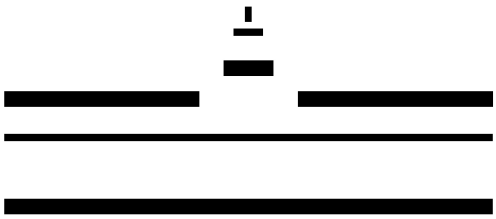

Biologie

# Genetic analysis of polysaccharide deacetylase encoding genes in *Bacillus licheniformis* with respect to spore germination and biosafety

**Inaugural-Dissertation**

zur Erlangung des Doktorgrades
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vorgelegt von

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"Surely with every difficulty there is a relief (ease).

Then when you are done (of business),

devote time and do earnestly the other (task).

And to the Lord turn (all) the attention" (QS 94: 6-8)

*To my parents,*

*sisters, wife and daughter*

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**Abbreviations**

| | |
|---|---|
| ATP | adenosine-5'-triphosphate |
| ADP | adenosine-5'-diphosphate |
| AGFK | asparagines, glucose, fructose and $K^+$ |
| *B.* | *Bacillus* |
| Bli/*Bli* | *B. licheniformis* |
| bps | base pairs |
| CE4 | carbohydrate esterase family 4 |
| CFLE | cortical fragment-lytic enzyme |
| CFU | colony forming units |
| DNA | Deoxyribonucleic acid |
| D-glu | D-glutamine |
| DPA | dipicolinic acid |
| EDTA | diaminoethane tetra acetic acid |
| e.g. | for example (lat. "exempli gratia") |
| 5FU | 5-fluorouracil |
| GFP | green fluorescent protein |
| NAG/GlcNAc | *N*-acetyl-D-glucosamine, |
| HHP | high hydrostatic pressure |
| IPTG | isopropyl-β-D-thiogalactopyranoside |
| kb | kilobases |
| Kin | kinase |
| LB | Luria Bertani (medium) |

| | |
|---|---|
| L-lys | L-lysin |
| L-orn | L-ornithine |
| MPa | mega Pascals |
| MCS | multiple cloning site |
| MδL | Muramic–δ–Lactam |
| NaCl | Natrium chloride |
| NAM/MurNAc | *N*-acetylmuramic acid |
| Nod | nodulation |
| OD | optical density |
| ORF | open reading frame |
| ori | origin of replication |
| PCR | polymerase chain reaction |
| PDA | polysaccharide deacetylase |
| PGNG-dacs | Peptidoglycan GlcNAc deacetylases |
| pH | potential hidrogenii (potential /lat. "potency") |
| $\sigma^E$ | sigma E subunit of RNA polymerase |
| SASP | small, acid-soluble spore protein |
| SCLE | spore cortex-lytic enzyme |
| SDS | sodium dodecyl sulphate |
| 2xSG | 2 x Schaeffer's Glucose Sporulation Agar (medium) |
| SOE | splicing by overlap extention |
| Tris | tris (hydroxymethyl) aminomethane |
| ts | temperature sensitive |

## SUMMARY

*Bacillus licheniformis* is one of the major workhorses for industrial production of extracellular enzymes. However, the endospores produced routinely by all members of the genus constitute a frequent and scary source of contamination in large scale fermentations. Thus, strains impaired in sporulation or germination are the preferred industrial organisms. As neither growth nor vegetative physiology should be negatively affected it is desirable to generate germination mutants.

This study focuses on polysaccharide deacetylase (PDA) encoding genes, the proteins of which being suspected to be possibly involved in spore germination.

From the seven PDA-encoding genes identified in *B. licheniformis* in this study, six were genetically knocked-out: two clean deletion mutants, four single disruption mutants and three double mutants were generated; i.e. altogether nine mutant strains were constructed.

Among the knock-out mutants, those hit in one specific gene (*yfjS*) displayed an obvious reduced spore germination, although the effect was not as striking as for other members of the genus, such as *B. subtilis* and *B. megaterium*. Mutants hit in another specific genetic locus (*Bli02451*) displayed a different

colony morphology. The mechanism leading to such phenotype remains obscure.

The growth of all mutants in complex and minimal media did not deviate from the wildtype parental strain. There is also no evidence for reduction in the capacity to produce extracellular enzymes, as exemplified for amylases, proteases and glucanolytic enzymes.

Since one gene was identified that – when knocked-out – resulted in the reduction of spore germination (Δ*yjfS*), this mutant may constitute the basis for a safe strain applicable in industry.

# 1. INTRODUCTION

## 1.1. The genus *Bacillus*

*Bacillus* is the largest and most prominent genus among the aerobic endospore-forming bacteria, which in total comprise 25 genera with more than 200 species (see also Figure 1.1). The genus harbors Gram-positive, rod shaped organisms, displaying a wide range of phenotypical, physiological and genotypical characteristics. There are not only aerobes, facultative anaerobes or obligate anaerobes, psychrotrophic, psychrophilic, mesophilic, thermotolerant and thermophilic representatives, but also acidophilic to alkaliphilic members; some are salt tolerant and others are halophilic. Catalase is produced by most species (such as *B. sphaericus*, *B. marinus, B. pasteurii)*; also there are oxidase positive species such as *B. laterosporus* and oxidative negative species (such as *B. megaterium, B. circulans*) whereas *B. subtilis* is oxidative indeterminate. Most of them are chemo-organotrophs, facultative chemolithotrophs, prototrophs, and some are auxotrophs requiring several growth factors. The colony morphology and sizes vary between and within a single species. *Bacillus* commonly dominates the culturable soil microflora and members of the genus are routinely found in animal faeces, vegetables, food, and in other natural and man-made environments (Barbosa *et al.* 2005, Claus and Barkeley 1986, Porwal et al. 2009, Postolleca, et al. 2012). Some *Bacillus* species grow as extremophiles in cold, arid, and remote locations and persist

perennially in ice covered Arctic and Antarctic regions, such as *B. firmus* and *B. aryabhattai,* the spores of which survive also in the upper stratosphere (such as *B. subtilis*, *B. pumilus*, *B. firmus*, *B. circulans*, *B. simplex*, *B. isronensis* and *B. flexus*). The ability to grow in plant leaves as indigenous bacterial endophytes (such as *B. subtilis*, *B. silvestris*) is another remarkable feature (Wainwright *et al.* 2003, Wainwright 2008, Smith *et al.* 2011, Shivaji, *et al.* 2012, Reiter and Sessitsch 2006, Gagne-Bourgue, *et al.* 2012, Antony, *et al.* 2012).

Cells are rod-shaped, straight or slightly curved, occuring singly or as pairs, some in chains and occasionally as long filaments. Endospores, formed exclusively one per cell, are very resistant against a number of harsh conditions (see below for details). Gram staining is routinely positive at least during early stages of growth. A *meso*-Diamino pimelic acid murein cross-linkage type is common, but L-lys-D-glu, L-orn-D-glu and L-orn-D-asp have occasionally been reported (Logan and de Vos, 2009).

Cells are motile by means of peritrichous flagella or nonmotile. *Bacillus* species display diverse respiration systems; the bacteria use oxygen as the terminal electron acceptor, which may be replaced by alternatives in some members. Most species will grow on common media such as nutrient and blood agar. The various species of *Bacillus* were divided into groups based on the phenotype of the endosporangia (e.g., swollen vs. non-swollen), the shape of mature spore (e.g., spherical, cylindrical, or ellipsoidal), and on biochemical tests (such as catalase formation and starch hydrolysis) as well as phsyiological

capabilities (e.g., growth with 7% NaCl). Within each group the members are highly heterogeneous (Zeigler and Perkins, 2009).

*B. subtilis* is the model species of the genus. It serves as the paradigm for the genetic architecture and specific traits, such as sporulation and genetic competence, however, less than one third of the *B. subtilis* genes are conserved across other *Bacilli.* The variation is predominantly due to genes needed to respond to environmental stimuli, suggesting that the *Bacilli* have genetically specialized to ensure occupation of diverse habitats and ecological niches (Alcaraz et al. 2010).

## 1.2. *Bacillus licheniformis*

*Bacillus licheniformis* is closely related to *B. subtilis* and, thus, known to thrive in a number of natural environments as a saprophytic organism (Logan and de Vos, 2009). As characteristic for the *B. subtilis* group, *B. licheniformis* is facultatively anaerobic, Gram-positive, motile, and able to form ellipsoidal to cylindrical central spores, sometimes paracentral or subterminal in usually unswollen sporangia. Cells grown on glucose stain evenly. They are 0.6–0.8 x 1.5–3.0 μm in size (see Figure 1.2), occur singly and in pairs, or chains. The colony morphology is variable within and between strains, and, as for *B. subtilis,* may appear like a mixed culture. Colonies are round to irregular in shape and after 24-48 hours cultivation on LB-medium or Standard I of

moderate (2–4 mm) diameter, with margins varying from undulate to fimbriate; upon prolonged cultivation they become opaque, with surfaces that are dull and may become wrinkled; the color is whitish, and may become creamy or brown, perhaps red on media containing sufficient iron; textures of

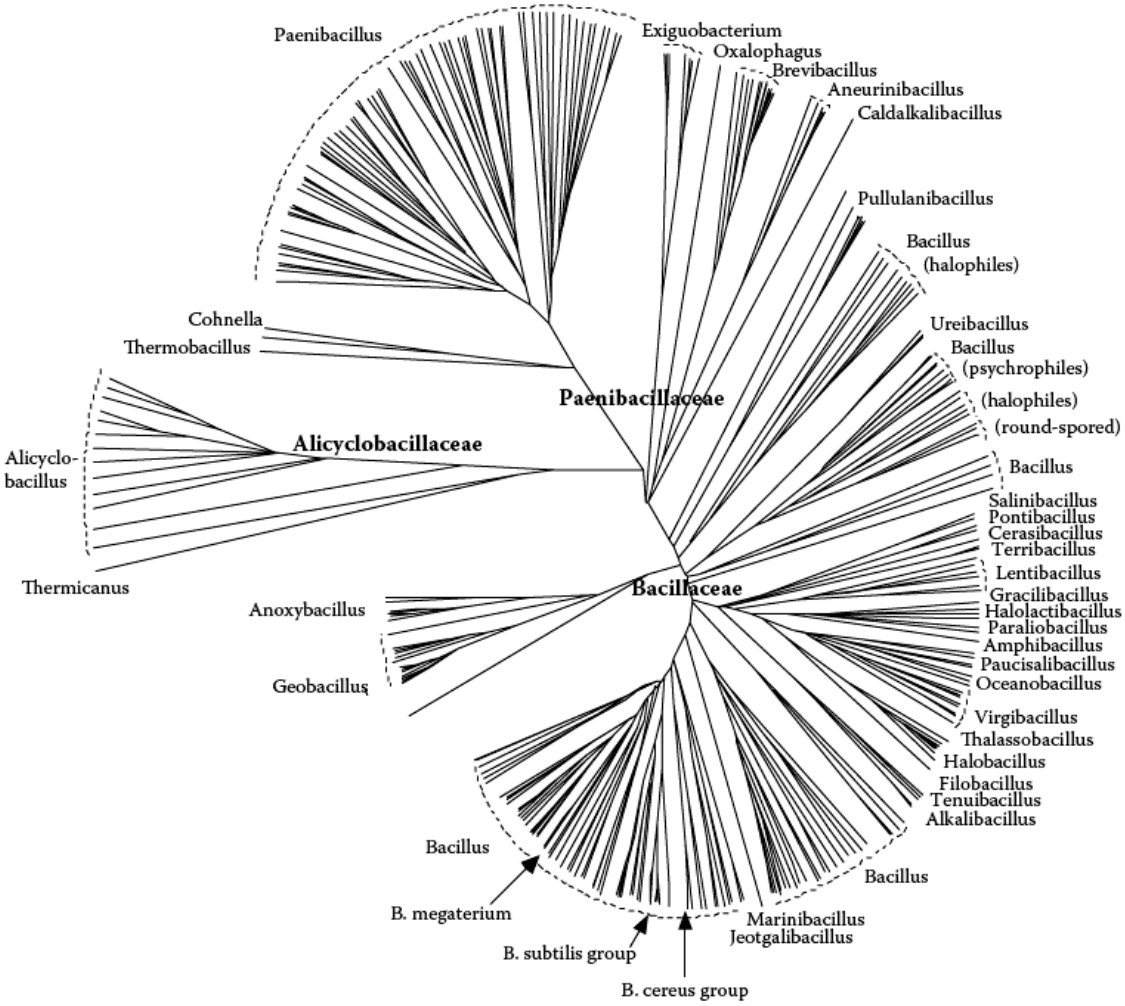



**Figure 1.1** Phylogeny of *Bacillus* sensu lato from 16S rRNA gene sequences. GenBank DNA sequences for species type strains were aligned with ClustalW. An unrooted phylogenetic tree was constructed from the ClustalW distance matrix with the PHYLIP Neighbour application and visualized with

PhyloDraw. Dashed lines group sequences that have been assigned to the same taxon. The rather close *"B. subtilis* group" consists of 14 species, including *B. licheniformis*. The *"B. cereus* group" consists of six species, including *B. anthracis* and *B. thuringiensis* (taken from Zeigler and Perkins 2009).

colony range from moist and butyrous with an underlying mucoid matrix, with or without mucoid beading at the surface, they appear rough to crusty when they get dry; such aponymous properties (licheniform) make the colony adhere to the agar. Though the minimum growth temperature routinely is 5 $^0$C, and the maximum 50 – 55 $^0$C, a geothermal isolate with a temperature maximum of 68 $^0$C has been reported (Llarch *et al.* 1997). Growth is optimal at pH 5.7 to 6.8, but limits have not been reported, and the species also has the capability to grow in rather high concentrations of salt (up to 7% NaCl). The species is catalase–positive but oxidase variable. Casein, esculin, gelatin and starch are hydrolyzed and occasionally strains can hydrolyze urea. The species is widely distributed in soil and many other environments, including milk and food, as well as clinical and veterinary samples. Indeed, vegetative growth may occur readily in food kept at 30–50 $^0$C and may be that is why there were food poisoning instances reported (Logan and De Vos 2009).

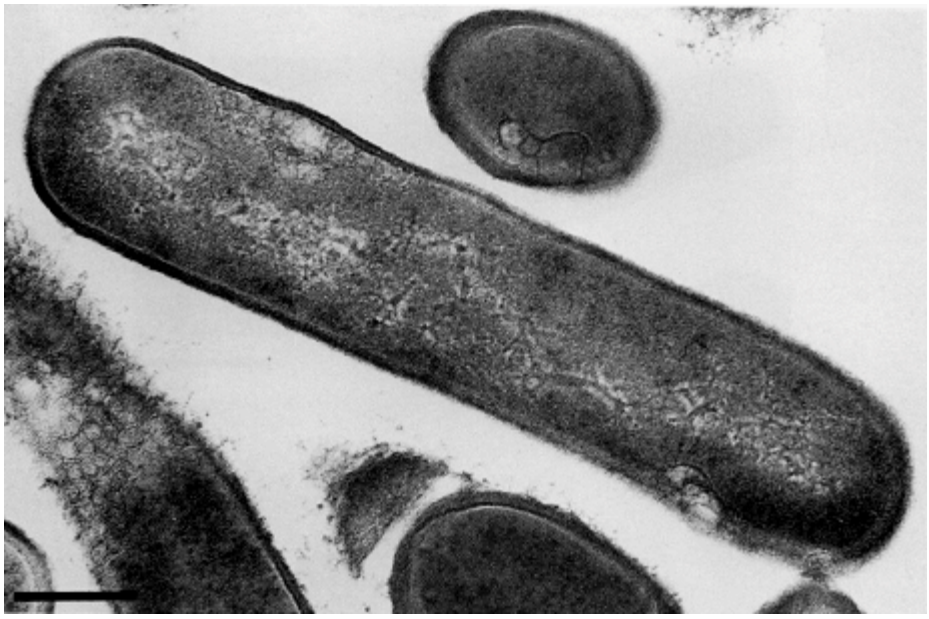


**Figure 1.2** The cell morphology of *B. licheniformis*, a longitudinal section of a vegetative cell, bar 0.25 μm. (the electron micrograph was taken from Williams *et al.* 1990)

## 1.3. Sporulation

The formation of highly resistant, non-reproductive, dormant structures, so called endospores, is a unique feature of the Firmicutes genera *Bacillus* and *Clostridium*. It was published that *Bacillus* spores have survived for approximately 250 million years in a salt crystal, (Vreeland *et al.*, 2000), which convincingly highlights the spore's potential to outlast extremely long periods of time under harsh conditions. The survival of endospores in harsh environments is attributed to distinct structural features observed only in the spore. The bacterial endospore contains the bacterium's DNA and part of its

cytoplasm, which is surrounded by a very tough outer layer structure and a protective protein coat. The function of the coat is to exclude harmful agents, but at the same time allowing for nutrients to enter and trigger germination. The layer is a complex structure containing at least 70 different proteins (in *Bacillus subtilis*). Although some display similarities to known enzymes, most of the coat proteins are only poorly understood (Alcaraz *et al.* 2010; Scheff *et al.* 2009).

The spore architecture highlighting the main structures is schematically depicted in the Figure 1.3.

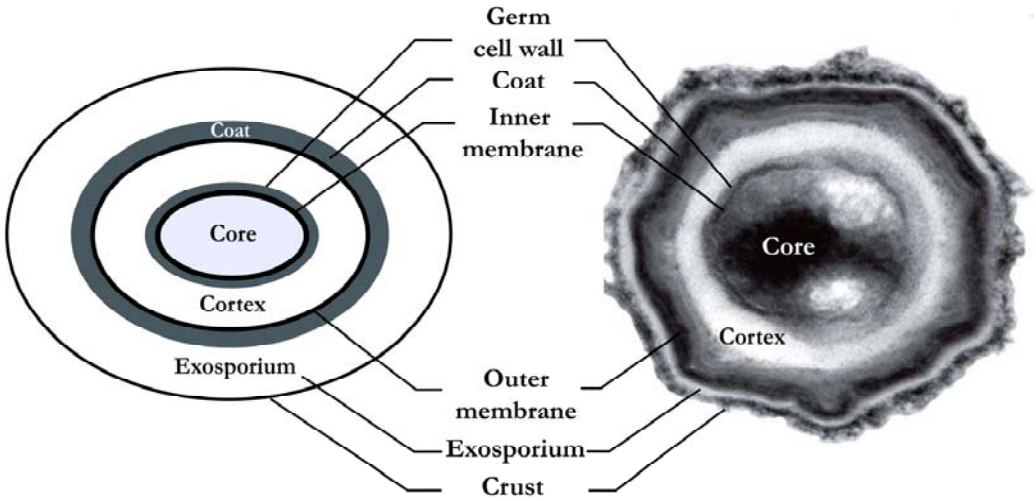



**Figure 1.3** The main structures of the *Bacillus* endospore (modified from Leggett *et al.* 2012, Imamura and Watabe 2012, and McKenney *et al.* 2013). The core not only consists of DNA, RNA, ribosomes, and enzymes, but also small molecules, i.e. dipicolinic acid [DPA], and the core is surrounded by the inner membrane which again is covered by the germ cell wall and cortex. The

cortex consists of specifically modified peptidoglycan, notably the complete absence of teichoic acids from the *N*-acetylmuramic acid (NAM) residues, and is surrounded by the outer membrane which is covered by the coat. The exosporium is the most outside part of the endospore which is covered by an un-characterized thin structure called "crust."

The final architecture of the spore results from the sporulation process which is decribed in more detail in the following chapter.

### 1.3.1. Sporulation Stages

As a strategy for surviving environmental uncertainties *Bacillus* cells enter the sporulation cycle, which is completed by germination when environmental conditions have changed to facilitate again survival and growth of vegetative cells.

Rapidly changing temporal and compartimental specific gene expression eventually gives rise to the formation of the endospores, the structure of which was detailed in the previous chapter. The most recent and thorough description of the sporulation and germination processes was recently done by McKenney *et al.* (2013), from which Figure 1.4 was taken.

*Bacillus* sporulation involves five main stages: asymmetric division, engulfment, late sporulation, mother cell lysis, and germination; starting in the stationary phase cells undergo asymmetric cell division during the so called spore

formation triggering phase. As spore formation is an extremely energy consuming process, the decision to form spores must be strictly controlled and should only happen as a response to most adverse conditions.

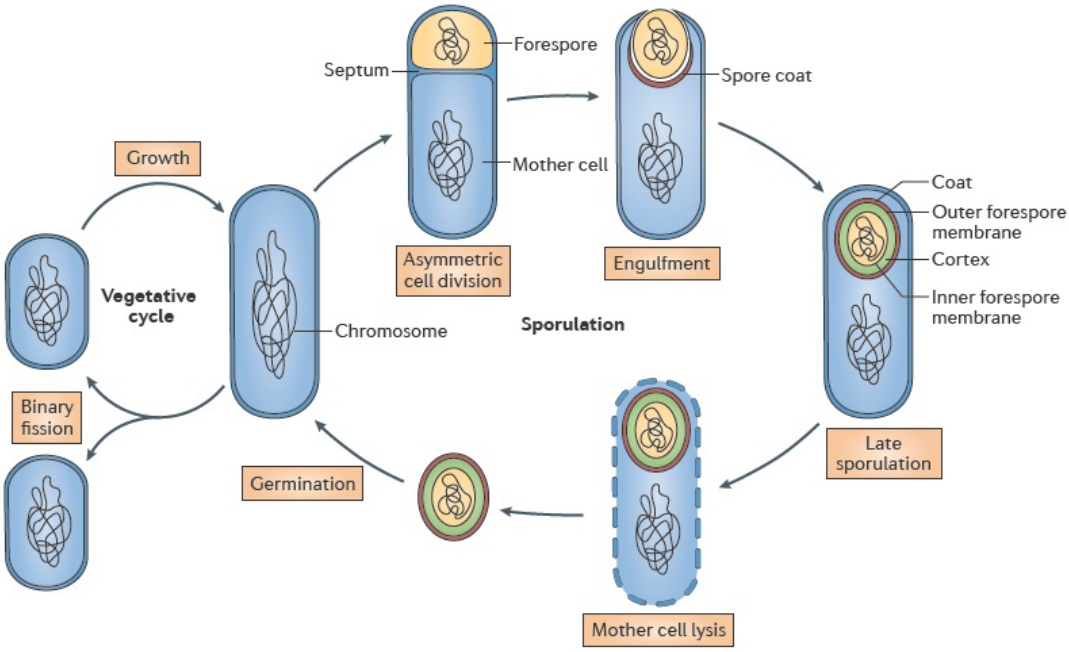



**Figure 1.4** The sporulation and germination cycle in *B. subtilis* (taken from McKenney *et al.* 2013). Sporulation begins when a cell divides asymmetrically to produce two compartments: the mother cell and the forespore, which are separated by a septum. Next, the mother cell engulfs the forespore, and following membrane fission at the opposite pole of the sporangium, a double-membrane bound forespore is formed. Coat assembly begins just after the initiation of engulfment and continues throughout sporulation. The peptidoglycan cortex between the inner and outer forespore membranes is assembled during late sporulation. In the final step, the mother cell lyses to release a mature spore into the environment. Spores are capable of quickly germinating and resuming vegetative growth in response to nutrients.

The decision the develop spores neccesitates signals from the environment, from the bacterial metabolism, and from the cell cycle. Although the nature of most of the signals still remains unresolved, signal recognition, integration and the activation of the gene expression program are getting more and more clear. Two major histidin sensor kinases are instrumental in triggering sporulation (KinA and KinB) as well as the two cognate response regulators Spo0F and Spo0A. However, the sporulation affecting signal transduction system, termed the phospho-relay does not only consist of the above two component systems, but – unique to the system – it makes use of Spo0B, a phosphotransferase that reversibly can transfer the phosphoryl group between Spo0F and Spo0A.

KinA and KinB receive environmental stimuli and undergo autophosphorylation, however they have distinct roles and different input signals. KinB, expressed during the exponential phase, ensures low levels of sporulation, whereas KinA is responsible for sporulation initiation at the onset of the stationary phase. Thus, sequential activation of kinases is central to the phosphorelay signal transduction system triggering such morphological differentiation *Bacillus*.

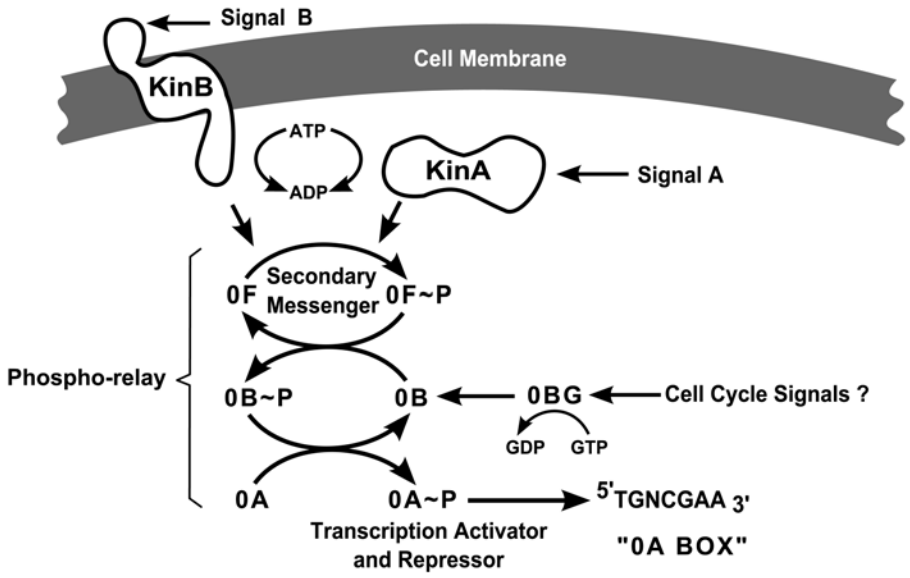



**Figure 1.5** Model for the signal inputs and information transfer of the phosphor-relay in the initiation of sporulation (taken and modified from Burbulys *et al.* 1991). KinA and KinB denote two protein kinases which serve phosphate as the input for the Spo0F secondary messenger; Spo0F~P phosphorylates Spo0B to form Spo0B~P and the phosphate is then transferred from Spo0B~P to Spo0A; Spo0A~P is a transcription factor that recognizes the 0A box (5'TGNCGAA3') in the respective promoter region; Spo0BG refers to a GTP-binding protein, which serves to recognize and possibly interpret cell cycle signals and possibly also influences the activity of Spo0B during the phosphate transfer.

Spo0A~P plays the most important role for the mutual exclusive decision of a cell to divide or sporulate. The level of Spo0A~P correlates to the activity of the phospho-relay and the phosphate flow through the system can be influenced by many parameters.

The active molecule Spo0A~P is involved in triggering the asymmetric cell division, which creates a sporangium containing the two compartments, i.e. the larger mother cell and the smaller forespore which are divided by a septum. In a process resembling phagocytosis the forespore is engulfed and subsequently covered by the spore coat. The engulfment stage is followed by a stage called late sporulation during which several layers are formed and mature, including the spore coat, the outer forespore membrane, the cortex, the inner forespore membrane and the spore core (containing less water than any other cytoplasmic solution and – most importantly – a copy of the genome). During the last sporulation stage termed mother cell lysis – while in the forespore the process to develop the mature spore proceeds – the mother cell expresses large amounts of lytic enzymes eventually leading to the lysis of the cell wall and cell membrane. Finally, the endospore is released.

## 1.4. Germination

Germination is a series of successive and degradative events triggered by specific germinants, which leads to the loss of the typical spore properties. The germinants comprise nutrient molecules with a low molecular weight, mainly amino acids, purine derivatives, and sugars. The molecules acting as germinants are species and strain specific. The signalling process, which occurs when the nutrient germinant binds to a receptor complex and subsequently

activates the spore germination-specific cortex lytic enzyme, is not yet known. Studies have shown that heat, combined with additional controlling factors (pH, organic acids, preservatives) affect *Bacillus* species spore viability, germination and outgrowth. Such understanding of the germination physiology is not only important for basic research but, is rather valuable for improving existing and the development of new sporicidal treatments (Atrih & Foster, 2002).

### 1.4.1. Germination Stages and Pathway

There are at least five stages in the activation of spore germination; maybe, however, upon thourough inspection, one has to add other initial triggering/induction stage prior to the five steps. In the very first step, spores are induced to germinate, in nature probably solely in response to nutrients (germinants). The germinants comprise not only single amino acids, sugars or purine nucleosides, but combinations of them can trigger spore germination, too, e.g. a mixture of asparagine, glucose, fructose and $K^+$ (AGFK) triggers *B. subtilis* germination. Within a few seconds after adding the germinants, spores are committed to germinate, and germination will proceed even after removal of the triggering germinant. Five immediate responses to the germinants were identified: first, the release of spore $H^+$, monovalent cations and $Zn^{2+}$ , probably from the spore core (release of $H^+$ leads to the increase of the core

pH from 6.5 to 7.7, a change, that is essential for activating the spore metabolism when the core hydration levels suffice for enzyme action); second is the release of the core's large depot (10% of spore dry weight) of pyridine-2, 6-dicarboxylic acid (dipicolinic acid [DPA]) and the associated divalent cations, predominantly $Ca^{2+}$; third is the replacement of DPA by water, resulting in an increase in core hydration and causing some decrease in the spore wet-heat resistance although this initial increase in core hydration is not sufficient for enzyme action in the spore core; fourth is the hydrolysis of the peptidoglycan spore cortex; and fifth concerns the swelling of the core by further water uptake and the rapid expansion of the germ cell wall. The first till the third steps are summarized as the first stage of spore germination, and the fourth and fifth step summarized as the second stage of the spore germination (Setlow 2003, Parades-Sabja and Sarker 2010). See also Figure 1.6 and Figure 1.8 for a schematic representation of the germination pathway.

The final stage of germination is the outgrowth, which is characterized by preliminary metabolism, leading to degradation of small, acid–soluble spore proteins (SASPs), macro molecules synthesis and the final escape from the spore coat. Due to the above cortex hydrolysis the spore core can expand without the need of membrane synthesis, allowing for alleviating DNA protection via the SASPs and finally start the cytoplasmic metabolism. The hydrolysis of SASPs represents a major step of outgrowth. The proteins are synthesised during spore development very lately. The function of the proteins

is to bind tightly to and saturate the DNA in the forespore cytoplasm to protect the DNA from hydrolysis by hydrolytic enzymes that could possibly damage the spore core. The presence of SASPs is one of the main characteristics of endospores (Setlow *et al.* 1998, Setlow, 2007).

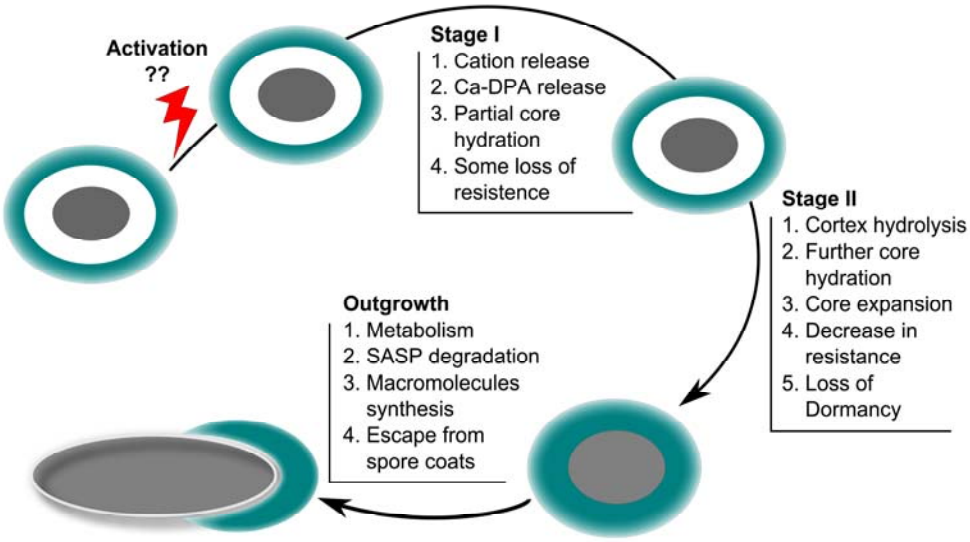



**Figure 1.6** The stages in spore germination (adapted from Setlow 2003). Four main stages are involved in spore germination: activation, stage I, stage II, and outgrowth. SASP refers to small, acid–soluble spore proteins. The protein is abundant and occupies almost 10-20% of the total spore proteins.

Recent studies, proved that not only chemical/nutrient germinants can trigger germination but also physical stimuli can initiate germination without the need of nutrient nor chemical germinants, and, moreover, it can also cause direct germination. Physical treatment such as high temperature and high hydrostatic

pressure (HHP) enables opening the spore's $Ca^{2+}$–DPA channel. There are several levels of pressure possibly affecting germination initiation and/or direct germination i.e.; low level of HHP (100–200 mega Pascals [MPa]) affect only the slow initiation of germination, medium level HHP (500–600 MPa) will trigger a rather rapid germination, and high level of HHP (100–600 MPa) will cause an immediate germination (Setlow 2003, Paredes-Sabja *et al.* 2011).

### 1.4.2. Germination Receptors

The activation of germination is presumbly the result of the interaction between the germinant with a specific site that functions as the germinant receptor in the spore and also by physical influences from the environment i.e. high temperature and high pressure (HHP). The chemical and physic stimuli constitute signals which must be received by the spore core.

The respective receptors for chemical germinants are known for *B. subtilis*, in which they are encoded by three rather homologous operons (*gerA*, *gerB* and *gerK*), collectively termed *gerA* homologs. Each of the above operons has three structural genes, which encode the respective receptor protein subunits A, B, C. The operons are expressed lately in the forespore; the proteins encoded are – due to the existence of membrane spanning domains – possibly located in the spore's inner membrane (see also Figure 1.7). The GerA receptor senses L-alanine, whereas GerB and GerK jointly act to trigger germination with

AGFK. Indeed, there is a rather simple model (as shown in Figure 1.7) proposing that the receptor proteins are located in the inner membrane, but nothing is known about possible interactions of the receptors themselves or with other proteins.

Indeed, the interaction of GerA with the single germinant (L-alanine) is inhibited by the presence of D-alanine, but at the same time D-alanine also binds to the germination receptor (GerB). The reaction implying D-alanine has a negative function (repressor) for GerA but positive (inducer) for the activation of GerB. When the environment is rich of germinants, all of the operons (*gerA*, *gerB* and *gerK*) respond jointly, which efficiently triggers spore germination (Makino and Moriyama 2002). In summary, binding of germinants to the germinant receptors can trigger spore germination due to one or a combination of several stimuli, e.g.: in a way that a single germinant receptor responds to a single nutrient germinant; a single germinant receptor responds to multiple germinants; or two or more receptors respond cooperatively to one or more germinants (Makino and Moriyama 2002; Paredes-Sabja *et al.* 2011).

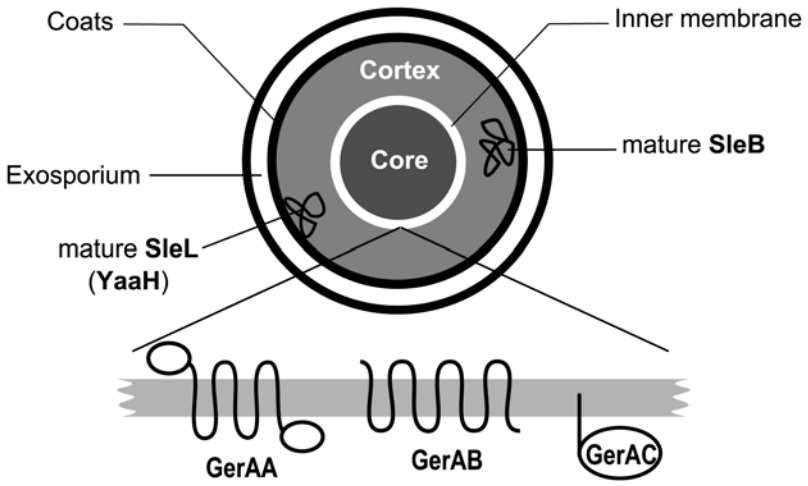



**Figure 1.7** Model of the germination receptor GerA consisting of GerAA, GerAB, and GerAC from *B. subtilis* (adapted from Moir *et al.* 2002, Makino and Moriyama 2002, Ross and Abel-Santos 2010, Wilson *et al.* 2011), highlighting the topology and localization of the GerAA, GerAB and GerAC proteins in the inner membrane of the spore. The enzymes SleB and SleL (YaaH) are instrumental in cortex lysis (see also Figure 1.8)

Besides the *gerA* operon homologs, there are several other genes known to be instrumental in germination i.e., *gerC*, *gerD*, *gerF*, *sleB*, *yaaH* (*sleL* homolog in *B. cereus*), *ypeB*, and *cwlJ*.

The *gerC* locus comprises a triscistronic operon, which encodes three binding subunit–proteins GerCA, GerCB and GerCC. Although the function of the gene products in germination is still rather unclear, suppressing gene expression led to a drastic change of the germination properties but only slightly influenced vegative growth (Yazdi and Moir 1990). Since the locus also encodes the catalytic subunit of heptaprenyldiphosphate synthase, the genes of

tricistronic operon were renamed: *hepA*, *menG* and *hepB*, respectively (Leatherbarrow *et al.* 1998).

Studies addressing an another germination receptor protein GerD encoded by *gerD*, proved that the protein is essential for germination and the receptor can bind to several specific amino acids i.e. to L-alanine or a mixture of L-asparagine, D-glucose, D-fructose, and Potassium. The gene product was also shown to be localized in the inner membrane. (Pelczar *et al.* 2007, Pelczar and Setlow 2008, Mongkolthanoruk *et al.* 2009, Korza and Setlow 2013).

From functional studies of *gerF*, another germinant receptor encoding gene, the gene product (GerF) was shown to have a similar function as for GerD and also there is a dependency on each other. When the gene (*gerF)* was disrupted (single knock-out), the mutant exhibited a very slow germination, implying that the gene is instrumental in germination. (Moir *et al.* 1990, Paidhungat and Setlow 2001, Iragashi *et al.* 2004, Setlow 2003).

### 1.4.3. Germination specific cortex-lytic enzymes

As mentioned in the previous chapter, spore germination comprises already several steps before the main process, the hydrolysis of the cortex, enables the spore core to extend and finally grow out.

Enzymes involved in cortex hydrolysis during germination have been identified and characterized at the molecular level. These enzymes are

classified into two categories by their substrate specificities: spore cortex-lytic enzymes (SCLEs) are capable of hydrolysing the peptidoglycan of the spore coat in vitro. Such action causes darkening of the phase bright decoated spores, similar to the spore darkening found during normal spore germination; contrary to SCLEs, the cortical fragment-lytic enzymes (CFLEs), act on cortical fragments solely in vitro, but not on decoated spores; thus, the spores will still be phase bright after application of the enzymes. The cortical fragment–lytic enzymes (CFLEs) were discovered by Chen *et al.* 2000 in *B. cereus* spores as new members of the group of spore lytic enzymes in *Bacillus*.

The presence of δ–lactam is the main requirement and the primary characteristic for SCLE and CFLE activity, as the enzymes neither act on the peptidoglycan of vegetative cell walls nor on the spore cortex peptidoglycan prepared from δ–lactam–defective mutants (Makino and Moriyama 2002).

The proteins SleB and CwlJ are spore cortex-lytic enzymes, which are essential for effective cortex hydrolysis. Localization studies suggested that SleB is situated in the outer membrane which directly allows interaction with the spore cortex surface; CwlJ is localized both in the inner membrane and in the spore coat. The *sleB* gene is the first one of a bicistronic operon (the second gene is *ypeB*), the gene product of the latter was suggested to be a partner and helper protein for correct localization of CwlJ and for its protection as it provided stabilization against proteolysis (Chirakkal *et al.* 2002, Li *et al.* 2013).

Functional studies of *yaaH* (*sleL* homolog in *B. cereus*) suggested an involment in the secretion of an epimerase that modifies the peptidoglycan during germination. A knock-out mutant of the latter did not show significant changes in the germination capability and that is why another assumption concerning the action of the enzyme was suggested: as a complementary factor in the lysis of the cortex or the depolymerization of the cortex peptidoglycan. However, the protein requires L-alanine during action on the spore cortex. Also, and in contrast to other spore cortex-lytic enzymes, the protein is present in abundant amounts in spore germination exudates. Anyway, there is no explanation why the enzyme is secreted in such large amounts during germination. According to the germination pathway (see Figure 1.8) and substrate specificities, the enzyme is classified as a cortical fragment specific glucosidase (CFLE) (Kodama *et al.* 1999, Chirakkal *et al* 2002, Lambert and Popham 2008, Popham *et al.* 2012)

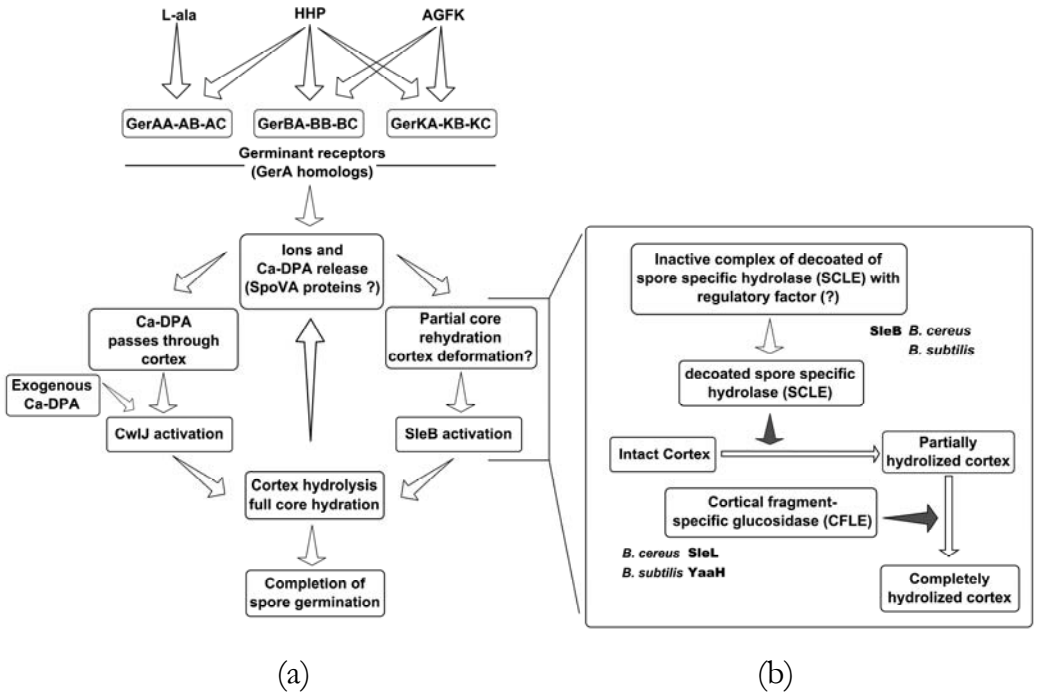



**Figure 1.8** Tentative model for the spore germination pathway, and a possible mechanism of cortex hydrolysis during nutrient-induced germination in *Bacillus* (adapted from Makino and Moriyama 2002, Paredes-Sabja *et al.* 2011). L-ala refer to the amino acid L-alanine; HHP, denote high hydrostatic pressure, presumptively of 200 – 400 MPa;

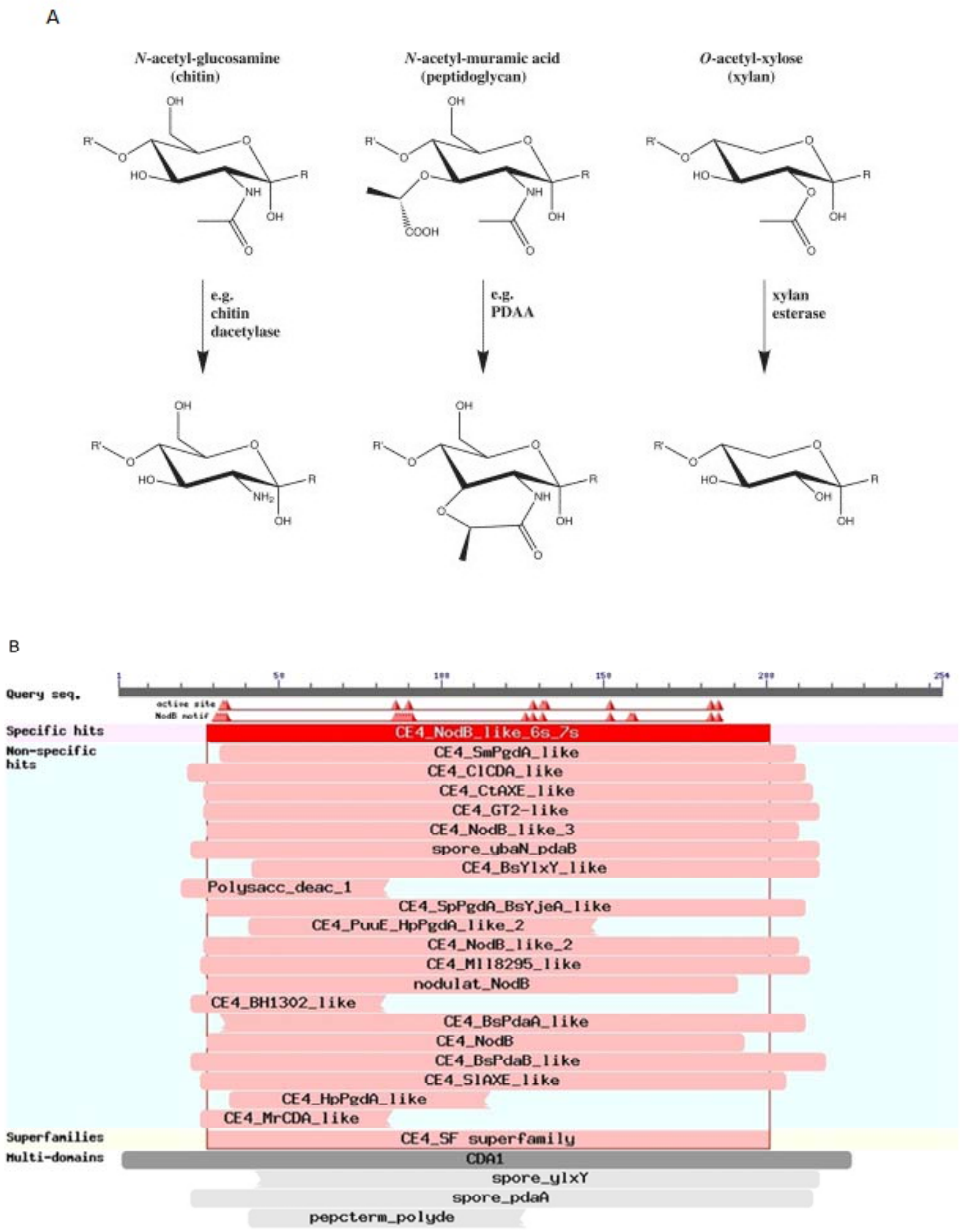
## 1.5. Polysaccharide deacetylases (PDAs)

Peptidoglycan deacetylases or Peptidoglycan GlcNAc deacetylases (PGNG-dacs for short) belong to the Carbohydrate Esterase Family 4 (CE4). Prominent members of CE4 are NodB, chitin-deacetylases and xylan esterases (http://www.cazy.org/ CE4_characterized.html) (Balomenou *et al.* 2013). The

CE4 family members display conserved regions of homology as outlined in Figure 1.10.

Members of the CE4 family display a conserved region highly homologous to the "NodB homology domain." The domain is very specific, and apart from this region, no other homologies were found between these enzymes and other proteins. NodB is a rhizobial protein that deacetylates Nod-factors, chitinous lipooligosaccharides that regulate the symbiotic relationships between leguminous plants and nitrogen fixing bacteria. (Tsigos, *et al.* 2000 and Caufrier, *et al.* 2003). To simplify the domain, the illustrative model of the NodB homology domain is also described in Figure 1.9

Eventhough the CE4 family members are originally described in the *Rhizobium* consortium bacteria and some fungi, the CE4 enzyme family was recently also found among the Firmicutes such as *Bacillus* and *Clostridium* species. Bioinformatic database analysis revealed that in those species the corresponding genomic regions encode microbial acetylxylan esterases, xylanases and several uncharacterized open reading frames (ORFs). Functional studies were done for several genes of the CE4 family by cloning and expression of the genes, however, the structure of any of these enzymes has not been elucidated.




(Figure 1.9, continued)

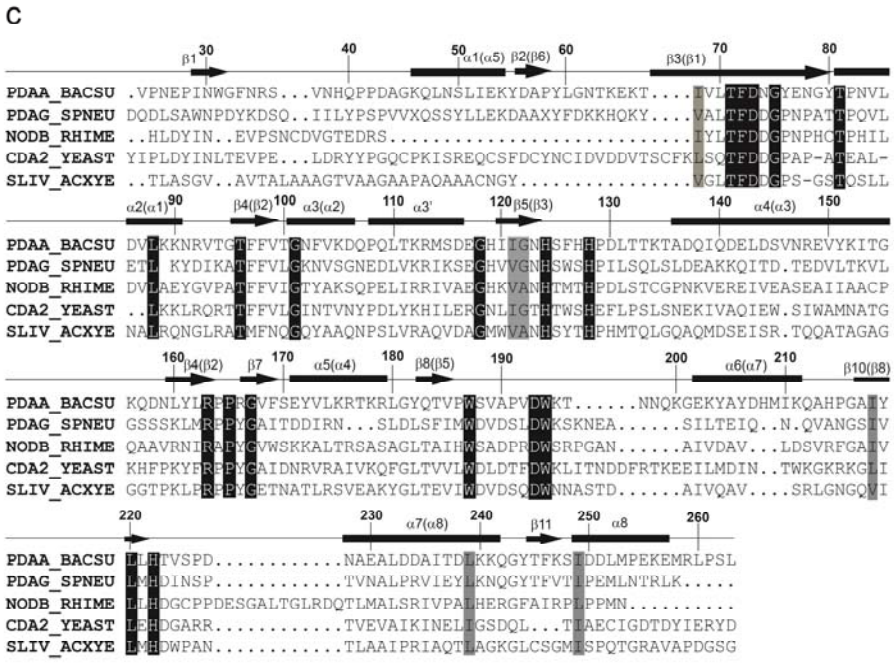



**Figure 1.9** Activity and sequence conservation of family 4 carbohydrate esterases: A) Substrates recognised and reactions performed by family CE-4 esterases; B) The illustrative model of NodB homology domain (taken from NCBI-CDD: http://0-www.ncbi.nlm.nih.gov.opac.acc.msmc.edu/Structure/cdd/wrpsb.cgi?seqinput=196049683). Black bars refer to the NodB domain; grey bar refer to NodB homolog/NodB ortholog; C) sequence alignment of four diverse representatives of the CE-4 family. PDAA_BACSU = *Bacillus subtilis* polysaccharide deacetylase, PDGA_SPNEU = *Streptococcus pneumoniae* peptidoglycan deacetylase, NODB_RHIME = *Rhizobium meliloti* nodulation protein B, CDA2_YEAST = *Saccharomyces cerevisiae* chitin deacetylase 2, SLIV_ACXYE = *Streptomyces lividans* acetyl xylan esterase. Fully conserved residues are shaded black, partially conserved residues are shaded grey. The secondary structure elements of PdaA are labelled, the labels between brackets refer to the secondary structure nomenclature in the canonical $(\beta/\alpha)_8$ fold.

Arrows indicate residues that interact with *N*-acetyl-glucosamine in the PdaA–GlcNAc complex (Taken from Blair and van Aalten 2004).

Recently, the open reading frames which encode putative polysaccharide deacetylases have been identified in large numbers in the genomes of Gram-positive and Gram-negative bacteria. Such PGNG-dacs and/or their corresponding genes have been identified and characterized in *Streptococcus pneumoniae* (*pgdA*), *Bacillus subtilis* (*pdaA*), *Bacillus cereus* (*bc1960* and *bc3618*), *Listeria monocytogenes* (*pgdA*) *Lactococcus lactis* (*pgdA*), *Streptococcus suis* (*pgdA*), *Shigella flexneri* (*sfpgdA*) and *Enterococcus faecalis* (*pgdA*). However, validation of the functional analysis of the open reading frames which were proposed to encode putative PGNG-dacs in bacteria must be performed as for the two main references, i.e. *pgdA* from *Streptococcus pneumonia* and *pdaA* from *B. subtilis*, since both genes were elucidated for enzyme activitiy, substrates and products as well as localization etc. (Psylinakis *et al.* 2005, Balomenou *et al.* 2013).

In *Bacillus* species, the PGNG-dacs are routinely functional active on the peptidoglycan of both, the forespore (especially on muramic δ-lactam synthesis in spore cortex) and the mother cell, but in some other species, such as *Staphylococcus epidermidis*, *Streptococcus mutans* and *Streptococcus iniae*, the PGNG-dacs must have other functions, since they are active on other non-peptidoglycan substrates such as β-1,6-linked N-acetylglucosamine (GlcNac) residues (named PIA, the polysaccharide intercellular adhesin); the

*S.epidermidis*, Pdi homolog in *S. iniae* act on unknown polysaccharides, PgdA of *S. mutans* strain SMU.623c acts on a hexamer of N-acetylglucosamine (Fukushima *et al.* 2002, Vuong *et al.* 2004, Deng *et al.* 2009, and Milani *et al.* 2010).

## 1.6. Aim of The Study

In a previous report (Fukushima *et al.* 2002), when the *B. subtilis* homolog of the *B. licheniformis yfjS* (renamed *pdaA*) was genetically knocked-out (Δ*yfjS*), it became evident that there is a drastic impact on spore function as it caused the complete loss of germination. Since *B. licheniformis* is one of major workhorses in the Biotech Industry, especially for extracellular enzyme production (Schallmey, 2004), such PDA-negative mutants would not only extend our knowledge about its function to another species but may be useful to generate biologically safe strains. Thus, the work presented here aimed at generation of several *B. licheniformis* mutants hit in putative PDA-encoding genes to check whether they can serve, alone or in combination, to establish biological containment. As biosafety should not negatively affect growth, physiology nor production, such parameters were experimentally addressed too.

Although *B. licheniformis* phylogenetically belongs to the *B. subtilis* group, recent studies (Hoffmann *et al.*, 2010) disclosed articulate differences to the latter, especially with respect to developing natural genetic competence and

subsequent transformation, eventhough the corresponding genome data are almost identical. Hence, it was not be excluded that, when studying the role of PDA-encoding genes for spore germination, again differences to the model organism may emerge.

Central to this study is the inspection of putative PDA encoding genes by creating the respective knock-outs and by the subsequent examination of their role in spore germination.

# 2. MATERIALS AND METHODS

## 2.1. Oligonucletides, Plasmids and Strains

**Table 2.1** Oligonucleotides used as PCR primers.

| No | Oligonucleotides | 5' - 3' Sequence |
|---|---|---|
| 1. | yjeA1BamHI | CGCGGTGGATCCCAGCGGGATCGACTTCAACC |
| 2. | yjeA4BamHI | GAAGGAGGATCCTGCCAAGCTGGCCAACGATG |
| 3. | yjeASOE2 | GTCAAGCAACAGCCAAGAGCCCGCAGATGCTGCTG AAGAG |
| 4. | yjeASOE3 | CTCTTCAGCAGCATCTGCGGGCTCTTGGCTGTTGCT TGAC |
| 5. | yjeA-T1 | CATCCACCGTGACGAAAC |
| 6. | yjeA-T2 | CCTAACTCTCCGTCGCTATTG |
| 7. | P5-yjeA | GACGCATGTCTTCTTCATCCTC |
| 8. | P6-yjeA | TCCCAATCCCAAGCCTTTCC |
| 9. | yjeA2Mutin2 BamHI | CGATCGAGCGGATCCATCAGGTTTGGCCTGAAG |
| 10. | yjeAMutin2 HindIII | CGAGCGAGCAAGCTTCCAGGCTCAGGATGAAGAC |
| 11. | yjeAN1-P5 | TCATGAAACAGGAGAGTGAGCAGCGATGTC |
| 12. | yjeAN1-P6 | AGATTGGAAAGCGAGCGGACGGAATCATTG |
| 13. | 02415Mutin2 HindIII | CGAGCGAGCAAGCTTAGATCATAAATTGAGCGCAC TAGG |
| 14. | 02451Mutin2 BamHI | ATCTATCTAGGATCCCGATACTTGATGCGTCACTCC |
| 15. | 02451N1-P5 | CCTGCTTGTTAACGCTCAATACCAGTACCC |
| 16. | 02451N1-P6 | AGGCGATCTGATCAAATGTGTGTCAACGTT |
| 17. | yheNMutin2 BamHI | GCGTGCGTGGATCCTGCAGCAGCCTTTGGCTTAC |
| 18. | yheNMutin2 HindIII | GCGCGCGTAAGCTTGAATCGGAACGAGAAAAATG |
| 19. | SC1-swYheN | CACTTGAAGATACAGCCCGCCTTCC |
| 20. | SC2-swYheN | AAATTTGTCAGCAATAACGGCAATG |
| 21. | ylxYMutin2 BamHI | GTGTGTGTGGATCCTGAACGTCAGGCTTCACTTG |
| 22. | ylxYMutin2 HindIII | TGTGTGTGAAGCTTTGAAAGGAACGATTGTTCGG |
| 23. | SC1-swYlxY | AACAAGACGGCACAATCTTCATTTC |
| 24. | SC2-swYlxY | GCCGTTATGTACTTTGCTTAATACC |
| 25. | yxkHMutin2 BamHI | GTGTGTGCGGATCCAGGCTTCCTCAGGTGTCAAC |

| | | |
|---|---|---|
| 26. | yxkHMutin2 HindIII | TTAGATGTAAGCTTGTAGATAGAAAGCGGGAATA |
| 27. | SC1-swYxkH | GTCATTTCTTCCGGGAGCGAGATTG |
| 28. | SC2-swYxkH | GCCGTCTTTCCTATTGTCTGCTGTG |
| 29. | lacZ-rev | CACGCTCATCGATAATTTCACCGCCGAAAG |
| 30. | Erm-for | GTTCGTGTTCGTGCTGACTTGCACCATATC |
| 31. | REGIO FL-1 | TATCGGCCTCAGGAAGATCGCACTC |
| 32. | REGIO FL-2 | TACACAGCCCAGTCCAGACTATTCG |

**Table 2.2** Plasmids

| Plasmid | Genotype | Reference |
|---|---|---|
| pJR1 | Mobilizable vector; Cam$^r$, *mob*, oriT, ori$^{ts}$, *sacB*, *rep* | Richhardt, *et al.* (2010) |
| pJR1Δ*yjeA* | pJR1 derivative with deletion flanks for *yjeA* | This study |
| pMMcomK | pMM1522 derivative, *Bacillus licheniformis* MW3 ComK expression vector | Hoffman, *et al.* (2010) |
| pUCBM20 | *E. coli* cloning vector; Ap$^r$ | Boehringer Mannheim, Germany |
| pUCBM20::*yjeA* | pUCBM20 derivative with *yjeA* insertion flank | This study |
| pUCBM20:: *Bli02451* | pUCBM20 derivative with *Bli02451* insertion flank | This study |
| pUCBM20::*yheN* | pUCBM20 derivative with *yheN* insertion flank | This study |
| pUCBM20::*ylxY* | pUCBM20 derivative with *ylxY* insertion flank | This study |
| pUCBM20::*yxkH* | pUCBM20 derivative with *yxkH* insertion flank | This study |
| pMutin2 | *B. subtilis* (Em$^r$ *lac/ lacZ*), *Pspac* promoter, *E.coli* (ApR), ColE1 ori, terminators $\lambda t_o$ | Vagner, *et al.* (1998) |
| pMutin2:: *yjeA* | pMutin2 derivative with *yjeA* insertion flank | This study |
| pMutin2:: *Bli02451* | pMutin2 derivative with *Bli02451* insertion flank | This study |

| | | |
|---|---|---|
| pMutin2:: *yheN* | pMutin2 derivative with *yheN* insertion flank | This study |
| pMutin2:: *yjlxY* | pMutin2 derivative with *yjlxY* insertion flank | This study |
| pMutin2:: *yxkH* | pMutin2 derivative with *yxkH* insertion flank | This study |

**Table 2.3** Strains

| Strain | Genotype | Reference |
|---|---|---|
| *Escherichia coli* DH5αF' | *endA1 hsd*R*17* ($r_K^-$, $m_K^+$) *supE44 thi-1 gyrA96 relA1* Δ(*lacIZYA-argF*)*U169 deo*R F'Φ80d*lacZ*ΔM15) | Woodcock, *et al. (*1989) |
| *Escherichia coli* S17-1 | Δ*recA*, *end*A1, *hsd*R17, *sup*E44, *thi*-1, *tra* $^+$ | Simon *et al.* (1983) |
| *Bacillus licheniformis* DSM 13 | Wild Type | DSMZ, Veith*, et al.* (2004) |
| *Bacillus licheniformis* MW3 | Δ*hsdR1*, Δ*hsdR2* | Waschkau, et al. (2008) |
| Bli MW3 Δ*yfjS* | Δ*hsdR1*, Δ*hsdR2,* Δ*yfjS* | Borgmeier (this laboratory, pers.comm.) |
| Bli MW3 Δ*yjeA* | Δ*hsdR1*, Δ*hsdR2,* Δ*yjeA* | This study |
| Bli MW3.01 | Δ*hsdR1*, Δ*hsdR2* :: *Bli02451*pMutin2 | This study |
| Bli MW3.02 | Δ*hsdR1*, Δ*hsdR2,* Δ*yfjS*:: *yjeA*pMutin2 | This study |
| Bli MW3.03 | Δ*hsdR1*, Δ*hsdR2,* Δ*yfjS*:: *Bli02451*pMutin2 | This study |
| Bli MW3.04 | Δ*hsdR1*, Δ*hsdR2,* Δ*yjeA*::*Bli02451*pMutin2 | This study |
| Bli MW3.05 | Δ*hsdR1*, Δ*hsdR2,* Δ*yfjS*:: *ylxYA*pMutin2 | This study |
| Bli MW3.06 | Δ*hsdR1*, Δ*hsdR2,* Δ*yfjS*:: *yheN*pMutin2 | This study |
| Bli MW3.07 | Δ*hsdR1*, Δ*hsdR2,* Δ*yfjS*:: *yxkH*pMutin2 | This study |

## 2.2. Molecular Techniques

The molecular, PCR and sequencing techniques are described below. Primers were deduced on the basis of the published *B. licheniformis* DSM13 (isogenic to ATCC 14580) genome sequence (Rey *et al.* 2004 and Veith *et al.* 2004). The oligonucleotides were ordered from and were delivered by Invitrogen (Life Technologies GmbH, Darmstadt, Germany) or Eurofins, MWG Operon (Ebersberg, Germany). PCR mixtures of 100 µl contained 200 µM deoxynucleotides, 100 ng template DNA, 1 pmol of each primer, and 1 U of *Taq* DNA polymerase or Phusion DNA polymerase (New England Biolabs, Frankfurt am Main, Germany). Purification of amplified fragments after gel electrophoresis was performed with a Perfectprep Gel Cleanup kit (Eppendorf AG, Hamburg, Germany). Sequencing was done with fluorescence-labeled dideoxynucleotides of the Big Dye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA) and an ABI Prism 3730 capillary sequencer (Applied Biosystems, Foster City, CA). Software for analysis sequencing: Clone Manager Pro.ver. 9.0 (Sci Ed Central, Cary NC, USA, 2007); BLAST (Altschul *et al.* 1990) in the NCBI server (http://blast.ncbi.nlm.nih.gov/ Blast.cgi); Edit-Seq ver. 5.05 and Seq-ManII ver. 5.05 (DNASTAR, Madison, USA, 2002); and GeneDoc (NRBSC, Pittsburgh, USA, 1997). For the alignment and for constructing pictures and figures: Enhance Map Draw ver.

4.00 (Sci Ed Central, Cary NC, USA, 2001), and Inkscape (Software Freedom Conservancy, Inc. Brooklyn, NY, USA,1991) or Adobe Illustrator CS2 (Adobe Systems Inc. San Jose, CA, USA, 1989) and Adobe Photoshop CS2 (Adobe Systems Inc. San Jose, CA, USA, 2005).

## 2.3. Plasmid Isolation from *E. coli*

One single colony of a plasmid carrying *E. coli* strain from a solidified Luria Bertani (LB) medium containing petri dish was inoculated and cultured in liquid LB broth containing the appropriate antibiotic by overnight cultivation at the required temperature in a rotary shaker (New Brunswick Scientific Shaker Innova 44 and Innova 42, Enfield, CT, USA). 1.5 ml were harvested in an Eppendorf tube by centrifugation (15.000 x g for 10 minutes at room temperature). The pellet was resuspended in 50 µl TE buffer using a pipet and gentle vortexing. Alkaline lysis was performed by adding 300 µl fresh TENS buffer and inverting the Eppendorf tube until the suspension became translucent. Neutralization was done by adding 150 µl of 3M Kalium Acetate (pH 4.8) and inverting shortly and gently. For removing the remnant proteins from the solution, 50 µl Chloroform ($CHCl_3$) were added and carefully inverted 5-6 times. Subsequently, the solution was centrifuged (15.000 x g for 10 minutes) at room temperature. The supernatants were transferred into a 1.5

ml Eppendorf tube and 500 µl cold isopropanol (-20 $^0$C) was added and kept on ice for 2-30 minutes to precipitate the plasmid DNA. The pellets were harvested by centrifugation for 30 minutes at 15.000 x g at 4 $^0$C and washed further by adding 500 µl 70% cold Ethanol followed by centrifugation for 10 minutes, 15.000 x g at 4 $^0$C. Supernatants were discarded and the plasmid DNA pellet vacuum dried for 5 minutes. To the dried plasmid DNA, 20 µl of TE buffer was added containing 1ng/ml RNase (Sigma-Aldrich, St. Louis, Missouri, USA). The DNA was short term stored at 4 $^0$C or at -20 $^0$C for longer periods of time.

**TE Buffer:**

| | |
|---|---|
| Tris-HCl | 10 mM |
| EDTA pH 8.0 | 1 mM |

**TENS Buffer:**

| | |
|---|---|
| 1 x TE | 10 ml |
| 10 mM NaOH | 100 ml |
| 10% SDS | 500 µl |

(Sambrook and Russel 2001)

## 2.4. Isolation of Chromosomal DNA from *Bacilli*

The applied chromosomal DNA isolation procedure was developed in this laboratorium by modifying the Plasmid DNA isolation method described for the JETQUICK plasmid purification spin Kit (GENOMED, Löhne, Germany; http://www.genomed-dna.com/) to make it more efficient.

A single colony of a *Bacillus* strain from Luria Bertani (LB) petri dish was inoculated and cultured in liquid LB broth without antibiotics overnight at the appropriate temperature and aeration (New Brunswick Scientific shaker innova 44 , Enfield, CT, USA). Cells from 1.5 ml of the overnight liquid cultures were harvested in an Eppendorf tube at 15.000 x g for 10 minutes at room temperature. The pellet was resuspended in 250 µl SET buffer containing 1 µg/ml RNase and 50 µl of 50mg/ml lysozyme in SET buffer was added, followed by incubation for 20 minutes at 37 $^{0}$C in a Thermomixer at 750 rpm (Eppendorf, Hamburg, Germany) . The GENOMED-JETQUICK Löhne, Germany**)** plasmid purification spin Kit was used. To the suspension 250 µl lysis buffer G2 was added, and mixed by inverting for 4 minutes. Buffer G3 (350 µl) was added to neutralize the solution, by continuing inversions and followed by strong shaking three to four times. Subsequently, centrifugation at maximum speed (15.000 x g) for 10 minutes was perfomed. The supernatant was transferred onto the spin column equipped with a receiver tube and centrifuged again at 15.000 x g for 1 minute. The flowthrough in the receiver

tube was discarded and the spin column was washed with 500 µl Buffer GX. After centrifugation (15.000 x g for 1 minute) the flowthrough Buffer GX in the receiver tube was again discarded. The spin column was refilled again with 500 µl Buffer G4 and the procedure was repeated, the flowthrough Buffer G4 in the receiver tube was again discarded. Centrifugation for 1 minute with the empty column removed the residual buffer. Subsequently, the column was tranferred onto a new 1.5 ml eppendorf tube and the column was filled with 30 µl dilution buffer/ aquadest followed by centrifugation for 2 minute at 15.000 x g. The flowthrough contained the chromosomal DNA and was stored at 4 $^{0}$C for short or stored at -20 $^{0}$C for longer.

**SET Buffer:**

| | | |
|---|---|---|
| Tris-HCl | 50 | mM |
| EDTA | 50 | mM |
| Sucrose (w/v) | 25 | % |
| pH | 8.0 | |

(The buffer composition was adopted from Voskuil and Chambliss 1993)

## 2.5. MIDI Plasmid Isolation from *Bacilli*

A single colony of a *B. licheniformis* strain containing a plasmid was inoculated and cultured overnight in 50 ml LB broth containing the appropriate antibiotic at the required temperature and aeration (170 rpm, 30 $^0$C, New Brunswick Scientific shaker innova 44 (Enfield, CT, USA). The cells of the overnight liquid culture were harvested in a Falcon tube by centrifugation (4000 x *g* for 10 minutes at 4 $^0$C). The pellet was then resuspended in 2.5 ml SET buffer. The cells were aliquoted into 250 µl samples in 1.5 ml eppendorf tubes. Lysozyme (50 mg/ml in SET) and RNAse were sequentially added (50 µl and 5 µl, respectively). The suspensions were incubated at 37 $^0$C at 300 rpm in an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany) for 20 minutes. Alkaline lysis and neutralization: to each aliquot 350 µl of lysis buffer was added and repeatedly and carefully inverted for 4 minutes. Subsequently, 250 µl neutralization buffer was added to the lysates and 3-4 times very slowly and carefully inverted. To the lysates 50 µl Chloroform was added, followed by very careful inversion 3-4 times and centrifugated at 15000 x g for 10 minutes. Subsequently, Phenol extraction was performed: the supernatant was transferred to a new 1.5 ml Eppendorf tube containing 250 µl Phenol and 250 µl of Chloroform **:** Isoamylalcohol (24:1), mixed and followed by

centrifugation at 15000 x g for 5 minutes. The upper phase was transferred into a fresh 1.5 ml Eppendorf tube containing 500 µl Chloroform : Isoamylalcohol (24:1), mixed again and centrifugated at 15000 x g for 3 minutes. Subsequently, all of the upper phases from each of the tubes were collected into a 50 ml Falcon tube and loaded onto an equilibrated spin column (JETSTAR Plasmid Purification Kits, Genomed GmbH, Löhne, Germany), and, after the liquid passed through the column (by gravitation), the column was washed twice with 10 ml of buffer E5. Afterwards, the cleaned column was placed into a 50 ml falcon tube (as receiver) and 5 ml elution buffer E6 was added. The throughflow was aliquoted into several 1.5 ml Eppendorf tubes and two volumes of ice-cold isopropanol were added to precipitate the DNA. The DNA-pellet was recovered by centrifugation at 15000 x g at 4 $^{0}$C for 30 min. The pellet was washed with ice-cold 70% ethanol, dried by vacuum centrifugation and dissolved in 50 µl Elution buffer (EB) for storage and further use.

**Buffer Composition:**

**Cell Resuspending Buffer (E1)**

| | | |
|---|---|---|
| Tris-HCl (pH 8.0) | 50 | mM |
| EDTA | 10 | mM |
| RNase A | 100 | µg/ml |

**Lysis Buffer (E2)**

| | | |
|---|---|---|
| NaOH | 200 | mM |
| SDS | 1% | (w/v) |

**Precipitation Buffer (E3)**

| | | |
|---|---|---|
| Potassium acetate | | |
| (pH 5.5 with acetic acid) | 3.1 | M |

**Equilibration Buffer (E4)**

| | | |
|---|---|---|
| Sodium acetate | | |
| (pH 5.0 with acetic acid) | 100 | mM |
| NaCl | 600 | mM |
| Triton® X-100 | 0.15 | % (v/v) |

**Wash Buffer (E5)**

| | | |
|---|---|---|
| Sodium acetate | | |
| (pH 5.0 with acetic acid) | 100 | mM |
| NaCl | 800 | mM |

**Elution Buffer (E6)**

| | | |
|---|---|---|
| Sodium acetate pH 5.0 | | |
| (adjusted with acetic acid) | 100 | mM |

| | | |
|---|---|---|
| NaCl | 1.500 | mM |

**RNase A solution**

| | | |
|---|---|---|
| RNase A | 20 | mg/mL |
| Tris-HCl, | 50 | mM |
| EDTA, pH 8.0 | 10 | mM |

## 2.6. Determination of the DNA concentration

The purity and concentration of the DNA samples were determined by measuring the absorption at 260 nm and 280 nm. An extinction $E_{260}$ = 1 corresponds to 50 µg dsDNA/ml or 38.5-40 µg ssDNA/ml (Davis *et al.* 1980; Sambrook and Russel 2001). For pure DNA preparations, the following ratio must be reached: $E_{260nm}$ / $E_{280nm}$ = 1.8. Following the rule, the DNA consentration was measured by Nanodrop ND 1000 (Peqlab Biotechnologie GmbH, Erlangen DE) (Sambrook and Russel 2001)

During the routine work with small volumes, the concentration of DNA was estimated by the fluorescence intensity of the bands within an agarose gel that had been stained with ethidium bromide.

## 2.7. Preparation of competent cells in *E. coli*

Two different methods have been used to prepare transformation competent *E. coli* cells:

### 2.7.1. Calcium chloride method

The *E. coli* strain to be transformed was cultivated in 10 ml LB medium (2.2.3) at 37 °C until the $OD_{600nm}$ reached 0.3-0.5. The cells were harvested by centrifugation at 2800 × *g* for 15 min at 4 °C. The cell pellet was washed once with ice cold 100 mM $CaCl_2$ and resuspended in 1 ml ice cold 100 mM $CaCl_2$. The cell suspension was divided in 200 µl portions and stored on ice until it was used (Sambrook and Russel 2001).

### 2.7.2. Preparation of permanent competent cells

The *E. coli* strain to be transformed was cultivated in 300 ml LB medium at 37 °C until the $OD_{578}$ reached = 1.0. After 15 min on ice, the cells were harvested by centrifugation at 2800 × *g* for 15 min at 4 °C. The pellet was resuspended in 100 ml RF1 solution. After 30 min on ice, the cells were harvested and the pellet was resuspended in 25 ml RF2 solution. After additional 15 min on ice,

the culture was divided into 200 µl portions and samples stored at –70 °C. (Hanahan, 1983)

**RF1 solution**

| | | |
|---|---|---|
| RbCl | 100 | mM |
| $MnCl_2$ | 50 | mM |
| Potassium acetate | 10 | mM |
| $CaCl_2$ | 10 | mM |
| Glycerol | 15 % | (v/v) |

The solution was sterilized by filtration (pore size 0.25 µm) after adjusting the pH to 5.8 with glacial acetic acid.

**RF2 solution**

| | | |
|---|---|---|
| RbCl | 10 | mM |
| MOPS, pH 6.8 | 10 | mM |
| $CaCl_2$ | 75 | mM |
| Glycerol | 15 % | (v/v) |

The solution was sterilized by filtration after adjusting the pH to 6.8 with NaOH.

## 2.8. DNA Transfer

Depending on the procedure used for the preparation of competent cells and on the recipient strains, different methods for the transfer of DNA were used.

### 2.8.1. Transformation of *E. coli* cells

200 µl competent cells were mixed thoroughly with 50 - 250 µg of the DNA and kept on ice for 30 min. During such step, the DNA was adsorbed at the surface of the competent cells. For the uptake of the adsorbed DNA, the cells were heated at 42 °C for exactly 90 seconds and subsequently kept on ice for 3-5 min. For regeneration of the cells and for expression of the plasmid encoded antibiotic resistance, 600 µl LB medium were added and the cells were subsequently incubated at 37 °C for 45 - 60 min. For selecting recombinant clones, 100 µl aliquots were spread on selective solid medium containing the respective antibiotic; plates were incubated overnight at 37 °C (Sambrook and Russel 2001)

### 2.8.2. Conjugation Protocols for *B. licheniformis*

The conjugation protocol for *Bacilli* established by Aquino de Muro and Priest (2000) was optimized and adjusted to the donor strain *E. coli* S17-1 carrying

the mobilizable vector pJR1 (Richhardt *et al.* 2010). The strains used for conjugation were *E. coli* S17-1 carrying plasmid pJR1.

*Bacillus* cells were grown in LB broth, and *E. coli* S17-1 pJR1 was cultivated in LB broth containing chloramphenicol; cultivations were performed at 37 °C overnight. Separate 250-ml Erlenmeyer flasks each containing 50 ml LB medium were inoculated with 1 ml of the above overnight *Bacillus* and *E. coli* S17-1 pJR1 cultures and grown at 37 °C until an $OD_{600\ nm}$ of 0.6–0.8 was reached. Cells were harvested by centrifugation (15 min, 3,220×*g*, 4 °C), washed twice in 15 ml holding buffer, pelleted by centrifugation (15 min, 3,220×*g*, 4 °C), and resuspended in 30 ml holding buffer. *Bacillus* cells were subjected to heat treatment (9 min at 49 °C) prior to mixing with the *E. coli* S17-1 pJR1 donor cells. The optimal mixing ratio of donor/recipient cells is 1:2. The mixture was then compressed on a sterile nitrocellulose filter with a pore size of 0.45 μm to ensure close contact between donor and recipient cells. According to the respective requirements for counter-selection, the filter was either placed on LB or on sporulation agar (Schaeffer et al. 1965) with the cells forming the top layer. Filter mating was either performed for 24 h at 30 °C on LB agar or for 48 h at 30 °C on sporulation agar.

### 2.8.3. Counter-selection by Pasteurization

When sporulating *Bacillus* strains were used in matings, the counter-selection against *E. coli* was done by pasteurization. For such purpose, the filter with the mixed mating partners was placed on the sporulation medium and incubated for 2 days at 30 °C to ensure complete sporulation. Cells on the filter were subsequently suspended in 900 µl holding buffer and incubated for 20 min at 80 °C, and then spread on LB agar plates containing chloramphenicol. Colonies growing after the heat treatment are considered to be transconjugant clones.

**Holding Buffer**:

| | | |
|---|---|---|
| $KH_2PO_4$ | 12.5 | mM |
| $K_2HPO_4$, | 12.5 | mM |
| $MgSO_4$, | 1.0 | mM |
| pH | 7.2 | |

### 2.8.4. *Bacillus licheniformis* Protoplast Transformation

An optimized Protoplast Transformation protocol originally developed by Chang and Cohen (1979) is available for transformation of *B. licheniformis* (Waschkau *et al.* 2008).

Cells were grown overnight at 37°C in 25 ml #416-medium supplemented with 5 ml glycerol-based electroporation regeneration medium (LBSPG). 35 ml of #416-Medium supplemented with 5 ml glycerol-based medium was inoculated with an overnight-culture (optical density of 0.25 at 600 nm) and grown at 37°C to an $OD_{600}$ of 0.85–0.9. Cells were then harvested by centrifugation (4°C, 3220 ×*g*, 15 min) in a 50 ml Falcon tube and resuspended in 5 ml SMMP. 130 µl lysozyme (10 mg in SMMP) were added to 5 ml of a cell suspension in a 50 ml-Falcon tube and incubated at 37°C with gentle shaking (90 rpm) for 20 min (New Brunswick Scientific shaker innova 4320, Enfield, CT, USA) until 85–90% of cells became protoplasts. Subsequently, 12 ml SMMP were added and gently shaken. Protoplasts were then pelleted by centrifugation (4 °C, 420×*g,* 12 min) and suspended in 3 ml SMMP. For transformation, 0.5 ml of the protoplast-suspension were transfered to a sterile 1.5 ml Eppendorf tube, containing 25 µl plasmid-DNA (100 ng/µl) and the same volume of 2× SMM. The protoplast–DNA–SMM mix was transferred to a new sterile 50 ml-Falcon tube, which contained 1.6 ml polyethylenglycol

(40% *w*/*v*), i.e. 40 g PEG 6000 (Sigma-Aldrich, St. Louis, Missouri, USA) and 50 ml 2× SMM in 100 ml. After 2 min of gentle mixing at room temperature, 5 ml filtersterilized SMMP supplemented with 2% bovine serum albumin was added. Protoplasts were harvested by centrifugation (8°C, 420×*g*, 8 min) resuspended in 1 ml filtersterilized SMMP supplemented with 2% bovine serum albumin and incubated for 135 min at 30°C with gentle shaking (100 rpm) in a New Brunswick Scientific shaker innova 44 (Enfield, CT, USA) prior precautious plating on antibiotic DM3 media. For selection, DM3 medium was supplemented with the appropriate antibiotic. Regeneration of protoplasts was checked on DM3 medium without antibiotics. For control purposes, solely protoplasts (without DNA) were plated on DM3 medium containing the respective antibiotic. Transformants were routinely discovered after incubation for 2–5 days at 30°C.

**Media composition:**

**#416-Medium**

| | | |
|---|---|---|
| Trypton | 20 | g |
| Yeast Extract | 10 | g |
| NaCl | 10 | g |
| 2M Sucrose | 100 | ml |

(Waschkau *et al.* 2008).

**LBSPG**

| | | |
|---|---|---|
| Peptone | 0.1 | g |
| Yeast extract | 0.05 | g |
| NaCl | 0.1 | g |
| Sucrose | 0.25 | M |
| Glycerol (v/v) | 10 | % |
| Potassium Phosphate Buffer (pH 7.2) | 50 | mM |
| Aquadest | 100 | ml |

(Xue *et al.* 1999)

**DM3 Regeneration Medium**

| | | |
|---|---|---|
| 4% (w/v) Agar | 200 | ml |
| 1 M Sodium Succinate (pH 7.3) | 500 | ml |
| 5% (w/v) Casamino acids | 100 | ml |
| 10% (w/v) Yeast extract | 50 | ml |
| 3.5% $K_2HPO_4$ + 1.5% $KH_2PO_4$ (w/v) | 100 | ml |
| 20% (v/v) Glycerol | 25 | ml |
| 1 M $MgCl_2$ | 20 | ml |
| 2% (w/v) BSA (filter-sterilized) | 5 | ml |

(Waschkau *et al.* 2008).

**2x SMM Buffer**

| | | |
|---|---|---|
| Sucrose | 0.5 | M |
| Maleic acid | 0.02 | M |
| $MgCl_2$ | 0.02 | M |
| pH | 6.5 (Adjusted with NaOH) | |

(Wyrick and Rogers 1973)

**SMMP. 2x SMM Buffer**

| | | |
|---|---|---|
| BSA (w/v) | 0.3 | % |
| 2M Sucrose (w/v) | 5 | % |
| 4x PAB * (w/v) | 25 | % |
| 2x SMM (w/v) | 50 | % |

*) PAB = Penassay Broth

(Wyrick and Rogers 1973)

### 2.8.5. *Bacillus licheniformis* Transformation by pMMcomK Driven Induced Competence

According to Hoffmann *et al.* 2010: 10-ml LB-tet (12.5 µg/ml tetracycline [Tet]), starter cultures were inoculated with single colony from a fresh LB plate

(12.5 µg/ml Tet) and grown overnight at 37°C and 170 rpm in a New Brunswick Scientific shaker innova 44 (Edison, NY). A 50-ml main culture (LB, 12.5 µg/ml Tet) was inoculated with 500 µl of the starter culture and grown in a four-baffle 300-ml Erlenmeyer flask at 37°C and 155 rpm until an OD546 of 0.5 reached. The main culture was divided into two 20-ml cultures, and each was transferred into separate 100-ml Erlenmeyer flasks. In culture A, ComK expression was induced with 0.25% xylose, while culture B served as the uninduced negative control. Both of the cultures (A and B) were grown for 1 to 3 h. For the transformation, 0.5 ml culture (of A and B) was transferred to an Eppendorf cup containing 1 to 5 µg DNA. Transformation mixtures were incubated for 30 min in a Thermomixer comfort (Eppendorf, Hamburg, Germany) at 37°C and 700 rpm, after the cells were harvested by centrifugation, subsequently resuspended in 500 µl M9 minimal medium, and incubated for another 30 min at 37°C and 700 rpm to stop comK induction. For obtaining integrative pMutin2 mutant, the cells were directly plated on LB containing erythromycin (0.75 µg/ml) for selecting the mutants.

**M9 Salt Solution:**

| | |
|---|---|
| $Na_2PO_4$ | 6 gr |
| $KH_2PO_4$ | 3 gr |

| | | |
|---|---|---|
| $NH_4Cl$ | 10 | gr |
| NaCl | 5 | gr |
| Aquadest | 1000 | ml |
| pH | 7.4 | |

**M9 Medium:**

| | | | |
|---|---|---|---|
| M9 Salt Solution | | 1 | ml |
| 20% (w/v) | Glucose | 10 | ml |
| 100 mM | $MgSO_4$ | 1 | ml |
| 10% (w/v) | Cassamino Acid | 1 | ml |
| 100 mM | $CaCl_2$ | 100 | µl |
| 10% (w/v) | Yeast Extract | 100 | µl |
| 2 mg/ml | $MnSO_4$ | 10 | µl |
| Aquadest | | 1000 | ml |
| pH | | 7.4 | |

(The M9 Medium was adopted from Nicholson and Setlow, 1990)

## 2.9. Sporulation and Germination

### 2.9.1. *Bacillus* Induction of Sporulation

A fresh *B. licheniformis* colony from overnight grown cells on an LB plate was tooth-picked and emulsified by vortexing in 5 ml Schaeffer's medium (2x SG). 100 µl of emulsified cells were plated onto Schaeffer's medium and incubated overnight at 30 $^0$C. The growing colonies were flooded by 4 ml of Schaeffer's medium and carefully suspended with a glass spreader. Subsequently, 50 ml Schaeffer's medium in a 250 ml Erlenmeyer flask was inoculated with the resuspended cells to give an $OD_{600}$ of about 1.0; cultures were incubated at 37 $^0$C with vigorous aeration in a New Brunswick Scientific shaker innova 44 (Enfield, CT, USA). After 30 hours, cells were harvested and followed by spore purification and germination examinations (Nicholson and Setlow 1990).

### 2.9.2. Spore purification

The Purification protocol was adopted from Ferguson *et al.* (2007) and modified. 100 ml spore suspension were pelleted by centrifugation 8.000 rpm/ 4000 g for 2 minutes at 4 $^0$C in two Falcon tubes, the pellets were washed twice with sterilized TE buffer (pH 8.0). To ensure the killing of the vegetative cells; the pellet was treated with 1 mg/ml lysozyme in TE buffer and

continued incubation at 37 $^{0}$C in an Eppendorf thermomixer (Eppendorf, Hamburg, Germany) for one hour. To the spore solution 140 µl of 2% SDS were added and continously shaken for further 45 minutes. The spores were then washed with 1000 µl sterilized water and pelleted by centrifugation at 4000 g for 10 minutes at 4 $^{0}$C. The washing step was repeated until the supernatant remained clear. All spores were free of growing cells and cell debris. To check the level of purity, spores were inspected by phase-contrast microscopy to ensure that phase-bright spores comprised at least 90% of visible particles. Spores were stored at -20 $^{0}$C in 960 µl sterilized water.

### 2.9.3. Determination of the Germination Efficiency

The spore solution (in water) was heated to 80°C for 20 minutes in order to heat kill negative cells and any spores that had germinated, cooled on ice for 15 minutes, and used in germination experiments. 5 µl of serial dilutions, were dropped onto LB plates to check the germination capabilities, 200 µl of the rest solution from each dilution series were plated on LB plates for quantitative analysis. The percentage of spores resistant to heat treatment was then calculated by determining the Colony Forming Units (CFU) after overnight incubation. These were divided by the total CFUs obtained without heating and multiplied by 100 to yield the percentage of germination. From

three independent experiments mean values and standard deviations were calculated.

**2 x Schaeffer's Glucose Sporulation Agar Medium (2xSG Agar)**

| | | |
|---|---|---|
| Nutrient Broth | 16.0 | g |
| KCl | 2.0 | g |
| $MgSO_4.7H_2O$ | 0.5 | g |
| 1 M NaOH (to adjust pH = 7,0) | ~ 0,5 | ml |

**Micro nutrients in 2 x SG**

| | | |
|---|---|---|
| 1 M $Ca(NO_3)_4$ | 1.0 | ml |
| 1 mM $FeSO_4.7H_2O$ | 1.0 | ml |
| 0,1 M $MnCl_2.4H_2O$ | 1.0 | ml |
| 50 % (w/v) Glucose | 2.0 | ml |

(The 2 x SG Medium was adopted from Nicholson and Setlow 1990)

# 3. RESULTS

This chapter deals with the search for the genes encoding polysaccharide deacetylases (PDAs) in *Bacillus licheniformis* MW3 and the construction of clean deletion mutants as well as the disruption by integration of selectable marker genes for the potential application as biological safety measures to be finally established in a producer strain. Hence, the subsequent phenotypic investigations did not only include the inspection of the colony morphology and growth in liquid media, but also the determination of possible impacts on enzyme formation and spore germination. In the last part of the thesis, the discussion, the relevance and potential of the respective (possibly germination deficient) mutants hit in the PDA encoding genes for biological containment and industrial application as well as environmental issues will be addressed.

Since PDAs play important roles in endospore formation and germination processes, especially in the formation of muramic δ–lactam of the spore peptidoglycan (Atrih *et al.* 1996, Fukushima *et al.* 2002, Gilmore *et al.* 2004), genes potentially encoding such enzymes were searched by scanning the respective literature and the genome of *B. licheniformis* MW3, Waschkau *et al.* 2008 (a restrictase deficient derivative of *B. licheniformis* DSM13; Veith *et al.* 2004) which is genetically almost identical to *B. licheniformis* ATTC 14850 (Rey *et al.* 2004).

The results of the search are depicted in Table 3.1.

**Table 3.1** Polysaccharide deacetylase (PDA) encoding genes in *Bacillus licheniformis*

| Gene designation | Protein name | Accession number | Locus tag | Function | Functional status | Strain | References |
|---|---|---|---|---|---|---|---|
| ***yjeA **** | **YjeA** | YP_090286.1 | BLi00655 | 1. Carbohydrate esterase fam. 4<br>2. Extracellular DNAse<br>3. Endo-1,4-beta xylanase<br>4. Similar to Chitooligosaccharide deacetylase | predicted | DSM 13/ ATTC 14580 and F11 | Rey, *et al.* 2004. Veith *et al.* 2004. Waldeck *et al.* 2006. Voigt *et al.* 2006. Voigt *et al.* 2009 |
| ***yfjS*** | **YfjS** | YP_090455.1 | BLi00827 | 1. Delta-lactam-biosynthetic de-N-acetylase<br>2. Similar to Polysaccharide deacetylase<br>3. Peptidoglycan GlcNAc deacetylase | predicted | DSM 13 /ATTC 14580 | Rey, *et al.* 2004. Veith *et al.* 2004. |
| ***yheN*** | **YheN** | YP_090640.1 | BLi01039 | 1. Similar to Endo-1,4-beta-xylanase | predicted | DSM 13 /ATTC 14580 | Rey, *et al.* 2004. Veith *et al.* 2004. Voigt *et al.* 2006. Voigt *et al.* 2009 |
| ***ylxY*** | **YlxY** | YP_091483.1 | BLi01895 | 1. Polysaccharide deacetylase family | predicted | DSM 13/ATTC 14580 | Rey, *et al.* 2004. Veith *et al.* 2004. |
| ***yxkH*** | **YxkH** | YP_093657.1 | BLi04151 | 1. Polysaccharide deacetylase<br>2. Carbohydrate esterase family 4 | predicted | DSM 13 /ATTC 14580 | Rey, *et al.* 2004. Veith *et al.* 2004. |
| ***Bli02451 /yheN2*** | **Bli02451 /YheN2** | YP_092021.1 AAU41828.1 | Bli02451 | 1. Chitin deacetyl-ase / xylanase<br>2. Peptidoglycan GlcNAc deacetylase | predicted | DSM 13 /ATTC 14580 | Rey, *et al.* 2004. Veith *et al.* 2004. Wiegand *et al.* 2012. |
| *yba*N | YbaN / PdaB | YP_089842.1 | BLi00175 | 1. Polysaccharide deacetylase | predicted | DSM 13 /ATTC 14580 | Rey, *et al.* 2004.. Veith *et al.* 2004. |

***** All PDAs are putative; the genes investigated in this study are in bold face. The data in the table were taken from the CAZY database (http://www.cazy.org/CE4_bacteria.html), the Combrex database (http: //combrex.bu.edu/) and the NCBI database (http://www.ncbi.nlm. nih.gov/). Accession numbers refer to unique identifiers given to the protein sequences in GenBank (http://www.ncbi. nlm. nih.gov/genbank/); Locus tags refer to synonyms for the loci in the genomes.

The anticipated use of the mutants for biological containment in biotechnological large scale fermentations implies that mutations should not interfere with vegetative growth and physiology and that is why we focused on genes putatively instrumental in spore germination (bold face in Table 3.1 ; Silvaggi *et al.* 2004 and Fukushima *et al.* 2002) as such mutations do a priori not interfere with vegetative growth.

## 3.1. Construction of mutants

### 3.1.1. Deletion of *yjeA*

From the bioinformatic analysis, especially from its predicted function, *yjeA* is suggested to encode a chitooligosaccharide deacetylase. Indeed, the majority of the other data bank entries (Carbohydrate esterase; 1,4 beta xylanase) in general agree with such function, even though the notion of an extracellular DNAse does not.

For constructing an *yjeA* clean deletion mutant, the upstream and downstream flanking regions (flankA, 721 bps and flankB, 708 bps of *yjeA,* respectively) were amplified by applying the *Phusion* DNA polymerase employing the primer pairs yjeA1BamHI/yjeASOE2 and yjeA4BamHI/yjeA3 with the total DNA of *B. licheniformis* MW3 as the template. The obtained amplification products (flankA and flankB) were combined to yield a fused fragment (flankA-B, 1.429 kb) via SOE-ing PCR (Heckman and Pease 2007). Since the outer primers were both equipped with BamHI restriction sites, the fusion product - after BamHI restriction - was cloned into the likewise cut shuttle vector pJR1 (Richardt *et al.* 2010), yielding pJRyjeA. The construction details are outlined in Figure 3.1 and the restriction analysis of the plasmid is shown in Figure 3.2.

The conjugative transformation of plasmid pJRyjeA from *E. coli* S17-1 into the cells of *B. licheniformis* MW3 was conducted by the transconjugation method developed by Richardt, *et al.* (2010).

Subsequent to the mating, the *B. licheniformis* MW3 transconjugants were obtained by applying a counter selection procedure which makes use of the capability of the *Bacillus* species to develop heat resistant endospores. After cultivation on 2xSG solid medium (sporulation medium) for approximately 2-3 days at the appropriate temperature of 30 $^{0}$C, selection was done by pasteurization of the scraped off cells grown on the 2xSG plates, a procedure, that ensured that only the *Bacillus* cells survived which had sporulated.

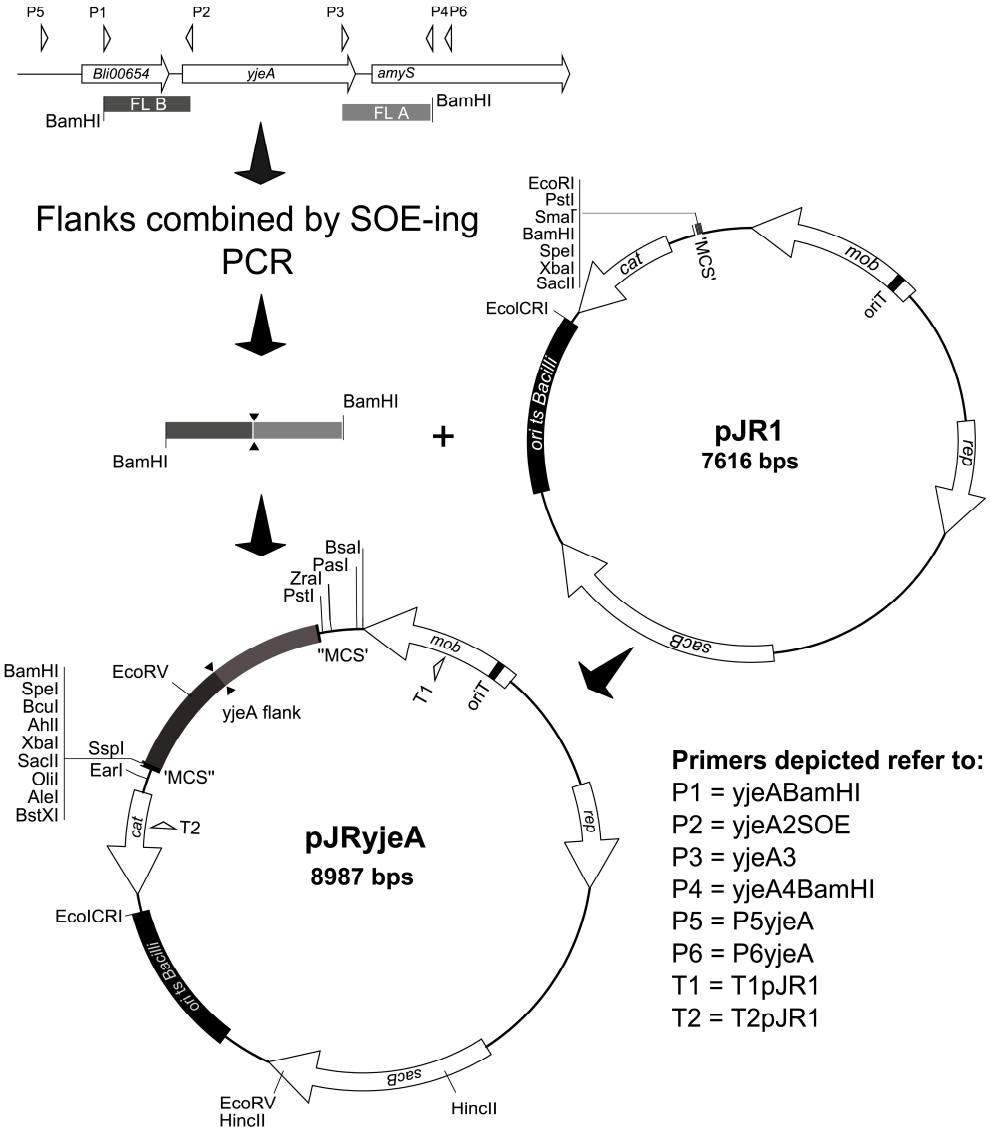



**Figure 3.1** Construction strategy of a deletion vector for the *yjeA* gene of *B. licheniformis* MW3. Open reading frames of the *B. licheniformis* MW3 genome are depicted as arrows, the direction of which corresponds to the transcriptional orientation; different shaded grey bars refer to the deletion flanks A and B, respectively, and are denoted as such. When known, gene designations are given: *yjeA* is the predicted PDA-encoding gene; *amyS* is an Amylase encoding

gene; *Bli00654* corresponds to the respective locus tag. Primers and their positions are depicted as triangles. The deletion cartridge along with the flanking BamHI sites is shown underneath. The black boxes in the mobilizable pJR1 and pJRyjeA refer to the *Bacillus* temperature sensitive (*ts*) origin of replication and the origin of transfer (*oriT*) of *E. coli*. MCS denotes the multiple cloning site, applicable for the respective resctriction enzymes, *sacB* refers to the *B. subtilis* levansucrase encoding gene. The *mob* region in mobilizable plasmids pJR1 and pJRyjeA code for the mobilization protein and *rep* encodes the Rep helicase of *E. coli*.

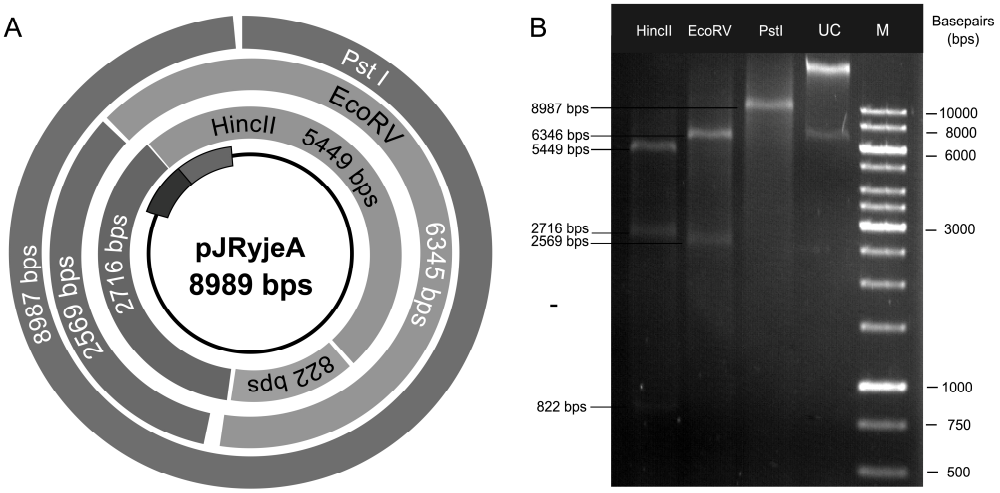



**Figure 3.2** Restriction analysis for checking the deletion cartridge insertion for *yjeA* in plasmid pJR1. A) Scheme of the plasmid pJRyjeA, showing several restriction sites used in the analysis; B) Agarose gel electrophoresis to detect the fragments from digestion with the three restriction enzymes HincII, EcoRV and PstI; the sizes of the bands in bps are denoted on the left site of the gel. UC refers to the uncut plasmid and M contains the 1 Kb DNA Ladder (Fermentas, Pittsburgh PA, USA).

The putative transconjugant *Bacillus* strains were subsequently checked for a defective *yjeA* gene by analytical PCR. For such purpose, the primer pair P5yjeA (P5) and P6yjeA (P6) was applied. The result of the PCR-check for the defective (deleted) *yjeA* is depicted exemplarily in Figure 3.3. (MT) along with the check of the corresponding genomic regions of the wild type (WT).

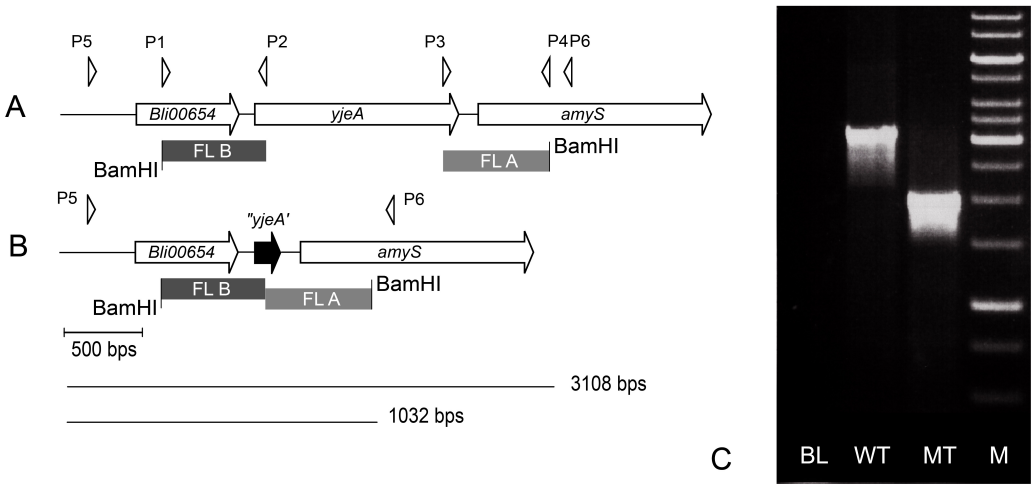



**Figure 3.3** Genetic organization of chromosomal DNA of *B. licheniformis* MW3 and the deleted knock out mutant of *yjeA*: A) Wild type situation in MW3 before deletion of the large internal part of *yjeA*; B) After the deletion of the internal part of *yjeA*. Empty arrows depict open reading frames, black arrows refer to the remaining part of *yjeA* gene after the clean deletion; different shaded light and grey bars depict the deletion flanks A and B; C) PCR analysis of chromosomal DNA of the wild type (WT), and the *yjeA*

deleted mutant (MT), BL is the mock control (blind lane); M refers to the 1 Kb DNA ladder (Fermentas, Pittsburgh PA, USA).

Subsequent to the PCR–screening the *B. licheniformis* MW3 Δ*yjeA*, i.e. the putative mutant strain, was checked by an additional analytical PCR to ensure that there is no plasmid pJRyjeA left in the mutant cells and for verifying that the construction of the *B. licheniformis* MW3Δ*yjeA* clean deletion is indeed correct; the plasmid–based primers used are T1pJR1 (symbolized as **x**) and T2pJR1 (symbolized as **y**) in combination with genomic–based primers P5yjeA (symbolized as **5**) and P6yjeA (symbolized as **6**). The results of the analytical PCR including the appropriate controls are shown in Fig. 3.4.

Three combinations of primer pairs were used to address the genomic DNA (see the illustrative Figure 3.4) to find an amplicon that should be produced solely by the pair x and y with the genomic DNA of the transconjugant as the template, to exclude that the strain still contains plasmid pJRyjeA; whereas the genomic DNA of the wild type and also genomic DNA of the mutant should not.

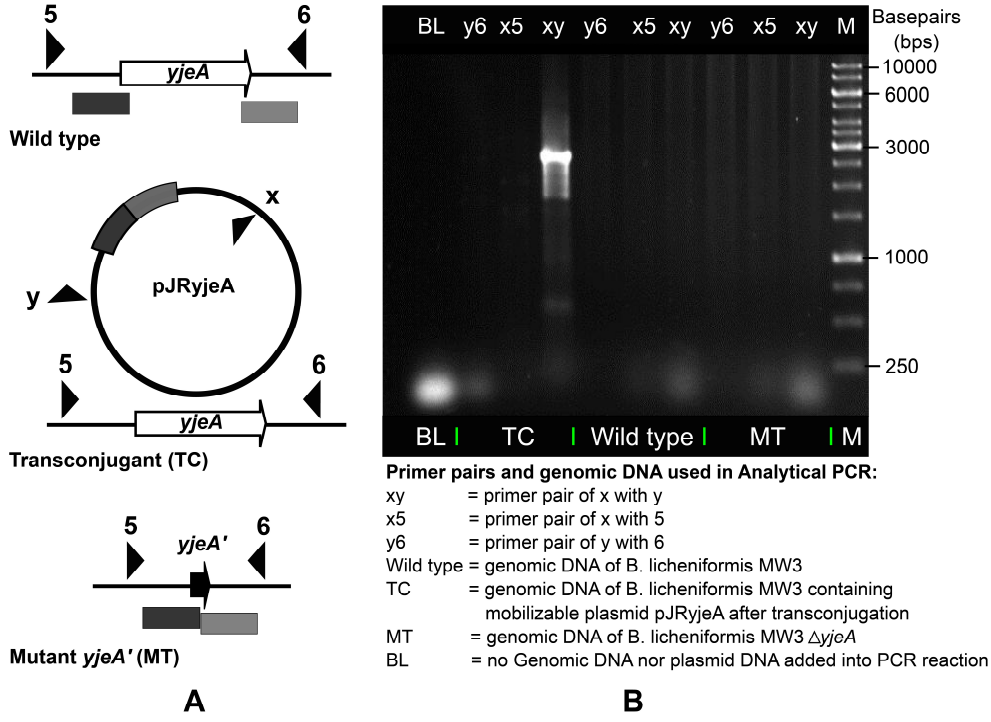



**Figure 3.4** Analytical PCR for checking the clean deletion mutant of *B. licheniformis* MW3 Δ*yjeA*. A) Genomic illustration of the three possible situations and the corresponding analytical PCR; empty arrows depict open reading frames along with the gene name, the black arrow refers to the remaining part of *yjeA* gene after the clean deletion; different shaded light and grey bars depict the deletion flanks and black triangles denote the primers ; B) The gel picture shows the fragment obtained by PCR using the primer pairs described in part A. Lane M contains the 1 Kb DNA ladder (Fermentas, Pittsburgh PA, USA). The Wild type (WT), BL, TC and MT denote the isolated genomic DNA used as the respective PCR template: Wild type (WT), refers to the genome of *B. licheniformis* MW3; BL refers to the mock control (Blind lane); TC denotes the lane in which genomic DNA of *B. licheniformis* MW3 transconjugants containing pJRyjeA were used as the template; MT refers to the genome of *B. licheniformis* MW3 Δ*yjeA* as the template. For each of

the genomic DNAs noted in the gel, PCRs were performed with three primer pairs decribed as xy, x5 and y6. The primer pairs used are: **xy**, refers to primers x and the primer y; **x5**, refers to the primer pair x and 5; and **y6** refers to the pair of primers y and which is additionaly noted underneath the gel picture.

## 3.2. Construction of *B. licheniformis* knock out mutants by gene disruption.

There are various methodologies to produce knock out mutants by gene disruption. One of the most frequently performed procedures, which is highly reliable for *Bacillus* strains involves pMutin vectors (Vagner *et* al. 1998) and derivatives of the latter. The vector does not only comprise several functional genes for tracking the integration into chromosomal loci, such as the Tetracyclin resistance, but also elements for functional studies of non-characterized open reading frames. All pMutin vectors carry a *lacZ* reporter gene and the inducible Pspac promoter, which is tigthly regulated and can be induced 1000-fold by the addition of IPTG (Vagner *et al.* 1998). In this study, the respective vector named pMutin2 was used. For more details and the plasmid map; I refer to the protocol in Material and Methods and the schematic representation of the vector in figure 6.2 in the addendum, respectively.

### 3.2.1. Generation of a mutant with an integrated pMutin2 in the *Bli02451* locus

The gene with the locus tag *Bli02451* encodes a predicted protein which is a putative chitin deacetylase (see also the list of the genes given in Table 3.1). In more detail, the Combrex (http://combrex.bu.edu/) as well as the NCBI (http://www.ncbi.nlm.nih.gov/) database (Marchler-Bauer *et al.* 2013) confirm that the putative polypeptide encoded by the gene possesses a conserved chitin deacetylase domain (CDD) (also known as CDA1); the encoded polysaccharide deacetylase is according to NCBI classified more specifically as a peptidoglycan GlcNAc deacetylase. The latter annotation made the locus highly relevant to our goal, as the database entry suggests that the gene product might be involved in spore germination.

Since a *B. licheniformis* MW3 Δ*yfjS* strain was already available at the beginning of the work (constructed by Claudia Borgmeier, in this laboratory) as well as the firstly constructed *B. licheniformis* MW3 Δ*yjeA* (see previous chapter), it was not only anticipated to construct a single integrational *B. licheniformis* MW3 ::*Bli02451*pMutin2 mutant, but also the two respective double mutants *B. licheniformis* MW3 Δ*yfjS* ::*Bli02451*pMutin2 and *B. licheniformis* MW3 Δ*yjeA* ::*Bli02451*pMutin2. The scheme detailing the construction of the pMutin2 integration into *Bli02451* is depicted in Figure 3.5. The first step involved the PCR amplification of the region of the gene into which the integration by

homologous recombination is to be targeted.

For this purpose, the primer pair (02451-pMutin2-HindIII and 02451-pMutin2-BamHI) was used to generate a fragment consisting of 433 bp, termed 02451' in Figure 3.5. Subsequently, the fragment was treated with the restriction enzymes HindIII and BamHI, resulting in a 409 bp spanning DNA piece. The fragment was cloned into plasmid pUCBM20 that was cut with the same restriction enzymes. From the resulting pUCBM20-02451', the respective HindIII and BamHI fragment was cut out, electro–eluted and cloned into pMutin2 as outlined in the figure, eventually resulting in the integrative hybrid plasmid pMutin02451' that was finally used for transformation of *B. licheniformis* MW3 cells.

The strategy for the construction of the strains with a chromosomally integrated pMutin in the *Bli02451* locus, is outlined in Figure 3.6.

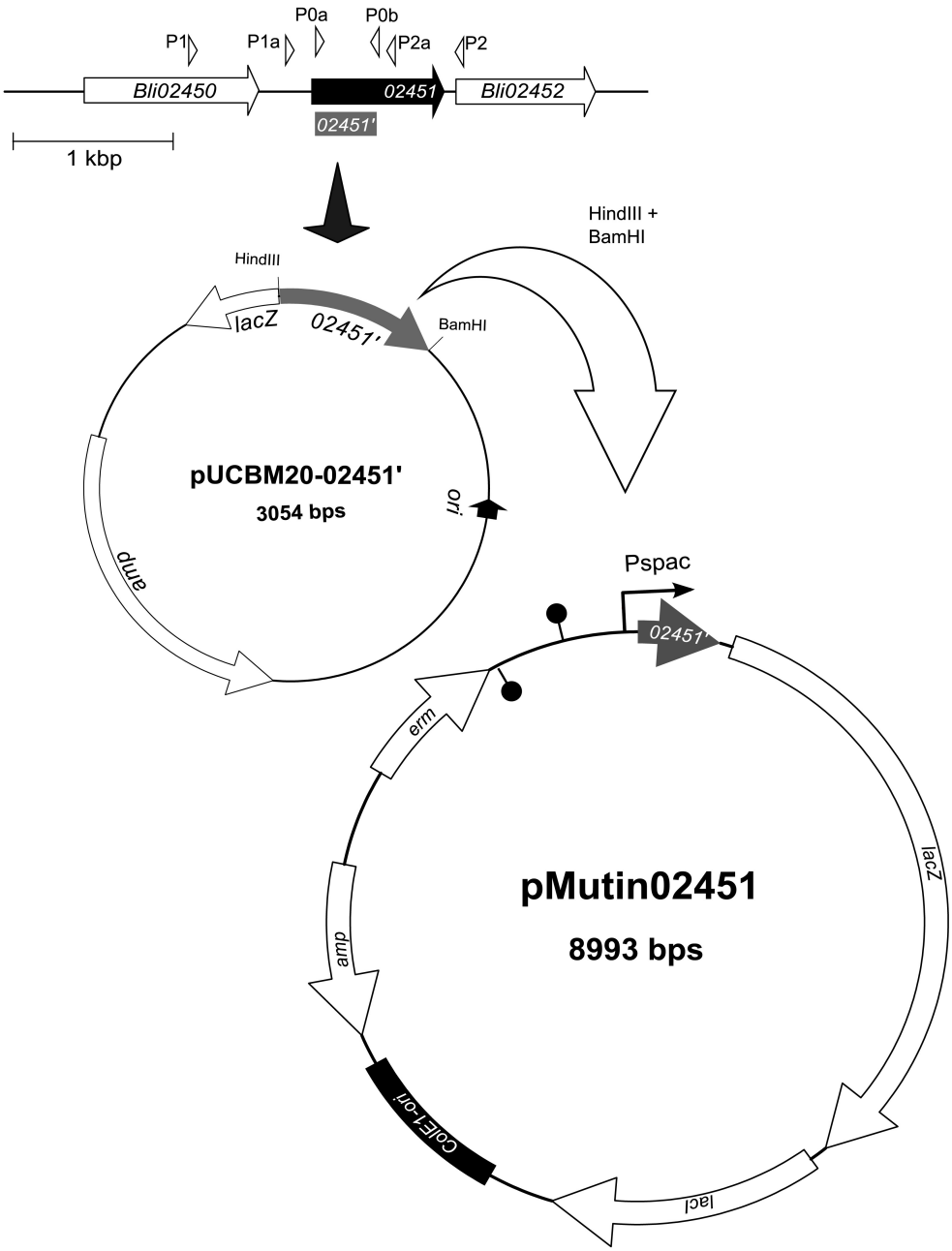



**Figure 3.5** Construction scheme for plasmid pMutin02451, obtained by ligating HindIII/BamHI cut pMutin2 and the HindIII/BamHI fragment obtained by PCR from *Bli245*. Open reading frames are depicted as arrows. The black hairpins refer to transcriptional terminators; the grey arrow refers to the amplified recombination flank 02451'. The right–angle–arrow flags the

Pspac promoter and is denoted as such; the triangles on top of the figure refer to primers used to amplify the region of interest; the black box designated as *ColE1* represents the part that ensures replication in *E. coli*; *erm* refers to the gene encoding Erythromycin resistance in *Bacillus*; *amp* denotes the gene encoding the Ampicillin resistance in *E. coli*; *lacZ* refers to the gene encoding the β-galactosidase and *lacI* is the gene coding for the Lac repressor.

To transform the above integrative vector into *B. licheniformis* MW3, the cells were made genetically competent by applying a system previously developed in this laboratory (Hoffmann *et al.* 2010). Briefly, the cells used for transformation carry an episomal plasmid that harbours a xylose inducible *comK* gene. The latter encodes the key transcriptional regulator for competence development. With the aid of such transformation tool it was possible to generate a *B. licheniformis* MW3:: *Bli02451*pMutin2 mutant strain. Analogously, in two other separate transformation approaches the integrative plasmid pMutin02451' was used to establish the respective construction in the *B. licheniformis* MW3Δ*yfjS* and in the *B. licheniformis* MW3Δ*yjeA* background, eventually resulting in the two double mutants *B. licheniformis* MW3Δ*yfjS*::*Bli02451*pMutin2 and *B. licheniformis* MW3Δ*yjeA* ::*Bli02451*pMutin2, respectively. The genotype of the strains with respect to the pMutin02451' insertion is schematically depicted in Figure 3.6 along with the PCR check.

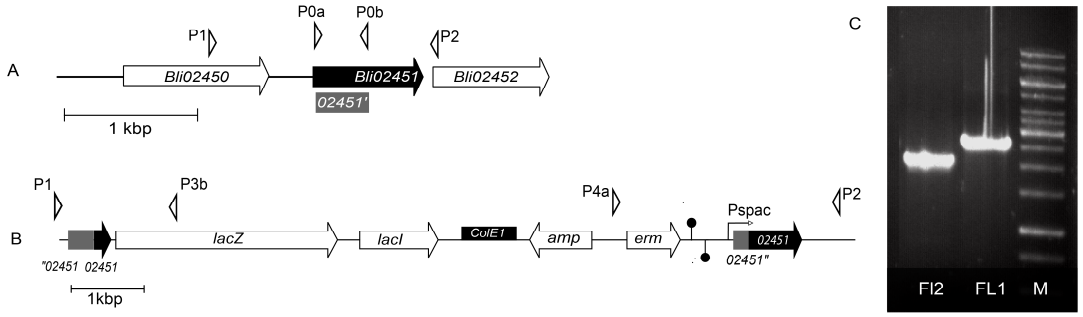



**Figure. 3.6** Genetic organization of the chromosomal region of *B. licheniformis* MW3 strains with pMutin2 integrated in the *Bli02451* locus: A) Wild type MW3; B) The genetic knock out situation in *Bli02451*; arrows depict open reading frames (ORF); other symbols and designation of pMutin genes as in Figure 3.5; C) Agarose gel stained with Ethidium bromide (EtBr) for verification of the knock-out mutant by PCR; FL1 refers to the amplicon obtained with primers 0251N1-P5 (P1) and lacZ-rev (P3b); FL2 refers to the amplicon obtained with primers 02451N1-P6 (P2) and Erm-for (P4a); lane M contains the 1 kb DNA ladder (Fermentas, Pittsburgh PA, USA).

### 3.2.2. Generation of a *yjeA* disruption

In order to combine the Δ*yfjs* mutation with a *yjeA* disruption, the respective integrative plasmid was constructed.

The target flank, namely *yjeA'* (502 bps), was produced using the primer pair yjeA-pMutin2-HindIII and yjeA-pMutin2-BamHI and chromosomal DNA as the template. Subsequently, the restriction enzymes HindIII and BamHI were

applied to produce sticky ends at both sides of the flank, resulting in a 478 bps fragment that was cloned into plasmid pUCBM20 to produce pUCBM20-yjeA'. The above flank was then excised with the restriction enzymes HindIII and BamHI revealing *yjeA'* that was finally cloned into plasmid pMutin2, yielding the integrative plasmid pMutinyjeA, ready for transformation into strain *B. licheniformis* MW3Δ*yfjS*.

The schematic representation of the construction along with respective genotype of *B. licheniformis* MW3Δ*yfjS* ::*yjeA*pMutin2 is depicted in Figure 3.7.

Transformation of plasmid pMutinyjeA into MW3 Δ*yfjS* was performed by applying again the the *comK* competent induction system based on the pMMcomK vector that carries a *comK* gene that can be induced by xylose.

The correct integration of pMutinyjeA into the anticipated locus was checked by applying two primer pairs (one for the left side and the other for the right side of the integrated pMutin2). Both PCR approaches yielded the fragments of the expected sizes (see Figure 3.7).

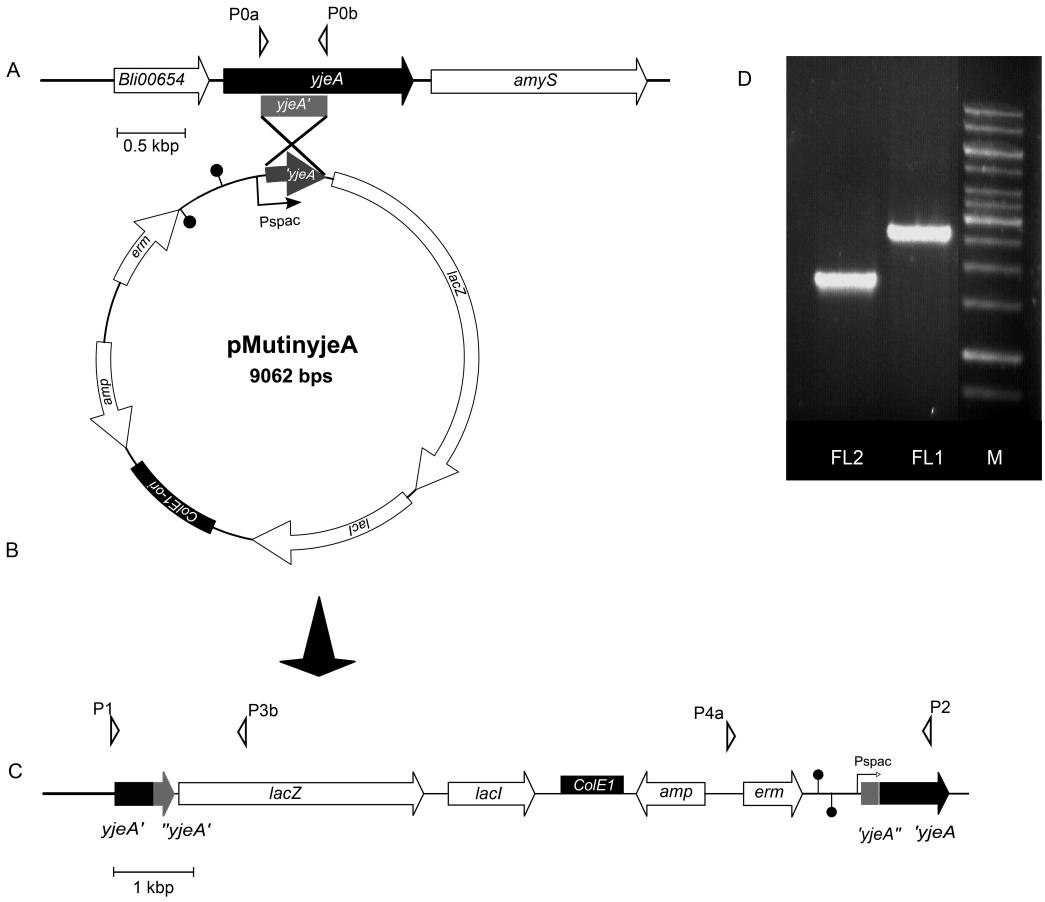



**Figure 3.7** Genetic organization of chromosomal DNA of *B. licheniformis* MW3 strains with pMutin2 integrated in the *yjeA* locus: A) Wild type MW3; B) Plasmid pMutinyjeA, a plasmid which was constructed by inserting a flank (*'yjeA*) into the BamHI and HindIII restriction sites; C) Genetic organization after the genetic knock out of *yjeA*; arrows depict open reading frames; other symbols and designation of pMutin genes as in Figure 3.5; D) Agarose gel stained with Ethidium bromide (EtBr) for verification of the knock-out mutant by PCR; FL1 refers to the amplicon obtained with the primers yjeAN1-P5 (P1) and lacZ-rev (P3b); FL2 refers to the amplicon obtained with

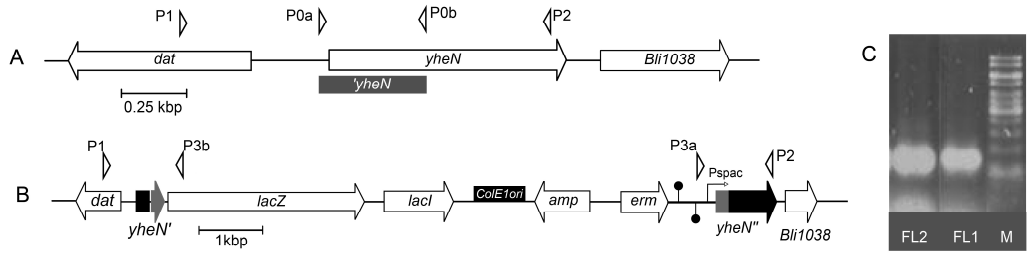
primers yjeAN1-P6 (P2) and Erm-for (P4a), while both flanks resulted from PCR reaction using genomic DNA of mutant MW3::*yjeA*pMutin2 as the template; lane M contains 1 kb DNA ladder (Fermentas, Pittsburgh PA, USA).

### 3.2.3 Construction of single integrative disruption mutants of *yheN*, *ylxY*, and yxkH

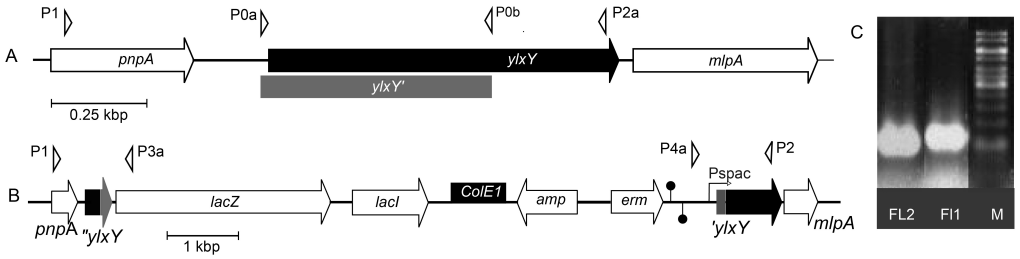
A fragment containing the promoter region of the *yheN* gene was amplified with a pair of primers (SW-yheNHindIII and SW-yheNBamHI) resulting in a 438 bps flank, which was subsequently treated with the restriction enzymes HindIII and BamHI to produce the smaller (416 bps) *'yheN* flank. Similarly, the plasmid pUCBM20 was cut with both of the restriction enzymes and the *'yheN* flank was ligated to the cleaved vector giving rise to pUCBMyheN. The latter was isolated from an *E. coli* transformant. The strain carrying the plasmid was grown overnight at the appropriate temperature and shaker speed in LB medium containing Ampicillin for the isolation of sufficient amounts of the plasmid. After isolation and purification, the plasmid was subjected to digestion with the restriction enzymes HindIII and BamHI, and the obtained small band (*'yheN*) was subsequently inserted into pMutin2, resulting in plasmid pMutin2'yheN. The construction of two other pMutin2 derivates, i.e. plasmid pMutin2'ylxY, and pMutin2'yxkH followed the same scheme as for the hitherto described pMutin2 integrational mutants (see also previous Figure

3.5.). Due to the rather similar approaches, the details of the construction are not explicitly shown here; anyway, the results from the final mutant screenings for the three single pMutin2 integration mutants are presented in Figure 3.8.

I.




II.




III.

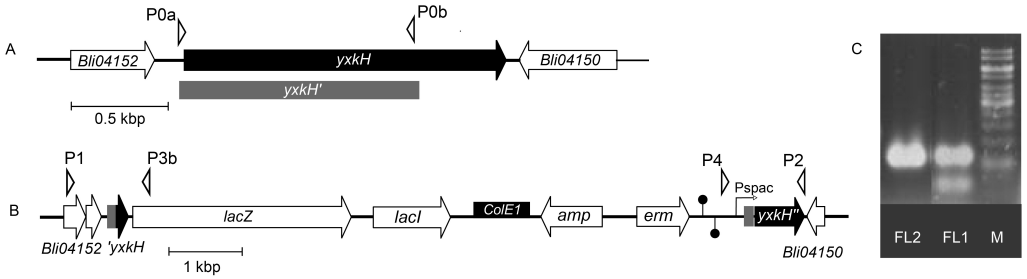


**Figure 3.8** Genetic arrangement of the chromosomal DNA of *B. licheniformis* MW3 strains at the pMutin2 integration sites: A) before the knock out and; B) after the genetic knock out of the genes *yheN* (I), *ylxY* (II) and *yxkH*.(III); C). Anticipated amplification products for the knock-out mutants FL1 and FL2 refer to the amplicons obtained with two primer pairs anticipated to obtain bands of such size, lane M contained the 1 kb DNA ladder (Fermentas, Pittsburgh PA, USA).

## 3.3. Inspection of the colony phenotype of *B. licheniformis* PDA knock-out strains

As mentioned in the previous chapter of the Results section, in addition to the *B.licheniformis* MW3Δ*yfjS* mutant generated by Claudia Borgmeier in this laboratory, eight additional knock–out mutants of *B. licheniformis* MW3 hit in genes predicted to encode an enzyme with PDA activity were constructed: 1) Δ*yjeA*; 2) MW3::*Bli02451*pMutin2; 3) Δ*yfjS*::*yjeA*pMutin2; 4) Δ*yfjS*::*Bli02451* pMutin2; 5) Δ*yjeA*::*Bli02451* pMutin2; 6) MW3::*yheN*pMutin2; 7) MW3 ::*ylxY*pMutin2; and 8) MW3::*yxkH*pMutin2.

Since one of the main aims of the study was to check and potentially generate biotechnologically contained strains useful for application in the Biotech industry, several industrially important features were addressed in addition to the colony morphology, i.e. growth pattern, extracellular enzyme production and spore germination.

To examine the phenotypic appearance of the strains, the mutants were grown overnight in 7 ml LB medium supplemented with the appropriate antibiotic; and subsequently, the cells were serially diluted till the dilution factor of $10^{-4}$ and for each of the strains the final dilution was spotted (5 µl) on an LB plate and incubated for 72 hours at 30 $^0$C. The experiment was repeated 3 times for each strain to exclude variations resulting from ungovernable environmental influences. The phenotypic appearance of the different mutants strains and the parental MW3 are exemplarily depicted in Figure 3.9. The shown phenotypes were equal in all experiments.

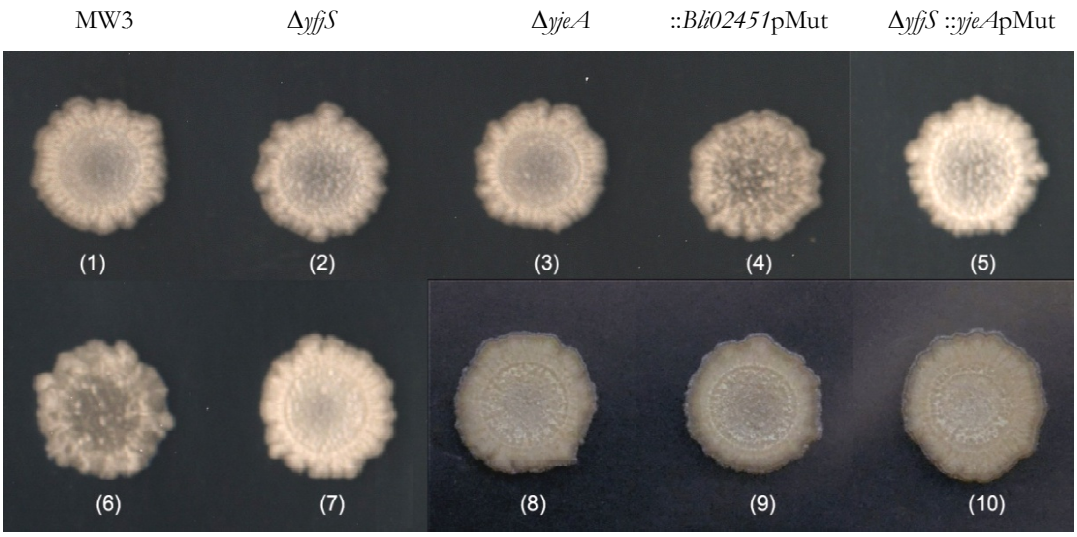



**Figure 3.9** *B. licheniformis*; Colony phenotype of the wild type MW3 and the mutants strains after 72 hours at 30 $^0$C grown on LB agar–medium. The relevant genotype of each mutant strain is given in close proximity to the

respective colony. The colonies formed by strains formed by strains ::*Bli02451*pMut and Δ*yfjS*::*Bli02415*pMut differ from all other colonies by their dotted appearance. The colony number of (5) and (7) are whitish.

### 3.4. Growth Inspection of *B. licheniformis* knock-out strains

In industrial biotechnology, fermentation is often performed as submerged batch cultivation in liquid medium. Thus, mutants that can potentially be applied in industrial large scale fermentation must be checked in liquid media to ensure that growth is not negatively affected.

Hence, the *B. licheniformis* PDA mutant strains were grown in two commonly used media, one of which is a complex medium (CM, LB-medium) and the other is a minimal medium (MM, M9 medium). For monitoring the growth in both of the media and to evaluate whether there are side effects as the consequence of the gene knock-outs both, single and double mutants, were grown for 72 hours at 37 $^{0}$C at 165 rpm (Innova 44, New Brunswick-Eppendorf, Hamburg, DE). Erythromycin at low concentrasion (0.75 mg/ml) was added to the cultivations of the strains carrying an integrative pMutin vector. The cultures were inoculated to an optical density of 0.1 at the OD 547 nm. The growth patterns are shown in Figure 3.10 and Figure 3.11, for CM and MM, respectively

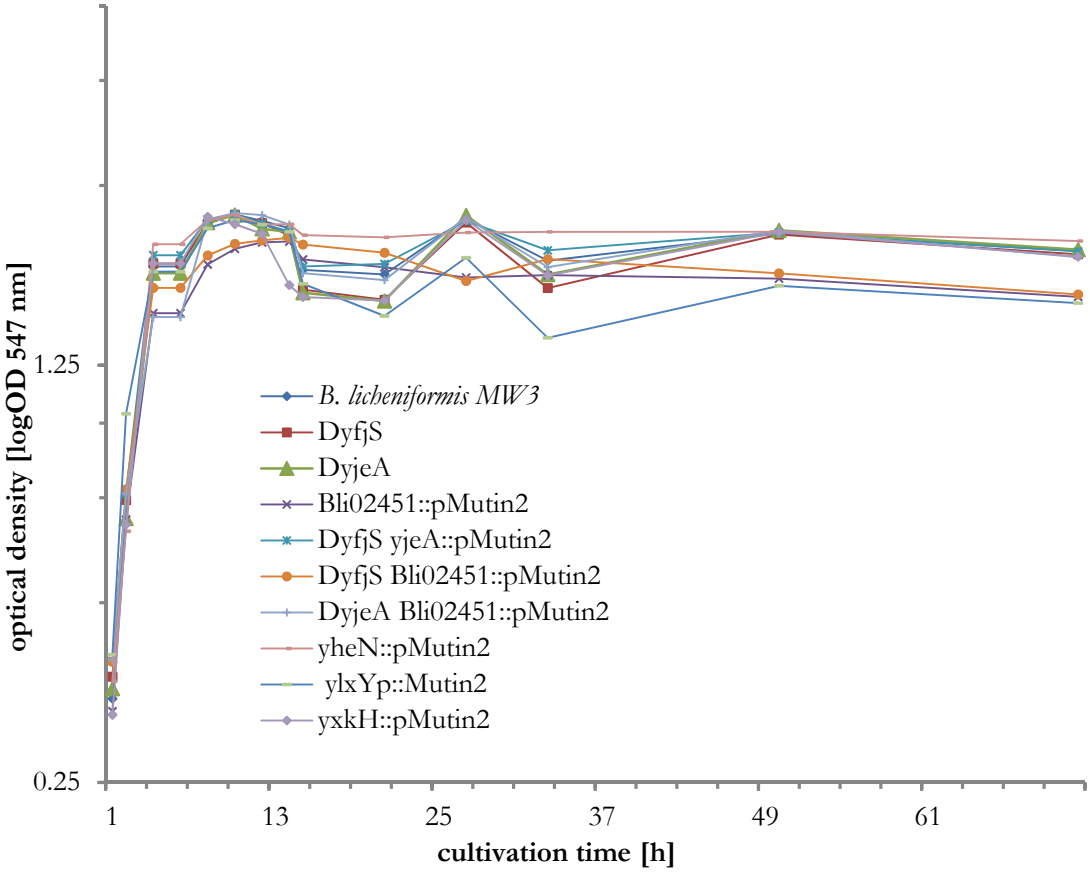



**Figure 3.10** Growth curves of the *B. licheniformis* MW3 knock–out mutants in complex media in comparison to the wild type MW3. Cultivation was done in LB medium at the initial pH 7.5. Strain designations along with the corresponding graphs are mentioned as such. The data presented resulted from two different experiments (mean values).

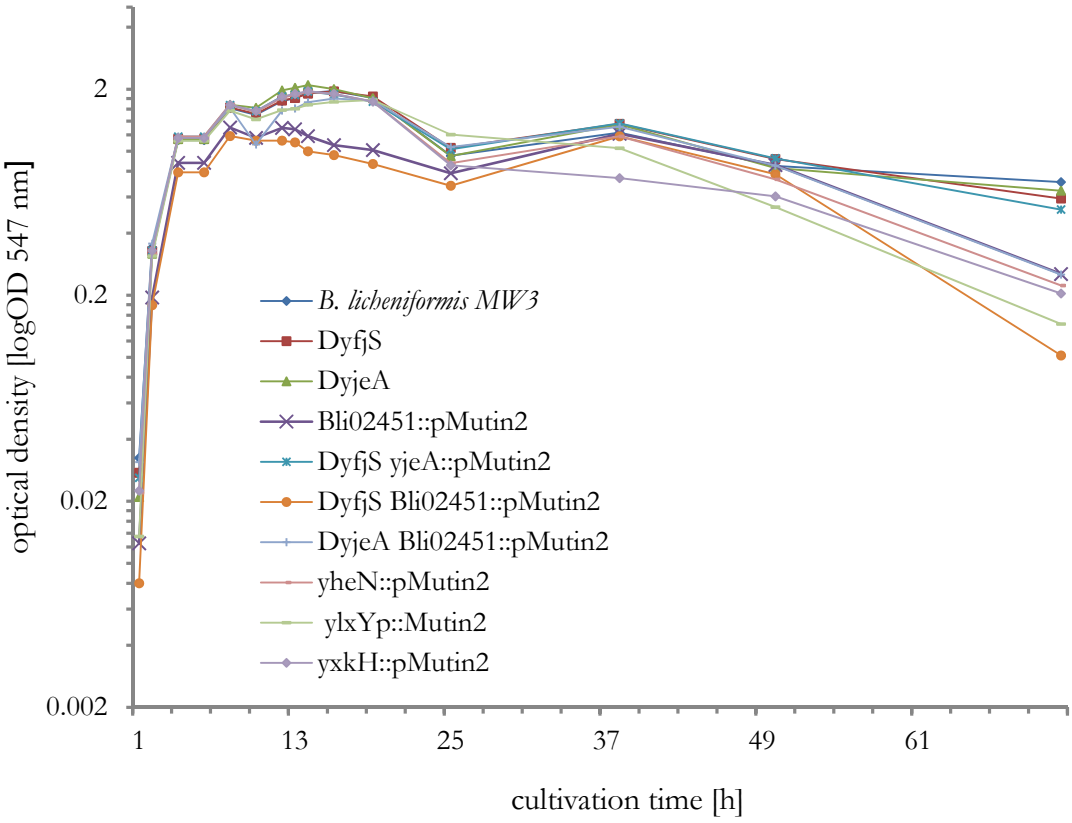



**Figure 3.11** Growth curves of the *B. licheniformis* MW3 knock–out mutants in minimal media in comparison to the wild type MW3. Cultivation was done in M9 medium at the initial pH 7.4. Strain designations as in Figure 3.10. The data resulted from two different experiments (mean values).

## 3.5. Extracellular Enzymatic Activities of the *B. licheniformis* knock–out strains

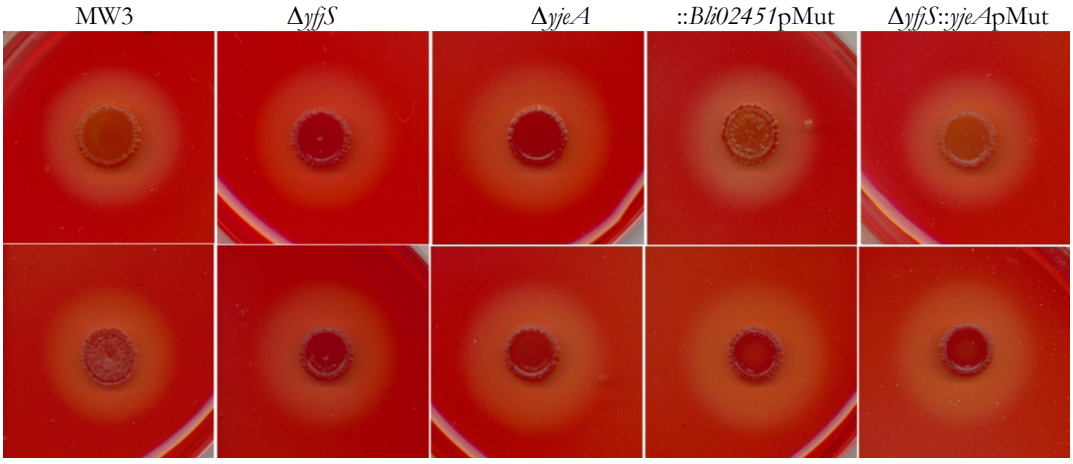
Since the members of the species *B.licheniformis* represent bacterial workhorses in the enzyme industry (Schallmey *et al.* 2004), any mutation that affects the capability to produce extracellular enzymes negatively, can hardly be applied as

a means to ensure biological containment as the biotechnologically most important aspect concerns the productivity.

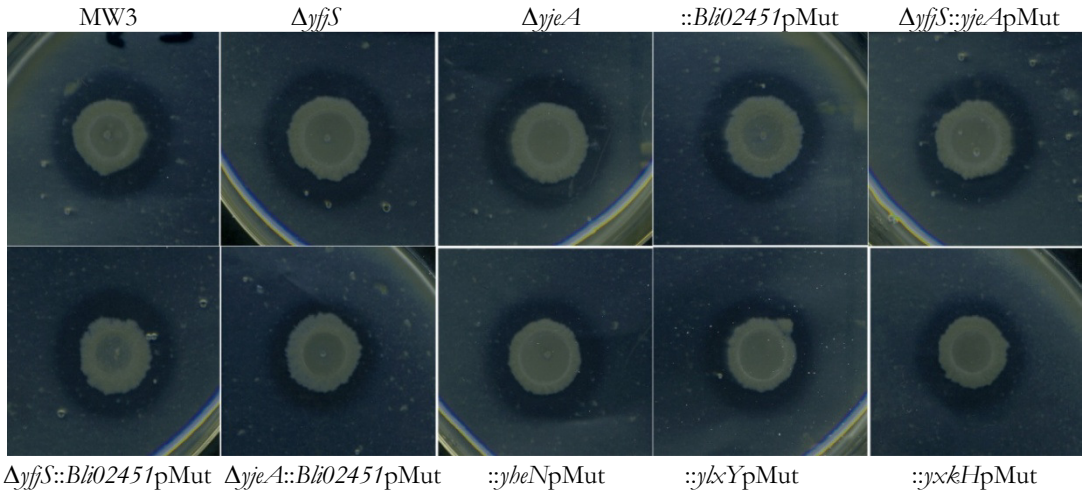
Exemplarily, for the great number of secreted degradative enzymes, the activity of glucanolytic, amylolytic, and proteolytic enzymes were monitored. The results are depicted in Figure 3.12.A, B, C respectively.

Although the conducted plate assays may not allow for the detection of minor deviations, they are well suited to monitor mutations that can cause relevant production losses (Waldeck *et al.* 2007).

## A. Glucanolytic activities



**B. Proteolytic activities**




**C. Amylolytic activities**

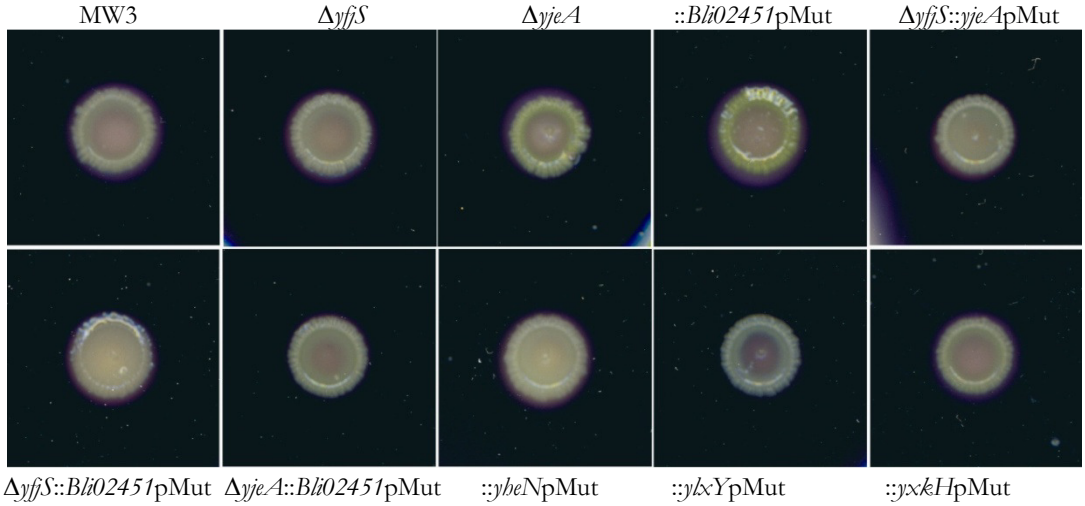



**Figure 3.12** *B. licheniformis;* A) glucanase activities of the mutant strains after 36 hours at 37 $^{0}$C grown on M9 minimal agar medium supplemented with 0.02% lichenin; to visualize the glucanolytic activity plates were stained with Congo Red. B) protease activities of the mutant strains after 36 hours at 37 $^{0}$C grown on M9 minimal agar medium supplemented with 2% skim milk; C) amylase activities of the strains after 36 hours at 37 $^{0}$C grown on LB agar

medium supplemented with 1% soluble starch and stained with Lugol's solution. Clearing halos refer to the glucanolytic (A), proteolytic (B) and amylolytic activities (C), respectively. Relevant genotypes of the strains are depicted as such.

### 3.6. Sporulation of the *B. licheniformis* PDA knock-out Mutants

#### 3.6.1 Microscopic investigation

Since *Bacillus* endospores can be readily visualized by phase contrast microscopy (they appear as phase bright particles), the spore formation of the constructed mutant strains was investigated by this technique. The result of the examination are shown in Figure 3.13.

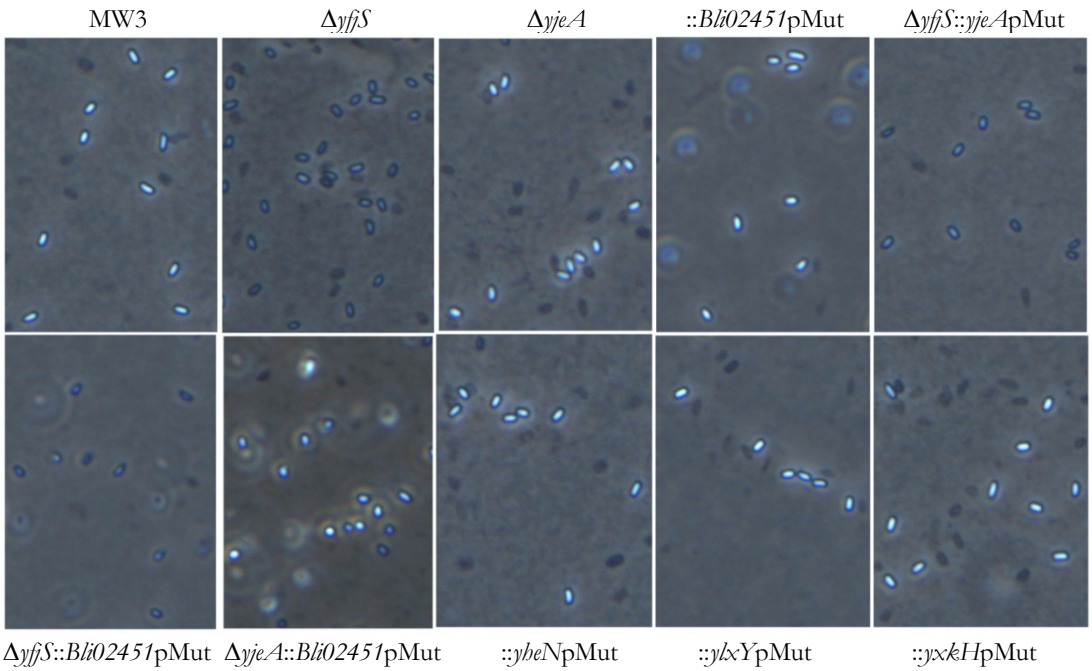


**Figure 3.13** Spore formation of the *B. licheniformis* PDA knock-out mutants. The spores were obtained from cultures on Schaeffer's sporulation medium (2 x SG medium). The respective genotype of the mutants is shown above/underneath of each picture.

Note, that Δ*yfjS,* Δ*yfjS* ::*yjeA*pMut and Δ*yfjS*::*Bli02451*pMut display grey rather than bright spores.

### 3.6.2 Spore Germination of Spores of the *B. licheniformis* PDA knock-out Mutants

As for the growth inspection and the enzyme productivity assays, the germination examinations were perfomed with three biological replicates. The experimental procedures included drop dilution assays for semi-quantitative recording (shown in Figure 3.14), and plating assays for verification and true quantitavive determination; for the latter, the last three dilutions ( $10^{-6}$ – $10^{-8}$) were plated on LB agar medium followed by incubation at 37 $^{0}$C overnight. The colony forming units were counted and related to the spore numbers calculated prior to both experiments. The calculated numbers obtained from the spore germination experiments are shown in Figure 3.14 and Figure 3.15, respectively.

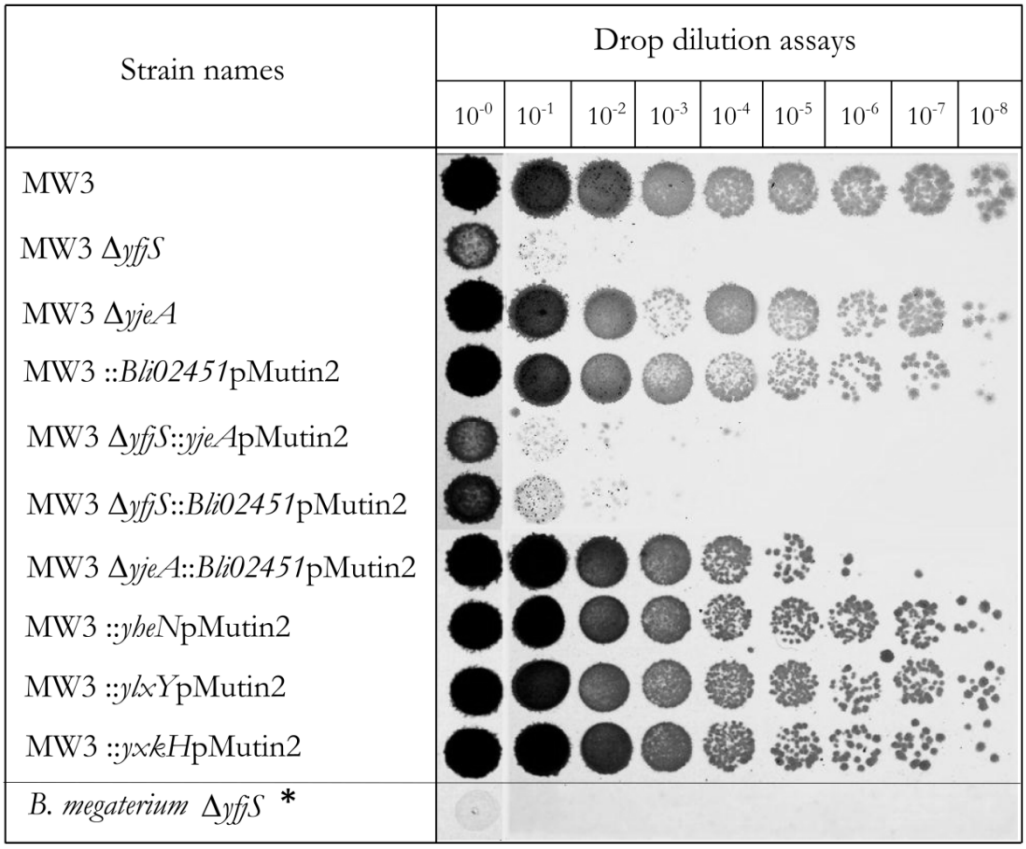



**Figure 3.14** *B. licheniformis,* drop dilution assays for recording the impacts on spore germination of the knock out mutations. The *yfjS* deletion mutant of *B. megaterium* (obtained from this laboratory, Borgmeier and Meinhardt, unpublished) served as an internal control. The cultures were normalized to $10^8$ spores per ml and subsequently heated for 20 minutes at 80 $^0$C to kill remaining vegetative cells. Strain designations are mentioned as such. The asterisk (*) refers to the reference strain which was constructed by Borgmeier and Meinhardt (unpublished).

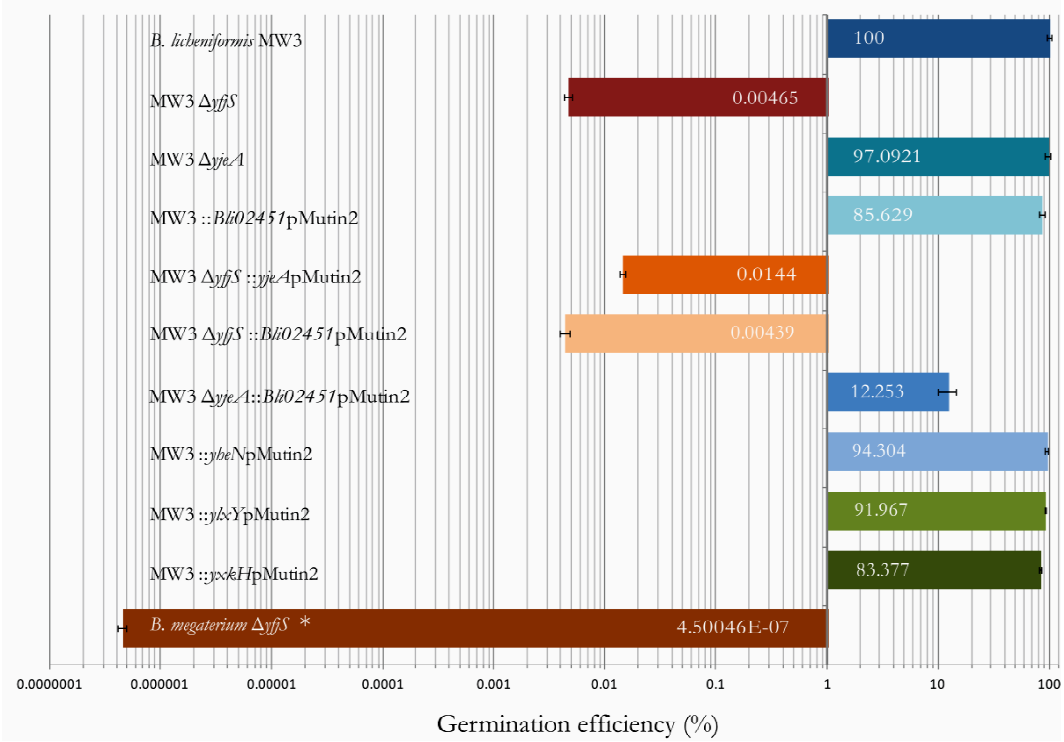



**Figure 3.15** *B. licheniformis*; Relative germination efficiencies (%) of knock out mutants compared to the wild type (100%) and a Δ*yfjS* deletion mutant of *B. megaterium***.** Mean data and standard deviations were calculated from three biological replicates. The asterisk (*) refers to a *B. megaterium* strain constructed by Borgmeier and Meinhardt (unpublished). Strain designation as in Figure 3.14.

# 4. DISCUSSION

## 4.1. Generation of mutants and strain development

Any genetic knock-out involves either the deletion of the entire or at least a part of the corresponding open reading frames or, alternatively, the disruption of the respective gene by integration of a selectable marker. Both strategies were employed in this study.

From the seven genes of *B. licheniformis* that were initially suspected to encode proteins with a possible polysaccharide deacetylase activity, 6 were chosen for inactivation; *ybaN*, a *pdaB* homolog of *B. subtilis* was excluded from our work, since it was not considered to confer an effect on spore germination because of the information available from bioinformatic databases (NCBI, Combrex and CAZY, see the Result chapter for details) and because of the work on *pdaB* of *B. subtilis* (Fukushima *et al.* 2004, 2005) and *B. thuringiensis* (Hu *et al.* 2006). All of the data made highly unlikely that *ybaN* and its protein product is involved in spore germination, however, the latter became disputable soon after finishing the experiments, see the discussion (4.2 Phenotype and Germination).

Although clean deletions are preferred in strains designed for industrial use, the generation of such mutants is rather time consuming due to the routine lack of counterselection possibilities in industrial microorganisms.

Only quite recently, the adaptation of a counterselection strategy based on the uracil salvage pathway (developed for *B. subtilis*, Fabret *et al.* 2002) was adopted for *B. megaterium* and *B. licheniformis* (Borgmeier *et al.* 2012). The system makes use of a mutant with a deleted *upp*–gene (encoding the Uracil phosphoribosyl transferase), which normally enables the bacteria to use external uracil. But also 5-Fluoro-Uracil (5FU) is taken in, the latter being toxic for the cells. By using the wild type gene along with an antibiotic resistance as the selectable integration cassette in an *upp*-mutant, it is possible to screen for strains carrying the integration; when the resistance marker is subsequently lost again (by recombination), clean marker-free mutants can be selected as those strains grow on 5FU-containing media.

As such an *upp*-mutant was not available, the clean deletion mutant (i.e. Δ*yjeA*) was generated by a methodology that involved rather time-consuming repeated rounds of sub cultivations and PCR-screenings for enrichment of the deletion carrying cells, which, however, finally (after 5 months) sufficed for obtaining the respective mutant. In principle, the applied experimental procedure, followed the methodology previously applied in this laboratory for generating restriction negative mutants of *B. licheniformis* DSM 13 (Waschkau *et al.* 2008), as well as sporulation and DNA-repair mutants (Nahrstedt *et al.* 2005 and Waldeck *et al.* 2007).

However, while in the above mentioned publications the DNA was introduced via protoplast transformation, in this study the DNA-transfer for deleting *yjeA* was accomplished by a transconjugation procedure that was originally developed for *B. megaterium* (Richardt *et al.* 2010). At any rate, as the procedure for generating and screening the Δ*yjeA* mutant consumed such a long period of time, the necessity of applying a faster procedure became evident.

The integrative plasmid system pMutin2 (Vagner *et al.* 1998) was indeed faster, mainly because of the fact that the pMMcomK based efficient competence inducing method was developed for *B. licheniformis* (Hoffmann *et al.* 2010). Enforcedly, the construction of clean deletion mutants could no longer be followed. However, disruption mutants can help to identify whether the gene has the potential for safety services. In an industrial producer strain, the clean deletion as the prefered mutation can later be installed based on the knowledge acquired in such experiments.

The pMutin-plasmids as versatile genetic tools have widely been applied to generate disruption mutants mainly in *B. subtilis* (Perego, 1993) as well as for *B. licheniformis* for genetic competence analysis (Hoffmann *et al.* 2010) and for *degSU* operon analysis (Borgmeier *et al.* 2012) in this group. Although some ectopic integrational events leading to false positive clones may occur, it was

possible via induced competence to generate the single and double mutants as shown in the result section.

## 4.2. Phenotype and Germination

As the biotechnological objective was to identify genetic loci to be useful for construction of a biologically contained safety strain, the search for the candidates concentrated on genes presumably being active "late" in germination, as it was presumed that the PDA knock-out mutants (see the Results chapter) are only hit in spore germination. The above presumption was supported by findings that neither the growth on minimal and complex media nor the capability to produce and secrete extracellular enzymes was negatively influenced when the selected genes were mutated. Rather unexpectedly, however, there was only one candidate gene which is probably involved in germination, namely *yfjS*, a *pdaA* homolog of *B. subtilis*. The mutants of this gene and its double mutant derivatives (Δ*yfjS*, Δ*yfjS* ::*02451*pMutin2, and Δ*yfjS*::*yjeA*pMutin2) displayed different spore morphologies as the spores are phase–grey rather than phase–bright. Wittchen *et al.* (1998) also found a similar phenotype with respect to the microscopic picture of the knock-out mutant Δ*yqfD* in *B. megaterium*. The gene encodes spoIV (a YqfD homolog in *B. subtilis*), a protein which is functional during a late sporulation phase (stage IV) (Eichenberger *et al.* 2003).

Although the generated Δ*yfjS* mutants displays the microscopic phenotype as for the *B. megaterium* Δ*yqfD* mutant, only the latter is a totally sporulation defective mutant, the former (Δ*yfjS* mutant) and its derivatives are still able to form viable spores. Apparently, however, in most cells of the latter the sporulation cannot finalize and reveal mostly phase-grey spores (after 72 hours when grown in Schaeffer's sporulation medium, see Figure 3.13). Suprisingly, considering the germination efficiency, Δ*yfjS* still germinates, though poor, i.e. 0.00465, and Δ*yfjS*::*02451*pMutin2 has 0.0144 and Δ*yfjS*::*yjeA*pMutin2 has 0.00439 spore germination efficiencies. Such values show that the spores still have the ability to germinate, though in low numbers. On the other hand, as for *B. subtilis* (Fukushima *et al.* 2002), the *B. megaterium* Δ*yfjS* (Borgmeier and Meinhardt, unpublished) used as reference strain displays an extremely low germination efficiency (calculated 4.50046 x $10^{-7}$), which infact means that there was no germinating spore on the plates (Borgmeier and Meinhardt, pers. comm.).

The answer to the question why the spore germination of the knock-out mutant Δ*yfjS* (a *B. subtilis pdaA* homolog) in *B. licheniformis* is rather different when compared to its relatives *B. subtilis* and *B. megaterium* still remains obscure. Maybe one or several other gene products can complement the germination defect caused by Δ*yfjS* in *B. licheniformis* or *yfjS* in *B. licheniformis* has

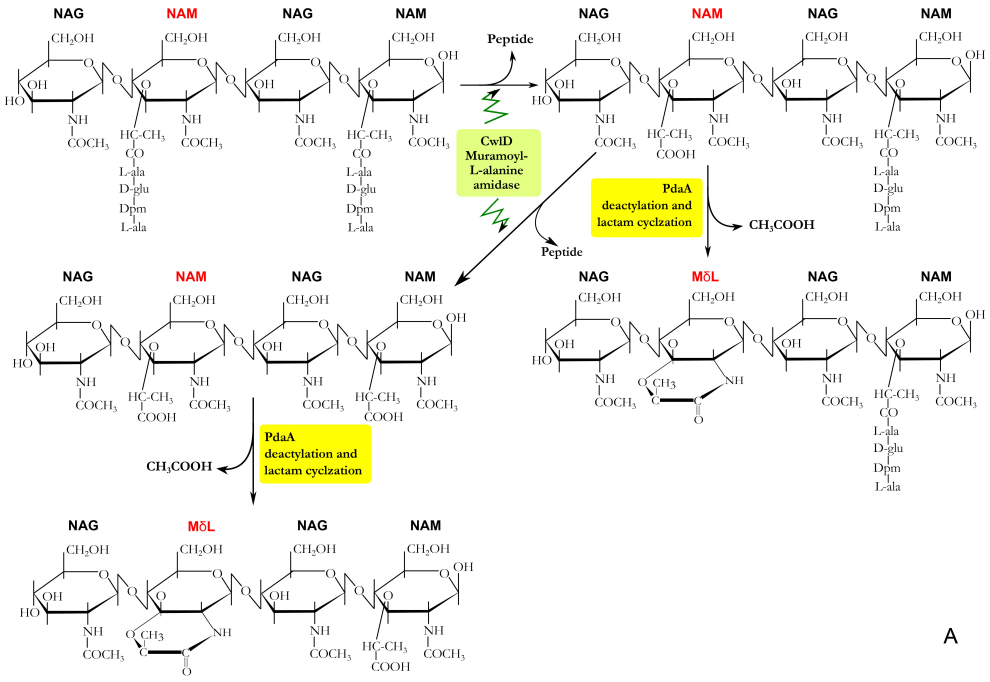
yet several unknown roles in cell development and sporulation especially in spore cortex formation and also in spore germination.

There is another PDA named YbaN (PdaB homolog in *B. subtilis*) encoded by *yba*N (*pdaB*) predicted to work in peptidoglycan formation during sporulation. In *B. subtilis*, PdaB is expressed in the mother cell and transported into the cortex, where it acts in the morphological change to bright spores in the late stages of sporulation. Thus, *B. subtilis*, PdaB is suggested to act in cortex synthesis (Eichenberger *et al.* 2003, Fukushima *et al.* 2004). Because of the prediction of a functionally similar YbaN in *B. licheniformis* (similar to PdaB in *B. subtilis*) it is conceivable that YbaN may display a redundant function which allows for the recovery or compensation of functional inactive YfjS during spore cortex formation; possibly, this protein is also involved in germination along with other spore cortex lytic enzymes. The presence of YbaN would it make possible to restore recognition of Muramic acids lacking δ–Lactam during the late stages of germination, as YbaN recognize N-acetyl-muramic acid (NAM) which – by the loss of *yfjS* – failed to be converted to Muramic–δ–Lactam (MδL). Another explanation for the experimental findings may be offered by CwlD, a germination-specific N-acetylmuramoyl-L-alanine amidase (peptidoglycan amidase, Veith *et al.* 2004). CwlD in *B. subtilis* was originally considered to be a sporulation and germination protein (Eichenberger *et al.* 2003), however, in the mean time, the gene is likely to act only during spore

formation (Silvaggi *et al.* 2004, Fukushima *et al.* 2004). However, this does not a priori exclude a different role in *B. licheniformis* as there are proven differences between the species concerning competence regulation and –proteins (Hoffmann *et al.* 2010). Another supporting work in *B. subtilis*, also suggested that some amount of solely CwlD which is produced in the forespore was enough to enable germination at low rate (Gilmore *et al.* 2004). Hence, it might well be, that CwlD is active in spore germination in *B. licheniformis*. Another candidate gene to explain germination in a Δ*yfjS* genetic background is *cwlJ* (*BLi00345*) (Veith et al. 2004). In *Bacillus* species, CwlJ is not only a spore cortex lytic enzyme (SCLE) which works along with SleB (See Figure 1.9 in the previous chapter), but also functions as a sporulation specific enzyme. The enzyme is expressed in the mother cell under control of $\sigma^E$ and then transported into the forespore to modify the spore peptidoglycan structure (Ishikawa *et al.* 1998, Heffron *et al.* 2009). In the other part of the sporulation cycle (see Figure 1.5 in Introduction), during spore cortex degradation, this enzyme is associated with and classified as a spore coat protein, and functionally acting as a transglycosylase and muramidase to modify peptidoglycan strands in the spore cortex. The enzyme is rather inefficient but measurable in its action on cortex material (muropetides) (Popham *et al.* 2012). Those dual functions, spore peptidogylcan modification during sporulation and degradation of peptidoglycan during spore cortex degradation, this enzyme is supposingly not only involved in subtituting the missing YfjS for

finalization of the spore cortex formation but also allowing the degradation of uncompleted (lacking Muramic-δ-lactam) spore cortex structure, and thus, germination can still happen at a low rates.

Concerning the connection between the spore cortex formation and its degradation, Fukushima *et al.* (2002), Gilmore *et al.* (2004) and Popham *et al.* (2012) proposed a scheme and a reaction pathway involving enzymes for cortex formation as well as spore cortex lytic enzymes. Fukushima *et al.* (2002) proposed the involment of a novel gene *yjfS* (renamed as *pdaA*) in *B. subtilis*, which was suggested to function in the final reaction of the synthesis of Muramic–δ–Lactam (MδL) in the spore cortex, the key event for ensuring spore dormancy for long period of times. The chemical nature of the spore cortex is also most important for enabling recognition by the spore cortex lytic enzymes (SCLEs) such as SleB. During the formation of the spore cortex, PdaA (encoded by *pdaA*) works sequentially and simultaneously with CwlD, an enzyme belonging to the amidase family (See Figure 4.1).


NAG
NAM
Peptide
CwlD
Muramoyl-
L-alanine
amidase
PdaA
deactylation and
lactam cyclzation
CH3COOH
MδL
L-ala
D-glu
Dpm
L-ala
A

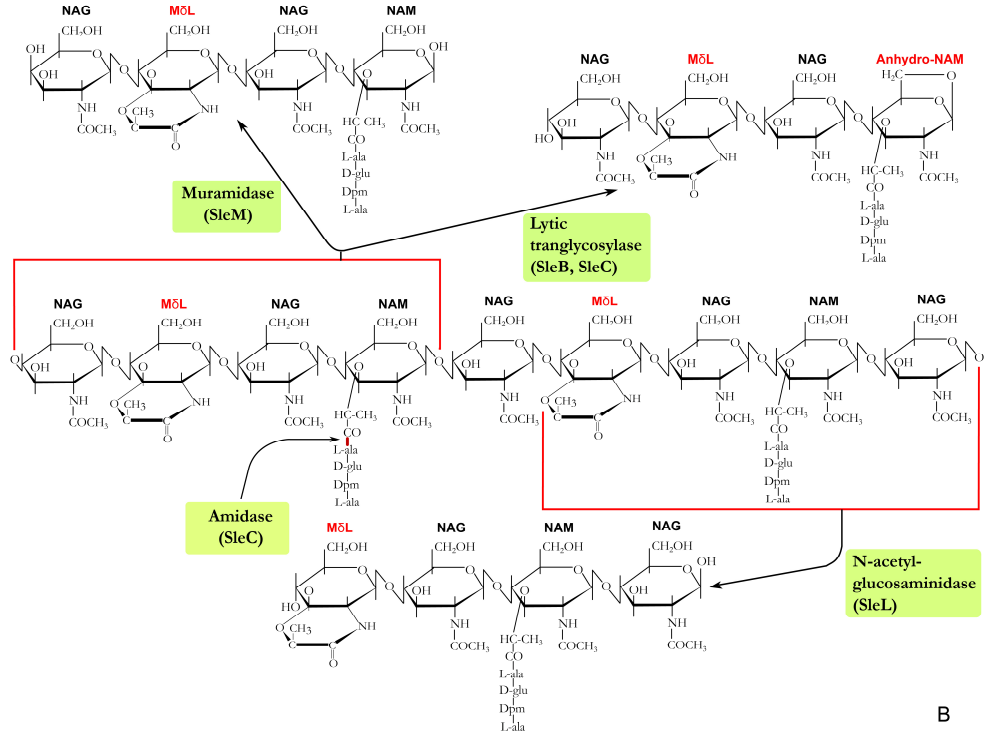

NAG
MδL
NAM
Anhydro-NAM
Muramidase
(SleM)
Lytic
tranglycosylase
(SleB, SleC)
Amidase
(SleC)
N-acetyl-
glucosaminidase
(SleL)
L-ala
D-glu
Dpm
L-ala
B

**Figure 4.1** Central mechanism for the formation of the Muramic–δ–Lactam ring of the endospore cortex peptidoglycan catalyzed by CwlD and PdaA (YfjS) (A), and degradation of the endospore cortex peptidoglycan (containing Muramic–δ–Lactam ring) by several spore cortex lytic enzymes (SCLEs) (B), (Adapted from Gilmore *et al.* 2004 and Popham *et al.* 2012). Recognition and degradation of the Muramic–δ–Lactam ring (which was produced during sporulation by PdaA), is the main process during germination. NAM refers to N-acetyl- muramic acid, NAG is N-acetyl glucosamine and MδL refers to Muramic–δ–Lactam.

The germination assays for mutants hit in *yjeA, yheN, yheN2* (*Bli02451*), *ylxY* and *yxkH* revealed no influence on germination, both, in single and double mutants of *yjeA* and *yheN2* (*Bli02451*). Considering the *yjeA* mutant, there are several previous investigations. Bisicchia *et* al. (2007) and Dubrac *et al.* (2008) discuss the cell wall metabolism function of *yjeA* of *B. subtilis* and noted that YjeA modifies the cell wall peptidoglycan thereby making it susceptible to lysozyme attack. Supported by proteome analysis (Voigt *et al.* 2006, 2009), YjeA was presumed to be an intracellular enzyme rather than extracellular. Kobayashi *et al.* (2012) concluded that the enzyme in *B. subtilis* is involved in cell wall metabolism and cell wall modification and able to deacetylase *N*-acetylmuramic acid. The ability to modify the peptidoglycan, leading to deacetylation of *N*-acetylmuramic acid, gives us a clue how YjeA might be

involved in functional recovery of lacking YfjS in the forespore. Assumingly, the protein is also produced separately in the spore core at early times of sporulation, making it possible to restore the lacking YfjS during muramic–δ–lactam synthesis, although the MδL is produced less efficiently.

Only little is known about above genes in *Bacillus*, (*yheN, yheN2*, *ylxY*, and *yxkH*). The only proteomic analysis (Voigt *et al.* 2006, 2009) reported the presence of YheN in the late stationary phase of *B. licheniformis* in the secretome. Recently, Traag *et al.* (2013) proposed *ylxY* as a novel sporulation gene in *B. subtilis*. They found a phenomenon in which the expression of YlxY appears only during sporulation. The expression was not seen during normal growth. However, experimental data is not available for *yheN2* and *yxkH* at present.

Thus, to explore further the function of the genes in more depth additional experiments including bioinformatic and molecular work are necessary.

With respect to colony morphology there was a special phenotype of the *yheN2* (*Bli02451*) mutants (see Figure 3.9 in Results). *B. licheniformis* MW3 ::*02451*pMutin2 and *B. licheniformis* MW3 Δ*yfjS* ::*02451*pMutin2 displayed a rather different colony morphology than the other strains.

Advanced searches performed with the protein (YheN2) by applying SignalP, TargetP, and Psortb (Petersen *et al.* 2011, Emanuelsson *et al.* 2007) revealed

that YheN2 has a length of 276 amino acids and the value for a signal peptide is 0.450, thus, the probability for a signal peptide structure is rather low (max. C = 0.222, max. Y = 0.386, max. S = 0.866). Another analysis using TMHMM (Trans membrane Hidden Markov Model, Erik *et al.* 1998 and Krogh *et al.* 2001), revealed that the protein is located inside rather than outside of the membrane. Physicochemical parameter analysis using SOSUI (developed by Hirokawa *et al.*, 1998), suggested that YheN2 is a membrane protein with only a single transmembrane helix (see Figure 4.2).

Protein transmembrane analysis applied for all of the PDAs of *B. licheniformis*, revealed that only YheN and YheN2 are membrane proteins. However, concerning the colony morphology there is no effect for the knock-out mutant *B. licheniformis*::*yheN*pMutin2. As mentioned Voigt *et al.* (2006), found YheN during the late stationary phase in the secretome (1 hour after entering stationary growth) when cells start to lyse. Such finding agree with a presumed transmembrane protein. Above mutants show different colony morphologies when the respective genes were knocked-out, suggesting that both proteins have different roles in the mother cell structure development. Anyway, the answer to the question why the *yheN* gene knock-out (*B. licheniformis* MW3::*yheN*pMutin2) did not display the same colony morphology as for *B. licheniformis* MW3::*02451*pMutin2 (*yheN2* knock-out mutant) remains obscure at present.

Taken into consideration the spore phenotype (phase-bright) and the germination assay in which no effect was found on spore germination for the respective knock-out strains, it is assumed that YheN2 and YheN are located in the mother cell rather than in the forespore. However, such assumption needs further experimental proof.

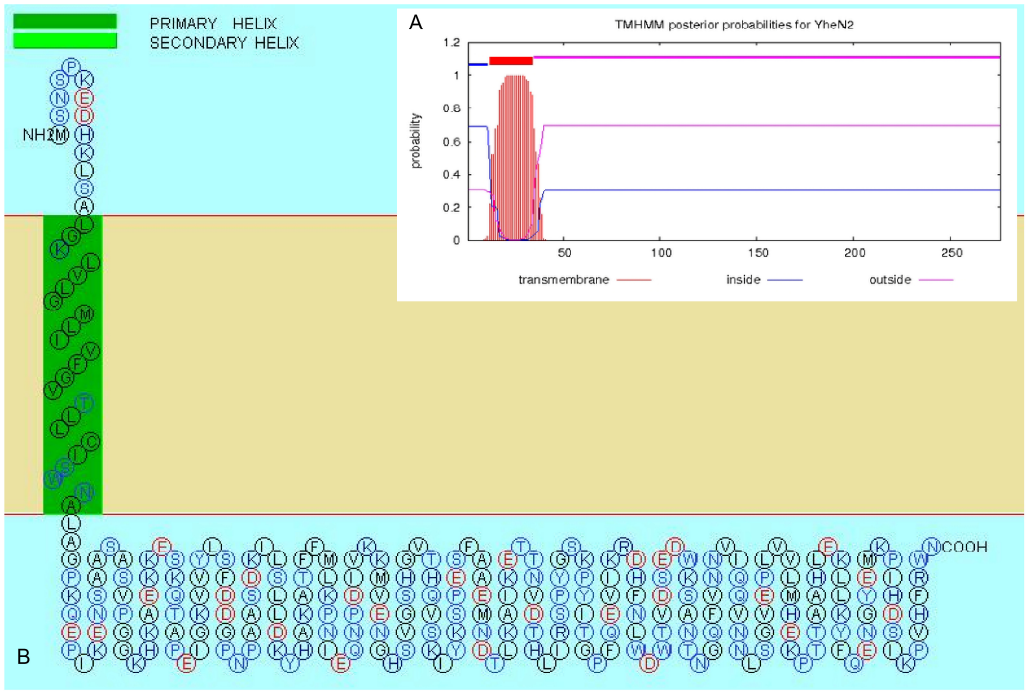



**Figure 4.2** Transmembrane predicted analysis of YheN2 of *B. licheniformis* DSM 13. A) TMHMM (transmembrane Hidden Markov Model) prediction of YheN2 location in the cell; B) Discriminating transmembrane helical protein and predicting membrane-spanning segment of YheN2 by SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html).

At any rate, only two genes out of the six PDA encoding loci – upon knock-out – influenced either colony morphology (*yheN2*, originally *Bli02451*), or germination (*yfjS*) of *B. licheniformis* MW3. As none as the mutant strains were affected in growth (in both complex medium and minimal medium) as well as enzyme production (exemplified for glucanase, amylase and protease), it is suggested that the knock-out mutant *B. licheniformis* Δ*yfjS* is the first choice candidate for use as a biological containment device in industrial large scale fermentation for extracellular enzyme production.

# 5. OUTLOOK

Members of the genus *Bacillus* are major workhorses in the biotech-industry. However, the endospores routinely produced by the *Bacilli* constitute a major challenge in large scale fermentation. Out of the six PDA encoding genes experimentally inactivated in this study exclusively *yfjS* is a suitable target to create a strain with a greatly reduced spore germination capacity.

However, there are still a number of questions that need to be experimentally addressed. It is challenging and most interesting why *B. licheniformis* Δ*yfjS* behaves so differently when compared to the respective mutants of *B. subtilis* and *B. megaterium* (reference strain in this study).

The generation of further single and double knock-out mutants (e.g. in combination with *yba*N), the heterologous complementation with genes from *B. subtilis* and *B. megaterium*, and localization studies using gfp–reporter constructs may contribute to solve such problem.

With respect to the colony morphology, *B. licheniformis* MW3::*yheN2*pMutin2 and *B. licheniformis* MW3Δ*yfjS*::*yheN2*pMutin2 are different from the other mutants. Assumingly, the disruption of the genes cause damages in cell wall development, which agree with the predicted peptidoglycan modification activity of the encoded proteins. *B. licheniformis* MW3Δ*yjeA*::*yheN2*pMutin2 double mutant displayed no such colony morphology deviation. Apparently,

the *yjeA* gene-product and the *yheN* functioning during cell wall development interfere in a way that does not lead to change in the phenotype.

Localization studies (e.g. with GFP-fusion) of both gene products as well as complementary mutations can help to find out whether the colony phenotype in the double mutants is due to an interaction or due to other mutations that have concomitantly occurred during the experiments.

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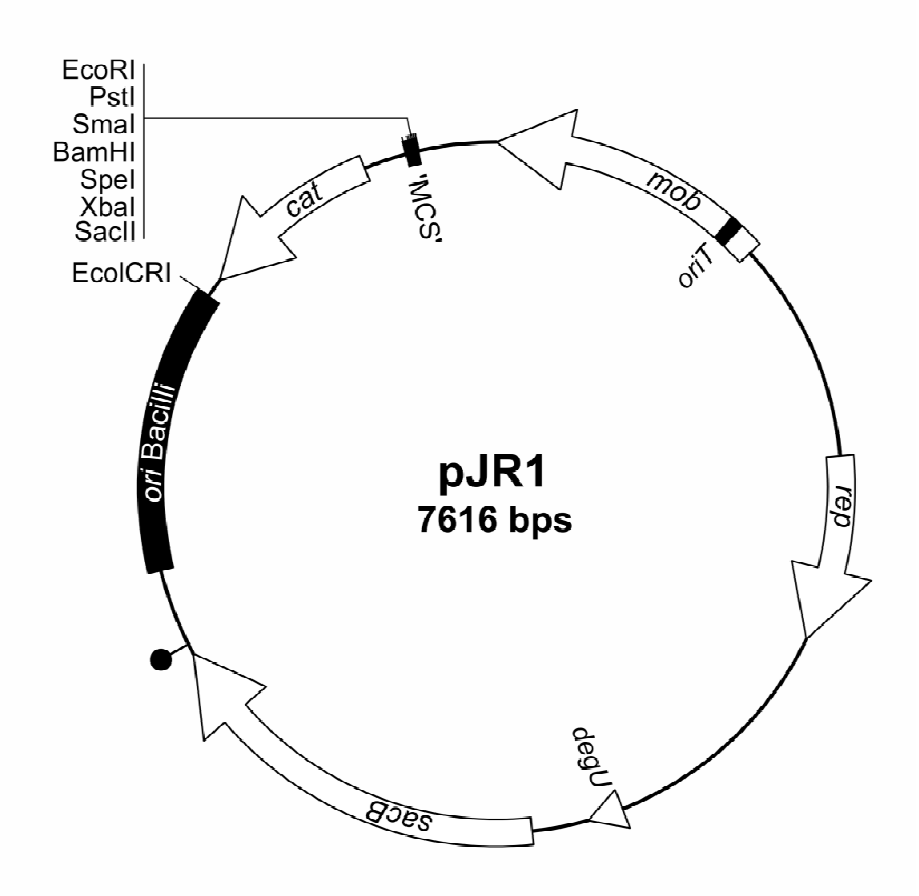



**Figure 6.1.** pJR1, schematic representation of the corresponding replicating temperature-sensitive plasmid according to Richardt *et al.* (2010). Open reading frames are depicted as arrows, the black hairpin refers to transcriptional terminator, and the black areas refers to the *Bacillus* temperature sensitive (ts) origin of replication and the origin of transfer (oriT) of *E. coli*. MCS denotes the multiple cloning site, applicable for the respective resctriction enzymes, *sacB* refers to the *B. subtilis* levansucrase encoding gene. The *mob* region code for the mobilization protein and *rep* encodes the Rep helicase of *E. coli*, and *cat* refers to a gene encoding chloramphenicol acetyltransferase.

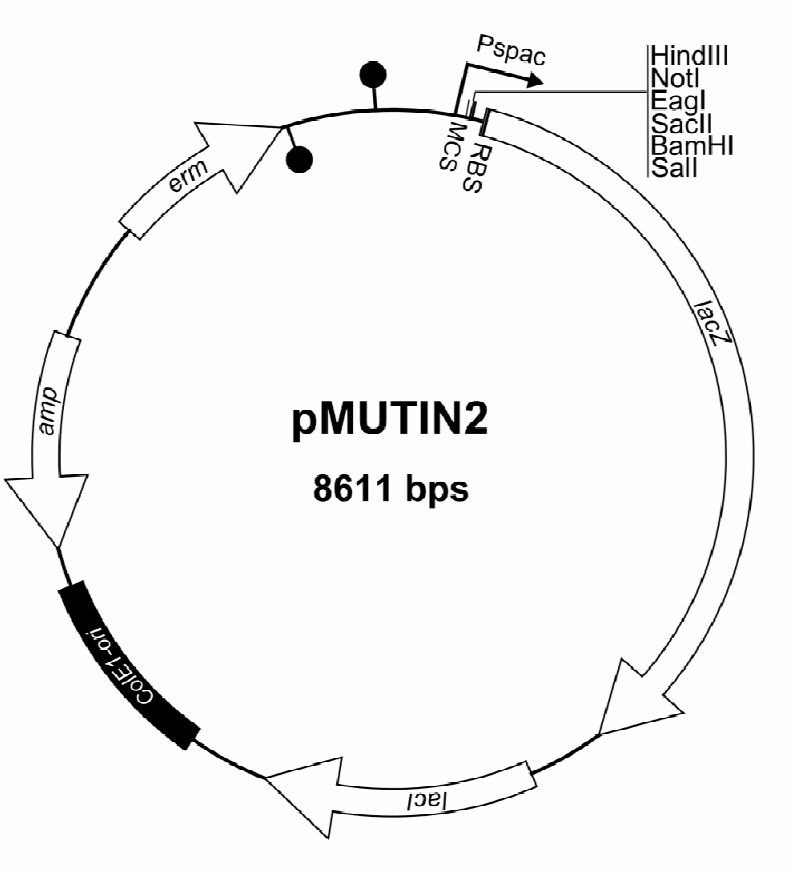



**Figure 6.2**. pMutin2, schematic representation of the integrative plasmid according to Vagner *et al.* (1998). Open reading frames are depicted as arrows. The black hairpins refer to transcriptional terminators. The right–angle–arrow flags the Pspac promoter and is denoted as such; the black box designated *ColE1* represents the part that ensures replication in *E. coli*; *erm* refers to the gene encoding Erythromycin resistance in *Bacillus*; *amp* denotes the gene encoding the Ampicillin resistance in *E. coli*; *lacZ* refers to the gene encoding the β-galactosidase and *lacI* is the gene coding for the Lac repressor; RBS denotes the ribosome binding site for the *lacZ* operon and MCS refers to the multiple cloning site.

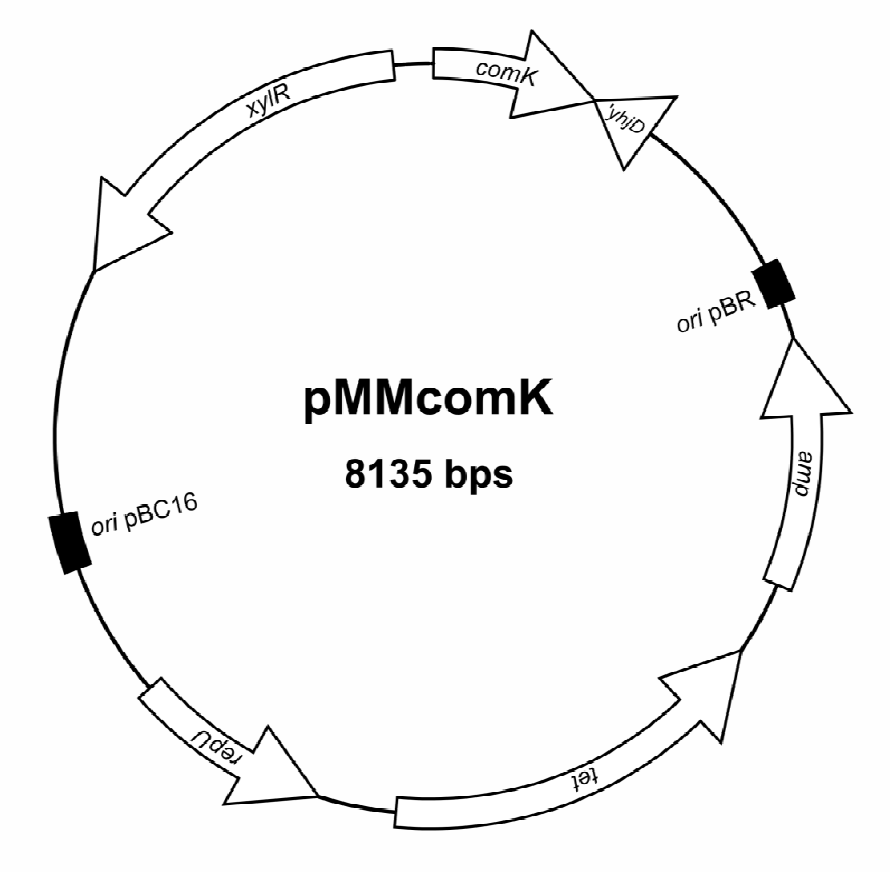


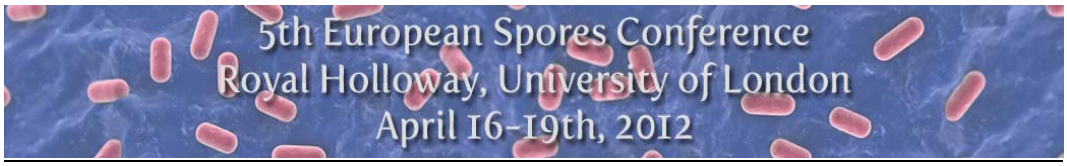

**Figure 6.3.** pMMcomK, a vector for xylose-inducible ComK expression according to Hoffmann *et al.* (2010). Open reading frames are depicted as arrows. Restriction enzymes are abbreviated. *comK* encodes the main activator for natural competence; *'yhjD*, gene downstream of *comK*, encoding part of a hypothetical protein; *xylR* refers to xylose repressor gene; *repU* depicts the replication protein encoding gene; *tet* refers to Tetracycline resistance gene (*Bacillus*); *amp* refers to Ampicillin resistance gene (*E. coli*); *ori* pBC16 depicts the *Bacillus* origin of replication; *ori* pBR refers to the ColE1 origin of replication.

**Scientific meetings attended**

1. The Annual Meeting of the German Microbiological Society (Vereinigung für Allgemeine und Angewandte Mikrobiologie /VAAM), March 8 – 11, 2009, Ruhr–Universität Bochum, Bochum, Germany.
   *Participant.*

2. The Annual Meeting of the German Microbiological Society (Vereinigung für Allgemeine und Angewandte Mikrobiologie /VAAM), March 28 – 31, 2010, Convention Center Hannover – Deutsche Messe , Hannover, Germany.
   *Participant.*

3. 5th European Spores Conference, April 16 – 19, 2012, Royal Holloway, University of London, London, Great Britain.
   *Poster presentation* (see next page for abstract).

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## The impact of polysaccharide deacetylase (PDA) encoding genes in *Bacillus licheniformis* on spore germination

In the Gram-positive model organism *Bacillus subtilis* PDA encoding genes, such as *yfjS* (*pdaA*) have a drastic impact on spore germination.
To check the possible role of PDA encoding genes on spore germination in the biotechnologically most important *B. licheniformis*, we generated several knock out mutants. We addressed *pdaA (yfjS)*, *yjeA*, *Bli02451*, *yheN, ylxY* and *yxkH*.
The generation of the mutants along with drop dilution assays addressing sporulation efficiency will be presented and compared to the respective *B. subtilis* mutants. As we observed a limited impact of PDAs on germination efficiency other yet unknown factors are likely to be involved in spore germination in *B. licheniformis*