Functional characterization of the germination receptors in the spore-forming species *Bacillus licheniformis*

Philosophiae Doctor (PhD) Thesis

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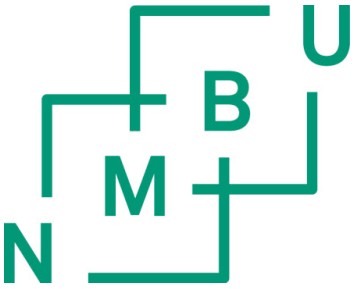

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Sincerely,

Kristina Borch-Pedersen
September, 2017

Abbreviations

ACES	N-(2-acetamido)-2-aminoethanesulfonic acid
AGFK	L-asparagine, D-glucose, D-fructose, K^+
ATCC	American type culture collection
CFU	Colony-forming unit
CLEs	Cortex-lytic enzymes
CaDPA	Calcium dipicolinic acid (pyridine-2,6-dicarboxylic acid)
DSM	Deutsche Sammlung von Mikroorganismen
FCM	Flow cytometry
FDA	U.S. Food and Drug Administration
GRs	Germination receptors
HP	High pressure
HPP	High-pressure processing
HPTS	High-pressure thermal sterilization
IM	Inner membrane (referring to spore IM)
mHP	Moderately high pressure (in this thesis: 150 MPa)
MPa	Megapascal (1MPa = 10 bar = 9.869 atm)
OM	Outer membrane (referring to spore OM)
PATS	Pressure-assisted thermal sterilization
PG	Peptidoglycan
PI	Propidium iodide
SASP	Small, acid-soluble proteins
vHP	Very high pressure (in this thesis: 550 MPa)
UHT	Ultra-high temperature

Summary

The food industry experience extensive losses worldwide due to food spoilage caused by microbial activity. Endospore-forming bacteria, such as members of the genera *Bacillus* and *Clostridium*, are becoming increasingly problematic to the food industry due to a growing consumer demand for mildly processed foods with a fresh quality. Bacterial endospores (spores) exist in a morphological unique life state in which they are extremely resistant to environmental insults (e.g. nutrient deprivation, heat, irradiation and harmful chemicals). Therefore contaminating spores will readily survive food-processing temperatures of ≤ 100 °C, which kills most vegetative bacteria. The spore-former *Bacillus licheniformis* is commonly isolated from both ingredients and the finished food products, and because of its high capacity to produce and excrete enzymes it is an important spoilage bacterium. It has also been reported to cause food poisoning related to the consumption of meat and vegetable dishes, rice dishes and infant formula.

Spore germination is the essential step that allow spore-related food spoilage or disease, and coincidentally, it can be the key to spore elimination. There is currently little knowledge about spore germination in *B. licheniformis* despite its economic importance. The main goal of this thesis work was therefore to expand our understanding of how germination is triggered in *B. licheniformis* spores. Germination is generally triggered by the activation of germination receptors present in the inner membrane of the spore by specific nutrient molecules (often amino acids). This will initiate the cascade of events transforming the spore into a vegetative cell. The germination receptors are present in most endospore-forming bacteria, and are encoded by closely related *gerA* family genes. The work described in this thesis characterize the germination receptors found in *B. licheniformis* ATCC14580/DSM13 encoded by the *gerA*, *gerK*, *ynd* operons and the *yndF2* orphan gene. By screening 20 different L-amino acids, we showed that L-alanine, L-cysteine or L-valine induced the strongest germination response in *B. licheniformis* spores. We found that both the GerA and Ynd germination receptors were necessary to induce an effective germination response to the tested L-amino acids, indicating cooperative interaction between these receptors. The GerA germination receptor was found to be essential for L-amino acid-induced germination, as any disruption in this operon completely abolished germination. The Ynd germination receptor contributed substantially to germination but was not essential, the germination efficiency was reduced by 40-60 % when the *ynd* operon was disrupted. GerK did not have a function in L-amino acid-induced

germination, however, this receptor was necessary for the weak glucose-induced germination we observed.

A closer study of the Ynd receptor was initiated, as the *ynd* operon differ from the most common, tricistronic operon organization of the *gerA* family genes, encoding the receptor's A-, B- and C subunit. The *ynd* operon in *B. licheniformis* is pentacistronic, encoding the A-, B_{3}-, B_{2}-, C- and B_{1} subunit, respectively, and this atypical organization of the Ynd operon is highly conserved in *B. licheniformis*. In this study, we showed that deleting of one or two of the B subunits rendered the receptor non-functional. However, the Ynd receptor was capable of functioning when all three B subunits were deleted, demonstrating that the A subunit was sufficient for the cooperative interaction between Ynd and GerA.

High pressures (80 MPa–600 MPa ≈ 790–5900 atm) induce spore germination in *Bacillus* spores. Moderately high pressures (80-300 MPa) induce germination by activating the germination receptors, however, different germination receptors display different high-pressure responsiveness. In *B. licheniformis* spores, the Ynd germination receptor contributed the most to high pressure-induced germination, unlike what was observed for nutrient-induced germination where GerA germination receptor seemed to be dispensable for high-pressure-induced germination. Pressure above 300 MPa are of commercial interest, and are known to trigger germination independent of the germination receptors. When *B. licheniformis* spores were exposed to very high pressures (550 MPa), the ability of the pressure to induce germination was temperature dependent. That is, only increasing the temperature to 60°C would result in pressure-induced spore germination and inactivation.

Sammendrag

På verdensbasis må en stor andel av den industriproduserte maten kastes på grunn av kvalitetsforringelse som følge av mikrobiell aktivitet. Dagens forbrukere ønsker i større grad matvarer som fremstår fersk og har lite tilsetningsstoffer. Dette fører til at såkalte sporedannere utgjør en økende problem for matindustrien. Sporedannere er bakterier, særlig fra slektene *Bacillus* og *Clostridium*, som danner endosporer (sporer). Sporene befinner seg i et unikt morfologisk stadium hvor de er ekstremt motstandsdyktige mot miljømessige utfordringer (f. eks næringsmangel, varme, stråling og kjemikalier). De er svært utbredt i naturen og finnes i store mengder i jord, derfor vil de enkelt kunne forurense matvarer og produksjonsutstyr. De aller fleste vegetative bakterieceller vil dø under mildere prosesseringsbetingelser med temperaturer ≤ 100 °C, men sporene vil overleve, vokse og føre til forringelse av matvarene og i verste fall matforgiftning. *Bacillus licheniformis* er en art som ofte finnes i råvarer og ferdige produkter. På grunn av artens evne til å danne store mengder enzymer, er *B. licheniformis* ofte knyttet til kvalitetsforringelse av matvarer. Arten har også vært knyttet til tilfeller av matforgiftning etter inntak av blant annet kjøtt og grønnsaksretter, risretter og morsmelkerstatning.

Spiring (germinering) av sporene er helt essensielt for at sporene skal kunne forårsake kvalitetsforringelse av matvarer eller matbåren sykdom. I denne prosessen entrer sporene ut av det motstandsdyktige stadiet, og vil igjen bli sårbare for varme og andre påkjenninger. Hovedmålet i dette doktorgradsarbeidet var å beskrive hvordan germinering av *B. licheniformis* sporer initieres, ettersom det fra før av finnes svært begrenset kunnskap om denne artens germinering. Germinering trigges vanligvis når germineringsreseptorer i sporens indre membran aktiveres av næringsstoffer (oftest aminosyrer), og det igangsettes en prosess som omdanner sporen til en bakteriecelle igjen. Disse germineringsreseptorene finnes i nesten alle sporedannere, og kodes av nært beslektede gener tilhørende *gerA* familien. I våre studier har vi undersøkt funksjonen til germineringsreseptorene hvis gener finnes i genomet til *B. licheniformis* stamme ATCC14580/DSM13. Germineringsreseptorene kodes av operonene *gerA*, *gerK* og *ynd*, i tillegg til enkeltgenet *yndF2*. Aminosyrene L-alanin, L-cystein og L-valin trigger en effektiv germineringsrespons hos *B. licheniformis*, og våre funn viser at denne responsen er avhengig av at de to germineringsreseptorene GerA og Ynd er intakte i sporene, noe som tyder på at et samarbeid mellom disse to reseptorene. GerA reseptoren var den viktigste germineringsreseptoren for germinering i nærvær av aminosyrer ettersom sporene mistet

evnen til å germinere dersom vi slo ut *gerA* operonet. Ynd reseptoren var ikke essensiell for at sporene skulle germinere, men var viktig for at germineringen skulle være optimal. GerK reseptoren hadde ingen funksjon i germineringsresponsen til aminosyrer, men var derimot viktig for germineringsresponsen i nærvær av glukose.

Siden Ynd reseptoren viser en atypisk oppbygning sammenlignet med andre germineringsreseptorer, igangsatte vi videre studier av denne reseptoren. De fleste germineringsreseptorer er bygd opp av tre komponenter, nemlig A, B og C subenhetene. Ynd reseptoren derimot, kodes av *ynd* operonet som inneholder tre B subenhetgenene i tillegg til A og C subenhetgenene. Når *ynd* operonet manglet en eller to av de tre B subenhetgenene, opphører funksjonen av Ynd helt. Om alle tre B subenhetene mangler derimot, vil A subenheten fungere i samarbeid med GerA reseptoren.

Høytrykk mellom 80 og 600 MPa (790–5900 atm) kan fremkalle sporegerminering i *Bacillus* arter. Moderat høytrykk (150 MPa) vil i likhet med næringsindusert germinering, aktivere germineringsreseptorene. Ulike germineringsreseptorer utviser ofte forskjellige grader av trykkfølsomhet. I motsetning til hva som ble observert i forsøkene med næringsindusert germinering, viste det seg at Ynd reseptoren var den mest trykkfølsomme reseptoren og bidro mest til høytrykksindusert germinering av sporer. I industriell sammenheng er høytrykk over 500 MPa interessante, og kan indusere en germineringsrespons i sporer som skjer uavhengig av germineringsreseptorene. I våre forsøk kunne *B. licheniformis* sporer kun germinere under trykk på 550 MPa, om vi økte temperaturen til 60 °C. Disse betingelsene ga også en effektiv inaktivering av sporene.

List of papers

Paper I

The cooperative and interdependent role of GerA, GerK and Ynd in germination of *Bacillus licheniformis* spores

Kristina Borch-Pedersen, Toril Lindbäck, Elisabeth H. Madslien, Shani W. Kidd, Kristin O'Sullivan, Per Einar Granum and Marina Aspholm.

Applied and Environmental Microbiology (2016) 82, pp. 4279-87

Correction for Borch-Pedersen *et al*,"The cooperative and interdependent role of GerA, GerK and Ynd in germination of *Bacillus licheniformis* spores"

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Paper II

The effects of high pressure on *Bacillus licheniformis* spore germination and inactivation

Kristina Borch-Pedersen, Hilde Mellegård , Kai Reineke, Preben Boysen, Robert Sevenich, Toril Lindbäck and Marina Aspholm

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Applied and Environmental Microbiology: Accepted

Paper III

Dissecting the cooperative interaction between the GerA and Ynd germination receptors in *B. licheniformis* spore germination

Toril Lindbäck, Kristina Borch-Pedersen, Kristin O'Sullivan, Siri Fjellheim, Inger-Helene Aardal, Per Einar Granum and Marina Aspholm.

Manuscript

1. Introduction

1.1 Endospore-forming bacteria

The ability to enter a state of low metabolism for a period of time in order to persist under unfavorable conditions is common across the bacterial kingdom (Rittershaus et al., 2013). Dormancy is regulated by environmental signals, and is a strategy that facilitates survival during periods of nutrient depravation and during exposure to environmental stressors such as high temperature, irradiation, host-defenses and antibiotics (Dworkin and Shah, 2010). Bacterial endospores (spores) are capable of surviving an array of environmental stressors that would normally kill vegetative cells, and are among the most resistant dormant forms known (Setlow, 2003, 2006, 2014b). The most prominent spore formers belong to the genera *Bacillus* and *Clostridium,* which members range from harmless soil bacteria to dangerous pathogens such as *Bacillus anthracis*, the causative agent of anthrax that has plagued mankind for centuries (Torred et al., 2012), the agents of tetanus (*Clostridium tetanii*), botulism (*Clostridium botulinum*) and food-borne or antibiotic-induced gastrointestinal illness (*Clostridium perfringens*, *Bacillus cereus* or *Clostridium difficile*, respectively) (Logan and Vos, 2015).

Several members of the bacterial phylum Firmicutes (low G+C Gram-positive bacteria) are able to produce spores. Bacterial spores were first described in 1870s independently by Ferdinand Cohn (Cohn, 1876) and Robert Koch (Koch, 1876). They are formed inside the mother cell in a process called sporulation, which is the best studied cell differentiation in prokaryotes (further elaborated upon in section 1.2.2). Sporulation likely evolved about 2.5-3 billion years ago, before the rise in oxygen levels about 2.3 billion years ago, in a common ancestor of the Clostridia and Bacilli classes (Battistuzzi et al., 2004). The ability to undergo sporulation is conserved in certain members of the classes Bacilli, Clostridia and Negativicutes, but seem to have been lost in several phylogenetic lines within these classes, such as the staphylococci, streptococci and lactobacilli (Galperin, 2013). The loss of this ability is likely a consequence of the numerous gene products and complex regulatory pathways involved in sporulation that can be disrupted by mutation, and due to the high energy cost of sporulation (Battistuzzi et al., 2004; Galperin et al., 2012). Spore formers are ubiquitously distributed in nature and are present in large numbers in soil (Logan and Halket, 2011). Spores can remain in the dormant spore state for very long periods of time, and reports claim to have revived spores after isolation from the

abdominal content of extinct bees preserved in 25-45 million-years-old amber (Cano and Borucki, 1995), and from a 250 million-years-old salt crystal (Vreeland et al., 2000).

Spore formers are of great concern to the food industry, as their perseverance and omnipresence allow them to readily contaminate food and survive common food-processing methods (Andersson et al., 1995). Conventional thermal food-processing strategies kill most vegetative bacteria while spores survive. Subsequently, the spores can germinate, resulting in growth of vegetative cells, which in turn can result in food spoilage and sometimes food-borne disease. The spores can remain in the food-production environments for long periods of time, and eradication is difficult.

The main focus of this thesis work is the spore-forming species *Bacillus licheniformis* (presented in section 1.6). The following sections will summarize relevant literature available on *Bacillus*.

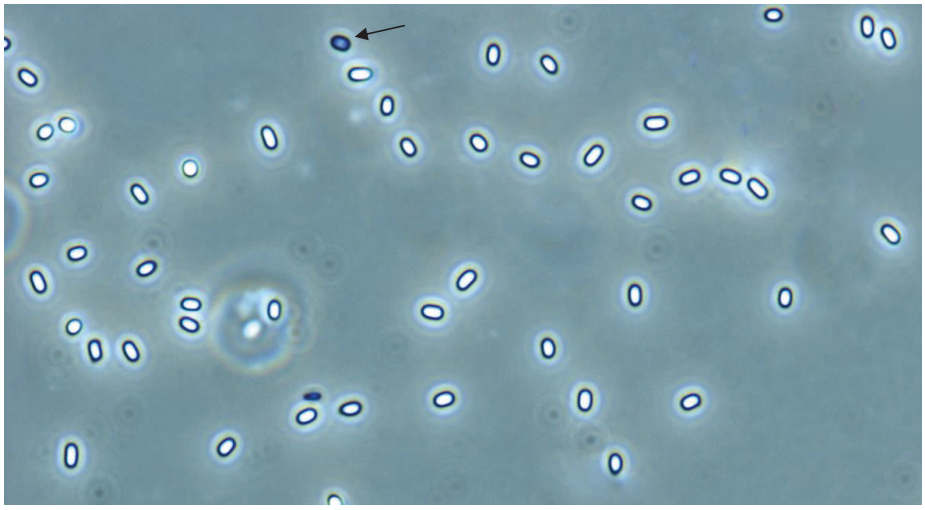

Fig. 1: Bacterial spores. Phase-contrast micrograph showing oval and refractive structures which are spores of the *B. licheniformis* strain MW3. Spores lose their phase-bright appearance upon germination, and will then turn phase-dark (arrow). Photo: Kristina Borch-Pedersen, 2014.

1.2 The *Bacillus* genus

Bacillus is a diverse genus comprised of over 300 species at the time of writing (LPSN, www.bacterio.net, accessed 08.2017). The *Bacillus* genus was established by Ferdinand Cohn (Cohn 1872), and originally described aerobic, spore-forming bacilli. Today it

encompasses endospore-forming, rod-shaped (although *Bacillus saliphilus* may be strictly coccoid), aerobic, facultative or strictly anaerobic bacteria that stain Gram-positive to Gram-variable (sometimes even Gram-negative) (Logan and Halket, 2011; Logan and Vos, 2015). Members of the *Bacillus* species are ubiquitously distributed in the environment and soil has traditionally been considered to be the main habitat for most *Bacillus* species. They excrete a wide range of degradative enzymes consistent with a saprophytic lifestyle. However, the intestinal tract of invertebrates and mammals has also been suggested as an important habitat for *Bacillus* species (Hong et al., 2009).

The *Bacillus* genus contains two commonly referenced, informal groupings with significant economical and clinical importance; the *B. subtilis* group and the *B. cereus* group. The *B. subtilis* group includes the closely related species *B. subtilis*, *B. licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus mojavensis*, *Bacillus atropheus*, *Bacillus vallismortis*, *Bacillus sonorensis* and *Bacillus pumilus* (Fritze, 2004). *B. subtilis* is the model organism for a number of cellular processes, and much of the knowledge we have about spore formation and germination comes from the studies of the *B. subtilis* strain 168 and its derivative strains.

The *B. cereus* group (*Bacillus cereus sensu lato*) includes the eight closely related species; *B. cereus* (*sensu stricto*), *B. anthracis*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bacillus mycoides*, *Bacillus pseudomycoides* (Fritze, 2004), *Bacillus toyonensis* (Jiménez et al., 2013) and *Bacillus cytotoxicus* (Guinebretière et al., 2013). The members of this group are of great importance to the food industry, human health and agriculture. The *B. cereus* group contain the two important pathogens *B. cereus* causing food poisoning, and *B. anthracis* causing anthrax (Logan and Vos, 2015). *B. thuringiensis* is an insect pathogen, commercially utilized as a biopesticide in agriculture (Logan and Vos, 2015). Despite their phenotypical differences, *B. thuringiensis*, *B. cereus* and *B. anthracis* are so closely related that they can be considered pathotypes of the same species (Helgason et al., 2000).

There is a commercial interest for *Bacillus* species, especially for members of the *B. subtilis* group, due to their large capacity to produce hydrolytic enzymes such as amylases and proteinases (Priest, 1977; Schallmey et al., 2004; van Dijl and Hecker, 2013). Some species are also used for antibiotic production, such as *B. licheniformis* that produces the topical antibiotic Bacitracin (Katz and Demain, 1977). *Bacillus* probiotics are popular both in veterinary medicine and as dietary supplements for humans. The spores permit a long shelf life for the products, will survive the stomach barrier and are thought to

germinate in the lower intestine (Casula and Cutting, 2002; Cutting, 2011; Hong et al., 2005).

1.2.1 Relevance of *Bacillus* species in food spoilage and food-borne illness

Food spoilage

Microbial spoilage of food relies on the alteration of the sensory quality of a food product to the extent that it is unacceptable for consumption (Gram et al., 2002). This happens by the degradation of polymers, gas production, production of off-odors and flavors or by visible microbial growth in the products and results in reduced shelf-life and economical losses (Gram et al., 2002). Because of their ubiquitousness, *Bacillus* species can easily enter the food production chain at multiple stages; they can contaminate raw material, ingredients and production equipment through soil, feed, dust or fecal matter (Carlin, 2011). Conventional food processing strategies acts selectively towards heat-resistant, spore-forming bacteria, which can survive and later multiply in the products without competition. Spores can survive cooking at temperatures up to 100 °C for several minutes to hours (Berendsen et al., 2016a), and may germinate if conditions (i.e. temperature, a_w and pH) are favorable.

Bacillus species are of particular concern in dairy products because they readily contaminate milk via soiled udders, bedding or fecal matter and the pasteurization process selects for spore formers and reduces the competition from other species (Andersson et al., 1995). *B. cereus*, *B. weihenstephanensis* and *B. lichenformis* are the most prevalent *Bacillus* species present in raw milk (Crielly et al., 1994; Heyndrickx, 2011). Psychotropic *B. cereus* and *B. weihenstephanensis* grows in pasteurized milk at low temperatures (≥8°C) and decreases the shelf life by due to enzymatic activities that results in defects such as sweet curdling, coagulation and bitty cream (Gopal et al., 2015). Growth of *Bacillus* species such as *B. amyloliquefaciens* and *B. licheniformis* can also cause spoilage of bread, so called "ropiness" spoilage, under warm, moist conditions (Rosenkvist and Hansen, 1995; Sorokulova et al., 2003; Valerio et al., 2012). Low-acid canned food (pH > 4.5) can be spoiled during storage after thermal processing by thermophilic, highly heat-resistant spore formers (André et al., 2013).

Today, consumer preferences lean towards ready-to-eat, minimally processed foods (Heyndrickx, 2011). As the products are heat treated at temperatures ranging from 65-95°C, most spores will not be killed, but rather, activated for germination. If outgrowth and

proliferation is allowed (growth of psychotropic species, temperature abuse etc.), spoilage and food poisoning may occur.

Food poisoning

The most important member of the *Bacillus* genus in regards to food poisoning is *B. cereus. B. cereus* can cause two types of food poisoning; the emetic type and the diarrheal type (Logan, 2012; Stenfors Arnesen et al., 2008). The emetic type is an intoxication caused by the pre-formed toxin cereulide, a highly stable, ring-formed peptide synthetized by a non-ribosomal synthease encoded by the plasmid-borne cereulide synthase cluster (*ces*) (Agata et al., 1995; Ehling-Schulz et al., 2006; Ehling-Schulz et al., 2005). Symptoms occur 0.5-6 h after consumption and includes nausea and vomiting, sometimes accompanied by stomach cramps and fever, lasting for 6-24 h (Stenfors Arnesen et al., 2008). Although symptoms are often mild and self-limiting, fatal cases have been reported (Dierick et al., 2005; Mahler et al., 1997; Shiota et al., 2010). The diarrheal type is caused by an infection of *B. cereus* in the small intestine, causing watery diarrhea and abdominal pain and lasting between 12-24 h (Stenfors Arnesen et al., 2008). The three enterotoxins Nhe, CytK and Hbl are implicated in the diarrheal disease (Logan, 2012; Stenfors Arnesen et al., 2008). Both types of *B. cereus* food poisoning is associated with inadequate cooling of cooked food, and consumption after longer storage at temperatures above 8-12 °C. *Bacillus* species outside the *B. cereus* group, most commonly *B. subtilis*, *B. licheniformis* and *B. pumilus*, are sporadically associated with food poisoning (Logan, 2012). Symptoms differ depending on the species responsible, but usually include vomiting, stomach cramps and diarrhea. In addition, symptoms such as sweating, flushing sensations and headache has also been reported in *B. subtilis* and *B. pumilus* food-poisoning incidents and *B. pumilus* has been reported to cause dizziness (Kramer and Gilbert, 1989). Incubation periods for the three types of food poisoning range from 10 minutes to 14 hours with symptoms lasting for up to 24 hours (can be longer for *B. pumilus*), and the infectious dose is $>10^6$ bacilli (Kramer and Gilbert, 1989). Although the exact virulence factors are unknown, implicated toxins are most probably the cyclic lipopeptides lichenysin (*B. licheniformis*), surfactin and amylosin (*B. subtilis*) and pumilacidin (*B. pumilus*) (From et al., 2007; Mikkola et al., 2000).

1.3 The structure and formation of the endospore

1.3.1 The structure and resistance of the endospore

The key to the spores´ unique resistance properties lies in their complex and multilayered structure (**Fig 2**). The spore core corresponds to the cell protoplast, and contains the genome, proteins, ribosomes and RNA. The core is highly dehydrated, with a water content corresponding to 25 - 55 % of the cores wet weight (Setlow, 2006, 2014b). About 5 - 15 % of spores dry weight consists of pyridine-2,6-dicarboxyl acid (dipicolinic acid [DPA]). DPA chelates with Ca^{2+} and other divalent cations in a 1:1 ratio (CaDPA), resulting in a mineralization of the core (Setlow, 2006, 2014b). The CaDPA and the low water content results in rotationally immobilized core proteins, contributing to the spores resistance to wet heat (Moir, 2003; Setlow, 2006, 2014b). The chromosome is highly condensed and packed with small, acid-soluble proteins (SASPs) that contributes to the resistance to DNA damage from irradiation, dry heat or chemicals (Setlow, 2006; Setlow, 2007; Setlow, 2014b).

A highly compressed and immobile inner membrane (IM) surrounds the spore core and functions as a major permeability barrier for small molecules (Cortezzo et al., 2004a; Cowan et al., 2004). The IM is enveloped by the germ cell wall which is similar to the vegetative germ cell wall. The germ cell wall is in turn surrounded by a thick cortex that consists of spore-specific modified peptidoglycan (PG) (Popham, 2002; Popham and Bernhards, 2015). The outer membrane (OM) separates cortex from the spore coat, and is not likely to act as a permeability barrier (Setlow, 2006, 2014b). No specific function in spore resistance has been described for the PG or OM (Setlow, 2006, 2014b).

The multilayered coat which encases the spore, is made up of at least 70 different proteins and consist of the following layers; the basement layer, inner coat, outer coat and crust (McKenney et al., 2013). The individual coat layers consist of several sheets with distinct morphologies (Plomp et al., 2014). The coat is impenetrable for larger molecules, and protects the spore from toxic chemicals and enzymatic degradation (Driks, 1999; Setlow, 2014b). The coat may also provide protection against predation (Klobutcher et al., 2006; Laaberki and Dworkin, 2008; Setlow, 2014b). Pigments (usually carotenoids or melanin) present in the coat likely offer additional protection against UV irradiation. However, not all *Bacillus* species have pigmentation in their spore coat (Hullo et al., 2001; Moeller et al., 2005; Setlow, 2006). A silicon layer, which increases acid resistance, is also present in the outer layers of *B. cereus* spores (Hirota et al., 2010; Setlow, 2014b)

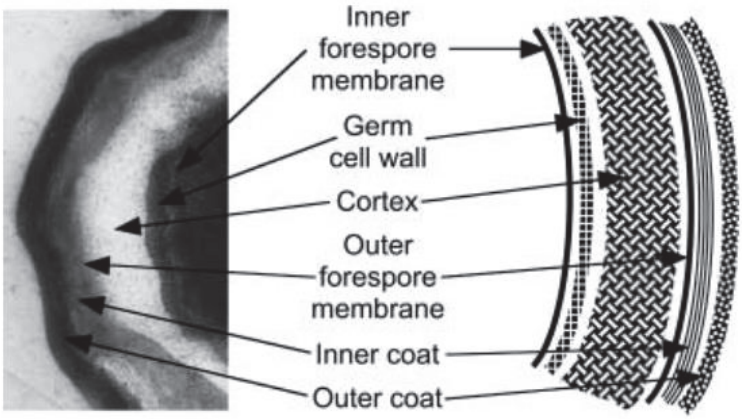

Fig. 2: The structure of the endospore. The dehydrated spore core is surrounded by several protective layers contributing to the spore's resistant nature. In addition, several species have an exosporium outside the coat (not shown). Figure from Popham et al., 2002. Printed with permission from Springer.

A loose-fitting, sac-like outer layer called exosporium is present in spores of several *Bacillus* species such as *B. cereus* and *B. anthracis*, but it seems to be absent in most *B. subtilis* spores (Stewart, 2015). The double-layered exosporium is comprised of a protein basal layer with a honeycomb-like structure of concave cups and channels, and an outer layer sometimes covered with a hair-like nap made up of glycoproteins (**Fig. 3A**) (Stewart, 2015). Small molecules can pass through the exosporium but its exact function is still unknown. It has been suggested to function in adhesion of spores to favorable surfaces, and be important in pathogenesis (Oliva et al., 2009; Stewart, 2015; Williams et al., 2013). Spores of some species carry multiple long, hair-like structures (appendages) on the surface. The appendages vary in number and shape between different strains and species; there are spore appendages shaped as ribbons, pili (hair-like), brushes, tubes and swords and they are structurally distinct from the short protein fibers in the nap layer that covers the exosporium of some species (Driks, 2007; Hachisuka et al., 1984; Walker et al., 2007). Very little is known about the composition, architecture, genetic identity, and biological functions of the appendages, but the variety in morphology likely reflect niche adaptation (Walker et al., 2007). Although it is generally accepted that the *B. subtilis* group species lacks an exosporium, SEM analysis of *B. licheniformis* spores revealed an exosporium-like structure carrying appendage-like protrusion, tightly surrounding the spore (**Fig. 3B**) (Madslien, 2013; Rönner et al., 1990).

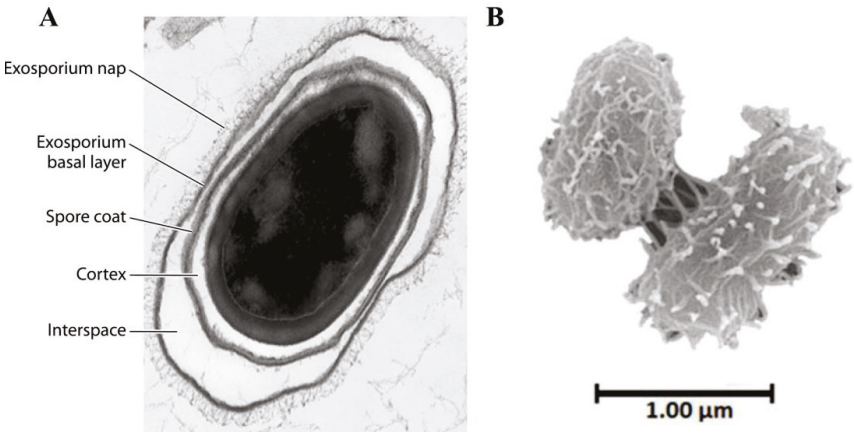

Fig. 3: (A) TEM micrograph of a *B. anthracis* spore showing the loose-fitting exosporium with the glycoprotein nap. Figure from Steward, 2015 and used with permission from American Society for Microbiology. (B) SEM micrograph of *B. licheniformis* strain NVH-1032 spores showing an exosporium-like layer with appendages. Photo: E.H. Madslien and A. Hoenen, Electron Microscopy Lab, Department of Biosciences, University of Oslo. Used with permission.

External factors during sporulation such as temperature, pH and salt concentration have also been shown to influence the spores' resistance properties (Baweja et al., 2008; Melly et al., 2002; Nguyen Thi Minh et al., 2011; Nguyen Thi Minh et al., 2008). Recently, it has been shown that *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* strains carrying a Tn1546-like transposon in their genome produce spores with increased wet-heat resistance and that the heat-resistant phenotype of these strains is associated with a *spoVA*^{2mob} operon encoded within the transposon (Berendsen et al., 2016a; Berendsen et al., 2016b).

1.3.2 Sporulation

Initiation of sporulation

The model organism *B. subtilis* has been intensely studied for its ability to differentiate into several different cell types (Lopez et al., 2009). Even clonal cells can express widely different characteristics ranging from sporulation, motility, natural competence for transformation, dormancy, enzyme or toxin secretion and extracellular matrix production (Cairns et al., 2014; Lopez et al., 2009). Sporulation is an irreversible, energy-costly and

time-consuming process (lasting 8-10 hours in *B. subtilis*), and is the "last resort" option when *Bacillus* cells experience nutrient deprivation. To avoid inappropriate initiation of sporulation this process is tightly regulated.

In *B. subtilis*, sporulation is initiated when sensor kinases (KinA-E, where KinA is the major kinase responsible for sporulation initiation) phosphorylate the Spo0A regulator (Spo0A-P) indirectly via Spo0F and Spo0B (a phosphorelay system). This process is likely activated by environmental stimuli such as nutrient deprivation and high cell density (Higgins and Dworkin, 2012; Hilbert and Piggot, 2004). Spo0A-P regulate both sporulation and biofilm formation, and the actual cell fate is determined by the cellular level of phosphorylated Spo0A (Spo0A-P). When Spo0A-P reaches high levels, the sporulation process is initiated (Lopez et al., 2009). Spo0A-P regulates expression of the sigma factor H (σ^H), which, in a positive feedback loop, stimulates the phosphorylation of Spo0A. The activity of σ^H will, together with Spo0A-P, activate genes responsible for the polar septum formation, marking the "point of no return", where cells become committed to sporulate, and also regulates the transcription in the cell prior to the asymmetrical cell division (Parker et al., 1996)

Prior to commitment to sporulation (before the formation of the polar septum), lower levels of Spo0A-P triggers the expression of a spore-delaying-protein (*sdp*) and a spore-killing-factor (*skf*) that lyse non-sporulating cells (Gonzalez-Pastor, 2011; González-Pastor et al., 2003). The lysed cells provide nutrients that delay, or even reverse, the energy-costly sporulation process (Gonzalez-Pastor, 2011). It should be noted that in a natural environment with a mixed community, the excreted killing protein could aid in predatory behavior rather than cannibalism, as the Skf is also active against other species (Gonzalez-Pastor, 2011; Nandy et al., 2007).

Sporulation stages

The sporulation process is characterized by a number of morphological changes, and is often divided into 7 distinct stages (Setlow and Johnson, 2013). The sporulation stages are briefly summarized in this section (see also **Fig. 4**). Stage 0 corresponds to the vegetative cell containing two chromosomes. At the first stage, the two chromosomes condenses, and forms an axial filament. A septum, which separates the cell into a forespore compartment and a mother cell compartment, is then formed close to one of the cell poles, and represents entry into stage 2. In stage 2, one of the condensed chromosomes are completely translocated into the forespore compartment. The mother cell engulfs the forespore in stage

3, resulting in a forespore with an inner- and an outer membrane. During transition from stage 3 to stage 4, the spore core starts to dehydrate and there is a decrease in pH and volume. There is also synthesis of SASPs that bind to the chromosome arrange it into a ring-like structure. A germ cell wall and a modified peptidoglycan layer (cortex) forms between the inner and outer membrane in stage 4. In stage 5, the spore coats are constructed outside the outer membrane and immediately prior to stage 6, DPA is synthesized in the mother cell compartment and transported into the forespore. Uptake of divalent cations (Ca^{2+}, Mg^{2+} and Mn^{2+}) also occurs, and the dehydration of the spore core is finalized. The spore matures in stage 6, as the outer layers are completed and the permeability of the spore core is decreased. Finally, in stage 7, the mother cell lyses and releases the mature spore into the environment. The process of sporulation is under a complex regulative control, governed in large by compartment-specific RNA polymerase subunits capable of binding to DNA promoter regions (σ factors).

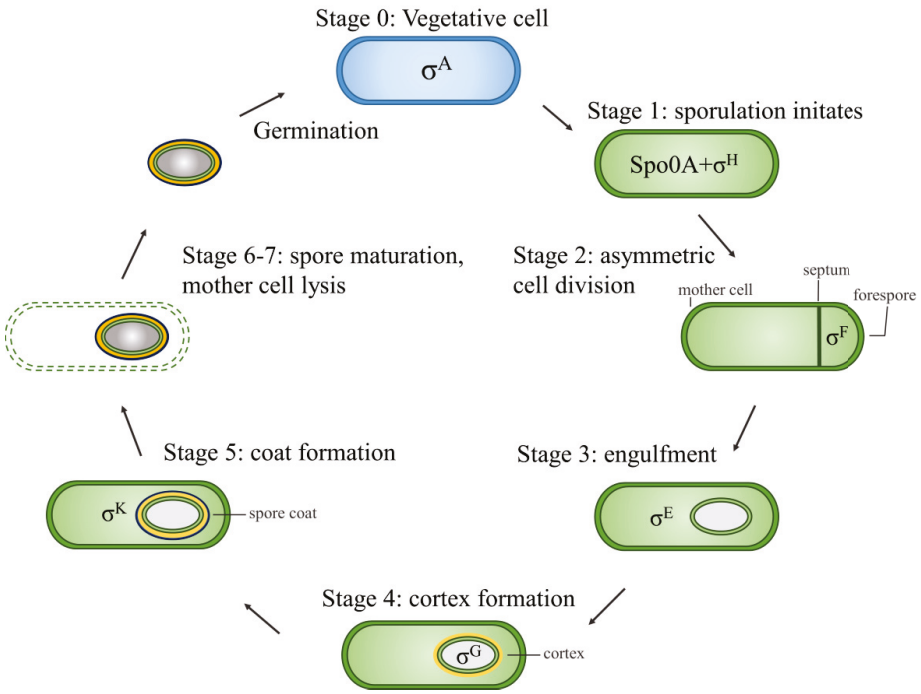

Fig. 4: The sporulation cycle and major gene regulators in *B. subtilis*. The figure is adapted from González-Pastor, 2011 and printed with permission from Oxford University Press.

Genetic regulatory network of sporulation

The process of sporulation is regulated by a cascade of events that rely on step-wise activation of four sigma factors active in their respective compartment; σ^F (early forespore), σ^E (early mother cell), σ^G (late forespore) and σ^K (late mother cell) (Hilbert and Piggot, 2004; Piggot and Hilbert, 2004). The cascade is possible because of extensive "cross-talk" between the forespore and mother cell compartments, and can be briefly summarized as follows: Upon asymmetrical cell division, σ^F is activated in the early forespore compartment. Subsequently, σ^E is activated in the early mother cell, where it orchestrate the engulfment of the forespore. Next, σ^G is activated in the late forespore and initiates the expression of genes important for cortex synthesis and synthesis of SASPs. It is also responsible for the expression of genes of proteins that are involved in spore germination, such as the germination receptors (GRs), DPA channel components (SpoVA proteins) and the cortex lytic enzymes (CLEs). Finally, σ^K is active in the late mother cell where it regulates the completion of the spore coat and activates the transfer of DPA into the forespore through the DPA channels. Mother cell lysis is also controlled by σ^K.

Following separation of the cell into two distinct compartments, there is a need for communication between these entities to facilitate the activation cascade that drives sporulation, and to maintain the metabolic activity throughout the process. The "feeding tube" model suggests a gap junction-like feeding tube through which the mother cell can supply the developing spore with small molecules (Camp and Losick, 2009).

1.4 Germination – exiting dormancy

Dormancy is only beneficial as a survival strategy if the microorganism can return to the vegetative state when environmental conditions turn more favorable for growth. The exit of spore dormancy happens in a process called germination, which, in contrast to sporulation that takes several hours, is completed within minutes (Setlow, 2003). Despite their inert state, the spores are capable of sensing environmental signals, and can rapidly germinate when triggered by certain environmental stimuli that indicate advantageous conditions (Moir and Cooper, 2015; Setlow, 2003). Nutrients, including amino acids, sugars, purine nucleosides and inorganic salt are important triggers of spore germination and are hereafter called "germinants" (Moir and Cooper, 2015; Ross and Abel-Santos, 2010a). The germinants are recognized by so-called germination receptors (GRs) present in the spore IM. Upon binding to germinant molecules, the GRs trigger a cascade of events that ultimately results in vegetative growth (Hudson et al., 2001; Moir and Cooper, 2015; Paidhungat and Setlow, 2001; Setlow, 2003). A number of non-nutrient triggers of germination have also been identified (described in section 1.4.6). An overview of the different germination pathways presented in the following sections is illustrated in **Fig. 5.**

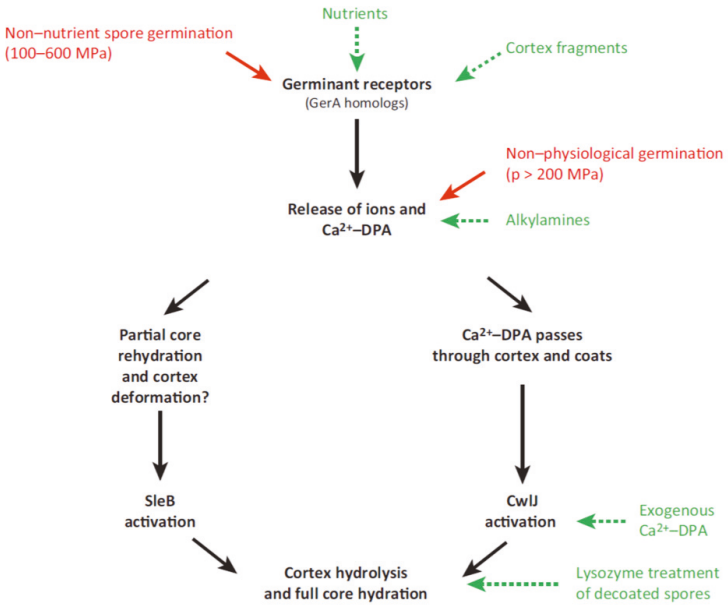

Fig. 5: Overview of the different germination pathways in *Bacillus* that will be presented below. The figure is modified from Reineke et al., 2013 and reprinted with permission from Elsevier.

1.4.1 Activation and commitment

Exposure of *Bacillus* and *Clostridium* spores to sublethal temperatures for a short time (10-20 min) results in a more synchronized and efficient germination response compared to non-heat treated spores (Rodriguez-Palacios and Lejeune, 2011; Setlow, 2003). This treatment is referred to as spore activation. Other conditions that also activate spores are high or low pH, certain chemicals and ionizing radiation (Keynan et al., 1964). The exact mechanism for how these factors activate spores are not understood, however, it has been suggested that activation works by improving the permeability of the coat (Behravan et al., 2000; Moir and Cooper, 2015; Setlow, 2014a). Spores stored for longer periods of time are more prone to germination than fresh spores, due to a process described as "aging" (Keynan et al., 1964). Aging has been proposed to be heat activation at lower temperature over long periods of time, where the ability to reverse the activation is lost (Keynan et al., 1964). The storage temperature for spore solutions have been shown to influence the germination efficiency, as *Bacillus* spores stored at refrigeration temperatures (3-8°C) for one week germinated slower than spores stored for one week at 30-33°C (Løvdal et al., 2013).

Spores that are committed to germinate by nutrient-induced germination will continue to germinate even when the nutrients are removed (Stewart et al., 1981; Yi and Setlow, 2010). The accepted model of spore commitment suggests that a threshold number of GRs need to be "activated" by recognizing their cognate germinant before germination commences (Yi and Setlow, 2010).

1.4.2 Stages in germination and proteins involved

Germination can be divided into distinct two stages (Setlow et al., 2001), followed by the outgrowth stage (**Fig. 6**). Stage I is characterized by increase in IM permeability, leading to a rapid release of the core's depot of cations (H^+, K^+ and Na^+), amino acids and CaDPA (Moir and Cooper, 2015; Setlow, 2003; Swerdlow et al., 1981). This results in increased core pH and core hydration, and the spore loses some of its heat resistance (Moir and Cooper, 2015; Setlow and Setlow, 1980; Swerdlow et al., 1981). CaDPA release occurs through channels in the IM (Moir and Cooper, 2015; Setlow, 2003). In *B. subtilis*, the heptacistronic *spoVA* operon is most likely involved in CaDPA transport, both in CaDPA uptake into the forespore, and in CaDPA release during germination (Li et al., 2012; Setlow, 2003; Tovar-Rojo et al., 2002; Vepachedu and Setlow, 2007b).

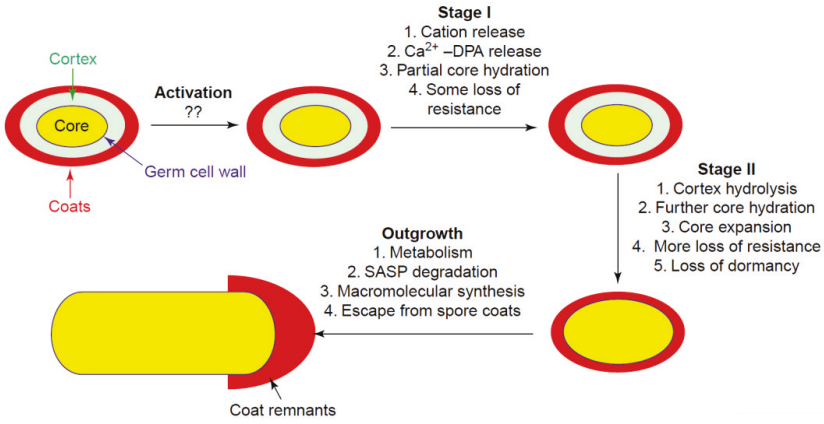

Fig. 6: Depiction of the different stages in *Bacillus* spore germination. The figure is from Setlow, 2003 and is reprinted with permission from Elsevier.

The importance of the *spoVA* operon in sporulation and germination is emphasized by the presence of at least a minimal set of *spoVA* genes in all *Bacillales* and *Clostridiales* (Moir and Cooper, 2015; Paredes-Sabja et al., 2011) In stage II, cortex-lytic enzymes (CLEs) recognize cortex-specific peptidoglycans and will hydrolyze the cortex (Moir and Cooper, 2015; Popham et al., 1996; Setlow, 2003). Cortex hydrolysis allow for complete hydration of the core and core expansion. Two enzymes, CwlJ and SleB, play important roles in initiation of cortex hydrolysis, SleB is localized in the IM and integument, whereas CwlJ is located in the spore coat (Chirakkal et al., 2002). CwlJ is activated by CaDPA and can trigger cortex hydrolysis independent of GR activation (Paidhungat et al., 2001; Setlow, 2003). The mechanism for SleB activation following nutrient induction of germination is still unknown. However, germination can still occur in mutants lacking one of the CLEs, albeit at a slower rate than in the wild-type background strain (Boland et al., 2000; Ishikawa et al., 1998). Stage II is also characterized by the degradation of the DNA-binding proteins SASPs, allowing the spore to resume metabolism and start the process of outgrowth (Moir and Cooper, 2015; Setlow, 2003).

Spore germination is assumed to take place without the need for metabolic activity (Setlow, 2003). In contrast to this assumption, Sinai et al. showed that protein synthesis occurs during the early stages of germination, and suggested this is essential for germination to proceed (Sinai et al., 2015). A recent paper, however, refutes this by showing that even spores depleted of rRNA will germinate normally (Korza et al., 2016).

1.4.3 Inhibition of germination

The enzyme alanine racemase is active in *Bacillus* spores and is likely present in the spore coat (*B. subtilis*) or in the exosporium (*B. cereus* and *B. thuringiensis*) (Stewart and Halvorson, 1953; Todd et al., 2003; Yasuda et al., 1993). Alanine racemase converts the common germinant L-alanine to its D-enantiomer D-alanine, which likely acts as a competitive inhibitor by binding to the GRs (Yasuda et al., 1993). By changing the ratio of L-/D-alanine, the alanine racemase inhibits spore germination during unfavorable growth conditions, such as during high spore densities, low germinant concentrations or when the pH is high (Yan et al., 2007; Yasuda et al., 1993). Other compound that inhibit germination includes alcohols, enzyme inhibitors like $HgCl_2$, ion channel blockers and organic acids (Cortezzo et al., 2004b).

1.4.4 Germination receptors

GRs are present in both Bacilli and Clostridia spores, but are best described in *B. subtilis*. The *B. subtilis* genome encodes the functionally characterized GerA, GerK and GerB GRs. The GerA GR is cognate to L-valine and L-alanine, whereas the GerB and GerK GRs cooperate to trigger a response to a mixture of L-asparagine and cogerminants glucose, fructose and KCl (AGFK) (Atluri et al., 2006; Paidhungat and Setlow, 2000).

The GRs consist of three to four subunits, the A, B and C subunit (depicted in **Fig. 7)**, and the more recently described D subunit (Moir and Cooper, 2015; Moir et al., 2002). The A subunit is an integral membrane protein and consist of 5-8 transmembrane helices in addition to a larger N-terminal hydrophilic domain exposed on the outer surface of the IM, and a small C-terminal hydrophilic domain which is exposed on the inner surface of the IM (Setlow, 2014a). The C subunit is a hydrophilic lipoprotein, anchored to the IM outer surface (Li et al., 2011; Li et al., 2010). The peptide sequences of the A and C subunits share no homology with any other known protein family. The B subunit, however, shows a weak homology with the amino acid-polyamine-organocation (APC) family of amino acid transporters (Setlow, 2014a). It consists of 10 transmembrane helices, and has never been shown to be capable of transporting amino acids across the IM (Jack et al., 2000). The B subunit has been suggested to contain the germinant binding site, as amino acid substitutions in the *Bacillus megaterium* B subunits GerVB changed the germinant specificity (Christie et al., 2010; Christie et al., 2008). However, amino acid substitutions in both the A and B subunits of the *B. subtilis* GerB GR changed the germinant specify of

the receptor, and no ligand-binding sites have so far been identified (Christie et al., 2008; Paidhungat and Setlow, 1999). Genes encoding the putative D subunit have been identified in close proximity to the genes encoding the A, B and C subunits of the GRs in several *Bacillus* species, and the encoded proteins are commonly small with two transmembrane helices (Paredes-Sabja et al., 2011). The putative D subunit has been shown to modulate germination (Ramirez-Peralta et al., 2013). GerD is a lipoprotein located both in the IM and in the outer layers of the spores and it has been shown to be required for efficient germination of both *B. subtilis* and *B. megaterium* spores, and is conserved in *Bacillales*, but not *Clostridiales* (Mongkolthanaruk et al., 2009; Paredes-Sabja et al., 2011; Ramirez-Peralta et al., 2013).

The GRs are present at low levels in the spore IM. Paidhungat and Setlow estimated that the number of GRs in the IM was only < 40 per spore, but improved methods showed that the number is approximately 1000 GR per spore (Paidhungat and Setlow, 2001; Stewart and Setlow, 2013). Presumably, the sensitivity of the spores to germinants should not be too high, as that could lead to premature germination in an unfavorable environment (Ross and Abel-Santos, 2010a). However, combinations of germinants in low concentrations have been shown to exhibit synergistic effects on *B. subtilis* spore germination, likely by simultaneously activating several GRs (Yi et al., 2011). Some GRs are dependent on another receptor to trigger a germination response. As mentioned above, GerB and GerK of *B. subtilis* cooperate in triggering germination when exposed to a mixture of germinants (Atluri et al., 2006). By visualizing *B. subtilis* GRs using fluorescence protein fusions, Griffiths et al. showed that the GRs colocalize into a single foci in the IM, called the germinosome, and that the colocalization is facilitated by the GerD protein (Griffiths et al., 2011).

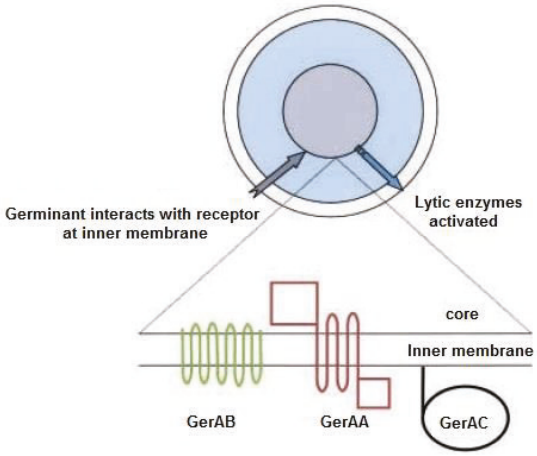

Fig. 7: The organization of the GerA receptor in the inner membrane of *B. subtilis* spores. The figure is derived from Moir, 2002 and reprinted with permission from Springer.

1.4.5 The genetics of germination receptors: *gerA* family genes

The *gerA* gene cluster was first described to be essential for L-alanine-induced germination of *B. subtilis* spores, and has become the paradigm for GRs (Moir et al., 1979; Moir and Smith, 1990; Ross and Abel-Santos, 2010a). Indeed, the *gerA* family genes are present in almost all investigated spore formers, except *C. difficile* (Paredes-Sabja et al., 2011). Their wide distribution in the orders *Bacillales* and *Clostridiales* suggests that they likely arose in a common ancestor, subsequently, paralogs which have diverged functionally have evolved by gene duplication and mutations and been selected for during speciation and niche adaption (Moir, 2006). The *gerA* family genes are most commonly organized in tricistronic operons, encoding A, B and C subunits. However, the *gerA* family genes can also be organized in di-, tetra- and pentacistronic operons or be present as orphan genes encoding single subunits, some of which can function in germination by associating with other GR subunits (Paredes-Sabja et al., 2011). This is described for the orphan B subunits *gerVB* and *gerWB* present in the *B. megaterium* QM B1551 genome that can be used interchangeably in the GR encoded by the tricistronic *gerU* operon, each subunit recognizing different germinants (Christie et al., 2010; Christie and Lowe, 2007). The role of multiple A, B or C subunits in the same operon or that of orphan subunits, is not completely understood. In proteolytic strains of *C. botulinum*, a pentacistronic *gerA*

orthologue encoding three B subunits have been identified, but no function has been assign to this operon (Brunt et al., 2014).

The number of *gerA* orthologues encoded within a genome varies among species. The *B. subtilis* genome encodes five *gerA* family operons; *gerA* (ABC), *gerB* (ABC), *gerK* (ACB), *ynd* (DEF) and *yfk* (QRST) (Kunst et al., 1997; Setlow, 2003). The *B. cereus* strain ATCC 14579 has seven *gerA* family operons, designated *gerG*, *gerI*, *gerK*, *gerL*, *gerQ*, *gerR* and *gerS* (Hornstra et al., 2006; Ivanova et al., 2003). *B. anthracis* Ames encodes six *gerA* family operons (*gerA*, *gerH*, *gerK*, *gerL*, *gerY* and *gerS*), in addition to a *gerX* operon present on the virulence plasmid pX01 (Fisher and Hanna, 2005). The *gerX* operon is less homologous to the *gerA* family genes encoded by *Bacillus* species, and seems to be more similar to *gerA* orthologues in other genera, such as *Geobacillus* (Ross and Abel-Santos, 2010b).

1.4.6 Nutrient-independent germination

Although nutrient-induced germination is the most important germination pathway in nature, spores can also exit the dormant state in response to other stimuli. After initiating germination in response to the presence of nutrients, spores release their core content of CaDPA, which in turn activates the CLE CwlJ, initiating cortex hydrolysis. Exogenous CaDPA can also trigger cortex degradation leading to spore germination (Paidhungat et al., 2001). CaDPA can thereby trigger germination of spores with low levels of GRs (superdormant spores) or lacking all GRs (Ghosh and Setlow, 2009; Paidhungat and Setlow, 2000). Cationic surfactants such as the alkylamine dodecylamine can also induce germination, likely by acting on the DPA channels themselves (SpoVA proteins) (Setlow et al., 2003; Vepachedu and Setlow, 2007b). In de-coated spores, which have been treated to remove the outer proteinaceous layers, lysozyme can degrade the PG cortex leading to CaDPA release and core hydration (Moir and Cooper, 2015; Setlow, 2003).

Murapeptides have been shown to induce germination through a novel germination pathway separate from the nutrient-induced pathway (independently of GRs). The murapeptides are recognized by the eukaryotic-like serine/threonine kinase PrkC (Shah et al., 2008). This kinase is present in both *Clostridiales* and *Bacillales*, and trigger germination in response to species-specific peptidoglycan fragments, which signals the growth of kin bacteria (Paredes-Sabja et al., 2011; Shah et al., 2008).

A small proportion of the spore population will germinate spontaneously in the absence of germinants and independently of any known GR (Paidhungat and Setlow, 2000; Sturm and Dworkin, 2015). Spontaneous germination has been suggested to be due to stochastics events leading to phenotypic variation, perhaps to "scout" for beneficial conditions (Buerger et al., 2012; Epstein, 2009; Paidhungat and Setlow, 2000; Sturm and Dworkin, 2015).

An important non-nutrient trigger of germination with commercial potential is high pressure. High pressure is currently used for inactivation of vegetative microbes. Isostatic pressures in the rage of 80-600 MPa, corresponding to ~790–5900 atm, will trigger spore germination, which will be discussed in the next section.

1.5 High pressure

1.5.1 High-pressure processing

Killing spores can be achieved by exposing spores to harsh conditions, most commonly 121 °C for 3 minutes (botulinum cook), strong chemical substances (i.e. hypochlorite) or irradiation for prolonged periods of time (Anderson et al., 2011; Wohlgemuth and Kämpfer, 2014). High temperatures (>120 °C) are sufficient to kill most spores, but will negatively affect the taste and nutritional quality of many food products. Consumers demand for fresh-tasting, nutritious foods without additives are increasing, bringing an incentive for the food industry to explore alternative processing methods. High-pressure processing (HPP) of food products is a more gentle processing method that better maintains the sensory and nutritional properties of the food, while still inactivating spoilage and pathogenic organisms (Balasubramaniam and Martínez-Monteagudo, 2016). The earliest description of high pressure (HP) as a food-processing method was more than 100 years ago for "keeping milk sweet for longer" (Hite, 1899). However, HPP was first used commercially in Japan in the 90's to treat jams (Yamamoto, 2017). In the following 20 years the number of industrial HP systems and application have steadily increased and HPP products are manufactured in Asia, USA and Europe. Today, HPP is used as a cold pasteurization method to improve food safety in products such as jams, juices, seafood, ready-to-eat meals and sliced meats.

Two scientific principles are important for the use of HPP to inactivate microorganisms. Le Chatelier's principle states that in a system influenced by HP, the equilibrium will shift towards the lowest volume. This will induce, among other phenomena, molecular conformational changes, phase transitions and chemical reactions (Hogan et al., 2005). According to the isostatic principle, the pressure is uniformly and instantly transmitted throughout the sample during HP treatment (Hogan et al., 2005). In addition, when HP is applied to a system, a temperature rise, so-called adiabatic heat of compression, will occur. The amount of heat developed will depend on the matrix, but for water, the temperature increases approximately 3 °C per 100 MPa (Georget et al., 2015).

Two important targets for HP in microorganisms are proteins and the cell membrane (Rivalain et al., 2010). Proteins will undergo non-uniform conformational changes when exposed to HP, which can lead to reversible or irreversible changes in the native structure. HP will not affect covalent bonds to a substantial degree, but will cause changes to hydrophobic interactions, cavities and hydrogen bonds (Knorr et al., 2006; Rivalain et al.,

2010). The cell membrane is likely the most pressure-sensitive biological structure in the cell, and HP exposure has been shown to cause rupture of the cell membrane (Huang et al., 2014; Rivalain et al., 2010). Lipid bilayer membranes have been shown to undergo a phase transition from a liquid-crystalline phase to a gel phase (Rivalain et al., 2010).

1.5.2 Spore inactivation by high pressure

There are large variations in HP sensitivity among microorganisms. In general, eukaryotic parasites are more sensitive to HP than yeast or molds. Gram-negative bacteria are more sensitive than Gram-positive bacteria, and the HP resistance of viruses relies on whether they are enveloped or not. Bacterial spores are highly resistant to HP compared to other microorganisms, but can be inactivated under certain HP conditions (Reineke et al., 2013b; Rivalain et al., 2010; Smelt, 1998). The exact mechanism for how HP kills spores is not understood, but spore inactivation is dependent on the pressure level and processing temperature, and HP inactivation of spores is generally accepted to be a two-step process dependent on prior spore germination (Margosch et al., 2004; Reineke et al., 2013b). Higher temperatures (>60 °C) during HPP above 500 MPa will act synergistically on both spore germination and inactivation (Reineke et al., 2013b). Under these conditions there is a rapid loss of CaDPA and an increase in heat sensitivity (Reineke et al., 2013b; Reineke et al., 2011).

To achieve effective spore inactivation using HPP, two concepts for commercial sterilization is currently of interest to researchers. Pressure-assisted thermal sterilization (PATS) is a FDA (U.S. Food and Drug Administration) approved technology that utilizes the adiabatic heat of compression to rapidly reach sterilization temperatures in preheated low-acid foods, which lowers the processing time. Alternatively, spore inactivation can be achieved at lower temperatures by high-pressure thermal sterilization (HPTS), using the synergetic effects of high temperatures and HP to inactivate spores (Reineke et al., 2013b; Reineke et al., 2011). None of these strategies are so far being used by the food industry to combat spores, likely due to technical limitations and inadequate knowledge about the inactivation kinetics and killing mechanism of spores during high pressure and high temperature treatments (Lenz and Vogel, 2015).

1.5.3 Moderately high pressure-induced germination

Moderately high pressures (mHP) of 80 - 300 MPa are known to initiate spore germination (Clouston and Wills, 1969; Gould and Sale, 1970). In *B. subtilis*, mHP activates the GRs and triggers a germination response similar to that induced by nutrients (Paidhungat et al., 2002). The individual GerA, GerK and GerB GRs in *B. subtilis* demonstrated a hierarchical HP responsiveness. GerA was the most HP-responsive GR, followed by GerB and lastly GerK (Black et al., 2005). Interestingly, the HP responsiveness of the GRs could be altered by increasing the level of GRs in the IM, however, the GR level was not the only factor that affected HP responsiveness (Black et al., 2005; Doona et al., 2014). A GerB variant, containing amino acid alterations in either the A or B subunit, was more HP responsive than the native GerB (Black et al., 2005). In *B. cereus*, which encode 7 GRs, the loss of a single GR did not significantly affect mHP-induced germination, however, loss of the cation antiporters GerN or GerT resulted in decreased germination (Wei et al., 2009).

1.5.4 Very high pressure-induced germination

At very high pressures (vHP), above 300 MPa and upwards to 600 MPa, germination is initiated through a GR-independent pathway (Reineke et al., 2012). Germination likely rely on the pressure-assisted opening of the DPA-channels, resulting in CaDPA release (Black et al., 2007b; Paidhungat et al., 2002). Spores germinated through this pathway are likely arrested at stage I of germination, as there is no SASP degradation (Wuytack et al., 1998). This can explain why vHP-germinated spores retain their pressure resistance, while mHP germinated spores are inactivated by prolonged HP treatments (Wuytack et al., 1998).

1.5.5 High pressure equipment

HP can be achieved either by decreasing the volume of a pressure chamber using a piston (direct compression), or by pumping pressure transmitting fluid (i.e. water, silicon oil, castor oil, glycol) into a pressure vessel (indirect compression) (Elamin et al., 2015). The HP conditions are maintained by stopping the piston or closing the valves, without further energy input, for the desired holding time (Hogan et al., 2005). Both batch and semi-continuous HPP systems are now available, however, the batch system with an external hydraulic pump and pressure intensifier is most commonly used in the industry (Elamin et al., 2015; Yamamoto, 2017). For semi-continuous systems, liquids can be pressurized in a pressure chamber by reducing the volume with a piston. After the HP treatment, the product

must be discharged to a sterile holding tank and packed under aseptic conditions (Hogan et al., 2005).

The HP experiments performed in this thesis were done using a monovessel and multivessel system of the model U111 (Unipress, Warsaw, Poland) (**Fig. 8**). These systems both contain a hydraulic pump and an external intensifier. The pressure-transmitting fluids used in these systems were di-2-ethylhexyl-sebacate for the monovessel unit and silicon oil for the multivessel unit. The pump (Mannesman Rexroth Pilska Ltd, Warsaw, Poland) produced a pressure of up to 70 MPa in the low-pressure part of the intensifier, which multiplied the pressure ~11 times in the high-pressure part.

Fig. 8: High pressure equipment used for the work presented in paper II. (a) U111 HP Monovessel unit with manual pump (UniPress, Warsaw, Poland) (b) U111 HP Monovessel unit chamber that is submerged in silicon oil bath for temperature regulation. (c) U111 HP Multivessel unit (UniPress, Warsaw, Poland) with an automatic pump. Photo: Kristina Borch-Pedersen, 2015.

1.6 *Bacillus licheniformis*

B. licheniformis (L. lichen-shaped) is a facultative anaerobic member of the *B. subtilis* group capable of growing at temperatures from 15 - 55 °C (Logan and De Vos, 2009). *B. licheniformis* is of economic value as it is used as an industrial workhorse for production of enzymes such as amylases, proteinases and the topical antibiotic bacitracin (de Boer et al., 1994; Katz and Demain, 1977). On blood agar, *B. licheniformis* forms whitish to yellowish, dry colonies that adhere strongly to the agar. Some strains demonstrates β-hemolysis on blood agar, although the type strain DSM13 does not (**Fig. 9**) (Madslien et al., 2013). For some *B. licheniformis* strains, colony morphology varies even within pure cultures, giving the impression of contamination. *B. licheniformis* are commonly isolated from soil, decomposing plant material, feces and bird plumage (Burtt and Jann, 1999; Logan and Vos, 2015). The complete genomes of type strain *B. licheniformis* DSM13 and isogenic strain ATCC14580 were published in 2004 (Accession no. CP000002.3). The genome is 4.2 Mb with a G+C ratio of 46.2 % (Rey et al., 2004; Veith et al., 2004). In 2015, Dunlap *et al*. identified a new species through phylogenetic analyses, namely *B. paralicheniformis*. Consequently, some strains previously annotated as *B. licheniformis* are now renamed as *B. paralicheniformis*, for example the biotechnologically important strain ATCC 9945a (Dunlap et al., 2015).

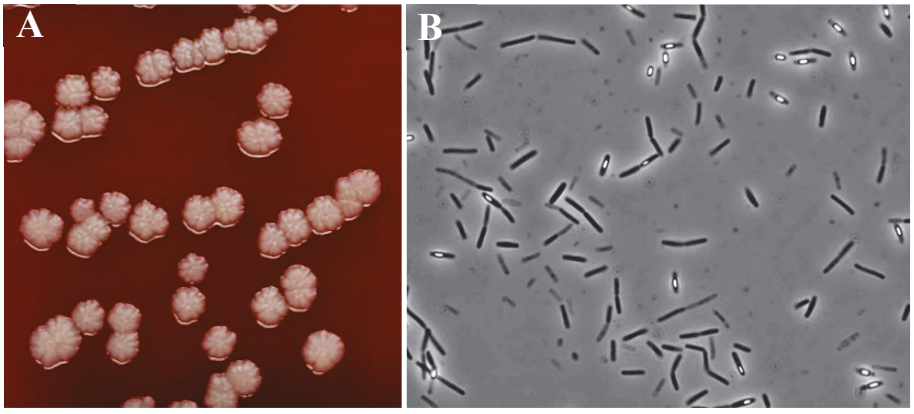

Fig. 9: *B. licheniformis*. (a) Colony morphology of *B. licheniformis* MW3 (type-strain derivative) after 20 hours incubation on blood agar. (b) Micrograph of *B. licheniformis* vegetative cells with spore formation. The phase-bright forespores are visible in the vegetative (phase-dark) *B. licheniformis* cells. The vegetative cells often appear in pairs (as seen above) or chains. Photo: Kristina Borch-Pedersen, 2015.

1.6.1 Pathogenic potential of *B. licheniformis*

B. licheniformis is generally regarded as a non-pathogenic species, however, it has occasionally been reported to cause food poisoning and systemic infections. A fatal case of *B. licheniformis* food poisoning related to infant formula has been reported and lichenysin A was the only substance identified from the *B. licheniformis* cell extracts that showed toxicity to boar spermatozoa (Mikkola et al., 2000; Salkinoja-Salonen et al., 1999). In nature, lichenysin A is the most abundant of several isoform of lichenysin (Rønning et al., 2015; Yakimov et al., 1999; Yakimov et al., 1995). Lichenysin is a heat-stable, small, cyclic lipopeptide, belonging to the surfactin family, and is synthesized by the lichenysin synthase encoded by genes *lchAA-C* (Tapi et al., 2010; Yakimov et al., 1998). The mechanism of action of lichenysin and other surfactins is unknown but their amphipathic nature indicates that they destabilize the lipid membrane and may form channels in the cell membrane, resulting in leakage and cell lysis (Carrillo et al., 2003; Heerklotz, 2008). Madslien *et al*. showed that all 53 tested *B. licheniformis* strains harbored the *lchA* operon and produced lichenysin. However, not all of these strains demonstrated cytotoxicity towards Vero cells or boar spermatozoa (Madslien et al., 2013). The high occurrence of lichenysin-producing strains and the prevalence of *B. licheniformis* in raw materials and food products, is not reflected by the frequency of *B. licheniformis*-related food poisoning incidents. Reports on food poisoning caused by consumption of milk powder, desserts, meat and vegetable dishes indicate that *B. cereus* is by far the most common *Bacillus* species associated with food poisoning (Kramer and Gilbert, 1989; Logan, 2012). The toxicity of lichenysin has never been demonstrated *in vivo*, and the potency of lichenysin is likely to be low (Madslien et al., 2013). *B. licheniformis* is sporadically reported to cause systemic infections such as septicemia and bacteremia in immunocompromised patients (Blue et al., 1995; Haydushka et al., 2012; Lépine et al., 2008; Sugar and McCloskey, 1977). However, there is no support for *B. licheniformis* being invasive in humans. In veterinary medicine, *B. licheniformis* has been associated with bovine abortions (Agerholm et al., 1995).

1.6.2 *B. licheniformis* spore germination

Martin and Harper identified L-alanine, L-cysteine and L-valine as the three most potent germinants of *B. licheniformis* spores in 1963 (Martin and Harper, 1963). After this, however, the germinative behavior of this species was left un-studied until recently. The construction of the more easily transformable DSM13 derivative strain MW3 has made in-

depth, genetic studies of *B. licheniformis* germination mechanisms possible (Waschkau et al., 2008).

Three *gerA* family operons *gerA*, *gerK* and *ynd* and an orphan, truncated *yndF2* gene have been identified in *B. licheniformis* strain ATCC14580/DSM13 (Rey et al., 2004; Veith et al., 2004; Xiao et al., 2011). The *gerA* and *gerK* operons show a similar gene organization as described for the orthologues in *B. subtilis*, ABC and ACB, respectively. The *ynd* operon does, however, encode five genes, including three B subunits (ABBCB). This organization is seen in *ca*. 0.2 % of spore formers investigated (Paredes-Sabja et al., 2011). Løvdal *et al*. showed that the *gerA* operon was essential for L-alanine-induced germination, and germination can be inhibited by the addition of D-alanine (Løvdal et al., 2012). *B. licheniformis* has a pentacistronic *spoVA* operon, containing the *spoVAF* gene which shares homology with the *gerAA* gene. An additional, orphan *spoVAF2* (*spoVAFA*) gene is also present in the *B. licheniformis* strain ATCC14580/DSM13 genome (Rey et al., 2004; Veith et al., 2004; Xiao et al., 2011). However, the function of the two latter-mentioned genes has not been investigated in this thesis.

1.7 Background of the study and knowledge gaps

Spore formers of the genera *Bacillus* and *Clostridium* are of great concern to the food industry. Spores are highly resilient to processing strategies because they can survive long heat exposures, chemical treatments and nutrient deprivation. As consumers demand fresh-tasting, nutritious food, the food industry needs to provide mildly heat-treated, ready-to-eat products. Therefore, spore formers will continue to be an increasing challenge in food production, as spore contamination is almost impossible to avoid.

Although spores are successful vehicles for perseverance and dispersion, it is by the process of germination that the spore formers can grow and cause food spoilage or food-borne disease. Even though spore germination has been studied for decades, the signal transduction and molecular events leading to germination and outgrowth are still largely unknown. The majority of the knowledge on spore germination comes from studies of the model organism *B. subtilis* 168 and closely related strains. By extending our research to more spore-forming species, we expand our insight into the diversity of germination behaviors.

A modified Tyndallization (double-heat treatment) is used commercially in Norway for spore control in semi-preserved food products. The success of this method rely on sufficient activation and germination of spores. In 2004, a slow-germinating *B. licheniformis* strain (NVH-1032) was isolated from a batch of spoiled canned meat from a Norwegian producer. Due to its slow germination response, the strain survived the Tyndallization treatment. This problem was solved by increasing the time between the two heat treatments. Following this incidence a research project on the germination and pathogenic potential of *B. licheniformis* was initiated. Despite the importance of *B. licheniformis* as a food-spoilage bacterium and its role in food poisoning incidents, very little is known this species' spore germination. Genetic studies are needed to functionally characterize the *gerA* family homologs (*gerA*, *gerK* and *ynd* operons) further, and to understand the role of the *gerA*, *gerK* and *ynd* operon in spore germination in response to multiple germinants. The *ynd* operon has so far been uncharacterized, despite being present in the genome of several *Bacillus* species, both within the *B. subtilis* and *B. cereus* groups. *In B. licheniformis ynd* is a pentacistronic operon encoding multiple B subunits. Similar cistronic organizations occur among other spore formers, however, there is a need for studies on the functional importance of duplicate or triplicate homologous A, B or C subunits encoded in the same *gerA* family operon.

2. Aims of the study

The main objective of this study was to genetically and functionally characterize the GerA, GerK and Ynd GRs present in *B. licheniformis* spores.

Sub-objectives

1. To identify nutrients that induce germination of *B. licheniformis* spores (Paper I).

2. To describe the role of the GerA, GerK and Ynd GRs in nutrient-induced germination (Paper I).

3. Compare the effect of HP on spores of *B. licheniformis* and the model organism *B. subtilis* (Paper II).

4. To understand the role of the GerA, GerK and Ynd GRs in HP-induced germination of *B. licheniformis* spores (Paper II).

5. To characterize the role of individual GR subunits in the cooperative function between the Ynd and GerA GRs in *B. licheniformis* spore germination (Paper III).

6. To determine the potential function of the orphan *yndF2* gene in *B. licheniformis* spore germination (Paper III).

3. Summary of results

Paper I: The cooperative and interdependent role of GerA, GerK and Ynd in germination of *Bacillus licheniformis* spores

Kristina Borch-Pedersen, Toril Lindbäck, Elisabeth H. Madslien, Shani W. Kidd, Kristin O'Sullivan, Per Einar Granum and Marina Aspholm.

Applied and Environmental Microbiology (2016) 82, pp. 4279-87

A screening of 23 nutrient compounds for their ability of to induce germination identified the amino acids L-alanine, L-cysteine, L-valine, L-serine, L-aspartic acid and L-isoleucine as germinants of *B. licheniformis* MW3 spores. Mutational analyses showed that a complete *gerA* and *ynd* operon was required for an effective spore germination in response to L-amino acids. Disruption of the *gerA* operon completely abolished L-amino acid-induced germination, whereas spores with the *yndD*-null mutation showed a reduction in the germination efficiency. D-glucose was also found to induce a weak germination response, which was dependent on a complete *gerK* operon. Interestingly, the germination response to glucose was also dependent on the presence of the GerAB and/or the GerAC subunits in addition to GerK, as *gerAA* null mutant-spores, but not the *gerAA-C* null-mutant spores, were able to germinate in response to glucose.

Paper II: The effects of high pressure on *Bacillus licheniformis* spore germination and inactivation

Kristina Borch-Pedersen, Hilde Mellegård , Kai Reineke, Preben Boysen, Robert Sevenich, Toril Lindbäck and Marina Aspholm

Applied and Environmental Microbiology (2017) 83, doi:10.1128/AEM.00503-17

Exposure to moderately high pressure (mHP) of 150 MPa resulted in efficient germination of *B. licheniformis* MW3 spores. This effect was similar to that observed for *B. subtilis* PS832 spores. However, spores of the *B. licheniformis* strain NVH-1032 showed a slower germination response compared to the above-mentioned strains. HP treatment of *ger*-null mutant spores allowed us to map the contribution of individual GRs to mHP-induced germination. We observed similarities in the interaction between GRs, between nutrient-induced and mHP-induced germination. The Ynd GR did not require the presence of another GR to function in mHP-induced germination. In contrast to nutrient-induced

germination of *B. licheniformis* MW3 spores where GerA plays a major role, Ynd appears to contribute most to mHP induced spore germination, as *gerAA gerKAC* null-mutant spores (Ynd^+) germinated almost as efficiently as wild-type spores under the tested conditions. We also exposed *B. licheniformis* spores to very high pressure (vHP) of 550 MPa. At moderate temperatures, no *B. licheniformis* spore germination was seen, although *B. subtilis* PS832 germinated efficiently under the same conditions. Increasing the temperature to 60°C acted synergistically with vHP to induce effective germination in *B. licheniformis* spores.

Paper III: Dissecting the cooperative interaction between the GerA and Ynd germination receptors in *B. licheniformis* spore germination

Toril Lindbäck, Kristina Borch-Pedersen, Kristin O'Sullivan, Siri Fjellheim, Inger-Helene Aardal, Per Einar Granum and Marina Aspholm.

Manuscript

The pentacistronic *ynd* operon was found to be conserved in all *B. licheniformis* genomes investigated. The *ynd* operon consists of the *yndD, yndE3, yndE2, yndF* and *yndE1* genes encoding A-, B-, B-, C- and B subunits. Phylogenetic analysis of the *yndE* genes from 20 *B. licheniformis* and *B. paralicheniformis* strains showed that the *yndE1*, *yndE2* and *yndE3* diverged into three separate branches, indicating that the three genes have different functions. The orphan gene *yndF2* was found in 12 out of the 20 strains. Interestingly, while the *yndF2* gene product was N-terminally truncated in the type strain ATCC14580/DSM13 genome, most other strains carried intact *yndF2* genes. To assess the functions of the *yndE* genes and the *yndF2* orphan gene, in-frame deletion mutants were constructed in the *B. licheniformis* strain MW3. The Ynd GR could not function with an incomplete set of *yndE* genes, as deleting the *yndE3* or the *yndE3* and *yndE2* genes disrupted the GR function. However, when all *yndE* genes were deleted, the Ynd A (*yndD*) and C (*yndF*) subunit were functional in germination demonstrating that Ynd GR subunits A and C were sufficient for the cooperative interaction with the GerA GR. Further analysis suggested that only the Ynd A subunit was necessary for an effective germination response when GerA was present.

4. Discussion

4.1 General discussion

Although *B. licheniformis* is frequently involved in food spoilage and has been implicated in food-poisoning incidents, detailed understanding of the molecular biology of spore germination in this species was previously very limited. By studying the germination behavior of *ger* null-mutant spores in response to various nutrient compounds and high pressures, this thesis work functionally characterizes the GerA, GerK and the Ynd GRs in *B. licheniformis* spores.

4.1.1 The *gerA* family of genes and germinant recognition in *B. licheniformis*

The *B. licheniformis* ATCC14580/DSM13 genome encodes the *gerA* family operons *gerA*, *gerK* and *ynd* in addition to the orphan gene *yndF2* (**Fig. 10**) (Rey et al., 2004; Veith et al., 2004). A study by Martin and Harper identified the three amino acids L-alanine, L-cysteine and L-valine as germinants of *B. licheniformis* spores (Martin and Harper, 1963), and Løvdal *et al*. showed that the *gerA* operon was essential for L-alanine induced germination of *B. licheniformis* spores (Løvdal et al., 2012).

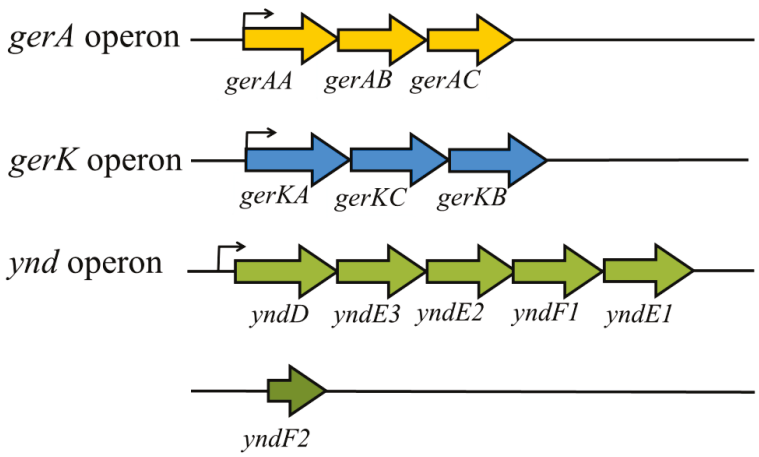

Fig. 10: The *gerA*-family gene clusters identified in the *B. licheniformis* ATCC14580/DSM13 genome. Arrows signify putative promoter positions. The *yndF2* orphan gene is found separate from the *ynd* operon. Modified from Borch-Pedersen et al., 2016 and printed with permission from American Society for Microbiology.

In **paper I** of this thesis we identified 8 amino acids in addition to D-glucose that induced germination (>10 % phase-dark spores after 2 h germinant exposure) of *B. licheniformis* spores. L-alanine, L-cysteine and L-valine were the most potent germinants, but the L-amino acids L-aspartic acid, L-serine and L-isoleucine could also induce a moderate germination response. Furthermore, glucose could induce a weak germination response. The germinant mixture AGFK (the amino acid L-asparagine with cogerminants glucose, fructose and KCl) did not increase the germination levels compared to when germination was induced by glucose alone, likely because *B. licheniformis* lacks a *gerB* homolog. GerB is known to act cooperatively with the GerK GR to induce a germination response to AGFK in *B. subtilis* spores (Atluri et al., 2006). D-alanine, a stereoisomer of L-alanine, inhibited spore germination in response to the three most effective germinants, L-alanine, L-cysteine and L-valine. This indicates a relaxed affinity of the GRs to these three germinants. The inhibitory effect of D-alanine on germination has previously been shown for spores of *B. subtilis, Clostridium sporogenes* and some strains of *C. botulinum*, which are also germinated by L-alanine (Atluri et al., 2006; Brunt et al., 2014; Yi and Setlow, 2010).

L-alanine was the strongest germinant to *B. licheniformis*, and is nearly a universal germinant among *Bacillus* and *Clostridium* species, likely due to its high occurrence in nature (Ross and Abel-Santos, 2010a). L-cysteine, a slightly less efficient germinant to *B. licheniformis* spores compared to L-alanine, is an uncommon germinant within the *Bacillus subtilis* group. It is, however, known to trigger germination in strains of *B. cereus*, *C. sporogenes* and *C. botulinum* spores (Brunt et al., 2014; Hornstra et al., 2006). Cysteine is a reducing agent, indicative of anaerobic conditions, which coincides with the strictly anaerobic growth requirements of *Clostridium* species (Brunt et al., 2014). L-cysteine is also an important constituent of gluten proteins and keratins (components of feathers and hair) as disulfide bonds are important for the proteins elasticity and strength (Shewry and Tatham, 1997; Wieser, 2007). The germinant L-cysteine might thus reflect the common habitats of *B. licheniformis*. *B. licheniformis* is facultative anaerobic and is often isolated from spoiled (ropy) bread and bird feathers (Logan and Vos, 2015). Due to its production of keratinase and the ability to degrade bird feathers *in vitro*, it has been suggested that *B. licheniformis* has an important ecological role. Some authors have suggested that feather-degrading bacilli such as *B. licheniformis* may be a factor driving the evolution of feather color, as more pigmented feathers were shown to be more resistant to bacterial

degradation (Burtt and Ichida, 2004; Goldstein et al., 2004; Grande et al., 2004; Gunderson et al., 2008).

The concentration of germinants used in this work was set to 100 mM. In natural settings, spores are likely to be exposed to mixtures of various germinants at once, in most habitats the germinants may also be present in significantly lower concentrations than used in our experiments. Multiple germinants, which act on different GRs, have been shown to exert a synergistic effect and induce germination of *B. subtilis* spores even at µM concentrations. The synergistic effect was attributed to simultaneous activation of multiple GRs, with the assumption that there is a threshold level of activated GRs needed for spores to be committed to germinate (Yi et al., 2011). In our experiments, we could not detect any synergistic effects when exposing spores to low concentrations of L-alanine and glucose (**Paper I**) or L-cysteine and glucose (Toril Lindbäck, *unpublished results*).

4.1.2 Cooperativity between germination receptors

The work presented in this thesis supported the previous results from our laboratory, which showed that the *gerA* operon has an essential role in L-alanine-induced germination of *B. licheniformis* spores (Løvdal et al., 2012). We showed that *gerA* is imperative for spore germination in response to L-cysteine and L-valine as well. Deleting the first gene in the *ynd* operon (*yndD*), which encodes the A subunit, had a negative effect on both the rate and efficiency of the germination in response to L-alanine, L-valine or L-cysteine (**Paper I**). However, germination was not completely abolished, as 40-60 % phase-dark spores were observed after 2 h germinant exposure. These results suggested that both the GerA and the Ynd receptor are needed for an efficient germination response to either L-alanine, L-valine or L-cysteine. The need for two GRs to induce germination to single-trigger germinants has been described for the GerI and GerQ GR in *B. cereus* 569 in response to inosine (Barlass et al., 2002).

Deletion of the A and C subunit encoded by the *gerK* operon reduced the germination to L-amino acids only slightly compared to the wild-type strain, but a complete *gerK* operon was necessary for the weak germination response to glucose observed in the wild-type spores. The mutant spores encoding complete *gerK* and *ynd* operons, but carrying a *gerAA-C* (*gerAABC*) deletion, displayed a reduced germination response to glucose compared to the wild-type spores and spores expressing the *gerK* operon and *gerAB* and *gerAC*. These results indicates that the B and C subunits of GerA GR are somehow

functional in glucose-induced germination, perhaps cooperatively interacting with GerK. Alternatively, GerA could be important for the formation or the integrity of the germinosome, and not directly cooperating with GerK in glucose-induced germination. In *B. subtilis,* GerK does not trigger germination alone, but act cooperatively with GerB to induce germination in response to AGFK (Atluri et al., 2006). GerK can also act together with GerA in *B. subtilis* to "stimulate" the germination response to L-alanine when cogerminants GFK are added, even if glucose alone does not trigger germination (Atluri et al., 2006; McCann et al., 1996). Disruption the *ynd* operon did not affect germination in response to glucose.

Cooperative protein-protein interactions in the IM

Exactly what the cooperative interactions between GRs entail are not understood. There is no direct evidence for physical interaction between GRs. However, a genetic study concluded, based on overexpression of GerAC or GerBC, that there was an interaction not only between the C subunits and the A and B subunits within individual GRs, but also an interaction between GerBC and GerAA or GerAB (Igarashi and Setlow, 2005). Studying protein-protein interactions of spore proteins is challenging, mainly due to difficulties purifying integral membrane proteins from the spore's IM for structural and functional studies. Additionally, recombinant expression of the GRs in an *E. coli* system has, to the best of our knowledge, so far failed. The lipoproteins GerKC and GerD are the only GR-associated proteins which structures have been published (Li et al., 2014; Li et al., 2010). Vepachedu and Setlow used the yeast two-hybrid system and Far-Western analysis to study protein-protein interaction, but could not detect any interaction between subunits of the GerA and GerB GRs (Vepachedu and Setlow, 2007a). Taking membrane proteins out of their natural environment may hamper with their native structure, and therefore also, any interaction possible. This does, however, not necessary mean that such interactions does not exist, as we know that GRs assemble into germinosomes (Griffiths et al., 2011).

GerA can function alone

An *yndD gerKAC*–null mutant constructed and published in **Paper I** and **II** displayed no germination in response to nutrients and moderately high pressure (mHP), which led us to conclude that GerA could not induce germination in the absence of either the GerK or Ynd GR. However, more recent results showed that spores of a mutant in which the complete *ynd* and *gerK* operons were deleted, germinated at a similar efficiency as mutant spores where only the *ynd* operon was deleted (**Paper III**). Therefore, the GerA

GR *can* function alone, however, to achieve optimal germination efficiencies the Ynd GR is also necessary. A reevaluation of the *yndD gerKAC*-null mutant showed that it mistakenly contains a spectinomycin resistance cassette in the *gerAA* gene. Hence, this mutant is in reality a *gerAA yndD gerKAC*-triple mutant and the lack of germination is as expected.

4.1.3 Characterization of the Ynd GR in *B. licheniformis*

We have shown that the Ynd receptor is functional in *B. licheniformis* spore germination (**Paper I**). The pentacistronic organization of the *ynd* operon was evident in all investigated *B. licheniformis* strains (**Paper III**), and differs from the organization of the uncharacterized *ynd* operon in *B. subtilis* (Kunst et al., 1997; Paidhungat and Setlow, 2000). The *B. licheniformis ynd* operon contains the genes *yndD*, *yndE3, yndE2*, *yndF* and *yndE1* encoding A-, B-, B-, C- and B subunits, while the tricistronic *ynd* operon of *B. subtilis* consist of genes *yndD*, *yndE* and *yndF,* encoding A-, B- and C subunits (Kunst et al., 1997; Rey et al., 2004; Veith et al., 2004). There is also an orphan *yndF2* gene in the *B. licheniformis* ATCC14580/DSM13 genome, which is further discussed in section 4.1.5.

The Ynd GR was classified into the GerA clade by Ross and Abel-Santos, a clade that also include the GerB GRs of *B. subtilis* and *B. amyloliquefaciens*, and the uncharacterized Ynd GRs of *B. cereus* and *B. anthracis* (Ross and Abel-Santos, 2010b). In accordance with their close relatedness, *B. licheniformis* GerA and Ynd both recognize L-alanine, L-cysteine and L-valine (**Paper I**). L-alanine and L-valine are known to also induce germination via the GerA GR in *B. subtilis* (Paidhungat and Setlow, 2000; Setlow, 2003).

B. licheniformis is so far the only species known to encode a functional Ynd GR. The *ynd* operon is highly conserved in the *B. licheniformis* strains we investigated in our study, including the *B. paralicheniformis* strains included (**Paper III**). In contrast, the *ynd* operon was not conserved in all wild strains of *B. subtilis*, indicating that the selective pressure to maintain this operon was significantly lower in this species (Alzahrani and Moir, 2014). The interdependence between the GerA and the Ynd GR in *B. licheniformis* could be a major determinant for the maintenance of the *ynd* operon. When the entire *ynd* operon was deleted, the germination efficiency was considerably reduced (ca. 50-65 % reduction: **Paper III**). As mentioned in section 4.1.1, *B. licheniformis* spores germinate in response to L-cysteine, an uncommon germinant among the *B. subtilis* group members. Ynd is particularly important for L-cysteine-induced germination, as deletion of the

complete *ynd* operon results in a poor spore germination when spores are exposed to L-cysteine (36 % germinated spores after 2 h exposure to high concentrations of L-cysteine, compared to 98 % germinated spores for wild-type spores, **Paper III**).

The tricistronic organization of the *gerA*-family genes is the most common operon organization, based on a screening of previously published of *Bacillales* and *Clostridiales* genomes (Paredes-Sabja et al., 2011). Nevertheless, orphan *ger* genes as well as *ger* operons with multiple A, B or C subunits exist, but the significance of the number of subunits in a *ger* operons is not understood. In *B. megaterium*, the germination receptor *gerU* tricistronic operon (ABC) is flanked by an orphan *gerVB* gene. Studies have shown that the B subunits GerUB and GerVB can be used interchangeably (Christie and Lowe, 2007). In our study, *gerAB* null-mutant spores germinated very poorly in response to nutrients, indicating that the B subunits of the *ynd* operon cannot be utilized interchangeably with B subunits of other GRs, and that the GerA GR cannot function when the B subunit is removed (**Paper III**). The B subunits of the *B. megaterium* GerUB and GerVB showed high amino acid sequence identity (80 %), which could be a prerequisite for interchangeability (Christie and Lowe, 2007). In comparison, the *B. licheniformis* GerAB and YndE subunits only demonstrate sequence identities of approximately 35 % (results not shown).

Phylogenetic analysis showed that the YndE proteins of *B. licheniformis*, *B. paralicheniformis*, *B. subtilis* and *B. cereus* all formed a monophyletic clade, in which the *B. licheniformis* $YndE_1$, $YndE_2$ and $YndE_3$ formed distinct monophyletic groups (**Paper III**). Because the *B. licheniformis* $YndE_1$, $YndE_2$ and $YndE_3$ separate into three distinct phylogenetical clusters, this suggests that they could have different functions in germination (**Paper III**). Therefore, we originally hypothesized that the different YndE subunits recognize different nutrient compounds, and thereby contribute to a broader germinant recognition profile.

4.1.4 The consequences of *yndE* gene deletions for *B. licheniformis* spore germination (Paper III)

To unravel the role of the three B subunits encoded by the *ynd* operon, we analyzed the germination behavior of mutant spores where one or more of the *yndE* genes were deleted (**Paper III**). The *yndE3* and *yndE3E2* null-mutant spores germinated with a similar efficiency as the *yndD* single-null mutant spores and the spores lacking the entire *ynd*

operon. This indicated that the Ynd GR could not function without the $YndE_3$ and/or the $YndE_2$ proteins. Notably, the mutant lacking of all *yndE* genes (*yndE3E2E1* null-mutant spores) germinated a lot better than the single or double *yndE* null-mutant spores. Hence, the YndD and YndF GR components (A- and C subunits) are clearly functional in germination when all YndE subunits are absent, but their role in germination is not understood. This finding also indicates that an incomplete set of *yndE* genes can negatively affect the cooperation between the Ynd and GerA GR. Difficulties in creating *yndE2* and *yndE1* single null-mutants made it necessary to *cis*-complement *ynd*$^-$ and *yndE3E2E1* null-mutants with construct carrying *yndE* genes and the putative promoter region. Interestingly, complementing the *yndE3E2E1*-null mutant ($YndDF^+$) with the *yndE3* did not reduce the germination levels compared to the *yndE3E2E1* null-mutant spores, however, we cannot be sure from our experiments that the *yndE3* gene product in this strain is functional in germination. Complementing the same *yndE3E2E1*-null mutant with the *yndE3E2* gene complex, however, reduced the spore germination efficiency to levels indicating that the Ynd GR was no longer functional. Hence, the presence of the *yndE2* seem to obstruct the Ynd GR integrity (**Paper III**). This is similar to the inhibitory effect of the *B. megaterium* $GerK_2$ exhibit on the GerA GR function, although $GerK_2$ is a complete GR (Gupta et al., 2013). An incomplete set of YndE genes could somehow destabilize the germinosome. No studies have been done on germinosome formation in *B. licheniformis*, but a *gerD* gene is present in the *B. licheniformis* ATCC14580/DSM13 genome and all other investigated *Bacillus* species (Paredes-Sabja et al., 2011). GerD likely functions as a scaffolding protein in the colocalization of GRs into a germinosome, indicating that germinosome formation is universal at least within the *Bacillus* genus (Griffiths et al., 2011; Li et al., 2014).

When all *yndE* genes are deleted, the remaining YndD and YndF proteins can function successfully, and germination efficiencies approaching wild-type levels were achieved. A similar observation was done in *C. perfringens*, where the GerK GR does not require all three subunits in order to function, but can induce effective germination in the presence of only GerKA and GerKC (Paredes-Sabja et al., 2009). The deletion of *yndF* has so far not been successful. However, complementing an *ynd*$^-$ null mutant with the *yndD* gene alone (including the promoter region), restored germination to the levels seen for the *yndE3E2E1* null-mutant spores ($YndDF^+$) (**Paper III**). This indicates that the YndD subunit alone is functional in germination when GerA is present, and might be the GR component necessary the cooperative interaction with GerA. In *Clostridium* species monocistronic *ger* operons encoding A subunits do occur, however, the function of these

genes is unknown (Paredes-Sabja et al., 2011). *C. perfringens* encodes a single *gerAA* gene, however, the gene does not seem to be necessary for the germination response (Paredes-Sabja et al., 2011; Paredes-Sabja et al., 2008).

4.1.5 The role of the orphan *yndF2* gene in spore germination

The orphan gene *yndF2* encodes a C subunit homologous to the *yndF* gene of the *ynd* operon (Rey et al., 2004; Veith et al., 2004). However, the $YndF_2$ in *B. licheniformis* ATCC14580/DSM13 is truncated at the N-terminal, resulting in a putative gene product of only 184 amino acids, compared to the 399 amino acids long YndF protein. Analysis of the relative expression of *yndF2* compared to *rpoB* in strain MW3, showed that *yndF2* was expressed at approximately the same level as the *yndE* genes (**Paper III**). Despite this, we were unable to identify a promoter region of this gene corresponding to the σ^G consensus (**Paper III**). It should be noted, however, that the putative promoter of the *ynd* operon shared less similarity with the σ^G consensus compared to the putative promoters of the *gerA* and *gerK* operons, and it was located more than 100 bp upstream of the *yndD* start codon (**Paper I**). Therefore, it could be difficult to locate the *yndF2* promoter. Genome mining using the nBLAST function at the NCBI website (blast.ncbi.nlm.nih.gov) and *yndF2* from strain ATCC14580/DSM13 as a seed for searches, revealed that the majority of the sequenced *B. licheniformis* strains possessed the orphan *yndF2* gene. Interestingly, most of these strains carried a full-length *yndF2* gene (**Paper III**).

The C subunits of the *gerA* family operons are lipoproteins (Setlow, 2003). Lipoproteins contain a signal sequence with a lipobox motif and a conserved cysteine residue. This allows for diacylglycerol addition at the N-terminal, enabling the glycosylation of the pre-prolipoprotein, which, after cleavage to the prolipoprotein, aid the anchoring of the lipoprotein to the IM (Hutchings et al., 2009; Igarashi et al., 2004). We could not identify any signal sequence or cysteine residue in the translated *yndF2* sequence in the *B. licheniformis* ATCC14580/DSM13 genome (**Paper III**). Therefore, even if *yndF2* of strain MW3 is expressed, the absence of the signal sequence might obstruct its localization to the IM. However, diacylglycerol addition is not necessary for the function of some *B. subtilis* lipoproteins (Kempf et al., 1997; Leskelä et al., 1999). A previous study has shown that although lipoprotein modification is necessary for the function of the *B. subtilis* GerA and GerB GR, it is not necessary for the function of GerK (Igarashi et al.,

2004). In our study, however, deletion of *yndF2* had no effect on the germination in response to L-cysteine, L-valine or L-alanine (**Paper III**).

4.1.6 Moderately high pressure-induced spore germination in *B. licheniformis*

High pressure (HP) is an established non-nutrient trigger of spore germination in *Bacillus* species and the mechanism of how germination is induced depends on the level of pressure administered (Clouston and Wills, 1969; Gould and Sale, 1970; Paidhungat et al., 2002; Reineke et al., 2013b; Wuytack et al., 2000). In **paper II** we investigated the effect of HP on *B. licheniformis* spores using two pressure levels (moderately HP (mHP) of 150 MPa and very HP (vHP) of 550 MPa). Exposure of *B. licheniformis* spores to mHP induced a GR-dependent germination response (**Paper II**). By analyzing *ger* double-null mutant spores' response to mHP we found that the Ynd GR alone was sufficient for induction of a strong germination response and was the GR that contributed most to mHP-induced germination. However, Ynd was not essential for the mHP-induced germination, as GerK alone also triggered a slightly less effective response to mHP compared to Ynd. Because of the error with the NVH-1376 strain (supposed $GerA^+$ strain) (see section 4.1.2), we have not analyzed how efficiently GerA alone can induce germination in response to mHP. However, the disruption of GerA did not significantly reduce the germination response, indicating only a minor role for GerA in mHP-induced germination (**Paper II**). A study of *B. subtilis ger* mutants have shown a hierarchical ordering of GRs contribution to mHP-induced germination, as the GerA GR alone triggered the strongest germination response, followed by GerB and lastly GerK, which also coincides with the GRs relative efficiency in nutrient-induced germination (Black et al., 2005). What determines the individual GRs sensitivity to mHP is not understood, however, published reports indicate that both GR levels in the IM and their structure are important determinants for their HP sensitivity (Black et al., 2005; Doona et al., 2014). In **paper I** we show that the *yndD* and *gerKA* genes are expressed at approximately 10 times higher levels relative to the *gerAA*. By assuming cotranscription of all genes in the *ger* operons, this could coincide with their relative efficiency in mHP-induced germination. However, attempt in our laboratory to determine the levels of GR proteins in the *B. licheniformis* IM using Western Blot have so far been unsuccessful and therefore, we cannot confirm the association between GR levels in the spores IM and their relative importance in triggering mHP-induced germination.

4.1.7 Germination in response to very high pressure was inefficient at moderate temperatures

Whereas the GRs are well-established molecular targets for mHP-induced germination, the mechanism of how vHP (>300 MPa) triggers germination is not completely understood. Exposure of *B. subtilis* spores to vHP at ambient to moderate temperatures, results in release of CaDPA and germination of spores, although the germination is less efficient than during exposure to mHP (Reineke et al., 2013a). The GRs are redundant in this process. Instead, vHP permeabilizes the IM independent of GRs and there is strong evidence that vHP acts on a protein target in the IM, rather than on the IM itself (Black et al., 2007b; Paidhungat et al., 2002; Wuytack et al., 1998). The SpoVA proteins likely form mechanosensitive DPA channels in the IM (Velásquez et al., 2014). Doona *et al*. reported that deletion of the transcription factor *ylyA* reduced the vHP-induced germination rate, likely because *ylyA* spores had reduced levels of SpoVA proteins in their IM (Doona et al., 2014). In **paper II**, we show that exposure to 550 MPa at 37°C was much less effective in triggering germination of *B. licheniformis* compared to *B. subtilis* spores. Interspecies differences in the IM composition or differences in the structure of the protein target(s) could explain the variability in spores' sensitivity to HP. The pressure level of 550 MPa used in our study induced efficient germination of *B. subtilis* spores in previous studies (Doona et al., 2014; Reineke et al., 2012; Reineke et al., 2013a), but might not be optimal for activating the target structure(s) in *B. licheniformis* spores. Increasing the temperature from 37 °C to 60 °C during the vHP treatment resulted in efficient germination and inactivation of *B. licheniformis* spores. The effective inactivation of *B. licheniformis* spores by combining vHP and high temperatures is in line with previous reports (Margosch et al., 2004; Mathys et al., 2007). The ability of the fluorescent DNA dyes Syto16 (membrane permeant) and propidium iodide ([PI] membrane impermeant) to bind in *B. subtilis* and *B. licheniformis* spores treated at vHP and ≥60 °C has previously shown to be reduced compared to spores treated with HP at 37 °C (Reineke et al., 2013a; Reineke et al., 2013c). A less efficient cortex degradation at higher temperatures has been suggested as an explanation for the reduced staining (Mathys et al., 2007; Reineke et al., 2013a; Reineke et al., 2013b; Reineke et al., 2013c). Comparisons between data from the flow cytometry (FCM) analyses and the plate counts showed that staining with Syto16 and PI was less efficient for spores treated with vHP at 60 °C than for spores treated at mHP or vHP at 37 °C also in our experiments, as a subpopulation of the vHP/60 °C-treated spores remained unstained even if the level of spore inactivation was high (**Paper II**).

4.1.8 Spores of the *B. licheniformis* food-isolate NVH-1032 germinated slowly in response to high pressure

Margosch *et al.* reported large inter- and intraspecies variations in the level of spore inactivation after treatment with vHP and high temperature (Margosch et al., 2004). Similarly, we also detected differences in germination behavior in response to mHP and vHP, between the *B. licheniformis* strains MW3 and NVH-1032 (**Paper II**). The *B. licheniformis* strain NVH-1032 was originally isolated from a spoiled batch of double-heat-treated canned meat. This strain has previously been shown to germinate slowly in response to nutrients (Madslien et al., 2014) and the slow-germinating phenotype of this strain was likely the factor enabling it to survive the double-heat treatment regime. Spores of strain NVH-1032 also germinated less efficiently in response to mHP compared to spores of the *B. licheniformis* strain MW3 (type-strain derivative) and the *B. subtilis* strain PS832. As expected based on results from analysis of strain MW3, exposure of NVH-1032 spores to vHP at 37 °C did not induce germination. Additionally, NVH-1032 spores germinated at a slower rate, compared to spores of the other investigated strains, in response to vHP at 60 °C, even if germination was more efficient at these conditions than at 150 MPa (37 °C) **(Paper II**).

4.1.9 Why do some strains germinate slowly?

We, as well as others, have observed a large heterogeneity in germination behavior, even in clonal populations. Some spores in a population will germinate quickly after exposure to nutrients or mHP, while others will germinate only after some time (Chen et al., 2006; Kong et al., 2014; Vary and Halvorson, 1965; Zhang et al., 2010; Zhang et al., 2014). Studies have shown that the time between germinant addition or mHP exposure and the beginning of CaDPA release vary, and this lag time seems to be dependent on previous heat activation, nutrient concentration and level of GRs in the IM (Zhang et al., 2010). An important event during lag time is that the spores become committed to germination (Setlow et al., 2012). A proposed model for commitment is that a threshold level of GRs must be activated for the spores to commit to germination, and this model is supported by the relationship between the level of GRs in the IM, and germination rate (Setlow et al., 2012; Stewart et al., 2012; Yi et al., 2011).

The level of GR expression is assumed to be determined by stochastic events and sporulation conditions, but any role of hereditary factors is not well understood (Setlow et

al., 2012). The stochastic and environmental factors coincides with the heterogeneity within a clonal population and the existence of superdormant spores, but in the case of slow-germinating strains there must be hereditary factors involved that affect the germination ability. Several studies have shown that sequence characteristics of germination receptors (i.e. amino acid substitutions, the presence of pseudogenes) affected the germination phenotype observed in different strains of the same species to some degree, however, the sequence characteristics were not sufficient to explain the existence slow-germinating strains (Krawczyk et al., 2017; Madslien et al., 2014; Warda et al., 2017). By complementing the type-strain derivative MW3*ΔgerAA* with different *gerA* variants from slow-germinating strains, Madslien *et al.* demonstrated that the slow-germinating phenotype of strain NVH-1032 and other slow-germinating strains could not entirely be explained by sequence characteristics of the *gerA* operon (Madslien et al., 2014). No correlation between *ger* gene expression and germination phenotype has been found (Krawczyk et al., 2017; Madslien et al., 2014). Therefore, more studies are needed to establish the role of *ger* gene sequence characteristics and germination efficiency.

Recently, a transposon carrying, among other genes, a *spoVA*^{2mob} operon, was associated with slow germination and increased heat resistance. This transposon was found in *B. subtilis* spores, as well as in *B. licheniformis* and *B. amyloliquefaciens*, and spores of strains carrying this transposon exhibited a slower CaDPA release than spores of strains without the transposon (Berendsen et al., 2016b; Krawczyk et al., 2016). A study of several *B. subtilis* strains concluded that germination efficiency was likely determined both by the existence and copy number of the *spoVA*^{2mob} operon, and the sequence characteristics of *ger* genes (Krawczyk et al., 2017). It remains to be determined if slow-germinating NVH-1032 and other slow-germinating *B. licheniformis* food isolates carries this transposon. Reineke *et al*. found that spores' CaDPA-release rate was the limiting factor to spore inactivation during treatment high-pressure thermal sterilization [HPTS] (Reineke et al., 2013c), and slow-germinating strains pose a problem for effective spore inactivation in food products using methods relying on spore germination. Slow germination (inefficient CaDPA release) seems to be connected with higher thermal- and HP resistance (Krawczyk et al., 2016; Reineke et al., 2013c). In food processing environments slow-germinating strains may therefore survive thermal or HP processing and later cause food spoilage or disease.

4.1.10 Spore inactivation by high-pressure treatment

High-pressure processing (HPP) has the potential to inactivate spores while retaining the nutritional and sensorial qualities of the food. To achieve successful spore inactivation by HPP, germination must first take place during the pressure holding time, in order to make the spores susceptible to this treatment. *B. licheniformis* spore inactivation by mHP treatment was evident only after a holding time of 30 min (**Paper III**). Studies of the physiological state of mHP-treated spores using flow cytometry, showed that Syto16 fluorescence intensity decreased with longer holding times and that the majority of spores exhibited low Syto16 and PI fluorescence intensities when plate counts showed a high level of spore inactivation (uncultivable spores). The mechanisms for HP inactivation is not known, but the low PI fluorescence intensities indicate that there are considerably less IM damages in mHP-treated spores than in heat-inactivated germinated spores (**Paper II**). The loss of Syto16 fluorescence intensity observed in mHP-treated spores after holding times >5 min may imply irreversible damages to cellular components (**Paper II**). Previous studies suggest that *de novo* protein synthesis occurs early in germination (Keijser et al., 2007; Sinai et al., 2015). It has been proposed that HP exposure could result in lethal protein misfolding of newly synthesized proteins, or denaturation of already present proteins necessary for outgrowth of the vegetative bacteria (Reineke et al., 2012).

Increasing the pressure to 550 MPa resulted in minimal germination and inactivation of *B. licheniformis* spores (**Paper II**). *B. subtilis* spores germinated under these conditions, but high-pressure inactivation of the spores was inefficient. *B. subtilis* spores exposed to 550 MPa seems to have degraded the cortex as they stain with Syto16 (**Paper II**) (Reineke et al., 2013a). However, binding of Syto16 seems to be slower, likely because the SASP are not completely degraded (Kong et al., 2010; Reineke et al., 2013a). The synergistic effect of vHP and high temperatures has the best potential for commercial use (Reineke et al., 2013b). Even if the variable Syto16 and PI staining behavior indicates that the cortex hydrolysis is ineffective in spores treated at these conditions, the spores rapidly release CaDPA and becomes heat-sensitive (Reineke et al., 2013c).

4.1.11 Can high-pressure processing be used commercially to combat spores?

At the time of writing, HPP has not yet been commercially implemented for spore control. Spore inactivation is inefficient during HPP at ambient to moderate temperatures (Daryaei et al., 2016). In our study, we achieved a 4-log spore inactivation only after more than 30 min mHP exposure, likely due to high-pressure inactivation of germinated spores (**Paper**

II). Such long treatment times are not ideal for the food industry. The potential to use mHP to induce spore germination, and subsequent treatment to inactivate spores in a Tyndallization-like approach has been considered (Gould, 2006; Lenz and Vogel, 2015). For instance, the application of pressure cycling (multiple, subsequent pressure treatments), utilizing the mHP-induced germination, could result in effective spore inactivation (Black et al., 2007a). However, mHP induction of germination and subsequent spore inactivation are sensitive to spore-to-spore variations in GR levels, the presence of superdormant spores and the variation in germination efficiencies between strains and species (Gould, 2006; Lenz and Vogel, 2015). Additionally, continuous HP cycling would reduce the lifetime of the processing equipment, which results in increased costs for the food producers. The two concepts of HP sterilization that has received researchers' (and commercial) interest are HPTS and the FDA approved PATS (pressure-assisted thermal sterilization). HPTS utilizes the synergistic effect of vHP and elevated temperature on spore inactivation, whereas PATS exploits the adiabatic heat of compression to rapidly achieve a sterilization temperature of 121 °C (Daryaei et al., 2016; Lenz and Vogel, 2015; Reineke et al., 2013b). Both concepts suffer from the lack of understanding of the mechanism of spore inactivation and the difficulties modelling (predicting) the inactivation kinetics (Lenz and Vogel, 2015; Reineke et al., 2013b).

4.2 Methodological considerations

4.2.1 Strain selection

In contrast to the highly transformable model organism *B. subtilis* 168 and its derivatives, *B. licheniformis* ATCC14580/DSM13 contains a restriction modification system (RMS) making genetic manipulation difficult and time consuming (Rey et al., 2004; Veith et al., 2004). The availability of the readily transformable ATCC14580/DSM13 derivative strain MW3, in which the RSM genes *hsdR1* and *hsdR2* have been knocked out, has made genetic manipulation of *B. licheniformis* possible (Waschkau et al., 2008). Strain MW3 was used for the majority of experimental work in this thesis and facilitated characterization of the *gerA*, *ynd* and *gerK* operons. For **paper II**, the *B. licheniformis* strain NVH-1032 and the *B. subtilis* strain PS832 was also included. NVH-1032 was chosen because of its slow-germinating phenotype and it responded well to the standardized sporulation and growth conditions optimized for the MW3 strain. *B. subtilis* strain PS832 is a trp^+ derivate of the commonly used strain 168 (Paidhungat and Setlow, 2000). It has been used in in a number of publications studying spore germination with nutrients and HP, and served as a basis for comparison in our HP experiments (Black et al., 2005; Black et al., 2007b; Doona et al., 2014; Paidhungat et al., 2002; Paidhungat and Setlow, 2000; Reineke et al., 2012; Reineke et al., 2013a; Reineke et al., 2011; Reineke et al., 2013c; Yi and Setlow, 2010).

4.2.2 Construction of *ger*-deletion mutants and complementation tests (Paper I and III)

The majority of mutants used in this thesis was constructed using a markerless gene-replacement method originally developed for *Bacillus anthracis*. This method relies on double cross-over events that ultimately replaces the target gene with a start and a stop codon (Janes and Stibitz, 2006). The only exception was NVH-1307, the Δ*gerAA* mutant, in which most of the *gerAA* gene had been replaced with a spectinomycin-resistance cassette (Løvdal et al., 2012). The presence of this cassette did not affect sporulation efficiency of the NVH-1307 compared to the wild type, but any effect on the transcription of downstream genes cannot be ruled out.

The *ynd* operon proved more difficult to manipulate than the other *gerA* family operons. Several attempts were necessary in order to construct deletions of the *yndE3*, *yndE2* and *yndE1* genes of the *ynd* operon. Problems seemed to arise at the second cross-over stage. By increasing the erythromycin concentration from 1 µg/ml to 5 µg/ml in the

selective plates, during the second cross-over event, the yield of correct recombinants was enhanced. The presence of several subsequent genes with high sequence homology (the *yndE* genes) may have been causing problems with achieving the wanted crossovers. For unknown reasons, construction of *yndE2*- and *yndE1* single-null mutant has so far failed, despite the successful construction of an *yndE3*-null mutant (**Paper III**).

Inadequate control of the mutant strain NVH-1376 lead to the results from germination experiments using *gerAA yndD gerKAC* null-mutant spores being presented as the results of *yndD gerKAC* null-mutant spores in **paper I** and **II**. This could have been avoided by testing for spectinomycin resistance of the NVH-1376 strain, however, the mistake was not discovered until after the publication of two articles. The successful construction of an *yndDE3E2FE1 gerKACB*-null mutant provided accurate insights into the GerA function (**Paper III**).

Complementation of the *yndD* mutation in strain NVH-1335 was done *in trans* using the vector pHT315 in **Paper I**, in accordance with the previous work at our laboratory (Løvdal et al., 2012). Re-introduction of the *yndD* gene resulted in restoration of the germination efficiency to near wild-type levels. However, complementation pHT315_*yndDF* in the NVH-1387 strain (Δ*yndDE3E2FE1*) could not restore the germination level to that of the strain expressing *yndDF* (Toril Lindbäck, *unpublished results*). The copy number of pHT315 is expected to be moderate; approximately 15 per chromosome in *B. thuringiensis* (Arantes and Lereclus, 1991). Hence, the levels of *yndD* and *yndF* proteins are likely higher than the levels of the native protein in the wild type, which could affect the germination efficiency. There could also can be an issue with plasmid distribution in the mother cell and forespore compartments, resulting in spores with little to no plasmid present. Attempts to construct *cis*-complemented mutants which carried the complemented gene in the *amyL* locus were done for **Paper III**. All complementing gene constructs carried the respective promoter sequence for the gene(s), in addition to the *amyL* flanking sequences, and was cloned into the thermosensitive shuttle-vector pMAD (Arnaud et al., 2004). The complete plasmid did, however, integrate into the chromosome, either in the *amyL* locus or the promoter region for *ynd* (**Paper III**). This method of complementation restored germination a lot better than *trans*-complementation, and the construction of more *cis*-complemented strains are currently being performed in our laboratory.

4.2.3 Sporulation and spore purification

Bacillus species sporulate in response to nutrient deprivation, likely as a last resort based on a multi-step, bimodal decision-making system. In natural systems, only a subpopulation will undergo sporulation. The rest of the population may take on other roles; production of biofilms, becoming competent, engaging in enzyme secretion or lysis. In the laboratory, we need sporulation to be more synchronized and to achieve high spore yields. The conditions for *B. licheniformis* spore production was optimized in previous projects on *B. licheniformis* spores done in our laboratory (Løvdal, 2012; Madslien, 2013).

In the thesis work, we produced spores by using a rich, complex sporulation medium and achieved nutrient exhaustion due to cell growth. All spores were produced at 37 °C at a pH in the range of 7.1-7.4. The Bacto-MS medium, which was used for sporulation, is based on a complex medium developed for *B. cereus* (van der Voort et al., 2010), and later optimized for *B. licheniformis* by Løvdal *et al*. By omitting maltose the spore yield was dramatically increased, furthermore, by reducing the concentration of $FeSO_4$ from 1 mM to 1μM the spore production was improved. (Løvdal, 2012; Løvdal et al., 2012). The *B. subtilis* spores used in **Paper II** were produced on 2X SG agar, which is also a rich and complex medium (Nicholson and Setlow, 1990). Both media are based on Difco nutrient broth (Becton, Dickinson and Company, NJ, USA) with addition of trace minerals, and glucose was also added to the 2X SG media. A suspension with at least 80 % phase-bright spores, was achieved after incubation for 3-5 days. Spores made in rich media will have high GRs levels, this will result in faster germination of the spores made in rich media, compared to if the spores were made in minimal media (Ramirez-Peralta et al., 2012). The *trans*-complemented strains carrying the pHT315 vector were produced in the presence of 1 μg/ml erythromycin in order to maintain the plasmid. These strains showed a slower sporulation and reduced spore yields. However, the effect of pHT315 was not studied further in this thesis work.

After harvesting, *B. licheniformis* spores were purified by use of Nycodenz® density gradient. No effect of Nycodenz® was found on the germination behavior of *B. licheniformis* spores (E.H. Madslien, *unpublished results*). *B. subtilis* spores were purified only with deionized water to not divert from protocols used in previous studies (Reineke et al., 2012; Reineke et al., 2013a; Reineke et al., 2013c). During spore purification, spores are exposed to mechanical stressors such as centrifugation and resuspension. To the best of our knowledge, the centrifugation and resuspension steps are necessary for purification of spores and therefore are unavoidable. Spores were stored dark

at 4°C in sterile MilliQ water before use. Spores were used within 3 months, as older spore batches can germinate spontaneously. Hence, the effect of aging on the germination behavior were not accounted for. Despite standardized protocols for spore production, some batch variation was seen in the germination behavior with both nutrients and mHP. Spore characteristics, such as germination efficiency and wet-heat resistance, can be influenced by several factors during sporulation. The use of a complex medium could result in some batch variation, which could have been avoided by the use of a defined medium. Segev *et al.* showed that spores continue to mature after the spore is released from the mother cell, as there is a period of RNA degradation and synthesis. These processes are temperature dependent, and may be a way for the spore to further adapt to the surrounding environment (Korza et al., 2016; Segev et al., 2012). Because sporulation is not completely synchronized, this could mean that spores that were released early, would have more time to mature at 37°C than spores that were formed later. Therefore, spores in the same population can have different characteristics depending on how early they were formed (Hornstra et al., 2009). Additionally, the sporulation protocol has been optimized for the type strain DSM13 and its derivatives. Therefore, it may not be optimal for studying the germination behavior of the food isolate NVH-1032, the strain that showed the most batch variation.

4.2.4 Nutrient-induced germination (Paper I and III)

To induce germination, single nutrient compounds were added at high concentrations to spore suspensions before incubation at 37°C for 2 h, unless otherwise stated. 37°C has been described as the optimal germination temperature for *B. licheniformis* spores (Halmann and Keynan, 1962).We performed a screening of 23 nutrient compounds in **Paper I**, to find the most potent germinants. In agreement with the 1963 paper by Martin and Harper, L-alanine, L-cysteine and L-valine was found to be the strongest germinants (Martin and Harper, 1963). These germinants, in addition the weak germinant glucose, were used for most nutrient-induced germination assays presented in this thesis. The germinant concentration of 100 mM, which was used in most germination assays, is unnaturally high, but allowed for the identification of weak germinants. For the germination assays the spores were suspended in potassium phosphate buffer (pH 7.2), as described previously (Martin and Harper, 1963). Because potassium was always present in the nutrient-germination assays,

it could positively impact the germination with glucose as described for *B. subtilis* (Atluri et al., 2006). However, potassium alone could not induce germination.

4.2.5 Detection of germination

As the spores germinate, they undergo a number of physiological changes. Some of these changes are measureable and used to detect germination. Below, different methods used for detecting germination in the thesis work are discussed.

Germination assays (OD_{600} measurements)

As spores germinate the OD_{600} of the spore suspension decreases. The decrease in OD_{600} corresponds to the level of germination in the whole spore population (Powell, 1950). In **papers I** and **III**, the optical density at 600 nm (OD_{600}) was measured over a period of 2 h, after addition of the germinant solution. The maximum rate of germination (G_{max}) was determined using the dynamic modeling fit (DMFit) (browser.combase.cc/DMFit.aspx). DMFit was developed to fit bacterial growth curves where a linear phase is preceded by a lag phase and followed by a stationary phase, using the Baranyi and Roberts model (Baranyi and Roberts, 1994). The ability of the germination assays to detect low-levels of germination is limited. Clumping and spore adhesion to the well walls in the 96-well plates used in these assays, can also interfere with the measurements.

Phase-contrast microscopy

Using phase-contrast microscopy, we can observe the change in the refractive index of the spore as it germinates. Dormant spores appear as phase-bright, but changes to phase-dark spores during germination. By counting phase-dark and phase-bright spore in 10 fields of view (approximately 300-500 spores), we calculated the percentage of germinated spores, to complement the results from the OD_{600} measurements (**Paper I** and **III**). Due to the manual counting of spores, there were limitations to how many spores could be counted for each individual experiments.

Wet-heat-sensitivity assays (plate counts)

In **Paper II** we chose the plate-count method to determine the amount of germinated spores in response to HP. As spores germinate they lose their heat resistance, and will be inactivated by a heat treatment at 80°C for 20 min. We spread appropriate dilutions of a spore suspension on nutrient agar before any treatment (N_0), after HP treatment (N_1) and following an additional heat treatment at 80°C for 20 min (N_2). By calculating the log ratio

(log (N_0/N)), we could determine the log HP inactivated spores and the log HP-germinated spores.

Flow cytometry analyses

During stage II of spore germination, cortex hydrolysis will take place. This will make the IM accessible to the membrane-permeant nucleic-acid dye Syto16, and we can monitor the level of germinated spores by measuring the green fluorescence intensity using flow cytometry (Black et al., 2005; Mathys et al., 2007).

The membrane-impermeant nucleic-acid dye PI, which gives red fluorescence, cannot stain spores unless the IM permeability is compromised. PI was therefore used as an indicator of spore inactivation, as described in previous publications (Mathys et al., 2007). PI could only stain spores with IM damages, and was not ideal to detect the viability of mHP-treated spores. Even though we detected high numbers of uncultivable (inactivated) spores in the plate counts after >30 min mHP exposure, the PI-fluorescence intensity still remained low. This phenomena has also been described before, with *B. subtilis* and *B. licheniformis* spores (Mathys et al., 2007).

Determining the gating strategy for the FCM analyses were done on the basis of previous reports on *B. licheniformis,* as well as FCM analyses of spore suspensions controlled by plate counting and phase-contrast microscopy. However, the borders of regions R1-R5 were set manually using the Kalzua 3.1 analyzing software and are therefore affected by subjective considerations.

For logistic reasons, the FCM analyses were performed on two different systems. The majority of analyses presented in **Paper II** were performed on the CyFlow ML cytometer that had the best resolution and could measure a total of 200 000 events. The, the FACSCalibur™, which was the other system used in our analyses, measured total of 10 000 events. We chose to compare the results from the non-germinating NVH-1376 strain from the FACSCalibur™ instrument, with the results from the CyFlow ML in **Paper II**, Fig. 4. Simple comparisons of FCM analyses of strains MW3 and PS832 treated at mHP at 37 °C done on both instruments showed that, although the resolution was much enhanced in the CyFlow ML, the results from the two instruments are comparable (results not shown).

In some cases, FCM analyses showed that a subpopulation of spores had germinated, while this was not detected by plate counting. When comparing FCM data and plate count data one has to take into account that FCM data is expressed as percentage of the total spore population, while plate count data is expressed as log spores. For instance,

for NVH-1368 spores treated with mHP **(Paper II)** the FCM data show that a large percentage of spores have germinated, more specifically 91.5 % spores, after 2 h HP exposure. This is not visible to the same degree in the plate counts. However, if 91.5 % of the spore population is heat-sensitive after the 2 h of mHP treatment, this would corresponds to 1 log (N_0/N) heat-sensitive spores detected in the plate counts when the initial spore titer is 8 log spores/ml. Hence, this is more difficult to detect by plate counts, especially when we consider a certain uncertainty in the measurements both for FCM analyses (10-15 %; K. Reineke, *personal communication*) and the plate counts.

4.2.6 High pressure experiments (Paper II)

To achieve high-pressure conditions, two separate HP units were used for spore treatment. Logistic reasons lead to the use of two units, a monovessel unit and a multivessel unit. Both units are made by the same company and are of the same model (U111), but differed in the number of chambers and whether there was a manual (monovessel unit) or automatic pump (multivessel). Additionally, the vessel volume differed. In the monovessel unit approximately 230 µl of spore suspension was transferred into shrinking tubes and four of these tubes were placed into a Nunc Cryotube (**Fig. 11**). This allowed for treatment of several technical replicates and strains at once. For the multivessel unit, spore suspension was filled into 1-ml Nunc Cryotube. Any effects of the differences in volume of the treated spore suspensions were not accounted for in these experiments. However, by comparing of wild-type spores HP treated in both units, we could not detect differences that exceeded the batch variations.

Fig. 11: Shrinking tubes used in HP experiments done in the monovessel unit. Four shrinking tubes (left) were placed in a Cryotube (right) before HP treatment.

Upon pressurization, the adiabatic heat of compression of the pressure-transmitting medium and the spore suspension medium will result in a temperature increase. Sebacate (di-2-ethyl-hexyl-sebacate) or silicon oil were used as pressure-transmitting liquids. The adiabatic heat of compression was utilized to quickly reach the target temperatures (37 °C or 60 °C), by initiating compression at an empirically determined starting temperature. The temperature of the spore samples was measured continuously throughout the HP treatments, and stayed constant over time. Heat loss was avoided by submerging the pressure chambers in a thermostatic bath with silicon oil. The spore suspension buffer for all HP experiments were N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer (pH 7.0). HP and high temperatures can lead to a shift in the disassociation equilibrium in aqueous systems that will result in a pH shift. ACES buffer was chosen due to its stable pH during HP treatments (Mathys et al., 2008).

Compression rates were 25 MPa/min for the multivessel unit and approximately 12 MPa/min for the monovessel unit, due to the manual pump. By determining the HP-induced spore germination and inactivation after compression (1 sec holding time) by plate counting, we were able to determine any effect of compression on the spores.

Due to time constraints, most HP experiments were only done using two independent spore batches, whereas the in experiments presented in **Paper I** and **III** we use three independent spore batches. As a consequence, the interpretation of the results in **Paper II** will be more sensitive to batch variation. Therefore, we chose to show results for each individual spore

batch in the germination and inactivation plots and not perform statistical analyses. However, the standard error of estimate (SE_{est}) for the regression analyses performed is presented, as it gives a measurement of the accuracy of the regression line prediction.

5. Conclusions

This thesis work has provided the following conclusions regarding *B. licheniformis* spore germination.

- Multiple L-amino acids, as well as glucose, initiated germination of *B. licheniformis* spores.
- The GerA, GerK and Ynd GRs acted together to facilitate an efficient germination response with the L-amino acids germinant L-alanine, L-cysteine and L-valine.
- Cooperation between GerA, GerK and Ynd increased the germination efficiency, but were not essential for GR function.
- The *ynd* operon encodes three B subunits (the *yndE* genes), contrary from the canonical *Bacillus gerA* family operons, which encode one B subunit. When we deleted one or two *yndE* genes, the Ynd receptor was not functional. However, if all three *yndE* genes were deleted, the remaining A and C subunits were functional in germination. Further analyses suggested that it was the A subunit that functioned in germination, and that the C subunit was dispensable. Hence, the Ynd A subunits seem to be sufficient for cooperation with GerA.
- In nutrient-induced germination, GerA was the central germination receptor, whereas in germination triggered by pressures of 150 MPa, the Ynd receptor contributed the most to germination.
- Spores of *B. licheniformis* did not germinate in response to 550 MPa when treated at 37 °C. However, when the temperature was increased to 60 °C during HP treatment, spore germination and inactivation was effective. However, strain variation regarding germination and inactivation at these conditions will be a challenge for implementation of high-pressure thermal sterilization regimes for spore control.

6. Future perspectives

- The construction of more *ynd* deletion mutants will further dissect the role of the different Ynd subunits in germination. Construction of an *yndE3 yndE1* double-null mutant carrying only an intact *yndE2* gene will determine if $YndE_2$ can function alone in the Ynd receptor. Additionally, an *yndF* null-mutant will determine the necessity of C subunit for receptor function.

- Introducing a complete *yndF2* gene from a closely related strain to the MW3 strain could determine if the orphan C subunit is functional in spore germination.

- In close proximity to the GerA and GerK operons there are small, uncharacterized open reading frames. The gene following the GerK operon shows strong homology to *mtaD*, a MerR family transcriptional regulator, not playing a role in germination. However, the gene upstream of *gerA* is still unknown. Further study into the potential D subunits should be done in *B. licheniformis*.

- The *ynd* and *gerK* operons should be sequenced in the slow-germinating strains of *B. licheniformis*, including strain NVH-1032, that were published by Madslien *et al*., 2013. Further study to determine if the slow-germinating phenotypes can be associated with deletions, frame shifts or point mutations in the GR operons, or possibly the presence of the newly described Tn159-like transposon.

- The protocol for IM protein isolation from *B. licheniformis* spores needs optimizing, to allow us to determine the levels of GerA, GerK and Ynd in the IM.

- There is a need to determine the 3-dimensional structure of GR components and the protein-to-protein interactions occurring between GRs, to further our understanding of spore germination.

7. References

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8. Scientific papers I-III

I

Author Correction for AEM00594-16

Correction for Borch-Pedersen et al., "The Cooperative and Interdependent Roles of GerA, GerK, and Ynd in Germination of *Bacillus licheniformis* Spores"

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Volume 82, no. 14, p. 4279--4287, 2016, https://doi.org/10.1128/AEM.00594-16. The strain NVH-1376, which was presented as a Δ*yndD* Δ*gerKA-C* double mutant, was found to contain a spectinomycin resistance cassette inserted into the *gerAA* gene, making it a *ger*-null mutant, and the lack of germination of this strain is because there are no functional germination receptors present.

Hence, there is no evidence indicating that the GerA germinant receptor cannot function alone in nutrient-induced germination, as was originally argued in our published paper. All statements in the Abstract, Introduction, Results, and Discussion that GerA cannot induce germination when present as the single, intact germination receptor should be disregarded.

Page 1, Abstract, lines 10 and 11: “Neither GerA nor Ynd could function alone” should read “Ynd could not function alone.”

Page 2, Table 1, column 2, row 7: “Δ*yndD* Δ*gerKA-C*” should read “Δ*gerAA*::*spc* *ΔyndD* Δ*gerKA-C*.”

Page 2, column 2: Lines 3 and 4 should read as follows. “...Furthermore, we showed that GerK was essential for D-glucose-induced germination.”

Page 5, Table 4, column 1, row 7: “Δ*yndD* Δ*gerKA-C*” should read “Δ*gerAA* Δ*yndD* Δ*gerKA-C*.”

Page 5, column 2: Lines 25 -- 27 should read as follows. “…To further examine whether there is a functional interplay between GerK and other GRs, we analyzed the germination efficiency of *gerAA gerKA-C* double-null mutant spores, expressing only GerA.”

Page 5, column 2: Lines 27 -- 33: The sentence beginning with “Spores expressing only GerA…” and the sentence beginning with “This is in contrast…” should be deleted.

Page 5, column 2: Lines 33 -- 37 should read as follows. “Spores of the *gerAA gerKA-C* double-null mutant, which only expresses Ynd, demonstrated a low level of germination in response to L-cysteine and L-alanine, but not to L-valine, which is very similar to the germination of the *gerAA*-null mutant expressing both Ynd and GerK (Table 4).”

Page 7, column 1: Lines 19 -- 27: The sentence beginning with “Mutant spores…”, the sentence beginning with “ Further analyses of …” and the sentence beginning with “The cooperative function…” should be deleted.

Supplemental material: In Fig. S4, top right panel, “Δ*gerKA-C* Δ*yndD*” should read “Δ*gerAA* Δ*yndD* Δ*gerKA-C*.” Strain names were included to clarify. Revised supplemental material is posted at http://aem.asm.org/content/82/14/4279/suppl/DCSupplemental.

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The Cooperative and Interdependent Roles of GerA, GerK, and Ynd in Germination of *Bacillus licheniformis* Spores

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ABSTRACT

When nutrients are scarce, *Bacillus* species form metabolically dormant and extremely resistant spores that enable survival over long periods of time under conditions not permitting growth. The presence of specific nutrients triggers spore germination through interaction with germinant receptors located in the spore's inner membrane. *Bacillus licheniformis* is a biotechnologically important species, but it is also associated with food spoilage and food-borne disease. The *B. licheniformis* ATCC 14580/DSM13 genome exhibits three *gerA* family operons (*gerA*, *gerK*, and *ynd*) encoding germinant receptors. We show that spores of *B. licheniformis* germinate efficiently in response to a range of different single L-amino acid germinants, in addition to a weak germination response seen with D-glucose. Mutational analyses revealed that the GerA and Ynd germination receptors function cooperatively in triggering an efficient germination response with single L-amino acid germinants, whereas the GerK germination receptor is essential for germination with D-glucose. Mutant spores expressing only GerA and GerK or only Ynd and GerK show reduced or severely impaired germination responses, respectively, with single L-amino acid germinants. Neither GerA nor Ynd could function alone in stimulating spore germination. Together, these results functionally characterize the germination receptor operons present in *B. licheniformis*. We demonstrate the overlapping germinant recognition patterns of the GerA and Ynd germination receptors and the cooperative functionalities between GerA, Ynd, and GerK in inducing germination.

IMPORTANCE

To ensure safe food production and durable foods, there is an obvious need for more knowledge on spore-forming bacteria. It is the process of spore germination that ultimately leads to food spoilage and food poisoning. *Bacillus licheniformis* is a biotechnologically important species that is also associated with food spoilage and food-borne disease. Despite its importance, the mechanisms of spore germination are poorly characterized in this species. This study provides novel knowledge on germination of *B. licheniformis* spores. We characterize the germinant recognition profiles of the three germinant receptors present in *B. licheniformis* spores and demonstrate that the GerA germinant receptor cooperates with the Ynd and GerK germinant receptors to enable an effective germination response to L-amino acids. We also demonstrate that GerK is required for germination in response to the single germinant glucose. This study demonstrates the complex interactions between germinant receptors necessary for efficient germination of *B. licheniformis* spores.

Endospore formation is a phenotypic adaptation to unfavorable environmental conditions, which allows bacteria to persist in the environment in a dormant and extremely resistant state. Nevertheless, spores are able to continuously monitor the environment for conditions favorable for growth. Many members of the bacterial orders *Bacilliales* and *Clostridiales* are able to survive starvation by forming endospores, which are much more resistant to heat, chemicals, irradiation, and desiccation than the vegetative cells (1). However, upon exposure to nutrient germinants, spores can return to active growth within minutes in the process of germination (2–5).

The nutrient-induced germination is initiated when specific nutrients are recognized by their cognate germinant receptors (GRs) located in the spores inner membrane (6–8). Multiple GR isoforms, with distinct nutrient specificities, have been characterized in different spore-forming species of the genera *Bacillus* and *Clostridium* (4, 9–15).

The genetic organization and knowledge gained from functional studies suggest that the GRs are heterotrimeric complexes consisting of A, B, and C subunits, and at least in *Bacillus*, they are all required for the formation of a functional receptor (3, 16). Genes encoding GR homologs are often organized in polycistronic so-called *gerA* family operons, encoding the A, B, and C subunits (8). These operons are only expressed in the developing spore under the control of the forespore specific RNA polymerase sigma factor σ^G (17–19). The individual A, B, and C subunits show significant intra- and interspecies homology with the corresponding subunits of other GRs (4, 10, 20), but the organization of GR operons varies between species and between different operons

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within the same genome (4). In *Bacillus subtilis*, five different tricistronic *gerA* family operons (*gerA*, *gerB*, *gerK*, *ynd*, and *yfk*) encode three functional GRs and two putative GRs (5). Furthermore, it has been reported that at least some GRs have an additional D subunit (4). Currently, the specific role of the individual subunits in the GR function is poorly understood.

Specific amino acids and ribonucleosides, either independently or in mixtures, act as powerful germinants of *Bacillus* spores. Among *Bacillus* species, L-alanine seems to be the most common germinant (10). In *B. subtilis*, GerA is required for L-alanine germination, whereas GerB and GerK act cooperatively to trigger germination with a mixture of L-asparagine, D-glucose, D-fructose, and K^+ called AGFK (21, 22). GerK is necessary for recognition of glucose (21, 23), whereas GerB seems to recognize several L-amino acids in addition to L-asparagine (21). A study of wild *B. subtilis* strains showed that the *gerA* operon was more conserved than the *gerK* and *gerB* operons and that the *ynd* and *yfk* operons were truncated or absent in many strains, suggesting that the selection pressure for maintaining these genes is low in this species (24).

Bacillus licheniformis is a facultative anaerobic sporeformer that is closely related to *B. subtilis*. It is economically valuable due to its production of various compounds such as enzymes, antibiotics, and surfactants that are used for various industrial applications. However, besides its beneficial properties, it is also a common food spoilage bacterium in milk, meat products, bread, and canned foods (25–33). *B. licheniformis* is also an occasional pathogen in humans and animals (34–36). It has been isolated from cases of food poisoning and there are reports linking *B. licheniformis* to severe diseases such as bacteremia or recurring sepsis in both immunocompromised and clinically healthy individuals (37–40). Despite its importance, detailed functional studies of *B. licheniformis* GRs and their germinant recognition patterns are still scarce. *B. licheniformis* has previously been described to germinate in response to L-alanine, L-cysteine, and L-valine (41). Three different *gerA* family operons and one single gene member of this family have been identified in the genome of the type strain *B. licheniformis* ATCC 14580/DSM13 (42–44). These are the *gerA*, *gerK*, and *ynd* operon orthologues and the orphan *gerAC* homologue *yndF2*. No homologue to the *gerB* operon is present in the genome of ATCC 14580/DSM13.

In *B. licheniformis*, *gerA* is required for germination induced by L-alanine and, similar to the *B. subtilis* 168 *gerA*, this response is strongly inhibited by D-alanine (45). Analysis of spore germination in *B. licheniformis* suggests that some *B. licheniformis* isolates germinate remarkably slowly when exposed to L-alanine and that the slow germination phenotype is, at least partly, explained by specific amino acid substitutions in GerA (46). Such slow-germinating strains pose a challenge to the food industry, which wants to implement induced germination as a strategy to eliminate spores during processing. The roles of *gerK*, *ynd*, and *yndF2*, if any, are still unknown.

We assessed the roles of amino acids, ribonucleosides, and AGFK in initiating *B. licheniformis* spore germination. We also characterized the functions of GerA and the putative germination receptors Ynd and GerK in more detail by constructing mutants in all three GR operons. By analyzing the germination responses of wild-type and mutant spores to amino acids and glucose, we could assign functional roles for the Ynd and GerK GRs, and we demonstrated their cooperative and interdependent role with GerA in triggering efficient germination responses to L-amino acids. Furthermore, we showed that GerK was essential for D-glucose-induced germination and that the cooperative function with GerA was independent of glucose activation of GerK.

TABLE 1 *B. licheniformis* strains used in this study

B. licheniformis strain	Genotype	Source or reference(s)
ATCC 14580/DSM13	Type strain	42, 43
MW3[a]	Δ*hsdR1* Δ*hsdR2*	47
NVH-1307	Δ*gerAA*::*spc*	45
NVH-1324	Δ*gerKA-C*	This study
NVH-1335	Δ*yndD*	This study
NVH-1368	Δ*gerAA*::*spc* Δ*yndD*	This study
NVH-1376	Δ*yndD* Δ*gerKA-C*	This study
NVH-1323	Δ*gerAA*::*spc* Δ*gerKA-C*	This study
NVH-1370	Δ*gerAA*::*spc* Δ*gerKA-C* Δ*yndD*	This study
NVH-1348	Δ*gerAA-C*	This study
NVH-1377	Δ*yndD*/pHT315_*yndD*$^+$	This study

[a] MW3 was used as a background strain for the construction of all mutants.

MATERIALS AND METHODS

Strains and culture conditions. The strains used in this study are listed in Table 1. The *B. licheniformis* strain MW3 was used as background for all gene-deletion mutants. *B. licheniformis* is difficult to manipulate genetically due to the presence of type 1 restriction modification systems (T1rm), which target foreign DNA. Two T1rm loci have been identified in ATCC 14580/DSM13 (42, 43). In the ATCC 14580/DSM13 derivative strain MW3, both T1rms are deleted, resulting in a significantly higher transformation frequency compared to the wild-type background (47). All *B. licheniformis* strains were cultured aerobically in brain heart infusion broth or Luria-Bertani broth at 37°C with agitation (225 rpm).

Spore preparation. Spores were prepared, harvested, washed, and stored as described previously (46). The spores were stored for at least 7 days prior to use. Spores of the *yndD* complementation strain NVH-1377 were made in the presence of 1 μg of erythromycin ml^{-1}.

Germination assays and inhibition by D-alanine. Spore germination was assessed by monitoring the optical density at 600 nm (OD_{600}), which decreases in the spore suspension during germination (48), as described previously (46). The purity of the spore suspension was determined by phase-contrast microscopy prior to use. Spore batches used in the germination experiments contained at least 98% phase-bright spores. Spores were heat activated (65°C, 20 min) before use. Amino acids (Sigma-Aldrich, USA), D-glucose (Sigma-Aldrich, USA), and KCl (Merck, USA) were used at a concentration of 100 mM in the germination assays unless noted otherwise. For inhibition with D-alanine, 100 μl of buffered (0.2 M K-phosphate buffer [pH 7.2]) spore suspension (OD_{600} of ~2) was mixed with 50 μl of 100 mM D-alanine and preincubated for 15 min at 37°C with agitation. Subsequently, 50 μl of 100 mM L-amino acid germinant was added, which resulted in a final germinant and D-alanine concentration of 25 mM, and a spore suspension with an initial OD_{600} of 1 (~10^8 spores ml^{-1}). All germination assays were repeated three times using independent spore batches unless otherwise stated, and the results are presented as the means of all replicates. Spore suspensions with Milli-Q water were used as negative controls. Phase-contrast microscopy was routinely used to monitor the level of germinated spores after 120 min of exposure to germinant compound and was particularly useful for assessing low levels of germination. The number of phase-dark (germinated) spores was determined for approximately 200 to 500 spores in each experiment by counting spores in 10 random fields of view, and the average percentage of germinated spores was calculated from three independent spore batches.

The maximum germination rate (G_{max}) was calculated from the linear

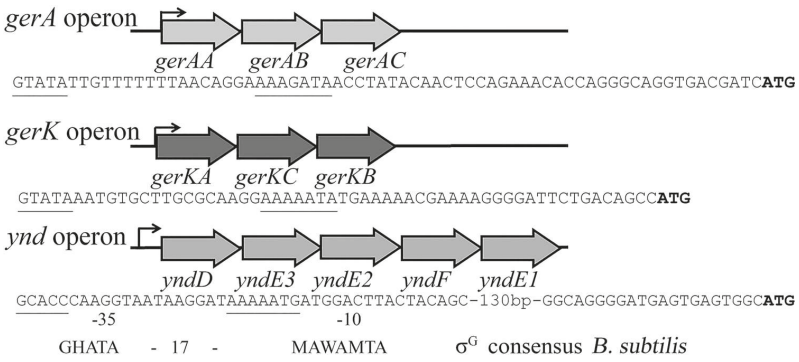

FIG 1 Organization of the *B. licheniformis* ATCC 14580/DSM13 *gerA* family operons. The putative promoter regions are indicated with arrows. IUPAC nucleotide ambiguity code: H = A, T, or C; M = C or A; and W = A or T.

segment of the curves of OD changes (presented in the supplemental material) using DMFit. DMFit (where "DM" stands for "dynamic modeling") fits curves where a linear phase is preceded by a lag phase and followed by a stationary phase (49). The germinant concentrations yielding 50% germination (C_{50}) were calculated using linear regression (www.geogebra.org) on the dose-response curves (see Fig. S1 in the supplemental material).

Mutant construction. According to the annotations in the NCBI database, *B. licheniformis* MW3 contains the following *gerA* family operons: the *gerA* operon (TRNA_RS38675, TRNA_RS38680, and TRNA_RS38685), the *gerK* operon (TRNA_RS23740, TRNA_RS23745, and TRNA_RS23750), the *ynd* operon (TRNA_RS32310, TRNA_RS32305, TRNA_RS32300, TRNA_RS32295, and TRNA_RS32290), and the orphan *gerAC* homologue *yndF2* (TRNA_RS32565).

The *ynd* operon of *B. licheniformis* MW3 contains five genes, *yndD*, *yndE3*, *yndE2*, *yndF1*, and *yndE1* (42, 43). The first gene in the operon, *yndD*, was deleted and replaced with 5′ATGTAG-3′ using a markerless gene replacement method (50) as described by Lovdal et al. (45). This method leads to an in-frame deletion of the target gene and ensures that the up- and downstream flanking sequences, including the promoter region, are intact.

To delete *yndD*, primers A and B (see Table S1 in the supplemental material) were used to amplify a 589-bp fragment upstream of *yndD*, and primers C and D were used to amplify a 530-bp fragment downstream of *yndD*. Primers B and C (see Table S1 in the supplemental material) carried a sequence overlap, and the resulting AD fusion PCR product thus contained the *yndD* upstream and downstream sequences. The AD product was ligated into the thermosensitive shuttle vector pMAD (51) containing an additional *I-SceI* site (kindly provided by Annette Fagerlund, Nofima, Norway). The pMAD-*I-SceI* Δ*yndD* plasmid was transformed into *B. licheniformis* MW3 electrocompetent cells as described previously (45). Integration of pMAD-*I-SceI* Δ*yndD* into the chromosome by homologous recombination was performed as described previously (51). After verification of the single crossover, pBKJ233 containing the gene encoding the *I-SceI* enzyme was introduced by electroporation. *I-SceI* makes a double-stranded DNA break at an 18-bp recognition site in pMAD-*I-SceI*. The repair of the strand break may lead to a second crossover, resulting in deletion of the target sequence. Deletion of *yndD* was verified by PCR using oligonucleotides located upstream and downstream of *yndD* (oligonucleotides A and D; see Table S1 in the supplemental material), followed by sequencing of the PCR product (Source BioScience Lifesciences, United Kingdom). All PCRs were conducted using an Eppendorf Mastercycler ep-Gradient S (Eppendorf, Germany) and Phusion high-fidelity DNA polymerase (Finnzymes, Finland) according to the manufacturers' instructions.

The *gerKA-C*-null mutant, wherein the two first genes of the *gerK* operon (*gerKA* and *gerKC*) were deleted in-frame, and the *gerAA-C*-null mutant, wherein all three genes of the *gerA* operon were deleted in-frame (*gerAA*, *gerAB*, and *gerAC*), were constructed according to the method described above and using the oligonucleotides listed in Table S1 in the supplemental material.

Complementation of *yndD*. The shuttle vector pHT315 (52) was used for transcomplementation of *yndD*. The *yndD* gene, including the promoter region, was amplified by PCR using the primers yndD-F and ynd-R (see Table S1 in the supplemental material) and Phusion high-fidelity DNA polymerase (Finnzymes, Finland) as described above. The amplicons were cloned into the pCR2.1-TOPO vector before introduction into the shuttle vector pHT315. The resulting construct was transformed into electrocompetent *B. licheniformis* NVH-1335, as described above. The presence of the correct plasmid construct was verified by PCR and sequencing. Complementation of the *gerA* operon has been described before (45).

RT-qPCR. The gene expression levels of *yndD* and *gerKA* were determined relative to *gerAA* by reverse transcription-quantitative PCR (RT-qPCR). A culture with ca. 50% sporulated *B. licheniformis* cells was harvested and stored in 1:1 methanol at −80°C. RNA extraction, cDNA synthesis, and RT-qPCR analysis were done as described by Madslien et al. (46). The quantity and purity of the RNA preparations were determined by measuring the absorbance at 260 nm and the ratio of the absorbances at 260 and 280 nm, respectively, by using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). The RT-qPCR analyses were performed in triplicates on at least three independent biological replicates. Melting-curve analyses were performed after each run in order to confirm amplification of specific transcripts. Amplification of serial dilutions of DNA template from strain MW3 enabled the estimation of the slope (s) of the standard curves for each primer. The PCR efficiency (E) was calculated by using the equation $E = 10^{-1/s}$. To quantify mRNA transcript levels, the C_T (threshold cycle) values of the target gene and the reference gene (*gerAA*) from the same sample were transformed using the following term E^{-CT}, and the expression levels of the target genes were normalized by dividing the transformed $C_{T\,\text{target}}$ by the transformed $C_{T\,\text{reference}}$ (53, 54).

RESULTS

Characteristics and relative expression levels of the *ger* operons in *B. licheniformis*. The gene organization of the *gerA*, *gerK*, and *ynd* operons in *B. licheniformis* strain ATCC 14580/DSM13 is presented in Fig. 1. The *B. licheniformis ynd* operon is pentacistronic, containing the D, E_3, E_2, F_1, and E_1 genes, encoding the germina-

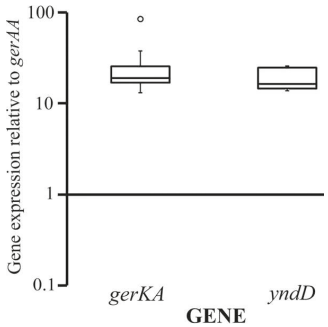

FIG 2 Relative expression levels of *gerKA* and *yndD* to *gerAA* in *B. licheniformis* MW3, as determined by qRT-PCR. Boxes represent 50% of the observations (first quartile to third quartile), and the horizontal lines mark the median. The ends of the whiskers show the 1.5× interquartile range (IQR) below the first quartile and 1.5× IQR above the third quartile. ○, outlier.

tion receptor subunits A, B, B, C, and B, respectively. The pentacistronic organization is different from the tricistronic organization of the *ynd* operon of *B. subtilis* (55). The *gerK* and *gerA* operons of *B. licheniformis* both contain three structural genes and demonstrate the same gene organization seen in their orthologues in *B. subtilis* (55). Putative −10 and −35 promoter sequences, with similarities to those recognized by σ^G, were identified upstream of the *gerA*, *gerK*, and *ynd* operons (Fig. 1). The −10 and −35 promoter regions of *gerA* and *gerK* match the *B. subtilis* σ^G consensus sequence, while the promoter region of *yndD* is less similar to the consensus sequence. Quantitative analyses of the relative transcription levels in sporulating cells of the ATCC 14580/DSM13 derivative strain MW3 showed that the *ynd* and the *gerK* operons were transcribed at approximately the same levels, 16.4 and 18.9 times higher relative to *gerAA*, respectively (Fig. 2).

Germinant recognition profile of *B. licheniformis* spores. Spores of strain MW3 have previously been shown to germinate in response to casein hydrolysate (a mixture of amino acids obtained from the hydrolysis of casein) and L-alanine as a single germinant compound (45). To further characterize the germinant recognition profile of this strain, we screened spores for germination in response to 18 standard L-amino acids, the ribonucleosides adenosine and inosine, and AGFK. A high concentration (100 mM) of each germinant compound (100 mM concentration of each AGFK component) was used for screening to also identify weak germinants. The germination efficiency was assessed after 120 min of germinant exposure by microscopic examination determining the percentage of phase-dark (germinated) versus phase-bright spores, and the results are listed in Table 2. L-Alanine, L-cysteine, and L-valine induced the most efficient germination responses, resulting in >90% germinated spores after 120 min of exposure to the germinant compound. L-Isoleucine, L-serine, and L-aspartic acid appeared as moderately efficient germinants (30 to 90% germination), while L-methionine and L-lysine appeared as weak germinants, resulting in <30% germinated spores. The most efficient germinants (L-alanine, L-cysteine, and L-valine) were selected for kinetic analyses. Kinetic analyses showed that spores induced by L-alanine demonstrated the highest germination rate, followed by L-cysteine and L-valine (Table 3). Assessment of the lowest concentration giving half-maximum rate of germination (C_{50}) confirmed that L-alanine was the most efficient germinant tested, followed by L-cysteine and L-valine (Table 3; see Fig. S1 in the supplemental material). AGFK acted as a weak germinant resulting in <30% germinated spores after 120 min of exposure to the germinant compounds (Table 2). Further experiments showed that D-glucose alone triggered the same level of germination as AGFK. This suggests that the germination response to AGFK is largely due to the D-glucose component. D-Fructose, L-asparagine, and K^+ induced germination responses comparable to that of the negative control (Table 3).

A number of studies have shown that the D-enantiomer of alanine functions as an efficient competitive inhibitor of L-alanine-induced germination (41, 56–58). D-Alanine has also been shown to inhibit L-valine-induced germination of *B. subtilis* and casein hydrolysate-induced germination in *B. licheniformis* (21, 45, 59). As shown in Table 3, preexposure of spores to D-alanine,

TABLE 2 Classification of nutrient germinants for *B. licheniformis* MW3 spore germinants

Germinant efficiency (%)[a]	Germinant compounds[b]
High (>90)	L-Alanine, L-cysteine, L-valine
Moderately (30–90)	L-Serine, L-isoleucine, L-aspartic acid
Weak (10–30)	AGFK,[c] D-glucose, L-methionine, L-lysine
Very weak (2–10)	D-Fructose, inosine, L-phenylalanine
None (<2)	Negative control, L-glutamic acid, L-arginine, L-histidine, L-leucine, L-proline, L-glycine, L-threonine, L-asparagine, adenosine,* L-tyrosine*

[a] The percentages of germinated (phase dark) spores were estimated after 120 min of exposure to germinant compounds.
[b] The germinant concentration was 100 mM. *, due to low solubility in water, the concentrations of the indicated compounds were set to 1 mM.
[c] AGFK, a mixture of asparagine, glucose, fructose, and K^+ (100 mM each).

TABLE 3 Germination responses of *B. licheniformis* MW3 spores[a]

Germinant	% germination[b]	% OD loss[c]	G_{max} (% OD loss min^{-1})[d]	C_{50} (mM)[e]	% OD loss D-alanine[f]
L-Alanine	98.3 (1.2)	56.8 (4.6)	1.4 (0.4)	0.7	27.3 (8.0)
L-Valine	90.4 (2.9)	56.3 (3.9)	1.0 (0.2)	8.1	15.2 (6.9)
L-Cysteine	97.6 (1.2)	57.0 (2.7)	2.0 (0.7)	2.4	13.5 (7.6)
AGFK	10.4 (5.3)	20.7 (3.8)	<0.5	ND	ND
L-Asparagine	<2.0	20.6 (4.8)	<0.5	ND	ND
D-Glucose	12.6 (8.7)	23.8 (3.0)	<0.5	ND	ND
D-Fructose	3.7 (2.1)	16.0 (5.0)	<0.5	ND	ND
KCl	<2.0	17.8 (3.5)	<0.5	ND	ND
Negative control	<2.0	15.3 (2.7)	<0.5	ND	ND

[a] All data are presented as means. The standard deviations, where applicable, are given in parentheses. ND, not determined.
[b] The percent germinated (phase dark) spores was determined after 120 min of exposure to 100 mM germinant compound.
[c] The percent loss of OD_{600} after 120 min of exposure to 100 mM germinant compound.
[d] G_{max} is the maximum rate of germination (ΔOD_{600} min^{-1}).
[e] C_{50} is the concentration of the germinant required to achieve 50% of the maximum germination rate.
[f] The percent loss of OD_{600} after 120 min of exposure to 25 mM germinant compound, when spores were pretreated with D-alanine. These experiments were only replicated in two different spore batches.

TABLE 4 Germination properties of *B. licheniformis* mutant spores with L-amino acids[a]

Genotype	Functional GR(s)	% germination[b]					G_{max} (% OD loss min^{-1})[c]		
		L-Alanine	L-Cysteine	L-Valine	D-Glucose	NC	L-Alanine	L-Cysteine	L-Valine
Wild type[d]	GerA, GerK, Ynd	98.3 (1.2)	97.6 (1.2)	90.4 (2.9)	12.6 (8.7)	<2.0	1.4 (0.4)	1.0 (0.2)	2.0 (0.7)
Δ*gerAA*	Ynd, GerK	8.3 (4.4)	10.7 (3.9)	3.0 (1.2)	13.7 (9.7)	<2.0	<0.5	<0.5	<0.5
Δ*yndD*	GerA, GerK	57.5 (2.0)	45.0 (12.3)	61.1 (1.2)	13.7 (8.6)	<2.0	<0.5	<0.5	<0.5
Δ*gerKA-C*	GerA, Ynd	90.4 (4.6)	86.6 (6.6)	75.1(5.6)	<2.0	<2.0	2.0 (0.2)	1.4 (0.2)	1.1 (0.3)
Δ*gerAA* Δ*yndD*	GerK	<2.0	<2.0	<2.0	17.4 (5.9)	<2.0	<0.5	<0.5	<0.5
Δ*gerAA* Δ*gerKA-C*	Ynd	7.3 (4.3)	11.0 (8.5)	<2.0	<2.0	<2.0	<0.5	<0.5	<0.5
Δ*yndD* Δ*gerKA-C*	GerA	<2.0	<2.0	<2.0	<2.0	<2.0	<0.5	<0.5	<0.5
Δ*gerAA* Δ*yndD* Δ*gerKA-C*	None	<2.0	<2.0	<2.0	<2.0	<2.0	<0.5	<0.5	<0.5
Δ*gerAA-C*[e]	Ynd, GerK	<2.0	2.8 (2.7)	<2.0	2.8 (1.5)	<2.0	ND	ND	ND
Δ*yndD*/pHT315_*yndD*$^+$	GerA, GerK, Ynd	ND	ND	ND	ND	ND	1.5 (0.1)	1.3 (0.3)	0.8 (0.1)

[a] All data are presented as means. The standard deviations, where applicable, are given in parentheses. NC, negative control; ND, not determined.
[b] The percentages of germinated (phase dark) spores were determined after 120 min of exposure to 100 mM concentrations of germinant compounds.
[c] G_{max} is the maximum rate of germination (ΔOD_{600} min^{-1}).
[d] Wild-type data have been transferred from Table 3 to aid in the interpretation of the data in Table 4.
[e] That is, deletion of the entire *gerA* operon.

before the addition of L-alanine, L-valine, and L-cysteine, strongly reduced germination induced by all three L-amino acids.

GerA and Ynd cooperate in triggering germination. To assess the functional role of the GerA and Ynd GRs, mutant spores, where the first gene in each GR operon was deleted in-frame, were analyzed for germination responses to alanine, valine, and cysteine (Table 4). Spores of the *gerAA*-null mutant showed severely reduced germination rates with all L-amino acids tested (Table 4). However, phase-contrast microscopy examination of spores after 120 min of exposure to L-alanine or L-cysteine revealed that 8.3 and 10.7% of the spores had germinated, respectively. The weak ability of the strain MW3 *gerAA*-null mutant spores to germinate in response to L-alanine has also been observed before (46). The germination response to L-valine was on the other hand similar to the negative-control levels (3.0% germinated spores, Table 4). Since the promoter region of *gerA* is still intact, the remaining weak germination of *gerAA*-null mutant spores may be explained by the expression of GerAB and GerAC subunits which could be functional in germination. To address this possibility, a *gerAA-C*-null mutant was constructed. Analysis of mutant spores, which lacks the complete *gerA* operon, revealed that the weak germination responses observed in the *gerAA*-null mutant was essentially eliminated. This suggests that the GerAB and GerAC subunits contribute to germination in the absence of the GerAA subunit (Table 4).

Analyses of spores lacking *yndD* revealed that they exhibit an ~40 to 55% reduced germination efficiencies with L-alanine, L-valine, and L-cysteine compared to the wild-type spores (Tables 4). Disruption of *yndD* in the *gerAA* background reduced the germination efficiency to a level similar to the negative control (Table 4). Transcomplementation of the *yndD*-null mutant with a plasmid carrying an intact copy of *yndD* restored the germination efficiency to wild-type levels (Table 4). The *gerAA*-null mutation in strain MW3 has been complemented before (45).

Role of GerK in germination. Having established by RT-qPCR that the *gerK* operon is expressed during sporulation (Fig. 2), we next constructed a *gerKA-C*-null mutant to assess its role in germination. The ability of the *gerKA-C*-null mutant spores to germinate in response to L-alanine, L-valine, and L-cysteine was slightly reduced (~10 to 25%) compared to wild-type spores (Table 4). However, the *gerAA yndD* double-null mutant spores, which only express GerK, demonstrated germination levels with L-alanine, L-valine, or L-cysteine similar to the negative control, suggesting that the function of GerK in germination with L-amino acids is dependent on cooperative interactions with other GRs.

Given the role of GerK in glucose-induced germination of *B. subtilis* spores, we tested *B. licheniformis gerKA-C*-null mutant spores for germination in response to glucose. As expected, the *gerKA-C*-null mutant did not germinate at all after 120 min of exposure to D-glucose (Table 4). The contribution of GerK to glucose-induced germination appeared to be independent of both GerA and Ynd since the *yndD gerAA* double-null mutant spores demonstrated no significant change in germination efficiency with D-glucose compared to the wild-type spores, and the *gerAA* and *yndD* single-null mutant spores (Tables 4). Altogether, this indicates that D-glucose interacts specifically with the GerK germination receptor. However, the *gerAA-C*-null mutant showed a reduced germination efficiency in response to glucose, indicating that the GerAB and GerAC subunits somehow contribute to the function of GerK in glucose-induced germination.

Functional dependence of GerA on Ynd or GerK. The results presented above indicate that there is a functional cooperation between the GerA, GerK, and the Ynd GRs. To further examine whether there is a functional interplay between GerK and the other GRs, we analyzed the germination efficiency of *yndD gerKA-C* and *gerAA gerKA-C* double-null mutant spores, expressing only GerA or Ynd, respectively. Spores expressing only GerA demonstrated germination efficiencies with L-alanine, L-valine, and L-cysteine similar to the negative control (Table 4). This is in contrast to the ability of the *yndD*-null mutant spores, which express both GerA and GerK, to germinate with either L-alanine, L-valine, or L-cysteine at an efficiency of ~45 to 60% of wild-type levels (Table 4). On the other hand, spores of the *gerAA gerKA-C* double-null mutant, which only expresses Ynd, demonstrated a low level of germination in response to L-cysteine and L-alanine, but not to L-valine, which is very similar to the germination of the *gerAA*-null mutant expressing both Ynd and GerK (Table 4). This indicates that there is no functional dependency between the GerK and Ynd GRs. Triple *gerAA yndD gerKA-C*-null mutant spores

TABLE 5 Effect of D-glucose and L-alanine spore germination[a]

Genotype [GR(s) present][b]	% OD loss[c]			G_{max} (% OD loss min^{-1})[d]		
	L-Alanine	D-Glucose	L-Alanine + D-glucose	L-Alanine	D-Glucose	L-Alanine + D-glucose
Wild type (GerA, Ynd, GerK)*	38.0 (7.7)	28.7 (9.3)	50.5 (7.1)	0.73 (0.1)	0.38 (0.2)	1.02 (0.04)
Δ*yndD* (GerA, GerK)†	40.4 (4.2)	23.5 (3.5)	53.2 (2.3)	0.58 (0.1)	0.48 (0.3)	0.80 (0.2)
Δ*gerAA* (Ynd, GerK)†	15.3 (5.6)	20.1 (3.1)	21.4 (2.1)	0.48 (0.2)	0.52 (0.1)	0.51 (0.1)
Δ*gerKA-C* (GerA, Ynd)*	42.0 (7.6)	21.5 (3.1)	40.0 (6.6)	0.99 (0.1)	0.46 (0.1)	0.96 (0.4)

[a] All data are presented as means. The standard deviations, where applicable, are given in parentheses.
[b] Symbols: *, 1 mM L-alanine and 10 mM D-glucose were used; †, 100 mM L-alanine and 50 mM D-glucose were used.
[c] The percentages of OD_{600} loss after 120 min of exposure to the germinant compound or germinant mixture are indicated.
[d] G_{max} is the maximum rate of germination (ΔOD_{600} min^{-1}).

demonstrated germination levels similar to the negative control with all of the germinants tested.

Germination with multiple germinants. Synergetic effects, where germination with mixtures of low concentrations of germinants, acting on different germinant recognition sites, is much higher that the sums of germination with individual germinant compounds, has been described in *Bacillus* species (12, 14). If the germination efficiency obtained with a mixture of germinants is similar to the sum of germination obtained with the individual components, the effect is additive. To test how complex signals trigger germination of *B. licheniformis* spores, wild-type spores were exposed to low concentrations of binary combinations of L-amino acids (0.2 mM L-alanine, 0.2 mM L-cysteine, and 2.0 mM L-valine). We could, however, not detect any significant synergistic or additive effects on germination with the binary combinations of germinant compounds compared to the effects with the individual compounds (see Table S2 in the supplemental material). In *B. subtilis*, the germination response initiated by GerA is stimulated by D-glucose activated GerK, although glucose activated GerK alone does not stimulate germination (21). However, exposure of strain MW3 to binary combinations of L-alanine and D-glucose did not result in a synergistic effect on the germination efficiency, but an additive effect was observed. This effect was also seen in the *yndD*-null mutant, but no additive effect was observed in the *gerAA*-null mutant. Deletion of *gerKA-C* abolished the additive effect of glucose plus L-alanine seen in wild-type and *yndD*-null mutant spores (Table 5; see also Fig. S6 in the supplemental material).

Status of *ger* operons in *B. licheniformis* strains. To investigate the status of the *gerA*, *gerK*, and *ynd* gene clusters in other *B. licheniformis* strains, a series of nBLAST searches were performed using the *ger* genes from *B. licheniformis* ATCC 14580/DSM13 as query sequences (Table 6). The output from the searches revealed that the *gerA* family operons *gerA*, *gerK*, and *ynd* are present in all 17 *B. licheniformis* genomes found in the NCBI Nucleotide and WGS (whole-genome shotgun contigs) databases. The atypical cistronic organization of the *ynd* operon, containing three *yndE* genes, where the second and third *yndE* genes are separated by the *yndF* gene, was found in all *B. licheniformis* genomes analyzed. However, three strains carried premature stop codons or gene deletions within the *ynd* operon. The *gerA* and *gerK* operons were intact in all strains. The orphan gene *yndF2* was found in 12 of 17 strains, while the *gerB* gene was not present in the genomes analyzed here.

TABLE 6 Presence of *gerA* family operons among *B. licheniformis* strains

Strain[a]	Accession no.	*gerA* family operon[b]					Note
		gerA (ABC)	*gerB* (ABC)	*gerK* (ACB)	*ynd* (ABBCB)	*yndF2* (C)	
ATCC 14850/DSM13	AE017333.1	+	–	+	+	+	
9945A	CP005965.1	+	–	+	+	–	
WX-02	CP012110.1	+	–	+	+	+	
BL-09	CP010524.1	+	–	+	+	–	
10-1-A†	AJLV01000023.1	+	–	+	+	+	
5-2-D†	AJLW01000029.1	+	–	+	+	+	
F1-1†	AZSL01000017.1	+	–	+	+	+	
GB2†	JYGX01000007.1	+	–	+	+	+	
CG-B52†	AVEZ01000049.1	+	–	+	+	+	*yndE2* disrupted
S16†	AZYP01000014.1	+	–	+	+	+	*yndE3* and *yndE2* disrupted
5NAP23 LG49†	JYBQ01000003.1	+	–	+	+	+	
F2-1†	AZSM01000012.1	+	–	+	+	+	
3F-3†	JFYM01000017.1	+	–	+	+	+	
CGMCC 3963†	AMWQ01000010.1	+	–	+	+	+	
12759 DJ88†	JMPZ01000014.1	+	–	+	+	–	*yndF* disrupted
G-1†	AZSK01000007.1	+	–	+	+	–	
S27†	LFIM01000005.1	+	–	+	+	–	

[a] †, whole-genome shotgun sequences.
[b] +, found in the genome; –, not found during search. The operons were identified using nBLAST on genomes available in the NCBI database, and the *gerA* family operons from strain DSM13/ATCC 14580 were used as query sequences. The operon organizations of the genes encoding the A, B, and C subunits are indicated in parentheses.

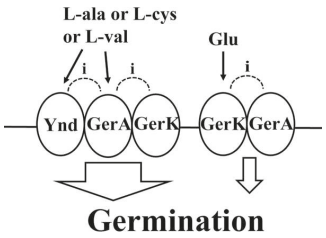

FIG 3 Schematic representation of spore germination pathways in *B. licheniformis* strain MW3. Both GerA and Ynd recognize L-alanine, L-cysteine, and L-valine, while GerK recognizes D-glucose. The glucose triggered germination pathway is separate from the germination pathway triggered by the L-amino acids. Cooperative interactions between the GRs are necessary for germination, since none of the GRs can initiate efficient germination on their own. i, interaction between GRs. Black arrows indicate germinant recognition.

DISCUSSION

This study provides a more complete characterization of germinants and GRs of *B. licheniformis* spores. *B. licheniformis* spores germinated in response to alanine, cysteine, valine, serine, isoleucine, aspartic acid, lysine, methionine, and glucose. Alanine and cysteine were the most potent germinants, stimulating germination at a lower concentration than the other L-amino acids tested. The germination responses of *B. licheniformis* spores to L-alanine, L-valine, and L-cysteine were strongly inhibited by D-alanine. The inhibitory effect of D-alanine on *gerA* dependent germination with other amino acids than L-alanine has previously been observed in *B. subtilis*, in which germination via L-valine is efficiently inhibited by D-alanine (21, 59).

Analyses of *ger* mutant strains revealed that the GerA, Ynd and GerK GRs were all functional in germination and that GerA functions as the primary GR in *B. licheniformis*. However, the most efficient germination responses to single germinant compounds were seen in wild-type spores containing intact GerA, GerK, and Ynd GRs.

Mutant spores, which express only GerA, demonstrated germination levels similar to the negative control, suggesting that GerA could not function alone. Further analyses of the *yndD*- and *gerKA-C*-null mutant spores revealed that GerA required either a functional Ynd or GerK GR to stimulate germination. The cooperative function between GerA and GerK in triggering germination in response to single L-amino acids did not require glucose activation of GerK, in contrast to what has been observed in *B. subtilis* (21). No functional interdependence between Ynd and GerK could be identified, but Ynd depended on GerA to induce efficient germination (Fig. 3). Cooperation between GRs for induction of germination in response to single germinant compounds has also been observed for inosine-induced germination in *B. cereus* 569 spores (60), for efficient L-alanine-induced germination in *B. anthracis* spores (13), and for the $GerA_2$ GR to function in *B. megaterium* spore germination (61).

We observed that L-alanine and L-cysteine induced a weak germination response in *gerAA*-null mutant spores, whereas *gerAA-C*-null mutant spores demonstrated germination levels similar to uninduced spores. The weak germination response was dependent on an intact *ynd* operon, but not on the *gerK* operon, since the *gerAA yndD*-null mutant spores showed a germination level similar to the negative control, whereas the *gerAA*- and *gerAA gerKA-C*-null mutant spores demonstrated similar, weak germination efficiencies. This findings suggests that the GerAB and GerAC subunits form functional interactions with the A subunit of the *ynd* operon (*yndD*). Functional interactions between GR subunits encoded by different *ger* operons have previously been reported to occur in *B. subtilis* spores (62, 63).

In *B. subtilis*, the disruption of *ynd* did not alter the germination phenotype of the *gerA gerB gerK* triple-mutant spores, suggesting that the *ynd* encoded proteins did not contribute significantly to nutrient-triggered spore germination (22). The importance of the *ynd* operon in *B. licheniformis* is reflected by the presence of intact *ynd* operons in most *B. licheniformis* genome sequences, whereas the selection pressure for maintaining a functional Ynd appears to be lower in *B. subtilis* (24).

GerK was essential for the weak germination response to D-glucose. This was not surprising given the glucose recognition described for the GerK orthologues in *B. subtilis* and *B. megaterium* (21, 23, 61, 64). The *gerAA-C*-null mutant spores showed a reduced ability to germinate in response to glucose, even if GerK was present, indicating that either GerAB or GerAC or both subunits are important for glucose-induced germination. The combination of L-asparagine, glucose, fructose, and K^+ did not stimulate a more efficient germination response in *B. licheniformis* spores compared to glucose alone. This could be explained by the absence of GerB, which, in cooperation with GerK, stimulates an efficient germination response to AGFK in *B. subtilis* (21).

In nature, bacterial spores are likely to encounter a mixture of potential germinants, and in many species some germinant combinations are able to trigger stronger germination responses than others, perhaps dependent on the niche preferences of the species (12, 14). It has been suggested that integrated signals from multiple activated germinant binding sites can synergistically increase the efficiency of germination (12). We were, however, unable to detect any synergistic effects with mixtures L-amino acid on spore germination. It is still possible that the GRs in *B. licheniformis* forms synergistic interactions under other conditions, or that they recognize and respond to other germinants; these possibilities were not tested here. However, exposure of spores to binary mixtures of D-glucose and L-alanine generated an additive effect on germination.

The mutational analyses further support that GerA and Ynd have overlapping germinant recognition profiles, since both the *gerAA*- and *yndD*-null mutant spores were affected in L-alanine-, L-valine-, and L-cysteine-induced germination. However, the atypical architecture of the *ynd* operon suggests that it contains more than one nutrient binding site. In *B. megaterium*, B subunits from different operons could be utilized interchangeably in the GerU GR and hence provided an extended range of recognized germinants (15). It is tempting to speculate that a similar mechanism is at work in *B. licheniformis*, where the three B subunits encoded in the *ynd* operon may have different germinant specificities and contribute to an extended germinant recognition profile. However, determination of the function of the multiple B subunits encoded by the *ynd* operon remains an important objective for further work in this area.

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Supplementary information

The cooperative and interdependent role of GerA, GerK and Ynd in germination of *Bacillus licheniformis* spores

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Revised, clean version

TABLE S1: Primers used in this work.

Operon	Primer	Sequence (5' – 3')
ynd		
	A yndDup	GCTTGAGCAGACATTGCTTG
	B yndD	CTTTCTATG**CTACAT**GCCACTCACTCATCC
	C yndD	AGTGGC**ATGTAG**CATAGAAAGG
	D yndDdown	ACGCAAGTTGTCCACATCAA
	yndD F	GCATTGAGCGGATCCGAAAACCGGAAT
	ynd R	CAGGATGTCAGTGATCTAGAGGTTGAC
	yndD int F	AGCCCGGGATGAAACGAAAT
	yndD int R	CGCGAATTCAACTTAGGGCG
gerK	**A** gerKAup	ACGAGGTTATCGGCAATACG
	B gerKAr	TCTCTTTCAT**TCACAT**ATTTTTCCTTGCGCAAGC
	C gerKCf	AAGGAAAAAT**ATGTGA**ATGAAAGAGAGAGGAGG
	D gerKCdown	TTTCCAAGAATGGGCAAAAG
	gerKE	AAGCGTCGCAAGTCTTCATT
	gerKF	TCGTCAGTCGATCAGCTTTG
	gerKnedr2	CGTCTGGATCGTACTCGCA
gerA	**A** gerAoperon	ACATCATCATTCGGGTGGAT
	B gerAoperon	GAGAA**CAACAT**GATCGTCACC
	C gerAoperon	ACGATC**ATGTTG**TTCTCGTATG
	D gerAoperon	CAAGAAAGCCGTTTTTCAGC
RT qPCR		
	gerAAF (E=2,01)	CCCTGTTCCTATCGGCGTTT
	gerAAR (E=2,01)	TCGGCAGCATGCCTTGA
	gerKAF (E=2,00)	CGGCAGGGAGGTTCTTGAG
	gerKAR (E=2,00)	GACACCGCCCAGTGCAA
	YndD F (E=1,99)	GCGCGTCGGCTTTACAGA
	YndD R (E=1,99)	CCGTGCTGCCTCAAAAGG
	rpoBF (E=2,00)	ACCTCTTCTTATCAGTGGTTTCTTGAT
	rpoBR (E=2,00)	CCTCAATTGGCGATATGTCTTG

Bold indicates START and STOP codon.

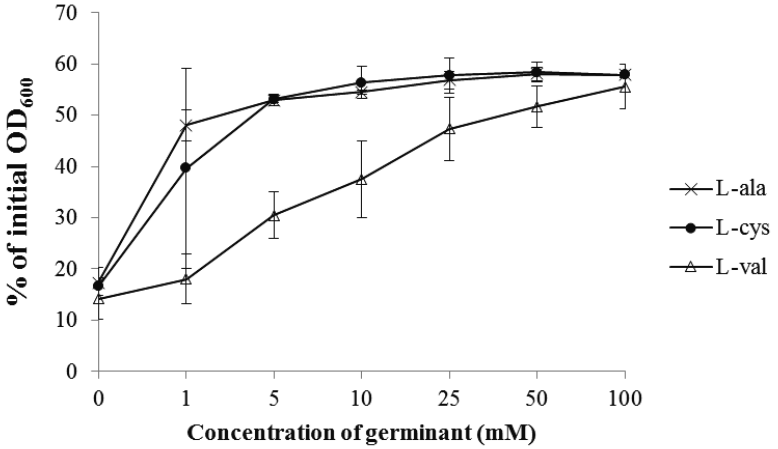

Figure S1: Dose-response curves for L-alanine, L-valine and L-cysteine for *B. licheniformis* MW3. Measurements of OD_{600} were done over 70 minutes exposure to germinant compound. Error bars show 95 % confidence intervals. Dose-response curves were determined for two independent spore batches.

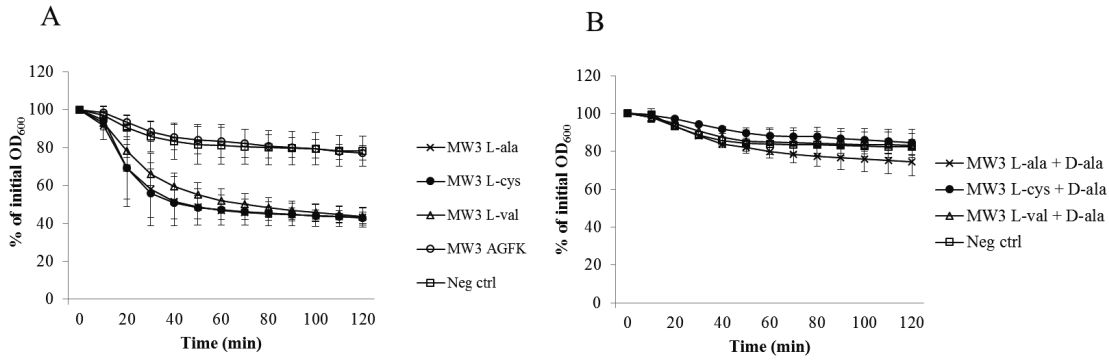

Figure S2: Germination of *B. licheniformis* MW3 spores to 100 mM L-alanine, L-cysteine, L-valine, and AGFK (A). Germination with binary combinations of 25 mM of either L-alanine, L-cysteine or L-valine and 25 mM D-alanine, (B). Spore germination was measured by following the decrease of the initial OD_{600} (%) of the spore suspension after addition of germinants. Error bars show 95 % confidence intervals.

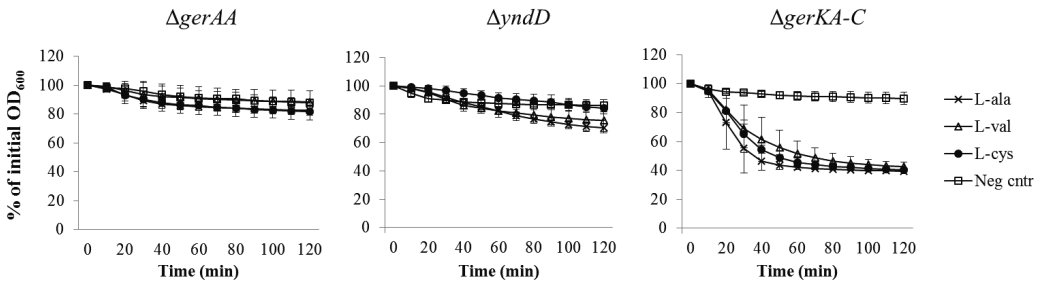

Figure S3: Germination responses of *B. licheniformis* single null mutant spores to 100 mM L-alanine, L-cysteine, and L-valine measured by following the decrease of the initial OD_{600} (%) of the spore suspension after addition of germinants. Error bars show 95 % confidence intervals.

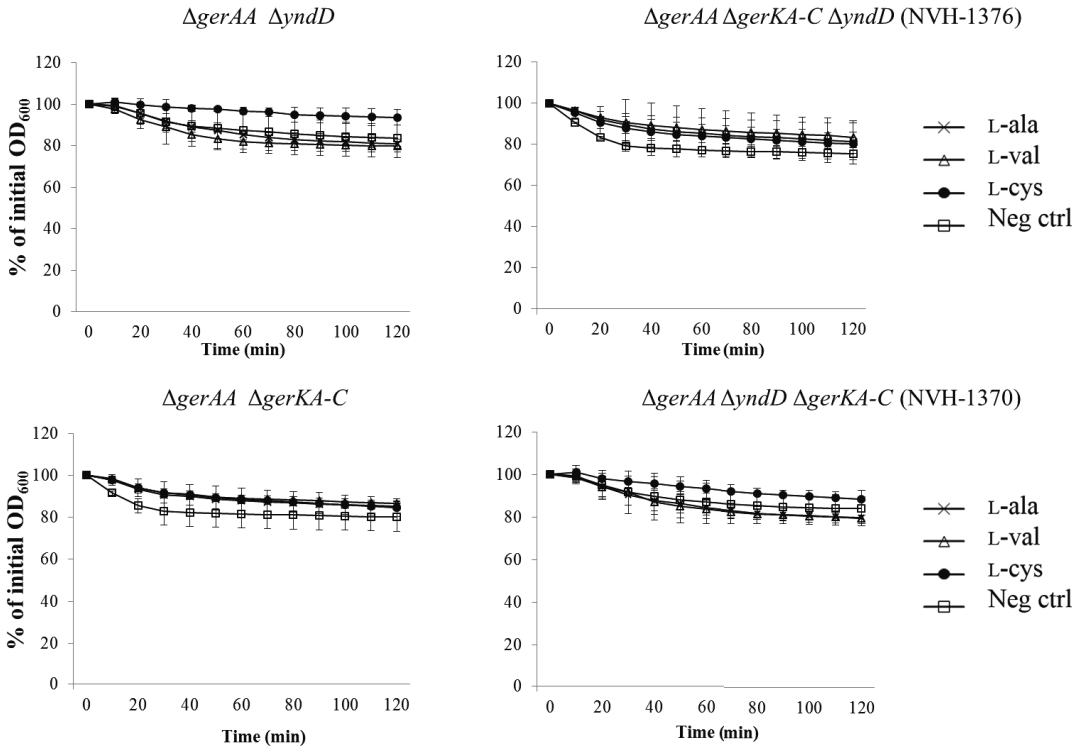

Figure S4: Germination of *B. licheniformis* double and triple null mutant spores with 100 mM L-alanine, L-cysteine, and L-valine measured by following the decrease of the initial OD_{600} (%) of the spore suspension after addition of germinants. Error bars show 95 % confidence intervals.

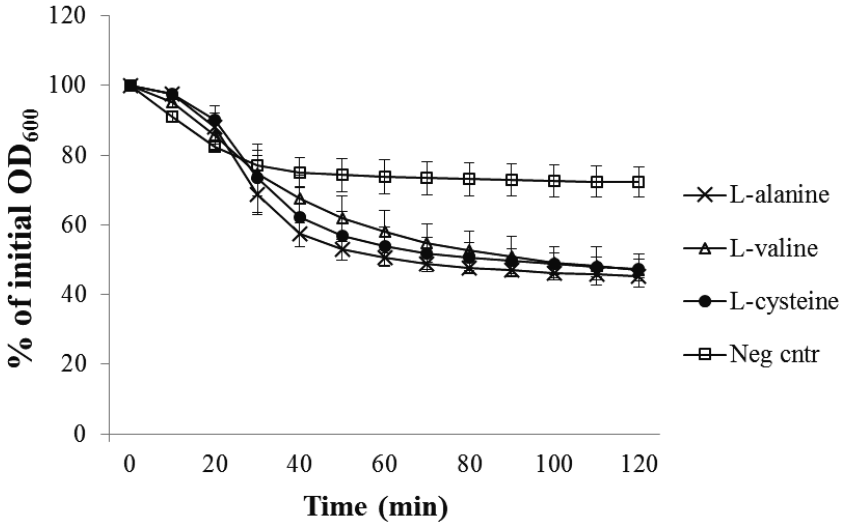

Figure S5: The effect of 100 mM L-valine, L-cysteine, and L-alanine on germination of *yndD* null mutant spores complemented with shuttle vector pHT315 containing the *yndD* gene with the upstream promoter sequence. The germination was measured by following the decrease of the initial OD_{600} (%) of the spore suspension after addition of germinants. Error bars show 95 % confidence intervals.

Table S2: The effect of binary combinations of L-amino acids on spore germination.

Germinant	% OD loss[a]	G_{max} (% OD loss min^{-1})[b]
L-ala 0.2 mM	29.04 (7.3)	0.71 (0.3)
L-cys 0.2 mM	16.33 (3.7)	0.34 (0.3)
L-val 2.0 mM	27.16 (3.9)	0.45 (0.3)
L-ala + L-val[c]	30.18 (3.01)	0.65 (0.1)
L-ala + L-cys[c]	38.78 (17.7)	0.93 (0.5)
L-cys + L-val[c]	29.6 (7.7)	0.50 (0.2)

[a] Spore germination measured as the decrease of the initial OD_{600} (%) after 120 min exposure to germinant compounds/ germinant mixtures.

[b] G_{max}, maximum rate of germination (ΔOD_{600} min^{-1}).

[c] The concentrations of L-amino acids used were 0.2 mM of L-alanine, 0.2 mM of L-cysteine, and 2.0 mM of L-valine. L-valine is a much weaker germinant compared to L-alanine and L-cysteine and was therefore used at a ten times higher concentration.

All data are presented as the mean (SD).

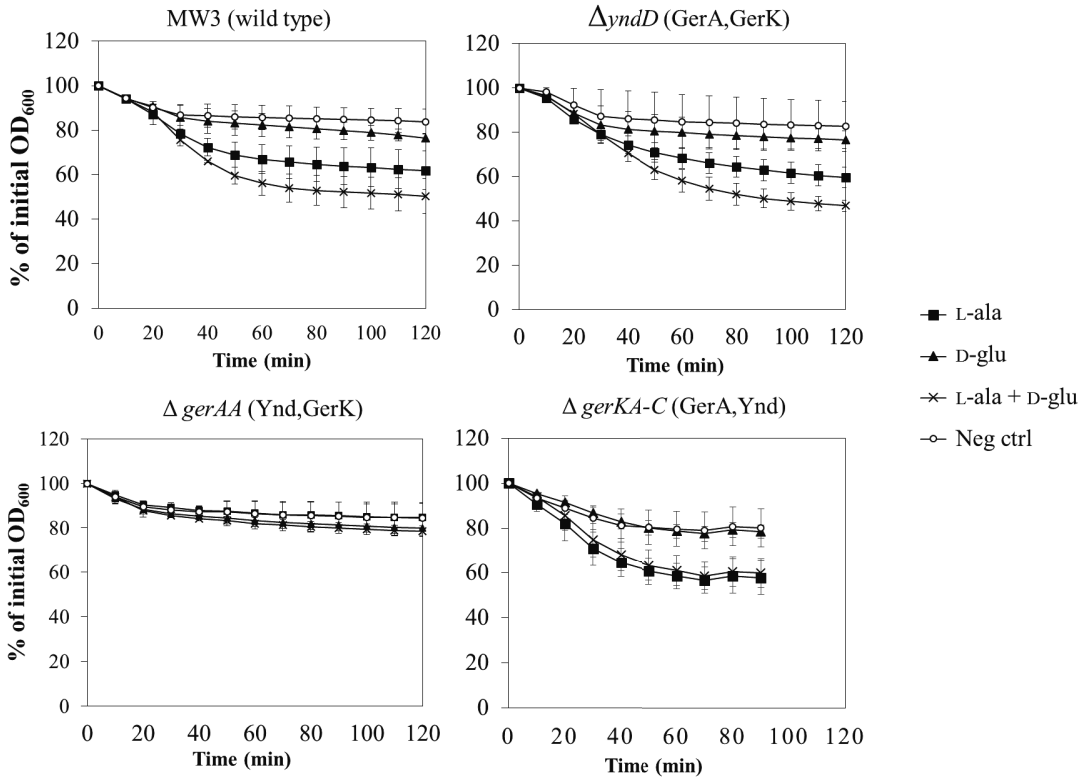

Figure S6: The effect of D-glucose on L-alanine induced germination of spores of the wild type, and the Δ*yndD*, Δ*yndD* Δ*gerKA-C*, and Δ*gerKA-C* mutant strains measured by following the decrease of the initial OD_{600} (%) after addition of germinant compounds. 50 mM of D-glucose was used for the mutant spores, while 10 mM was used for the wild type spores. L-alanine was used in concentrations of 1 mM for the wild type and the *gerKA-C* single null mutant spores, and 100 mM was used for the *yndD* and *gerAA* mutant spores. Error bars show 95 % confidence intervals.

II

Author Correction for AEM00503-17

Correction for Borch-Pedersen et al., "Effects of High Pressure on *Bacillus licheniformis* Spore Germination and Inactivation"

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Volume 83, no. 14, e00503-17, https://doi.org/10.1128/AEM.00503-17. The strain NVH-1376, which was presented as a Δ*yndD* Δ*gerKA-C* double mutant, was found to contain a spectinomycin resistance cassette inserted into the *gerAA* gene, making it a *ger*$^-$ mutant.

Hence, there is no evidence indicating that the GerA germinant receptor cannot function alone in inducing germination in response to moderately high pressures, as was originally argued in our published paper. All statements in the Abstract, Results, and Discussion that GerA cannot induce germination in response to moderately high pressure when present as the single, intact germination receptor should be disregarded.

Page 1, Abstract, line 11: “GerK also triggers” should read “GerK and GerA also trigger.”

Page 1, Abstract, lines 12 and 13: The sentence beginning with “GerA stimulates...” should be deleted.

Page 2, line 30: “*B. licheniformis* spore germination” should read “efficient *B. licheniformis* germination.”

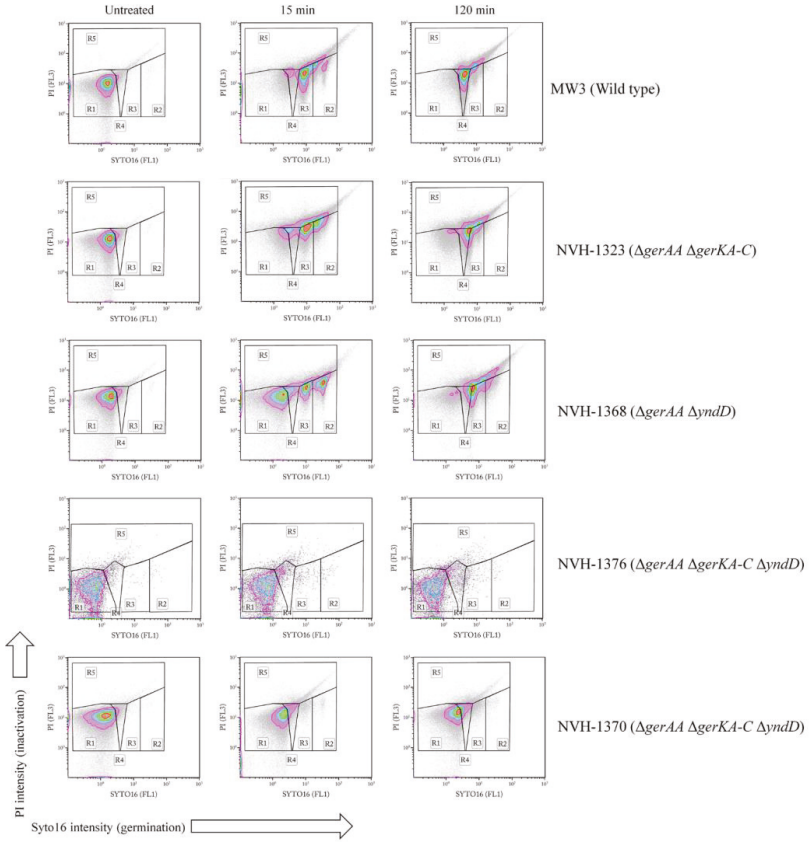
Page 3, Table 1, column 2, row 15: “Δ*yndD* Δ*gerKA-C* mutant” should “Δ*gerAA* Δ*yndD* Δ*gerKA-C* mutant.”

Page 5, legend to Fig. 3: “(C) NVH-1376 (Δ*gerKA-C* Δ*yndD*)” should read “(C) NVH-1376 (Δ*gerAA* Δ*yndD* Δ*gerKA-C*).”

Page 5: Lines 19--22 should read as follows. “...For spores of strain NVH-1370 (Δ*gerAA* Δ*gerKA-C* Δ*yndD*), which lack all functional GRs (Ger^{-}), no mHP-induced germination was detected, as determined by the plate counts (Table 1 and Fig. 3D).”

Page 6, legend to Fig. 4, line 4: “NVH-1376 (Δ*gerKA-C* Δ*yndD*)” should read “NVH-1376 (Δ*gerAA*::*spc* Δ*yndD* Δ*gerKA-C*).”

Page 6, Fig. 4 should appear as shown below:



Page 6, line 1: "(GerA^{+})" should read "(Ger^{-})."

Page 6, line 4: "(GerA^{+})" should read "(Ger^{-})."

Page 8, lines 31--34: The sentence beginning with "The importance..." should be deleted.

Page 11, Table 2, column 2, row 10: "*B. licheniformis* MW3 Δ*gerKA-C* Δ*yndD*" should read "*B. licheniformis* MW3 Δ*gerAA*::*spc* Δ*yndD* Δ*gerKA-C*."

Supplemental material: In Table S1, column 1, "NVH-1376 (Δ*gerKA-C* Δ*yndD*)" should read "NVH-1376 (Δ*gerAA* Δ*gerKA-C* Δ*yndD*)." Revised supplemental material is posted at http://aem.asm.org/content/83/14/e00503-17/suppl/DCSupplemental.

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FOOD MICROBIOLOGY

Effects of High Pressure on *Bacillus licheniformis* Spore Germination and Inactivation

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ABSTRACT *Bacillus* and *Clostridium* species form spores, which pose a challenge to the food industry due to their ubiquitous nature and extreme resistance. Pressurization at <300 MPa triggers spore germination by activating germination receptors (GRs), while pressurization at >300 MPa likely triggers germination by opening dipicolinic acid (DPA) channels present in the inner membrane of the spores. In this work, we expose spores of *Bacillus licheniformis*, a species associated with food spoilage and occasionally with food poisoning, to high pressure (HP) for holding times of up to 2 h. By using mutant spores lacking one or several GRs, we dissect the roles of the GerA, Ynd, and GerK GRs in moderately HP (mHP; 150 MPa)-induced spore germination. We show that Ynd alone is sufficient for efficient mHP-induced spore germination. GerK also triggers germination with mHP, although at a reduced germination rate compared to that of Ynd. GerA stimulates mHP-induced germination but only in the presence of either the intact GerK or Ynd GR. These results suggests that the effectiveness of the individual GRs in mHP-induced germination differs from their effectiveness in nutrient-induced germination, where GerA plays an essential role. In contrast to *Bacillus subtilis* spores, treatment with very HP (vHP) of 550 MPa at 37°C did not promote effective germination of *B. licheniformis* spores. However, treatment with vHP in combination with elevated temperatures (60°C) gave a synergistic effect on spore germination and inactivation. Together, these results provide novel insights into how HP affects *B. licheniformis* spore germination and inactivation and the role of individual GRs in this process.

IMPORTANCE Bacterial spores are inherently resistant to food-processing regimes, such as high-temperature short-time pasteurization, and may therefore compromise food durability and safety. The induction of spore germination facilitates subsequent inactivation by gentler processing conditions that maintain the sensory and nutritional qualities of the food. High-pressure (HP) processing is a nonthermal food-processing technology used to eliminate microbes from food. The application of this technology for spore eradication in the food industry requires a better understanding of how HP affects the spores of different bacterial species. The present study provides novel insights into how HP affects *Bacillus licheniformis* spores, a species associated with food spoilage and occasionally food poisoning. We describe the roles of different germination receptors in HP-induced germination and the effects of two different pressure levels on the germination and inactivation of spores. This study will potentially contribute to the effort to implement HP technology for spore inactivation in the food industry.

KEYWORDS *Bacillus licheniformis*, spore germination, germination receptor, endospores, high pressure, high-pressure processing, spore inactivation, flow cytometry

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To survive potentially lethal conditions, several members of the orders *Bacillales* and *Clostridiales* form metabolically dormant spores. The spores are much more resistant than vegetative cells to stressors such as UV irradiation, wet and dry heat, freezing, and chemical assaults (1, 3). Many spore-forming bacterial species are associated with foodborne illness and food spoilage, which, combined with their extreme resistance to traditional food-processing strategies, makes them a major concern to the food industry.

Several protective layers surround the dehydrated spore core, including the inner membrane (IM), peptidoglycan cortex, outer membrane, spore coats, crust, and sometimes a sac-like exosporium (2). The spore core contains large amounts of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) chelated in a 1:1 ratio with Ca^{2+} (CaDPA) and DNA-binding small acid-soluble proteins (SASPs), which also contribute to the extreme resistance of the spores (1, 3). Environmental signals trigger the spores to exit dormancy via the process of germination. These signals include exposure to specific nutrients (germinants) or to nonnutrient factors, such as exogenous CaDPA, cationic surfactants, and high pressures (2, 4–7). Nutrient germinants act on their cognate germination receptors (GRs) present in the spore's IM. The GRs are encoded by *gerA* family genes present in most *Bacillus* and *Clostridium* species (7, 8). The *gerA* genes are often organized in tricistronic operons encoding the A, B, and C subunits, which together compose the functional GR (9, 10). *Bacillus subtilis*, which is a frequently used model organism for studying bacterial spores, contains genes encoding functional GerA, GerK, and GerB GRs and the *ynd* and *yfk gerA* family operons with unknown functions (7). The GerA GR triggers germination when activated by L-valine or L-alanine, whereas the GerB and GerK GRs cooperate to trigger germination in response to a mixture of L-asparagine, glucose, fructose, and K^+ (AGFK) (7, 11). *Bacillus licheniformis*, a close relative of *B. subtilis*, frequently causes spoilage of foods, such as dairy products, bread, and canned meats, and it is also occasionally associated with foodborne disease (12–18). Three functional GRs, GerA, Ynd, and GerK, have been identified in the *B. licheniformis* type strain ATCC 14580/DSM 13 (8, 19–21). We have recently shown that *B. licheniformis* spore germination in response to nutrients depends on cooperation between the GerA GR and the Ynd or GerK GR, and that the most efficient nutrient germinants are L-alanine and L-cysteine (19, 22). Setlow et al. (23) divided the spore germination process into two distinct stages. During the first stage, the GRs are activated by nutrients, followed by a rapid release of the spores' depot of CaDPA, partial rehydration of the core, and partial loss of heat resistance. In the second stage, cortex lytic enzymes (CLEs) degrade the cortex, which leads to increased water influx to the core. As the core rehydrates and expands, metabolism resumes, and the spores enter the outgrowth phase (2, 23). During this process, the spores become fully susceptible to environmental stress (1, 3).

Because of spores' extreme resistance, traditional food-processing methods for spore inactivation often rely on harsh temperature treatments, which have the disadvantage of altering the food's nutritional and sensory qualities. Alternative processing methods, where inactivation can be achieved at lower temperatures, are therefore of interest to food manufacturers. One such technology is high-pressure (HP) processing, which reduces the microbial load without compromising the fresh quality of the food. Conditions of HP in the range of 200 to 600 MPa combined with low to moderate heat are currently used in the food industry to inactivate vegetative microorganisms or to modulate enzymatic reactions in food, but they have not yet been implemented for elimination of spores due to their high-pressure resistance at ambient temperatures (24, 25). To achieve HP inactivation of spores, it is generally accepted that spores must first germinate and that germinated spores are inactivated by subsequent heat or pressure exposure in a two-step process (26, 27). Recently, flow cytometric analysis of HP-treated *B. licheniformis* and *B. subtilis* spores has revealed that HP inactivation of spores is a three-step process, with an "unknown" physiological state between the germinated and the inactivated states (28–30). The mechanism whereby HP initiates germination differs depending on the level of pressure administered. At moderate HP

TABLE 1 Germination and inactivation kinetics for all *B. licheniformis* and *B. subtilis* strains, as determined by plate counts

Strain	Description	Functional GR(s)	HP treatment (MPa)	Temp (°C)	G_{max}[a]	Log germination[b]	Log inactivation[b]
MW3	Wild type	GerA, Ynd, GerK	150	37	0.21 (0.018)	4.9	3.4
			550	37	<0.01	−0.5	−0.6
			550	60	0.82 (0.050)	4.3	4.8
NVH-1032	Food isolate		150	37	0.029 (0.005)	3.3	3.1
			550	37	<0.01	0.01	−0.1
			550	60[c]	0.33 (0.035)	4.8	4.2
PS832	Wild type	GerA, GerB, GerK	150	37	0.26 (0.034)	5.1	4.3
			550	37	0.054 (0.007)	2.5	0.3
			550	60[c]	0.98 (0.007)	5.3	5.2
NVH-1307	Δ*gerAA* mutant	Ynd, GerK	150	37	0.17 (0.040)	3.6	1.0
NVH-1335	Δ*yndD* mutant	GerA, GerK	150	37	0.071 (0.028)	3.4	2.3
NVH-1324	Δ*gerKA-C* mutant	GerA, Ynd	150	37	0.20 (0.021)	4.9	2.9
NVH-1323	Δ*gerAA* Δ*gerKA-C* mutant	Ynd	150	37	0.16 (0.040)	2.7	1.2
NVH-1368	Δ*gerAA* Δ*yndD* mutant	GerK	150	37	<0.01	0.7	−1.8
NVH-1376	Δ*yndD* Δ*gerKA-C* mutant	GerA	150	37	<0.01	0.05	−0.3
NVH-1370	Δ*gerAA* Δ*yndD* Δ*gerKA-C* mutant	None	150	37	<0.01	−0.4	−0.6

[a]G_{max}, maximum germination rate (log heat-sensitive spores per minute). Standard error of the estimate (SE_{est}) is presented in parentheses.
[b]Log germinated or log inactivated spores after HP treatment determined by plate counts. Data are presented as arithmetic means.
[c]Only one spore batch.

(mHP) of 80 to 300 MPa, germination is triggered via the GRs, while very HP (vHP) of 300 to 600 MPa triggers GR-independent germination by directly opening the DPA channels causing CaDPA release (27, 31, 32). During vHP (>300 MPa) treatment, *B. subtilis* spores become heat sensitive but are not inactivated by the pressure treatment (33). High-pressure thermal sterilization (HPTS) is a strategy that exploits the synergetic effect of vHP (>500 MPa) and elevated temperatures (>60°C) on spore inactivation (24, 34). However, due to technical limitations, temperature inhomogeneity in industrial-scale vessels, and difficulties predicting spore inactivation kinetics, this method is not yet applied commercially (24, 25).

Currently, studies on the effect of HP on GR-induced spore germination have been limited to *B. subtilis* and *Bacillus cereus* spores (31, 36, 37). This study examines the effect of mHP on *B. licheniformis* mutant spores deficient in one or several of the GerA, Ynd, or GerK GRs. The effect of vHP at moderate and elevated temperature on *B. licheniformis* spores was also assessed.

RESULTS

Effect of mHP on spore germination. To explore the effect of mHP on *B. licheniformis* spores, we exposed 10^8 spores/ml of strain MW3 and the slow-germinating food isolate NVH-1032 to 150 MPa at 37°C and determined the level of germination and inactivation by plate counts. For comparison, spores of the *B. subtilis* strain PS832 were subjected to the same treatment. The results are presented as log germinated or inactivated spores (arithmetic mean of two or more spore batches). The exposure of MW3 and PS832 spores to mHP induced spore germination at a maximum rate (G_{max}) of 0.21 and 0.26 log spores/min. After a holding time of 2 h, 4.9 and 5.1 log germinated (heat-sensitive) spores were detected for strains MW3 and PS832, respectively (Table 1 and Fig. 1A and C). The MW3 and PS832 inactivation levels after 2 h of mHP treatment were 3.4 and 4.3 log, respectively (Table 1 and Fig. 1A and C). Spores of strain NVH-1032 displayed a germination rate of 0.029 log spores/min, which was lower than the G_{max} of MW3 and PS832 spores (Table 1 and Fig. 1B). However, NVH-1032 spores treated with mHP for 60 min and 2 h demonstrated a large (up to 3-log) difference between spore batches in the levels of germinated and inactivated spores (Fig. 1B, and Fig. S1 in the supplemental material). Notably, the germination rates were based on an average of the data collected over the first 15 min of mHP exposure, where there was little variation between spore batches.

Flow cytometry analysis of mHP-treated *B. licheniformis* spores. Flow cytometry (FCM) analysis provides information about the physiological state of the HP-treated

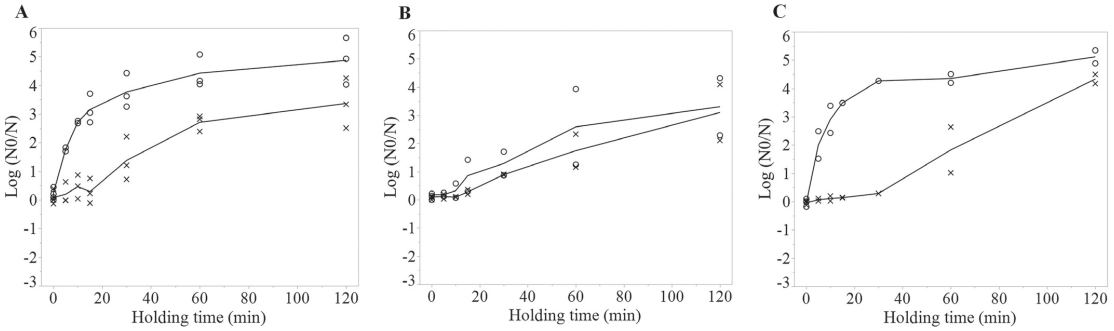

FIG 1 Plate count data showing the effect of mHP treatment (150 MPa at 37°C) on *Bacillus* spores. ○, germinated spores; ×, inactivated spores. Each symbol represents an independent spore batch. (A) *B. licheniformis* strain MW3. (B) *B. licheniformis* strain NVH-1032. (C) *B. subtilis* strain PS832.

spores. By studying staining characteristics of MW3 spore samples containing known physiological states, five subpopulations representing dormant (R1), germinated and viable (R2), unknown (R3 and R4), and inactivated (R5) states were identified (Fig. 2). The percentages of spores detected in each physiological state are shown in Table S1. The dormant spore population displayed low fluorescence intensities for both propidium iodide (PI) and Syto16 (Fig. 2, R1). As the spores germinated, more Syto16 entered the core of the spores, which increased the Syto16 fluorescence intensity, while the PI fluorescence intensity stayed low (Fig. 2, R2). At mHP holding times ≥5 min, two other subpopulations, both displaying staining characteristics different from those of dormant and germinated spores, started to emerge between R1 and R2. The subpopulation in the first "unknown" state (R3) displayed a lower Syto16 fluorescence intensity than the germinated spores, but the PI fluorescence intensity did not change. The second subpopulation (R4), which increased in size with longer holding times, displayed even lower Syto16 fluorescence intensity than the subpopulation in R3 (Fig. 2, R4). Heat treatment after HP exposure resulted in a 5th subpopulation that exhibited a strong PI fluorescence intensity indicative of damage to the IM of the spores (Fig. 2, R5). The great majority of the spore populations were present in the R5 region after an additional heat treatment following HP exposure, which suggests that the spores present in R2, R3, and R4 were heat sensitive (had germinated). When we compare FCM data and plate count data below, we have interpreted the spores in regions R2 to R4, as well as R5, as spores that have undergone germination and now possess different degrees of injuries (sublethal to lethal).

Sensitivity of individual germination receptors to mHP. To assess the role of individual GRs in mHP-induced germination, we exposed spores of MW3 *ger* double-null mutants to mHP. Spores of strain NVH-1323 (Δ*gerAA* and Δ*gerKA* Δ*gerKC* [Δ*gerKA*-

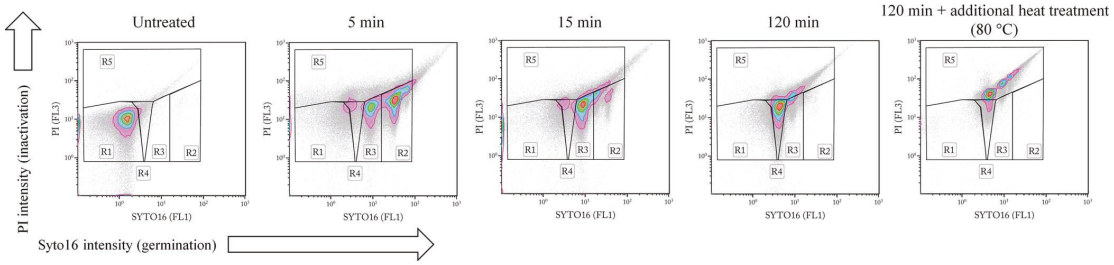

FIG 2 Contour-density plots of FCM data showing the effect of mHP treatment (150 MPa at 37°C) on the physiological state of *B. licheniformis* strain MW3 spores. Gating: R1, dormant spores; R2, germinated spores; R3, unknown state 1; R4, unknown state 2; and R5, inactivated spores. Contour-density plots depict the results from analyses performed on the CyFlow ML (~200,000 events). Instrument-specific gates were constructed as described in "Flow cytometry analysis of mHP-treated *B. licheniformis* spores," above.

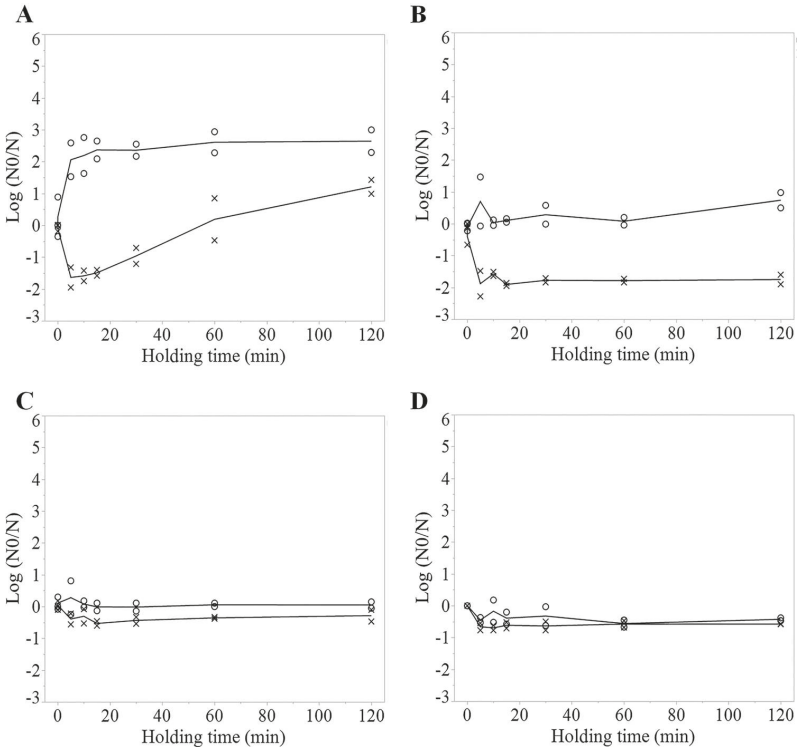

FIG 3 Plate count data showing the effect of mHP treatment (150 MPa at 37°C) on spores of *B. licheniformis ger* double- and triple-null mutants. ○, germinated spores; ×, inactivated spores. Each symbol represents an individual spore batch. (A) NVH-1323 (Δ*gerAA* Δ*gerKA-C*). (B) NVH-1368 (Δ*gerAA* Δ*yndD*). (C) NVH-1376 (Δ*gerKA-C* Δ*yndD*). (D) NVH-1370 (Δ*gerAA* Δ*gerKA-C* Δ*yndD*).

C]), in which the only intact GR expressed is Ynd (Ynd$^+$), demonstrated a G_{max} comparable to that of wild-type (MW3) spores (0.16 log spores/min compared to 0.21 log spores/min, respectively). Two hours of mHP treatment of NVH-1323 (Ynd$^+$) spores resulted in 2.7 log germination and 1.2 log inactivation (Table 1 and Fig. 3A), which was 2.2 log lower, with respect to both germination and inactivation, than observed for wild-type spores (Table 1 and Fig. 1A). This was also confirmed by FCM analysis (Fig. 4). NVH-1368 spores (Δ*gerAA* Δ*yndD*), in which the only intact GR expressed is GerK (GerK$^+$), also demonstrated a reduced level of germination (0.7 log) compared to the wild-type and NVH-1323 (Ynd$^+$) spores after 2 h of treatment (Table 1 and Fig. 3B). Inactivation of NVH-1368 (GerK$^+$) spores was −1.8 log after 2 h of treatment. The negative value for log inactivated spores is due to an increase in CFU per milliliter after mHP treatment compared to the untreated sample (N_0). This was also observed for other strains and resulted in a "dip" in the curves in Fig. 3A and B and 5A. FCM analyses of NVH-1368 (GerK$^+$) spores showed that a subpopulation germinated in response to mHP (Fig. 4). After 15 min of treatment, approximately 57% of the total population had germinated (entered R2, R3, R4, and R5 states), compared to approximately 94% and 89% of the wild-type and NVH-1323 (Ynd$^+$) spore populations, respectively (Fig. 4). After 2 h of mHP exposure, the majority of the NVH-1368 spore population had germinated (Fig. 4). For spores of strain NVH-1376 (Δ*gerKA-C* Δ*yndD*), in which the only intact GR expressed is GerA (GerA$^+$), and spores of strain NVH-1370 (Δ*gerAA* Δ*gerKA-C* Δ*yndD*), which lack all functional GRs (Ger$^-$), no mHP-induced germination was detected, as determined by the plate counts (Table 1 and Fig. 3C and D). However, it

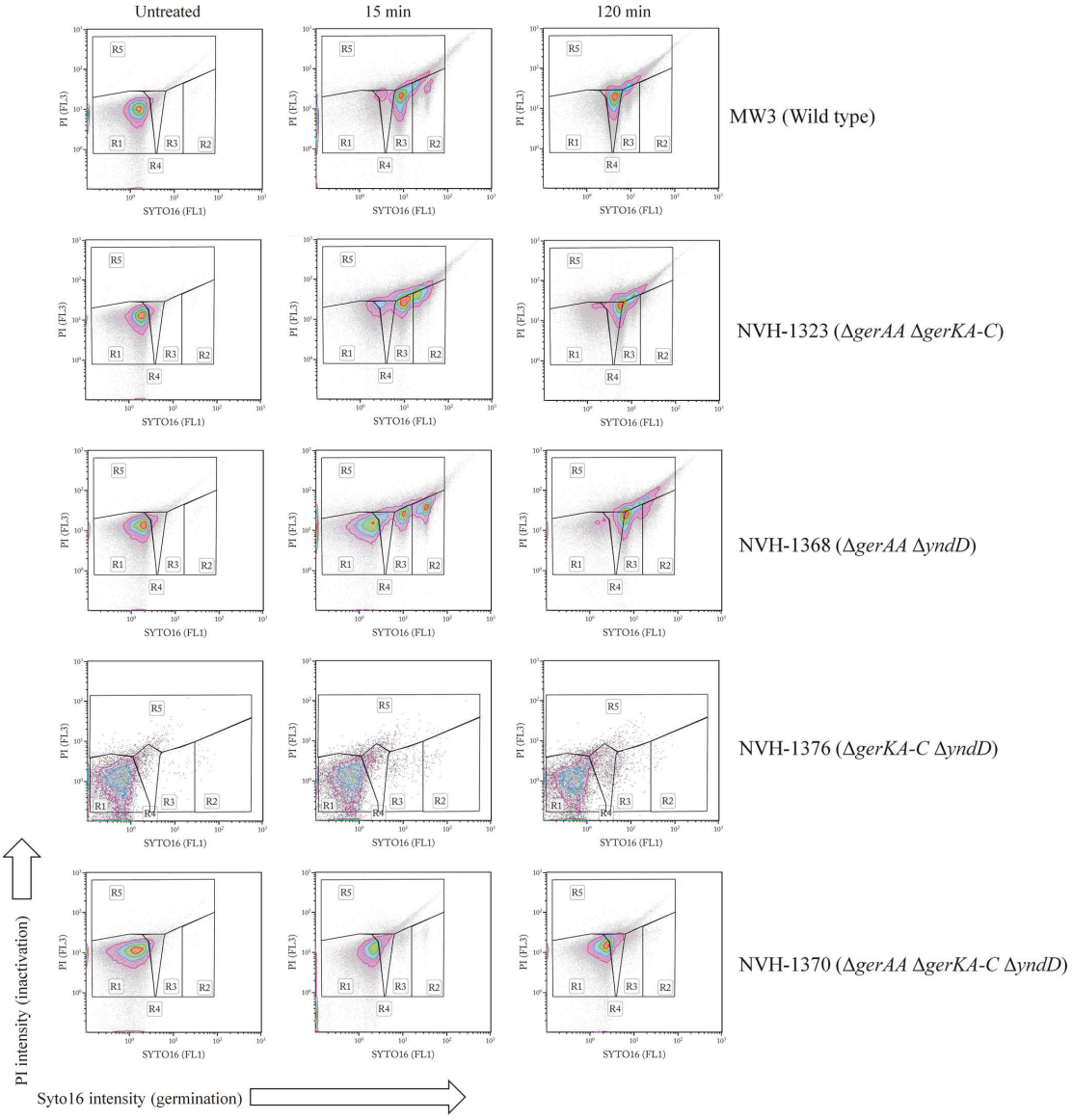

FIG 4 Contour-density plots of FCM data showing the effect of mHP treatment (150 MPa at 37°C) on the physiological state of wild-type and *ger* double- and triple-null mutant spores of the *B. licheniformis* strain MW3. Gating: R1, dormant spores; R2, germinated spores; R3, unknown state 1; R4, unknown state 2; R5, inactivated spores. Contour-density plots depict the results from analyses of MW3 (wild type), NVH-1323 (Δ*gerAA* Δ*gerKA-C*), NVH-1368 (Δ*gerAA* Δ*yndD*), and NVH-1370 (Δ*gerAA* Δ*gerKA-C* Δ*yndD*) spores performed on the CyFlow ML (~200,000 events). NVH-1376 (Δ*gerKA-C* Δ*yndD*) analyses were performed on the FACSCalibur (~10,000 events). Instrument-specific gates constructed as described in "Flow cytometry analysis of mHP-treated *B. licheniformis* spores," above.

should be noted that the NVH-1370 (Ger$^-$), NVH-1376 (GerA$^+$), and NVH-1368 (GerK$^+$) spores were still able to germinate and form colonies on nutrient agar, as approximately 0.1 to 1% of the total spore population germinated (data not shown). FCM analyses confirmed that NVH-1376 (GerA$^+$) and NVH-1370 (Ger$^-$) spores did not germinate in response to mHP treatment (Fig. 4).

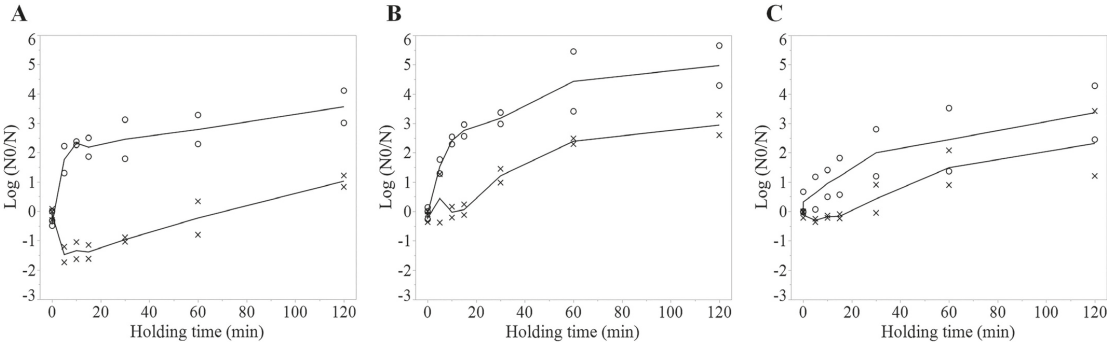

FIG 5 Plate count data showing the effect of mHP treatment (150 MPa at 37°C) on *B. licheniformis ger* single-null mutant spores. ○, germinated spores; ×, inactivated spores. Each symbol represents an individual spore batch. (A) NVH-1307 (Δ*gerAA*), (B) NVH-1324 (Δ*gerKA-C*), and (C) NVH-1335 (Δ*yndD*).

To address the possibility of functional cooperation between GRs in mHP-induced germination, we exposed spores of *ger* single-null mutant strains to mHP. Spores of strain NVH-1307 (Δ*gerAA* mutant), expressing intact GerK and Ynd GRs ($GerK^+$ Ynd^+), and spores of strain NVH-1324 (Δ*gerKA-C* mutant) expressing intact GerA and Ynd GRs ($GerA^+$ Ynd^+), displayed G_{max} values in response to mHP similar to spores of the wild-type strain, i.e., 0.17 log spores/min and 0.20 log spores/min compared to 0.21 log spores/min, respectively (Table 1 and Fig. 5A and B, compared to Fig. 1A). In contrast, spores of strain NVH-1335 (Δ*yndD* mutant), which expresses intact GerA and GerK GRs ($GerA^+$ $GerK^+$), demonstrated a reduced G_{max} (0.071 log spores/min) compared to the wild type, NVH-1307 ($GerK^+$ Ynd^+), and NVH-1324 ($GerA^+$ Ynd^+) spores (Table 1 and Fig. 5C). FCM results confirmed the plate counts, as a larger fraction of the NVH-1335 ($GerA^+$ $GerK^+$) spore population remained dormant (R1) after mHP exposure while large germinated subpopulations (R2, R3, and R4) were seen in the wild-type, NVH-1324 ($GerA^+$ Ynd^+), and NVH-1307 ($GerK^+$ Ynd^+) spore populations (Fig. S3).

Germination and inactivation of spores by vHP. In order to obtain information about the effect of vHP on *B. licheniformis* spores, spores of strains MW3 and NVH-1032 were exposed to 550 MPa at 37°C for holding times of up to 2 h. *B. subtilis* PS832 was included for comparison. Exposure to vHP did not trigger germination or inactivation of MW3 and NVH-1032 spores, even after a holding time of 2 h, as determined by plate counting (Table 1 and Fig. 6A and B). FCM analyses of NVH-1032 spores treated with vHP confirmed the plate count results (Fig. S4). However, FCM analyses of spores of strain MW3 revealed a subpopulation of germinated spores following this treatment

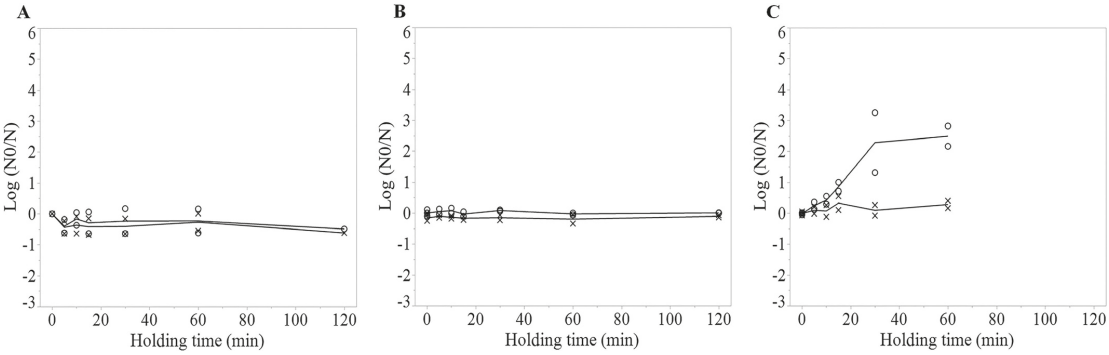

FIG 6 Plate count data showing the effect of vHP treatment (550 MPa at 37°C) on *Bacillus* spores. ○, germinated spores; ×, inactivated spores. Each symbol represents an individual spore batch. (A) *B. licheniformis* strain MW3. (B) *B. licheniformis* strain NVH-1032. (C) *B. subtilis* strain PS832.

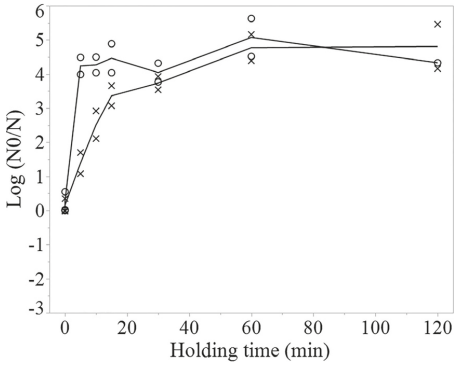

FIG 7 Plate count data showing the effect of vHP treatment (550 MPa at 60°C) on *B. licheniformis* strain MW3 spores. Each symbol represents an individual spore batch. ○, germinated spores; ×, inactivated spores.

(Fig. S4), which corresponded to approximately 58% of the total spore population after 2 h of vHP treatment (Fig. S4). Germination at this level can be difficult to detect accurately by plate counts, as it corresponds to ~0.4 log germinated spores. *B. subtilis* PS832 spores demonstrated 2.5 log germination after 60 min of vHP exposure, while 0.3 log spore inactivation was detected (Table 1 and Fig. 6C). This was also supported by FCM analyses. However, the formation of subpopulations in R3 and R4 following germination (R2) was slower than when spores were exposed to mHP (Fig. S4).

To study the effect of vHP combined with high temperature on spore germination and inactivation, spores of strain MW3 were exposed to 550 MPa at 60°C for holding times of up to 2 h. This treatment resulted in 4.3 log germination and 4.8 log inactivation after 2 h of vHP exposure (Table 1 and Fig. 7). In accordance with these results, FCM analyses showed that a large proportion of the spore population was inactivated after 2 h of exposure, i.e., displayed strong PI fluorescence intensities (Fig. S6). However, it should be noted that the DNA-binding abilities of Syto16 and PI are reduced in spores treated under these conditions compared to the HP treatments at 37°C (29, 30, 35). Similar results were obtained for spores of the *B. subtilis* strain PS832, as determined by plate counting (Table 1 and Fig. S5B). Spores of the *B. licheniformis* strain NVH-1032 exhibited lower G_{max} (0.33 log spores/min) than the spores of strains MW3 and PS832 (0.82 and 0.98 log spores/min, respectively) in response to this treatment (Table 1). However, after 2 h of exposure, the total levels of germinated and inactivated NVH-1032 spores were 4.8 log and 4.2 log, respectively, which is similar to the levels observed for strains MW3 and PS832 (Table 1 and Fig. S5A).

DISCUSSION

In the present study, we have investigated the effects of mHP and vHP on *B. licheniformis* spores and assessed the contribution of individual GRs to mHP-induced germination. We found that mHP treatment at 37°C induced *B. licheniformis* spore germination via the GerA, GerK, and Ynd GRs. The Ynd GR appeared to play the central role in mHP-induced germination, as spores of strain NVH-1323 (Ynd$^+$) germinated with an efficiency similar to that of the wild-type spores. In addition, NVH-1335 (GerA$^+$ GerK$^+$) spores, which carry a *yndD* deletion, demonstrated reduced germination efficiency. Spores of NVH-1368, expressing only GerK, also germinated in response to mHP, although not as efficiently as NVH-1323 (Ynd$^+$) spores or wild-type spores. The importance of the Ynd and GerK GRs in mHP-induced spore germination was supported by the inability of strain NVH-1376 (GerA$^+$) spores to germinate in response to mHP exposure. Spores of strain NVH-1335 (GerA$^+$ GerK$^+$) demonstrated a more efficient germination response to mHP than NVH-1368 (GerK$^+$) spores, and similarly, NVH-1324

(GerA$^+$ Ynd$^+$) spores demonstrated a more efficient germination response than NVH-1323 (Ynd$^+$) spores. This suggests that GerA facilitates mHP-induced germination in the presence of the GerK or the Ynd GR. This is similar to the cooperation between GerA, Ynd, and GerK GRs during nutrient-induced germination of *B. licheniformis* spores (19).

We have recently shown that the GerA GR appears to play the most prominent role in nutrient-induced germination (19). Gene depletions in the *gerA* operon were more detrimental to nutrient-induced germination than gene depletions in the *ynd* and *gerK* operons (19, 22). This differs from the role of the individual GRs in mHP-induced germination, as revealed by the results from the present study, where Ynd appeared to play a more prominent role than the other GRs. In *B. licheniformis*, the individual GRs' contribution to nutrient- and mHP-induced germination observed for *B. licheniformis* spores differs from that observed for *B. subtilis* spores, where the individual GRs' mHP responsiveness corresponds to their relative contribution to nutrient-induced germination (7, 37). However, a study on how mHP affects *B. cereus* spores lacking one of the seven GRs showed that loss of individual GRs did not affect mHP-induced germination (36). The reason for the differences in mHP sensitivity between GRs is not fully understood. It has previously been shown that the rate of mHP-induced germination can be increased by overexpressing individual *ger* operons (37, 38). We have shown that *yndD* and *gerKA* are expressed at ~10 times higher levels than *gerAA* during sporulation (19). If the expression levels reflect the levels of GRs in the IM, this could at least partly explain the importance of Ynd and GerK in mHP-induced germination. In addition to GR levels in the spores' IM, structural properties of the GR might play a role in its mHP responsiveness. In *B. subtilis*, variants of the GerB GR with single amino acid substitutions in the A or B subunits were more responsive to mHP than the native GerB GR (37). The gene organization of the *ynd* operon in *B. licheniformis* differs from that typically found in *Bacillus* species, as it contains three B subunit genes, in contrast to the more commonly occurring single B subunit gene (8, 20, 21). The potential significance of the multiple B subunits for the sensitivity to mHP and nutrient-induced germination is currently unknown but will be subjected to further studies.

Exposure to 550 MPa (vHP) at 37°C induced only low levels of germination of *B. licheniformis* spores, while *B. subtilis* spores germinated more efficiently under these conditions. The absence of substantial spore inactivation under these conditions has previously been described for *B. subtilis* spores (29), and this was confirmed in our study. The difference in vHP responsiveness between *B. licheniformis* and *B. subtilis* spores could be due to structural differences in the SpoVA proteins that form the mechanosensitive DPA channels (39). Alternatively, vHP could inactivate other proteins or pathways responsible for CaDPA release and subsequent germination. Differences in the IM, for instance, in the fatty acid composition, may also influence the sensitivity of the spores to vHP. However, in previous studies, spores of *B. subtilis* strains with altered levels of unsaturated fatty acids in the IM demonstrated no difference in vHP responsiveness compared to wild-type spores (32). Sporulation temperature has been shown to affect spore germination in response to HP, likely by affecting the fluidity of the IM. *B. subtilis* spores produced at higher temperatures displayed reduced vHP-induced germination rates, whereas mHP-induced germination rates increased with increased sporulation temperatures (32, 37). *B. licheniformis* spores have previously been reported to germinate in response to 600 MPa at 77°C (30), as high temperature and HP are known to act synergistically on germination and inactivation (24). In accordance with that study (30), we observed efficient germination and inactivation of MW3 and NVH-1032 spores after they were exposed to vHP at 60°C.

In this study, some of the *B. licheniformis* strains showed an approximately 100-fold increase in CFU per milliliter after mHP treatment, as detected by the plate counts. A similar effect has been described before, as a "shoulder formation" in the inactivation kinetics of HP-treated *B. subtilis* spores, a phenomenon that was assigned to spore agglomerates that disassociated during HP treatment (40, 41). In the present study, we observed an increase in CFU per milliliter for three strains (Fig. 3A and B and 5A). However, the FCM analyses supported the plate count data on germinated (heat-

sensitive) spores for these strains, and the shoulder formation observed in the inactivation kinetic curves was not investigated further. In our experiments, some variation in mHP responsiveness between spore batches was observed; particularly, the food isolate NVH-1032 demonstrated a large variation between the two spore batches used. Both sporulation conditions and stochastic events during sporulation have been shown to affect different spore properties, including germination behavior (42). In addition, heterogeneity in germination and inactivation behavior between spores of different species, strains, spore batches, and even within the same population has been described before (26, 42–44).

Approximately 0.1 to 1% of *B. licheniformis* spores lacking all functional GRs were still capable of forming colonies on nutrient agar. Similar observations have been made in *B. subtilis* and were attributed to spontaneous germination (7). Spontaneous germination may also explain our observations. There is an orphan *gerA* homolog (*yndF2*) encoding an N-terminally truncated C subunit in the *B. licheniformis* type strain ATCC 14580/DSM 13 genome (20, 21). However, deletion of this gene did not significantly affect L-alanine or L-cysteine-induced germination (our unpublished data) and is therefore not likely to explain the low level of germination observed in spores lacking all functional GRs.

The results from the FCM analyses largely supported the observations from the plate count data, and they also provided information about the physiological state of the spores. The FCM analyses identified two distinct subpopulations representing unknown states, which increased with increasing HP holding times. Previous FCM analyses of HP-treated *B. subtilis* and *B. licheniformis* spores have detected one such unknown state (28, 30, 40). The two unknown states seen in the present study displayed lower Syto16 fluorescence intensities than the germinated subpopulation. Mathys et al. (30) hypothesized that the lower Syto16 fluorescence in the unknown state could be due to the entry of some PI into the core, which partly displaces Syto16 or leads to quenching of Syto16 by fluorescence resonance energy transfer to PI. Syto16 is commonly used in eukaryotic cells as an indicator for apoptosis, as Syto16 fluorescence intensity is lost during cell death (45, 46). A loss of Syto16 fluorescence upon programmed cell death has also been reported for *B. subtilis* mother cells during sporulation (47). We compared data from the FCM analyses and plate counts to assess the viability of the spores in the two unknown states. After 15 min of mHP exposure of *B. licheniformis* wild-type spores, the FCM analyses showed a large subpopulation in R3, and the plate counts showed a high level of germinated (heat-sensitive) spores and low levels of inactivation. After 2 h of mHP exposure, FCM analyses showed a large subpopulation in R4, and the plate counts showed a high level of inactivation. This suggests that the subpopulation present in region R3 (unknown state 1) corresponds to viable spores capable of outgrowth on nutrient agar, whereas the subpopulation present in region R4 (unknown state 2) likely represents uncultivable spores. Syto16 does not bind exclusively to DNA, at least in eukaryotic cells, but also binds to cytoplasmic contents (46). The loss of Syto16 fluorescence could be due to DNA fragmentation, breakdown of cytoplasmic contents, or membrane leakage of cytoplasmic contents (45). As PI fluorescence intensities are low, even after prolonged HP treatment at both mHP and vHP (37°C), extensive membrane disruptions are unlikely. Therefore, the unknown regions likely correspond to spores with different degrees of injury. However, further studies using cell sorting are needed to better understand the physiological characteristics of HP-exposed spores.

MATERIALS AND METHODS

Strains and spore preparation. The strains included in this study are listed in Table 2. The *B. licheniformis* strain NVH-1032 was isolated from a batch of spoiled canned meat, and its spores have been shown to germinate significantly more slowly than spores of the type strain *B. licheniformis* DSM 13 (50). *B. licheniformis* strain MW3 is a more readily transformable derivative of the type strain (51). The *B. licheniformis ger*-null mutants are isogenic to MW3 and carry different in-frame deletions in the *gerA* family operons (19, 22). The *B. subtilis* strain PS832 is a *trp*$^+$ derivative of *B. subtilis* 168 (7). Spores of the *B. licheniformis* strains and *B. subtilis* PS328 were prepared as described previously (7, 50, 52). Sporulation of all strains was done at 37°C, and spores were stored in sterile water.

TABLE 2 Strains used in this work

Strain or isolate	Description	Reference
PS832	*B. subtilis* subsp. *subtilis* 168 *trp*$^+$	7
NVH-1032	*B. licheniformis* food isolate	55
MW3	*B. licheniformis* DSM 13 Δ*hsdR1* Δ*hsdR2*	51
NVH-1307	*B. licheniformis* MW3 Δ*gerAA*::*spc*	22
NVH-1323	*B. licheniformis* MW3 Δ*gerAA*::*spc* Δ*gerKA-C*	19
NVH-1324	*B. licheniformis* MW3 Δ*gerKA-C*	19
NVH-1335	*B. licheniformis* MW3 Δ*yndD*	19
NVH-1368	*B. licheniformis* MW3 Δ*gerAA*::*spc* Δ*yndD*	19
NVH-1370	*B. licheniformis* MW3 Δ*gerAA*::*spc* Δ*gerKA-C* Δ*yndD*	19
NVH-1376	*B. licheniformis* MW3 Δ*gerKA-C* Δ*yndD*	19

High-pressure treatment. For HP treatment of spores, a monovessel and a multivessel U111 unit (Unipress, Warsaw, Poland) were used. The multivessel unit had a compression rate of 12 MPa/s, whereas the hand-pump-driven monovessel had a compression rate of 20 to 25 MPa/s. The monovessel unit had a vessel volume of 3.7 ml, the pressure-transmitting medium used was di-2-ethyl-hexyl-sebacate, and 4 300-μl spore solutions were treated at the same time. The multivessel unit had five chambers with vessel volumes of 4 ml each, and these were used for the treatment of four spore samples at a time. The pressure-transmitting medium used in this system was silicon oil. Exposure of spores to sublethal temperatures before induction of germination with nutrients or high pressure is known to synchronize the germination response (53, 54). Therefore, *B. licheniformis* and *B. subtilis* spores were heat activated at 65°C for 20 min prior to HP treatment. Subsequently, the spores were resuspended in 50 mM *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer (pH 7.0), and the optical density at 600 nm (OD_{600}) was adjusted to correspond to a spore titer of 10^8 spores/ml. For the experiments using the monovessel unit, a volume of 300 μl of the spore suspension was transferred to a shrinking tube with an inner diameter of 3 mm and outer diameter of 3.6 mm (Schrumpfschlauch 3/1; DSG-Canusa, Meckenheim, Germany), and the tube was sealed with a soldering iron. Four shrinking tubes were packed into a 1.8-ml Nunc cryotube with a screw top (Nunc A/S, Roskilde, Denmark), and the tube was filled with water. The temperature was measured in the tube's geometrical center. For the multivessel unit experiments, spore suspensions were added to 1.6-ml Nunc cryotubes, ensuring that no air bubbles were present, and treated directly with HP. Temperature measurement was done in a dummy sample containing only water, which was HP treated together with the spore samples. A thermocouple (Unipress, Warsaw, Poland) was used to monitor temperatures in both units.

HP experiments were performed at 37°C and 60°C, and the pressure chambers were submerged in a thermostatic bath (cc2; Huber GmbH, Germany) with silicon oil. The HP treatments were performed at 150 MPa and at 550 MPa, with holding times ranging from 1 s to 2 h under isothermal conditions. The HP experiments were performed in technical duplicates using at least two independent spore batches.

Enumeration of inactivated and germinated spores. Following HP treatment, an aliquot of the spore suspension was diluted in Ringer's solution (Merck, Germany), and volumes of 50 μl or 100 μl were plated on Nutrient agar (Oxoid, England) and incubated overnight at 37°C before colony enumeration. The level of spore inactivation was calculated as the log N_o/N ratio of CFU per milliliter of untreated spores (N_o) and CFU per milliliter of the HP-treated spores (N). In each experiment, an aliquot of the HP-treated spore suspension was heat treated at 80°C for 20 min before plating to determine the level of heat-sensitive (germinated) spores. The level of spore germination was calculated using $\log(N_o/N)$, where N_o is the CFU per milliliter of untreated spores and N is the CFU per milliliter of heat-treated spores. To show the batch variation, the results for each individual spore batch were plotted, with a line representing the mean of the spore batches. The maximum rate of germination (G_{max}) was determined by linear regression of the steepest segment (between holding times of 0 to 15 min) of the germination plots. The lower limit of detection of G_{max} was set to 0.01 log spores/min. The data were analyzed using JMP Pro 12 (SAS Institute Inc., NC, USA). Plate count results from experiments using the two different HP units (multivessel and monovessel) are presented in the same plots, as the differences in HP effects on spore germination and inactivation between the two units were less than the differences observed between spore batches (results not shown).

Flow cytometry analyses. Flow cytometry (FCM) analysis provides information about the physiological state of the spores and was performed as described by Mathys et al. (30). Spore suspensions were double stained with the fluorescent DNA stains Syto16 (Invitrogen, CA, USA) and propidium iodide (PI) (Invitrogen, CA, USA) to distinguish between spore germination and inactivation. Syto16 is membrane permeant but cannot penetrate the spore core, and it is used to indicate breakdown of the cortex during spore germination (32, 37). PI is not membrane permeant and indicates IM rupture and spore inactivation (30). Syto16 and PI were added to the spore suspension at concentrations of 0.5 μM and 15 μM, respectively, and the spores were stored in the dark for 15 min at room temperature prior to FCM analysis. The analyses were carried out with either a CyFlow ML (Partec GmbH, Münster, Germany) or a FACSCalibur flow cytometry instrument (BD Biosciences, CA, USA). HP-treated and untreated spores were diluted in ACES buffer (0.05 M [pH 7.0]) to allow a flow rate of 2,000 events/s, and a total of ~200,000 events were measured for the CyFlow ML, or 1,000 events/s and a total of 10,000 events were measured for the FACSCalibur. The data were analyzed with the Kaluza 3.1 software (Beckman Coulter, USA), using gating strategies based on biological controls confirmed by plate counts and phase-contrast microscopy. The flow cytometry results for a single spore batch are presented in the Results.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00503-17.

SUPPLEMENTAL FILE 1, PDF file, 2.0 MB.

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Supplementary information

The effect of high pressure on *Bacillus licheniformis* spore germination and inactivation

Kristina Borch-Pedersen, Hilde Mellegård, Kai Reineke, Preben Boysen, Robert Sevenich, Toril Lindbäck and Marina Aspholm

Revised, clean version

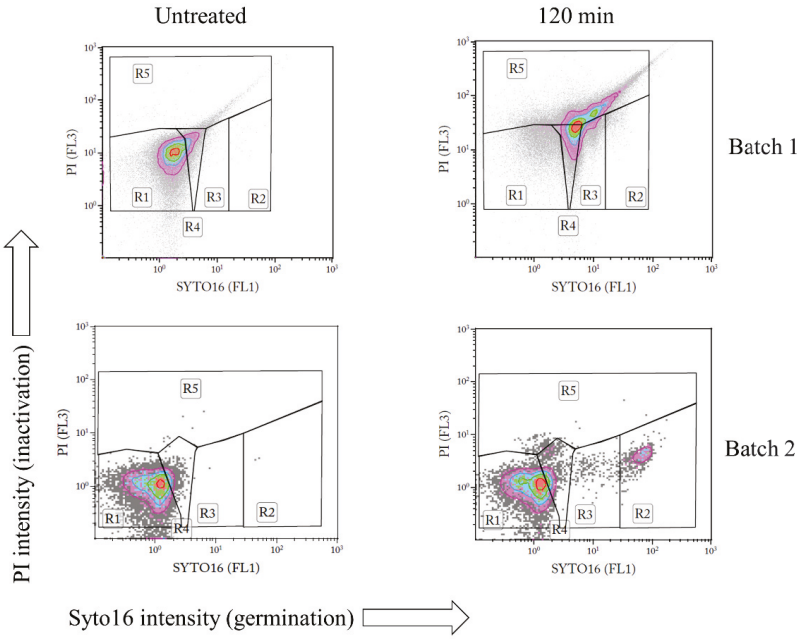

Fig. S1: Contour-density plots of FCM analyses showing the effect of mHP treatment (150 MPa at 37°C) on the physiological state of *B. licheniformis* strain NVH-1032, comparing two different spore batches. Gating: R1 – dormant spores, R2 – germinated spores, R3 – unknown state 1, R4 – unknown state 2 and R5 – inactivated spores. Contour-density plots of batch 1 depict the results from analyses performed on the CyFlowML (~200 000 events), whereas the contour-density plots of batch 2 depict the results from analyses performed on the FACSCalibur™ (~10 000 events).

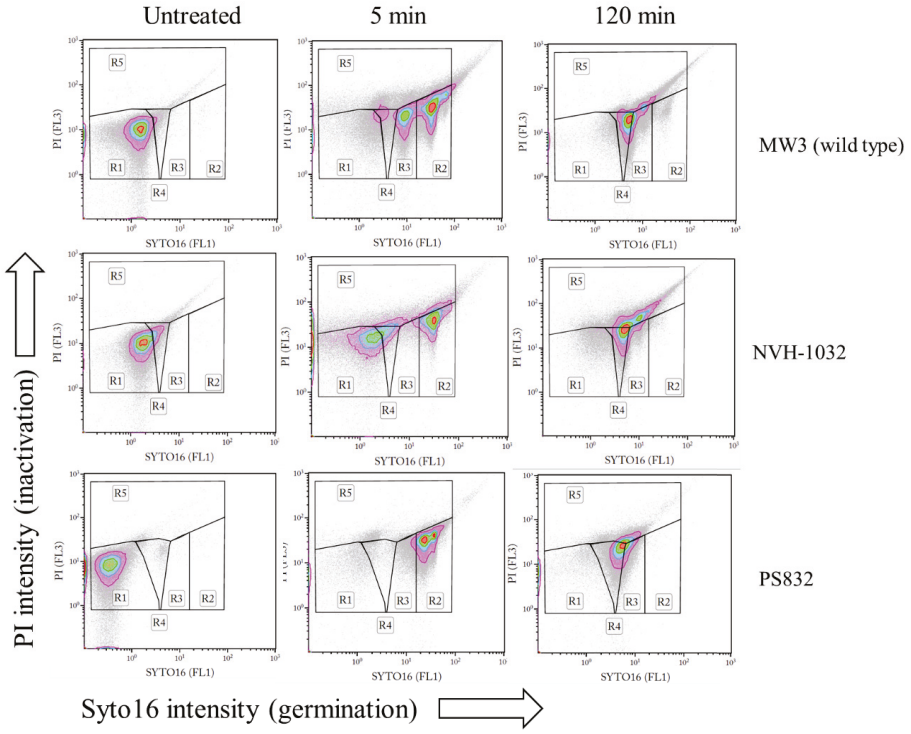

Fig. S2: Contour-density plots of FCM analyses showing the effect of mHP treatment (150 MPa) at 37°C on the physiological state of spores of the *B. licheniformis* strains MW3, *B. licheniformis* and NVH-1032 and the *B. subtilis* strain PS832. Gating: R1 – dormant spores, R2 – germinated spores, R3 – unknown state 1, R4 – unknown state 2 and R5 – inactivated spores. Contour-density plots depict the results from analyses performed on the CyFlowML (~200 000 events).

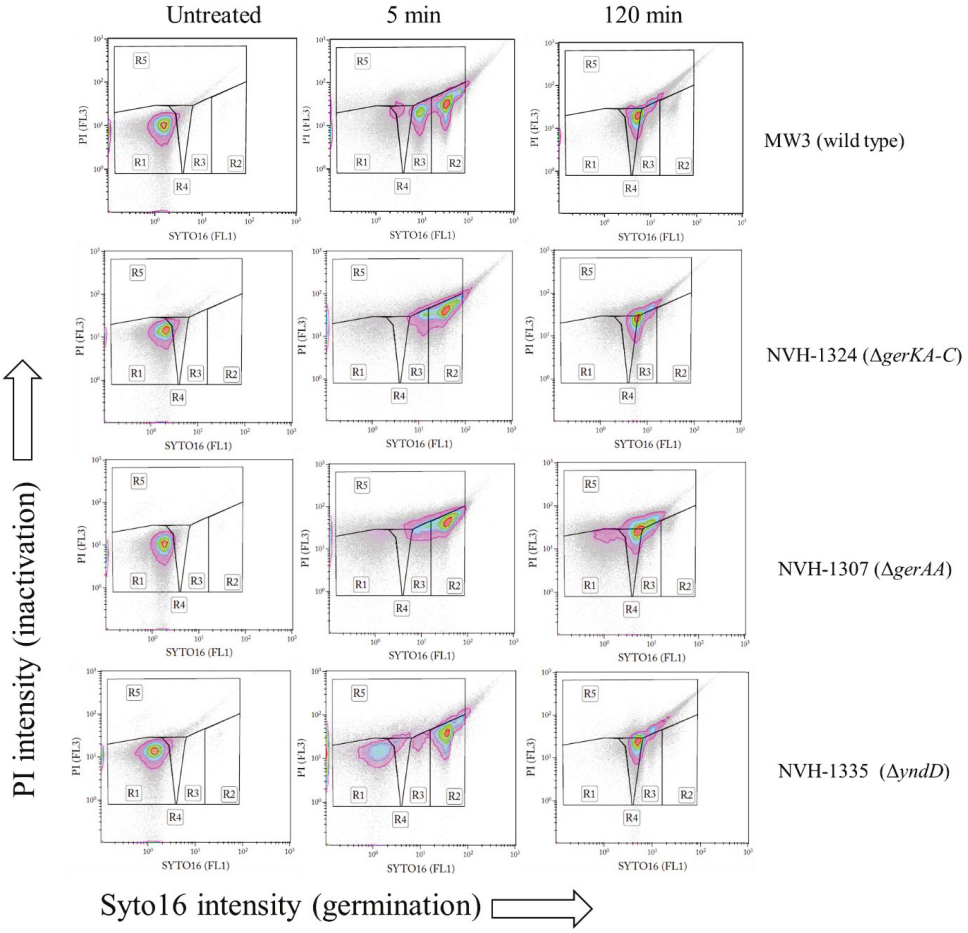

Fig. S3: Contour-density plots of FCM analyses showing the effect of mHP treatment (150 MPa at 37°C) on the physiological state of spores of the *B. licheniformis* strain MW3 and its single *ger*-null mutant derivative strains. Gating: R1 – dormant spores, R2 – germinated spores, R3 – unknown state 1, R4 – unknown state 2 and R5 – inactivated spores. Contour-density plots depict the results from analyses performed on the CyFlowML (~200 000 events).

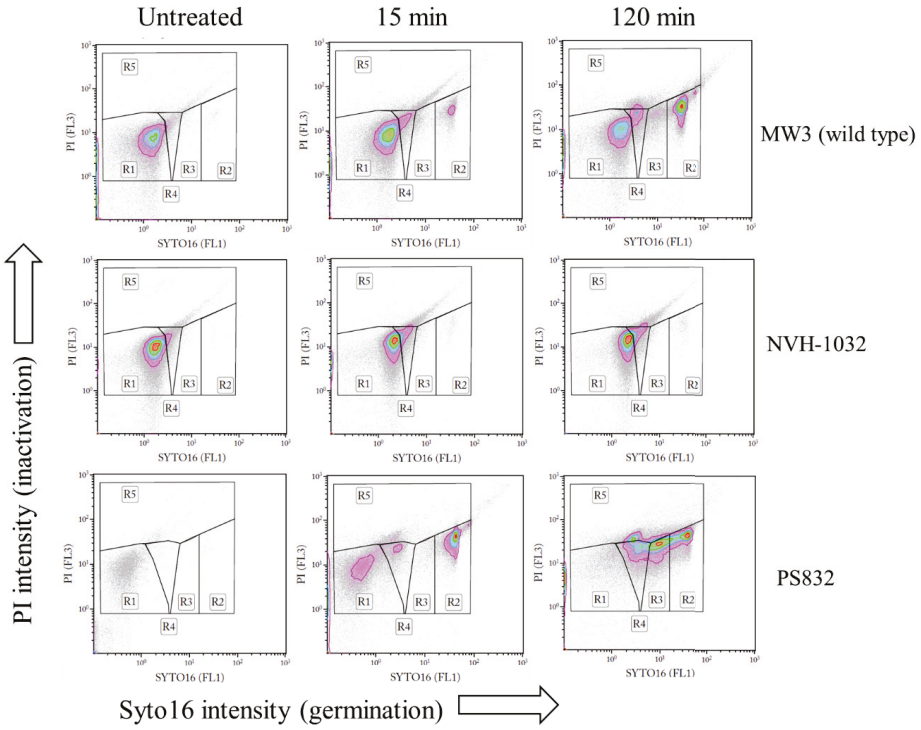

Fig. S4: Contour-density plots of FCM analyses showing the effect of vHP treatment (550 MPa) at 37°C on the physiological state of spores of the *B. licheniformis* strains MW3 and NVH-1032 and the *B. subtilis* strain PS832. Gating: R1 – dormant spores, R2 – germinated spores, R3 – unknown state 1, R4 – unknown state 2 and R5 – inactivated spores. Contour-density plots depict the results from analyses performed on the CyFlowML (~200 000 events).

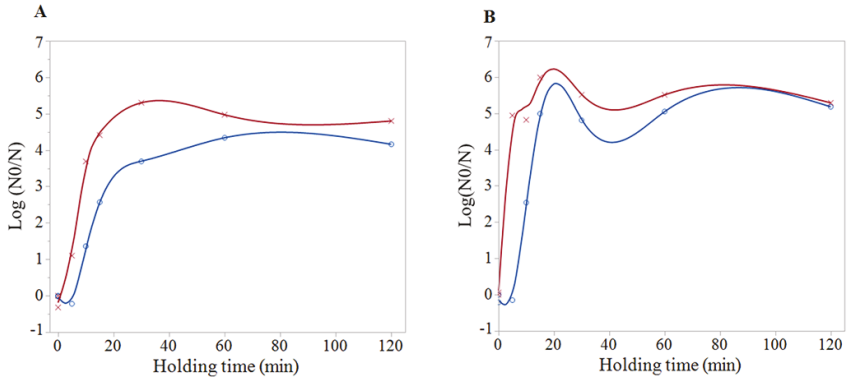

Fig. S5: Effect of vHP treatment (550 MPa) at 60°C on *Bacillus* spores for holding times up to 2 hours. (×) Germinated spores. (○) Inactivated spores. (A) *B. licheniformis* NVH-1032 and (B) *B. subtilis* PS832. Results represent a single spore batch.

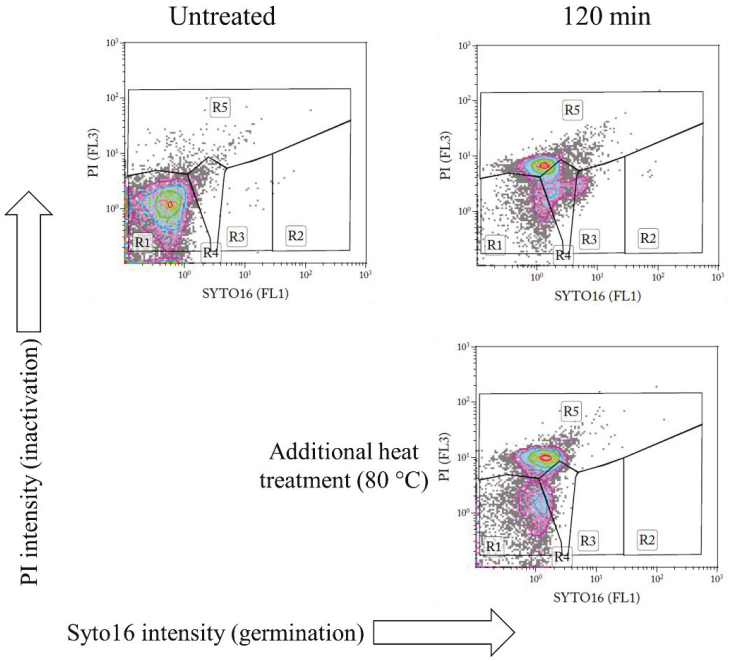

Fig. S6: Contour-density plots of FCM analyses showing the effect of mHP treatment (550 MPa at 60°C) on the physiological state of spores of the *B. licheniformis* strain MW3. Gating: R1 – dormant spores, R2 – germinated spores, R3 – unknown state 1, R4 – unknown state 2 and R5 – inactivated spores. Contour-density plots depict the results from analyses performed on the FACSCalibur™ (~10 000 events).

Table S1: Results of the FCM analyses presented as percentage of spores present in each region (R1-5).

Strain	**Treatment**		**% spores in region 5 min**	**% spores in region 15 min**	**% spores in region 120 min**
MW3 (wild type)	150 MPa	R1	6.19	5.67	5.19
		R2	54.51	13.23	0.31
		R3	26.29	61.6	9.01
		R4	5.14	8.55	64.41
		R5	7.86	10.95	21.07
	550 MPa	R1	83.95	79.86	41.94
		R2	5.26	5.56	39.62
		R3	0.91	1.75	4.33
		R4	8.03	9.81	9.53
		R5	1.85	3.03	4.58
***B. subtilis* strain PS832**	150 MPa	R1	2.32	2.15	2.04
		R2	84.50	81.69	0.09
		R3	8.44	9.56	19.06
		R4	1.47	1.93	54.03
		R5	3.27	4.67	24.79
	550 MPa	R1	47.80	23.71	4.30
		R2	42.81	67.46	24.89
		R3	0.45	0.65	30.34
		R4	6.21	5.38	25.25
		R5	2.73	2.80	15.22
NVH-1032 (food isolate)	150 MPa	R1	37.27	12.13	5.95
		R2	32.72	14.27	0.52
		R3	3.36	21.86	8.55
		R4	10.10	13.29	38.14
		R5	16.55	38.46	46.84
	550 MPa	R1	80.62	78.12	74.62
		R2	0.13	0.17	0.21
		R3	0.41	0.40	0.37
		R4	15.57	17.41	19.23
		R5	3.27	3.89	5.56

NVH-1323 (Δ*gerAA* Δ*gerKA-C*)	150 MPa	R1	65.31	10.91	9.55
		R2	10.1	17.32	0.62
		R3	2.52	35.27	21.65
		R4	13.52	10.29	34.99
		R5	8.56	26.22	33.19
NVH-1368 (Δ*gerAA* Δ*yndD*)	150 MPa	R1	ND	43.32	8.52
		R2	ND	19.79	5.32
		R3	ND	17.85	35.7
		R4	ND	8.34	20.98
		R5	ND	10.7	29.48
NVH-1376 (*ΔgerAA* Δ*gerKA-C* Δ*yndD*)	150 MPa	R1	ND	80.59	76.62
		R2	ND	0.84	0.35
		R3	ND	3.17	4.18
		R4	ND	9.47	12.43
		R5	ND	5.93	6.43
NVH-1370 (*ΔgerAA ΔgerKA-C ΔyndD*)	150 MPa	R1	ND	78.47	71.85
		R2	ND	0.55	0.40
		R3	ND	2.17	3.45
		R4	ND	15.02	19.54
		R5	ND	3.79	4.76
NVH-1324 (*ΔgerKA-C*)	150 MPa	R1	5.54	ND	3.88
		R2	62.19	ND	0.57
		R3	18.60	ND	30.09
		R4	2.34	ND	34.20
		R5	11.33	ND	31.25
NVH-1307 (Δ*gerAA*)	150 MPa	R1	9.05	ND	15.37
		R2	51.57	ND	0.82
		R3	17.50	ND	12.70
		R4	3.69	ND	30.55
		R5	18.20	ND	40.56
NVH-1335 (Δ*yndD*)	150 MPa	R1	30.45	9.70	2.19
		R2	49.48	33.46	0.78

		R3	5.44	27.29	11.44
		R4	4.57	6.56	54.65
		R5	10.06	22.98	30.95

ND, not determined.

III

Dissecting the cooperative interaction between the GerA and Ynd germination receptors in *B. licheniformis* spore germination

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MANUSCRIPT

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Running title: Dissection of the Ynd germination receptor

Key words: *Bacillus licheniformis*, spore germination, germination receptor, endospores

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ABSTRACT

Germination of *Bacillus* spores is triggered by the binding of specific nutrients to germinant receptors (GRs) located in the spore's inner membrane. The GRs usually consist of A, B and C protein subunits, encoded by tricistronic *ger* operons. Other cistronic organizations of *ger* operons are also seen, but the reason for this heterogeneity is not known. In contrast to the ABC (D) organization that characterizes *gerA* operons of many *Bacillus* species, all *Bacillus licheniformis* and *B. paralicheniformis* genomes analyzed here carry a pentacistronic *ynd* operon containing the *yndD*, *yndE3*, *yndE2*, *yndF*, *yndE1* genes encoding A, B, B, C and B subunits. We have previously shown that the Ynd GR depend on the GerA GR to induce efficient spore germination and that depletion of the A subunit of Ynd reduced the germination efficiency. Here we show that GerA triggers spore germination in the absence of the entire Ynd and GerK GRs, albeit at reduced efficiency. The absence of $YndE_3$ and/or $YndE_2$ resulted in severely reduced germination, while deletion of all three YndE subunits restored germination to near wild-type levels. This suggests that the presence of incomplete sets of YndE subunits exhibit a dominant negative effect on the cooperative function between GerA and Ynd. Re-introduction of *yndD* in a mutant lacking the entire *ynd* operon restored the cooperative function between Ynd and GerA indicating that only the A subunit of Ynd is essential for the cooperation between Ynd and GerA. This study provides novel insights into the cooperative functions between GRs in triggering spore germination.

INTRODUCTION

When starved for nutrients, many *Bacillus* species differentiate into the endospore (spores) form, which is a metabolically dormant, highly stress resistant and non-reproductive differentiation state (1, 2). The spores can stay dormant for long periods and survive environmental stressors that would normally kill the vegetative bacteria (3). When survival conditions improve, the dormant bacteria will initiate their metabolism and rapidly return into vegetative growth by the process of germination (3).

In *Bacillus* species, spore germination can be induced by exposure to biochemical and physical stimuli (3). However, in nature, spore germination is most likely triggered by exposure to various nutrient compounds (germinants) such as amino acids, nucleosides and sugars (3). In *Bacillus* spores, nutrient germinants are recognized by the GerA family of germinant receptors (GRs), located in the spore´s inner membrane (3). This recognition triggers subsequent germination events including release of spores large depot of Ca^{2+}-dipicolinic acid (CaDPA), core rehydration and hydrolysis of the spores peptidoglycan cortex (3).

A large diversity in germination receptors exist among *Bacillus* species and each species often express several GRs (2, 4, 5). Some GRs function alone while other require cooperation with another GR to trigger spore germination in response to a single germinant or combination of germinant compounds (5). In *Bacillus subtilis*, GRs colocalize into germinosomes, which appears to be essential for efficient spore germination (6). However, it is still largely unknown how the different GRs interact and how recognition of nutrient germinants ultimately leads to spore germination (3).

Molecular, genetic and biochemical data indicate that germinant receptors are composed of three subunits A, B and C (7). GRs are encoded by *gerA* family genes that usually are organized in tricistronic operons, encoding the A, B and C subunits. However, *gerA* genes are also found

as single genes, in dicistronic operons or in operons containing more than one gene encoding homologous GR subunits (2, 4). Some *Bacillus* species also encode an additional D subunit, but the function of the D subunit is currently unknown (2, 4). In *Bacillus* species, all A, B and C subunits appears to be required in the functional GR but the specific role of each subunit is poorly understood (8). The A and B subunits are predicted to contain five and ten transmembrane domains, respectively, and are therefore almost certainly integral membrane proteins. The B subunit show homology to proteins of the amino acid/polyamine/organocation (APC) superfamily of single component membrane transporters (9). There is, however, no evidence as to whether the B subunit functions in molecular transport. Evidence based on cross-homologue chimeric constructs and site directed mutagenesis suggests that the B subunit contains the germinant recognition site but the identity of the ligand-binding site and the mechanism of binding is currently unknown (10-12). In *B. megaterium*, B subunits encoded by different *ger* operons can be used interchangeably in the GerU GR complex, which provides an extended range of recognized germinants (13). In cases where GR operons consist of more than three genes, the B-subunit gene is often present in multiple copies. For example, in *B. megaterium* QM B1551 there is an atypical cluster of GR-associated genes, comprised of two B-subunit genes separated by a putative D-subunit gene (14). GR operons possessing multiple B-subunit genes have also been found in the genomes of *Bacillus cereus* E33, *Bacillus halodurans*, *Bacillus cytotoxicus*, *Bacillus licheniformis*, *Clostridium botulinum*, *Clostridium sporogenes* and *Clostridium acetobutylicum* (4). However, the functional significance of the multiple B-subunit genes remains to be elucidated. The C subunit is a membrane-anchored lipoprotein which structure has been resolved to 2.3 Å resolution but, unfortunately, this did not elucidate its function (15).

B. licheniformis is widespread in nature and a common food-spoilage bacterium (16-22). It is closely related to *B. subtilis* and has occasionally been associated with disease in humans and

abortions in cattle (23-29). *B. licheniformis* strains carry three *gerA* family operons (*gerA, gerK*, and *ynd*) and some isolates also carry an monocistronic *yndF2* gene (TRNA_RS32565) (4, 30, 31). In contrast to the ABC (D) organization that characterizes *gerA* operons in *B. subtilis*, the *B. licheniformis* type strain ATCC14580/DSM13 possesses a pentacistronic *ynd* operon with the gene organization *yndD* (TRNA_RS32310), *yndE3* (TRNA_RS32305), *YndE2* (TRNA_RS32300), *yndF* (TRNA_RS32295), *yndE1* (TRNA_RS32290), encoding GR A-, B-, B-, C-, B- subunits, respectively. *B. licheniformis* spores germinate in response to a range of different amino acids as well as glucose, and alanine, cysteine and valine are the most potent germinants (32) . Mutational analyses have shown that GerA, Ynd and GerK GRs are all functional in nutrient-induced germination of *B. licheniformis spores* (32). GerA appears to function as the primary GR in amino acid-induced germination but it seems to require an intact Ynd and GerK GR to stimulate efficient germination in response to the above-mentioned germinants (32). No functional interdependence between the Ynd and GerK GRs has been found, but similar to GerK in *B. subtilis* and *B. megaterium*, GerK is required for D-glucose induced germination of *B. licheniformis* spores. However, D-glucose only functions as a weak germinant of *B. licheniformis* spores (32).

The purpose of this study is to dissect the cooperative function between the Ynd and GerA GRs. In particular, it addresses the functional role of the three paralogous B-subunit genes encoded by the *ynd* operon in the functional interplay with the GerA GR. The functional importance of the orphan *yndF2* gene was also investigated.

MATERIALS AND METHODS

Strains

The *B. licheniformis* strains used in this study are listed in Table 1. Strain MW3 carries deletions in the *hsdR* loci encoding two type I restriction modification systems and is therefore a more readily transformable derivative of the *B. licheniformis* type strain DSM13 (33).

Bioinformatic analyses

The *ynd* gene sequences of the *B. licheniformis* type strain DSM13/ATCC14580, the *B. subtilis* strain 168, the *B. cereus* strain ATCC14579 and the panel of 20 *B. licheniformis* strains and *B. paralicheniformis* were acquired from the NCBI database including both complete and draft assembled genomes (www.ncbi.nlm.nih.gov/). The *ynd* genes were identified using nBLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the *ynd* genes from strain DSM13/ATCC14580 as query sequences. To investigate the evolutionary relationships between the three *B. licheniformis* YndE subunits we performed a phylogenetic analysis. The amino acid sequences of the three *yndE* genes from each of the 20 strains of *B. licheniformis* were aligned using Clustal X followed by manual inspection in BioEdit (34). We also included the amino acid sequences of the *yndE* genes of *B. subtilis* and *B. cereus*. As outgroup we used GerLB from *B. anthracis*, GerAB from *B. licheniformis* and GerBB from *B. subtilis*. Based on the Akaike Information Criterion calculated in SMS (35) we estimated maximum likelihood phylogenies from the data under the LG+G+F substitution model using PhyML (36) with 500 bootstrap replicates.

135 **Construction of deletion mutants**

136 To assess the role of each individual *ynd* genes, in-frame, deletion mutants were constructed
137 by replacing the target gene(s) with the nucleotide sequence 5'ATGTAR 3' using a markerless
138 gene replacement method (37), as described previously (38). This method results in in-frame
139 deletion of the target gene and ensures that the up- and downstream flanking sequences,
140 including the promoter region, are intact. Briefly, the deletion mutants were constructed by
141 amplifying a ~500 base pair segment upstream of the target gene, using primers A and B, and
142 a ~500 base pair segment downstream of the target gene, using primers C and D. Primers B
143 and C contain a sequence overlap enabling fusion of the AB and CD PCR products by Sequence
144 and Ligation Independent Cloning (SLIC)-PCR. The resulting AD fragment contain the
145 upstream and downstream sequences of the target gene. The AD fragment was then cloned into
146 a thermosensitive shuttle vector pMAD (39) containing an *I-SceI* restriction site (40). The
147 resulting pMAD-*I-SceI* carrying the gene deletion construct was then transformed into
148 electrocompetent *B. licheniformis* cells (38). Here, the whole plasmid construct was expected
149 to integrate into the chromosome by a single crossover. The plasmid pBKJ233, encoding the
150 *I-SceI* enzyme, was then introduced by electroporation. The *I-SceI* restriction enzyme makes a
151 double stranded cut at its recognition site, which allows for recombinational repair, resulting
152 in a second crossover where the target gene is deleted. Deletion of the target gene was
153 confirmed by PCR and sequencing (GATC Biotech). All primers used for construction of the
154 mutant strains are listed in Table S1. All PCR products used in making the deletion mutants
155 were produced using Phusion high-fidelity DNA polymerase (Finnzymes, Finland) according
156 to the manufacturer's instruction. All PCR reactions were run on an Eppendorf Mastercycler
157 ep-Gradient S (Eppendorf).

158

159 **Complementation tests**

160 The complementing constructs, comprising the *ynd* promoter-region (570 bp) followed by the
161 complementing gene(s), were cloned in pMAD-*NotI*. The *NotI* site was introduced into the
162 *EcoRI* site of pMAD by SLIC-PCR comprising an upstream (404 bp) and an downstream (252
163 bp) part of *B. licheniformis amyL* joined by a *NotI*-site (primers listed in Table S2). The purpose
164 of including the *amyL* sequence was to achieve a homologous recombination of the plasmid
165 into the *amyL* gene of *B. licheniformis*. The complementing sequences were constructed by
166 PCR (ordinary or SLIC) using AccuPrime high fidelity *Taq* polymerase (Invitrogen) and the
167 primers listed in Table S2. The pMAD vector, carrying the complementing sequence, was
168 transformed into *B. lichenformis* mutant strains by electroporation (38). The whole plasmid
169 construct was integrated into the chromosome by single crossover caused by a temperature
170 shift, which influences the temperature sensitive replicon of pMAD. All complementing
171 mutants were verified by PCR and sequencing.

172

173 **Germination assays**

174 Spores were prepared, washed and stored for at least seven days prior to use as described
175 previously (41). To assess spore germination we followed the fall in optical density of a spore
176 population at 600 nm (OD_{600}). The germination assays were performed on pure (>98%), heat
177 activated (20 min, 65°C) spore suspensions as described previously (41). L-amino acids
178 (Sigma-Aldrich, USA) were added to a final concentration of 100 mM. The maximum
179 germination rate (G_{max}) was calculated from the curves obtained from the germination assays
180 (Fig. S2), using DMFit (DM: Dynamic Modelling) (42).

181 **Analyses of relative gene expression using RT-qPCR**

182 The relative expression levels of *yndE1, yndE2, yndE3, yndF2* and *yndD* relative to *rpoB* was
183 determined using quantitative Reverse Transcriptase PCR (RT-qPCR). Sporulation for RNA
184 extraction, RNA extraction, cDNA synthesis and RT-qPCR analysis was performed as
185 described before (32, 41). RT-qPCR was performed in triplicates on three biological replicates
186 and the results were analyzed as described previously (32, 41). All primers used for RT-qPCR
187 analyses are listed in Table S3.

188

189

190 **RESULTS**

191 **The *ynd* operon is highly conserved within *B. licheniformis* strains**

192 When the status of *B. licheniformis ynd* operon was examined using the whole *yndD* nucleotide
193 sequence from *B. licheniformis* ATCC14580/DSM13 as a seed, we found homologs to the *ynd*
194 operon in all 20 strains investigated. Three of these strains were annotated as *B.*
195 *paralicheniformis* in the NCBI Nucleotide database (*B. paralicheniformis* strains 9945a, BL09
196 and 12759). Similar to the type strain, all *B. licheniformis* and *B. paralicheniformis* genomes
197 possessed atypical pentacistronic *ynd* operons with the *yndD*, *yndE3*, *yndE2*, *yndF*, *yndE1* gene
198 organization.

199 A neighbor-joining tree based on multiple amino acid sequence alignments revealed that the
200 YndE subunits form a monophyletic clade with three distinct branches corresponding to the
201 $YndE_1$, $YndE_2$ and $YndE_3$ (Fig. 1). The three *B. licheniformis* YndE subunits are resolved as
202 monophyletic groups, each with 100 % bootstrap support. The *B. cereus* YndE forms a well-

supported clade (91.2 %) with *B. licheniformis* $YndE_2$ and $YndE_3$. However, the monophyly of the *B. licheniformis* $YndE_2$ and $YndE_3$ clades receives relatively weak support (65.6 %), thus it might be that the *B. cereus* YndE is sister to either the $YndE_2$ or $YndE_3$ clade. The *B. subtilis* YndE also has an uncertain placement due to low support values, but is either sister to the *B. licheniformis* $YndE_3$ or $YndE_2$ genes and *B. cereus* YndE, or sister to the *B. licheniformis* $YndE_3$. The $YndE_2$ and $YndE_3$ appears to be more closely related to each other than to $YndE_1$, as determined by phylogenetic analyses of the YndE subunits of the *B. licheniformis* strains (Fig. 1). Accordingly, alignments of *B. licheniformis* DSM13/ATCC14580 amino acid sequences showed that $YndE_3$ and $YndE_2$ shared more identity (64 %), compared to $YndE_1$ and $YndE_2$ or $YndE_3$ (52 % and 54 %, respectively) (Table S4).

Importance of the individual YndE subunits in *B. licheniformis* spore germination

The separate phylogenetic clustering of $YndE_1$, $YndE_2$, and $YndE_3$ suggests that they have evolved distinct functions. To assess the role of the three *ynd*-encoded B subunits in *B. licheniformis* spore germination, we first determined their expression levels. RT-qPCR analyses revealed that all *yndE* genes are expressed at approximately the same level as *yndD* and *yndF1* (Fig. S3). Mutants carrying in-frame deletions of the *yndE* genes were constructed and tested for germination with a panel of amino acids, which have previously been shown to function as efficient germinants of *B. licheniformis* spores (32). A high concentration (100 mM) of each germinant compound was used for screening to also identify weak germination responses and the results are listed in Table 2. The germination rate was monitored by the fall in the optical density over time, and the total level of germinated spores after 120 min of germinant exposure was determined by counting the number of phase-dark (germinated) versus phase-bright spores (Table 2). Mutants lacking *yndE3* (NVH-1369) or *yndE3E2* (NVH-1378), expressing the $YndDE_2FE_1$ and $YndDFE_1$ subunits, respectively, demonstrated similar but

reduced germination levels with all tested amino acids (Table 2). Deletion of *gerKA*C in the Δ*yndE3E2* mutant background (NVH-1403) did not reduce the germination efficiency compared spores of the Δ*yndE3E2* background strain. This indicates that GerK is not involved in the remaining germination observed for Δ*yndE3E2* mutant spores. For an unknown reason, we were not able to construct an Δ*yndE1* mutant. However, we were able to construct an Δ*yndE3E2E1* mutant (NVH-1405), which produce spores carrying an Ynd GR lacking all three YndE subunits. Surprisingly, Δ*yndE3E2E1* mutant spores demonstrated a germination efficiency that was only slightly reduced (approximately 75-85 % germinated spores), compared to wild-type spores (90-98%) (Table 2). Complementation of the Δ*yndE3E2E1* mutant (NVH-1405) with *yndE3* resulted in spores that germinated as efficiently as spores of the non-complemented background strain (Table 2). However, when the Δ*yndE3E2E1* mutant was complemented with *yndE3E2* the germination efficiency was reduced compared to the background strain, indicating that $YndE_2$, in the absence of $YndE_1$, somehow disturbs the function of the Ynd GR. Complementation of the Δ*yndDE3E2FE1* Δ*gerKAC* mutant with the *yndDE2F* gene construct (strain NVH-1413) did not increase the germination rate compared to spores of the background strain, which suggests that $YndE_2$ alone is not able to support the function of the Ynd GR. Furthermore, complementation of the Δ*yndE3E2E1* mutant with *yndE3E2E1* genes (NVH-1417) restored the germination efficiency to levels similar to mutant spores expressing the $YndDE_3F$ and YndDF subunits (Table 2).

The Ynd A subunit (YndD) is functional in germination in the absence of the other Ynd receptor subunits

The efficient germination of the Δ*yndE3E2E1* mutant spores (NVH-1405) compared to mutant spores expressing $YndE_2$ and/or $YndE_1$ suggests that an incomplete set of B subunits exerts a deleterious effect on germination. The more efficient germination observed in the Δ*yndE3E2E1* mutant spores compared to Δ*yndDE3E2FE*1 mutant spores (NVH-1387), expressing only intact GerA and GerK GRs, suggest that YndDF are sufficient for the cooperative function between Ynd and GerA. To test whether both YndD and YndF are required for a functional cooperation with the GerA GR, we attempted to construct an *yndF* deletion mutant. This was, for an unknown reason, unsuccessful. However, complementation of the Δ*yndDE3E2FE1* Δ*gerKAC* mutant with the *yndD* gene (NVH-1410) resulted in similar levels of spore germination as seen when complementing the same mutant background with *yndDF* (NVH-1411). This indicates that the *yndF* encoded C subunit, is of less importance compared to the *yndD* encoded A subunit for the cooperative function between Ynd and GerA in nutrient-induced germination.

GerA functions in spore germination in the absence of other germination receptors

The Δ*yndDE3E2FE1 ΔgerKACB* mutant spores lacking both the Ynd and the GerK GR (NVH-1412) germinated to a certain extent (30-56 % phase dark germinated spores after 2 hours of nutrient exposure). Spores of an Δ*yndDE3E2FE*1 Δ*gerKAC* mutant (NVH-1404), expressing a functional GerA GR and the B subunit of the GerK GR, germinated as efficiently as Δ*yndDE3E2FE*1 Δ*gerKACB* mutant spores. Hence, the remaining B subunit present in Δ*gerKAC* mutant spores does not seem to disturb the germination process. However, it should be noted that the Δ*gerKAC* mutant strain ($GerKB^+$) produced some unstable spore batches with

a higher frequency of spontaneous germination. This is reflected by a higher percentage of germination in the negative controls (Table 2, results not shown).

Since the B subunits encoded by the Ynd operon appeared not to be essential for germination in the presence of an intact GerA GR the contribution of the GerAB subunit to the cooparative function between the GerA and Ynd GRs was tested. However, in contrast to spores lacking the B subunits of the Ynd GR, an in frame deletion of *gerAB* (NVH-1389) resulted in a severe reduction in germination rate and total germination levels (Table 2).

Functional analysis of YndF2

The orphan *yndF2* gene was present in 12 of the 20 *B. licheniformis* genomes analyzed here. The deduced sequence of $YndF_2$ from ATCC14580/DSM13 is 184 amino acids long and shows 60 % identity and 79 % similarity to the C- terminal region of YndF (amino acids 215 - 398) (Table S4). However, in the majority of the 12 genomes where it is present, it is 401 amino acids long, which close to the full length of YndF protein encoded by the *ynd* operon (399 amino acids). We were unable to identify a promoter upstream of *yndF2* in strain ATCC14580/DSM13. However, analysis of its expression by RT-qPCR revealed that it is expressed at approximately the same level as the *yndE* genes (Fig S4). Therefore, to evaluate its potential role in spore germination, we constructed a mutant strain carrying an in-frame deletion of *yndF2* in the wild-type background (Table 2). Analysis of Δ*yndF2* mutants spores for germination with L-alanine, L-cysteine and L-valine revealed germination levels similar to wild-type spores.

DISCUSSION

The main objective for the present study was to characterize the role of the paralogous $YndE_3$, $YndE_2$ and $YndE_1$ GR subunits (B subunits) in *B. licheniformis* spore germination. In addition, we wanted to increase the understanding on the functional interaction occurring between the Ynd and GerA GRs during nutrient-triggered spore germination.

The search for *ynd* operons in *B. licheniformis* and *B. paralicheniformis* genomes revealed pentacistronic *ynd* operons in all 25 strains investigated. However, five genomes carried *ynd* operons with premature stop codons in the *yndE* genes (4/5) or in the *yndF* gene (1/5) (results not shown). These genomes were excluded from the phylogenetic analyses in case of sequencing errors. Phylogenetic examination of the YndE subunits from the remaining 20 strains showed that $YndE_1$, $YndE_2$ and $YndE_3$ form three separate phylogenetic clusters, which suggest that the individual B subunits could play different functional roles. Interestingly, the *B. cereus* YndE subunit forms a clade with *B. licheniformis* $YndE_2$ and $YndE_3$. Two scenarios can explain the observed phylogeny: A putative horizontally transferred copy of the *B. cereus yndE* gene might have been acquired and subsequently duplicated into *yndE2* and *yndE3* in *B. licheniformis*. Alternatively, duplication of an ancestral *B. licheniformis yndE* gene resulted in *yndE1* and an ancestral paralog that subsequently duplicated into *yndE2* and *yndE3*. In this scenario *B. cereus* might have received the ancestral paralog from *B. licheniformis*.

Transcriptional analyses revealed that all the *yndE3*, *yndE2* and *yndE1* genes are expressed during sporulation, and mutational analyses conducted in the present study, revealed that all three YndE subunits play a functional role in spore germination. Deletion of *yndE3* or both *yndE3* and *yndE2* resulted in weaker germination responses with L-alanine, L-cysteine and L-valine, compared to wild-type spores. Remarkably, simultaneous deletion of all *yndE1*, *yndE2* and *yndE3* genes restored the germination rate to levels only slightly lower compared to wild-

type spores. Hence, an incomplete set of *yndE* genes had a negative impact on the function of the Ynd GR. A similar situation has previously been described for *B. megaterium* spores where the GerK2 GR conferred an inhibitory effect upon the GerA function in triggering spore germination (14). However, in *B. megaterium* it was the complete $GerK_2$ GR that conferred the inhibitory effect. Since all *B. licheniformis* genomes investigated in this study encode the GerD protein, which facilitate germinosome formation in *B. subtilis*, it is likely that the GerA, Ynd and GerK GRs are colocalized in germinosomes. There is, however, very little knowledge on the heteromeric organization of GRs in the germinosomes and therefore the interpretation of the physiological basis for our results remains speculative. Assuming that the GerA and Ynd GRs forms a heteromeric complex in the spores' inner membrane, the absence of one or more of the YndE subunits may obstruct the function of the complex. However, when all YndE subunits are absent, the remaining GerAB subunit may support the cooperative function between the Ynd and GerA GRs. The GerAB subunit was on the other hand indispensable for *B. licheniformis* spore germination with all tested germinant compounds. Mutant spores containing the GerA GR and the A and C subunits of the Ynd GR (Δ*yndE3E2E1*) demonstrated near wild-type rates of germination with L-alanine, L-cysteine and L-valine suggesting that the A and C subunits encoded by the Ynd operon is sufficient and required for the functional cooperation between the Ynd and GerA GRs. Further study of spores lacking the entire *ynd* operon (Δ*yndDE3E2FE1*), but complemented with the *yndD* gene, showed that it germinated with the same efficiency as Δ*yndE3E2E1* spores and more efficiently compared to Δ*yndDE3E2FE1* spores. This suggests that the A subunit is the only subunit of the of Ynd GR that is required for the cooperative function with GerA GRs. Monocistronic *gerA* family genes are found in several spore formers (2). The A subunit is the most common GR subunit to be encoded by a monocistronic *ger* gene, and are found in approximately 10 % of all investigated *Bacillales* and *Clostridiales* genomes (2). Whether the single A subunits are functional alone,

346 or if they form functional complexes with other GRs remains to be clarified. The *C. perfringens*
347 genome contain an orphan *gerAA* gene but its function remains to be elucidated (43). *C.*
348 *perfringens* also carry a bicistronic *gerK* operon that encode an A and a C subunit, which can
349 function in the absence of a B subunit (43, 44). Interestingly, in contrast to our results, the C
350 subunit was found to be essential for *C. perfringens* spore germination, whereas the A subunit
351 was dispensable (45).

352 The GerA GR could function alone in L-amino acid-induced germination, as mutant spores
353 lacking the complete *ynd* and *gerK* operons could germinate, although with approximately 50-
354 60 % reduced efficiency compared to wild-type spores. This was inconsistent with our previous
355 results using Δ*yndD ΔgerKAC* mutant spores, which did not germinate in response to exposure
356 to nutrients or high pressures (32, 46). However, recent results from our laboratory showed that
357 this strain contained an insertion in the *gerAA* gene, effectively knocking out the GerA
358 function. Hence, the strain is a *ger*$^{-}$ mutant.

359 The presence of the Δ*gerKAC* mutation seemed to increase spontaneous germination in the
360 negative controls. It is not unlikely that mutations in the *ger* genes can indirectly affect other
361 spore characteristics, as much is still unknown about the structure of the GRs, their organization
362 into germinosomes and their interaction with other components involved in downstream events
363 (3). Further studies are therefore needed to understand the precise function of the individual
364 GR subunits.

365 The $YndF_2$ subunit was found to be dispensable for *B. licheniformis* spore germination with all
366 tested germinant compounds. However, *yndF2* appears to be truncated in strain
367 ATCC14580/DSM13, while it is present as a full-length (1200 base pairs) gene in most other
368 *B. licheniformis* genomes. It is therefore possible that it plays a functional role in other *B.*
369 *licheniformis* strains, but this has yet to be determined.

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TABLES

TABLE 1: *B. licheniformis* strains used in this study

Strain	Relevant genotype	Source or reference
MW3	wild type	(33)
NVH-1369	Δ*yndE3*	This study
NVH-1371	Δ*yndF2*	This study
NVH-1378	Δ*yndE3E2*	This study
NVH-1405	Δ*yndE3E2E1*	This study
NVH-1387	Δ*yndDE3E2FE1*	This study
NVH-1389	Δ*gerAB*	This study
NVH-1403	NVH-1378Δ*gerKAC*	This study
NVH-1404	1387Δ*gerKAC*	This study
NVH-1410	NVH-1404 P*ynd*::pMAD_*yndD*	This study
NVH-1411	NVH-1404 *amyL*::pMAD_*yndDF*	This study
NVH-1412	NVH-1387Δ*gerKACB*	This study
NVH-1413	NVH-1404 P*ynd*::pMAD_*yndDE2F*	This study
NVH-1415	NVH-1405 P*ynd*::pMAD_*yndE3*	This study
NVH-1416	NVH-1405 *amyL*::pMAD_*yndE3E2*	This study
NVH-1417	NVH-1405 *amyL*::pMAD_*yndE3E2E1*	This study

All mutants are derived from MW3

514 **TABLE 2:** Germination properties of *B. licheniformis* mutant spores with L-amino acids

			% germination[ab]				G_{max} (% OD loss min^{-1})		
			L-ala	L-cys	L-val	NC[c]	L-ala	L-cys	L-val
STRAIN	**GENOTYPE**	**Remanining Ynd GR subunits**							
MW3[d]	wild-type	$YndDE_3E_2FE_1$	*98 (1)*	*98 (1)*	*90 (3)*	*< 2*	*1.4 (0.4)*	*1.0 (0.2)*	*2.0 (0.7)*
NVH-1369	Δ*yndE3*	$YndDE_2FE_1$	58 (6)	48 (8)	57 (1)	< 2	0.63 (0.05)	0.48 (0.05)	0.58 (0.07)
NVH-1378	Δ*yndE3E2*	$YndDFE_1$	50 (4)	41 (3)	53 (6)	< 2	0.71 (0.20)	0.57 (0.16)	0.56 (0.38)
NVH-1403	Δ*yndE3E2* Δ*gerKAC*	$YndDFE_1$	43 (7)	34 (14)	42 (7)	3 (3)	0.48 (0.09)	0.35 (0.05)	0.44 (0.10)
NVH-1405	Δ*yndE3E2E1*	YndDF	86 (5)	76 (7)	85 (5)	< 2	1.23 (0.56)	0.66 (0.27)	0.95 (0.39)
NVH-1415	Δ*yndE3E2E1* –*yndE3*	$YndDE_3F$	88 (5)	71 (15)	83 (8)	< 2	0.64 (0.22)	0.43 (0.17)	0.53 (0.12)
NVH-1416	Δ*yndE3E2E1* –*yndE3E2*	$YndDE_3E_2F$	40 (8)	32 (20)	48 (2)	< 2	0.59 (0.21)	0.36 (0.11)	0.46 (0.15)
NVH-1417	Δ*yndE3E2E1* –*yndE3E2E1*	$YndDE_3E_2FE_1$	73 (3)	68 (13)	76 (9)	< 2	1.06 (0.40)	0.68 (0.30)	0.68 (0.01)
NVH-1413	Δ*yndDE3E2FE1* Δ*gerKAC* -*yndDE2*	$YndDE_2F$	47 (5)	48 (5)	37 (3)	5 (5)	0.53 (0.07)	0.47 (0.04)	0.37 (0.04)
NVH-1387	Δ*yndDE3E2FE1*	Ynd^-	47 (7)	36 (10)	52 (5)	< 2	0.48 (0.14)	0.30 (0.05)	0.49 (0.18)
NVH-1412	Δ*yndDE3E2FE1* Δ*gerKACB*	Ynd^-	47 (2)	39 (0)	48 (1)	< 2	0.39 (0.04)[e]	0.34 (0.06)[e]	0.28 (0.07)[e]
NVH-1404	Δ*yndDE3E2FE1* Δ*gerKAC*	Ynd^-	57 (8)	58 (19)	62 (8)	5 (6)	0.45 (0.23)	0.30 (0.06)	0.56 (0.07)
NVH-1410	Δ*yndDE3E2FE1* Δ*gerKAC* -*yndD*	YndD	69 (9)	61 (12)	59 (2)	< 2	0.83 (0.12)	0.76 (0.10)	0.64 (0.05)
NVH-1411	Δ*yndDE3E2FE1* Δ*gerKAC* -*yndDF*	YndDF	70 (9)	67 (16)	64 (6)	2 (2)	0.85 (0.19)	0.86 (0.19)	0.69 (0.13)
NVH-1389	Δ*gerAB*	$YndDE_3E_2FE_1$	2 (2)	4 (4)	2 (1)	< 2	<0.1	<0.1	<0.1
NVH-1371	Δ*yndF2*	$YndDE_3E_2FE_1$	95 (4)	98 (1)	70 (12)	< 2	1.69 (0.30)	1.49 (0.29)	0.59 (0.25)

515 [a] All data are presented as means. The standard deviations, where applicable, are given in parantheses.
516 [b] The percentages of germinated spores (phase dark) were determined after 120 minutes exposure to 100 mM of germinant
517 compounds
518 [c] Negative control, no added germinant compound
519 [d] Data from Borch-Pedersen et al. 2016, included for comparison.
520 [e] Mean derived from only two independent spore batches
521

FIGURES

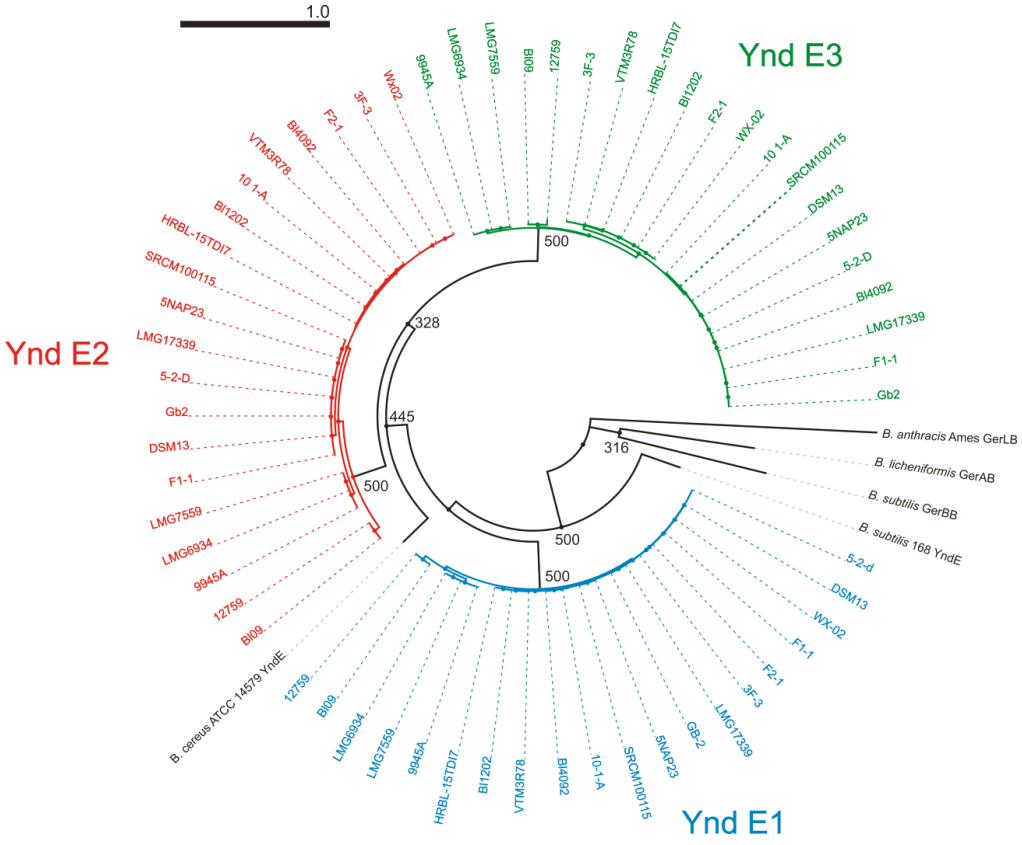

FIG. 1. Maximum likelihood phylogram for $YndE_1$ (B1), $YndE_2$ (B2) and $YndE_3$ (B3) of 20 strains of *B. licheniformis* as well as from *B. cereus* and *B. subtilis*. Bootstrap support values above 50 % are indicated at the nodes, however, no bootstrap support values for nodes within the clades for the three *B. licheniformis* Ynd genes are given. GerLB, GerAB and GerBB from *B. anthracis, B. licheniformis* and *B. subtilis*, respectively, were used as outgroup.

Supplementary information

Dissecting the cooperative interaction between the GerA and Ynd germination receptors in *B. licheniformis* spore germination

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TABLE S1 Primers used for construction of deletion mutants

Strain	Primer name	Primer sequence (5'-3')	Prepared in
NVH-1369			MW3
	1658	GCGCTCATTATGGAAGTGGC	
	1659	CAAGCGTCACATAAGCGTTTCCCT	
	1660	AACGCTTATGTGACGCTTGAAAAAGA	
	1661	GGCTTGATCCCTTTCCCGAG	
NVH-1371			MW3
	1628	GCCGTCACCGTGAATAAGGA	
	1629	TGCCTTTCAGATGACCTTTTTACC	
	1630	GATTAAGCTACATGATCCCG	
	1631	GGGATCATGTAGCTTAATCATGA	
NVH-1378			NVH-1369
	1658	GCGCTCATTATGGAAGTGGC	
	1841	TTCCTCCTCACTTACATAAGCGTTTCCCT	
	1842	AGGGAAACGCTTATGTAAGTGAGGAGGAAAAAGT	
	1665	CATTTTCAACGATTTTAAGC	
NVHΔ-1387			NVH-1378
	1586	GCTTGAGCAGACATTGCTTG	
	1799	GAACCTCTACATGCCACTCACTCATCC	
	1800	AGTGGCATGTAGAGGTTCAGCC	
	1669	TATGATCGTCGTCGGCTTGG	
NVH-1389			MW3
	1807	AGCGCTGATTATGGAAGTGACGAT	
	1808	CTTTTTTTTCATGAAGAACTACATGCGCCGAGCATTGTGCTGG	
	1809	CAATGCTCGGCGCATGTAGTTCTTCATGAAAAAAAAGGGAAT	
	1810	GCTCCTCTGAGAAAATCAGGACTTGC	
NVH-1403			NVH-1378
	1549	ACGAGGTTATCGGCAATACG	
	1562	TCTCTTTCATTCACATATTTTTCCTTGCGCAAGC	
	1563	AAGGAAAAATATGTGAATGAAAGAGAGAGGAGG	
	1564	TTTCCAAGAATGGGCAAAAG	
NVH-1404			NVH-1387
	1549	ACGAGGTTATCGGCAATACG	
	1562	TCTCTTTCATTCACATATTTTTCCTTGCGCAAGC	
	1563	AAGGAAAAATATGTGAATGAAAGAGAGAGGAGG	
	1564	TTTCCAAGAATGGGCAAAAG	
NVH-1405			NVH-1378
	1666	CATCGGAAAGGGGATTGGGG	
	1667	GAACCTCTACATTGCGCGTTTCTC	
	1668	CGCGCAATGTAGAGGTTCAGCC	
	1669	TATGATCGTCGTCGGCTTGG	
NVH-1412			NVH-1387

1549	ACGAGGTTATCGGCAATACG
1952	CGGGAAGGATCACATATTTTTCCTTG
1953	AGGAAAAATATGTGATCCTTCCCGCT
1954	CGAGCTCCCGATAAAACAAG

TABLE S2 Primers used for creation of complementation constructs

Primer name (orientation)		Primer sequence (5′-3′)
1838	(F)	TTCGTGCCTGACCATCATGCCACTCACTCATCCCCTGC
1839	(R)	AGGGGATGAGTGAGTGGCATGATGGTCAGGCACGAAGAG
1902	(R)	TGTTAGTTTTTTTCAATCAGCTGATTCCCTGTTTTC
1903	(F)	ACAGGGAATCAGCTGATTGAAAAAAACTAACACCATCCGT
1941	(F)	ACGGTATTACTGCCGTCTGG
1942	(R)	AATTGCGGCCGCCATGCCTTTCCTTGAAACT
1943	(F)	ATATGCGGCCGCGAGATGGGGAACGTGGTATG
1944	(R)	CCGTTCAGCAATTTCCTCAT
1945	(R)	AATTGCGGCCGCGTTCTTCTGTTACTGATCCC
1946	(R)	ATAGCGGCCGCTTCGTGCCTGACCATCATAA
1948	(F)	ATAGCGGCCGCACGCCTCAAGATCCGAAATA
1961	(R)	AATTGCGGCCGCCTAGCTTTGCACTGCCTTCTTT
1962	(R)	ATATGCGGCCGCTTAGTTTTTTTCAAAC
1963	(R)	TTATGCGGCCGCTCAGCTGATTCCCTGT

Underlined indicates NotI site

CONSTRUCTS			TEMPLATE STRAIN
yndDE$_2$F$_1$		1948-1945	NVH-1369
YndD		1948-1946	MW3
yndDF$_1$		1948-1945	NVH-1378
	SLIC-PCR		
yndE$_3$	AB	1948-1838	MW3
	CD	1939-1963	MW3
yndE$_3$E$_2$	AB	1948-1838	MW3
	CD	1839-1962	MW3
yndE$_3$E$_2$E$_1$	AB	1948-1938	MW3
	CD	1939-1959	MW3
	EF	1960-1961	MW3
amyL:	AB	1941-1942	MW3
	CD	1943-1944	MW3

TABLE S3 Primers RT-PCR

Gene (primer orientation) efficiency (E)	Primer name	Primer sequence (5´-3´)	Primer
gerAA (F)	1553	CCCTGTTCCTATCGGCGTTT	2.01
gerAA (R)	1554	TCGGCAGCATGCCTTGA	2.01
yndE3 (F)	1695	CGAAGAGCGCATCACCACTT	2.00
yndE3 (R)	1696	TGTCCCAAGCAGGAAATTGG	2.00
yndE2 (F)	1693	CCCCGACAAAACGCTATATCA	1.96
yndE2 (R)	1694	AAAGTCCTCCCGGCCACTT	1.96
yndE1 (F)	1691	TGCCAAGAACCGCTGTAGAAG	2.03
yndE1 (R)	1692	GCCTAATAGGACGGAAATCCATAC	2.03
yndF1 (F)	1699	ATTCGCTGTCGGAACTGCTAGA	1.99
yndF1 (R)	1700	TACTGCTCGGCCTGATTTCA	1.99
yndF2 (F)	1701	GCCATGTCAGTCGATCACCTT	2.00
yndF2 (R)	1702	TGAGAGGCGGTTTCCTTTGA	2.00
yndD (F)	1697	GCGCGTCGGCTTTACAGA	1.99
yndD (R)	1698	CCGTGCTGCCTCAAAAGG	1.99
rpoB (F)	1592	ACCTCTTCTTATCAGTGGTTTCTTGAT	2.00
rpoB (R)	1593	CCTCAATTGGCGATATGTCTTG	2.00

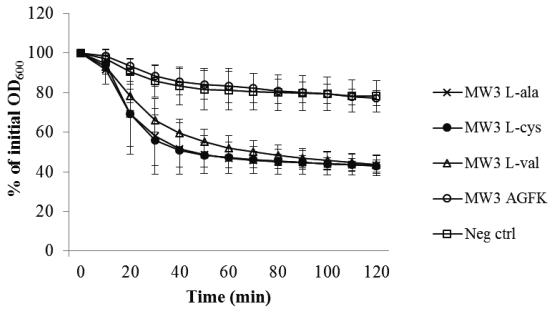

FIG S1 Germination of *B. licheniformis* MW3 (NVH-1289) spores to 100 mM L-alanine, L-cysteine, L-valine, or AGFK. FIG from Borch-Pedersen et al., 2016. Error bars show 95 % confidence intervals.

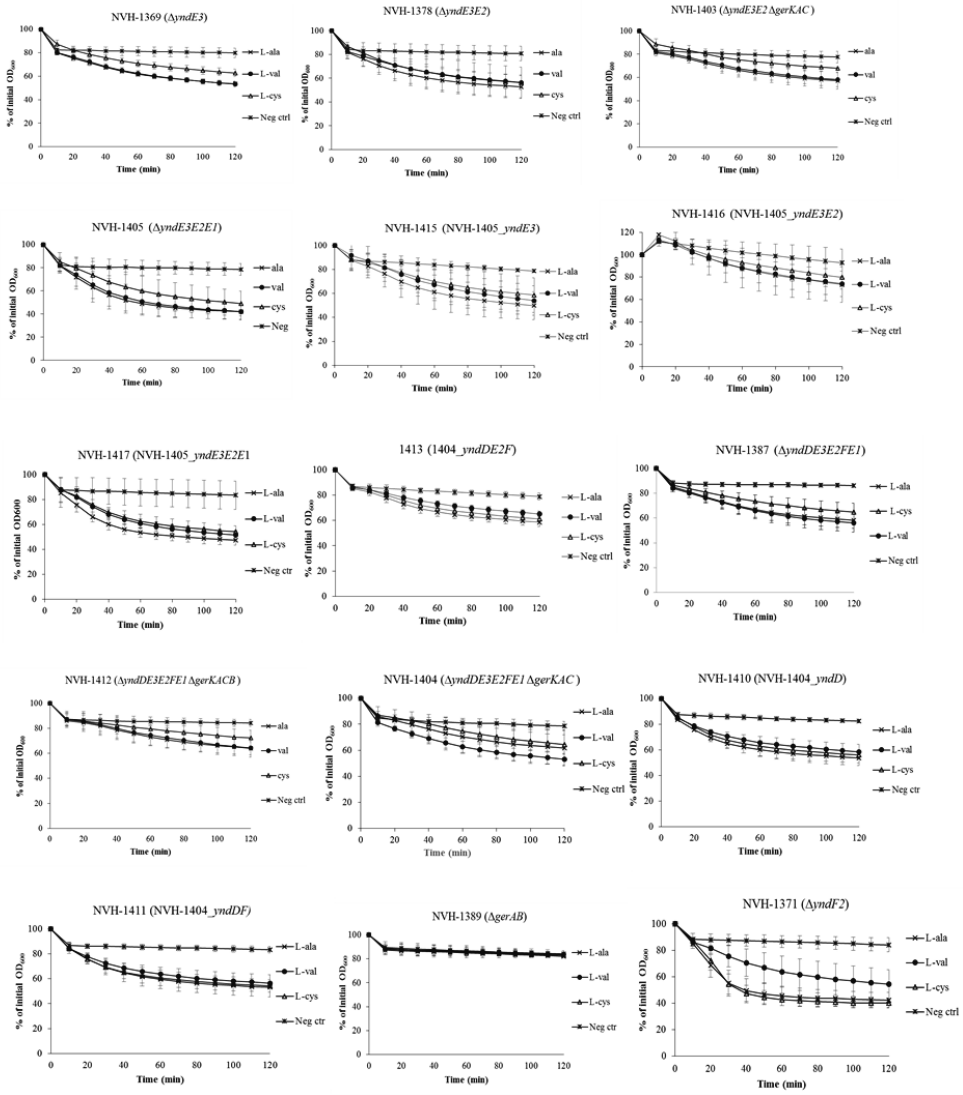

FIG S2 Germination response of *B. licheniformis* MW3 mutant spores to 100 mM L-alanine, L-valine or L-cysteine measured by following the decrease of the initial OD_{600} (%) of the spore suspension after addition of germinants. Error bars show 95 % confidence intervals.

TABLE S4 Percentage of amino acid sequence identity and similarity of Ynd subunits in *B. licheniformis* ATCC15480/DSM13.

Organism	Amino acid sequences alignments		% Identity	% Similarity
***B. licheniformis* ATCC14580/ DSM 13**	$YndE_1$	$YndE_2$	52	75
	$YndE_1$	$YndE_3$	54	75
	$YndE_2$	$YndE_3$	64	83
	YndF (aa 215 →)	$YndF_2$	60	79

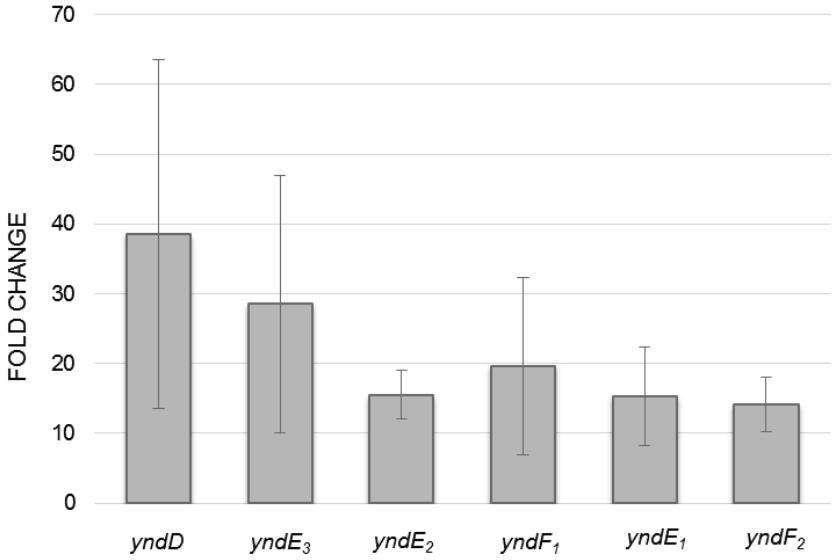

FIG S3 Increase in expression of *ynd* genes from mid exponential growth to start of sporulation relative to *rpoB*.

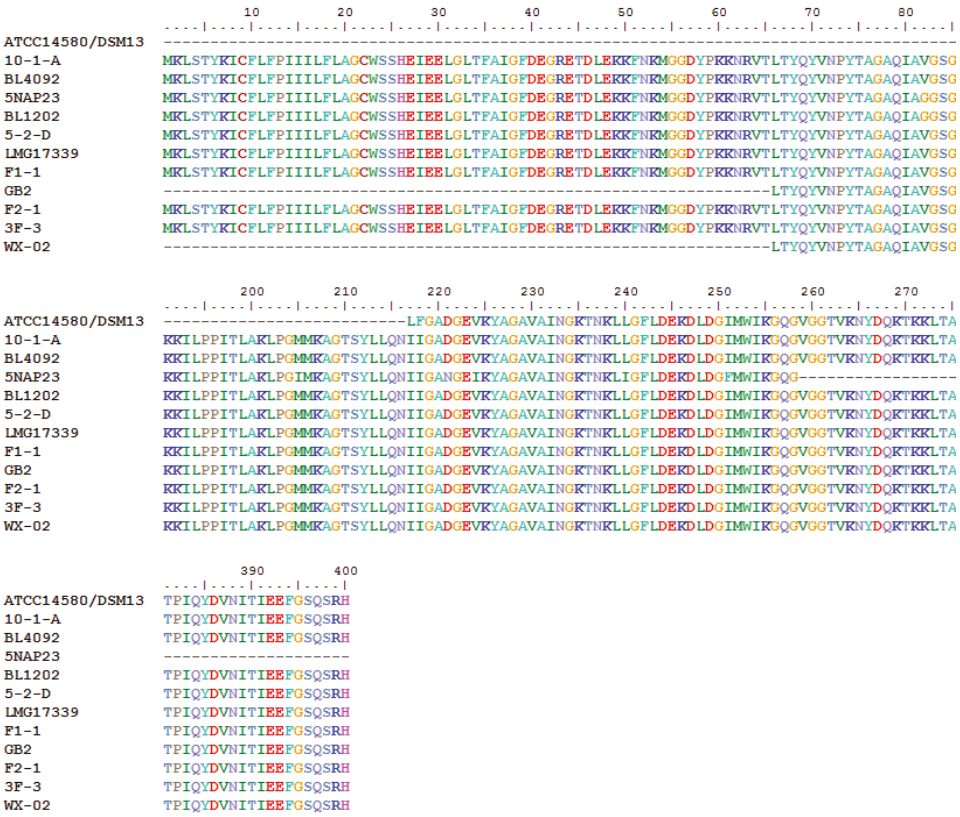

FIG S4 ClustalW alignment of amino acid sequences of $YndF_2$ of the 12 *B. licheniformis* strains found to carry the *yndF2* orphan gene.

Errata

p. v, L 22	**stammens** replaced by **artens**
p. vi, L 8 and 9	**subenhetene** replaced by **subenhetgenene**
p. 3, L 32	**is** replaced by **are**
p.4, L 1	redundant **in the** removed
p. 4, L 10	**it** replaced by **they**
p. 11, L 11	**of proteins** added
p. 12, L 4	**spore** added
p. 16, L 15	**low** replaced by **high**
p. 17, L 7	**with** replaced by **which have**
p. 30, L 17	**and *B. paralicheniformis*** added
p. 34, L 1	**in glucose-induced germination** added
p. 38, L 13	**from** replaced by **upstream of**
p. 42, L 4	**of germination receptors** added
Manuscript III, p. 2, L 26	**and *B. paralicheniformis*** added
Manuscript III, p. 6, L 126	***yndE* genes** replaced by **YndE subunits**
Manuscript III, p. 9-10, L 199-209	***yndE* genes** replaced by **YndE subunits**
Manuscript III, p. 9-10, L 199-208	***yndE1* gene** replaced by **$YndE_1$**
Manuscript III, p. 9-10, L 200-207	***yndE2* gene** replaced by **$YndE_2$**
Manuscript III, p. 9-10, L 200-207	***yndE3* gene** replaced by **$YndE_3$**
Manuscript III, p. 9-10, L 200-207	**genes** is removed
Manuscript III, p. 9-10, L 201-206	***yndE gene*** replaced by **YndE**
Manuscript III, p. 14, L 304	***yndE* genes** replaced by **YndE subunits**
Manuscript III, p. 14, L 307	***yndE* gene** replaced by **YndE subunit**
Manuscript III, p. 14, L 307	***yndE2* and *yndE3*** replaced by **$YndE_2$ and $YndE_3$**