Norwegian University of Life Sciences
Faculty of Vetrinary Medicine and Bioscience
Department of Food Safety and Infection
Biology

Master Thesis 2015
60 credits

Role of the *yndE* genes in the germination of *Bacillus licheniformis* spores

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Abstract

The risk of food poisoning and illness is an important area of research. How to prevent the presence of microorganisms in food products and the production of toxins causing illness is of large interest. Spore forming bacteria of the genera *Bacillus* cause problems in food production industry due to their role in pathogenesis in both humans and domestic animals. They also represent a significant food spoilage problem in the food industry where heat treatment is insufficient to kill the spores, while competition from other vegetative bacteria is eliminated. Later years, the demand for low processed refrigerated food with long durability has become a big issue. To maintain the quality of the food, the sterilization treatments need to be effective but have minimal impact on the foods sensory qualities. Knowledge of the germination receptors, present in the spores inner membrane, and their role in initiating the germination process has been shown of major importance for understanding the germination behavior of spores.

Bacillus licheniformis, has been associated with food borne outbreaks in cooked meats and vegetables, raw milk, and industrially produced baby food. One of the germination receptors of *B.licheniformis* is encoded by the *yndDEF* operon. Our unpublished results demonstrate that the Ynd germination receptor is involved in germination in response to L-alanine, L-cysteine and L-valine. In contrast to other *Bacillus* species, like *Bacillus subtilis*, *B.licheniformis* seems to have three homologous *yndE* genes, *yndE1*, *yndE2* and *yndE3*. The function of the *yndE* genes is unknown. This project aims to characterize the three homologous *yndE* genes in order to understand their role in germination of *B.licheniformis* spores.

Attempts were made to construct separate null mutants in all three *yndE* homologs but only the *yndE3* gene was successfully deleted. Analysis of the germination properties of the *yndE3* mutant in response to L-alanine, L-cysteine and L-valine showed that the mutant strain exhibited a strongly reduced germination rate in response to L-cysteine. The germination responses to L-alanine and L-valine were also slightly reduced. This result indicate that the *yndE3* gene is essential for efficient L-cysteine induced germination and suggest that the other *yndE* genes may be involved in Ynd dependent germination in response to L-alanine and L-valine.

To our knowledge this is the first functional characterization of the role of paralogous germination receptor subunits encoded by a single germination receptor operon.

Sammendrag

Sykdomsutbrudd relatert til matforgiftning er et viktig forskningsfelt. Hvordan man kan hindre vekst av mikroorganismer i matvarer, samt toksin-produksjon, som fører til sykdom, er av stor interesse. Bakterier i *Bacillus* slekten, som danner sporer, er et stort problem i matproduksjon grunnet den sykdomsfremkallende rollen de har hos mennesker og husdyr. Sporedannere representer også et stort problem for matvareindustrien, siden varmebehandling ikke bare er utilstrekkelig i forhold til å drepe disse, men i tillegg eliminerer konkurransen ved å drepe andre ikke sporedannende bakterier. I de senere årene har etterspørselen etter lite prosessert mat med lang holdbarhet økt. For å kunne imøtekomme disse kravene er det nødvendig med effektive metoder for sterilisering som ikke påvirker kvaliteten på maten. Kunnskap om germinerings reseptorer, som er lokalisert i den indre membranen hos sporer, og deres rolle i indusering av germinerings prosessen har vist seg å ha stor betydning for vår forståelse av germinerings prosessen.

Bacillus licheniformis, har blitt knyttet til bakterieutbrudd i kjøtt og grønnsaker som er kokt, melk og industrielt fremstilt barnemat. En av germinerings reseptorene til *B.licheniformis* er kodet av *yndDEF* operonet. Våre upubliserte resultater viser at Ynd germinerings reseptoren er involvert i germinering indusert av L-alanin, L-cystein og L-valin. I motsetning til andre *Bacillus* arter, som *Bacillus subtilis*, ser det ut til at *B.licheniformis* har tre homologe *yndE* gener, *yndE1*, *yndE2* og *yndE3*. Funksjonen til *yndE* genene er ukjent. Dette prosjektet har som mål å karakterisere de tre homologe *yndE* genene, for å forstå rollen de har germineringen av *B.licheniformis* sporer.

Det ble forsøkt å konstruere egne null-mutanter i alle tre *yndE* homologene, men bare delesjon av *yndE3* genet ble vellykket. Analysering av germinering i *yndE3* mutanten, indusert av L-alanin, L-cystein og L-valin, viste at mutant stammen fikk en sterkt redusert germinerings respons til L-cystein. Germinerings responsen til L-alanin og L-valin ble også svakt redusert. Dette resultatet indikerer at *yndE3* genet er avgjørende for effektiv germinering indusert med L-cystein og kan muligens tyde på at de andre *yndE* genene kan være involvert i *ynd* avhengig germinering indusert av L-alanin og L-valin.

Dette er den første funksjonelle kartleggingen av rollen til paraloge germinerings reseptor underenheter, kodet av ett enkelt germinerings respetor operon vi er kjent med.

Acknowledgements

This master thesis was performed at Department of Food Safety and Infection, Faculty of Veterinary Medicine and Bioscience, Norwegian University of Life Sciences, Oslo, from September 2014 until June 2015.

The thesis has been a project in a larger project which has involved several PhD and master students the last years, with PhD student Kristina Borch-Pedersen at present time. In this project I have gained an excellent opportunity to gain valuable knowledge regarding the principles of working in a research group, and solving challenges in plenum. The project gave me a lot of challenges, but also a great deal of knowledge. During a project there are several roles in a team, and all have their unique area of competence.

Firstly, I would like to give the attention to PhD student Borch-Pedersen for her support during the project period. Her knowledge regarding both practical and theoretical aspects of the project has been priceless. I would also like to thank her for good conversations this past year.

Also my supervisors Dr. Marina Aspholm and Dr. Toril Lindbäck deserves a good thank for their support at the practical laboratory work, and their sharing of both knowledge and support during the writing process, especially at the end of the project. Thanks to Tina O'Sullivan for all practical help on the lab, and for the valuable insight in the importance of routines when working in a research lab.

Another important aspect during this project is the social one, and I would like to thank master student Anne Kijewski and PhD student Hildegunn Iversen for good times, both inside and outside the laboratory. In relation to this, I would like to thank for a generally good working environment, and for the opportunity to finish my master's degree with this project.

My friends and family also deserves thanks for their support the last year. Last the two most important people in my life deserve the largest round of applause. My dearest husband Jostein, you are the most kind and patient man, especially during this year. And last my dear daughter Ingrid Emilie, you are my sunshine.

Oslo, June, 2015

Inger-Helene B. Aardal

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1. Introduction

The knowledge of food and drinking water contamination go back a long way in history. In the beginning of the 1900's the industrialized world still struggled with sickness related to food and water contamination. As people got aware of the importance of hygiene in food production and disinfection of drinking water, disease due to contamination of food products and drinking water has significantly decreased in the industrialized world (Kapperud, 2012). However, food and water contamination is still a major cause of disease outbreaks especially in developing parts of the world, where hygienic routines are still lacking. In developing parts of the world, food and water-borne diseases causes millions of deaths every year. In the industrialized part of the world, the hospitalization and treatment by antibiotics is sufficient in most cases, and even though the economic costs are large, the number of cases causing death and severe sickness is low (Kapperud, 2012). However, the demands and habits of consumers' in the Western world have changed, now they wish to have food with long durability, where pre heat treatments of the products has not affected the food's quality considerably. Another issue is the demand for food products low in additives as well as in sodium. Not at least, there is an increased international trade in food and agricultural products which causes spread of contaminants beyond borders (Kapperud, 2012).

To be able to produce safe food, methods to prevent contamination and growth of microorganisms during the processing and storing of food products are needed. One of the most effective ways to reduce the microbial load in foods is heat treatment, but it may affect the food quality, which in turn may affect the profit. Some bacteria are able to survive heat treatment and chemical disinfectants by formation of endospores. The bacterial endospores have a remarkable ability to survive in environments not suitable for growth and without access to nutrients. Spore forming bacteria of the genera *Bacillus* and *Clostridium* represent a group of especially challenging food contaminants.

Already in the 1800's bacterial spores were known as possible survivors of heat treatment. Cohn and Koch independently reported the discovery of bacteria that were able to grow after boiling (Torred *et al*., 2012). Later, Tyndall invented the process named Tyndallization where sequential heat treatment procedures are used to eliminate bacterial spores. The method is based on the knowledge that spores which survive the first heat treatment become vegetative cells which are effectively killed by a second heat treatment. (Torred *et al*., 2012). This has been shown to be a most effective method against many spore forming bacteria. However, it has been shown that some spore formers survive this double heat treatment, and represent a challenge to the food industry.

1.2 Genus *Bacillus*

The genus *Bacillus* belongs to the phylum *Firmicutes,* and consists of Gram-positive, endospore forming bacteria, both aerobic, facultative anaerobic and some strictly anaerobic species. The cells are rod-shaped, straight or slightly curved, and can appear as single bacterial cells, in pairs or as long filaments. They are ubiquitous in the environment and are frequently found in soil (Schraft & Griffths, 2006; Logan & De Vos, 2009).

1.2.1 Bacillus cereus group

The *B. cereus* group consists of 7 endospore forming bacterial species (Granum & Lindbäck, 2013), which primarily are aerobic and occasionally anaerobic. The cells are large (more than 0.9- 1 µm), non-swollen sporangia and forms ellipsoidal to cylindrical spores. Examples of species belonging to this group are *Bacillus anthracis* and *Bacillus cereus*, which both are important human pathogens. Another species in the *B. cereus* group is the psychrotolerant species *Bacillus weihenstephanensis* which is capable of growing at temperatures as low as 4-7 ^{o}C (Lechner *et al*., 1998; Fritze, 2002; Schraft & Griffiths, 2006; Logan De Vos, 2009).

1.2.2 Bacillus subtilis group

By description of Fritze (2002) and Hajmeer & Fung (2006) the *B. subtilis* group consists of 8 endospore forming bacteria with a diameter less than 1 µm, non-swollen sporangia, ellipsoidal spores and where most species are mesophilic and neutrophilic. Even though all species within this group display very similar 16S rRNA genes, some species demonstrate different DNA- DNA hybridization patterns.

B. subtilis has been known for a long time, and the characterization of *B. subtilis* is the most diverse among the Gram-positive bacterium (Kunst *et al*., 1997; Michna *et al*., 2013). In nature *B. subtilis* is common in soil and vegetation, and is often found in foods and feces (Kramer & Gilbert, 1989). *B. subtilis* is also considered a model organism for genetic studies (Michna *et al*., 2013).

Industrially *B. subtilis* is an important organism; one example of this is the secretion of enzymes like proteases and amylases in large amounts (Kunst *et al*., 1997).

B. licheniformis belongs to the *B. subtilis* group and is facultative anaerobic, endospore forming bacterium which has been isolated from soil, plants, from the plumage of birds and human and animal feces (Burtt & Ichida, 1999; Hoyles *et al*., 2012; Logan, 2012).

1.2.3 *B. licheniformis*

The colonies of *B.licheniformis* seems to be adherent on solid agar and microscopic examination shows the bacterial cells to appear as singles, in pairs or chains, and often give an impression of consisting of several species (Logan & DeVos, 2009). The colonies are white to cream in color or may appear yellowish when grown on blood agar (Madslien, 2013). The growth temperature may vary from 15 to 55 ^{o}C, with an optimum at 30-50 ^{o}C (Palmisano *et al*., 2001; Madslien, 2013). Growth at higher temperatures has also been reported (Warth, 1978).

Occasionally, *B. licheniformis* has been associated with disease, which will be described in section 1.3.1. *B. licheniformis* is better known for its industrial importance since it is able to produce several proteases which are used for production of biological laundry detergent, and abating and dehairing in the leather industry (Rao *et al*., 1998; Manczinger *et al*., 2003). In addition it produces the lipopetide surfactin which is a powerful antibiotic (Schallmey *et al*., 2004).

B.licheniformis demonstrates 84.5 % nucleotide identity to *B. subtilis* (Rey *et al*., 2004).Despite the high genetic similarity they differ on the ability to take up foreign DNA. As *B. subtilis* is naturally competent, this is not the case for *B.licheniformis* which transformation has been shown to be quite challenging and only possible with small plasmids (Gwinn & Thorne, 1964; Leonard *et al*., 1964). A type I restriction modification system has been identified by genome sequencing of *B. licheniformis* strain DSM 13 (Rey *et al*., 2004). This system prevents foreign DNA, which enters the cell, from being integrated into the bacterial genome. The *hsdR1* and *hsdR2* operons were suggested to encode the type I restriction system in *B.licheniformis* (Veith *et al*., 2004). These two operons were in a study of Waschkau *et al*., (2008) deleted to verify their functionality and to construct a mutant strain with better competence for uptake of foreign DNA compared to the wild type strain. The *hsdR1* and *hsrdR2* was deleted both independently and together, and mutant strains with both operons deleted demonstrated an increased transformability. The mutant strain which demonstrated the best transformation efficiency, also by *E.coli* shuttle vectors, was *B.licheniformis* MW3 (Waschkau *et al.,* 2008). By having this mutant at hand, the possibilities for transformation *of B.licheniformis* are significantly increased. This strain was used in this thesis as the “wild type” strain.

1.3 The presence of food contaminating spore formers

Bacillus species are often associated with foodborne illness and represent a challenge for the food industry. They also represent a huge problem when it comes to reduction of food quality. Large amounts of food are every year spoiled due to presence of spore forming bacteria. Microbial spoilage may appear as visible growth, structure changes or changes in flavor and odor (Gram *et al*., 2002). The contamination of food with bacterial spores represent a major challenge to all food producers and causes huge economic losses to the food industry.

The bacterial spores display several properties, in addition to their extreme thermoresistence, that makes them capable of contaminating a variety of food products. Andersson *et al*. (1995) refers to the fact that spore forming bacteria are highly frequent in soil and therefore very hard to eliminate as food contaminants. In soil, the amount of spores can reach 10^5-10^6 spores/g. Another important feature, described in *B. cereus* spores, is their ability to adhere to surfaces, as in production areas. One of the reasons for this strong adhesion is their high surface hydrophobicity (Andersson *et al.,* 1995).

Ultrahigh heat treatment (UHT) and different sterilization techniques have are often less effective towards spores, as at least some bacteria seems to survive these procedures as well. Clearly, as the techniques for killing the spores and preventing growth of vegetative cells improve, the bacteria alter their surviving mechanisms to overcome the physical obstacles they are exposed to (Heyndrickx, 2011). Heyndrickx (2011) also describes the increasing amount of ready-to-cook, ready-to-eat food products with extent durability (REPFED's) which have been heat treated at lower temperatures (65-95 ^{o}C) to reduce quality loss, but where the bacterial spores may survive.

B. cereus is often associated with food contamination. Although *B. cereus* is often associated with food spoilage there are also avirulent strains which serve as probiotics (Granum & Lindbäck, 2013).

B. cereus has been isolated from a large variety of foods like vegetables, rice, pasta and spices, which are products where cross contamination easily takes place. Dairy products are also often contaminated by *B. cereus* due the survival of *B. cereus* spores during heat treatment leading to germination when conditions are favorable for growth again (Kramer & Gilbert, 1989; Granum & Lindbäck, 2013).

As previously mentioned, there are many species which belong to the *B. cereus* group and they are all able to produce enterotoxins, although many species/strains do not produce detectable amounts (Granum & Lindbäck 2013). *B. cereus* is able to produce the enterotoxins hemolysin BL (Hbl)(Beecher &

Macmillan, 1991), the non- hemolytic enzyme (Nhe) (Lund & Granum, 1996), cytotoxin K (Cyt K) (Lund *et al*., 2000), and an emetic toxin named cerulide. The enterotoxin cause abdominal pain and diarrhea, while the emetic toxin causes vomiting, malaise and nausea (Kramer & Gilbert, 1989; Granum & Lindbäck, 2013).

There is less food poisonings reported related to the *B. subtilis* group compared to the *B. cereus* group. However, some cases have been linked to *B. subtilis*, *B.licheniformis* and *B. pumilis* (Salkinoja-Salonen *et al*., 1999; From *et al*., 2007; Logan, 2012). However, there are no identification routines for detecting these species in food products as there is for the more common contaminant *B. cereus*. This may easily lead to an underestimation of incidents related to the *B. subtilis* group (Madslien, 2013). In the few cases of sickness reported to be caused by *B. licheniformis,* the symptoms are milder compared to *B. cereus*, characterized by short incubation time, high infective dose and symptoms being present for only a short period of time (Madslien, 2013).

B. licheniforms has been isolated from a variety of different foods including ice cream, deserts, dairy products, dried milk (Rückert *et al*., 2004 ; Postollec *et al*., 2012), dried food ingredients (Postollec *et al*., 2012), traditional cacao fermentation (Schwan *et al*., 1986), pasteurized meat (Logan, 2012) and canned food (Postollec *et al*., 2012). *B. licheniformis* is able to survive in dairy products during pasteurization, and was in a study by Lücking *et al*., (2013) shown to be one of the dominant species surviving heat treatment (100^{o}C in 20 min). One of the most fatal outbreaks of illness related to *B.licheniformis* was associated to dried baby-milk powder, where a fatal case was reported (Salkinoja-Salonen *et al*., 1999).

1.3.1 Virulence factors in *B. licheniformis*

A known virulence factor in *B.licheniformis* is the production of the cyclic nonribosomally synthesized lipoprotein (NRP), lichenysin (Mikkola *et al*., 2000). The NRP's are a large group of lipopetides/peptides which are synthezised on large ribosomes. Most of the NRP's are partially or completely cyclic, branched or polycyclic, and many of the NRP's are non-proteinogenic amino acids (Caboche *et al*., 2010). Many of the NRP's have beneficial uses, as antibiotics (daptomycin (Tally *et al.*, 1999)), antitumor drugs (bleomycin) and as surfactants (Caboche *et al*., 2010). A large majority of the NRP's are produced by species of the genus *Bacillus* (Madslien, 2013).

Lichenysin is together with surfactin and pumilacidin in the group surfactins (Arima *et al*., 1968; Yamikov *et al*., 1999; From *et al*., 2007). The mechanisms of damage by the surfactins are based on membrane

damages by pore formation and destabilization of lipid membranes membrane which leads to cell lysis in the end (Maget-Dana & Ptak, 1995).

Lichenysin A was isolated from the food poisoning outbreak associated to *B. licheniformis* in dried baby-milk powder (Salkinoja-Salonen *et al*., 1999; Mikkola *et al*., 2000). Mikkola and coworkers (2000) showed that the toxicity of lichenysin A differ from that of cerulide and valinomycin produced by *B. cereus* and *Sterptomyces griseus*, by not damaging the mitochondrial membranes, but rather causing damage to the cell membrane. In the same study, the heat stability of lichenysin A was shown as well (Mikkola *et al*., 2000).

1.4 Bacterial spores

The spore morphotype is a survival mechanism found in many Gram positive bacteria of the phylum *Firmicutes*, and the spore formation is induced by environmental changes of a major character, such as nutrient starvation by lack of carbon, nitrogen or sometimes phosphorous (Piggot & Hilbert, 2004). The sporulation process involves morphological changes and altered gene expression and results in the production of metabolically dormant, non-reproductive, and highly resistant spores. The remarkable resistance of bacterial spores towards a large number of chemical, physical and environmental challenges is unique. They can withstand, both wet and dry heat, especially dry heat. The heat resistance is affected by the sporulation temperature (Fox & Eder, 1969; Condon *et al*., 1992). Spores are also 10 to 50 times more resistant towards UV-radiation and γ-radiation than growing cells (Setlow & Setlow, 1988). Spores may also survive multiple freezing's, cycles of desiccation, and they are resistant to many chemical disinfectants (Setlow, 2006; Nicholson *et al*., 2000).

The spore consist of several layers including the exosporium, spore coat, outer membrane, cortex, germ cell wall, the inner membrane and the innermost core (Figure 1) (Setlow, 2006).

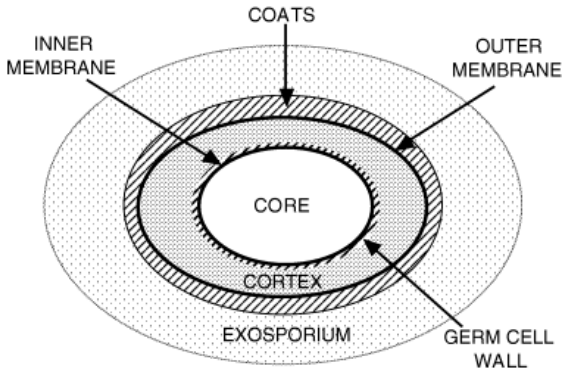

Figure 1. *The structure of a bacterial spore (Setlow, 2006). Reprinted with permission from John Wiley and Sons.*

The outer layer differs between spore formers; some species have exosporium (*B. cereus* and *B. anthracis*) while others do not (*B. subtilis*). In some cases, the exosporium is hard to differentiate from the rest of the spore structure. Species lacking the exosporium does not express the same number of exosporial proteins as the ones carrying exosporium (Aronson, 2012).

Beneath the exosporium layer is the spore coat, which in *B. subtilis* contains more than 50 spore coat proteins. Even though many of the coat proteins have been identified, little is known about their function (Lai *et al*., 2003). However, they have been reported to prevent hydrolyzation of the spore cortex by protecting the inner structures from lytic enzymes (Nicholson *et al*., 2000). In *B. subtilis,* mutants lacking the spore coat loose a significant part of their resistance towards lyzosyme. The spore coat protein CotE, is made early in the sporulation process and contributes to the spore coat assembly (Driks *et al*., 1994; Driks, 1999). The spore coat also functions as a permeable barrier where only smaller molecules get through. The size of molecules able to penetrate the spores varies between the different *Bacillus* species (Driks, 1999, Scherrer *et al*., 1971).

The outer spore membrane lies beneath the spore coat, and whether this is a functional membrane in dormant spores is not clear. However, Nicholson *et al*., (2000) reported that the function of the outer membrane was similar to the function of the spore coat.

The spore cortex is a peptidoglycan wall located between the inner and outer spore membranes. It consists of two distinct layers, one thin layer of germ cell wall synthesized at the inner spore membrane, and a thick peptidoglycan (PG) layer outside the germ cell wall. The cortex is important for maintaining the dehydrated state of the spore core and for maintaining the resistance towards environmental stressors (Popham, 2002). During spore germination the germ cell wall also remains as the cell wall in the resulting vegetative cell (Leggett *et al*., 2012), while the PG layer is degraded (Arith *et al*., 1998).

The inner spore membrane, which has a lipid composition similar to the plasma membrane of vegetative cells, constitutes a nearly impermeable barrier. It protects the core from chemicals, like DNA damaging compounds (Cortezzo & Setlow, 2005). Lipid probes attached to the inner membrane indicate a significantly compressed inner membrane structure during dormancy and an increased mobility of the lipids during germination (Cowan *et al*., 2004).

The spore core is in many ways composed in the same way as the protoplast of a growing cell, despite the fact that it has a water content which is lower than in a vegetative cell (25-50% in the spore core versus 75-80 % in the growing cell) (Nicholson *et al*., 2000). I addition, the core consists of a variety of enzymes, ribosomes, minerals, DNA and tRNA, most of which are also to be found in the growing cell (Setlow, 2006). However, the large amount (5-15 % of spore dry weight) of pyridine-2,6-dicarboxcylic acid (DPA) (Setlow, 2006) is only present in the spore core but not in the growing cell. The structure of DPA is shown in Figure 2. In the spore core, the DPA exists in a 1:1 chelate with divalent cations , mainly Ca^{2+}, but other cations may also be present, like H^{+}, Na^{+}, Mg^{2+} and Mn^{2+} (Nicholson *et al.*, 2000; Setlow, 2006).

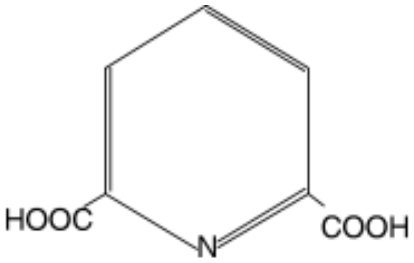

Figure 2. *Structure of pyridine-2,6-dicarboxcylic acid (DPA) (Setlow, 2003). Reprinted with permission from John Wiley and Sons.*

The small, acid soluble proteins (SASP) are also important constituents in the spore core. A high variety of α-/β- type SASP are found in all *Bacillus* spores (Setlow, 1992). The SASP's bind to the double helix DNA until saturation and induce a change in conformation of the DNA helix (Nicholson & Setlow, 1990).

1.4.1. Sporulation

B. subtilis is the most studied species regarding sporulation (de Hoon *et al*., 2010). And most genetic pathways described in sporulation is based on studies of *B. subtilis* (Barák *et al.,*2005; de Hoon *et al*., 2010; Higgins & Dworkin, 2012). However, analysis of sporulation in other *Bacillus* and *Clostridium* species has also generated valuable knowledge (deVries *et al*., 2004; Paredes-Sabja *et al*., 2009; Burns & Minton, 2011). Spore formation is regarded the last chance for the bacteria to survive, often after making other attempts to survive for example by taking up foreign DNA, or by cannibalism (González-Pastor *et al*., 2003; Veening *et al*., 2006).

1.4.2. Morphological changes during sporulation

The sporulation is initiated with the asymmetric division of the cell into a smaller forespore and a larger mother cell (Robleto *et al*., 2012).The DNA of the forespore is only partly located in the right forespore compartment, and the DNA translocase SpoIIIE is needed for the complete chromosome to enter the forespore (Wu & Errington, 1997). Further on, the mother cell engulfs the forespore, and a second membrane, surrounding the forespore, is made by a phagocytic-like process (Morlot *et al*., 2010). Then, the cortex is synthesized between the membranes surrounding the forespore (stage III to IV) (Popham, 2002). Thereafter, the highly DNA protective α-/β-SASP are expressed in the forespore (Robleto *et al.,* 2012). The outer proteinaceous coat outside the membrane is then formed (Henriques & Moran, 2007) and the next steps (steps V to VI) consist of dehydration of the spore core, synthesis of DPA, and uptake of divalent cations. At the end of the sporulation process (stage VII) the mother cell lyses and the spore is released (Setlow & Johnson, 2013). A schematic overview of the morphological changes during sporulation is shown in Figure 3.

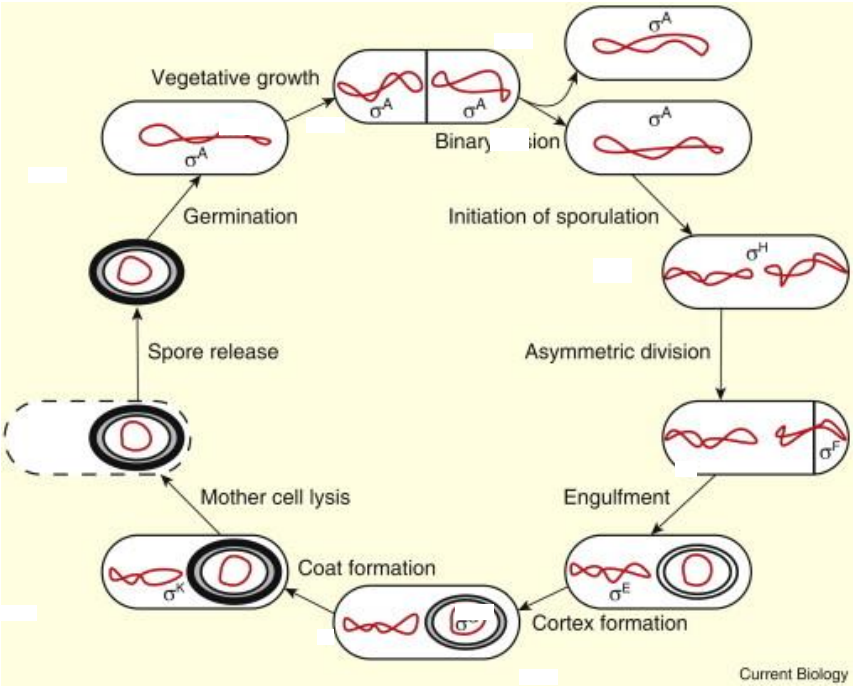

Figure 3. *An overview of the morphological changes occurring during sporulation* (modified from *De Hoon. et al., (2010). Reprinted with permission from Elsevier.*

1.4.3. Genetic regulation of sporulation

The regulation of gene expression during the sporulation is complex and involves several sigma factors and *spoO* gene products.

The sporulation is regulated by the six sigma factors, σ^A, σ^H ,σ^F, σ^E, σ^G and σ^K. The genetic regulation involves a close communication between the sigma factors, which operate both in the mother cell and in the forespore compartments (Hilbert & Piggot, 2004; Robleto *et al.,* 2012). Germination genes are regulated by σ^G (Robleto *et al*., 2012).

The initiation of the sporulation process involves the activation of the spo0A gene through phosphorylation. Spo0A is a DNA binding protein involved in the direct regulation of 121 genes (Molle *et al*., 2003). Spo0A is activated by being phosphorylated by Spo0F which transfers of the phosphate group via Spo0B to Spo0A (Setlow & Johnson, 2013).

The SpoVA proteins, regulated by σ^G, has been identified as important genes for transport of DPA (Tovar-Rojo *et al*., 2002; Li *et al*., 2012). By studying the transportation of $DPA_{2,6}$ and Ca^{2+}-$DPA_{2,6}$, Li and coworkers (2012) found that the SpoVAD protein is especially important in the uptake of $DPA_{2,6}$ and Ca^{2+}-$DPA_{2,6}$, due to its high affinity for both substances.

Recent studies have also revealed that the σ^G controlled gene, *ylyA* , which encodes an RNA polymerase binding protein, affects the efficiency of germination (Traag *et al*., 2013).

1.5 Germination

When the environmental conditions are favorable, the bacterial spores may break the dormant state and start metabolic processes. This process is called germination and involves a cascade of cytological changes and alters the chemical composition of the cell. Even though the spores have no measureable metabolism they constantly monitor the environment to be alert if the conditions changes and growth is possible again. Within minutes from when spores senses signals, indicative of favorable growth conditions, the germination process is irreversibly induced (Christie, 2012). The germination process is initiated when spores sense various germinant ligands which interact with germination receptors localized in the spores inner membrane (Paidhungat & Setlow, 2001; Hudson *et al*., 2001, Alberto *et al.*, 2005). To interact with the germination receptors the germinants need to migrate through the spores outer membrane and cortex (Christie, 2012).

There are a variety of germinants, both nutrient and non-nutrient, but in the environment the germination is usually initiated by the presence of nutrient germinants. Molecules such as single amino acids, sugars, purine nucleosides, or more complex nutrients like a mix of L- asparagine, glucose, fructose and K+ (AGFK) have been shown to trigger germination in *B. subtilis* (Setlow, 2003). Spores of different *Bacillus* species have been shown to initiate germination in response to different germinant compounds (Xiao *et al*., 2011).

What germinants different species responds to depends on the set of germination receptors expressed by the individual species (Gould, 1970). The nutrient germination receptors interact with nutrient germinants in a stereospecific manner. The amount of different germination receptors may vary between species ranging between 2 and 8. Most germination receptors are encoded by tricistronic *ger* operons encoding so called A, B and C subunits (Zuberi *et al*., 1985; Setlow, 2003; Paredes-Sabja *et al*., 2011). The A subunit consists of 5 or 6 transmembrane helices, and also a large hydrophilic N-terminal domain and a small hydrophilic C-terminal domain, while the B subunits on the other hand, consists of ten transmembrane helices. The C subunit is a lipoprotein anchored to the inner membrane by a lipid moiety in the N-terminal end (Moir *et al*., 2002; Christie, 2012). Comparison of the secondary structures of the different GR subunits from different species shows that their structures are highly conserved (Paredes-Sabja *et al*., 2011). Figure 4 illustrates the localization of the GR subunits in the inner membrane.

Another newly discovered feature of the GR is the fourth D subunit found in some species of both *Bacilliales* and *Clostridiales*, which seems to be connected to the other GR's but the function of this subunit is still unknown (Paredes-Sabja *et al*., 2011).

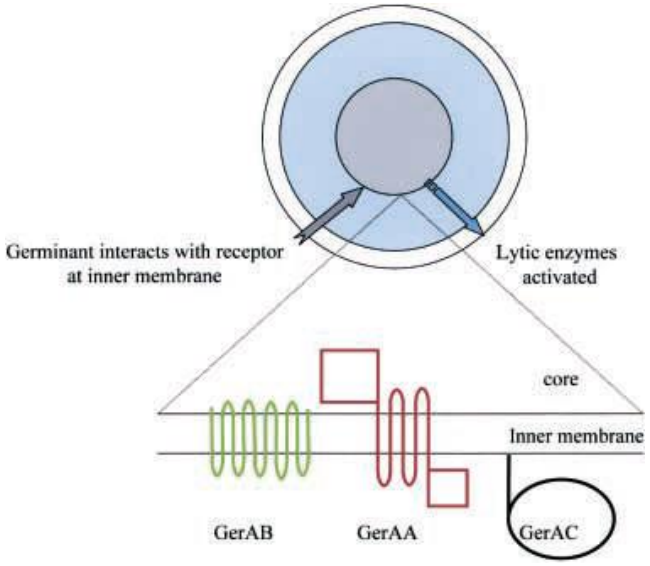

Figure 4 . *Localization of the GerA germination receptor subunits in the spore inner membrane of B. subtilis (Moir et al., 2002). Reprinted with permission from Springer.*

1.5.1 Stages during germination

Germination receptor mediated germination involves a cascade of physiological changes leading to reactivation into the vegetative state (Figure 5). In the first stage, the commitment to germinate is initiated. This activation includes the binding of nutrients to their specific GR. The germination process becomes irreversible within a short period of time and proceeds even if the germinants are removed (Stewart et al., 1981; Yi & Setlow, 2010). Next, the spores release their large pool of DPA and monovalent cations (H^+, K^+ and Na^+). This increases the pH in the spore core and starts the metabolism (Swerdlow *et al*., 1981). Changes in the inner membrane permeability makes partial core rehydration possible which reduces the spores heat resistance somewhat (Setlow *et al*., 2001).

The second stage of germination involves hydrolysis of the spore cortex which allows the spore core to expand and re-hydrate to levels found in vegetative cells. The loss of the cortex peptidoglycan layer and the increasing core hydration causes the spore to loose even more resistance and it is no longer defined as a dormant spore (Setlow, 2003). Cortex lytic enzymes (CLEs), with specifity for peptidoglycan containing muramic-δ-lactam, hydrolyze the spore cortex (Atrih *et al*., 1998; Setlow & Johnson, 2013). In *Bacillus subtilis* spores, there are two CLEs*, CwlJ* and *SleB*. CwlJ also functions in sporulation and in the synthesis of peptidoglycan containing muramic-δ-lactam (Ishikawa *et al*., 1998; Moir, 2006).

The set of cortex-lytic enzymes (CLE) or enzymes involved in cortex hydrolysis varies between different *Bacillus species*. The CLE's of *B. subtilis* are synthetized in the mother cell and in the forespore, and only during the sporulation process (Setlow, 2003).

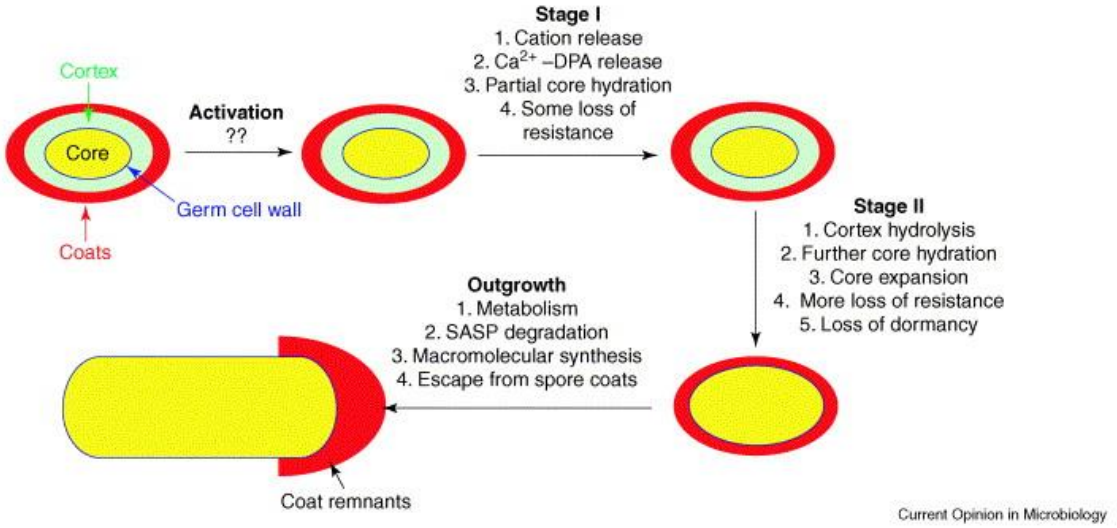

Figure 5. *Spore germination (Setlow, 2003). Reprinted with permission from Elsevier.*

During the 3rd (outgrowth) stage the enzyme activity and metabolism is high. The SASP molecules are inactivated and synthesis of amino acids is now possible. The synthesis of RNA starts, and the early RNA made consists, to a large extent, of mRNA's. The protein synthesis also starts early during the outgrowth, and is made possible by the presence of amino acids released during the degradation of SASP proteins. However, it is important to notice that exogenous nutrients are necessary for a complete outgrowth. Except for DNA repair, the synthesis of new DNA is not prioritized during early outgrowth (Setlow & Johnson, 2013).

1.5.2 Superdormant spores

An important feature to consider in the case of germination is the presence of spores that do not respond effectively to the nutrient germinants the other spores in the population respond to. This feature has been known for decades (Gould, 1970). Research has shown that spores that respond poorly to germinants, only activating a small amount of the germination receptors. By increasing the concentration and/or alter the mixture of germinants, a larger proportion of the germination receptors will be triggered, and the germination response will consequently increase (Gosh & Setlow, 2009).

The sporulation conditions seem to play a role in the formation of superdormant spores, but there is, so far, much more theories than evidence on why some spores become superdormant (Ghosh & Setlow, 2009).

1.5.3 Non-nutrient germination

The germination may be triggered by other processes than through the interaction between germinants and germination receptors. For example, lysozymes may induce germination in spores in which the spore coat has been removed (Setlow, 2003). The addition of exogenous Ca^{2+}-DPA may also induce germination by activating the CwlJ cortex lytic enzyme (Paidhungat *et al*.,2001). Exposure of spores to high pressure can activate spore germination; in *Bacillus subtilis* spores, pressures under 500 MPa induces germination through a process which is dependent on the presence of germination receptors. Pressure over 500-600 MPa activates germination by a process that is independent of germination receptors. Here, triggering of Ca^{2+}-DPA release seems like a likely mechanism, but this has not been proven (Wuytach *et al*., 1998).

Molecules such as metal ions, bicarbonate, n-Dodecylamine, initiator proteins and spore enzymes have also been shown to induce germination (Gould, 1970).

1.5.4 Germination receptors

The germination receptors present differs between *Bacillus* species and individual GR's have distinct ligand specificities. A lot of the research on GR's has been done on *B. subtilis*, and *B. subtilis* 168 is regarded as a model organism for studying spore germination mechanisms. The GR's and their components was first identified in *B. subtilis* where the *gerA* GR was found to consist of three subunits, named *gerAA*, *gerAB* and *gerAC* (Zuberi *et al*., 1985). Later on, the *gerA* GR homologoues *gerB* (Corfe, 1994) and *gerK* (Irie *et al*., 1996) were identified in *B. subtilis*. Later on several GerA protein orthologues have also been identified in other *Bacillus* and *Clostridium* species (Clements & Moir, 1998; Barlass *et al*., 2002; Ireland & Hanna, 2002; Paredes-Sabja *et al*., 2011; Xiao *et al*., 2011). Not all GR are encoded in tricstronic operons though. Some GR consists only of the A subunit. This is a feature seen in many *Clostridium spp*. In *Bacillus spp*., polycistronic operons are more common (Paredes-Sabja *et al*., 2011). But the variation is large, and GR containing several copies of one subunit have also been found during analysis of spore forming bacterial genomes (Paredes-Sabja *et al*., 2011; Christie, 2012).

One example of this is *B.licheniformis* which carries the a pentacistronic *ynd* operon with the gene organization *yndD*, *yndE3*, *YndE2*, *yndF*, *YndE1* (encoding GR A-, B-, B-, C-, B- subunits, respectively) (Figure 6) (Veith *et al.,* 2004).

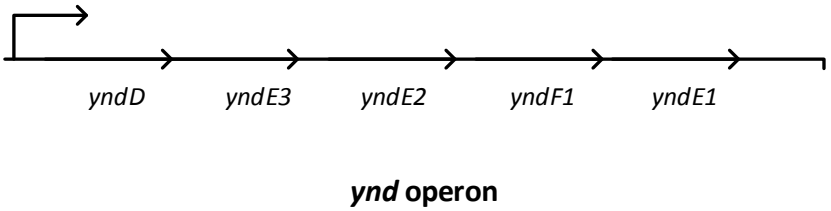

Figure 6. *Schematic overview of the ynd operon in B. licheniformis.*

Many GR's, including GerA, encoded by tricistronic operons, have indicated that deletion of one of the three cistrons gives a non-functioning GR. However, there are exceptions; like the *gerK* operon in *C. perfringens* which functions without a functioning B subunit (Paredes-Sabja *et al.*, 2009).

1.5.5 Germination in *B.licheniformis*

Sequence analysis of *B. licheniformis* ATCC 14580/DSM13 identified the *gerA*, *gerK* and *ynd* operons (Rey *et al*., 2004; Veith *et al*., 2004). In addition to these three GR operons, there is also an orphan *yndF2* gene (Madslien, 2013). It has been long known that *B.licheniformis* germinates in response to L-alanine, L-cysteine and L-valine, with the strongest germination response in the response to L-alanine (Martin & Harper, 1963). The germination response to L-alanine and L-cysteine and L-valine has also been demonstrated in later studies, with the strongest response to L-alanine and L-cysteine (Borch-Pedersen *et al*., unpublished results).*B. licheniformis* germination is inhibited by the presence substances like various salts, ethyl pyruvate and octyl alcohol (Halmann & Keynan, 1962).

The GRs in *B. licheniformis* have partly been functionally characterized. Løvdal *et al*., (2012) showed that the *gerAA* receptor initiate germination in response to L-alanine. Later on, the disruption of *gerAA* was shown to inhibit germination induced by L-alanine, L-cysteine and L-valine (Borch-Pedersen *et al*., unpublished results). Similarly, deletion of *yndD* also inhibited germination in response to L-alanine, L-cysteine and L-valine (Borch-Pedersen *et al*., unpublished results). This suggests that the y*ndDEF* operon functions in nutrient induced germination in *B.licheniformis*, which is distinct from *B. subtilis* where

disruption of the *yndDEF* operon had no effect on the germination (Paidhungat & Setlow, 2000). In contrast to *B. subtilis*, where *gerK* together with *gerB* induced germination by AGFK (Alturi et al., 2006), a mixture of AGFK had no effect on germination in *B.licheniformis* (Borch-Pedersen *et al.*, unpublished results). This may be seen in correlation with the lack of a GerB GR in *B. licheniformis*.

1.6 Aim of study

In 2004, there was an incident in Norway where a batch canned meat was spoiled by *B.licheniformis* NVH1032, but no illness due to this was reported. The spores had survived heat treatment by Tyndallization, and demonstrated a very slow germination rate. This leads to our interest in studying spore germination in *B.licheniformis*. The presence of three paralogous *yndE* genes in *B.licheniformis* opened questions regarding their functional role. This is also interesting considering the lack of knowledge on the function of paralogous germination receptor subunits, also in other species. Here, the role of the *yndE* gene homologues in germination will be investigated by knockout mutagenesis followed by measurements of the mutant spores germination abilities. We also aim to analyze the expression of *yndE* genes by quantitative Real Time PCR, and Western blot.

2. Method and materials

To study the function of the three germination receptor subunits *yndE1*, *yndE2* and *yndE3* (GenBank accession number: AAU23702, AAU23701 and AAU23699, respectively) in germination of *Bacillus licheniformis* spores several methods were used. Deletion mutants of the GR genes were created, using the markerless gene replacement method (Janes & Stibitz, 2006) with minor modifications, and optical density measurements were used to follow the germination process. Expression of germination receptor genes was examined by quantitative Real Time PCR (qRT-PCR). SDS-PAGE gel electrophoresis was used to separate the different proteins present in the spores based on their size. Western blot analysis, using antibodies raised against specific peptides in the *yndE* subunits, was performed in an attempt to visualize the expression of the GR subunits. Materials used, buffers and media made during this thesis are listed in Appendix I and II, respectively.

2.1 Construction of deletion mutants

Bacterial strains and plasmids

Bacterial strains and plasmids used for the construction of the deletion mutants, and bacterial strains constructed during the thesis are listed in Table 1. The plasmids are also displayed in Appendix III.

***Table 1**. Strains and plasmids used for construction of the deletion mutants*

Strains	**Description**	**Reference**
***B.licheniformis* MW3 (1289)**	*B.licheniformis* DSM13 Δ*hsdR1* and Δ*hsdR2*.	Waschkau *et al*., 2008
***B.licheniformis* MW3Δ*yndE3* (1369)**	Deletion of *yndE3* in *B.licheniformis* MW3	This thesis
Plasmids	**Description**	**Reference**
pBKJ223	Plasmid producing the *I-SceI* enzyme.	Janes & Stibitz 2006
pCR 2.1 TOPO	Cloning vector used for the PCR products.	Invitrogen™
pMAD-*I-SceI*	Shuttle vector carrying the *I-SceI* site	(Arnaud *et al*., 2004)/ Dr. Anette Fagerlund, University of Oslo, Norway.

Construction of plasmid for use in the markerless gene replacement method:

- To create the *yndE* deletion mutants the up-and down-stream regions of the *yndE* gene were amplified using high-fidelity fusion PCR.
- The up-and down-stream regions were fused by high-fidelity fusion PCR using the A and D primers and the up-and-down-stream fragments as template DNA. The resulting PCR fragments were treated with *Taq* polymerase (VWR) and dNTP's (VWR) to add a polyA tail by amplification by PCR.
- The resulting nucleotide fragment was cloned into a pCR 2.1 TOPO vector (Invitrogen™).
- The TOPO vector was digested with *EcoRI*, separated on agarose gel, excised from the gel, and isolated using QIAquick® Gel Extraction Kit (QIAGEN) (Appendix IX).
- Finally, the fused flanking regions were cloned into pMAD-*I-SceI*.

Markerless gene replacement:

- pMAD-*I-sceI* with insert was isolated using QIAprep Spin Miniprep Kit (QIAGEN) (Appendix IX) and transformed into electrocompetent *B.licheniformis* MW3.
- *B.licheniformis* carrying pMAD-*I-sceI* incorporated in the genome was isolated by QIAprep Spin Miniprep Kit (Appendix IX), and made electrocompentent. The *B.licheniformis* strains carrying the pMAD-*I-sceI* were thereafter, transformed with the plasmid pBKJ223 which encodes the *I-sceI* restriction enzyme.
- The restriction enzyme *I-sceI* encoded by pBKJ223, create a double strand break in the *I-sceI* site in the genome. The repair of this double strand break may lead to either reversal to wild type genotype, or excision of the *yndE* gene only leaving the start and stop codon of the deleted gene.

A schematic overview of the markerless gene replacement is illustrated in Figure 7.

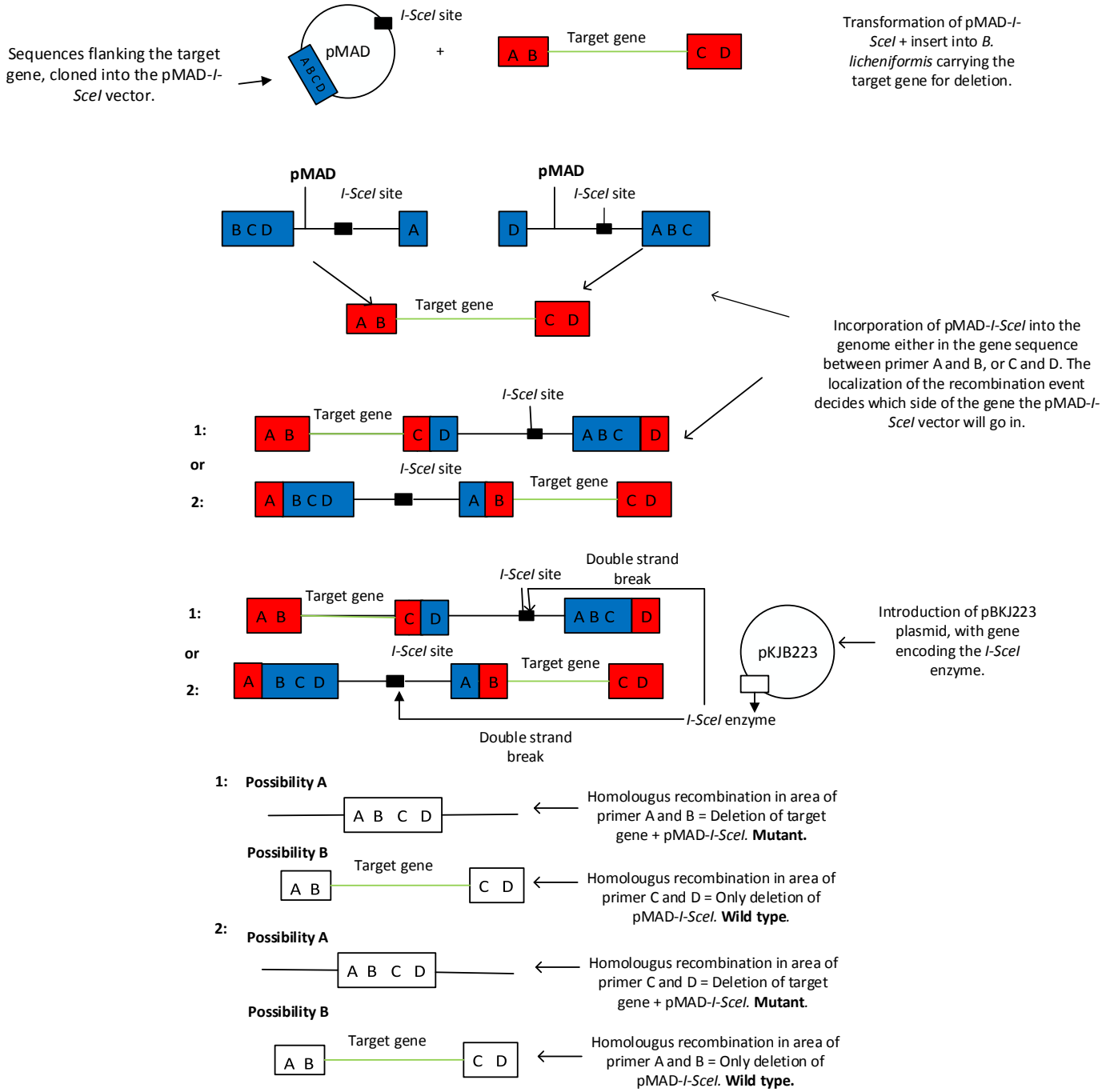

Figure 7. *A schematic overview of the markerless gene replacement procedure. The procedure results in either reversal to wild type, or deletion of the target gene only leaving the start and stop codon on the chromosome.*

2.2 Construction of pMAD-*I-sceI* carrying fused up-and down-stream regions of the gene deletion

2.2.1 DNA extraction

Genomic DNA from *B. licheniformis* was extracted using the protocol from Pospiech & Neumann (1995), with some modifications.

Briefly, *B.licheniformis* MW3 was streaked out on blood agar plates and incubated at 37^{o}C overnight. A single colony was isolated from the plates and cultured in 10 mL BHI overnight. The bacterial suspension was pelleted by centrifuging 6 mL suspension at 4500 rpm for 15 min. The supernatant was drained off, and the pellet was resuspended in 495 µl SET buffer, 50 µl lysozyme (100 µg/mL) and 10 µl RNase (10µg/mL) and incubated further for 1 hour at 37^{o}C.

After 1 hour, 50 µl (10 %) SDS and 5 µl Proteinase K (25 mg/mL) were added and the mixture was incubated at 55 ^{o}C for 2 hours. Following the incubation, 200 µl (5M) NaCl and 700 µl chloroform was added, and the mixture was incubated at room temperature for 30 minutes with inversions every 5 minute. The solution was then centrifuged for 25 minutes at 15.700 x g. After centrifugation, the aqueous phase on top was carefully transferred into a clean Eppendorf tube, precipitated with an equal amount of isopropanol, and centrifuged further at 15.700 x g for 10 minutes. After centrifugation, the pellet was washed in 100 µl (70 %) ethanol and dried at room temperature. The DNA was resuspended in 100 µl distilled H_2O (dH_2O) and frozen at -20 ^{o}C for later use.

The concentration of genomic DNA, isolated from MW3 (1289), was measured on NanoDrop, N1000 Spectrophotometer (Thermo Fisher Scientific). Genomic DNA was used as template in the PCR reactions amplifying upstream and downstream regions of the desired gene.

2.2.2 Primer design

The primers used for amplification of regions upstream and downstream of *yndE1*, *yndE2* and *yndE3* were designed by PhD student Kristina Borch-Pedersen, who also assisted in the design of the internal primers for the *yndE* gene homologues (List of primers: Table 4, Appendix III).

2.2.3 Construction of inserts by high fidelity fusion PCR

The flanking regions inserts made for deletion of each of the *yndE* genes, consisted of the up- and down-stream regions of the desired gene, which were fused together at the location of the start and stop codon of the deleted gene (Figure 8).

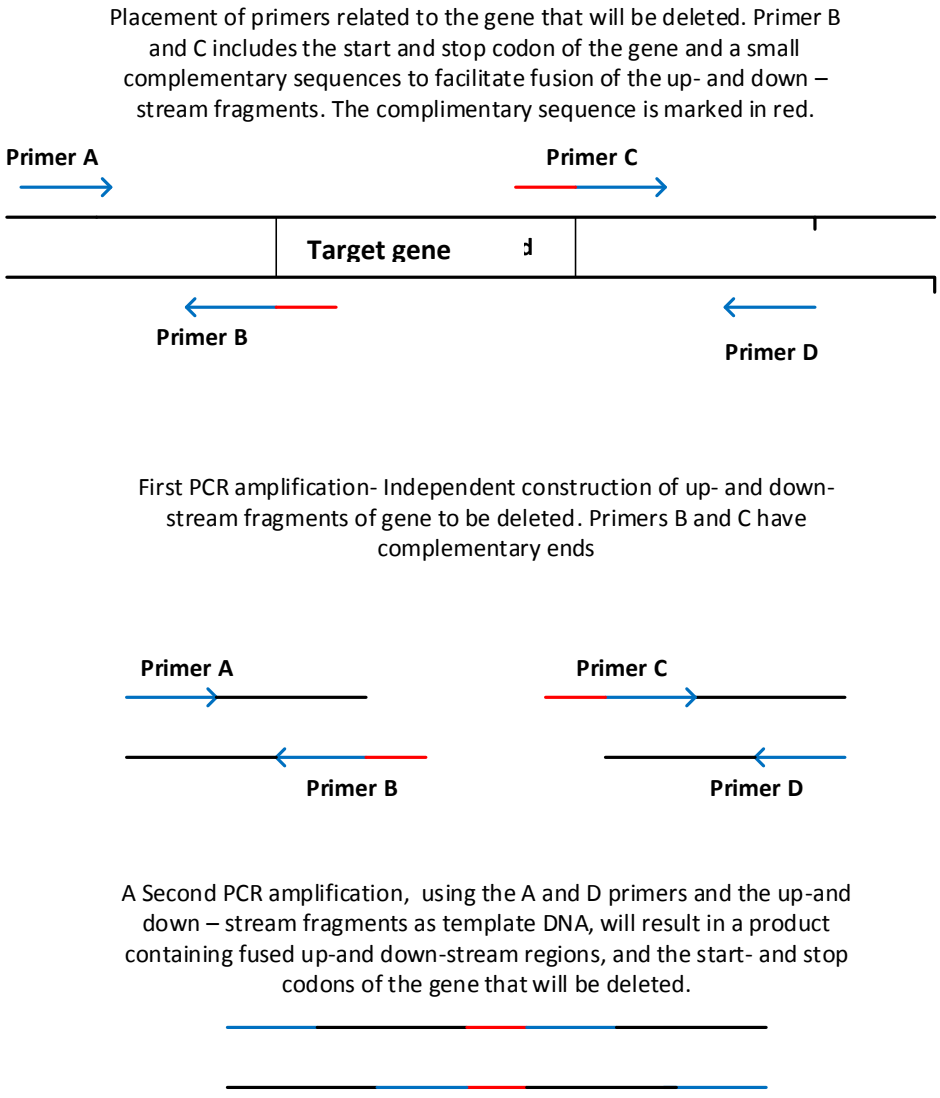

Figure 8. *Primer design for creation of the up-and down-stream fragments of the deletion gene, and fusion of the up- and down-stream fragments into one insert of flanking regions of the deletion gene.*

First, up- and down-stream regions of the gene were amplified separately by high fidelity fusion PCR, AccuPrime™ *Pfx* DNA Polymerase, (Invitrogen™) using A – B primers and C-D primers, respectively, with *B.licheniformis* MW3 genomic DNA as template in both amplifications. After PCR amplification, the resulting products were separated on a 1% agarose gel at 95 V for 40 minutes. TAE buffer were used for all agarose gel separations. The A-B and C-D PCR products were cleaned using the QIAqiuck® PCR Purification Kit (Appendix IX). The up- and down-stream PCR fragments were fused by high fidelity fusion PCR using the A and D primer pair, and the PCR products AB and CD as template DNA.

The B and C primers had, as shown in Figure 8, overlapping sequence enabling fusion of the up-and down-stream fragments.

PCR reaction mix:

5 µl - 10X AccuPrime™ *Pfx* Reaction mix (Invitrogen™)

1.5 µl - Primer 1 (Sigma-Aldrich)

1.5 µl – Primer 2 (Sigma-Aldrich)

1.0 µl Template DNA

0.5 µl AccuPrime™ *Pfx* DNA Polymerase (Invitrogen™)

40.5 µl – dH_2O

50 µl in total.

PCR conditions

Step	Temperature (°C)	Time
1 – Initial Denaturation	95	2 minutes
2 – Denaturation	95	15 seconds
3 – Annealing	55-64	30 seconds
4 – Extending	68	1 min per kb
5 – Final extension	68	5 minutes
6 – End cycle	4	∞
Step 2 -4 was repeated 30 times		

The annealing temperatures and the number of repeated denaturation, annealing and extension steps were optimized individually for the different PCR reactions used during creation of the different *yndE1*, *yndE2* and *yndE3* gene deletion constructs.

After construction of the insert, encompassing the fused *yndE* flanking regions, the insert was mixed with 0.5 µl *Taq* polymerase (VWR) and 0.5 µl dNTPs (VWR) and incubated at 72 °C for 15 minutes to construct the sticky, poly A tails at the 3' ends.

2.2.4 Cloning

The completed PCR product (AD) with sticky ends was cloned into the pCR 2.1 TOPO vector (Invitrogen™) and transformed into chemically competent *E.coli* One Shot TOP10 (Invitrogen™). The pCR 2.1 TOPO vector is shown in Appendix IV.

The ligation mix used for the cloning into pCR 2.1 TOPO vector:

2 µl PCR fragment

0.5 µl Salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) (Invitrogen™)

0.5 µl pCR 2.1 TOPO vector

The solution was incubated at room temperature for 5 minutes.

The *E.coli* One Shot TOP10 cells were thawed on ice for 10 minutes, and 2 µl of the ligation mix was added to the *E.coli* cells, mixed and incubated on ice for 30 minutes. The cells were then heat shocked for 30 seconds at 42 °C, incubated on ice for two minutes, 500 µl of room tempered, S.O.C. medium (Invitrogen™) was added and the mixture was incubated at 37 °C for 1 hour.

E.coli transformants carrying the TOPO vector with insertion were spread on LB plates containing 40 µl X-gal and 100 µg/mL ampicillin for screening. The plates were incubated at 37 °C overnight.

After incubation at 37 °C overnight, white colonies showing a non-functioning *lacZ* gene was checked for correct insert by PCR, using the A and D primer pair. A colony carrying insert of correct size was restreaked on LB plates containing 100 µg/mL ampicillin plates and cultured in 10 mL BHI for isolation of plasmid DNA. The TOPO vector with insert was isolated from *E.coli* by QIAprep Spin Miniprep Kit (QIAGEN),(Appendix IX).

2.2.5 Isolation of the pMAD-*I-SceI* vector from *E.coli*

The pMAD-*I-sceI* vector is a thermosensitive shuttle vector replicating both in *E. coli* and *B. licheniformis* (Arnaud *et al*., 2004). An extra restriction site, *I-sceI*, was inserted in the vector by Dr. Anette Fagerlund (University of Oslo, Norway) to make the second crossover occur at a higher rate. The pMAD-*I-sceI* vector is a kind gift from Dr. Anette Fagerlund, and is shown in Appendix IV.

Freeze culture of *E.coli* carrying the pMAD-*I-sceI* vector was streaked out on blood agar, and incubated overnight at 37°C before stored at 4 °C for later use.

For isolation of plasmid DNA, a single colony was inoculated in 10 mL BHI with 100 µg/mL ampicillin in a 100 ml Erlenmeyer flask, and incubated at 37°C overnight. The pMAD-*I-sceI* vector was isolated from the overnight culture by QIAprep Spin Miniprep Kit (Appendix IX).

The insert encompassing the *yndE* flanking regions were excised from the TOPO vector by digestion with *EcoRI,* separated on an agarose gel, cut out from the gel, *and* purified using the QIAquick® Gel Extraction Kit (QIAGEN), (Appendix IX), and ligated into pMAD-*I-sceI* digested with the same restriction enzyme.

2.2.6 Digestion of pCR2.1 TOPO vector with insert by EcoRI

Solution made for digestion of TOPO vector with insert by *EcoRI:*

40 µl plasmid DNA

10 µl 10 X NEBuffer *EcoRI* (New England BioLabs ®)

5 µl *EcoRI* enzyme (New England BioLabs ®)

45 µl dH_2O

The mixture was incubated at 37 °C for at least 2 hours, or overnight. In cases where the digestion mix was not separated on an agarose gel immediately, the *EcoRI* enzyme was deactivated at 65 °C for 10 minutes, and stored at -20°C for later use.

2.2.7 *EcoRI* digestion of pMAD-*I-sceI*

Solution made for digestion of pMAD-*I-sceI* by *EcoRI*:

30 µl DNA (isolated from the overnight culture)

10 µl 10 X NEBuffer *EcoRI*

5 µl *EcoRI* enzyme

55 µl dH_2O

The solution was incubated at 37 °C for at least 2 hours. After incubation, 10 µl Antarctic phosphatase buffer (New England BioLabs ®) and 3 µl Antarctic phosphatase (New England BioLabs®) were added to remove the phosphate group at the 5 ‘end, and thereby prevent the vector from religating.

The solution was incubated at 37 °C for 30 minutes, before the enzyme was deactivated at 65 °C for 10 minutes, and stored at -20°C for later use.

Ligation of insert into pMAD-*I-sceI:*

2 µl 5X Ligation buffer (New England BioLabs ®)

1 µl T4 Ligase (New England BioLabs ®)

4 µl pMAD-*I-sceI* digested with *EcoRI*

3 µl insert, digested with *EcoRI*

The reaction was incubated in room temperature for 1 hour.

2.2.8 Transformation of pMAD-*I-sceI* with insert into *E. coli*

A vial of chemically competent *E.coli* OneShot TOP10 cells were thawed on ice, before 6 µl of the ligation mix was added to the *E.coli* cells. The cells were heat shocked at 42°C for 30 seconds, before being transferred to ice for 1 minute. A volume of 500 µl of S.O.C. medium was added, and the cells were incubated in water bath for 1 hour at 37°C.

After incubation, the transformed *E.coli* cells were spread on LB plates with 40 µl X-gal and 100 µg/mL ampicillin, and incubated at 37 °C overnight. A PCR amplification, using the A and D primer pair, was performed to screen for correct transformants.

A colony containing the correct insert was grown overnight in 12 ml BHI containing 100 µg/mL ampicillin at 37 °C with agitation. The pMAD-*I-sceI* plasmid with insert was isolated from the overnight culture with the QIAprep Spin Miniprep Kit (Appendix IX).

2.2.9 Precipitation of pMAD-I-SceI plasmid with insert

For transformation of *B. licheniformis* MW3, a high concentration of plasmid DNA is needed. To increase the DNA concentration in the plasmid preparations, the plasmid DNA was precipitated with ethanol. A small amount of the precipitated plasmid DNA was treated with *EcoRI* and separated on an agarose gel to verify that the correct insert was present.

Precipitation reaction:

180 µl plasmid

20 µl (3M) NaAc, pH 5.2

550 µl (100 %) ethanol.

The solution was frozen for 1 hour at -20°C and then centrifuged at 15.700 x g for 15 minutes. After centrifugation and removal of the supernatant, 200 µl (70 %) ethanol was added, and the solution was centrifuged for 5 minutes at 15.700 x g. The supernatant was removed and the alcohol evaporated. The DNA was dissolved in 30 µl dH_2O.

2.3 Preparation of electrocompentent *B.licheniformis* cells

The preparation of electrocompetent *B. licheniformis* cells was done as described in Løvdal *et al*. (2012) with minor modifications.

B. licheniformis MW3 was streaked on LB agar and incubated overnight at 37 °C. A single colony was inoculated in 25 mL BHI in a 100 mL Erlenmeyer flask. The next day, 1 mL of the overnight culture was transferred to 200 mL BHI in a 500 mL Erlenmeyer flask, and incubated at 37 °C with a rotation speed of 200 rotations per minute (rpm) until A_{600} reached 0.6-0.7. The culture was harvested into two 200 mL centrifuge tubes by centrifugation using a Sorvall RC-5B refrigerated super centrifuge. The tubes were centrifuged in a GSA-rotor, at 20°C at 4500 rpm for 15 minutes. The supernatant was drained, and the pellet was resuspended in 100 mL dH_2O. The solution was centrifuged at 8000 rpm in 15 minutes at 20 °C. The washing step was repeated once.

After the second washing step, the pellet was resuspended in 5 mL freshly made (40%) PEG 6000, and centrifuged at 5000 rpm for 15 minutes. A loose pellet was formed after centrifugation and the spent PEG 6000 was carefully removed, and the pellet was resuspended in 750 µl (40 %) PEG6000. The electrocompetent cells were stored in 1.5 mL Eppendorf tubes at -80 °C, in aliquots of 100 µl.

2.4 Transformation of pMAD-*I-sceI* with insert into *B.licheniformis* MW3 by electroporation

A vial of electrocompetent *B.licheniformis* MW3 was thawed on ice before 4 µl plasmid was added and incubated on ice for one minute. 40 µl of the bacteria-plasmid mixture was transferred to a 0.2 cm electroporation cuvette and electroporated at 2.5 kV for 4 ms (Micropulser™, Biorad).

After electroporation, 500 µl S.O.C. medium was added to the cuvette, and the transformation mixture was transferred to a fresh Eppendorf tube. The tube was incubated in water bath at 30 ^{o}C for 4-5 hours. The transformed cells was spread on LB agar containing 3 µg/mL erythromycin and 40 µl X-gal, and incubated at 37 ^{o}C for 48 hours. Cells growing on erythromycin that had the ability to break down galactosidase and form blue colonies showed uptake of pMAD-*I-sceI* vector with insert. Blue colonies were spread on LB agar containing 3 µg/mL erythromycin.

The pMAD-*I-SceI* vector has a temperature sensitive replicon, meaning the plasmid cannot replicate without being incorporated into the genome when temperature reaches 37 °C. At 32 °C the plasmid can replicate. In both cases, whether the plasmid has been incorporated in the genome or not, the colonies turn blue. After transformation, the only way to verify whether the incorporation of the plasmid has taken place or not is by amplification using PCR. However, due to difficulties to predict the orientation of the plasmid after the transformation this was not done.

2.5 Transformation of pBKJ223 into electrocompetent B. licheniformis /cells carrying the pMAD-*I-sceI* plasmid with insert

For transformation of the pBKJ223 plasmid into the *B.licheniformis* cells, a batch of electrocompetent *B.licheniformis* MW3 with pMAD-*I-sceI* + insert was made as described in section 2.3.

The transformation of the pBKJ223 plasmid into electrocompetent *B.licheniformis* MW3 with pMAD-*I-SceI* + insert was done as described in section 2.4. After transformation, the cells where spread on LB agar containing 10 µg/mL tetracycline + 40 µl X-gal, and incubated at 37^{o}C overnight. White colonies indicating a loss of pMAD-*I-sceI* were restreaked on LB agar containing 10 µg/mL tetracycline, and incubated at 37 ^{o}C overnight. White transformants were screened with both external primers (A and D primers) and internal primers (forward and reverse primers) to verify the deletion of the *ynd* gene and exclude clones carrying the wild type gene. Light blue or blue colonies with a white halo were also screened, as they may be correct transformants as well (Juajun *et al*., 2011).When a colony where the second crossover had taken place was identified, the colony was grown in 25 mL LB medium without antibiotics to get rid of the pBKJ223 plasmid. The culture was incubated at 37 ^{o}C and 200 rpm for at least 5 hour, or overnight. The culture was spread on LB agar and incubated at 37 ^{o}C overnight. Single colonies were patched on three different LB agar plates: LB agar without antibiotics, LB agar with tetracycline and LB agar with erythromycin, to check for loss of both the pMAD-*I-sceI* (erythromycin sensitive) and pBKJ223 (tetracycline sensitive).

To screen for correct transformants, deletion mutant a high-fidelity fusion PCR was performed using primer pair AD, and whole cell lysates of the transformants as template DNA. Small amount of the resulting PCR products were separated on agarose gel to verify the correct mutant once again before the remaining PCR product was purified by the QIAquick® PCR Purification Kit (Appendix IX), and sent for sequencing at Source BioScience (Nottingham).

2.6 Sporulation

Bacteria from a frozen stock was streaked on LB agar and incubated at 37 ^{o}C overnight. A single colony was inoculated in 50 mL BHI and incubated at 37 ^{o}C overnight. A volume of 50 µl of the pre-culture was added to 100 mL Bacto-MS sporulation medium and incubated at 37 °C and 200 rpm for at least 3 days. The spore culture was incubated until > 70 % of the culture had sporulated (phase-bright spores in microscope). When the cells had sporulated the culture was divided into two 50 mL Falcon tubes and centrifuged in an Allegra X-22R centrifuge at 3900 x g for 20 minutes at 4 ^{o}C. Between centrifugations, the spore suspension was kept on ice. The spores were resuspended in 10 mL dH_20 and centrifuged at 3900 x g for 10 minutes at 4 ^{o}C. The supernatant was drained off and the pellet was resuspended in 3 mL 20 % Nycodenz (Axis Shield PoC). A Nycodenz gradient was made by adding 4 mL (50 %) Nycodenz in a 15 mL falcon tube, followed by 4 mL (45 %) Nycodenz and the spore solution in 3 mL (20 %) Nycodenz. The Nycodenz gradient was centrifuged at 3900 x g for 80 minutes at 4 ^{o}C.

After centrifugation, the spores were collected at the bottom of the tube (Figure 9). The upper Nycodenz layers were removed, to avoid contamination of the spores. A volume of 7-8 mL of Nycodenz was removed from the top, leaving the spore pellet in volume2-3 mL suspension at the bottom of the tube. To wash the spores, cold dH_20 was added to the tube to reach a total volume of 15 mL. The spores were resuspended in the water and centrifuged at 3900 x g for 10 minutes at 4 ^{o}C. This step was repeated three times, more if necessary to gain a solid pellet at the bottom of the tube. The pellet was resuspended in 2 mL of cold dH_20 and divided into two Eppendorf tubes.

***Figure 9**. The layers of Nycodenz after centrifugation, with the spores at the bottom of the tube. (Photo by: Kristina Borch-Pedersen*)

The Eppendorf tubes were centrifuged at 4500 x g for 5 minutes in a table top centrifuge. This step was repeated once. After the second wash, the pellet was resuspended in 1 mL cold dH_2O and set to sediment for at least five days at 4°C (Figure 10).

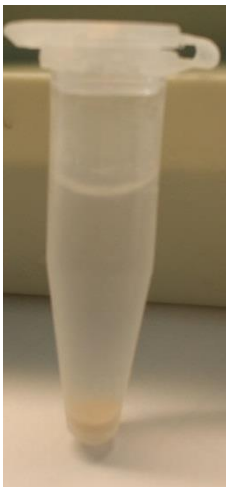

Figure 10. *The spore solution after sedimentation.*

2.7 Germination assay

The spores were checked in microscope to verify a spore content of >95 % spores (phase-bright) before they were prepared for germination assay. The protocol for germination assay described in Madslien *et al*. (2014) was used with minor modifications.

The spores were centrifuged at 1600 x g for 3 minutes at 4 ^{o}C, before the supernatant was removed. A volume of 1 mL cold dH_2O was added and the spores were resuspended. For heat activation, the spore solution was incubated in a water bath at 65 ^{o}C for 20 minutes. After heat activation the spores were centrifuged at 500 rpm for 5 minutes at 4 ^{o}C, resuspended in 900 µl (200 mM) K-phosphate buffer pH 7.2 and the OD_{600} was adjusted to 2.1 with K-phosphate buffer using the spectrophotometer (Shimadzu UV-160A, Shimadzu Europe GMBH). A volume of 100 µl of the spore -solution OD_{600} = 2.1 was added to the wells of a 96-well microplate. To the test wells, 100 µl (200 mM) germinant solution were added. As a positive control, 100 µl of MW3 were added to the wells. A volume of 100 µl of dH_2O was added to the negative control wells. All samples were run in duplicates, and no more than 16 wells were used in total. A microplate-reader (Tecan Infinite M200) was used with the Magellan 7.1 software to measure the germination response. The OD at 600 nM was measured every 2 minutes during incubation at 37 ^{o}C for 120 minutes with shaking. In this thesis, L-alanine, L-cysteine and L-valine were used as germinants. The germinants were sterile filtered before use, and L-cysteine was made fresh for each germination assay.

2.8 Quantitative Real time PCR

Quantitative Real Time PCR (qRT- PCR) was used to examine the expression of the *yndE* genes. RNA was isolated from sporulating cultures and cDNA was made from the RNA preparations. Three spore batches were made for each experiment.

2.8.1 RNA isolation

Three pre-cultures were made by inoculating a single colony of *B. licheniformis* MW3 in 25 mL of BHI in a 100 mL Erlenmeyer flask. A volume of 25 µl of the pre-culture was added to 50 mL Bacto MS sporulation medium, which was incubated at 37 ^{o}C at 200 rpm for about 15 hours until 50 % of the bacteria in the culture had sporulated. When making spore suspensions for isolation of RNA the OD_{600} of the cultures were supposed to be equal to 4 and about 50 % of the bacteria should have formed spores, which should be verified in microscope (Madslien *et al.,* 2014). During this work the OD_{600} did not, despite several attempts, reach 4 but rather 2-3 before it dropped again. Verification in microscope showed cultures with approx. 50 % spores, and the cultures was used for the isolation of RNA.

When 50 % sporulation was obtained, the spores were harvested. A volume of 750 µl of methanol was to 750 µl of spore solution in a 1.5 mL Eppendorf tube, and stored at -80 ^{o}C for further use. The culture was stored for less than 2-3 weeks before RNA isolation.

Two samples of each of the three spore suspensions were merged before isolation. The spore suspensions were thawed on ice, and centrifuged at 4 ^{o}C at 13.000 rpm for 1 minute. The supernatant was removed after the centrifugation, and the pellet was resuspended in 1 mL TRIzol® reagent (Ambion™) and transferred to a tube with pre-made silica beads (0,1 mm)(Lysing Matrix E, MP). The samples were shaken three times for 1 minute in a bead beater (Mini Bead Beater 8, Biospec) with one minute cooling on ice between the beatings. For further isolation the TRIzol® reagent protocol was followed, starting from step 2.

The samples were incubated at room temperature for 5 minutes, before centrifugation at 12.000 x g at 4 ^{o}C for 10 minutes. After centrifugation, the supernatant was transferred to a fresh tube, mixed with 100 µl of 1-bromo-3-chloropropane (BCP)(Sigma-Aldrich) per 1 mL TRIzol® reagent. The samples were incubated at room temperature for 10 minutes, before centrifugation at 12.000 x g for 15 minutes at 4 ^{o}C. After centrifugation, the aqueous top phase was transferred to a fresh tube without transferring any of the layers between the aqueous and organic phase. A volume of 500 µl of isopropanol per 1 mL TRIzol® reagent was added, the sample was vortexed and incubated at room temperature for 10

minutes. The samples were centrifuged at 12.000 x g for 8 minutes at 4 °C and the supernatant was discarded. A volume of 1 mL (75 %) ethanol was added per 1 mL Trizol® reagent, before the sample was centrifuged at 7500 x g for 5 minutes. The ethanol was removed and the samples were set to air dry. The RNA pellet was resuspended in 90 µl RNase free water, and DNase treatment was done immediately.

2.8.2 DNase treatment

To each sample, 10 µl (10X) TURBO Dnase buffer (Ambion™) and 2 µl Turbo Dnase (Ambion™) were added. The samples were incubated at 37 °C for 15 minutes before 1 additional µl of TURBO DNase was added and the samples were incubated for 15 minutes. A volume of 10 µl Dnase-inactivation Reagent (Ambion™) was added and the samples were mixed thoroughly, before incubation at room temperature for 2 minutes, with frequent vortexing. The samples were then centrifuged at 13 000 rpm for 90 sec, and a pellet of the Dnase and inactivation agent was visible at the bottom. The supernatant was carefully transferred to a fresh tube without transferring the loose pellet. Before storage at -80 °C, 0.1 volume (3M) NaAc pH 5.2 and 2.5 volumes of cold (100 %) ethanol was added to the supernatant.

2.8.3 cDNA synthesis

The RNA samples were thawed on ice and centrifuged at 13.000 rpm for 15 minutes at 4 °C. The supernatant was carefully removed, and 300 µl (80%) ethanol was added to wash the RNA. The samples were centrifuged at 13.000 rpm for 5 minutes. The supernatant was carefully removed, and the samples were set to air dry to remove ethanol. The pellet was resuspended in 20 µl RNase free water.

The RNA concentration was measured using a NanoDrop, N1000 Spectrophotometer (Thermo Fisher Scientific).

A High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems™) was used for synthesis of cDNA.

A 2X RT master mix was made:

2.0 µl 10x RT buffer

0.8 µl 25 x dNTP Mix (100 mM)

2.0 µl 10 x RT Random primers

1.0 µl Reverse Transcriptase

1.0 µl RNase Inhibitor

3.2 µl Nuclease free water

Total: 10 µl

10 µl of the 2x RT master mix and 10 µl RNA (50 ng/µl) were mixed in PCR tubes. The PCR tubes were centrifuged briefly to eliminate air bubbles.

Conditions for cDNA synthesis:

1. 25 ^{o}C – 10 minutes
2. 37 ^{o}C – 120 minutes
3. 85 ^{o}C – 5 minutes
4. 4 ^{o}C - ∞

2.8.4 Construction of standard curves for the quantitative Real Time PCR

Standard curves were made for each primer pair (*yndE1*, *yndE2* and *yndE3*) to check the binding efficiency of the synthesized primers. The cDNA was used in 5 fold dilutions (1, 1/5, 1/25, 1/125, 1/625 and 1/3125) and amplified in triplicates. The primers were designed, and the specific primers used for *yndE1*, *yndE2* and *yndE3* are given in Appendix III. The primers for the reference gene *rpoB* and *gerAA* were made to an earlier project (Madslien *et al*., 2014) (Table 5, Appendix III).

Master mix for qRT PCR:

12.5 µl Power SYBR® Green PCR Master Mix (Applied Biosystems™)

1 µl Forward primer

1 µl Reverse primer

5.5 µl MQ H_2O

20 µl in total

The amplification was performed using the StepOne Real Time PCR software (Applied Biosystems™) with thermal cycling conditions set at 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C.

2.8.5 Quantification of expression levels for *yndE1*, *yndE2*, *yndE3* and *gerAA* relative to the expression level of *rpoB* by quantitative Real Time PCR

For the quantification of *yndE1*, *yndE2*, *yndE3* and *gerAA* expression the cDNA was diluted 1:50 in MQ H_2O. *The rpoB* gene was used as reference, and the efficiency of the *rpoB* and *gerAA* primers was previously calculated to be 2.00 and 2.01, respectively (Madslien *et al*., 2014). The primers used for *rpoB* and *gerAA* samples are given together with the other qRT PCR primers in Appendix III.

A 48 well plate was used for sample setup and 20 µl master mix and 5 µl 1:50 fold diluted cDNA was added to each well. For the negative control samples, 5 µl MQ H_2O was added instead of cDNA. For the DNase control 5 µl RNA (50 ng/µl) diluted 1:50 was used. Three replicates and one negative control were used for each gene. The DNase control was performed for each cDNA batch. The same software as for the standard curves was used for quantification, but the ΔΔcT quantification program was used instead of standard curve program.

2.9 Western Blot for detection of YndE germination receptor proteins

To verify the expression of the germination receptors YndE1, YndE2 and YndE3 specific antibodies against the germination receptor peptides were constructed by Dr. Marina Aspholm. The spores were made as described earlier, and to find the optimal way to analyse the spores both sedimented whole spores and spore extract were used.

2.9.1 Preparation of spore extract

The spores were decoated to remove the spore coat and outer membrane, followed by extraction of the spores.

Decoating

Decoating was done as described by Vepachedu & Setlow (2005), with minor modifications. 1 mL spore solution was heat treated for 30 min at 70 ^{o}C in 1 mL (0.1M) NaCl, (0.1M) NaOH, (1%) SDS, (0.1M) dithiothreitol. After the heat treatment the spores was washed 3 times in 1.5 mL dH_2O.

Spore extracts

Extraction of the spores was done as described by Paidhungat & Setlow (2001) with minor revisions. The decoated spores were bead beated (Mini Bead Beater) three times for 1 minute with 1 minute cooling on ice between the beatings. A volume of 0.5 mL TEP buffer was added (50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% SDS and 0.15 M β-mercptoethanol), and the solution was incubated at 70 ^{o}C for 30 minutes. The insoluble material was removed by centrifugation at 13.000 x g for 5 minutes at 4 ^{o}C.

2.9.2 Protein-separation on SDS-PAGE

The spore samples were suspended in sample buffer. For the spore extract a pre made (4X) NuPAGE®LDS Sample Buffer (Novex®) was used. But for the negative control samples and spore suspensions another sample buffer were used (Dr. Marina Aspholm, personal communication) (Appendix II). A fresh overnight culture of *B. licheniformis* MW3 was used as negative control.

Prior to gel electrophoresis, all samples were heat treated for 15 minutes. The heat treatments were done at either 80 ^{o}C or 100 ^{o}C, to analyse the effect of the heating temperature on the extractability of spore proteins. The proteins were separated on a 12 % Bis-Tris precast gel (Criterion^{TM} XT, Bio Rad) with 18 wells, and 30 µl of sample was added to each well.

The SeeBlue Plus2 Prestained standard (Novex®) was used as molecular weight standard, and the MOPS SDS-PAGE buffer (Invitrogen^{TM}) was used as running buffer for the electrophoresis. The electrophoresis

was run at 180 - 200 V for 45 - 60 minutes, or until the samples almost reached the end of the gel. After the separation, the proteins were transferred to a PVDF-membrane (Sigma-Aldrich) by electro blotting.

2.9.3 Transfer of separated proteins from gel to PVDF membrane by electro blotting

The Transfer buffer (Novex®) for the electro blotting was cooled down before transfer to prevent heating of the blotting chamber. The PVDF-membrane was activated in methanol for 10 minutes, washed in water for 10 minutes and stored in Transfer buffer until use. A Criterion cell™ blotting chamber (BioRad) was used for the electro blotting. The blotting was run at 60 V for about 2 hours.

The membrane containing the separated proteins was incubated in blocking buffer (TBST containing 5 % non-fat milk) for ½ to 1 hour. After blocking, the membrane was incubated with primary antibody diluted 1:500 in TBST for at least 1 hour. The membrane was washed in TBST for 3x5 minutes and incubated with the secondary antibody, Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 546 (Life Technologies) , diluted in 1:3000 TBST, for 1 hour. The membrane was washed in TBST for 3x 5 minutes. The membrane was next on incubated in TBST buffer containing 1:3000 of Streptavidin and Alkaline phosphatase for at least 30 minutes. The membrane was washed in TBST for 3 x 5 minutes. To visualize the proteins, 10 mL of Tris-HCl pH 9.0, 100 µl Reagent A (NBT) and 100 µl Reagent B (BCIP) (AP conjugate substrate kit) (BioRad) was added. The membrane was washed in dH_2O to stop the development, and dried before storage.

3. Results

3.1 The pentacistronic *yndE* operon in *B.licheniformis* MW3

In contrast to the ABC (D) organization that characterizes the *gerA* operons in *B. subtilis* (Zuberi *et al*., 1985; Paredes-Sabja *et al*., 2011), *B. licheniformis* MW3 carries a pentacistronic *ynd* operon with the gene organization *yndD*, *yndE3*, *yndE2*, *yndF*, *yndE1* (encoding GR A-, B-, B-, C-, B- subunits, respectively)(Veith *et al*., 2004). Comparative analyses showed that the amino acid sequences of the three *yndE* homologs demonstrated between 52.2 % and 63.7 % identity and between 75.1 % to 82.8 % similarity to each other (Table 2). The pairwise amino acid sequence alignments between the *B.licheniformis* MW3 *yndE* genes are shown as Figure 17, 18 and 19 (Appendix V). A ClustalW multiple amino acid sequence alignment was performed for all three *yndE* subunits genes in *B.licheniformis* MW3 (Figure 11). A similar alignment also including the amino acid sequence for the *yndE* subunit gene in *B. subtilis* was done as well (GenBank accession number: KIX83039), (Figure 20, Appendix V).

Table 2. *Calculated identities and similarities from the pairwise amino acid sequence alignments of the yndE gene homologues yndE1, yndE2 and yndE3 in B. licheniformis MW3.*

Identities (%)		
	yndE1	***yndE2***
yndE1	100	52.2
yndE2	52.2	100
yndE3	54.3	63.7
Similarities (%)		
	yndE1	***yndE2***
yndE1	100	75.1
yndE2	75.1	100
yndE3	75.4	82.8

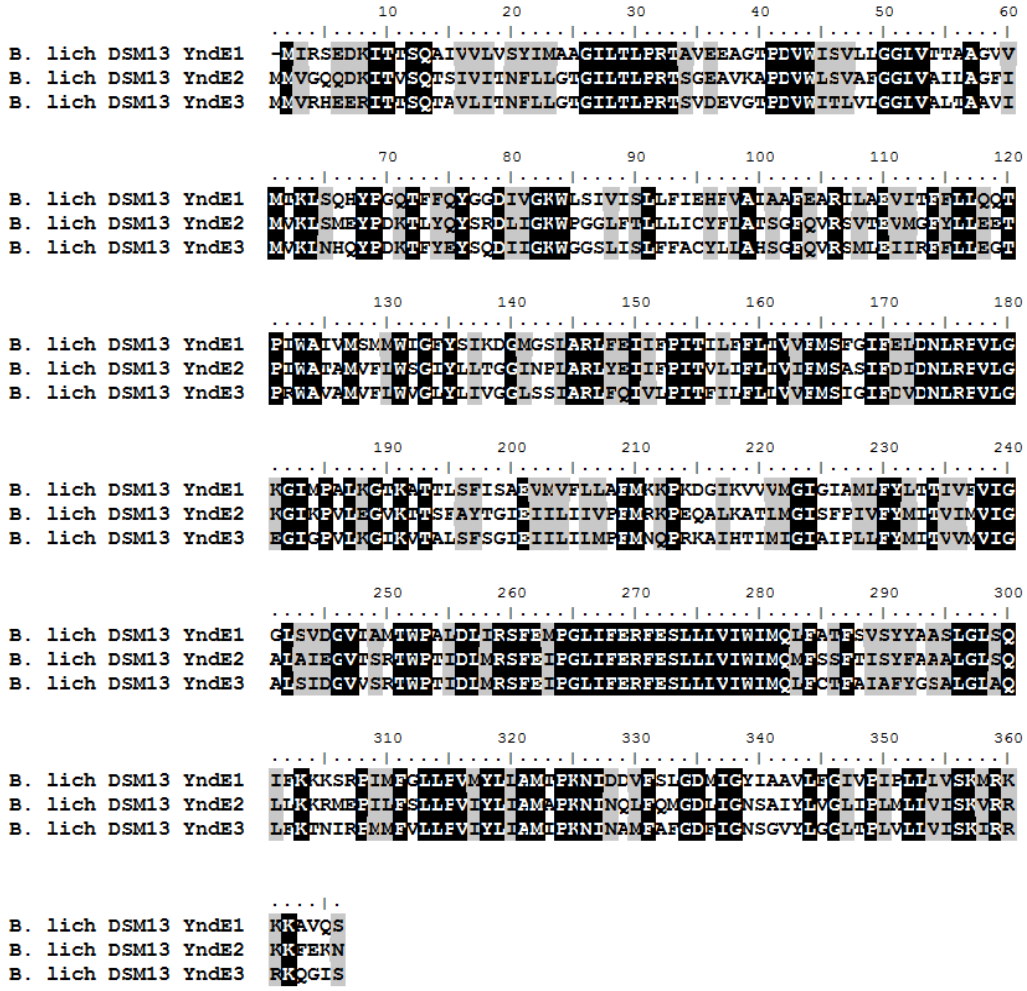

Figure 11. *ClustalW multiple amino acid sequence alignment by Bio Edit of yndE1, yndE2 and yndE3 in B. licheniformis MW3.*

The three YndE germination receptor subunits have similar lengths, both YndE2 and YndE3 consists of 366 amino acids (AA), while YndE1 has a sequence length of 365AA, lacking one methionine at the N-terminus. The differences in the amino acid sequences are spread along the peptide chains but there are also regions that are highly conserved between the three *yndE* genes, for example amino acids 255 and 284. *yndE2* and *yndE3* is also more similar compared to *yndE1,* as Table 2 indicates. The *B. licheniformis yndE* genes also demonstrate strong identity to the single *yndE* gene in *B. subtilis*. One AA at 274 is different in the middle of the conserved area from AA 264 to AA 282 in *B. licheniformis* differs *B. subtilis* from *B. licheniformis, where the B.licheniformis* yndE subunits encodes phenylalanine while *B.subtilis* encodes leucine. *B. subtilis* YndE also in correlation with YndE1 in *B.licheniformis* consists of 365 AA.

3.2 Quantitative Real Time PCR

The *ynd* operon has previously been reported to be poorly transcribed in *B. subtilis* and no function has so far been assigned to it (Paidhungat & Setlow, 2000).The expression level of the target genes *gerAA*, *yndE1*, *yndE2* and *yndE3* in *B. licheniformis* MW3 relative to the expression level of *rpoB* reference gene was calculated in Excel, and displayed in a boxplot. Together with the efficiency of the qRT PCR primers, the calculations, which the boxplot are based on, are shown in Table 6-10 (Appendix VI).

Analysis of the *yndE1*, *yndE2*, *yndE3* expression levels in *B. licheniformis* MW3 revealed that they are expressed at a significantly higher level than *gerAA* during sporulation. The relatively high expression level suggests that all the *yndE1*, *yndE2*, *yndE3* genes could be functionally active in *B. licheniformis.* By comparing the expression of the *yndE* genes to each other the expression of *yndE1* compared to the expression of *yndE2* or *yndE3* the expression was not significantly different. The expression of *yndE2* compared to *yndE3* was found to be significantly different. Expression levels of *gerAA*, *yndE1, yndE2* and *yndE3* relative to the expression level of *rpoB* in *B.licheniformis* MW3 are shown in Figure 12.

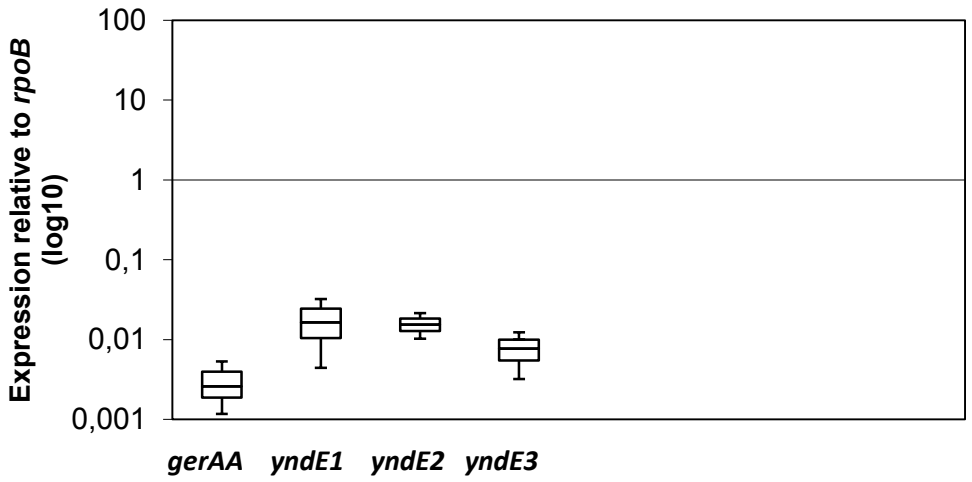

Figure 12. *Boxplot showing expression of gerAA, yndE1, yndE2 and yndE3 relative to rpoB in B.licheniformis MW3. The mean values are given as a line within the box. The whisper represent the 1,5 IQR above the third quartile (Q_3) and 1,5 IQR below the first quartile (Q_1).* Altogether, three biological replicates were used. For the first spore batch three measurements were done, all on cDNA from different RNA isolations. For the second spore batch, one RNA isolation was used and for the third spore batch two different RNA isolations were used.

3.3 Study of germination by measurements of optical density

To elucidate the potential role of yndE genes in germination, we tried to construct single gene deletion mutants in the *yndE1, yndE2 and yndE3* genes by using a markerless gene replacement method which eliminates the specific *yndE* genes while leaving the rest of the operon intact. However, we were only able to recover an *yndE3* deletion mutant (Δ*yndE3 mutant*). Spores were prepared from the wild type background strain MW3 (MW3) and from the MW3Δ*yndE3* mutant strain. The construction of the deletion mutants are given in Appendix VII.

To study the role of *yndE3* in germination, the germination kinetics of spores of the MW3 strain and the MW3Δ*yndE3* mutant strain was studied by measuring the decrease in OD_{600} the first 120 minutes after exposure to germinant. In addition, 120 minutes after germination was induced, the samples were analysed by phase contrast microscope to count the amount spores and germinated spores.

Surprisingly, lack of *yndE3* influences germination induced by all the single germinants L-alanine, L-valine or L-cysteine (Figures 13 to 15). The largest difference was observed on germination induced by L-cysteine where the MW3Δ*yndE3* mutant strain demonstrated a remarkably slower germination rate compared to the MW3 strain. The germination rate in response to L-alanine and L-valine was slightly reduced in the MW3Δ*yndE3* mutant strain, compared to the MW3 strain. However, since the results were based on two technical replicates from each of three spore batches we assume that the observed differences could be relevant and not due to technical variabilities or differences between spore batches.

The impact of the three germinants L-alanine, L-cysteine and L-valine on the germination of the MW3 strain and the MW3Δ*yndE3* mutant strain are given in Figures 13, 14 and 15, respectively. In addition, Table 3 and Figure 16 shows the results from phase contrast microscopy analysis of germinated MW3 and MW3Δ*yndE3* mutant spores. Together, these results suggest that *yndE3* is functionally active in *B. licheniformis* and influences germination in response to L-alanine, L-valine or L-cysteine with a particularly strong impact on the germination induced by L-cysteine.

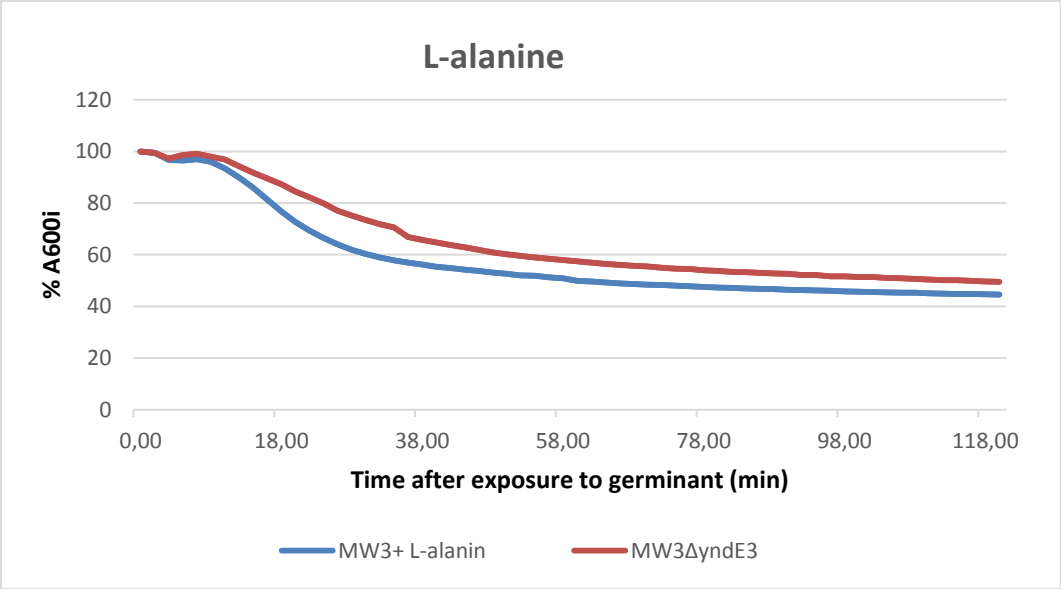

Figure 13. *Germination of the MW3 and the MW3ΔyndE3 mutant strains in the presence of 100 mM L-alanine. The germination was followed as a change in initial absorbance at 600 nm of phase bright spores after addition of 100 mM L-alanine. The results shown are representative of experiments performed on three individual spore batches tested in duplicates.*

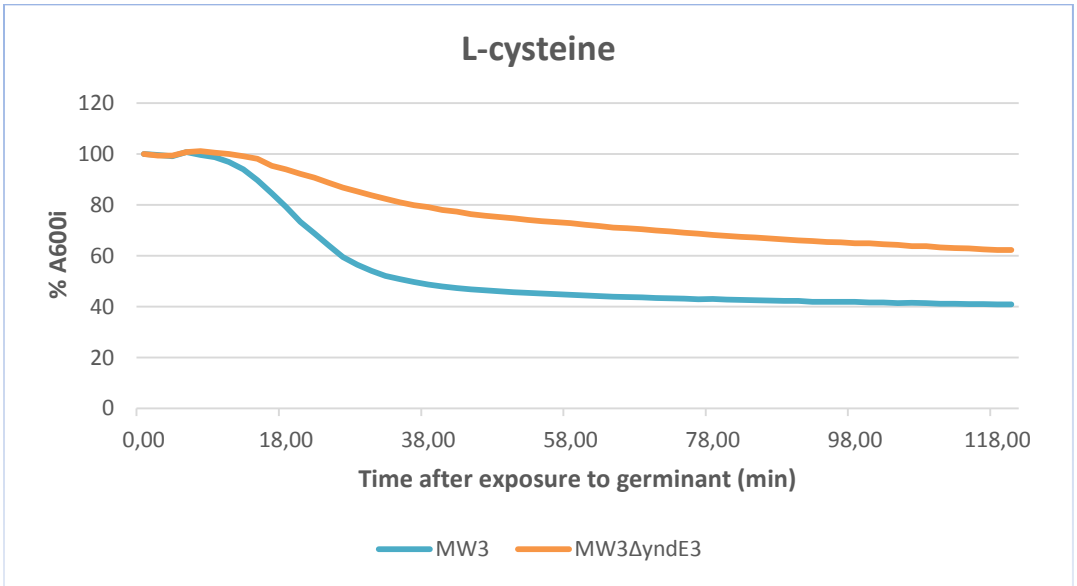

Figure 14. *Germination of the MW3 and the MW3ΔyndE3 mutant strains in the presence of 100 mM L-cysteine. The germination was followed as a change in initial absorbance at 600 nm of phase bright spores after addition of 100 mM L-alanine. The results shown are representative of experiments performed on three individual spore batches tested in duplicates.*

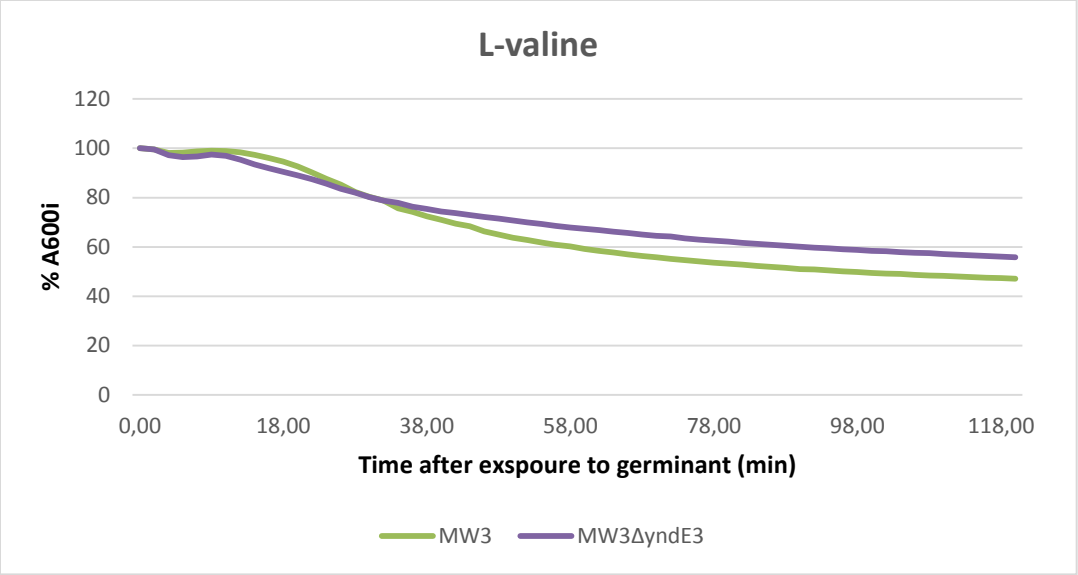

Figure 15. *Germination of the MW3 and the MW3ΔyndE3 mutant strains in the presence of 100 mM L-valine. The germination was followed as a change in initial absorbance at 600 nm of phase bright spores after addition of 100 mM L-alanine. The results shown are representative of experiments performed in on three individual spore batches tested in duplicates.*

Table 3. *Results from phase contrast microscopy analysis of germinated spores (phase dark) of the MW3 strain and the MW3ΔyndE3 mutant strains 120 minutes after induction with either L-alanine, L-cysteine or L-valine.*

Strain/Germinants	MW3 wild type	MW3 wild type, negative control	MW3*ΔyndE3* mutant	MW3Δ*yndE3* mutant, negative control
L-alanine	> 99%	1-2 %	70-80 %	< 5 %
L-cysteine	> 99 %	< 2 %	50-55 %	2-3 %
L-valine	75-80 %	< 2 %	60- 65 %	< 2%

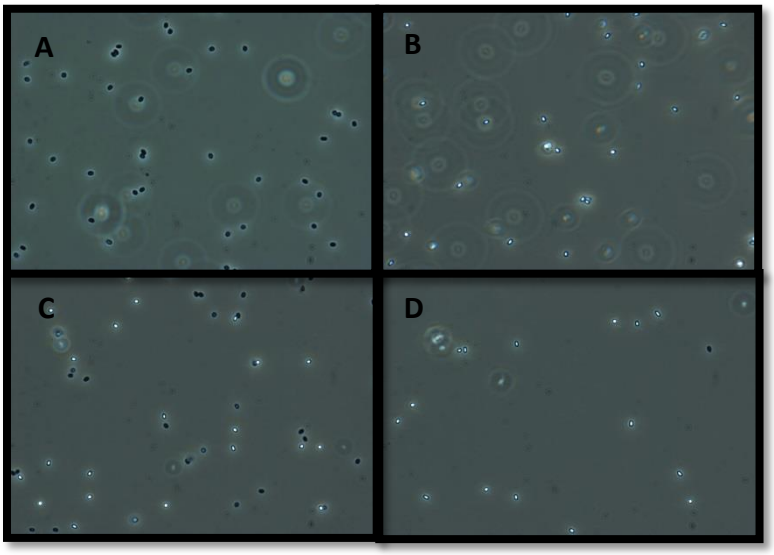

Figure 16. *Germinated (phase dark) and ungerminated (phase white) spores viewed by phase contrast microscopy 120 minutes after induction with L-cysteine.* ***A****: MW3 strain + L-cysteine,* ***B****: MW3 strain, negative control,* ***C****: MW3ΔyndE3 mutant strain+ L-cysteine,* ***D****: MW3 ΔyndE3 mutant strain, negative control.*

3.4 Western Blot

To examine expression of the YndE proteins we raised anti peptide antibodies specific for the three YndE proteins in rabbits (GenScript, USA) immunized with peptides from each YndE protein (personal communication, Dr. Marina Aspholm). The peptide sequences targeted by the peptide antibodies are presented in Figure 17. The following experiments are the first tests performed to test the antibodies.

YndE1

MIRSEDKITTSQAIVVLVSYIMAAGILTLPRTAVEEAGTPDVWISVLLGGLVTTAAGVVMTKLSQHYPGQTFFQYGG
DIVGKWLSIVISLLFIEHFVAIAAFEARILAEVITFFLLQQTPIWAIVMSMMWIGFYSIKDGMGSLARLFEIIFPIT
ILFFLTVVFMSFGIFELDNLRPVLGKGIMPALKGTKATTLSFISAEVMVFLLAFMKKPKDGIKVVVMGIGIAMLFYL
TTIVFVIGGLSVDGVTAMTWPALDLIRSFEMPGLIFERFESLLLVIWIMQLFATFSVSYYAASLGLSQIFKKKSRPI
MFGLLPVMYLLAMTPKNIDDVFSLGDMIGYIAAVLFGIVPIPLLLVSKMRKKKAVQS

YndE2

MMVGQQDKITVSQTSIVITNFLLGTGILTLPRTSGEAVKAPDVWLSVAFGGLVAILAGFIMVKLSMEYPDKTLYQYS
RDLIGKWPGGLFTLLLICYFLATSGFQVRSVTEVMGFYLLEETPIWATAMVFLWSGIYLLTGGINPLARLYEIIFPI
TVLIFLIVIFMSASIFDIDNLRPVLGKGIKPVLEGVKTTSFAYTGIEIILIIVPFMRKPEQALKATLMGISFPIVFY
MITVIMVIGALAIEGVTSRTWPTIDLMRSFEIPGLIFERFESLLLVIWIMQMFSSFTISYFAAALGLSQLLKKRMEP
ILFSLLPVIYLIAMAPKNINQLFQMGDLIGNSAIYLVGLIPLMLLVISKVRRKKFEKN

YndE3

MMVRHEERITTSQTAVLITNFLLGTGILTLPRTSVDEVGTPDVWITLVLGGLVALTAAVIMVKLNHQYPDKTFYEYS
QDIIGKWGGSLISLFFACYLLAHSGFQVRSMLEIIRFFLLEGTPRWAVAMVFLWVGLYLIVGGLSSIARLFQIVLPI
TFILFLLVVFMSIGIFDVDNLRPVLGEGIGPVLKGIKVTALSFSGIEIILILMPFMNQPRKAIHTIMIGIAIPLLFY
MITVVMVIGALSIDGVVSRTWPTIDLMRSFEIPGLIFERFESLLLVIWIMQLFCTFAIAFYGSALGLAQLFKTNIRP
MMFVLLPVIYLIAMIPKNINAMFAFGDFIGNSGVYLGGLTPLVLLVISKIRRRKQGIS

Figure 17. *Peptide sequences of YndE1, YndE2, YndE3 with the areas of which the antibodies targeted marked in yellow (Dr. Marina Aspholm, personal communication).*

Both untreated spores and spore extract of decoated spores from *B. licheniformis* MW3 were separated by SDS-PAGE and then blotted onto PVDF membranes to determine the most efficient treatment of spores for analysis by Western Blot.

The testing of spore preparations were done with YndE1 and YndE3 primary antibodies. As a negative control an overnight culture of the wild type strain was used. The overnight culture does not express the YndE GR proteins as these are not transcribed before the vegetative cells enters sporulation, and may be assumed to be a good negative control. The tests of the antibodies were done with different heating temperature of the spores and both YndE3 and YndE1 were tested as primary antibodies. One analysis of the pre-immunization serum for YndE3, against the MW3*ΔyndE3 mutant* and MW3, all figures from Western blot analysis are given as Figure 39 to 42 (Appendix VIII).

Based on the Western blot analysis we were not able to analyse the expression of the YndE proteins. No difference could be visualised between the MW3*ΔyndE3 mutant* or the MW3 spores, not even with use of YndE3 pre immunization serum as primary antibody.

4. Discussion

The aim of this thesis was to explore the function of the three *yndE* gene homologues in *B. licheniformis* on germination. The *yndE3* gene was successfully deleted, creating the MW3Δ*yndE3* mutant strain.

Quantitative Real Time PCR was used to examine the expression level of the *yndE* gene homologues and *gerAA* in *B.licheniformis* MW3 relative to the expression level of *rpoB*. The expressions of *yndE* genes relative to *rpoB* during sporulation were significantly higher than for the *gerAA* gene (Figure 12). Suggesting an expression of the *yndE* genes corresponding to a measurable function in *B. licheniformis* MW3. A previously study on the expression of *gerAA* showed a 10 fold higher expression of *gerAA* than in this study (Madslien *et al*., 2014). The difficulty in gaining the same OD_{600} of the sporulation cultures in this experiment compared to previous study needs to be taken into consideration when comparing these results. The expression of *yndE2* compared *yndE3* was found to be significantly different. *yndE1* was not found to be expressed significantly different than neither *yndE2* nor *yndE3*. Since the *yndE* genes are localized the same operon an expression with no significant differences as an equal gene regulation should be expected by theory. On the other side the expression of the *yndE* genes are measured in a biological system, which may lead to results different from the expectations. Another possibility for the significantly different expression in the *yndE* genes could be, as in all studies, due to experimental factors affecting the results of the study. *yndE* expression was only examined in the wild type *B.licheniformis* MW3 due to difficulties during RNA isolation and restricted time at the end of the project period.

The sequence identity for the *yndE* subunits in *B. licheniformis* MW3 was investigated. The *yndE* gene homologues in *B. licheniformis* MW3 show a high degree of amino acid sequence identity. As shown in Table 2 the *yndE* subunit genes contain over 50 % identity in the amino acid sequences, and over 75 % similarity. The *yndE* amino acid sequences also exhibit a large conserved area from AA 264 to AA 282, and both before and after this area in the AA sequence there is large areas of conservation or similarities, as shown in Figure 11. This large conserved area was also found in comparison analysis with the *yndE* subunit gene in *B. subtilis,* except for one alteration to the AA in position 274, where *B. subtilis* encodes leucine while all three *yndE* subunits in *B.licheniformis* encodes phenylalanine (Figure 20, Appendix IV). This alteration may also be of importance for the structure and function of the YndE subunit, but this is not known to our knowledge. The *yndE* subunit gene of *B. subtilis* also exhibits large identity and similarity with the rest of the *yndE* subunits in *B. licheniformis*, but the similarities are in some areas in correlation with one *yndE* subunit, and in other parts identical or similar to another of the

B. licheniformis yndE subunits. The length of the *yndE* genes in *B. licheniformis* MW3 and *B. subtilis* is also similar, with both *yndE2* and *yndE3* in *B.licheniformis* MW3 encoding 366 AA, while *yndE1* together with the *yndE* subunit in *B. subtilis* lack one methionine AA in the N-terminus compared to these. To our knowledge, the function of the *yndE* genes in *Bacillus* spp. is an unexplored field of research. Also the characterization of germination receptor operons exhibiting homologue genes has, to our knowledge, not been studied earlier.

Differences in the GR's between *B. subtilis* and *B. licheniformis* have been described earlier. Examples of this is the GerB GR found in *B. subtilis* which cannot be found in *B. licheniformis* (Corfe, 1994), and the germination response of *B. subtilis gerB* and *gerK* to AGFK (Moir, 2002; Atluri *et al*., 2006). As *B.licheniformis* lacks the GerB GR the same germination response as in *B. subtilis* cannot be expected since only the GerK GR is present. This was also confirmed by deletion of *gerK*, which had little effect on the germination response (Borch-Pedersen *et al*., unpublished results). Deletion of *yndD* in *B.licheniformis* reduces the germination in response to L-alanine, L-cysteine and L-valine (Borch - Pedersen *et al*., unpublished results). However, the reduction in germination was not as strong as for the *gerAA* mutant (Løvdal *et al*., 2012; Borch-Pedersen *et al*., unpublished results).These results suggests a different role of the *yndDEF* operon in *B.licheniformis* than in *B. subtilis*.

Germination assays was performed for the *B. licheniformis ΔyndE3* mutant, with L-alanine, L-cysteine and L-valine which have been shown to induce a strong germination response in *B. licheniformis* MW3 (Løvdal *et al*., 2012; Borch-Pedersen *et al*., unpublished results). Germination assays of the MW3Δ*yndE3* mutant showed a reduced germination rate compared to the MW3 background strain. The largest reduction in germination was with L-cysteine (Figure 14) as germinant, however, a small effect on germination in response to L-alanine (Figure 13) and minor effect in response to L-valine (Figure 15) was also observed. Comparing the decrease in germination in the MW3Δ*yndE3* mutant to the MW3Δ*yndD* mutant the *yndE3* gene was not shown as essential for germination as *yndD*, as the germination in the MW3Δ*yndD* mutant was strong in response to all three germinants. The effect in germination in response to L-cysteine for the MW3Δ*yndE3* mutant may also give an indication of separate role for the three *yndE* gene subunits. This needs to be verified by null mutations of the two other *yndE* gene as well.

In a study made on the laboratory strain *B. subtilis* PB832, a mutant with large deletions in *gerA*, *gerB* and *gerK* operons, and a mutant with deletion of the *yndDEF* and *yfkQRT* gene clusters in addition to the deletions in the three *gerA*, *gerB* and *gerK* operons, demonstrated identical levels of colony forming

units after heat activation of spores followed by overnight growth on LB agar. This study suggested a non-functional *yndDEF* operon in *B. subtilis* (Paidhungat & Setlow, 2000). Even though no function has been found for the *B. subtilis ynd* operon the operon is still present in the organism, which may suggest an undiscovered function of the operon. Another suggestion in relation to this is the alteration of the *ynd* operon in *B. licheniformis* by deletion of the original operon and replacement with the *ynd* operon of *B. subtilis*, would this gain the same *B. licheniformis* phenotype. Would that in this case indicate an undiscovered feature in *B. licheniformis* which makes the *yndDEF* operon a functional germination receptor. Another suggestion in this case is the alteration of the *ynd* operon in *B. licheniformis* by deletion of the original operon and replacement with the *ynd* operon of *B. subtilis*, would this gain the same *B. licheniformis* phenotype. Does in this case *B. licheniformis* exhibit an undiscovered feature which stimulates germination by the *ynd* operon.

The YndE subunits in *B. licheniformis* correspond to the B subunit in the GerA GR (Xiao *et al*., 2011). The B subunit has been suggested as one of the subunits that recognizes the germinants after studies of *Bacillus megaterium* (Christie & Lowe, 2007).

Western blot analysis was performed to visualize the expression of YndE proteins. However, the results showed that SDS-page separated whole cell protein from the MW3 wild type strain and MW3Δ*yndE3* mutant strain cells under sporulation demonstrated identical protein patterns. This was also the case when comparing blots incubated with the pre-immunization serum from the rabbit and antisera against YndE3. In the case of antibodies targeting the YndE3, revisions needs to be considered before further analysis. There is important to notice that only the pre-serum from production of the YndE3 antibody was tested and the pre serums from before YndE1 and YndE2 immunization have not been tested. This needs to be done to see whether these antibodies can be used in analysis of respectively *yndE1* and *yndE2* deletion mutants. The production of the antibodies also needs to be considered, and the peptides chosen for immunization may need some alterations to achieve the desired outcome of analysis. The expression of proteins may be weak as well, and further isolation of the proteins could be necessary if this is the case.

Even though the Western blot results cannot be used for verification of mutants and size determination of the YndE proteins, this work has developed a method for preparation of whole spore protein extracts for Western blot analysis. The spore extracts were decoated before initial heating seemed to give a higher solubility of spore proteins. Decoating of spores before heating should be done in the future testing of the YndE antibodies in Western blot.

5. Conclusion and future prospects

The expression of the three yndE genes in *B. licheniformis* MW3 was shown to be significantly higher than the expression of *gerAA*, suggesting the role of the *yndE* genes as functionally active genes in *B.licheniformis* MW3.

The homology between the *yndE* genes in *B.licheniformis* MW3 have been investigated, and showed a high degree of amino acid identity between the *yndE* subunits, together with a highly similar amino acid sequence compared to the *yndE* subunit in *B. subtilis*. Even though some differences separates the YndE subunit of *B. subtilis* from the ones in *B. licheniformis* MW3*,* the consequences of these differences when it comes to the function of the YndE subunits is not known.

Only the MW3Δ*yndE3* mutant was completed during this thesis, due to difficulties in the deletion of *yndE1* and *yndE2*. The challenges related to deletion of the *yndE* genes may be seen in correlation with the sequence homology of the *yndE* genes, but the DNA sequences for the *yndE* subunits needs to be compared to verify a possible high nucleotide sequence identity as well.

Measurements of germination by loss in optical density suggest a role of the *yndE3 gene* in *B.licheniformis* in germination in response to L-cysteine, as the germination response in presence of L-cysteine was reduced by deletion of *yndE3*.

Further work is needed for a complete characterization of the YndE GR in *B. licheniformis.* To be able to characterize the complete function of all the *yndE* genes in germination in *B.licheniformis* all three *yndE* genes needs to be deleted and analyzed as single mutants, double mutants lacking two *yndE* genes and as a triple mutant lacking all three *yndE* genes. These null mutations also need to be complemented to ensure that the mutant phenotypes are related to deletion of the *yndE* genes. A mutant where the complete *yndDEF* operon in *B. licheniformis* is deleted is another phenotype to study.

The difficulties with deletion of the *yndE* subunits need to be investigated to develop a new method for deletion of homologue genes. One suggestion in this case is to increase the length of the primers, and the complimentary parts of primer B and C. By doing so the specifity of the primers may be increased.

Western blot was also performed investigating the expression of whole cell YndE proteins the MW3 and MW3Δ*yndE3* mutant strains. No differences in the protein expression were found between the strains and neither size of the YndE proteins nor verification of deletion mutants could be done. Even though a method for extraction of whole spore proteins was developed and may be used for Western blot in the

future. In further analysis the primary antibodies targeting YndE1 and YndE2 need to be tested together with the pre serums to test the antibodies against YndE1 and YndE2 as well. Alterations in the peptide sequence targeted for immunization may needed as well, together with methods for yielding a higher expression of YndE proteins.

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Appendices

Appendix I – Materials and equipment

Materials and equipment used during the thesis subdivided based on the three subparts of the project and basic equipment.

Construction of deletion mutants

Material/Equipment	Manufacturer	Description
AccuPrime™ *Pfx* DNA Polymerase High Fidelity	Invitrogen™, Life Technologies	High-fidelity fusion PCR
AccuPrime™ *Pfx* Reaction Mix, 10X	Invitrogen™, Life Technologies	High-fidelity fusion PCR
Acetic acid	Fermentas	
Agar, bacteriological no.1	Oxoid	
L-alanine	Sigma-Aldrich	Germinant
Ampicillin	Sigma-Aldrich	Antibiotic
Antarctic phosphatase	New England Biolabs®	
Antarctic phosphatase Buffer	New England BioLabs®	
Bacto Brain Heart infusion medium, (BHI)	Becton, Dickinson and Company	
$Ca(NO_3)_2$	Merck	
Chloroform	VWR	
$CoCl_2$	Sigma-Aldrich	
$CuCl_2$	Sigma-Aldrich	
L-cysteine	Sigma-Aldrich	Germinant
Difico™ Nutrient Broth	Becton, Dickinson and Company	
dNTP's	VWR	PCR
EcoRI enzyme	New England BioLabs®	Restriction enzyme
Edetic acid, EDTA	Sigma-Aldrich	

Electroporation cuvette, 0,2cm	Bio Rad	
Ethanol, EtOH	Kemetyl Norge	
Ethidium Bromide	Sigma-Aldrich	
Erythromycin	Sigma-Aldrich	Antibiotic
Iron (II)Sulphate, $FeSO_4$	Merck	
Isopropanol	VWR	
Key buffer, 10X	VWR	PCR
K_2HPO_4	Sigma-Aldrich	K- phosphate buffer
KH_2PO_4	Sigma-Aldrich	K-phosphate buffer
L-valine	Sigma-Aldrich	Germinant
Ladder, Gene Ruler™ , 1 kb DNA Ladder	Thermo Scientific™, Life Technologies	
Ligation Buffer	New England BioLabs®	
Lysozyme	Sigma-Aldrich	
$MgCl_2$	Merck	
Micropulser™	Bio Rad	
$MnSO_4$	Merck	
NaAc	May & Baker Ltd. Dagenham, UK	
NaCl	Merck	
Na_2MoO_4	Sigma-Aldrich	
NEBuffer *EcoRI* buffer (10x)	New England BioLabs®	
$(NH4)_2SO_4$	Merck	
Nycodenz	Axis Shield PoC	
OneShot TOP10 Chemically competent *E.coli*	Invitrogen™, Life Technologies	
pCR™2.1 TOPO Vector	Invitrogen™, Life Technologies	
pEXP5-TOPO TA expression kit	Invitrogen™, Life Technologies	

Phosphatase buffer	New England BioLabs®	
pMAD-*I-SceI*	Anette Fagerlund, University of Oslo, Norway	pMAD shuttle vector (Arnaud *et al*., 2004) with and additional *I-SceI* site
Polyethylene glycol 6000	Sigma-Aldrich	
Proteinase K	Sigma-Aldrich	
QIAquick® PCR Gel Extraction Kit	QIAGEN, Venlo, The Nederlands	
QIAquick® PCR Purification Kit	QIAGEN	
QIAprep Spin Miniprep Kit	QIAGEN	
RNase A	Thermo Scientific™, Life Technologies	
SDS	Sigma-Aldrich	
SeaKem LE Agarose	Lonza, Basel, Switzerland	
S.O.C. medium	Invitrogen™, Life Technologies	Cell recovering
T4 Ligase	New England BioLabs ®	
Taq DNA Polymerase	VWR	PCR
Tetracycline	Sigma-Aldrich	Antibiotic
Tris	Sigma-Aldrich	
Tryptone	Oxoid	
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)	Sigma-Aldrich	
Yeast extract	Oxoid	
$ZnCl_2$	Sigma-Aldrich	

Gene expression by quantitative Real Time PCR

Materials/ Equipment	**Manufacturer**	**Description**
1-Bromo-3-chloropropane, BCP	Sigma-Aldrich	
Ethanol, EtOH	Kemetyl Norge	
High-Capacity cDNA Reverse	Applied Biosystems™, Life	cDNA synthesis

Transcription Kit	Technologies	
Isopropanol	VWR	
Lysing Matrix, pre-made silica beads (0,1 mm)	MD	
Methanol	VWR	
Mini Bead Beater	Biospec	
NaAc	May & Baker Ltd. Dagenham, UK	
Power SYBR® Green PCR Master Mix	Applied Biosystems™, Life Technologies	
TRIzol® Reagent	Ambion™, Life Technologies	RNA isolation
TURBO DNA-free™ Kit	Ambion™, Life Technologies	Dnase treatment
RNase free water	Ambion™, Life Technologies	
StepOne Real Time PCR System	Applied Biosystems™, Life Technologies	

Protein expression analysis by Western Blot

Materials/ Equipment	**Manufacturer**	**Description**
Alexa Fluor 546 Donkey anti-Rabbit IgG (H+L)	Life Technologies	Secondary antibody
AP conjugate substrate kit	Bio Rad	
Biotinylated Alkaline Phosphatase, Blotting Grade	Bio Rad	
Blotting casette	Bio Rad	
Blotting-Grade Blocker, Nonfat dry milk	Bio Rad	Blocking buffer
Criterion™ Blotter	Bio Rad	
Criterion™ cell	Bio Rad	
Criterion™XT Bis-Tris Precast Gels, 12 %	Bio Rad	Protein separation
DL - Dithiothreitol	Sigma-Aldrich	
EDTA	Sigma-Aldrich	
Filter paper	Bio Rad	
Glycerol	VWR	
HCl	Merck	
Immobilon®-P, Polyvinylidine difluoride membranes	Sigma-Aldrich, USA	
Lysing Matrix, pre-made silicabeads, 0,1 mm	MD	

Mini-Beadbeater	Biospec products	
Methanol	VWR, France	
β-mercaptoethanol	Sigma-Aldrich	
NaCl	Merck	
NaOH	Sigma-Aldrich	
NuPAGE® MOPS SDS-PAGE (20X)	Novex®, Life Technologies	
NuPAGE® Transfer Buffer (20X)	Novex®, Life Technologies	
NuPAGE® LDS Sample Buffer (4X)	Novex®, Life Technologies	
PVDF-membrane	BioRad	
Sea Blue® Plus2 Prestained Standard	Novex®, Life Technologies	
SDS	Sigma-Aldrich	
Tris	Sigma-Aldrich	
Tween	Merck	

Basic equipment

Basic equipment	**Manufacturer**	**Description**
Centrifuge, Allegra™ X-22R	Beckman Coulter, Brea CA, USA	
Centrifuge, Sorvall RC-5B	Sorvall, Buckinghamshire, UK	Refrigerated super speed centrifuge
Centrifuge, Heraeus Pico21	Thermo Scientific	Tabletop centrifuge
Centrifuge, 5451D	Eppendorf	Tabletop centrifuge
Disposable cyvettes, 1,5 mL	Brandtech, Männedorf, Switzerland	
Erlenmeyer flask, 50 mL, 100 mL, 200 mL, 500 mL		
Falcon tubes, 50 mL	Falcon BD	
Falcon 96 well microplate, flat bottom with lid	Becton Dickinson	
Incubator, Autoflow DH Automatic CO_2 Incubator	NUAIRE, Plymuth NM, USA	Heating incubator
Incubator, Minitron	HT Ingfors, Bottmingen, Switzerland	Heating incubator with agitation
Microplate reader, Tecan Infinite M200	Gröding, Austria	
NanoDrop N1000,	Thermo Scientific	

spectrophotometer		
pH-meter	Methrohm, Herisau, Switzerland	
Spectrophotometer, Shimadzu UV-VIS 160A	Shimadzu Europa GMBH	
Sterile filters, Minisart, 0,2 µm, 0,45 µm	Sartorius, Goettingen, Germany	
Tubes, 15 mL	VWR	
Thermal Cycler	Eppendorf	PCR
Thermal Cycler T100	Bio Rad	PCR
Waterbath, Tempette® Junior TE-8J	Techne	

Appendix II - Media made during the project

Construction of deletion mutants

All media listed below were autoclaved at 121 ^{o}C in 20 minutes after mixing.

- Luria Bertani (LB) medium
- LB-agar
- Bacto Brain Heat Infusion (BHI) medium
- BactoMS sporulation medium

Luria Bertani (LB) medium (450 mL):

- 4.5 g Tryptone (Oxoid)
- 4.5 g NaCl (Merck)
- 2.25 g Yeast extract (Oxoid)
- 450 mL dH_20

Luria Bertani agar (450 mL):

- LB medium
- 6.75 g Agar bacteriological No. 1 (Oxoid)

In case of adding antibiotic this where done after autoclaving, and not in to hot media (>60 ^{o}C).

Bacto Brain Heart Infusion (BHI) medium (450 mL):

- 16.65 g Brain Heart Infusion (BD)
- 450 mL dH_2O

Bacto MS sporulation medium (200 mL) (van der Voort *et al*., 2010)

- 1.6 g (8 g/L) Difico^{TM} Nutrient Broth (BD)
- 2 µl (2.5 µM) Na_2MoO_4 (Sigma-Aldrich)
- 200 µl (2.5 µM) $CoCl_2$ (Sigma-Aldrich)
- 5 µl (2.5 µM) $CuCl_2$ (Sigma-Aldrich)
- 40 µl (12.5 µM) $ZnCl_2$ (Sigma-Aldrich)
- 5µl (1 mM) $MgCl_2$ (Merck)
- 5 µl (5 mM) $(NH_4)_2SO_4$ (Merck)

The solution was autoclaved, cooled, and the following chemicals were added after they had been sterile filtering.

- 25 µl (1 µM) $FeSO_4$ (Merck)
- 1 mL (66 µM)$MnSO_4$ (Merck)
- 132 µl (1mM) $Ca(NO_3)_2$ (Merck)

S.O.C. medium (Invitrogen™, Life Technologies)

Supplied with One Shot TOP 10 chemically competent *E.coli.*

- 2% Tryptone
- 0.5 % Yeast extract
- 10 mM NaCl
- 2.5 mM KCl
- 10 mM $MgCl_2$
- 10 mM $MgSO_4$
- 20 mM Glucose
-

Buffers made during the project

Construction of deletion mutants

SET buffer (Pospiech & Neumann, 1995)

- 75 mM NaCl (Merck)
- 25 mM EDTA, pH 8.0 (Sigma-Aldrich)
- 20 mM Tris, pH 7,5 (Sigma-Aldrich)

Western Blot

Sample buffer (Dr. Marina Aspholm, personal communication)

- 6 mL (10%) SDS (Sigma-Aldrich)
- 3 mL glycerol (VWR)
- 1.9 mL (1M) Tris-HCl pH 6.8
- 0.3 mL (0.2 M) EDTA (Sigma-Aldrich)
- 3 mL β-mercaptoethanol (Sigma-Aldrich)
- 0.8 mL dH_20
  + bromphenol blue
-

TEP buffer (Paidhungat & Setlow, 2001)

- 50 mM Tris-HCl (pH 7.4)
- 5 mM EDTA (Sigma-Aldrich)
- 1% SDS (Sigma-Aldrich)
- 0.15 M β-mercptoethanol (Sigma-Aldrich)

NuPAGE ®MOPS SDS-PAGE, 20X (Novex®, Life Technologies)

- 50 mL 20X MOPS SDS-PAGE buffer added to 950 mL dH_2O

NuPAGE®Transfer buffer, 20X (Novex®, Life Technologies)

- 50 mL 20X Transfer buffer and 100 mL methanol (VWR) added to 850 dH_2O

TBS, Tris buffered saline

- 50 mM Tris (Sigma-Aldrich)
- 150 mM NaCl (Merck)

  pH adjusted to 7,6.

TBST

- TBS added 0.05 % Tween (Merck).

Blocking buffer

- TBST buffer added 5% Non-fat milk (Bio Rad).

Appendix III –Primers for construction of deletion mutants and quantitative Real Time PCR

For the construction of the deletion mutants and for the verification of the mutants primers listed in Table 4 were designed in cooperation with PhD student Kristina Borch-Pedersen. The primers for quantitative Real-Time PCR for the *yndE* genes were also designed in this thesis, in cooperation with Borch-Pedersen. The qRT PCR primers for *gerAA* and *rpoB* were made for an earlier project (Madslien *et al*., 2014). All primers for qRT PCR are listed in Table 5.

Table 4. *Primers designed to delete yndE1, yndE2 and yndE3, and internal primers to verify the deletion. The B and C primers contain an overhang to make sticky ends to join the up - and down-stream fragments by high-fidelity fusion PCR, marked blue with start and stop codon in red.*

Primer	Primer database number	Sequence (5'-3')
YndE1 A	1666	CATCGGAAAGGGGATTGGGG
YndE1 B	1667	GAACCTCTACATTGCGCGTTTCTC
YndE1 C	1668	CGCGCAATGTAGAGGTTCAGCC
YndE1 D	1669	TATGATCGTCGTCGGCTTGG
YndE2 A	1662	CTGTCTTAAAAGGAATAAAGGTG
YndE2 B	1663	TCCTCACTTACATGGAGAAATCACCCCTTTC
YndE2 C	1664	TTTCTCCATGTAAGTGAGGAGGAAAAAGT
YndE2 D	1665	CATTTTCAACGATTTTAAGC
YndE3 A	1658	GCGCTCATTATGGAAGTGGC
YndE3 B	1659	CAAGCGTCACATAAGCGTTTCCCT
YndE3 C	1660	AACGCTTATGTGACGCTTGAAAAAGA
YndE3 D	1661	GGCTTGATCCCTTTCCCGAG
YndE1 int forward	1673	AGCATTATCCCGGGCAAACA
YndE1 int reverse	1674	GACAGCCCTCCGATCACAAA
YndE2 int forward	1675	CCGGATGTCTGGCTTTCTGT
YndE2 int reverse	1676	AATGACCGGAAGCAAGCTGA
YndE3 int forward	1677	TTCAGGTCCGCTCAATGCTT
YndE3 int reverse	1678	CCCAGTACAGGACGCAAGTT

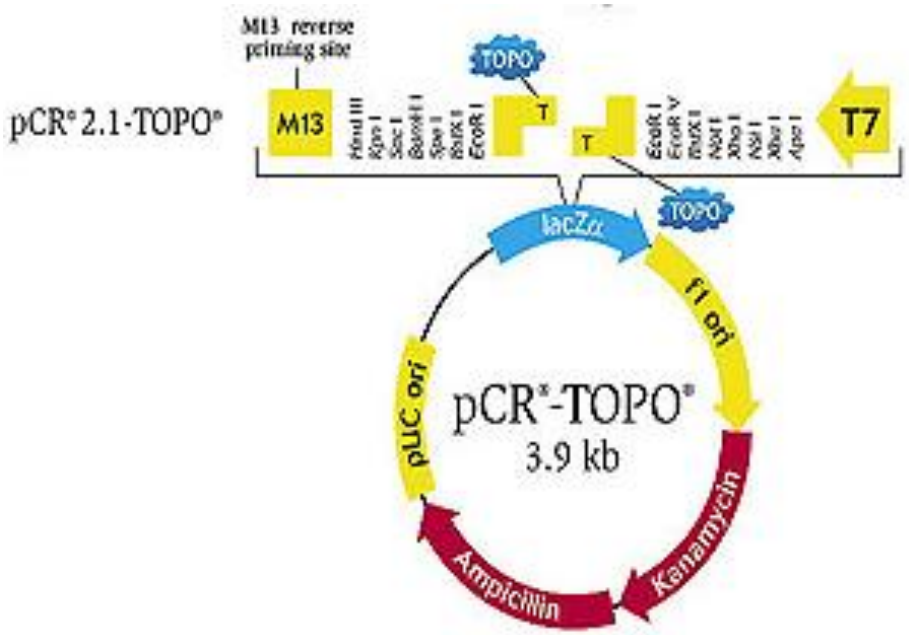
Table 5. *Primers for quantification of yndE expression by quantitative Real Time PCR. The yndE primers were designed using the StepOne RealTime PCR system. The rpoB and gerAA primers from an earlier project were used.*

Primer	Primer database number	Sequence (5'-3')
RT-YndE1 forward	1691	TGCCAAGAACCGCTGTAGAAG
RT-YndE1 reverse	1692	GCCTAATAGGACGGAAATCCATAC
RT-YndE2 forward	1693	CCCCGACAAAACGCTATATCA
RT-YndE2 reverse	1694	AAAGTCCTCCCGGCCACTT
RT-YndE3 forward	1695	CGAAGAGCGCATCACCACTT
RT-YndE3 reverse	1696	TGTCCCAAGCAGGAAATTGG
rpoB Forward	1592	ACCTCTTCTTATCAGTGGTTTCTTGAT
rpoB Reverse	1593	CCTCAATTGGCGATATGTCTTG
gerAA Forward	1553	CCCTGTTCCTATCGGCGTTT
gerAA Reverse	1554	TCGGCAGCATGCCTTGA

Appendix IV – Cloning vectors and plasmid

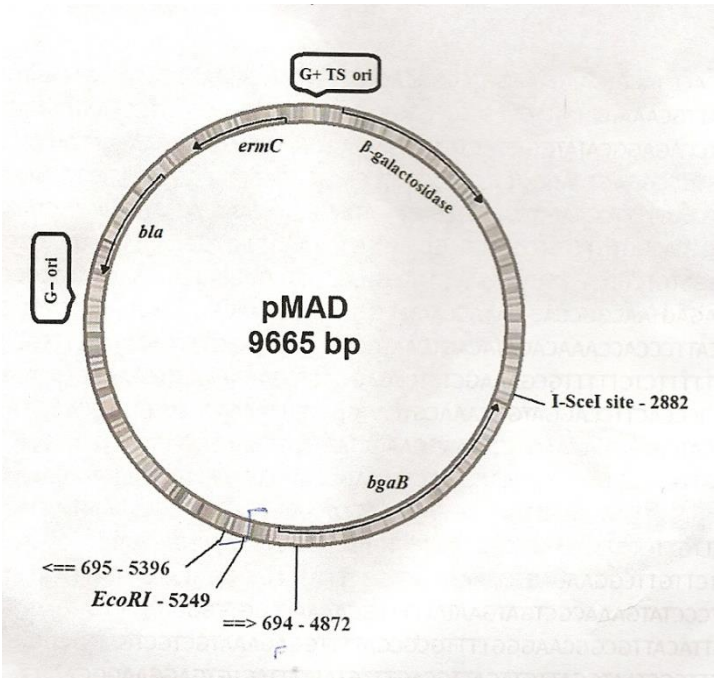
The cloning vectors and plasmid used to create the deletion mutants.

pCR 2.1-TOPO (Invitrogen™)



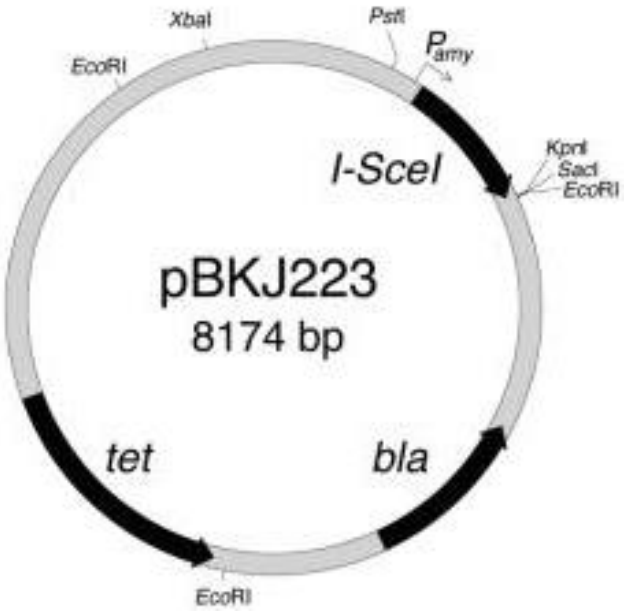
pMAD-*I-SceI*

The pMAD-*I-SceI* vector is a thermosensitive vector constructed from E194ts:: BR322 by Arnaud and coworkers (Arnaud *et.al*., 2004). To increase the frequency of double crossovers an extra restriction site, the *I-sceI* site, was inserted by Dr. Anette Fagerlund, (University of Oslo, Norway). The pMAD-*I-SceI* vector is a kind gift from Dr. Anette Fagerlund.



pBKJ223

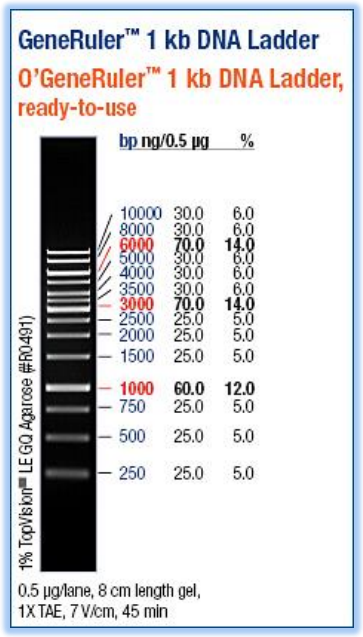
The pBKJ223 plasmid was created by Janes & Stibitz (2006) during Routine Markerless Gene Replacement**.**



Ladders

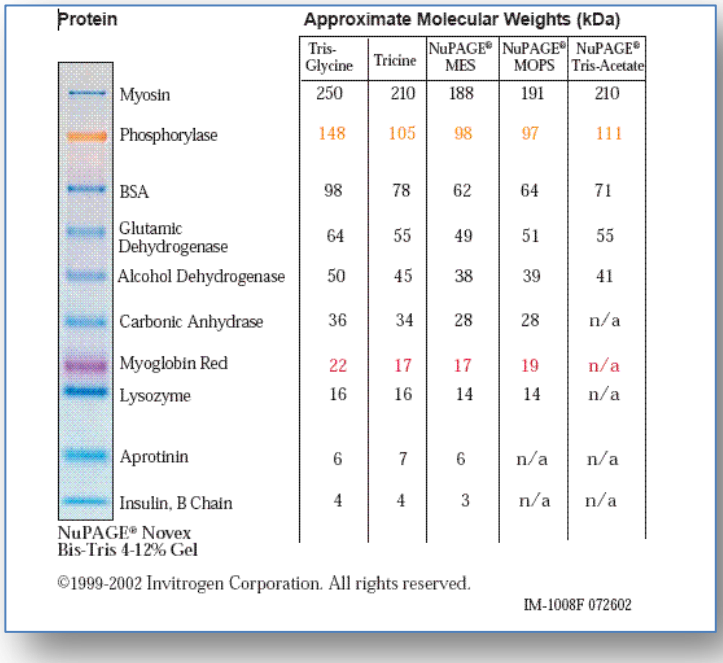
Construction of deletion mutants

The GeneRuler™ 1kb DNA Ladder (Thermo Scientific™) was used for all agarose gel electrophoresis experiments.



Western Blot

The SeeBlue Plus2 Pre-stained Protein Standard was used in a NuPAGE® MOPS buffer system (Life Technologies).



Appendix V – The pentacistronic *ynd* operon in *B. licheniformis* MW3

Comparison of the YndE germination receptors in *B. licheniformis* MW3, and comparison of the YndE subunits in *B. licheniformis* MW3 and the YndE subunit in *B. subtilis* (Figure 17, 18, 19 and 20).

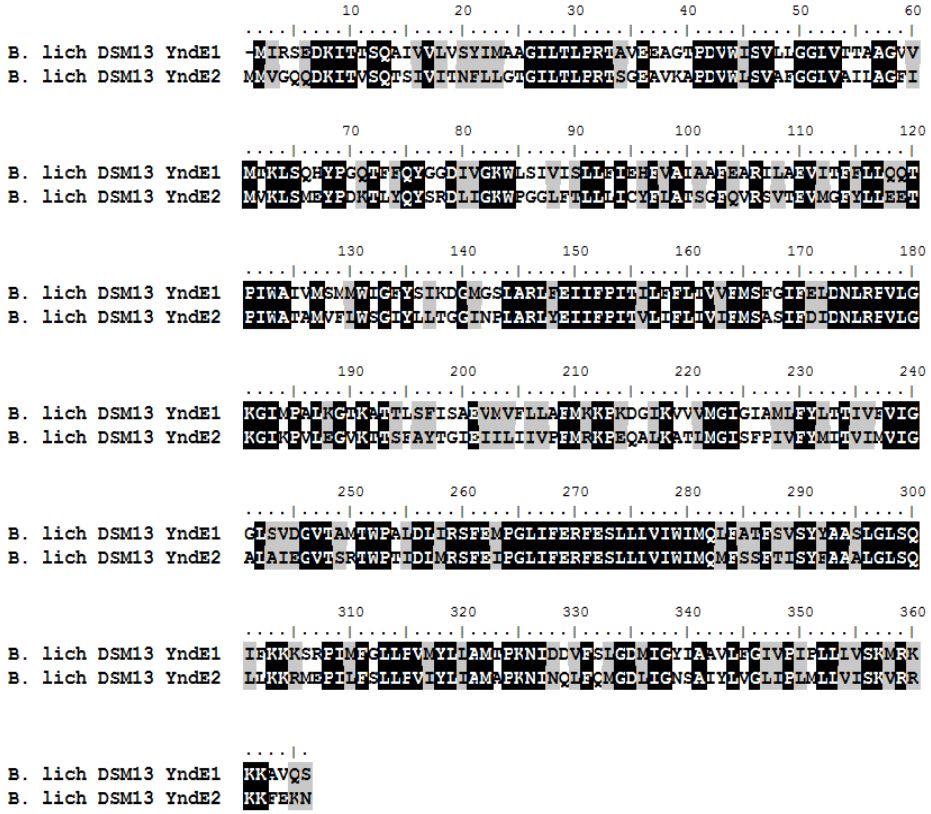


Figure 17. *Pairwise amino acid sequence alignment (Bio Edit) of YndE1 and YndE2 in B. licheniformis.*

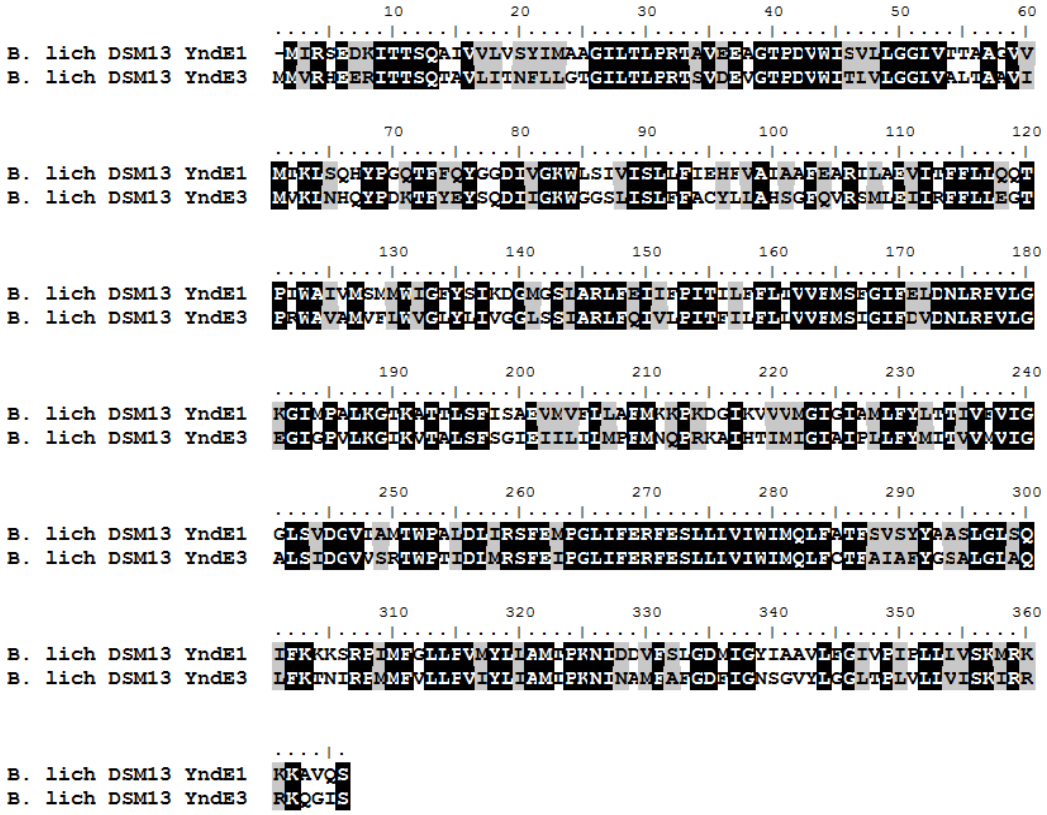


Figure 18. *Pairwise amino acid sequence alignment (Bio Edit) of YndE1 and YndE3 in B. licheniformis.*

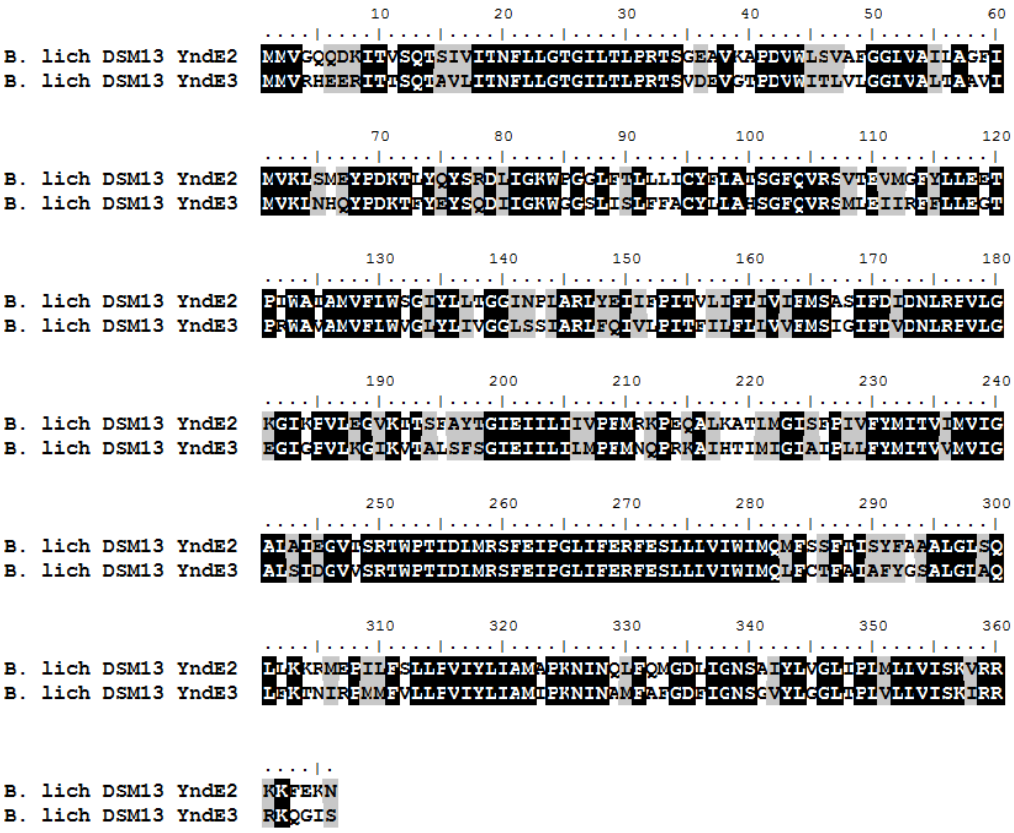


Figure 19. *Pairwise amino acid sequence alignment by Bio Edit of YndE2 and YndE1 in B. licheniformis.*

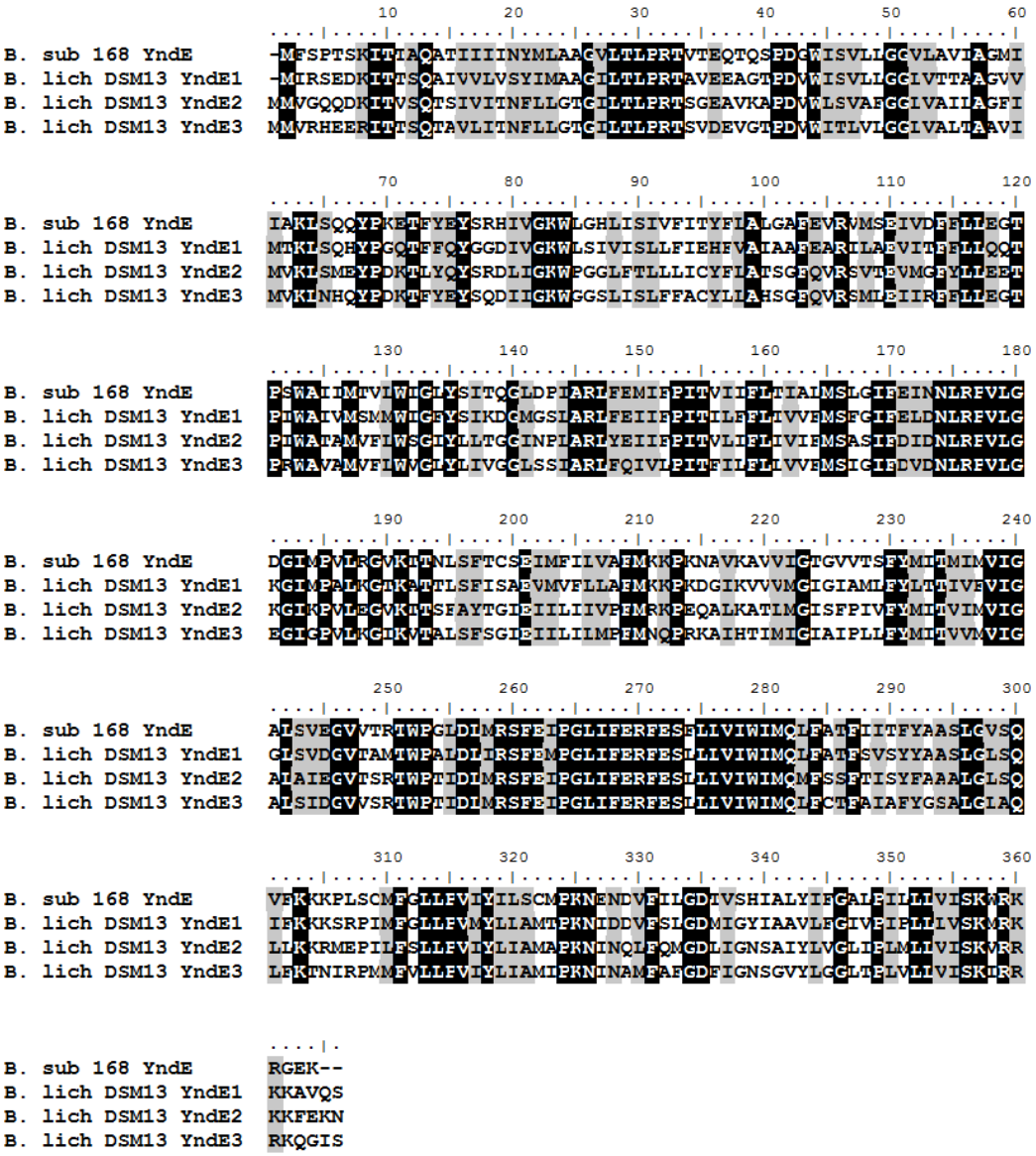


Figure 20. *ClustalW multiple amino acid sequence alignment by Bio Edit of YndE1, YndE2, YndE3 in B.licheniformis and YndE in B. subtilis.*

Appendix VI – Quantitative Real Time PCR

Calculations of expression of the target genes gerAA, yndE1, yndE2 and yndE3 relative to rpoB from quantitative Real Time PCR data.

Efficiency of the *rpoB, gerAA, yndE1, yndE2* and *yndE3* genes from the standard curve is given in Table 6. The calculations of mean expression values for the target genes *gerAA*, *yndE1*, *yndE2* and *yndE3* was done in Excel and are shown in Table 7 to 10.

Table 6. *Efficiency from the standard curves for rpoB, gerAA, yndE1, yndE2 and yndE3.*

Gene	Efficiency, E
rpoB	2.02
gerAA	2.01
yndE1	2.03
yndE2	1.96
yndE3	2

Table 7. *Efficiency calculated from the standard curves and mean cT for all replicates from all three RNA isolations for rpoB, gerAA, yndE1, yndE2 and yndE3.*

		RNA Isolation 1		RNA isolation 2			RNA isolation 3
Gene	Efficiency, E	cT (mean) (*replicate 1*)	cT (mean) (*replicate 3*)	cT (mean) (*replicate 1*)	cT (mean) (*replicate 2*)	cT (mean) (*replicate 3*)	cT (mean) (*replicate 1*)
rpoB	2.02	23.5146	18.702	18.3032	19.9951	21.7792	17.9134
gerAA	2.01	32.5911	28.5039	27.4736	27.6369	30.5858	26.4541
yndE1	2.03	28.7018	24.1976	25.8372	26.2060	26.4781	24.4301
yndE2	1.96	30.2853	25.9409	25.4652	27.4568	28.4774	25.5220
yndE3	2	30.1995	26.6627	25.3659	27.6369	28.6203	26.4541

Table 8. *Efficiency from the standard curves and mean E^{-cT} for all replicates from all three isolations from rpoB, gerAA, yndE1, yndE2 and yndE3*.

		RNA isolation 1		RNA isolation 2			RNA isolation 3
Gene	**Efficiency, E**	**E^{-cT} (*replicate 1*)**	**E^{-cT} (*replicate 3*)**	**E^{-cT} (*replicate1*)**	**E^{-cT} (*replicate 2*)**	**E^{-cT} (*replicate 3*)**	**E^{-cT} (*replicate 1*)**
rpoB	2.02	6.6037E-08	1.9468E-06	2.5769E-06	7.8428E-07	2.2371E-07	3.3894E-06
gerAA	2.01	1.3137E-10	2.2789E-09	4.6786E-09	4.1745E-09	5.3273E-10	9.5328E-09
yndE1	2.03	1.4939E-09	3.6252E-08	1.1355E-08	8.7450E-09	7.2126E-09	3.0750E-08
yndE2	1.96	1.4091E-09	2.6219E-08	3.6111E-08	9.4533E-09	4.7567E-09	3.4757E-08
yndE3	2	8.1104E-10	9.4130E-09	2.3126E-08	4.7914E-09	2.4234E-09	1.0877E-08

Table 9. *Efficiency from the standard curves and mean E^{-cT} relative to E^{-cT} rpoB for all replicates from all three isolations for gerAA, yndE1, yndE2 and yndE3*.

		RNA isolation 1		RNA isolation 2			RNA isolation 3
Gene	**Efficiency, E**	**E^{-cT} relative (*replicate 1*)**	**E^{-cT} relative (*replicate 3*)**	**E^{-cT} relative (*replicate 1*)**	**E^{-cT} relative (*replicate 2*)**	**E^{-cT} relative (*replicate 3*)**	**E^{-cT} relative (*replicate 1*)**
rpoB	2.02	1	1	1	1	1	1
gerAA	2.01	0.001989	0.001171	0.001816	0.005323	0.002381	0.002813
yndE1	2.03	0.02262	0.018622	0.004406	0.01115	0.03224	0.009072
yndE2	1.96	0.02134	0.013468	0.01401	0.01205	0.02126	0.01025
yndE3	2	0.01228	0.004835	0.008975	0.006109	0.01083	0.003209

Table 10. *Mean E^{-cT} relative to E^{-cT} rpoB for all replicates from all three isolations for, gerAA, yndE1, yndE2 and yndE3.*

Gene	**E^{-cT} relative (mean of all 6 samples)**
rpoB	1
gerAA	0.002582
yndE1	0.01635
yndE2	0.01540
yndE3	0.007707

Box plot

The box plot is based on the median values for the 6 samples for each target gene, and the upper and lower value for each gene. The first (Q1) and third (Q3) quartile for each gene was calculated and the upper and lower end of the whisper for each gene represent all samples inside 1,5 IQR on each side of the median value. The IQR is the difference in Q_3 and Q_1 (Q_3-Q_1). See Table 11 for calculation of quartiles – and IQR – values.

Table 11. *Calculation of minimum, maximum, Q1, Q3, median and IQR values for gerAA, yndE1, yndE2 and yndE3 genes relative to the rpoB gene.*

Labels	*gerAA*	*yndE1*	*yndE2*	*yndE3*
Min	0.001171	0.004406	0.01025	0.003209
Q_1	0.001876	0.01038	0.01283	0.005458
Median	0.002582	0.01635	0.01540	0.00771
Q_3	0.003952	0.02430	0.01837	0.00999
Max	0.005323	0.03224	0.02134	0.01228
IQR	0.002076	0.01392	0.005542	0.004536
Upper Outliers	0	0	0	0
Lower Outliers	0	0	0	0

Appendix VII – Construction of deletion mutants

The purpose with this thesis was to separately delete the three homologous *yndE* genes in *B.licheniformis* MW3, and to study the spore germination properties of the resulting mutant strains. During this work, only one deletion mutant Δ*yndE3* was obtained. The work creating the two other mutants, Δ*yndE1* and Δ*yndE2,* were not completed.

Amplification of yndE up- and down-stream regions

When amplifying the *yndE3* up and downstream fragments (AB and CD products) the high-fidelity fusion PCR was optimized to produce a single PCR product without non-specific products. The optimization was done by adjustment of the annealing temperature to 65°C, and by increasing the $MgCl_2$ concentration. Figure 21 show the optimized PCR products for *yndE3* up-and down-stream fragments. Addition of extra $MgCl_2$ in the PCR mix is presented in well 3 and 5. The AB and CD products for *yndE3* gave the expected products at 475 base pairs (bp) and 583 bp, respectively. The addition of extra $MgCl_2$ did not improve the results. Results for construction of *yndE1* and *yndE2* up-and down-stream fragments are given in Figure 22 and 23, respectively.

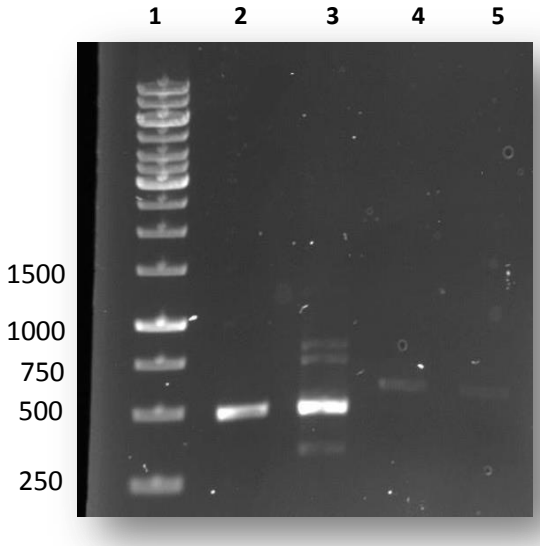


Figure 21. Agarose gel electrophoresis separation of the yndE3 upstream fragment (AB primer pair) and downstream fragment (CD primer pair) products, using high-fidelity fusion PCR. Well 1: *1 kB ladder,* ***Well 2:*** *Primer 1658 (A) and 1659 (B),* ***Well 3:*** *Primer 1658 (A) and 1659 (B), added extra $MgCl_2$,* ***Well 4:*** *Primer 1660 (C) and 1661 (D),* ***Well 5:*** *Primer 1660 (C) and 1661 (D), added extra $MgCl_2$.*

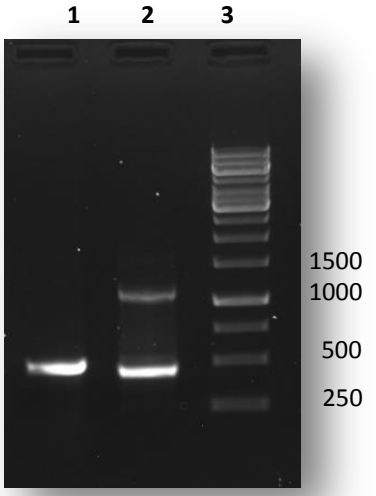


Figure 22 a. ***Agarose gel electrophoresis separation of the yndE1 upstream fragment (AB primer pair) and downstream fragment (CD primer pair) products, using high-fidelity fusion PCR. Well 1:*** *Primer pair 1666 (A)-1667 (B),* ***Well 3:*** *Primer pair 1668 (C)-1669 (D),* ***Well 4:*** *1 kb ladder.*

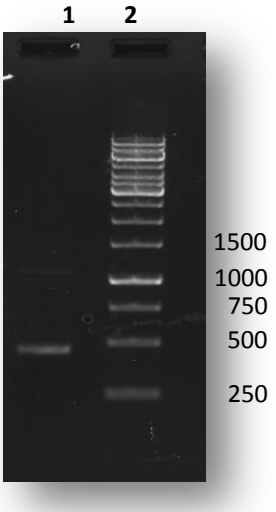


Figure 22 b. ***Agarose gel electrophoresis separation of the yndE1 downstream fragment (CD primer pair) products, using optimized high-fidelity fusion PCR. Well 1:*** *Primer pair 1668 (C)-1669 (D),* ***Well 3:*** *1 kb ladder.*

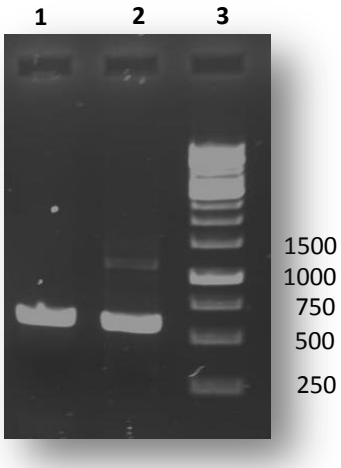


Figure 23 a. ***Agarose gel electrophoresis separation of the yndE2 upstream fragment (AB primer pair) and downstream fragment (CD primer pair) products, using high-fidelity fusion PCR.*** **Well 1:** Primer pair 1662 (A)-1663 (B), **Well 2:** Primer pair 1664 (C)-1665 (D), **Well 3:** 1 kb ladder.

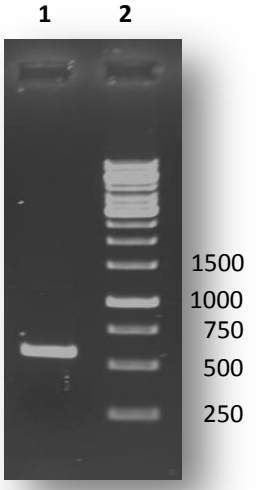


Figure 23 b. Agarose gel electrophoresis separation of the yndE2 downstream fragment (CD primer pair) products, using optimized high-fidelity fusion PCR. **Well 1:** Primer pair 1664 (C)-1665 (D), **Well 3:** 1 kb ladder.

Construction of the yndE AD fusion inserts

PCR amplification of the *yndE3* flanking regions insert of the up- and down-stream regions resulted in a few unspecific products in addition to the desired product of 1064 bp. The high-fidelity fusion PCR amplifying the complete insert, using the two up-and down-stream PCR fragments as template, are shown in Figure 24.

Nevertheless, the complete PCR reaction was used for cloning without further purification. This was done due the difference in size between the desired gene and non-specific products present. Also the amount of the desired product was much higher than the non-specific ones.

The PCR reactions for creation of the fusion fragments encompassing the *yndE1* and *yndE2* flanking regions inserts generated several unspecific products in addition to the desired products of 907 bp and 1122 bp, respectively. The PCR products with the expected size were excised from the agarose gel and purified using the QIAquick® Gel Extraction Kit. Figure 25 and 26 shows the agarose gels containing separated high-fidelity fusion PCR products for *yndE1* and *yndE2*, respectively. Figure 26 shows only the separation of a small amount of the PCR product from A and D primers, and not the whole PCR product used for excision from the agarose gel.

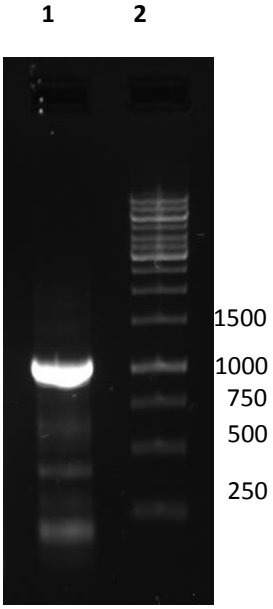


Figure 24. ***Agarose gel electrophoresis separation of the complete yndE3 flanking regions insert (AD primer pair) products, using high-fidelity fusion PCR. Well 1:*** *Primer pair 1658 (A)-1661 (D),* ***Well 2:*** *1 kb ladder.*

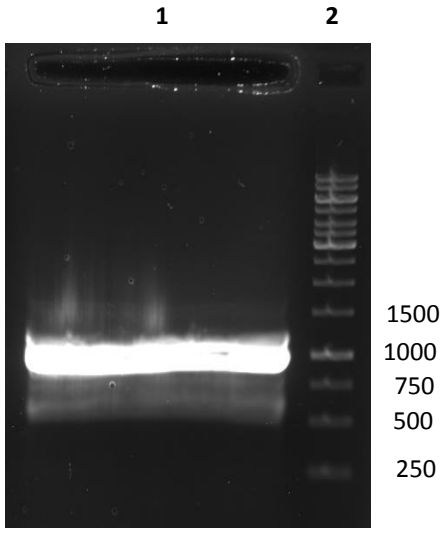


Figure 25. ***Agarose gel electrophoresis separation of the complete yndE1 flanking regions insert (AD primer pair) products, using high-fidelity fusion PCR. - Cut out product from gel.*** **Well 1:** Primer pair 1666 (A)-1669 (D), **Well 2:** 1 kb ladder.

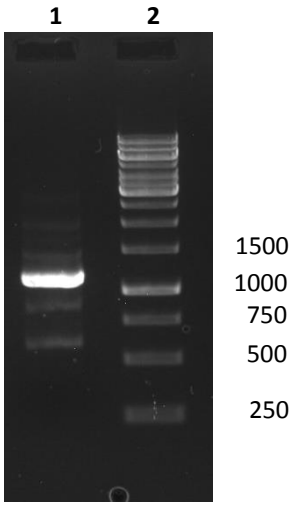


Figure 26. ***Agarose gel electrophoresis separation of the complete yndE2 flanking regions insert (AD primer pair) products, using high-fidelity fusion PCR.*** **Well 1:** Primer pair 1662 (A)-1665 (D), **Well 2:** 1 kb ladder.

Cloning of the fused yndE flanking regions into the TOPO vector and transformation into E. coli

After ligation of the *yndE* flanking regions insert into the TOPO vector, the TOPO vector + insert was transformed into chemically competent *E.coli*. White colonies growing on LB agar containing ampicillin was screened by PCR to verify the transformation of TOPO vector with desired insert. The PCR screening for the flanking regions insert of *yndE3* are shown in Figure 27.

All colonies screened for *yndE3* flanking regions insert ligated into TOPO vector were positive, containing the insert of 1064 bp. The *E.coli* clones from the samples separated in wells 4, 5, 8 and 10 were chosen for further work. All the screened colonies were positive for carriage of TOPO vectors containing *yndE1* or *yndE2* flanking regions inserts as well, (907 bp and 1122 bp, respectively). For *yndE1* and *yndE2*, four random *E.coli* clones for each of the genes were chosen for further work. The screening of the flanking regions insert for *yndE1* and *yndE2* are shown in Figure 28 and 29, respectively.

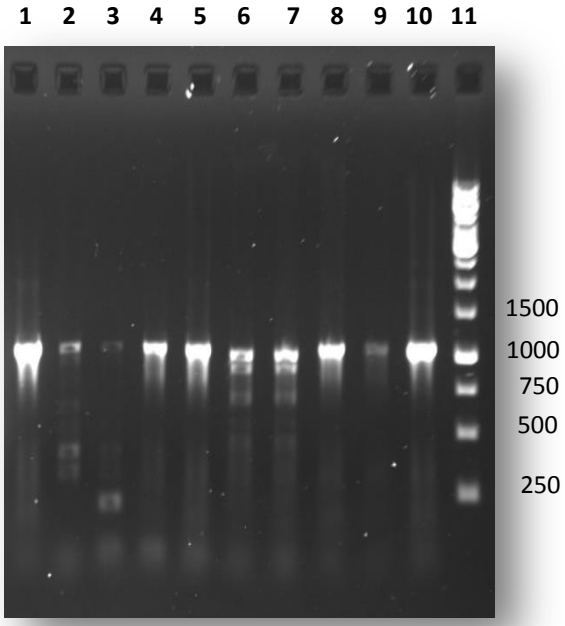


Figure 27. ***Verification of the yndE3 flanking regions insert ligated into the TOPO vector in E.coli by agarose gel electrophoresis separation. Well 1-10:*** *Primer pair 1658 (A)-1661 (D),* ***Well 11:*** *1 kb ladder.*

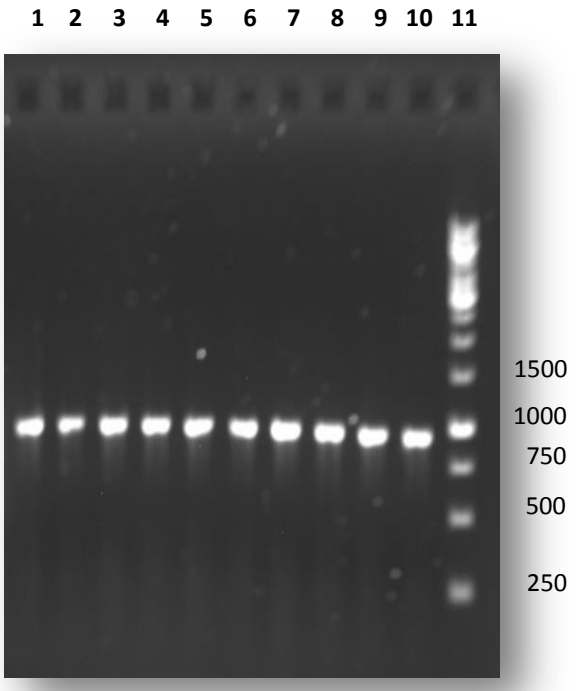


Figure 28. ***Verification of the yndE1 flanking regions insert ligated into the TOPO vector in E.coli, by agarose gel electrophoresis separation. Well 1 -9:*** *Primer pair 1666 (A)-1669 (D),* ***Well 11:*** *1 kb* ladder.

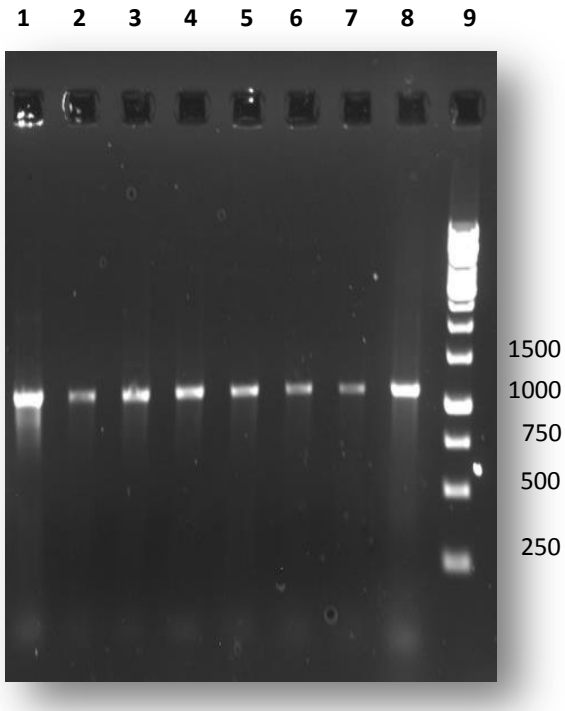


Figure 29. ***Verification of the yndE2 flanking regions insert ligated into the TOPO vector by agarose gel electrophoresis separation. Well 1-8:*** *Primer pair 1662 (A)-1665 (D),* ***Well 9:*** *1 kb ladder.*

Cloning of the fusion AD insert into pMAD-I-SceI and transformation into E. coli

The TOPO vector was isolated from *E.coli* by QIAprep Spin Miniprep Kit. Then, the plasmid was treated with the restriction enzyme *EcoRI* to achieve sticky ends compatible with the pMAD-*I-SceI* vector. The *EcoRI* digested TOPO vector was separated on an agarose gel to purify the insert. The separation of the *yndE3* flanking regions insert from the TOPO vector after *EcoRI* digestion is shown in Figure 30. For *yndE1* and *yndE2* the separation on agarose gel are shown in Figure 31 and 32, respectively.

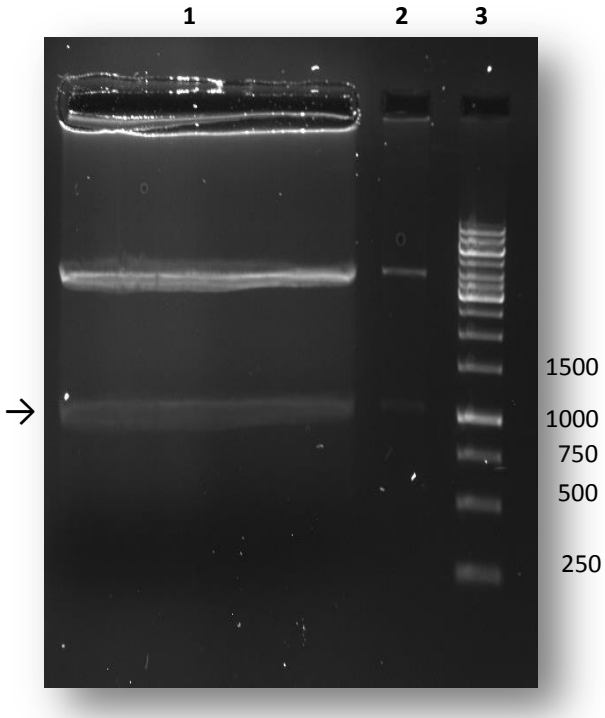


Figure 30. ***Agarose gel electrophoresis separation of isolation of the yndE3 flanking regions insert from the TOPO vector. The insert was excised from the TOPO vector by digestion with EcoRI. The band marked with an arrow was excised from the gel and purified. Well 1-2:*** *Primer pair 1658 (A)-1661 (D),* ***Well 3:*** *1 kb ladder.*

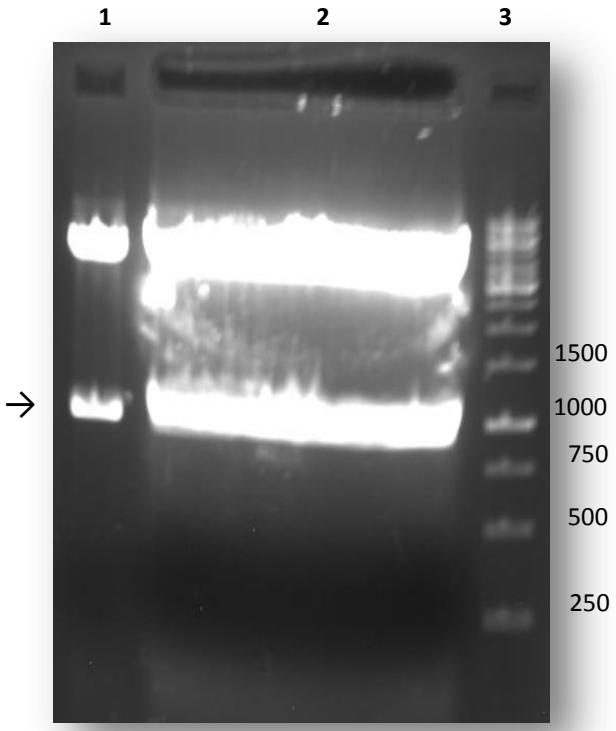


Figure 31 ***Agarose gel electrophoresis separation of isolation of the yndE1 flanking regions insert from the TOPO vector. The insert was excised from the TOPO vector by digestion with EcoRI. The band marked with an arrow was excised from the gel and purified. Well 1-2:*** *Primer pair 1666 (A) -1669 (D),* ***Well 3:*** *1 kb ladder.*

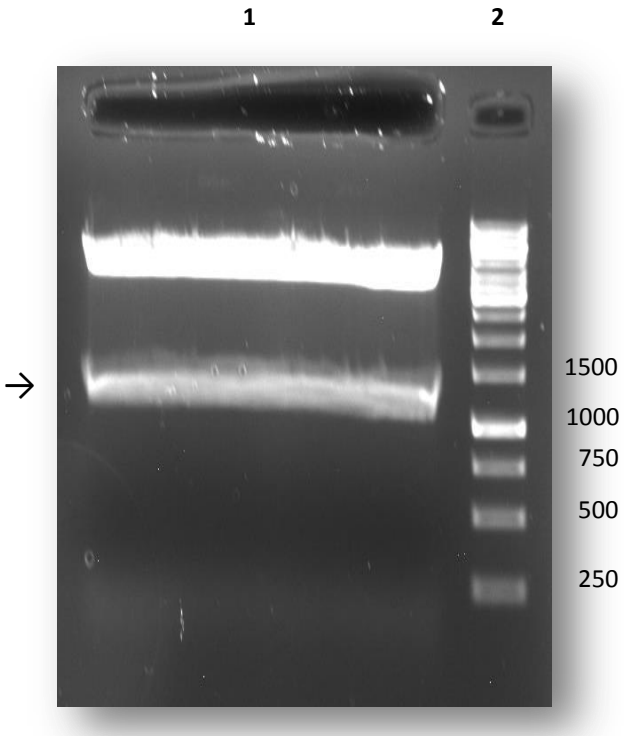


Figure 32. ***Agarose gel electrophoresis separation of isolation of the yndE2 flanking regions insert from the TOPO vector. The insert was excised from the TOPO vector by digestion with EcoRI. The band marked with an arrow was excised from the gel and purified. Well 1:*** *Primer pair 1662 (A) – 1665 (D),* ***Well 2:*** *1 kb ladder.*

The purified *EcoRI* digested *yndE* flanking regions inserts was ligated into the pMAD-*I-sceI* vector and transformed into chemically competent *E.coli* TOP 10 One Shot cells. The transformants were screened for presence of the correct insert by PCR. Figure 33 show that the *yndE3* flanking regions insert in clone 4 and 5 had the correct size at 1064 bp. The transformation of the insert into the pMAD-*I-SceI* vector produced a lower number of transformants than the transformation of inset into the TOPO vector. To find transformants, more colonies had to be screened for *yndE1* and *yndE2* and correct inserts of 907 bp and 1122 bp, respectively, were identified in one *E.coli* clone for each gene. Screenings done on *yndE1* and *yndE2* are shown in Figure 34 and 35, respectively.

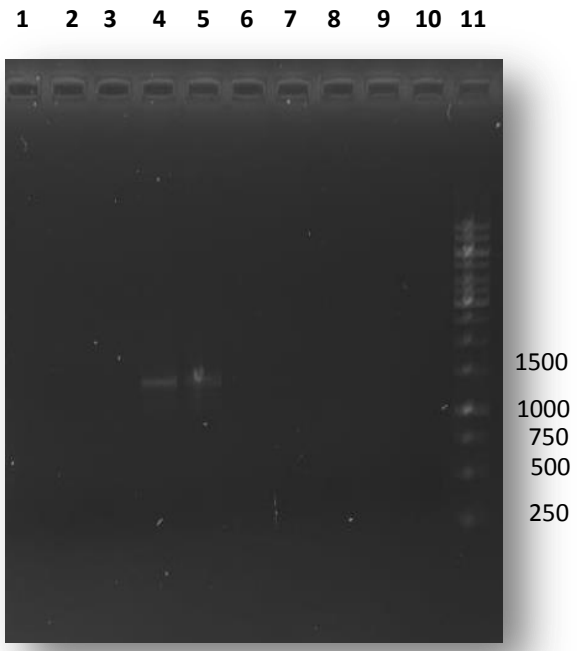


Figure 33. ***Verification of the yndE3 flanking regions insert ligated into the pMAD-I-SceI vector in E.coli, by agarose gel electrophoresis separation. Well 1-10:*** *Primer pair 1658 (A)-1661 (D),* ***Well 11:*** *1 kb ladder.*

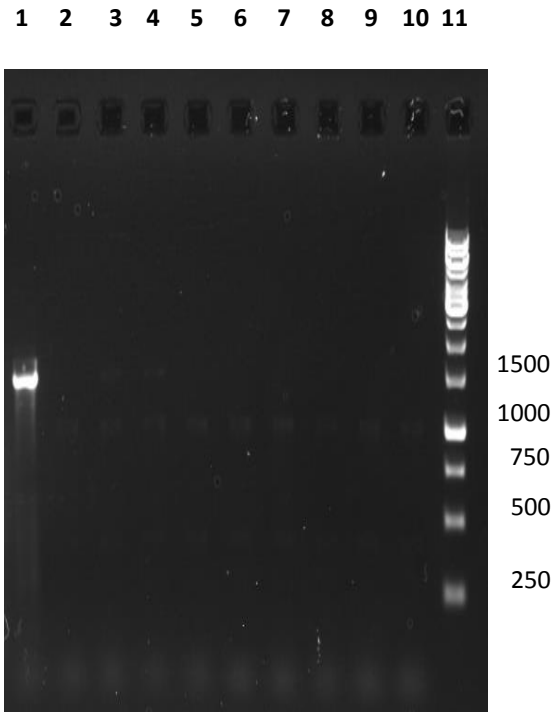


Figure 34. ***Verification of the yndE1 flanking regions insert ligated into the pMAD-I-SceI vector in E.coli, by agarose gel electrophoresis separation. Well 1-10:*** *Primer pair 1666 (A)-1669 (D),* ***Well 11:*** *1 kb ladder.*

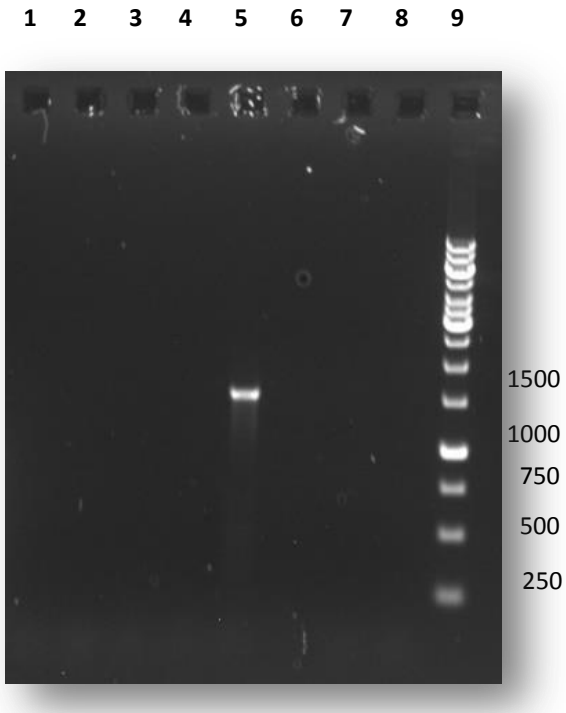


Figure 35. ***Verification of the yndE2 flanking regions insert ligated into the pMAD-I-SceI vector in E.coli, by agarose gel electrophoresis. Well 1-8:*** *Primer pair 1662(A)-1665 (D),* ***Well 9:*** *1 kb ladder.*

The pMAD-*I-sceI* vector carrying the desired *yndE* insert was isolated from *E.coli* after screening. A small amount of the vector was digested with *EcoRI* a second time to ensure that the correct insert was present.

Construction of the B. licheniformis MW3 ΔyndE3 mutant

After transforming the pMAD-*I-sceI* vector into electrocompetent *B.licheniformis,* one blue colony growing on erythromycin was chosen for further work and grown at 37 °C to force the pMAD-*I-SceI* plasmid to integrate into the chromosome.

The blue erythromycin resistant *B. licheniformis* described above was made electrocompetent and transformed with the plasmid pBKJ223 to achieve a double strand break and a following repair by homologous recombination. After plating the transformants on X-gal, white colonies and light blue colonies with a white halo colonies with a blue halo, showed loss of β-galactosidase gene and probably also a loss of pMAD-*I-SceI*. The white colonies, and the white colonies with a light blue halo were screened by PCR to verify loss of *yndE3*. Both external primers, A and D and *yndE3* internal primers were used for screening.

The putative *B. licheniformis* MW3 Δ*yndE3* mutant were compared to *B.licheniformis* MW3 wild type, and are shown in Figure 36. One deletion mutant was found for *yndE3*. When using the *yndE3* external primers the wild type strain was expected to produce a product of 2159 bp, and a 1064 bp for a mutant strain. When using the internal primers the wild type strain was expected to produce a PCR product of 232 bp, while the mutant strain should not produce any PCR product. The MW3 wild type strain produced a product of 232 bp using the internal primers, while the MW3 Δ*yndE3* deletion mutant did not give any product.

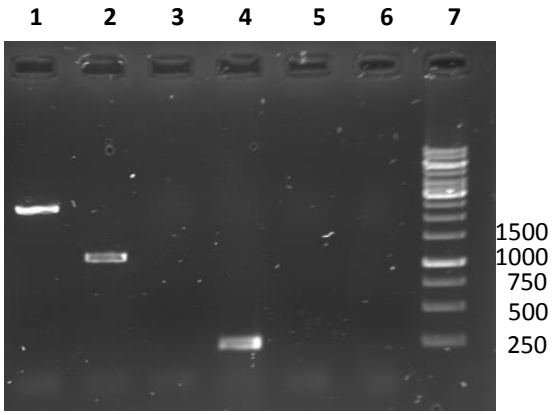


Figure 36. A*garose gel electrophoresis separation of PCR products for verification of a MW3 ΔyndE3 mutant. Well 1:* *MW3, primer pair 1658 (A) and 1661 (D),* ***Well 2:*** *MW3 ΔyndE3 mutant with primer pair 1658 (A) and 1661 (D)****, Well 3:*** *MW3ΔyndE3 mutant with internal primers 1677 (F) and 1678 (R),* ***Well 4:*** *MW3 with internal primers 1677 (F) and 1678 (R),* ***Well 7:*** *1 kb ladder.*

When using the *yndE1* and *yndE2* external primers the wild type strains was expected to produce products of 2022 bp and 2235 bp, respectively. Or a 907 bp and 1122 bp for mutant strains of *yndE1* and *yndE2*, respectively. When using the internal primers the wild type strains was expected to produce a PCR product for *yndE1* and *yndE2* of 532 bp and 831 bp, respectively, while the mutant strains should not produce any PCR product.

A number of colonies were screened for deletion of *yndE1* and *yndE2* genes but no correct transformants were identified (Figure 37 and 38, respectively). The internal primers gave PCR products of 532bp and 831 bp for *yndE1* and *yndE2* respectively, suggesting the gene is still present in both cases.

Due to time issues, the experiments involving the *yndE1* and *yndE2* mutants were finished at this point.

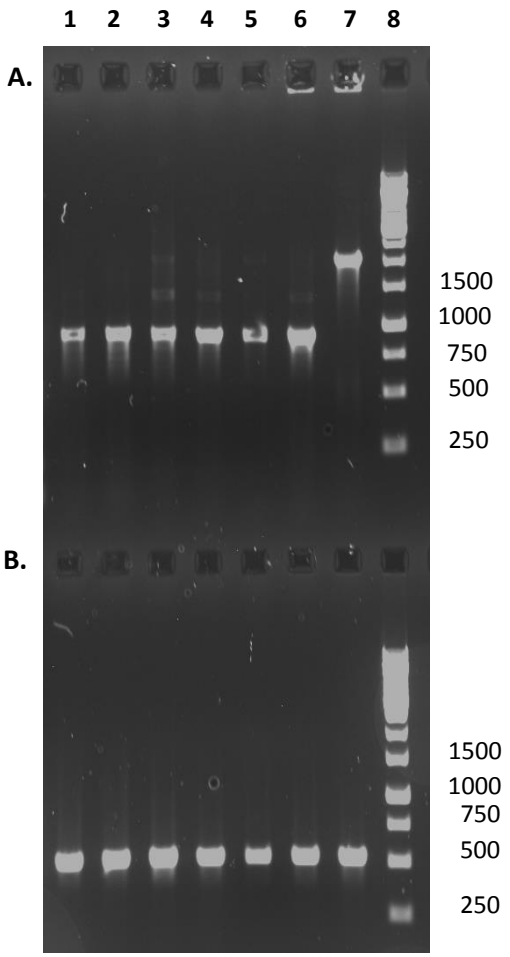


Figure 37. ***Agarose gel electrophoresis separation of PCR products for verification of possible MW3 ΔyndE1 mutants with (A.) primer pair, 1666 (A)-1669 (D), and (B.) primer pair, 1673 (F)-1674 (R) – yndE1. Well 1-6:*** *Possible mutants****, Well 7:*** *Negative control MW3,* ***Well 8****: 1 kb ladder.*

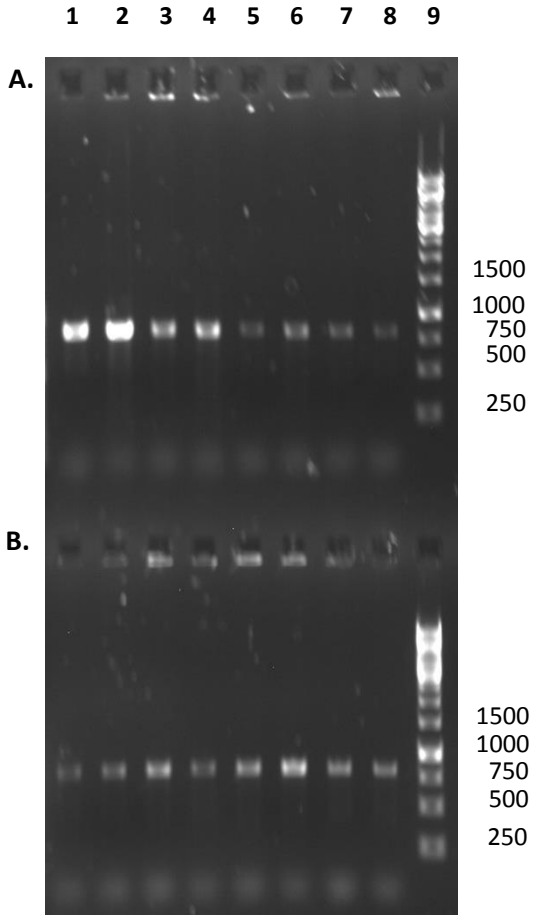


Figure 38. ***Agarose gel electrophoresis separation of PCR products for verification of possible MW3 ΔyndE2 mutants with (A.) primer pair, 1662 (****A****)-1665 (****B****), and (B.) primer pair, 1675 (F)-1676 (R) – yndE2. Well 1-8:*** *Possible mutants****, Well 9****: 1 kb ladder.*

B.licheniformis MW3Δ*yndE3* mutant was cultivated to loose the pBKJ223 plasmid and the clone was tested for sensitivity towards erythromycin and tetracycline. Sequence analysis was performed to verify that the *yndE3* gene was deleted, without any other alterations of the surrounding genes. The amplified PCR product using the A and D primers was used for sequencing. Sequencing was done by Source BioScience (Nottingham, UK), and confirmed that the deletion mutant was correct.

After the *yndE3* deletion mutant was verified by sequencing, the mutant was propagated and stored at -80 ^{o}C, and marked as strain no. 1369.

Appendix VIII – Western Blot

Spores from the *B.licheniformis* MW3 strain (1289) were pre heated at 80 ^{o}C for 15 minutes. The blot was incubated in primary antibodies against YndE3 (Figure 39).

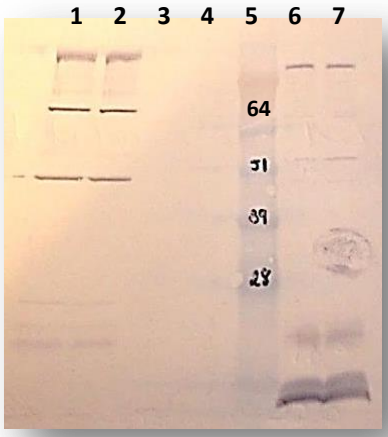


Figure 39. ***Western Blot analysis of whole spore extracts and untreated spores from the MW3 strain. Well 1, 2:*** *Spore extract,* ***well 3 and 4:*** *Negative control (overnight culture),* ***well 5:*** *SeeBlue ladder,* ***well 6 and 7:*** *Whole spores.*

The heating temperature of the spores was increased to 100 ^{o}C, and the analysis was repeated with primary antibodies against YndE1 (Figure 40).

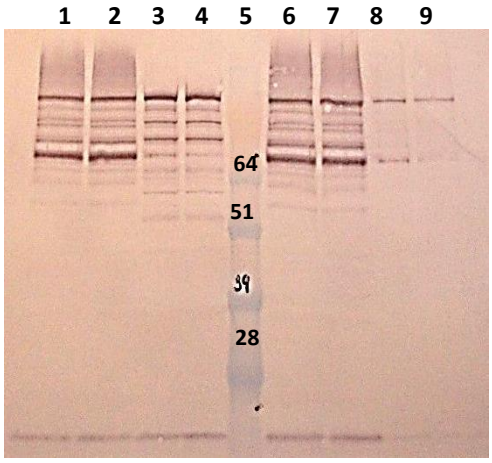


Figure 40. ***Western Blot analysis of whole spore extracts from strain MW3. Well 1 and 2:*** *Spore extract,* ***well 3 and 4:*** *Negative control (overnight culture),* ***well 5:*** *SeeBlue ladder,* ***well 6 and 7:*** *Untreated spores,* ***well 8 and 9:*** *Negative control (overnight culture).*

Comparison of Figure 39 and 40 with heating of the spores at respectively 80°C and 100 °C show that heating to 100 °C gives a higher solubilisation of spore proteins.

Spores and spore extracts (100 °C) of the MW3 Δ*yndE3* mutant strain were analysed on the next membrane, together with spore extract and untreated spores of the MW3 strain which was incubated with primary antibodies against YndE3 (Figure 41).

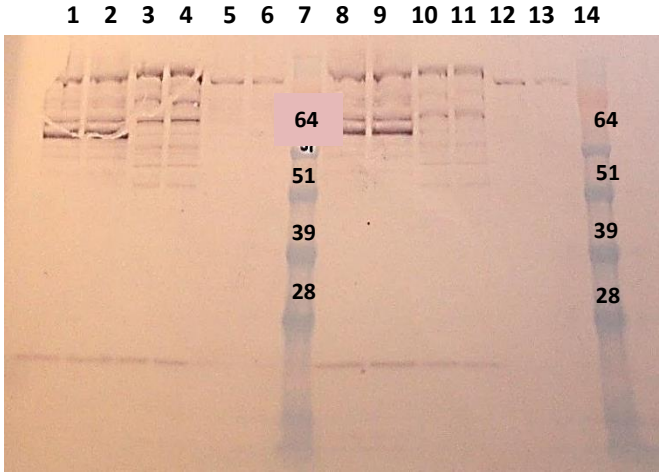


Figure 41. ***Western Blot analysis of whole spore extracts and untreated spores from the MW3 strain and the MW3 ΔyndE3 mutant strain****. **Well 1 and 2:** Spore extract MW3, **well 3 and 4:** Untreated spores MW3, **well 5 and 6:** negative control (overnight culture), **well 7:** SeeBlue ladder, **well 8 and 9:** Spore extract MW3ΔyndE3 mutant, **well 10 and 11:** Untreated spores MW3ΔyndE3 mutant, **well 12 and 13:** Negative control (overnight culture), **well 14:** SeeBlue ladder.*

To test if we could observe any specific reactivity against YndE3, two identical blots were incubated with anti YndE3 and pre-immunserum, respectively using spore extract from the MW3 strain and the MW3 Δ*yndE3* mutant strain. Figure 42 show the comparison between antibody YndE3 and pre serum YndE3 as primary antibodies.

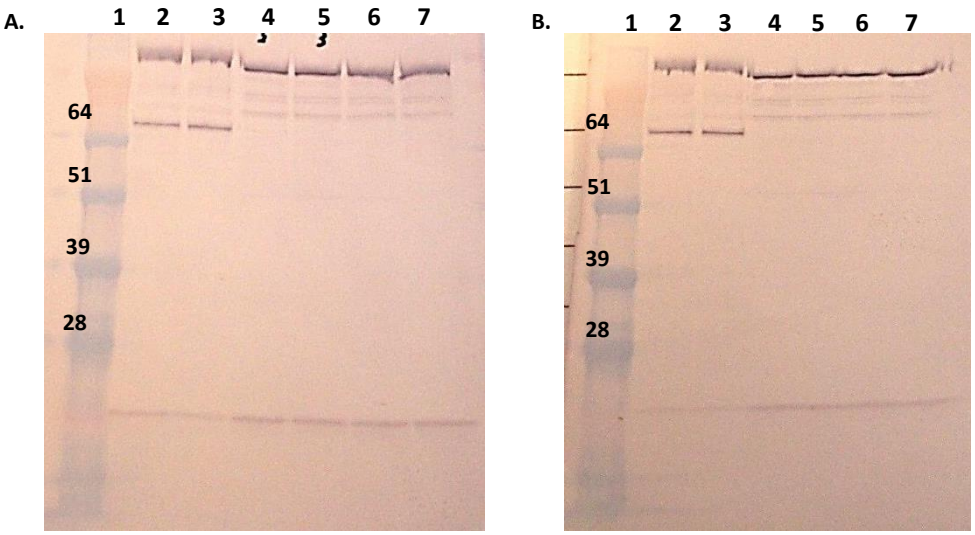


Figure 42. Western Blot analysis of whole spore extracts from the MW3 strain and the MW3 ΔyndE3 mutant strain. (A.): *Antibody YndE3****, (B.):*** *Pre Serum YndE3.* ***Well 1:*** *SeeBlue Ladder****, well 2 and 3:*** *Negative control (overnight culture),* ***well 4 and 5:*** *Spore extract MW3,* ***well 6 and 7:*** *Spore extract MW3ΔyndE3 .*

As Figure 42 A and B describes, the antibody against YndE3 and the pre-immunserum demonstrate identical recognition patterns. Also spores of the MW3 strain and the MW3Δ*yndE3* mutant strain gave the same protein pattern, suggesting that the protein bands on the blot are not the YndE3 protein.

Appendix IX - QIAGEN protocols

Quick-StartProtocol

QIAprep® Spin Miniprep Kit

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

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

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

Notes before starting

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

1. Pellet 1–5 ml bacterial overnight culture by centrifugation at >8000 rpm (6800 x g) for 3 min at room temperature (15–25°C).
2. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 min. If using LyseBlue reagent, the solution will turn blue.
4. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. If using LyseBlue reagent, the solution will turn colorless.
5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.

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

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

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

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

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

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

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

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.

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QIAquick PCR Purification Kit Protocol

using a microcentrifuge

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

Important points before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5.
- Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

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

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

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

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

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

   Collection tubes are re-used to reduce plastic waste.

6. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**
7. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.**

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

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

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

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

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

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

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

QIAquick® Gel Extraction Kit

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

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

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

Notes before starting

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

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg ~ 100 µl). For >2% agarose gels, add 6 volumes Buffer QG.
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
5. Add 1 gel volume of isopropanol to the sample and mix.

Step 5 was not used in the work for this thesis

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

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

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

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

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

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

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

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

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

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.

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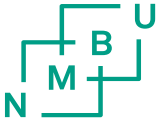

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