the genome by homologue recombination. When grown with erythromycin at 37 ˚C, cells that divide without the vector integrated in the genome will not survive since it does not replicate freely in the cytocol.

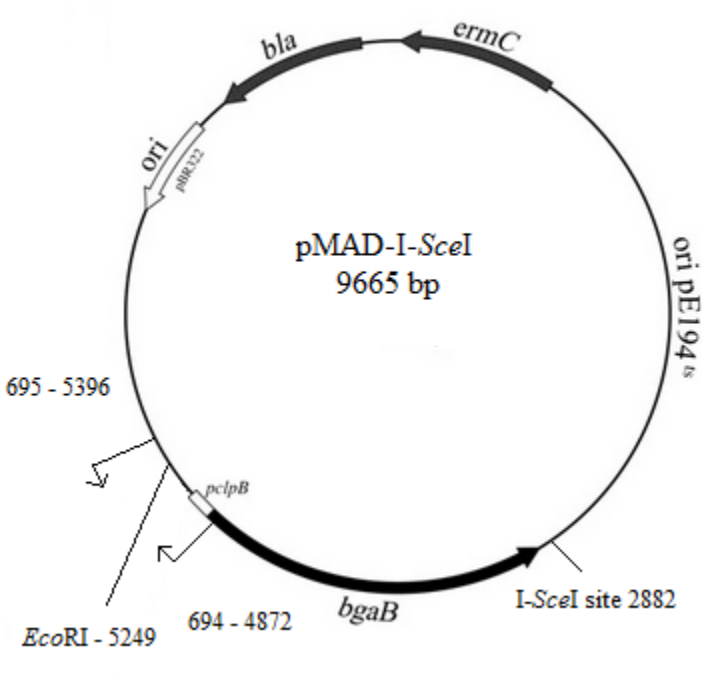


Figure 13: pMAD(Arnaud*, et al.*, 2004) with I-*Sce*I site introduced by Dr.Annette Fagerlund. Also shown is primer pair 695-694.

The plasmid used in this technique is the pBKJ223 (figure 14) (Janes & Stibitz, 2006). This was originally used in *B.anthracis* but was successfully applied to *B.cereus* (Lindback*, et al.*, 2012). The plasmid contains *I-SceI,* which encodes the enzymes that cuts the I-*Sce*I restriction site on the pMAD-I-*Sce*I. The principals of the markerless gene replacement methods are described in details in figure 15.

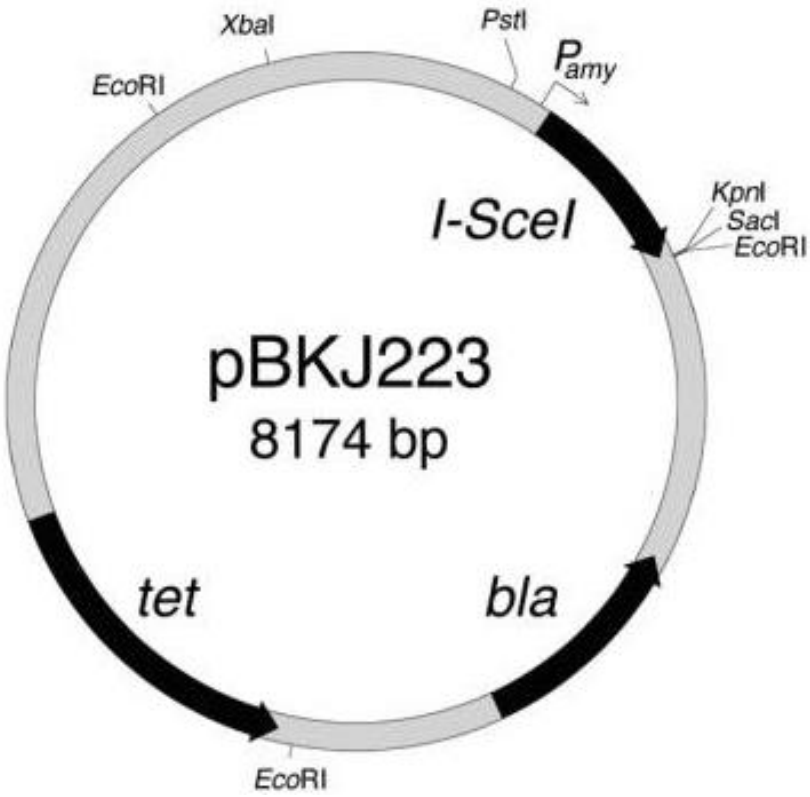
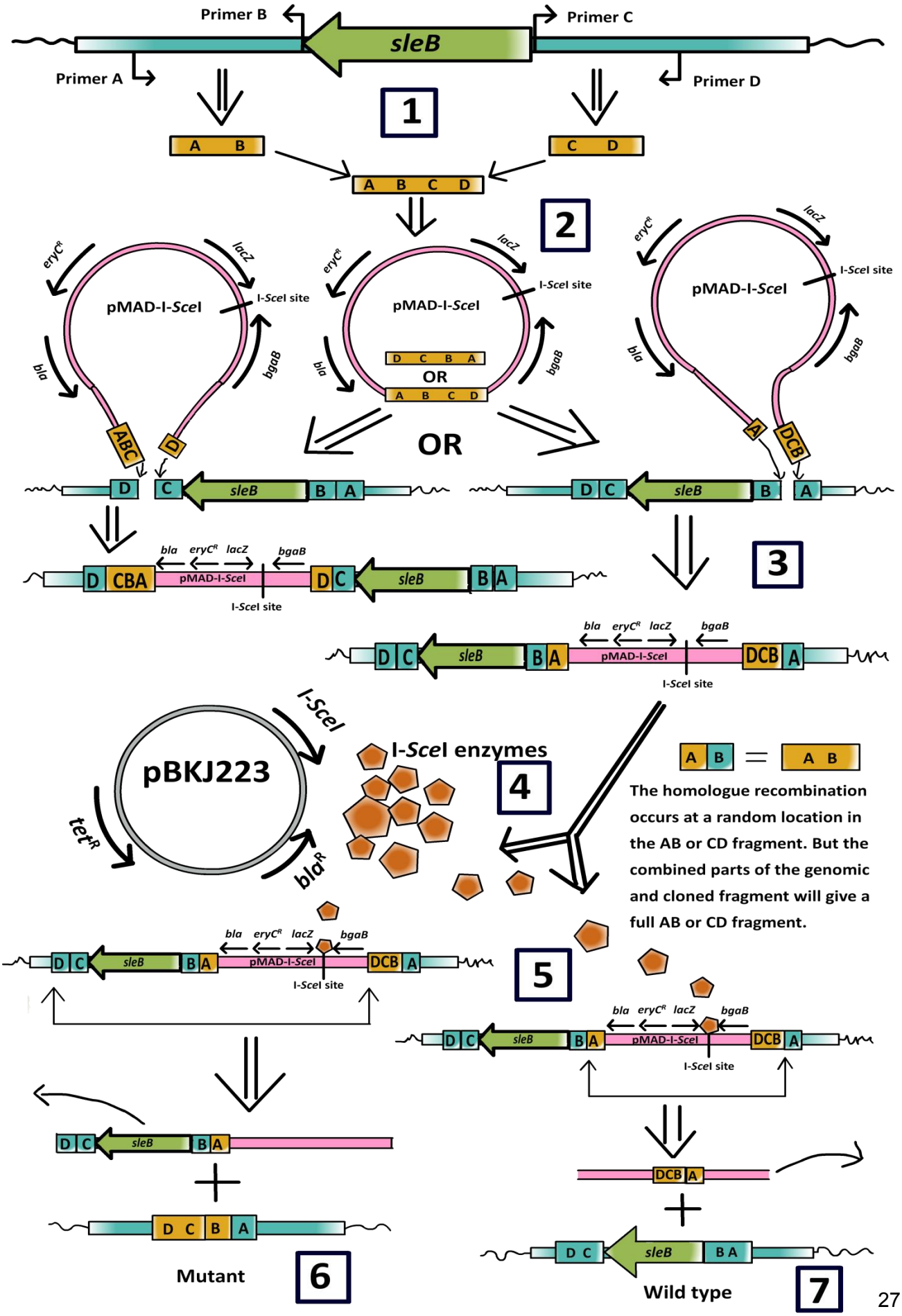


Figure 14: pBKJ223 as shown in (Janes & Stibitz, 2006).

Figure 15: Markerless gene replacement. 1 The flanking regions of the target gene is amplified and spliced together by high fidelity PCR, yielding gene fragment ABCD. Sticky end extension needed for cloning is made by treating the product with Taq polymerase (see Figure 16 p 30 for further details). The ABCD gene fragment is ligated into a pCR 2.1 TOPO vector (not shown) for amplification and digested with *Eco*RI to be cut out with overhangs (not shown). 2 The gene fragment is ligated into an *Eco*RI treated pMad-I-*Sce*I containing the I-*Sce*I restriction site. 3 The plasmid is transformed into cells by electroporation. It is then integrated into the chromosome by single crossover upstream or downstream of the target gene. 4 The vector pBKJ223, containing the I-*Sce*I restriction enzyme gene, is introduced by electroporation 5 and the enzyme will cut at the target I-SceI restriction site. This will lead to a double crossover leading to two possible scenarios depending on where the crossover takes place. In the scenario on the left, the recombination is done on the other side of the gene, and both the pMAD-I-*Sce*I and the target gene is removed 6. In scenario on the right, the recombination is done on the pMad-I-*Sce*I side of the target gene and only the pMAD-I-*Sce*I is removed 7.


Primer B
Primer C
sleB
Primer A
Primer D
1
A B
C D
A B C D
2
eryC^R
lacZ
I-SceI site
pMAD-I-SceI
bla
bgaB
D C B A
OR
A B C D
OR
ABC
D
A
DCB
D C sleB B A
bla eryC^R lacZ bgaB
D CBA pMAD-I-SceI D C sleB B A
I-SceI site
3
D C sleB B A pMAD-I-SceI DCB A
I-SceI site
I-SceI
pBKJ223
tet^R
bla^R
I-SceI enzymes
4
A B = A B
The homologue recombination occurs at a random location in the AB or CD fragment. But the combined parts of the genomic and cloned fragment will give a full AB or CD fragment.
5
6
Mutant
7
Wild type

2.2.1 General experiment methods

For each PCR done in this experiment, the following reaction was set up unless otherwise stated:

- 5µl 10x Buffer (15 mM $MgCl_2$)
- 1 µl dNTP (10 mM)
- 1 µl Forward primer (10 mM)
- 1 µl Revers primer (10 mM)
- 1 µl Taq polymerase
- 1 µl Template DNA
- 40 µl MQ

- Total: 50 µl

Step 1: 95 ˚C 4 min

Step 2: 95 ˚C 1 min

Step 3: 55 ˚C 1 min

Step 4: 72 ˚C 1 min

Step 5: 72 ˚C 5 min

Step 6: 4 ˚C ∞

Step 2 – 4 was repeated 30 times.

- 5 min at 72˚C as a final step at the very end to ensure complete elongation of all DNA strands.

Incubation of cultures were done at 37 ˚C and with shaking at 200 rpm (liquid medium) unless stated otherwise.

Each step in the creation of the double mutation was checked by gel electrophoresis with 1% Seakem Agarose at 115V for 40 min and viewed with a Gel Logic 200 imaging system (Kodak).

Cells that were electroporated was grown on BHI agar plates as it is more nutritious and yields more colonies. LB agar plates were otherwise used for better white blue screening. X–galactose in the experiment was suspended in dimethylformamide to a concentration of 40 mg/ml.

For every genetic extraction, Nanodrop was used to determine relative purity and ng/µl. When the amount of genetic material was not sufficient, a protocol from "Molecular Cloning: A laboratory manual 3rd edition (Sambrook and Russell)" was used to increase the concentration:

- 0,3 M NaAcetat (May & Baker)
- x 3 times the volume 100% ethanol.

Mix well and incubate at -20 ˚C for 30-60 min. Centrifuge at max speed on a table top centrifuge (13000 rpm or more) for 15 min, and siphon off the supernatant. Carefully add 150 µl 70% ethanol or more to wash the pellet. Repeat the centrifugation step and siphon off the supernatant and let the pellet dry completely. Resuspend the genetic pellet in the wanted amount of water.

2.2.2 Extraction of Genomic DNA

The extraction of genomic DNA method was based upon the protocol "A versatile quick-prep of genomic DNA from Gram-positive bacteria"(Pospiech & Neumann, 1995). *B. Licheniformis* MW3 freeze stock (- 80 ˚C) was inoculated in BHI-medium and incubated overnight. 3 ml was spun down for 10 min at 5600 rpm and the supernatant was carefully removed. The pellet was then resuspended in 495 µl SET buffer and added a lysozyme concentration of 1mg/ml (50 µl) and incubated at 37˚C for 1 hour. Afterwards a 1/10 volume of 10 % SDS (50 µl) and 0.5 mg/ml proteinase K (5 µl) was added and gently mixed before incubation at 55 ˚C for 2 hours with occasional inversion.

200 µl of a 5 M stock (final volume of 0,2M), and 800 µl of "chloroform:isoamyl alcohol" at a ratio of 24:1 was added and incubated at room temperature for 30 min with frequent inversion.

The solution was centrifuged at 13.000 rpm for 30 min in a table centrifuge, and the resulting aqueous solution was carefully transferred to a new Eppendorf tube. An equal volume of isopropanol was added and gently inverted so the DNA would properly precipitate, before a centrifugation at 13.000 rpm for 15 min was done. The supernatant was carefully removed and the precipitated DNA was washed with 70 % ethanol and centrifuged at 13.000 rpm for 10 min. After the ethanol was removed and the pellet had been dried up, the DNA was resuspended in 50 µl distilled water. The concentration of DNA in the solution was measured with Nanodrop.

2.2.3 Creation of DNA fragment Δs*leB*

The fragments of interest are located at the flanking gene regions of *sleB,* and primers designed by Kristin O' Sullivan were used (table 3, section 2.1.4) to amplify and splice (Figure 16 and 17)

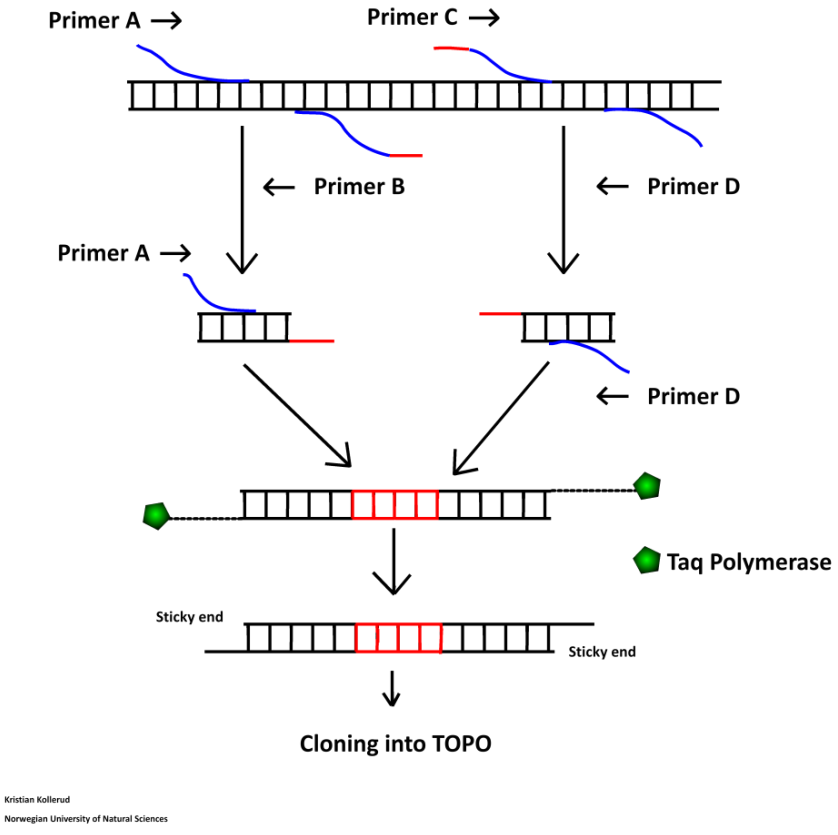


Figure 16: The principle of gene splicing by high fidelity polymerase PCR. Primer pair A-B and C-D will each yield a PCR product. Primer B and C have an extension at the end, that is complimentary to each other (marked as red).A second PCR with primer pair A-D will splice the two PCR products. A final treatment of Taq polymerase produces sticky ends to the PCR product and makes it ready to be cloned into a pCR 2.1 TOPO vector.

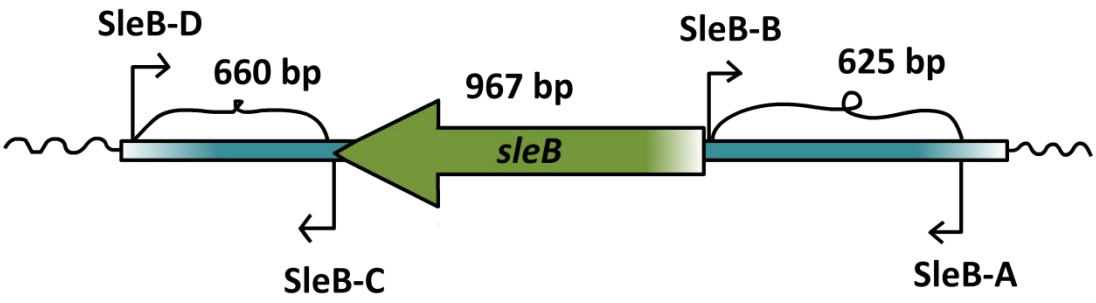


Figure 17: Gene map showing the expected PCR products.

This PCR product needs to be accurate with as few replication errors as possible. Thus "AccuPrime™ *pfx* DNA Polymerase High Fidelity" (Invitrogen™) was used, which is more accurate and possesses proofreading in addition.

The following PCR reactions were used:

- 5 µl AccuPrime™ PCR buffer x10 (includes dNTP)
- 1 µl SleB-A primer (10 mM)
- 1 µl SleB-B primer (10 mM)
- 1 µl Genomic DNA
- 1 µl Accuprime™ *pfx* Polymerase (Invitrogen™)
- 41 µl MQ

- Total volume: 50 µl

An equal PCR was simultaneously set up but with primer pairs SleB-C and SleB-D.

The parameters for the AccuPrime™ *pfx* polymerase (Invitrogen™) PCR reaction was as follows:

Step 1: 95 ˚C 4 min

Step 2: 95 ˚C 30 seconds

Step 3: 58 ˚C 30 seconds

Step 4: 68 ˚C 40 seconds

Step 5: 4 ˚C ∞

Step 2 – 4 was repeated 30 times.

The product was purified using “QIAquick PCR Purification Kit” (section 7. II p.65)

A different PCR reaction was used:

- 5 µl AccuPrime™ PCR buffer x10 reaction mix (Invitrogen™) (includes dNTP)
- 1 µl SleB-A primer (10 mM)
- 1 µl SleB-D primer (10 mM)
- 0,5 µl SleB-AB PCR product
- 0,5 µl SleB-CD PCR product
- 1 µl Accuprime™ *pfx* Polymerase (Invitrogen™)
- 42 µl MQ

- Total volume: 50 µl

As a final step, Taq polymerase was added to the PCR product and incubated at 72 ˚C for 15 min to create sticky ends for cloning into a pCR 2.1 TOPO vector (figure 17). The product was purified using “QIAquick PCR Purification Kit” (section 7. II p.65)

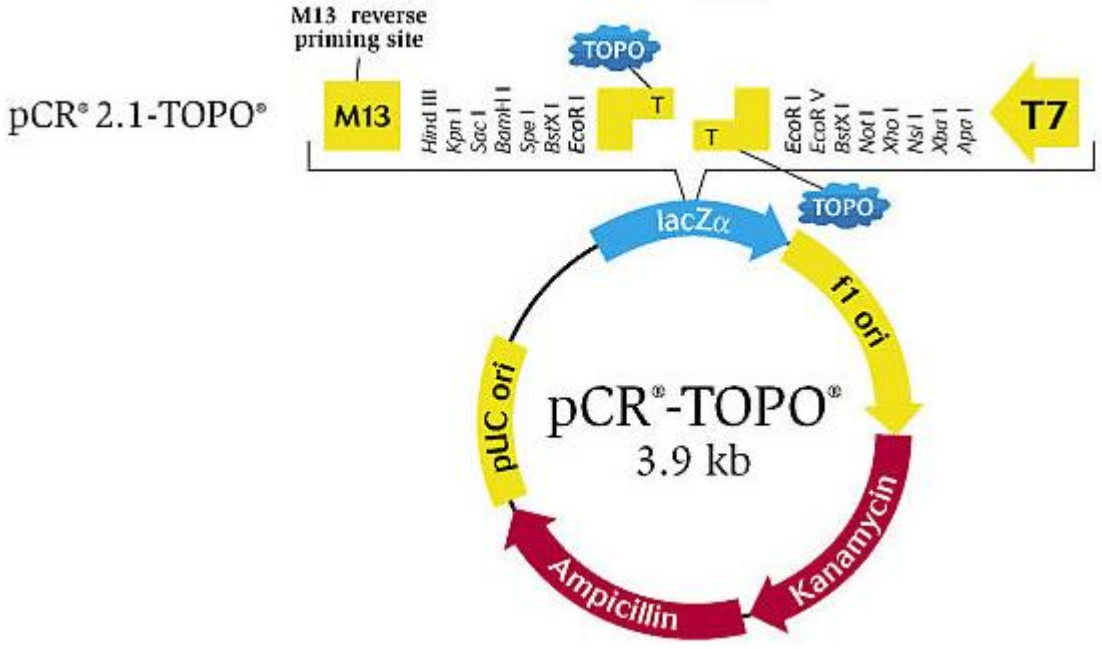


Figure 18: Invitrogen pCR 2.1 TOPO vector (Invitrogen)

2.2.4 Vector and Plasmid insertion

The SleB-AD fragment was ligated into pCR 2.1 TOPO vector via the following ligation reaction using "pEXP5 - TOPO® TA expression kit":

- 2 µl SleB-AD PCR product
- 0,2 µl salt solution (Invitrogen™)
- 0,5 µl TOPO vector (Invitrogen™)

The reaction was set to incubation at 21˚C for 5 min.

The ligated product was then transformed into *E.coli* One Shot TOP10 chemically competent cells (Invitrogen™). This was done by thawing the competent *E.coli* on ice and adding 2 µl of the pCR 2.1 TOPO, followed by very gently stirring with the pipette. The cells were kept on ice for 30 min before shock heating them at 42 ˚C for 30 sec, and immediately put back on ice again. After 1 min on ice, the cells were added 500 µl room temperate S.O.C medium and incubated at 37 ˚C for 1 hour. The cells were spread out on LB agar plates containing 100 µg/ml ampicillin and 40 µl X-gal.

The gene fragment is cloned in the *lacZ* gene, which will disrupt the blue color product. Cells that grew on X-gal and turned blue would have the TOPO vector but not the insert. White colonies were thus to be screened for the gene using the sleBAD primer pair. A white colony which had the insert was incubated overnight in 25 ml LB containing 100 µg/ml ampicillin. This was done in order for the vector with insert to be isolated in large concentration, so the fragment could be cut out with *Eco*RI and get the desired *Eco*RI overhang.

To isolate the pMAD-I-*Sce*I shuttle vector, an *E. coli* freeze stock stored at -80 ˚C containing the vector was inoculated overnight in 25 ml LB containing 100 µg/ml ampicillin.

12 ml of an overnight *E.coli* containing pMAD-I-*Sce*I was used for purified using "QIAprep spin miniprep kit", with some modifications (see section 7.II p.66).

4 ml of overnight culture of *E.coli* containing the pCR 2.1 TOPO vector with the SleB-AD insert, was purified using the same procedure as with the pMAD-I-*Sce*I.

Both the pMAD-I-*Sce*I and the TOPO with insert were digested with *Eco*RI with the following set up:

Digestion of TOPO with insert:

- 40 µl vector (miniprep product)
- 10 µl 10X *Eco*RI buffer (New England Biolabs®)
- 5 µl *Eco*RI enzyme (New England Biolabs®)
- 25 µl sterile distilled water

The reaction was incubated for 4 hours at 37 ˚C

Digestion of pMAD-I-*Sce*I:

- 30 µl pMAD-I-*Sce*I (miniprep product)
- 10 µl 10X *Eco*RI buffer (New England Biolabs®)
- 5 µl *Eco*RI enzyme (New England Biolabs®)
- 25 µl sterile distilled water

The reaction was incubated for 3 hours at 37 ˚C

To ensure that the pMAD-I-*Sce*I does not self-ligate into a whole vector again, phosphatase is added to remove the phosphate group from the 5’ end.

Phosphatase (New England Biolabs®) treatment of 70 µl (1400 ng/µl) pMAD-I-*Sce*I *Eco*RI digestion:

- 10 µl phosphatase buffer (New England Biolabs®)
- 3 µl arctic phosphatase (New England Biolabs®)

The Reaction was set to incubate at 37 ˚C for 30 min.

To ensure that neither *EcoRI* nor the phosphatase interferes in later ligation, the solution was incubated at 65 ˚C for 10 min to inactivate them.

The gene fragment has now been cut out of the TOPO vector. To purify the fragment from the digested solution, it was run on a gel electrophoresis. Since it was to be cut out and purified from the gel, the entire solution was put in one single large well for easier purification. Two fragments appeared on the gel, one that is TOPO (3900 bp) and a shorter one that is the gene fragment of interest (1229 bp). The small gene fragment was cut out using a scalpel with as little excessive gel as possible, and put into a 2 ml tube (Axygen Scientific®) and purified using QIAquick Gel Extraction kit (Qiagen) with some modifications (see section 7.II p.67). The gene fragment was now ligated into the pMAD-I-*Sce*I, digested with *Eco*RI, using T4 ligase (Invitrogen). The following reaction was set up:

Ligation of gene fragment into pMAD I-*Sce*I:

- 2 µl 5X T4 ligation buffer (Invitrogen)
- 1 µl T4 ligase (Invitrogen)
- 4 µ pMAD I-SceI treated with *Eco*RI and phosphatase
- 2 µl gene fragment treated with *Eco*RI

The reaction was incubated at 23 ˚C for 1 hour.
For an optimal ligation reaction a ratio of 3:1 mol of "gene fragment: pMAD I-SceI" could be used.

4 µl of the resulting product was ligated into *E. coli* One Shot TOP10 (Invitrogen) using the DNA concentration technique mentioned in section 2.2.4. The colony was spread out on a LB plate containing 1 µg/ml erythromycin and 40 µl X-gal.

pMAD-I-*Sce*I contains *bgaB* which make the colonies turn blue in the presence of X-gal. Colonies that turned blue should thus have the pMAD-I-*Sce*I. An overnight colony that had turned blue was quality checked for the insert using primer pair 694 and 965. These primers are flanking the insertion site in the pMad-I-*Sce*I (figure 13 p.25). A positive result should have 1809 bp (the gene

fragment is 1285 bp and flanking regions 524 bp) and a negative should have 524 bp. An overnight culture, containing the pMad-I-*Sce*I with insert, was incubated in 25 ml LB with 100 µg/ml ampicillin. The vector was then extracted from the overnight culture using "QIAprep spin miniprep kit" with modifications (see section 7.II p.66), and concentration of DNA was checked with Nanodrop.

2.2.5 Preparation of electrocompetent cells

A freeze stock of strain NVH-1333 (Δ*cwlJ*) (-80 ˚C) was inoculated in 25 ml LB and incubated overnight. 1 ml of the overnight culture was transferred to 100 ml (1 % culture transfer) BHI medium at 37 ˚C with shaking at 200 rpm. The OD_{600} of the medium was checked regularly with a UV-160A Spectrophotometer (Shimadzu) until the cells reached early log phase of about 0,5 - 0,9 OD_{600}. This is the moment when the cells are most susceptible to transformation through electroporation. The BHI was transferred to two 50 ml falcon tubes and centrifuged at 4 ˚C in an Allegra™ X-22R centrifuge at 4500 rpm for 15 min. The supernatant was removed, and the pellet in each tube was resuspended in 8 ml sterile distilled water and divided into 2 ml tubes (Axygen Scientific®), before being centrifuged at 8000 rpm for 15 min. This washing step was repeated with the pellet resuspended in 2 ml sterile distilled water. The pellet was then resuspended in 1 ml freshly made 40 % PEG 6000 and centrifuged at 8000 rpm for 10 min. The supernatant was drained off, the pellet was resuspended in 100 µl 40 % PEG 6000 and stored at – 80 ˚C.

2.2.6 Transformation and single crossover

A tube of electrocompetent cells stored at – 80 ˚C was thawed on ice and 5 µl of pMAD-I-*Sce*I was added and stirred very gently with a pipette. The cells were left on ice for 5 min before transformation. 100 µl of the cells were transferred to a – 20 ˚C chilled Gene Pulser® 0.2 cm (BIO-RAD) cuvette. The cuvette was then quickly put into a Micropulser™ (BIO-RAD) preset to 2.5 V at 4.0 msec and electroporated. The cells were then recovered in 500 µl room temperate

S.O.C medium (section 2.1.1), and incubated at 37 ˚C for at least 1 hour before being plated out on BHI-agar containing 1 µg/ml erythromycin. The pMAD-I-*Sce*I is a temperature sensitive vector and so the project was from this point kept at 37 ˚C until the desired deletion has been made. This is a vector that has proved difficult to transform into the cell, so to increase the transformation rate, the DNA concentration technique in section 2.2.0 with resuspension in 6 µl sterile distilled water was used. The concentration of DNA was raised from 68 ng/µl to 140 ng/µl (Nanodrop)

The plates where screened for blue/white colonies where blue colonies was positive for the pMAD-I-*Sce*I vector. Since insertion into the gnome does not always happen at first attempt, a blue colony was put into a LB medium containing 1 µg/ml erythromycin, and incubated overnight. A 10^{-5} dilution was made and 50 µl spread out on LB plates containing 1 µg/ml erythromycin. To verify the insertion into the genome and to know which direction the vector had been inserted, a colony was screened with primer pairs: 1283/964, 1283/965, 1282/965 and 1282/954. Electrocompetent cells were made from a verified colony using the protocol in section 2.2.5.

2.2.7 Double crossover and gene deletion

A freeze stock of *E. coli* containing the pBKJ223 was inoculated in 25 ml LB containing 100 µg/ml ampicillin and incubated overnight. 24 ml were used to isolate pBKJ223 using the "QIAprep spin miniprep kit", as it is a low copy plasmid. The plasmid was transformed into the pMAD-I-*Sce*I electrocompetent cells following the transformation protocol in section 2.2.6, with the exception that the cells where plated out on BHI plates containing 10 µg/ml tetracyclin. The plates were left to incubate for 48 hours as blue colonies are slow to appear.

White colonies had gotten the desired double crossover where the deletion has taken place. However, light blue and blue colonies with a white halo could also be positive. This is perhaps

due to *B. licheniformis* ability to break down β-galactose (Juajun*, et al.*, 2011). When the double crossover has taken place, the pMAD-I-*Sce*I is removed. Therefore it was important to keep some cells where the double crossover had not taken place yet. This is because if the double crossover did not occur, continuing growing the cells on tetracyclin would result in a loss of pMAD-I-*Sce*I since it contains erythromycin resistance. A plate was therefore made with both 1 µg/ml erythromycin and 10 µg/ml tetracyclin, and a colony was streaked out to save the status of the project. Since it is difficult to spot a potential mutant colony, a colony of the saved plate was incubated overnight in LB containing 10 µg/ml tetracyclin. By using the same dilution method as in section 2.2.6, several LB plates containing 10 µg/ml tetracyclin was plated out and incubated for 48 hour. The plates were once more looked for white colonies and checked with primer pair 1282/1283. A mutant should yield a PCR product of 1913 bp. This process was repeated using colonies from the saved plate until a mutant colony was found.

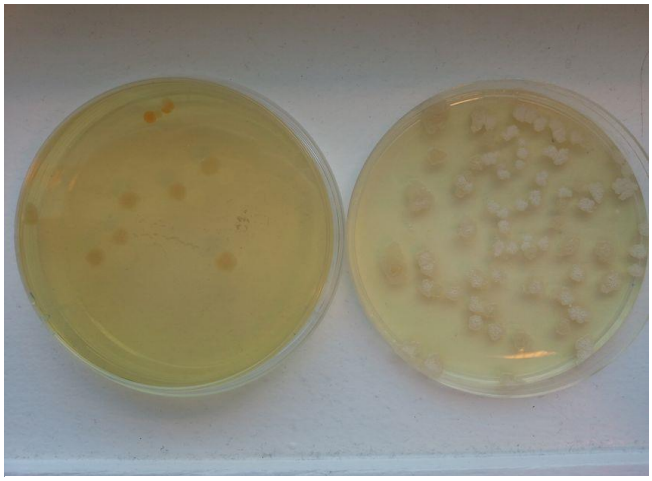
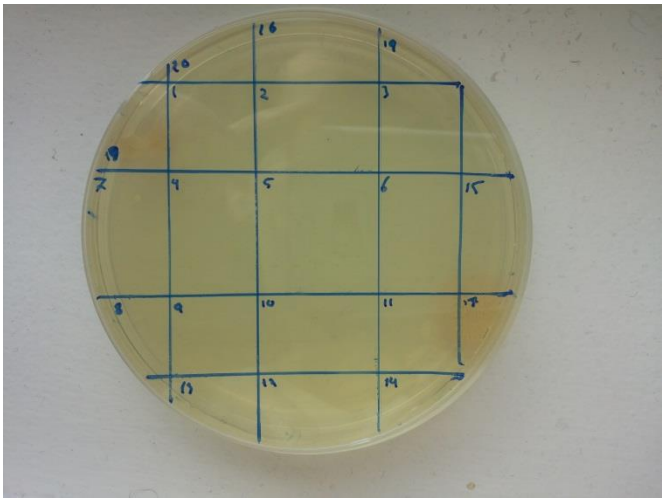


Figure 19: LB plates containing 10 µg/ml tetracyclin. Left: 3 cycles of growth without antibiotics. Right: 1 cycle of growth without antibiotics

A mutant Tet^R colony was put in LB medium without antibiotics and incubated overnight. This was repeated one more time. It was then plated out on one LB plate containing 10 µg/ml tetracyclin and one LB plate without antibiotics to check for Tet^S. This was repeated until the there were significantly less colonies on the tetracyclin plate compared to the plate without tetracyclin (figure 19).

Once this difference became apparent, single colonies from the plate without tetracyclin were marked and plated out on a plate that was grid lined containing 10 µg/ml tetracycline. This is to check many colonies at the same time (figure 20).



A colony that did not grow in a square on the tetracyclin plate was re-plated from the marked colony on the original plate (the one without tetracyclin) on a plate containing 10µg/ml tetracyclin. This was to quality check that the plasmid was gone. When no growth was confirmed, the cells were spun down and stored in a Minibank™ vial and stored at -80 ˚C.

Figure 20: Grid lined LB plate containing 10 µg/ml tetracyclin.

2.2.8 Preparation of spores

The protocol used was developed by Irene S. Løvdal with some modifications (Lovdal*, et al.*, 2013).
A freeze stock (-80˚C) were inoculated in 200 ml freshly made Bacto-MS broth (section 2.2.1) and incubated at 37 ˚C with shaking at 200 rpm. The broth was checked after 4 days in a phase contrast microscope, and checked daily thereafter until >80% spores were observed. The broth was divided and transferred to 50 ml Falcon tubes and centrifuged at 4500 rpm for 20 min in an Allegra™ X-22R centrifuge at 4 ˚C. The spore pellet was then washed with 10 ml sterile distilled water and centrifuged with the same speed and temperature for 10 min.

Figure 21: Nycodenz concentrations layered with spore pellet added on top.

Freshly made Nycodenz solutions of 20 %, 50 % and 70 % were made and sterile filtrated. 4 ml of the 70 % where layered in the bottom of a 15 ml Falcon tube, and carefully layered by 4 ml 50 % Nycodenz. 3 ml of the 20 % Nycodenz was mixed with

the spore pellet, and layered on top of the Nycodenz solutions (figure 21). The tubes were centrifuged at 4500 rpm at 4 ˚C for 90 min with minimal acceleration and no breaks at deceleration.

After centrifugation, four layers appear. The middle opaque layer consists of lighter spores and other contaminations (figure 22). The layers where siphoned off down to and parts of the bottom clear layer and the tubes was filled with sterile distilled water (15 ml) with resuspension of the spore pellets. The tubes were centrifuged at 3900 x g for 10 min at 4 ˚C, and this washing step was repeated twice after that. After the last wash, the pellet was resuspended in 2 ml sterile distilled water and divided into two Eppendorf tubes before being centrifuged at 4500 rpm at 4 ˚c for 5 min in a table top centrifuged. The supernatant was discarded, and the pellet was resuspended in fresh cold 1 ml MQ and stored at 4 ˚C for 5 days until the spores had settled. A quality check was made by looking at the spores in a light phase microscope. If the spores had a lot of aggregation into tightly packed clusters, it would interfere with measurement of optical density later on, and was discarded.

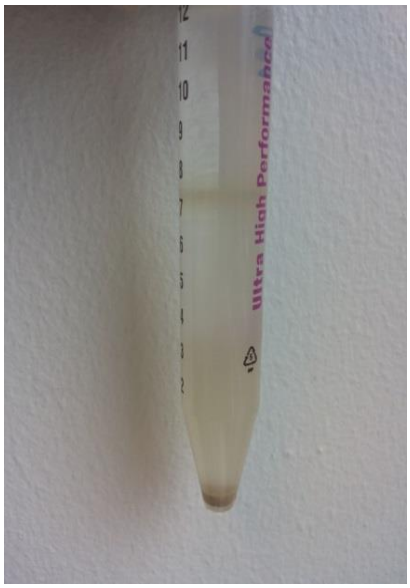


Figure 22: Different layers after centrifugation of spores in Nycodenz. A faint layer in the middle is seen containing vegetative cells and other impurities. An opaque layer is seen towards the bottom and a pure spore pellet is seen in the bottom.

2.2.9 Germination

For the germination experiments, spores of the following strains were used: Δ*cwlj*, Δ*sleB*, Δ*cwlj*Δ*sleB* and MW3.

A tube of spores were spun down at 500 rpm for 3 min, and then resuspended and incubated at 65 ˚C for 20 min before being centrifuged at 4600 rpm at 4 ˚C for 5 min. The optical density

(OD_{600}) was adjusted to 2.1 in $KPHO_4$, or with water when measuring germination by CaDPA as phosphate will bind Ca^{2+}. Three different assays where used: OD_{600}, Terbium Chloride ($TbCl_3$) and CaDPA.

Optical density A_{600}:

Optical density was used as a measure to survey germination over time, and the relative amount of spores that germinates. Spores go from phase bright to phase dark under the microscope when hydrating during germination, which is what is measured by the spectrophotometer. On a 96-well Falcon plate 100 µl of spores (adjusted in $KHPO_4$ pH 7.6) was added whilst preheating the TECAN machine. When reaching 37 ˚C, the spores were added 100 µl (100 mM) L-alanine of a 200 mM stock. MW3 spores were used as a positive control and water was used as a negative control. The program was set to cycles of 2 min over 100 min. In each cycle there was 1 ½ min of orbital shaking, 20 sec settling time and 10 sec of measurement.

Terbium Chloride ($TbCl_3$):

Terbium Chloride was used to detect the release of dipicolinic acid during germination which gives a more precise picture of when germination is committed. On a 96-well plate with light isolated wells and bottoms, 100 µl spores (adjusted in $KHPO_4$ pH 7.6) were added whilst preheating the TECAN machine. When reaching 37 ˚C the spores where added 10 µl (to a final concentration of 7.2 mM) $TbCl_3$ from a 150 mM stock and 100 µl (100 mM) L-alanine of a 200 mM stock.

100 µl of spores where boiled at 100 ˚C for 2 hours and added 10 µl $TbCl_3$ to measure the total content of DPA in the spores. Spores of MW3 with $TbCl_3$ were used as a positive control, and water with $TbCl_3$ was used as a negative control and to deduct background measurement. The program was set to cycles of 2 min over 24 hours. In each cycle there was 1 ½ min of orbital shaking, 20 sec settling time and 10 sec of measurement.

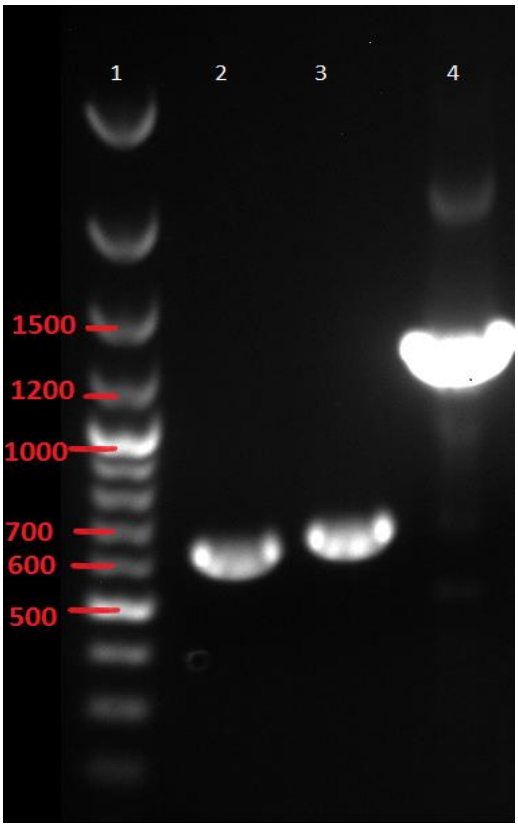
Dipicolinic acid - Calciumchloride (DPA-$CaCl_2$):

100 µl of spores (adjusted in distilled autoclaved water) was added 160 mM DPA and 160 mM $CaCl_2$ from a 0.5 mM stock to a total volume of 360 µl, and incubated in a water bath at 37 ˚C with shaking. The spores were checked and counted every 5 min for 20 min and then every 20 min till 100 min in a light contrast microscope. The spores where checked once more after 24 hours. The counting was done by taking a picture and counting the phase bright/dark ratio. MW3 spores were used as a positive control.

3 Results

3.1 Creation of the double mutant Δ*cwlJ*,Δ*sleB*

3.1.1 Spliced fragment of *sleB*



The PCR product of the flanking regions of *sleB* and the subsequent spliced product (figure 16 p.30 section 2.2.3) was run on a gel electrophoresis to confirm the correct PCR product (figure 23). SleB-AB had a band size of 625 bp and SleB-CD had a band size of 660 bp. The band size of the spliced product SleB-AD was 1285 bp. It was then cloned into the pCR 2.1 TOPO vector.

Figure 23: PCR product of the flanking regions of *sleB* and the following spliced product**:** Well 1: 100 bp ladder. Well 2: SleB-AB fragment of 625 bp. Well 3: SleB-CD fragment of 660 bp. Well 4: SleB-AD fragment of 1285 bp.

3.1.2 Cloning into TOPO

The pCR 2.1 TOPO with the 1285 bp insert was chemically transformed into chemically competent *E.coli* by heat shock. The cells were incubated overnight and a checked for blue/white colonies. White colonies had the fragment inserted because of cloning into the *lacZ* region. About 30 colonies were observed and approximately 2/3 of the colonies were white. A white colony was screened for the TOPO with the insert by PCR using primer pair SleB-A – SleB-D (picture not shown due to poor quality).

The verified colony was grown overnight and the TOPO vector with the cloned insert was purified and digested with *Eco*RI. The entire reaction mix was cast on a gel electrophoresis in a large well. Two bands appeared (figure 24). The short band is the gene fragment and the large band is the TOPO vector. The short band was cut out from the gel and purified.

The gene fragment now had *Eco*RI sticky ends. pMAD-I-*Sce*I was digested with *Eco*RI as well, and had the complimentary *Eco*RI sticky ends. This made it possible to ligate the gene fragment into the pMAD-I-*Sce*I vector.

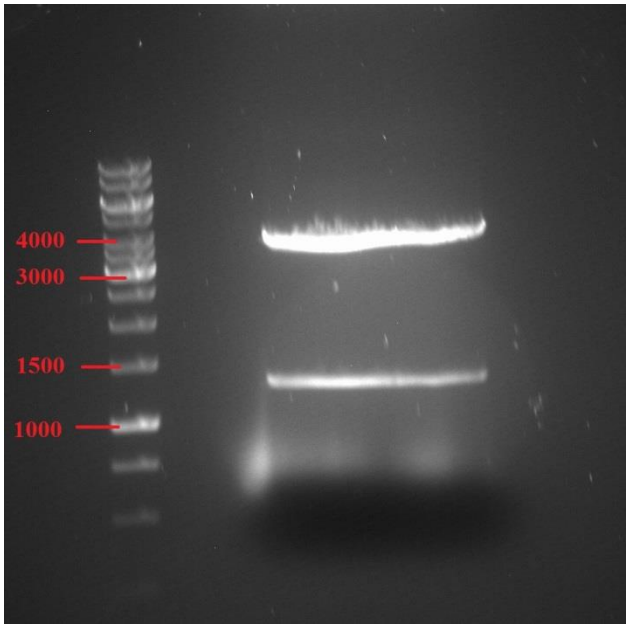


Figure 24: *Eco*RI disgested pCR 2.1 TOPO vector with desired gene fragment. The top band is the TOPO vector without the gene fragment. The smaller bottom band is the gene fragment.

3.1.3 Ligation into pMAD-I-*Sce*I

Ligation into the pMAD-I-*Sce*I was done by T4 ligase, and transformed into chemically competent *E.coli* by heat shock. Single colonies were checked by PCR using primer pair 694-695. The primer sequence is located in pMAD-I-*Sce*I flanking the insertion site. A PCR product where

the cloned fragment was present gave a band of 1809 bp. A pMAD-I-*Sce*I without the gene fragment gave a PCR product of 524 bp (figure 25)

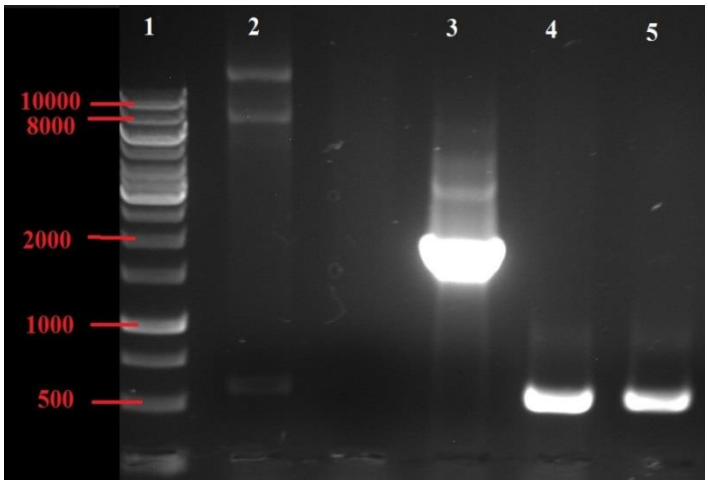


Figure 25: Verification of gene fragment insertion in pMAD-I-*Sce*I using primer pair 694-695.Well 1: 1kb ladder (Fermentas) Well 2: Negative control. Well 3: Colony 1. Well 4: Colony 2 Well 5: Colony 3

3.1.4 Transformation of pMAD-I-*Sce*I into *B.licheniformis*

The pMAD-I-*Sce*I with the insert was purified from the *E.coli,* and transformed into *B.licheniformis* Δ*cwl* by electroporation. Colonies containing the pMAD-I-*Sce*I turned blue. Only 3 of 7 colonies turned blue. One of these colonies was screened for the direction in which pMAD-I-*Sce*I with the insert had ligated into the genome. Primers in pMAD-I-*Sce*I (694 and 695) were used in combination with different primers (1582-1583) in the genome. Figure 26 shows the PCR products when using primer pair 1582-694 and 1583-695. 1582-694 has a band of 2880 bp due to *sleB* position in between the primer pair, while 1583-695 has a band of 1817 bp. This indicates the direction of pMAD-I-*Sce*I which

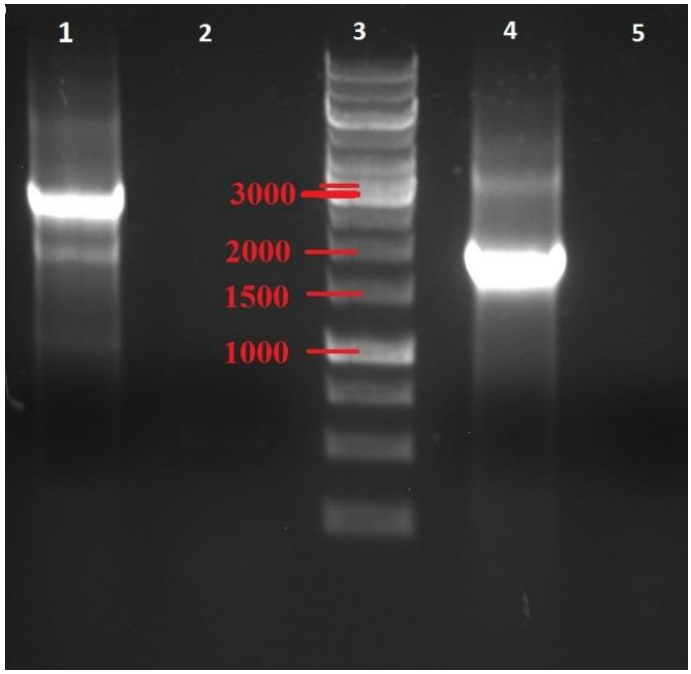


Figure 26: Direction of pMAD-I-*Sce*I with insert into the genome. Well 1: Primer pair 1582-694. Well 2: Primer pair 1582-695. Well 3: 1 kb ladder (Fermentas). Well 4: Primer pair 1583-695. Well 5: Primer pair 1583-694.

is visualized in a gene map in figure 27. The recombination had occurred within the CD part of the cloned fragment. It is not possible to determine exactly where in the CD region the recombination takes place.

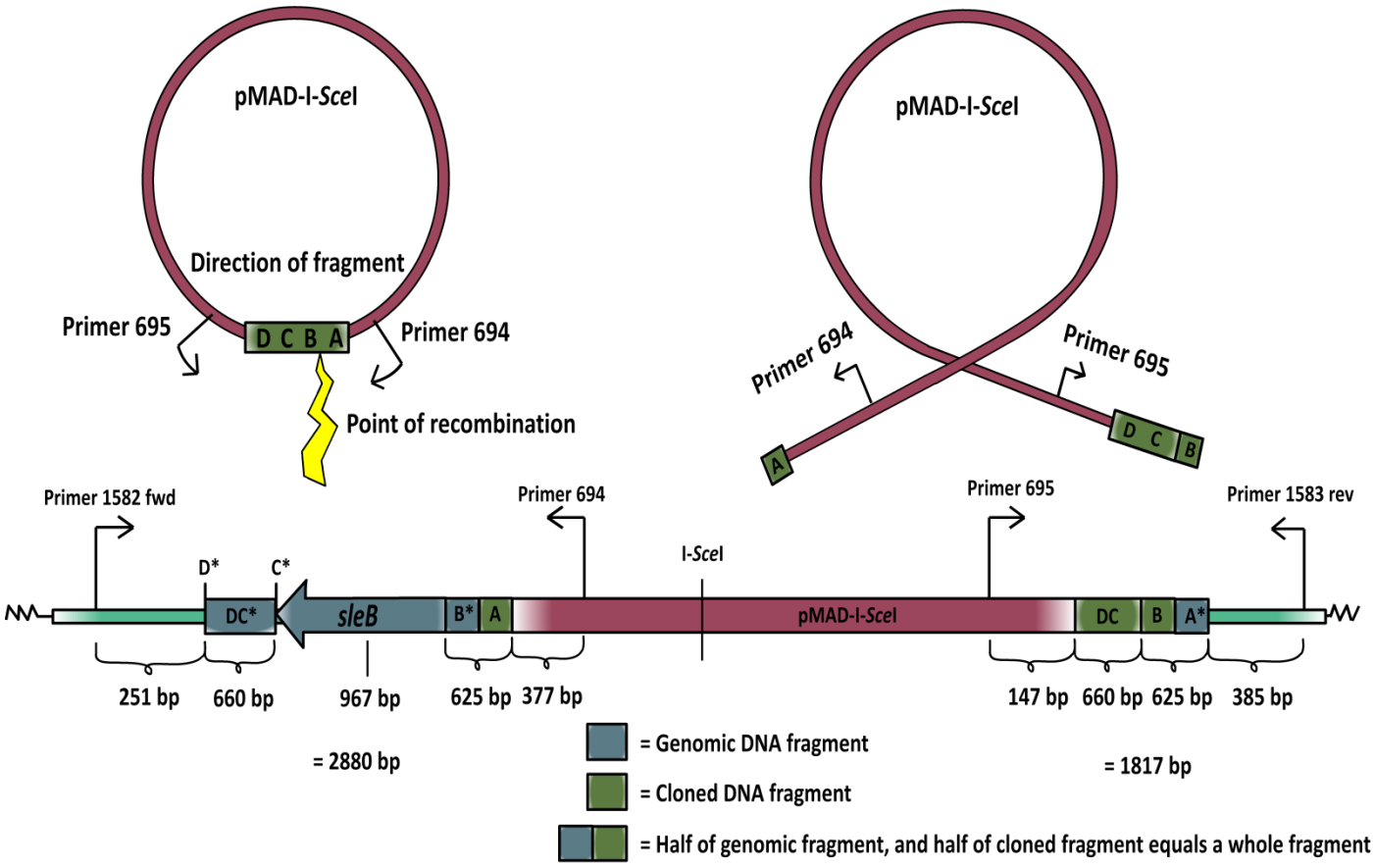


Figure 27: Gene map after single crossover of pMAD-I-*Sce*I has occurred. The fragment size between primer 694 and 1582 is 2880 bp. Fragment size for primer 695 and 1583 is 1817 bp. A successful excision of *sleB* would yield a PCR product of 2880 bp – 967 bp – 377 bp + 385 bp = 1921 bp.

3.1.5 Transformation of pBKJ223 into *B.licheniformis* and double crossover

The pBKJ223 plasmid, which encodes the I-*Sce*I enzyme, was electroporated into electrocompetent cells of the clone containing the pMAD-I-*Sce*I-*sleb*-AD inserted into the chromosome. This subsequently caused a double crossover. Approximately 200 colonies were observed when plating out 20 µl of a 10^{-5} dilution. Cells that have had the homologue

recombination had lost the pMAD-I-*Sce*I and were supposed to turn white, but this was not the case. After two days, all the colonies had turned blue. However, light blue and blue colonies with a white halo were screened using primer pair 1582-1583. The transformation efficiency was not possible to verify due to the difficulty of telling the mutant colonies from those who restored *sleB* (see figure 15 p.27 for the possible outcomes of the double crossover). A colony which gave a PCR product of 1921 bp was also checked with primers internal in *sleB* (1568-1569). If *sleB* has been removed, these primers would yield no PCR product. Figure 28 shows the PCR product of a colony screened with primer pair 1582-1583 and 1568-1569. This colony shows no PCR product when using primer pair 1568-1569 internal to *sleB* compared to the control. The PCR product when using primer pair 1582-1583 was 1921 bp. The double mutant was named NVH-1341.

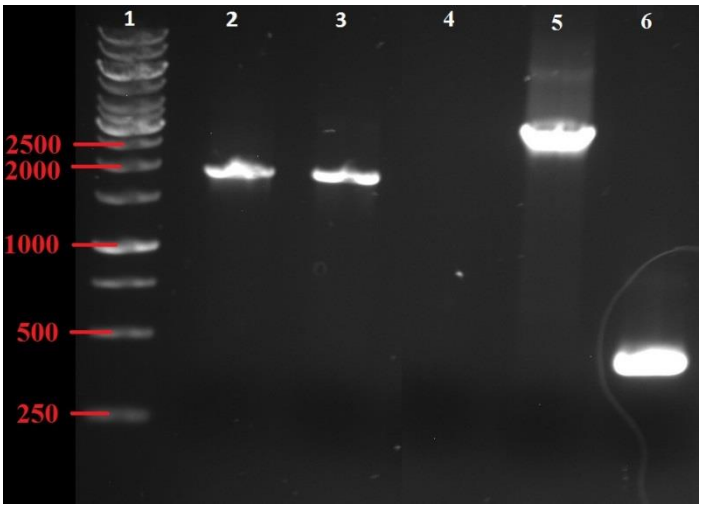


Figure 28: Control of white colony after transformation of pBKJ223. Well 1: 1kb ladder (Fermentas). Well 2: Δ*sleB,*Δ*cwlJ* with external primers 1582-1583 Well 3: Δ*sleB* external primers 1582-1583 Well 4: Δ*sleB,*Δ*cwlJ* with internal primers 1568-1569 Well 5: Negative control MW3 1583-1582. Well 6: Negative control MW3 1568-1569

3.2 Germination assays

3.2.1 Drop in OD_{600}:

Three spore batches of MW3, Δ*cwlJ*, Δ*sleb* and Δ*cwlJ,*Δ*sleB* were prepared with 2 days interval. However, one of the Δ*sleb* batches turned out not to be viable. Due to time and the necessity to have the same parameters for every batch, this batch was not replaced. The spores were stored for a week at 4 ˚C before germination.

The spores were adjusted to OD_{600} 2.1 in 50mM $KHPO_4$ buffer and transferred to a micro titer plate (96 well BD). Each well on the micro titer plate could hold a maximum of 250 µl. 100 µl

spore solution was therefore mixed with 100 µl L-alanine of a 200 mM stock to a total volume of 200 µl (Lovdal*, et al.*, 2013). Spores would not germinate unless they are in a $KHPO_4$ buffer. The experiment ran at 37 ˚C for 100 min with an OD_{600} measurement every 2 min (figure 29)

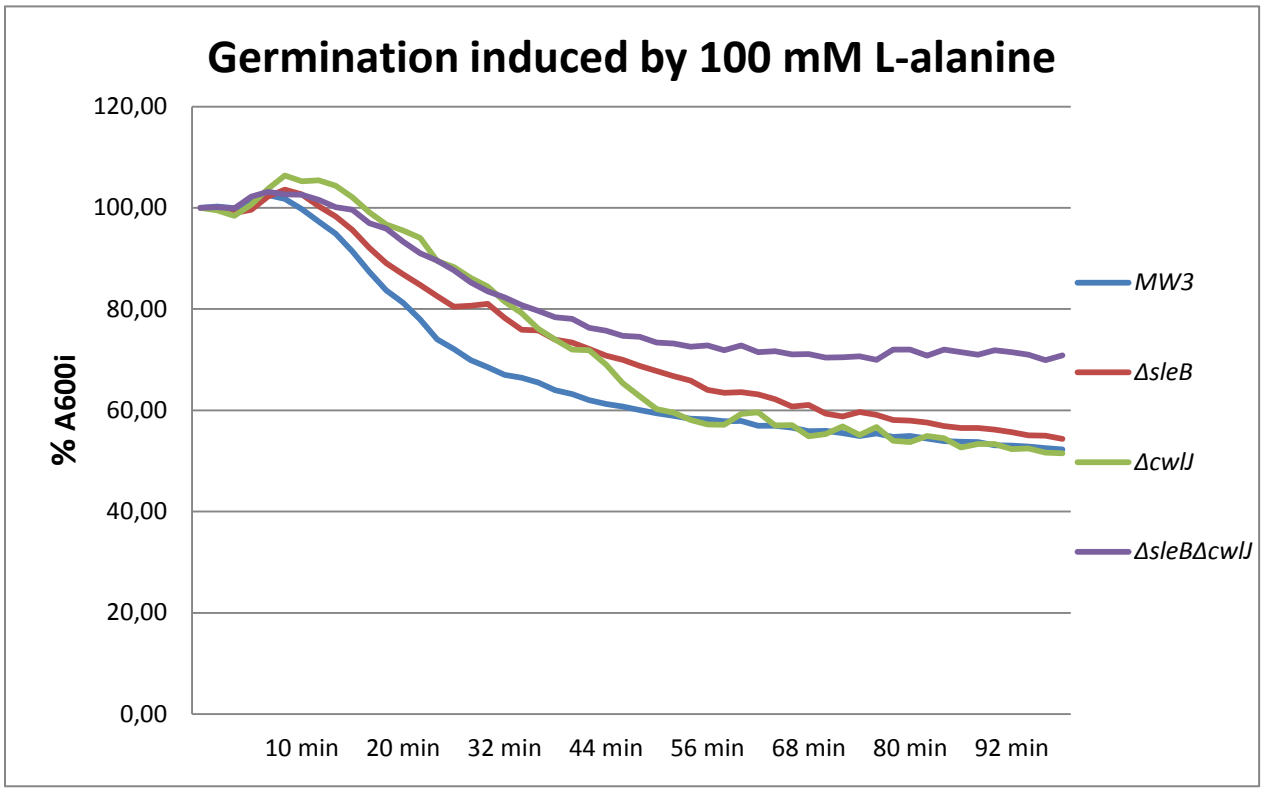


Figure 29: Germination induced by 100 mM L-alanine in 50mM phosphate buffer (pH 7.1). Each curve is an average of three batches except for Δ*sleB*, which is an average of two batches. The graphs display a relative drop in OD_{600} as a function over time.

With the exception of the double mutant, the single mutant and the wild type spores dropped about 50 % in OD_{600}. The double mutant ended at a relative drop of 30 % in OD_{600}. The drop rate of OD_{600} differs for the single mutants contrary to the wild type but the end OD_{600} was the same. Spores were checked in a phase microscope to confirm germination. Spores which hydrate the core as they germinate goes visually from a phase bright to phase dark state (figure 30).

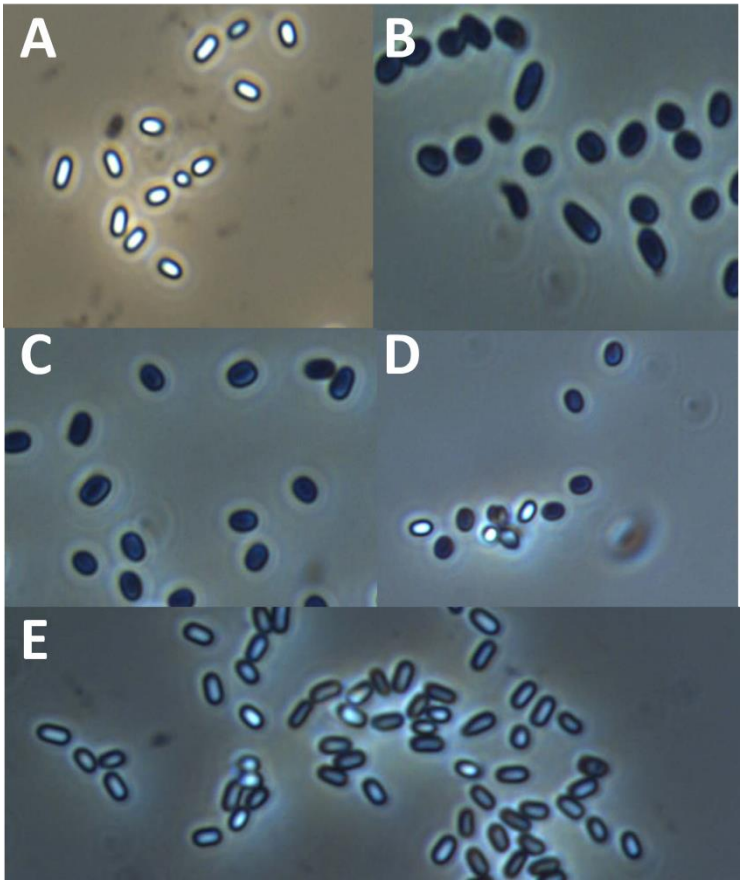


Figure 30: Phase microscopy of spores. A: Dormant phase bright spores of *B.licheniformis*. B-E: Germination has been induced by 100 mM L-alanine. Pictures are taken 100 min after being induced. B: MW3 C: Δ*sleB* D: Δ*cwlJ* E:

Pictures of the spores were taken immediately after germination with a phase microscope (the camera was an Olympus BX51). As seen in figure 30, the relative "colour" of the spores corresponds with the graphs in figure 29. All of the spores that dropped by 50 % in OD_{600} had turned phase dark after 100 min. This includes the single mutants *ΔsleB* and Δ*cwlJ*, and the wild type MW3 (figure 30 B-D). Spores of the double mutant dropped only 30 % in the graph and turned phase gray in the microscope after 100 min (figure 30 E). The spores were checked again by microscopy after 24 hours but there was no change in appearance. To see if the spores could complete the germination by vegetative outgrowth, they were plated on LB-plates. After being incubated overnight, there was almost no growth observed on LB-plates containing spores of the double mutant Δ*sleB*,Δ*cwlJ*. The very few colonies that appeared were a source of error. When looking at the spore batches of the double mutant in a phase microscope, there was a vegetative cell every now and then. Full growth was observed of spores of the single mutants Δ*sleB* and Δ*cwlJ* when plated out.

3.2.2 Release of DPA via Tb^{3+} assay

This assay was based upon experiments done by Yi and Setlow (Yi & Setlow, 2010) on *B.subtilis*, but was modified to fit this experiment (see discussion p.54-55).

The same spore batches used in measuring drop in OD_{600} (section 3.1.1) were used in this assay. Spores were adjusted to 2.1 OD_{600} in 50 mM $KHPO_4$. 100 µl spore solution was added 10 µl $TbCl_3$ (7.2 mM final concentration) before adding 100 µl L-alanine from a 200 mM stock. A wavelength of 545 nm was emitted with excitation 270 nm. The emission of 545 nm can only occur when Tb^3 forms a complex with DPA. Tb^3 cannot enter the spore and will thus bind to DPA complex once the CaDPA is released from the core during germination. As more TbDPA complex is formed, the fluorescence signal will get stronger and so the CaDPA release can be tracked during the germination (figure 31).

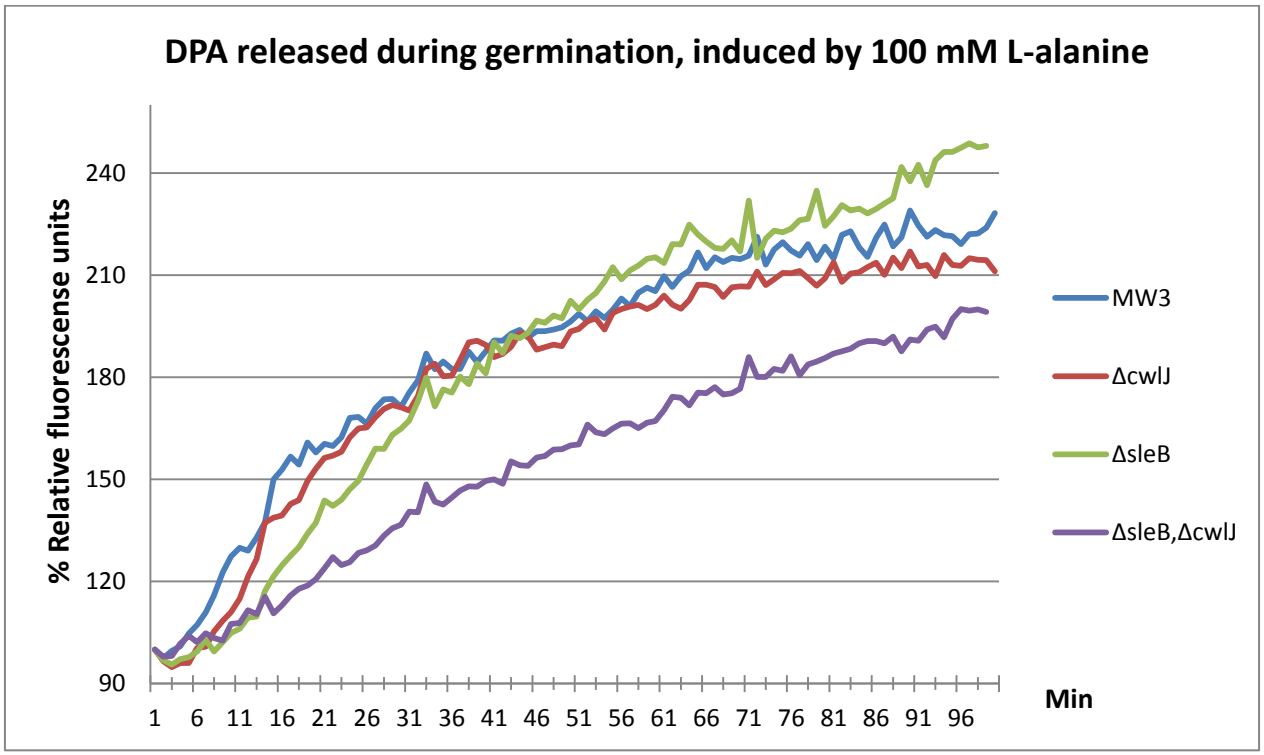


Figure 31: DPA released during germination induced by 100 mM L-alanine in 50 mM $KHPO_4$ (pH 7.1). Each curve is an average of three batches, with the exception of Δ*sleB* which is two batches. The graph displays fluorescence emitted by Tb^{3+} when reacting with DPA 1:1. Emission was 545 nm and excitation was 270 nm.

The graph shows that DPA is being released just 3-4 min after exposing the spore to L-alanine. For MW3 and the single mutants Δ*cwlJ* and Δ*sleB,* the rate of DPA release seem to have few differences. But for the double mutant Δ*cwlJ*,Δ*sleB,* the rate of release is significantly slower.

The final release of DPA is not shown in figure 31. Total concentration of DPA in the spores was measured after taking 100 µl of the same prepared spore solution, and boiling it for 2 hours at 95 ˚C without L- alanine. The values were approximately 400 % relative fluorescence units. When measuring the DPA release over 24 hours, the graphs reached this value after approximately 15 hour. This includes the double mutant Δ*cwlJ*,Δ*sleB*.

Because of the fluorescence, the micro titer plates needed to have wells that were non – transparent. This also meant that there could not be a lid on the micro titer plates. When measuring the DPA release over 24 hour, the solution would vaporize and the graph would show spikes and drop. The solution was to stretch a plastic film over the micro titer plate. It was important that the plastic film did not have any folds that could distort the fluorescence.

3.2.3 Germination by exogenous CaDPA

This assay looks for cortex lytic activity after activation by exogenous CaDPA. The spores were adjusted to 2.1 OD_{600} in sterile distilled water instead of $KHPO_4$ buffer as phosphate reacts with divalent cations. Ca^{2+} is therefore inhibited by the buffer and no the spores will not germinate by this method. Since CwlJ is directly activated by CaDPA, there is neither any need for the $KHPO_4$ buffer as a cofactor. When mixing Ca^{2+} (is added in the form as $CaCl_2$) and DPA together, they will form crystals which interfere with the mechanical measurement. This required the experiments to be done manually by counting phase dark spores in a phase microscopy. The amount of DPA and $CaCl_2$ needed to induce optimal germination was needed to be clarified. Different concentrations of both DPA and $CaCl_2$ were added as well at different ratios. Spores determine which concentration was the best, phase dark vs phase bright spores were counted after 40 min. The optimum amount of DPA and $CaCl_2$ needed to induce germination in spores of *B.licheniformis* MW3, was 1:1 150 mM (figure 32).

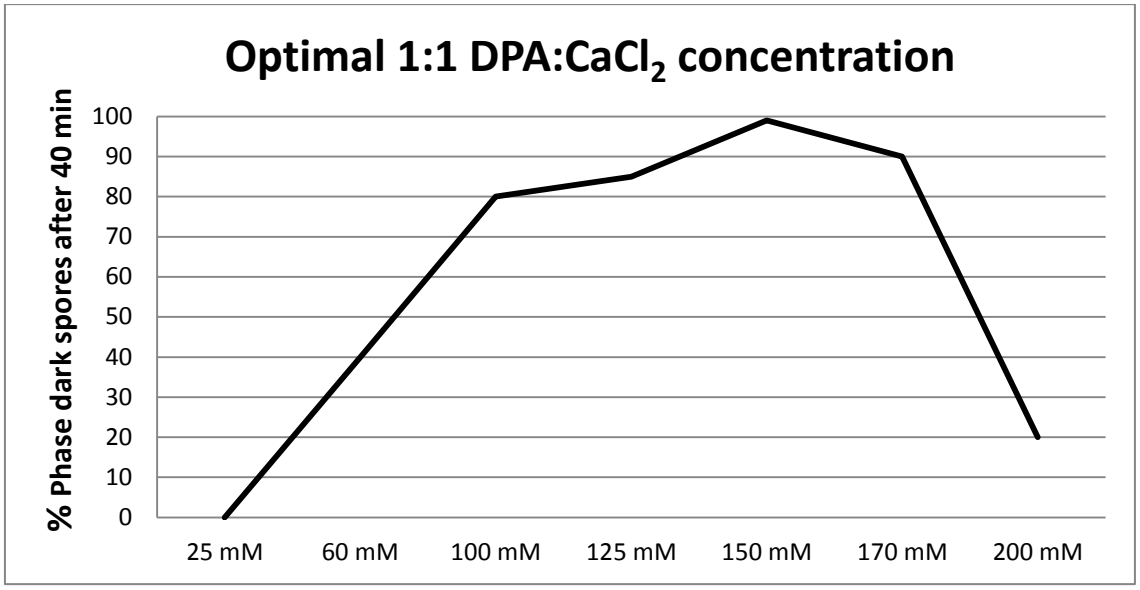


Figure 32: Optimal concentration of $CaCl_2$ and DPA based on % germinated spores after

It is not clear why concentration of 1:1 DPA: $CaCl_2$ higher than 170 mM hampered the germination, but salts are known to inhibit spores at a certain level (Yi*, et al.*, 2011). The germination assay was done by counting phase bright vs phase dark spores at a set of time intervals (Table 4)

Table 4: Amount of phase dark vs phase bright spores when exposed to 1:1 150 mM DPA: $CaCl_2$ in distilled autoclaved water.

Tid (min)	1289 MW3	1331 Δ*sleB*	1333 Δ*cwlJ*	1341 Δ*sleB*,Δ*cwlJ*
0	1 %	5 %	0 %	0 %
5	5 %	5 %	0 %	0 %
10	30 %	20 %	0 %	0 %
15	60 %	50 %	0 %	0 %
20	80 %	70 %	0 %	0 %
40	90 %	80 %	0 %	0 %
60	90 %	90 %	0 %	0 %
100	95 %	90 %	0 %	0 %
3600	99 %	95 %	5 %	0 %

Spores of Δ*cwlJ* and Δ*sleB,*Δ*cwlJ* did not turn phase dark in the microscope. A few spores of Δ*cwlJ* did germinate after 24 hours but this is likely due to spontaneous germination which happens for reasons unknown. Spores located in areas where crystal concentration was high showed a higher frequency of germination. This made it difficult to make an exact picture of the germination rate.

4.0 Discussion

4.1 Creation of deletion mutant Δ*sleB*, Δ*cwlJ*

The markerless gene replacement method was initially developed for *B.anthracis* (Janes & Stibitz, 2006), but has been modified to be used in *B.cereus* (Lindback*, et al.*, 2012). The method used in *B.cereus* was successfully transferred in gene replacements in *B.licheniformis*. However, there were some difficulties when making the double mutant. The first obstacle was that pMAD-I-*Sce*I was hard to transform into *B.licheniformis*. This problem was solved by isolating the vector in large quantities and transforming 100 µl instead of 50 µl (which has been used in other thesis by the germination project). Once transformed, pMAD-I-*Sce*I was incubated at 37 ˚C to perform a homologue recombination. pMAD-I-*Sce*I cannot replicate at this temperature due to its temperature sensitive replicon pE194^{ts}, but shows a wild-type copy number of the vector at temperatures lower than 32 ˚C (Villafane*, et al.*, 1987, Arnaud*, et al.*, 2004). The project was thus kept at 37 ˚C.

The second issue was the screening for the mutant after the second crossover. pMAD-I-*Sce*I contains *bgaB*, which will make the cells look blue when grown on X-gal. This gene is removed when the double crossover takes place, and the colonies are supposed to be white when grown on X-gal. This was not the case. The colonies turned blue instead after a few days. The reason for this may be because *B.lichenformis* has a β-galactosidase gene (*lacA*) (Juajun*, et al.*, 2011). Colonies that was near the edge of the petri dish remained white, but that was probably due to how X-gal is spread out (from the center and out). One or two days after transformation of

pBKJ223, colonies started to turn blue whilst others remained white. Those that remained white were taken note of. After two to three additional days (in the incubator at 37 ˚C) the marked colonies had turned blue or light blue. Those that had turned light blue or had a white halo were screened by PCR. Since this was a vague way to look for the mutant, there was no way of saying how many percent of the colonies had turned into the mutant vs returned to wild type. But theoretically 50 % of the colonies should be mutants (Arnaud*, et al.*, 2004).

4.2 Sporulation:

One of the major sources of error in this study was that spores behave differently from batch to batch. This behavior could be aggregation, slower germination, faster germination, non-viability and rate of spontaneous germination. It is speculated that spores differ in properties depending on the environmental factors during sporulation. For example cells that have an increased amount of CaDPA in the core have less water content and can sustain higher temperatures (Church & Halvorson, 1959).

Because this thesis is part of a bigger germination project, it was necessary to follow the same protocols as used by the germination research group. That meant growing the bacteria at 37 ˚C in Bacto-MS medium even though the optimum growth temperature for *B.licheniformis* is 50 ˚C (Warth, 1978). 37 ˚C was chosen to mimic the body temperature, as *B.licheniformis* is food borne and can germinate in the gut. Spores were also stored at 4 ˚C to for a week before testing to see if the spores were stable. Before running the germination test, the spores were heat treated at 65 ˚C for 20 min to imitate heat treatment used in food industry to get spores to germinate (Tyndallization figure 3 p 10). However, there was no observed difference in germination rate in spores that were not heat treated vs heat treated at 65 ˚C in *B.licheniformis* in this project. The sensitivity towards heat activation can differs between strains and species. Two species examples are *B.weihenstephanensis* and *B.pumilus* which readily germinates at a faster rate when heat treated at 80 ˚ C (Lovdal*, et al.*, 2013). The reason for why there was no observation of heat activation in *B.licheniformis* in this thesis, might be due to that spores were made at 37 ˚C, or because of spore batch variation. What was observed in this thesis was that spores up to two years old (supplied by Elisabeth H. Madslien) germinated at a slightly slower

rate (data not shown). This illustrates the importance of repeating the tests using different batches. The batches used in this thesis were made at a 2 day time interval in a total of three batches.

4.3 Germination:

4.3.1 Optical density

Since this assay is widely used by the spore germination project at "Department of Food Safety and Infection Biology", there were no issues regarding measurement by OD_{600}. The only noteworthy comment is the preheating vs no preheating of spores which did not show any difference in germination rate. The double mutant Δ*sleB,*Δ*cwlJ* did not drop to the same OD_{600} as the wild type and the single mutants. The question is what is observed? Is this due to that the spores did not rehydrate, or is it because the cortex does not lyse? It is generally accepted that the spores go from phase bright to phase dark as a result of rehydration. However, there might be that rehydration only makes the spores go phase gray, and that the remaining gray we observe is the cortex. It is thus possible that the spores in fact do fully hydrate even though lacking both SleB and CwlJ.

What is clear though, is that these spores cannot enter a vegetative state and outgrowth. This was shown by spreading the spores on LB plates. It was only the double mutant who failed to produce colonies.

4.3.2 $TbCl_3$ assay

For the $TbCl_3$ assay, protocols from experiments done by Yi and Setlow were used as a basis (Yi & Setlow, 2010, Yi*, et al.*, 2011). However, it became apparent that the molar concentration used in that paper where they experimented on *B.subtilis*, did not apply for this experiment with *B.licheniformis*. They used 60 µM $TbCl_3$ which did not give any clear data when applied here.

The thought was therefore that the more you have of a substance, the faster it will react. So by running a number of experiments it was clear that with increasing molarity of $TbCl_3$, the graph

rose higher and more rapid. The concentration that worked best was 7.2 mM, which is the equivalent of 10 µl in the germination mix (a final volume of 210 µl). A slightly higher concentration of $TbCl_3$ then 7.2 mM did not implicate the result. However, the germination was hampered if the concentration exceeded 10 mM. It is thus the opinion of this thesis that a far higher concentration of $TbCl_3$ gives a more accurate picture of the DPA released in *B.licheniformis*, than what is used in papers using *B.subtilis*.

The Δ*sleB,*Δ*cwlJ* did behave similar in *B.licheniformis* as has been described in papers investigating the germination in *B.subtilis* (Ishikawa*, et al.*, 1998, Yi & Setlow, 2010, Setlow, 2014). The common observation is that the first step of germination is triggered in the double mutant as seen in the wild type and the single mutants Δ*cwlJ* and Δ*sleB*. DPA is released, but then there is no activation of the cortex lytic enzymes. The rate of DPA released from the spore, based upon the $TbCl_3$ assay, was released at a continuously slow, but steady pace (figure 31 p 49). The wild type MW3 and the single mutants Δ*cwlJ* and Δ*sleB* did accelerate their release as SleB and CwlJ break down the cortex. This was observed in *B.subtilis* as well. After 15 hours, almost the same amount of DPA was released from all the strains. This indicates that CwlJ and SleB are not necessary for the release of DPA. Why DPA is released at all in the initial step is probably to activate these enzymes (Bagyan & Setlow, 2002).

4.3.3 Exogenous CaDPA

Germination was also induced by exposure to exogenous CaDPA, but was partly inaccurate as the measurement had to be done manually. When $CaCl_2$ and DPA are mixed together, they form crystals which will create spikes and inaccurate results when measuring by spectrophotometry. So by doing it manually, there was a need for speed and precision as the samples had to be prepared for microscopy while germination took place. It was important to try to keep the temperature at 37 ˚C, so samples could not stay long out of incubation. In addition there was a need to be on time for each interval when counting phase bright vs phase dark spores in the microscope. If a sample was to be looked at after 5 min, the time between sampling and counting could not exceed 1 min or the data would be misrepresentative. Lastly there was the issue with the actual counting. Spores that were inside an area of many crystals

seem to germinate quicker than those that were not in a crystal cluster. Given these reasons, only one to two batches of spores could be germinated at the time. Therefore it might be some small differences when comparing the germination rate between batches. However, the main point was to check if CwlJ was essential for germination induced by CaDPA, which it was.

The germination system by CwlJ may possibly work as quorum sensing. Even though this strain of *B.licheniformis* needs about 150 mM CaDPA for optimum germination compared to 60mM in *B.subtilis,* lower concentrations also induce germination. It is also not unthinkable that local CaDPA released from other spores can reach very high concentrations. The question is of course if this is the reason why CwlJ is there, since it is redundant because of SleB.

Sometimes the environment can be favorable for germination even though the germinants such as L-alanine are not in sufficient supply. Spores that spontaneously germinates, or germinating spores of another species can release CaDPA and might trigger CwlJ in neighboring spores. These spores will in turn release their CaDPA, increasing the concentration and a cascade might start. This system can thus work as a backup germination system based on other spores germination.

B. licheniformis is food borne and it is not unthinkable that there is a selective pressure to avoid heat treatment by the food industry. The strain that was discussed as a basis for this project was a slower germinant surviving the heat sterilization process. *B.licheniformis* needs almost twice as much CaDPA to germinate then spores of *B.subtilis*, so the spores may have evolved to be pickier when to germinate. It is thus not unthinkable that we can find more examples of spores surviving sterilization methods in the future. This is why map the genes involved in germination of *B.licheniformis* is important.

5 Conclusion

The basis for this thesis was to investigate the effect(s) of Δ*cwlJ*Δ*sleB* in *B.licheniformis* spores. These experiments have previously been done in *B.subtilis* (Ishikawa*, et al.*, 1998) where the spores did not germinate. It is important to investigate the difference between the two species as differences in germination behavior have been observed (Lovdal*, et al.*, 2013). Based on the data obtained in this study, there are no differences in germination when comparing Δ*cwlJ*, Δ*sleB* and Δ*cwlJ,*Δ*sleB* in *B.licheniformis* to *B.subtilis*. It is apparent that the cortex lytic enzymes are conserved and essential for a complete germination in both species. Germination was observed in Δ*cwlJ* and Δ*sleB* spores induced with L-alanine. Spores of Δ*cwlJ,*Δ*sleB* did however partly germination when induced with L-alanine. Δ*sleB* was the only mutant that would germinate with exogenous CaDPA. This is in accordance with the theoretical property that CwlJ can be activated by DPA (Bagyan & Setlow, 2002, Setlow, 2014). Commitment to germination and further germination analysis was possible using the terbium chloride essay. The data obtained from this analysis indicated that germination in spores of Δ*cwlJ,*Δ*sleB* is initiated and DPA is released, but outgrowth does not occur. This further supports the notion that the cortex remains intact.

This project was part of a larger project investigating germination in *B.licheniformis* MW3. Future prospects could be to investigate whether germination in Δ*cwlJ*, Δ*sleB* and Δ*cwlJ,*Δ*sleB* mutants can be induced by pressure. It would also be interesting to see if SleB and CwlJ can be practically applied as an agent to use against spores.

This project has added another piece in the *B.licheniformis* germination jigsaw puzzle which might be helpful in the food industry or other appliances.

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7 Appendices

Appendix I Gene Rulers

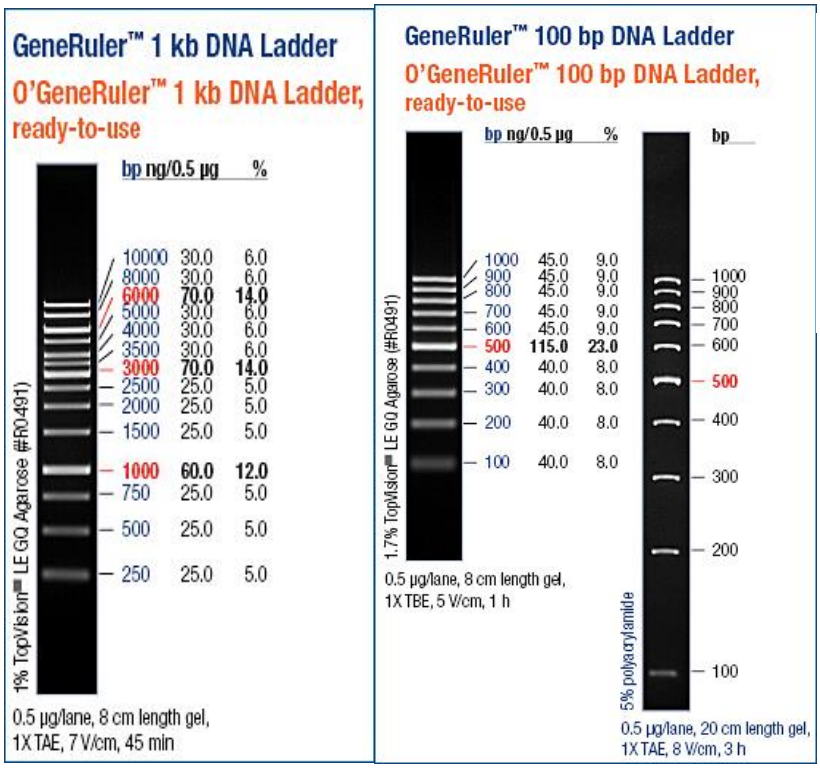


Figure Q: Left: Fermentas GeneRuler™ 1 kb DNA ladder. Right: Fermentas GeneRuler™ 100 bp DNA ladder.

Appendix II Protocols

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 new 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.

Notes:
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifuge steps are at ≥10,000 x *g* (~13,000 rpm) in a conventional tabletop microcentrifuge.

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. **Place a QIAquick spin column in a provided 2 ml collection tube.**
3. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**
4. **Discard flow-through. Place the QIAquick column back into the same tube.**

   Collection tubes are re-used to reduce plastic waste.
5. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**
6. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min at maximum speed.**

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
7. **Place QIAquick column in a clean 1.5 ml microcentrifuge tube.**
8. **To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or H_2O 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 (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

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.

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.

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.
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 0.5 ml 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 bacteria strains with high nuclease activity or carbohydrate content.
8. Wash the QIAprep spin column by adding 0.75 ml Buffer PE. **Let it stand for 1 min** ▲ 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 stand for **4** min, and centrifuge for 1 min.
    Reapplying the flowthrough may increase the yield of DNA.

QIAquick® Gel Extraction Kit

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.~~
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. **Let it stand 1 min before spin and repeat the step.**

   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 **4** 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.

Appendix III: List of Chemicals and equipment

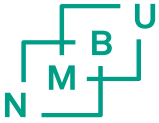
Table E: List of chemicals and equipment sorted by type usage.

Equipment/Chemical type	Product name	Supplier
Agar	Agar Bacteriological (Agar no1)	Oxoid
Agar	Agarose	SeaKem®
Amino Acid	L-Alanine	Sigma-Aldrich®
Amino Acid	L-Tryptophan	Sigma-Aldrich Cell culture™
Antibiotic	Ampicillin	Sigma-Aldrich®
Antibiotic	Erythromycin	Sigma-Aldrich®
Antibiotic	Tetracycline	Sigma-Aldrich®
Autoclavator	Multicontrol	CertoClav®
Broth	Bacto™ Brain Heart Infusion	BD
Broth	Difco™ Nutrient Broth	BD
Broth	Tryptone	Oxoid
Broth	Yeast Extract	Oxoid
Camera	Gel Logic 200 imaging system	Kodak
Camera	Olympus BX51	Olympus
Centrifuge	5415 D Tabletop	Eppendorf
Centrifuge	Allegra™ X-22R	Beckman Coulter™
Centrifuge	Heraeus Rico 21	Thermo Scientific
Chemical	$CaCl_2$	Merck
Chemical	$Ca(NO_3)_2$	Sigma-Aldrich®

Chemical	$CoCl_2$	Sigma-Aldrich®
Chemical	$CuCl_2$	Sigma-Aldrich®
Chemical	Dimethylformamide	Sigma-Aldrich®
Chemical	dNTP Mix	Quant Bioscience
Chemical	Dipicolinic acid	Sigma-Aldrich®
Chemical	EDTA	Sigma-Aldrich®
Chemical	Ethidium Bromide	GeneChoise™
Chemical	$FeSO_4$	Merck
Chemical	Glucose	Sigma-Aldrich®
Chemical	KCl	Merck
Chemical	K_2HPO_4	Merck
Chemical	KH_2PO_4	Merck
Chemical	Iso-Amylalkohol	Merck
Chemical	$MgCl_2$	J.T Baker
Chemical	$MgSO_4$	Merck
Chemical	$MnSO_4$	Sigma-Aldrich®
Chemical	NaAcetate	May & Baker
Chemical	NaCl	Emsure®
Chemical	NaOH	Sigma-Aldrich®
Chemical	$(NH_4)_2SO_4$	Emsure®
Chemical	Nycodenz	Axis-Shield POC
Chemical	PEG 6000	Sigma-Aldrich®

Chemical	Propanol	VWR
Chemical	$TbCl_3$	Sigma-Aldrich®
Chemical	Tris	Sigma-Aldrich®
Chemical	$ZnCl_2$	Sigma-Aldrich®
Cloning	Cpcr® 2.1-TOPO®vector	Invitrogen
Cloning	One Shot®TOP10 E.Coli	Invitrogen
Elektroporation	Gene Pulser® 0.2 cm	BIO-RAD
Elektroporation	Micropulser™	BIO-RAD
Enzyme	Antarctic Phosphatase	New England Biolabs®
Enzyme	Accuprime™ pfx DNA polymerase	Invitrogen™
Enzyme	EcoRI	New England Biolabs®
Enzyme	Proteinkinase K	Sigma-Aldrich®
Enzyme	T4 DNA Ligase	Invitrogen
Enzyme	Taq DNA polymerase	VWR
Filter	0.20 µm	Minisart®
Filter	0.45 µm	Minisart®
Inkubator	AG-CH-4103 Bottmingen	HT INFORS
Inkubator	DH Autoflow	NuAire™
Spectrophotometer	Nanodrop 1000	Thermo Scientific
Spectrophotometer	Inifinite M200	Tecan
Spectrophotometer	UV-160A Spectrophotometer	Shimadzu
Thermal cycler	Mastercycler gradient	Eppendorf®

Thermal cycler	PTC-100® Peltier	MJ Research®
Thermal cycler	T100™ Thermal cycler	BIO-RAD
Thermal cycler	Thermal cycler	Eppendorf®
Tube	1,5 ml	Eppendorf®
Tube	2 ml	Axygen Scientific®
Tube	15 ml	VWR™
Tube	50 ml	BD Falcon™



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