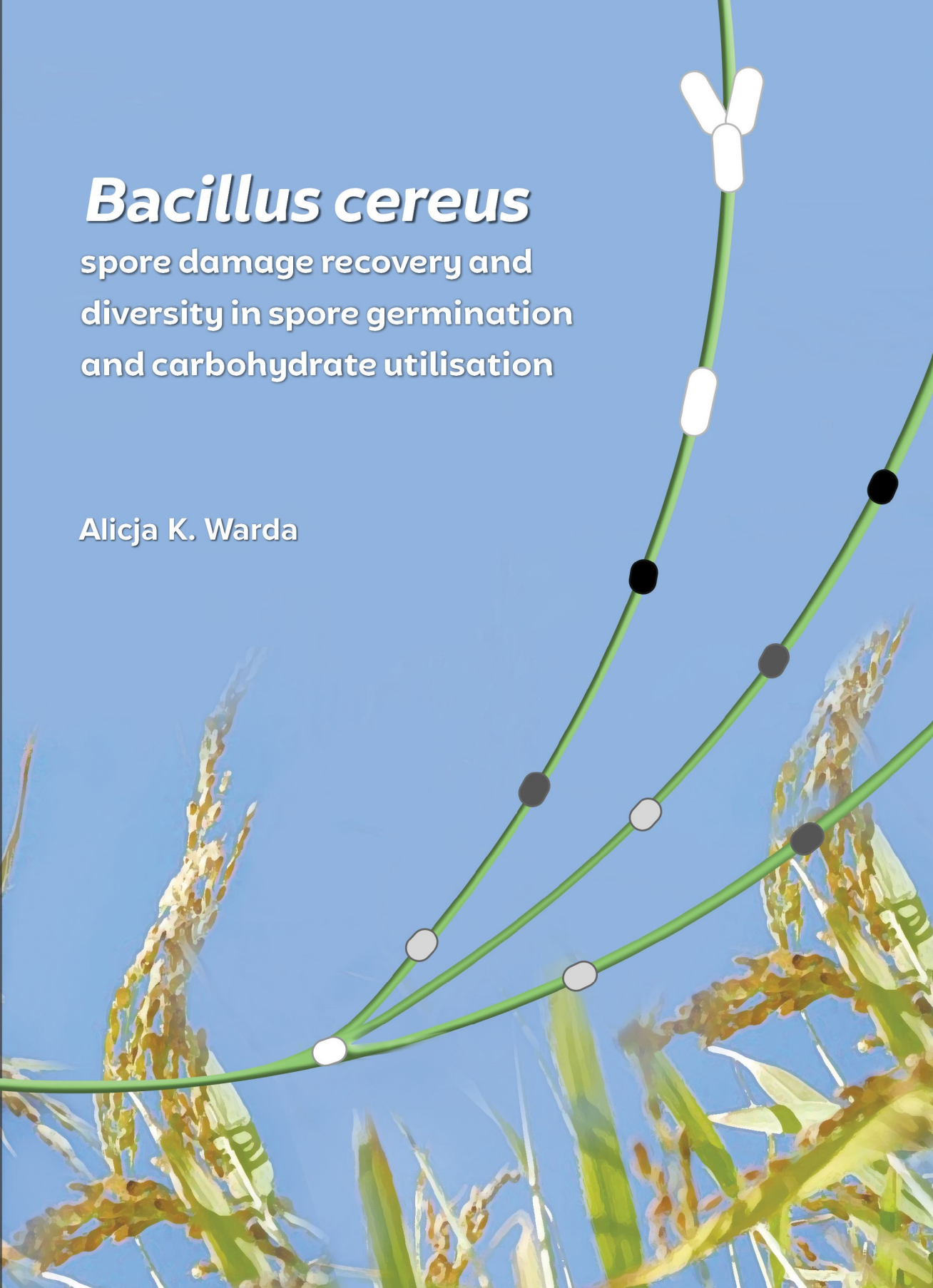
Bacillus cereus

spore damage recovery and diversity in spore germination and carbohydrate utilisation

Alicja K. Warda



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Thesis

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Abstract

Bacterial spores are extremely robust survival vehicles that are highly resistant towards environmental stress conditions including heat, UV radiation and other stresses commonly applied during food production and preservation. Spores, including those of the toxin-producing food-borne human pathogen *Bacillus cereus*, are ubiquitously present in a wide range of environmental niches such as soil, plant rhizosphere, intestinal tract of insects and animals, and it is virtually impossible to prevent contamination at the primary production level. Heat treatments are conventionally applied in food processing to reduce the microbial load of food products, however, to comply with consumer desire for products with higher sensory and nutritional values, the treatment intensity may become milder. Consequently, subpopulations of spores may emerge that are sublethally damaged rather than inactivated conceivably causing quality and safety issues following repair and outgrowth. In this thesis, a functional genomics approach was used in combination with subpopulation and single spore analysis to identify factors involved in recovery of heat damaged spores, and to link *B. cereus* genotypes to nutrient-induced germination capacity and carbohydrate utilisation capacity.

Using comparative analysis of *B. cereus* ATCC 14579 wild type and targeted mutants, putative damage repair factors were identified such as putative transcriptional regulator CdnL, that supported recovery of spores in a range of conditions including model foods. The majority of identified genes encoding putative damage repair factors appeared to be unique for *B. cereus* group members. This novel information on spore recovery adds to further insights in versatility of survival strategies of *B. cereus.*

Different types of foods may contain different types and levels of nutrients including amino acids and carbohydrates, that can affect spore germination capacity and subsequent outgrowth performance of vegetative *B. cereus* cells. Nutrient germinants present in food products can trigger specific germinant receptors (GRs) located in the spore inner membrane leading to spore germination, a critical step before growth resumes. Combined analysis of genotypes and nutrient-induced germination phenotypes using high throughput flow cytometry analysis at the level of individual spores, revealed substantial diversity in germination capacity with a subset of strains showing a very weak germination response even in nutrient-rich media containing high levels of amino acids. Phylogenetically, these *B. cereus* strains grouped in subgroup IIIA encompassing strains containing pseudogenes or variants of some of the Ger clusters and two strains containing the recently identified SpoVA^{2mob} transposon, that induced heat resistance with concomitant reduced germination response in *Bacillus subtilis* spores. The same *B. cereus* isolates were also used to link genotypes with carbohydrate utilisation clusters present on the genomes, and this revealed representatives of subgroup IIIA to lack specific carbohydrate utilisation clusters (starch, glycogen, aryl beta-glucosides; salicin, arbutin and esculin) suggesting a reduced capacity to utilise plant-associated carbohydrates for growth. Since these *B. cereus* subgroup IIIA representatives contain host-associated carbohydrate utilisation gene clusters and a subset of unique Ger clusters, their qualification as poor germinators may require revision following assessment of spore germination efficacy using host-derived compounds as germinants.

The research described in this thesis has added novel insights in *B. cereus* capacity to cope with spore damage and provided novel overviews of the distribution and putative functionality of (sub)clusters of GRs and carbohydrate utilisation clusters. Knowledge on spore damage repair, germination and metabolism capacity adds to further understanding of *B. cereus* ecology including niche occupation and transmission capacity.

Chapter 1

Introduction and outline of the thesis

Abstract

This chapter provides an introduction to the world of spore forming bacteria, starting with a brief description of the life cycle of spore formers from spore formation, via germination to outgrowth and vegetative cell growth. The highly stress resistant nature of spores, their structure and other specific properties are described together with their impact on quality and safety issues in food industry. In particular, the role of *Bacillus cereus*, a causative agent of foodborne illnesses and food spoilage, is discussed. Aspects affecting the predictability of spore behaviour including strain diversity, population heterogeneity and damage recovery are introduced. Finally, the outline of this thesis is provided.

Impact of spore forming bacteria on food quality and safety

Spore forming bacteria are ubiquitously present in a wide range of environmental niches such as soil, plant rhizosphere, intestines of insects and animals, and this facilitates transmission of their spores to food processing environments. Once spores enter the food chain, they are difficult to eradicate since spores are highly resistant towards environmental stress conditions including heat, UV radiation and other stresses commonly applied during food production and preservation [1-5]. Since spores act as extremely robust survival vehicles, it is not surprising that even today, the challenge remains to develop and implement efficient (minimal) processing and preservation strategies to prevent food quality and safety issues [6]. Although metabolically inactive, dormant spores are able to monitor their environment and respond to availability of nutrients by activation of the germination process as a first step towards resumption of vegetative growth. Favourable conditions for germination and outgrowth can be encountered in food products, and therefore additional preservation measures are required [6, 7].

Spore forming bacteria belong to the Firmicutes phylum, which encompasses a highly diverse group including pathogenic and non-pathogenic organisms. *Bacillus cereus*, *Clostridium perfringens* and *Clostridium botulinum* are representatives of the pathogenic group and are traditionally associated with a number of food borne outbreaks in Europe [8]. Non-pathogenic spore formers can cause product defects or spoilage i.e. gas production and strong off flavours leading to severe economic losses [9]. Due to the high diversity in metabolic capacity of spore forming bacteria, and despite measures taken at the level of packaging and storage conditions, many types of food products are vulnerable to spoilage and safety issues. Anaerobic Clostridia are more likely to cause spoilage in vacuum packed or canned products while *Bacillus* species can also cause spoilage in the presence of oxygen and in modified atmosphere packaged (MAP) products. Notably, even mildly acidic and acidic food products are at risk of spoilage by acidophilic spore formers such as *Bacillus coagulans* or *Alicyclobacillus acidoterrestris* [10, 11], whereas refrigerated products with neutral pH are challenged by psychrotrophic spore formers including *Bacillus weihenstephanensis*, some strains of *B. cereus* [12] and *C. perfringens* [13], *Clostridium tyrobutyricum* [14] and other members of the *Bacillus* or *Paenibacillus* species [15]. Thermophilic species i.e. *Geobacillus* spp. [16] require higher growth temperatures encountered in specific environmental niches including silage, and can form heat resistant spores that may cause spoilage of dairy products and a range of other food products [17].

Spore structure and resistance

In response to unfavourable conditions, spore forming bacteria can initiate the sporulation process in which the cell transforms into a dormant, highly resistant form, called spore or endospore (Figure 1.1). Spore formation is initiated by asymmetric cell division resulting

in a smaller forespore that is engulfed by the mother cell. The correct engulfment of the forespore and formation of spore specific components requires compartment specific gene expression under the control of several spore specific sigma factors, and inter-compartmental communication [5]. During the steps following engulfment, the forespore (at this stage called prespore) prepares for dormancy, the internal pH drops (indirectly leading to accumulation of 3-phosphoglycerate (3PGA)) while the prespore accumulates spore specific proteins, α/β-type small acid soluble proteins (SASPs), and pyridine-2,6-carboxylic acid (dipicolinic acid, DPA) chelated with divalent cations thereby lowering the spore water content. At the same time, the typical multi-layered spore structure is formed, followed by spore maturation. Finally, the spore is released into the environment upon lysis of the mother cell [18]. The resultant dormant spores are generally considered to have no (or very low) metabolic activity [19, 20] and are highly resistant to environmental stresses including heat, salinity, acidity, radiation, oxygen and/or water depletion [1-3].

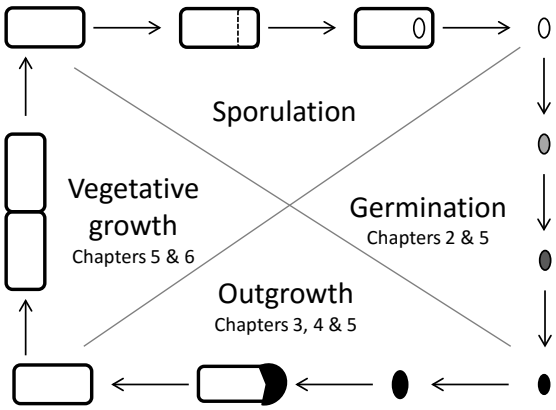


Figure 1.1. Life cycle of spore forming bacteria. Four main processes, sporulation, germination, outgrowth into a vegetative cell and vegetative growth, are depicted. Processes for *B. cereus* covered in the thesis research chapters are indicated.

The multi-layered structure of bacterial spores (Figure 1.2) provides resistance against environmental insults and, combined with the limited metabolic activity provides the basis for spores longevity [2]. Several specific characteristics provide resistance properties to the spores. Firstly, the genetic material in the spore core is protected by dehydration, which largely immobilizes the proteins present in the spore [21]. The integrity of the spores genetic material is furthermore protected by SASPs and the presence of DPA chelated with divalent cations (mainly calcium) in the spore core [2]. The inner membrane surrounds the spore and provides protection against chemicals [22] and contains specific proteins required for germination (discussed below) [7]. Outside the inner membrane, the germ cell wall, which becomes the vegetative cell wall after germination, and the cortex, which consists of spore-specific peptidoglycan and is required for the development of

full resistance towards wet heat [7] are located. The cortex is surrounded by an outer membrane for which no clear role in resistance is known to date [23]. The outer layer, the spore coat, contains various proteins that provide protection against different threats including lysozyme, toxic chemicals, and grazing protozoa [3]. Finally, in some species including *B. cereus* and *Clostridium* spp., the spore is covered by an exosporium which is in direct contact with the environment and is potentially involved in pathogenicity [24, 25].

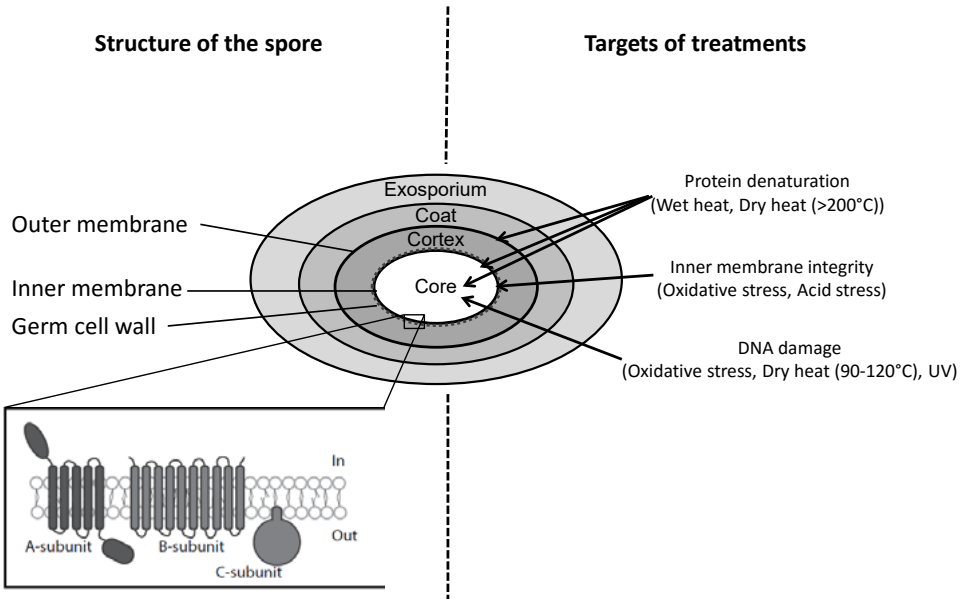


Figure 1.2. Schematic representation of the spore structure. On the left side: the different spore components are depictured. The thickness of the layers can vary between species, and not all species are equipped with an exosporium. The magnified box shows the schematic structure of the three subunits forming the typical germinant receptor in *Bacillus* spp. On the right side: spore components targeted by commonly applied treatments are shown (Adopted from PhD thesis of Hornstra [26] and van Melis [27]).

Spore germination and outgrowth

Dormant spores are able to monitor the surrounding environment for conditions that may be favourable for growth, as for example availability of amino acids, sugars, nucleosides, and salts typically present in food products. These compounds, also called germinants, can trigger specific germinant receptors (GRs) initiating the germination process (Figure 1.1). In addition, the presence of peptidoglycan fragments, indicative for bacterial growth, can also trigger germination [28]. Germination is a physical process during which the spore rapidly loses its dormancy and resistance properties concomitant with the resumption of metabolic activity. Next, spores grow out and eventually resume vegetative cell growth [29, 30].

Genomes of almost all spore formers contain at least one, but usually several GRs [30, 31]. The GRs of the model strain *B. subtilis* 168 are best studied. This strain carries three functional GRs, namely GerA, GerB and GerK. GerA responds to L-alanine while GerB and GerK both respond to a mixture of L-asparagine, D-glucose, D-fructose, and K^+ (AGFK) [29]. *B. cereus* strains usually carry a higher number of GRs [31, 32] with the *B. cereus* ATCC 14579 genome encoding seven GRs. *B. cereus* germinates fast in response to the combination of L-alanine and inosine. Notably, only a limited number of germinant-GR relations have been identified. GRs can act either individually, with a single GR responding to a given germinant, but also interaction of two or more GRs might be required for a germination response [33, 34]. Moreover, some GR require multiple germinants to trigger germination in a ration and concentration dependant manner [20, 30, 33]. Nowadays, the exact mechanism of GR-germinant(s) interactions remain to be elucidated.

GRs are usually composed of three subunits (A, B, and C) (Figure 1.2), but for some species, more subunits may be present as for example in *Clostridia*, operons of 4 or 5 subunits occur [35]. The spore GRs subunits A and B are integral membrane proteins composed of 5 to 8 and 10 to 12 predicted membrane spanning domains, respectively [20, 32]. Subunit B belongs to a subfamily of single component membrane transporters and it is speculated that this unit is involved in germinant recognition [32]. Subunit C is membrane associated and conceivably bound to the A and B subunits [32]. Until now it is not clear how the GR subunits interact and what is their individual function [20]. In *B. subtilis*, GRs were shown to co-localize together with SpoVA proteins and GerD in the inner membrane of the spore in a structure termed the germinosome [20]. GerD is a lipoprotein required for maintaining GR-dependent germination rate, while SpoVA proteins are suggested to form SpoVA channels involved in DPA movement during sporulation and germination [20]. The signalling from the GR(s) to the putative SpoVA channel(s) remains to be elucidated. Notably, the presence and functionality of germinosomes in other *Bacillus* spp. and *Clostridium* spp. remains to be elucidated, especially, since the number and types of GRs can vary significantly across the different species and even within strains [31, 36].

Shortly after the GR is triggered by its germinant, the spore is committed to the germination process (Figure 1.1). At first, spores release the monovalent cations (H^+, Na^+, and K^+) and DPA and partially rehydrate, and thereby loose most of their wet heat resistance [20, 37]. The germination event can be recognized by the transition of spores from phase bright to phase dark with phase contrast microscopy [38]. Subsequently, the cortex lytic enzymes CwlJ and SleB are activated in Bacilli, allowing for degradation of the cortex, further water uptake and spore swelling. After full core rehydration, additional enzymes reactivate that are involved in ATP synthesis and degradation of SASPs. The resulting free DNA, allows the onset of RNA and protein synthesis during the outgrowth phase. The initial germination step does not require energy, while during the early outgrowth (after increase in pH) energy is conceivably obtained from metabolism of 3-phosphoglycerate

(3PGA) present in the spore. In contrast, the late outgrowth phase is an energy demanding process requiring utilisation of extracellular carbon and energy sources to complete the emergence of the new cell (Figure 1.1) [19, 29, 30, 39].

Germination of spores can be enhanced by an activation step. Usually this is achieved by a short mild heat treatment ranging from 60 to 100°C, depending on the species, and this can result in a dramatic increase in the fraction of germinating spores and enhance homogeneity of this process [40, 41]. Specific targets affected by heat activation have not been identified, but super-dormant spores were shown to require higher activation temperatures than dormant ones [20, 42].

Besides nutrient induction, germination can be initiated by chemical treatments with the cationic surfactant dodecylamine and Ca^{2+}DPA, or by exposure to high hydrostatic pressure [29]. In addition, lytic enzymes such as lysozyme [29] or the presence of peptidoglycan fragments (also referred to as muropeptides) [28] may trigger germination. Such non-nutrient triggers were shown to by-pass GRs and target directly release of ions and Ca^{2+}DPA or activation of cortex lytic enzymes in *B. subtilis* and several other spore formers [29, 30], with the muropeptide signalling pathway conceivably depending on a eukaryotic-like Ser/Thr membrane kinase [28]. Notably, the underlying mechanism of the latter activation pathway still remains to be elucidated.

So called super-dormant spores, that respond slower or not at all to conditions commonly triggering germination may evoke extended heterogeneity during germination. Moreover, heterogeneity in germination may arise from the sporulation conditions. Sporulation conditions might affect gene expression and translation efficiency conceivably resulting in changes in the number of functional GRs and in more general terms, the protein composition of individual spores [43-46]. In practice, sporulation conditions may differ greatly as e.g. water activity, pH, oxygen availability, and nutrient availability can affect the properties of the resulting spores [47, 48]. Such intrinsic heterogeneity within the spore population could be further enhanced by exposure to sublethal treatments that cause spore damage. The degree of damage and/or repair capacity may vary for individual spores. Obviously, optimum recovery conditions can differ for different spore forming species, strains and conceivably even individual spores.

Bacillus cereus as food contaminant

B. cereus is a spore forming, gram positive, rod shaped, and facultative anaerobic food-borne pathogen belonging to the genus *Bacillus*. *B. cereus* displays high adaptive capacity to different environmental condition making it a survival expert in diverse environments. Historically, *B. cereus* has been considered a soil dwelling organism, however, the available genomic sequences of *B. cereus* strains [49, 50] reveal an extended capacity

for amino acid and peptide utilisation, indicating adaptation towards a symbiotic or parasitic life cycle within animals and insects. Even though *B. cereus* seems to be better adapted to growth on protein sources, it carries an extended repertoire of substrate transport and utilisation systems to survive and grow in soil matter [51], rhizosphere [52], insect gut [53], and in the mammalian gastrointestinal tract [54, 55]. The diverse metabolic capacity also enables *B. cereus* to grow in a variety of food products including rice [56], vegetables [57], meat, milk and dairy products [58] and consequently, this bacterium is frequently associated with food spoilage and food-borne disease [9, 55]. Nowadays, a wide range of food products is stored at low temperatures ranging from 4 - 7°C. In particular, spoilage of dairy products stored at refrigeration temperatures is often associated with psychrotolerant *B. cereus* strains able to grow below 7°C, thereby causing "sweet curdling" and "bitty cream" defects [59, 60]. The ability of *B. cereus* to grow anaerobically poses also a risk of spoilage for vacuum or modified atmosphere packed (MAP) foods [61].

Association of *B. cereus* with food-borne diseases was first recognized in 1906 when staff and patients of a sanatorium developed diarrhoea and vomiting linked to presence of *Bacillus* ssp. in the incriminated meal [62]. *B. cereus* is recognised as one of the leading causative agents of food-borne diseases among spore formers [8], its symptoms are usually mild and self-limiting resulting in under reporting of the number of *B. cereus* cases, but in rare instances fatal outcomes can occur [63-65]. The vegetative cells of *B. cereus* can produce a range of virulence factors associated with two gastro-intestinal syndromes, namely emetic and diarrhoeal syndrome [54, 55, 66-68].

The emetic syndrome can be caused by consumption of food, particularly starchy products, in which a heat-stable toxin, cereulide, has been produced [55, 66, 68]. Cereulide is a cyclic peptide, resistant to low pH and proteolytic enzymes, toxic to mitochondria by acting as a potassium ionophore and it has been reported to inhibit human natural killer cells [68]. The emetic syndrome presents itself 0.5 – 5 h after ingestion, and usually mild symptoms like nausea, vomiting and malaise can last up to 24 h [54, 66, 67]. In contrast, the diarrhoeal syndrome is a toxico-infection, caused by enterotoxin secretion by vegetative cells present in the small intestine. Symptoms like abdominal pain, watery diarrhoea and occasionally nausea occur usually 8 to 16 h after ingestion and can last one up to several days. Meat products, soups, vegetables, dried herbs and spices, puddings, sauces and milk products were most frequently associated with the diarrheic syndrome [55, 66]. Several *B. cereus* enterotoxins have been identified that affect the integrity of membranes of epithelial cells including the well-studied haemolysin BL (Hbl) [69], the non-haemolytic enterotoxin (Nhe) [70] and the single component cytotoxin K (CytK) [64]. *B. cereus* can also be the causative agent of non-food related diseases, such as local or systemic infections like periodontitis, eye infections, fulminant endophthalmitis, and meningitis in immunocompromised patients, neonates and drug addicts [54, 55, 71].

Notably, rather high numbers (10^5 - 10^8 cfu/g) of *B. cereus* cells have been found in food products linked to reported cases [8]. Next to safety issues, *B. cereus* can also cause food spoilage and affect production due to equipment failure [9]. Already in 1930, a cheese spoilage study led to isolation of *B. cereus* ATCC 10987 [72], a *B. cereus* type strain with high biofilm forming capacity [73]. A biofilm, is a complex multicellular structure in which cells are protected by matrix components from cleaning and disinfection procedures. Eventual dispersal of the cells or spores from the biofilm into the production line may lead to contamination or recontamination of the food products. Additionally, *B. cereus* spores are highly hydrophobic which may facilitate the initial attachment of the spores to the surfaces, supporting *B. cereus* persistence.

B. cereus group

B. cereus belongs to the *Bacillus cereus* group, also called *Bacillus cereus sensu lato* group, which is composed of seven closely related but diverse species of spore formers. Besides *B. cereus sensu stricto*, the *sensu lato* group includes *Bacillus mycoides*, *Bacillus pseudomycoides*, *B. weihenstephanensis*, *Bacillus thuringiensis*, *Bacillus anthracis* [74], and more recently, *Bacillus cytotoxicus* [75]. Classification of species within the *B. cereus* group is still under debate [49, 50, 76-78], species within *B. cereus* group can generally be distinguished based on morphology, physiology and/or virulence characteristics including genes encoding toxins that are often located on plasmids.

B. mycoides and *B. pseudomycoides* can be phenotypically recognised by the formation of a rhizoidal colony morphology, further phenotypic distinction between those two species requires analysis of fatty acids composition [79]. *B. weihenstephanensis* is characterised as a psychrotolerant species able to grow below 7°C, but not at 43°C. Not all psychrotolerant strains within the *B. cereus* group belong to this species, also *B. cereus* strains able to grow at low temperatures have been identified [12]. *B. thuringiensis* is an insecticidal bacterium producing crystal proteins. *B. thuringiensis* is used in biological pest control directly or by introducing insecticidal protein-encoding genes into transgenic crops. Even though *B. thuringiensis* is considered harmless to humans, cases of infections in mammals have been reported [80]. By contrast, *B. anthracis*, is a causative agent of anthrax, a fatal illness in animals and humans [81]. Finally, *B. cytotoxicus* is a thermotolerant species that contains a gene encoding for the production of CytK-1, a cytotoxic variant of cytotoxin K [75].

Notably, diversity in characteristics of strains of the same species has been studied extensively and revealed differences in growth performance [82, 83] and stress resistance [84], but also in spore properties. The strain diversity in spore heat resistance was recently shown by Berendsen et al. for *B. subtilis* spores [85]. Spores derived from *B. subtilis* food isolates displayed a high diversity in resistance to one hour exposure to 100°C, ranging from 0.1 to 10.2 log reduction [85]. The germination capacity of *B. subtilis* [86] and *B. cereus*

spores [32, 87, 88] is also highly divers, and minimal concentrations of germinants required for full spore germination are ranging for example from 1 to above 200 mM of L-alanine for different *B. cereus* strains [87]. This illustrates the importance of taking strain diversity into account to understand and predict microbial behaviour in designing new products and processes, or when performing challenge tests.

In recent years, growing attention was devoted to classification of species/strains within the *B. cereus* group based on phylogeny of group members. Guinebretière et al. [74] introduced seven phylogenetic groups (Figure 1.3), namely *B. pseudomycoides* belonging to group I, *B. anthracis* belongs to group III, *B. cytotoxicus* belongs to group VII, *B. mycoides* and *B. weihenstephanensis* belong to group VI, and groups II, III, IV, V and VI encompass *B. cereus* and *B. thuringiensis* strains. The seven phylogenetic groups cover the entire spectrum of growth temperatures observed for strains within the *B. cereus* group [74].

Spores in the era of mild food preservation processes

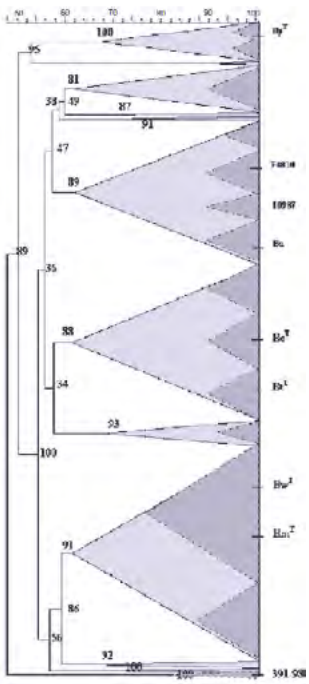
Ideally, spores are prevented from entering the food chain, but this is difficult to achieve in practice due to the ubiquitous presence of spore formers in the environment, and given their robustness towards stress conditions, control strategies aim for both reduction of microbial load of ingredients and prevention of vegetative growth (secondary stresses). Traditionally, thermal sterilisation processes have been applied to target heat resistant spores, aiming for production of commercially sterile foods with extended shelf life at ambient conditions. Hence, highly heat resistant spores may still survive such processes and may cause spoilage [16, 89]. Next to high heat treated products such as sterilised canned foods (e.g. meat, fish, vegetables) and ultra-high temperature (UHT) treated liquids (juices and milk) [7], there is a demand for fresher, more natural and nutritious products [90]. Consequently, milder processing techniques are applied often including an inactivation treatment (e.g. heat) to reduce microbial loads, in combination with factors that control growth of microbes throughout shelf life [91]. Control measures include intrinsic foods factors such as water activity, pH, presence of organic acids but also storage and packaging conditions such as temperature and oxygen limitations in e.g. MAP - packaging [91]. At the same time, consumers as well as governments desire a reduction in use of salt and/or sugar which are traditionally applied preservatives [90]. Increasing complexity of applied strategies poses new challenges in assuring the stability and safety of a variety of foods, and particularly in predicting combined effects of individual treatments.

Damaged but viable spores: repair prior to outgrowth

Spores are well protected against injury, nevertheless they can be damaged during most commonly used food processing and preservation treatments. Exposure to chemical (e.g. disinfectants) or physical (e.g. high hydrostatic pressure) treatments to inactivate

dormant spores in foods or on food contact materials can increase the inherent variation (resulting from the genetic makeup and conditions during sporulation) in the capacity of spores to germinate. Spores may survive such treatments but encounter damage to DNA and/or proteins (Figure 1.2). Generally, it is assumed that repair cannot take place during long lasting dormancy owing to lack of (or very low) metabolic activity in the spore [20, 93, 94], consequently, damage accumulates until repair processes become active once metabolic activity resumes [7].

A



B

Phylogenetic group	Species	Range of growth [C]	% of food poisoning strains	Carried toxin genes
I	*B. pseudomycoides*	10 - 43	0 0	*nhe, hbl*
II	*B. cereus* II *B. thuringiensis* II	7 - 40	21	*nhe, hbl, ctK-2*
III	*B. cereus* III	15 - 45	13	*nhe, cytK-2, hbl*
	B. thuringiensis III		53	*nhe, ces/cytK-3, hbl*
	B. anthracis		21	*nhe, cytk_2, hbl, (ces)*
			18	*nhe, cytK-2, hbl*
IV	*B. cereus* IV *B. thuringiensis* IV	10 - 45	22	*nhe, hbl, cytK-2*
			31	*nhe, hbl/cytK-2*
			10	*nhe, hbl, cytK-2*
V	*B. cereus* V *B.thuringiensis* V	8 - 40	12	*nhe, hbl, (cytK-2)*
VI	*B. weihenstephanensis*	5 - 37	0	*nhe, hbl*
	B. mycoides			
	B. thuringiensis VI		0	*nhe, hbl*
VII	*B. cytotoxicus*	20 - 50	50	*nje, cytK-1*

Figure 1.3. Genetic diversity of the *B. cereus* group. (A) Simplified dendrogram showing the genotypic relationship between bacterial strains of the *B. cereus* group, based on fAFLP data analysis. BpT, *B. pseudomycoides* DSM 12442; Ba, *B. anthracis* CEB 94–0040; BcT, *B. cereus* ATCC 14579; BtT, *B. thuringiensis* CIP 53.137; BwT, *B. weihenstephanensis* WSBC 10204; BmT, *B. mycoides* CIP 103472. Used with permission from Guinebretière et al. [74] (B) Characteristics of strains in each genetic group based on data from Guinebretière et al. [74, 92].

Molecular mechanisms involved in repair of damaged DNA in germinating spores have been studied, capitalizing on knowledge available for vegetative cells (reviewed in [95, 96]). Apurinic/apyrimidinic (AP) endonucleases (Nfo and ExoA) are thought to play a role, as their absence in *B. subtilis* severely delayed spore revival [93]. DNA repair and outgrowth processes appear to be aligned, as the DNA integrity scanning protein (DisA) was reported to delay *B. subtilis* spore outgrowth until oxidative damage repair of DNA was completed [93]. Exposure of *B. cereus* spores to DNA-damaging treatments such as pulsed light or irradiation

resulted not only in longer lag times to spore outgrowth but also in reduced growth rates, possibly owing to mutations [97]. DNA damage in *Bacillus* spores has furthermore been reported to result from UV exposure or application of dry heat at moderately high temperatures of 80 to 100°C [98]. At temperatures exceeding 200°C, dry heat also appears to lead to protein damage, in particular damage to the spore core proteins, which points to the temperature-dependent damaging effects of dry heat [98]. Wet heat, on the other hand, is thought to cause protein damage that may include the germination machinery of spores. In contrast, acid treatment inactivates spores due to inner membrane damage resulting in spore rupturing during germination. While oxidative stress results in damage to the inner membrane or DNA damage, depending on the compound used [2]. Various processing conditions may lead to spore damage, however, in most cases location and underlying mechanisms of damage remain to be elucidated [2, 7, 99]. More general information on protein damage and repair is provided in reviews by Chondrogianni et al. [100] and Visick & Clarke [101].

Although most repair processes are thought to be activated upon germination, spores may already be equipped with repair enzymes (produced during endospore formation) that allow for quick repair upon germination. Recently, it was suggested that protein synthesis already occurs during the early stages of germination [102], although it remains to be elucidated if factors involved also can play a role in repair processes [7].

Notably, the extent of spore injury can be quantified by studying spore recovery on optimal media and selective media containing antimicrobials, such as organic acids, sodium chloride or nitrite, as sublethally injured spores have an increased sensitivity to these antimicrobials [99, 103]. Moreover, certain antimicrobials may act in the spore outgrowth phase, including nisin [104] and sorbic acid [105] by dissipation of ion and pH gradients. The variety of conditions and antimicrobials affecting the outgrowth phase offer opportunities to study underlying mechanisms of repair in a range of recovery media including (model) foods [57].

Individual spores may vary in the degree of damage, resulting in a heterogeneous population. Single cell approaches including Anopore, time-lapse microscopy and flow cytometry have been applied to study behaviour of individual spores [7, 20, 105-107]. For example, application of Anopore technology, revealed that heat treated *B. cereus* spores showed more heterogeneous germination and outgrowth than non-heat stressed spores [105]. Such findings highlight the impact of different (model) food recovery media on outgrowth efficiency and heterogeneity of non-heat treated and heat damaged *B. cereus* spores.

Thesis outline

In this thesis, factors contributing to the germination and outgrowth capacity of *B. cereus* spores derived from genome sequenced food isolates and the model strains, *B. cereus* ATCC 14579 and ATCC 10987, were assessed in a range of conditions. Selected functional

genomics approaches were used including comparative analyses of full genome sequences and transcriptome analysis of untreated and heat treated outgrowing spores of *B. cereus* ATCC 14579. Moreover, performance was assessed at single spore/cell and subpopulation level using Anopore technology and flow cytometry. Special attention was devoted to assess the impact of strain diversity on nutrient-induced germination efficiency and carbohydrate utilisation capacity. Using *B. cereus* ATCC 14579 wild type and selected mutants, genes encoding putative functions in spore damage repair and recovery were identified.

To assess the impact of strain diversity in *B. cereus* spore germination, a high throughput flow cytometric approach was used (Chapter 2) to analyse nutrient-induced germination of individual spores from 17 *B. cereus* strains and link responses to the genomic content of the strains. The approach revealed diversity in germinant receptors (GRs) and their contributions to the observed germination responses.

Next, outgrowth of untreated and heat-treated spores was evaluated in Chapter 3 using the direct-imaging-based Anopore approach which allows monitoring of initial outgrowth and microcolony formation from individual spores on selected media including model foods. Conditions were selected such that a high fraction of damaged spores were obtained allowing for investigation of the effect of recovery/food media on the outgrowth heterogeneity of damaged spores.

In Chapter 4, both germination and outgrowth of heat damaged spores of *B. cereus* ATCC 14579 were studied at the population level, revealing delayed and heterogeneous germination and outgrowth compared to untreated spores. Transcriptomic approaches were applied to identify cellular parameters involved in spore damage repair and recovery. The role of identified genes in recovery of heat damaged *B. cereus* ATCC 14579 spores was assessed in this chapter and Chapter 5 and identified factors such as putative transcriptional regulator CdnL and BC5242, a gene encoding a membrane protein with C2C2 zinc finger, that supported recovery of spores in a range of conditions including model foods.

Spore recovery was shown to depend on recovery media (Chapter 3) and ability to repair (Chapters 4 and 5). Additionally, recovery and outgrowth capacity may be associated with carbohydrate transport and utilisation capacity which conceivably links to transmission efficiency and occupation of environmental niches. Therefore, Chapter 6 focuses on the ability of 20 *B. cereus* food isolates to grow on various carbohydrate sources. The genomes and carbohydrate utilisation capacities were evaluated revealing a core set of compounds that could be used by all *B. cereus* strains, whereas utilisation of other carbohydrates, particularly related to specific niches such as the human host, was less widespread and could be linked to specific phylogenetic groups previously proposed by Guinebretière et al. [74].

Finally, Chapter 7 presents a general discussion, concluding remarks, and future directions based on the research described in this thesis.

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Chapter 2

Linking germination capacity of genome sequenced *Bacillus cereus* environmental isolates and model strains to germinant receptor (sub)clusters

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Abstract

Germination of bacterial spores is a critical step before vegetative growth can resume. Food products, may contain nutrient germinants that trigger germination and outgrowth of *Bacillus* spp. spores possibly leading to food spoilage or foodborne illness. Prediction of spore germination behaviour is however very challenging, especially for spores of natural isolates that tend to show more diverse germination responses than laboratory strains. In this study, we evaluated *Bacillus cereus* spore germination using flow cytometry analysis in combination with fluorescent staining at a single spore level. This approach allowed for rapid collection of germination data from 17 whole genome sequenced *B. cereus* food isolates and reference strains, in over 20 conditions. Tested variables included heat activation of spores and germination in either complex media (BHI and TSB) and exposure to saturating concentrations of single amino acids and the combination of alanine and inosine. Whole genome comparisons revealed a total of eleven clusters encoding germinant receptors (GRs), with GerK, GerI and GerL shared by all strains, whereas the presence of GerR, GerS, GerG, GerQ, GerX, GerF, GerW and GerZ (sub)clusters was distributed among different strains or groups of strains. The spores of tested strains displayed high diversity with regard to their sensitivity and responsiveness to selected germinants and heat activation. The two laboratory strains, ATCC 14579 and ATCC 10987, and 11 food isolates showed a good germination response in a range of conditions, whereas four other strains (B4085, B4086, B4116 and B4153) showed a very weak germination response even in BHI and TSB media containing high levels of mixtures of amino acids. Phylogenetic grouping clustered these strains in group IIIA. Those four group IIIA strains contained either pseudogenes or variants of subunit C in their GerL cluster combined with a pseudogene in the GerK cluster and sub-cluster $GerR_I$ (B4086, B4153), or combined with the presence of $SpoVA^{2mob}$ transposon (B4085, B4116), that was recently shown to induce heat resistance with concomitant reduced germination response in *Bacillus subtilis* spores. High diversity between tested strains in the number of Ger clusters (5 - 10) and a possible role for other factors in germination, such as cortex enzymes, conceivably hampered assessment of positive correlations between Ger cluster(s) and germination response(s). Further studies are required to elaborate on these interactions. In conclusion, this study provided information on the genetic diversity in GRs and corresponding sub-clusters encoded by *B. cereus* strains as well as their germination behaviour and possible associations with GRs.

Introduction

Dormant bacterial spores are able to monitor the environment for conditions that favour growth, indicated by the presence of specific nutrients such as amino acids, which can trigger spore germination. Germination is a relatively fast biophysical process required to resume vegetative growth that can be initiated by activation of the germinant receptors (GRs) located in inner membrane of the spore [1, 2]. The majority of spore formers contain at least one and usually several GRs that may differ in their specificity for different nutrients [3, 4]. The model spore former *Bacillus subtilis* 168 carries three functional GRs, of which GerA responds specifically to L-alanine, whereas GerB and GerK cooperate to respond to the mixture of asparagine, glucose, fructose and K^+ (AGFK) [1]. Notably, spores of the toxin-producing food-borne human pathogen *Bacillus cereus* ATCC 14579 germinate most efficiently in response to a mixture of alanine and inosine and are equipped with seven GRs (GerG, GerI, GerK, GerL, GerQ, GerR, and GerS) that show limited similarity to the GRs present in *B. subtilis* [5]. *B. cereus* ATCC 14579 GerR plays a dominant role in germination, as its disruption affected germination in response to many amino acids, purine ribosides and food products [5, 6]. GerG appears specifically required for germination with glutamine [5]. Additionally, it has been suggested that GRs can respond to more than one germinant, and that cooperation of multiple GRs could enhance the germination response with specific individual germinants [4, 7-10]. Despite attempts of Ross [11] to standardise the nomenclature used for the GR's, the annotation and naming of GR's is inconsistent across and within spore forming species, this complicates comparative analysis and prediction of the GRs specificity.

GRs are usually composed of three subunits (A, B, and C), and genes encoding these subunits are typically arranged in tricistronic operons [12, 13] with some exceptions more frequently found in anaerobic Clostridia. The spore GRs subunits A and B are integral membrane proteins composed of 5 - 8 and 10 - 12 predicted membrane spanning domains, respectively [4, 10]. Subunit B belongs to a subfamily of single component membrane transporters and is speculated to be involved in germinant recognition [4]. On the other hand, subunit C is membrane associated and conceivably bound to the A and B subunits [4]. So far, function of the individual GR subunits and their interaction is not clear [10]. GRs are thought to cluster in complexes, so-called germinosomes, involving the GerD lipoprotein that influences GR-dependent germination rates [14, 15] and possibly the SpoVA channels (located in the spore inner membrane), involved in release of small molecules, mainly dipicolinic acid (DPA) and monovalent cations, and uptake of water during germination [10]. Recently, it was shown that heat resistance and germination rate of *B. subtilis* spores could be attributed to the number of $spoVA^{2mob}$ copies on the genome, with a higher number of copies (up to three) correlating with increased heat resistance and reduced germination rate [16, 17].

The spore germination process follows well described sequential steps [9, 10, 18]. Spore swelling of germinating spores and full rehydration of the core requires hydrolysis of the peptidoglycan cortex layer. In *B. subtilis*, two germination-specific cortex-lytic enzymes, namely, CwlJ and SleB, are responsible for cortex peptidoglycan degradation [1, 13]. After full rehydration, metabolic activity is regained, and spore outgrowth is initiated, followed by vegetative growth. Heat activation is commonly applied to enhance fast and homogeneous spore germination however, the processes involved remain unknown. Spore germination can also be initiated by non-nutrient germinants, including chemical triggers (Ca-DPA, the cationic surfactant dodecylamine), mechanical triggers (high hydrostatic pressure), enzymatic treatment (lysozyme) or bacterial cell wall fragments (muropeptides). These non-nutrient triggers usually bypass GRs and directly target either release of ions and Ca-DPA or activate cortex lytic enzymes [1, 2].

Traditionally, germination is monitored by measurement of optical density of spore suspensions at 600 nm (OD_{600}), with % decrease in OD_{600} correlating to germination efficacy assessed by phase contrast microscopy and/or plate counts [5, 19]. Additional methods include measurement of Ca-DPA content by Raman spectroscopy, sometimes combined with laser tweezers [20-22], automated phase-contrast or differential interference contrast (DIC) microscopy, and time lapse microscopy (for review see Wells-Bennik et al. [18]). Alternatively, spore staining approaches can be applied in combination with high throughput analysis of individual spores using flow cytometry (FCM) [23-25]. Germinated spores, but not dormant spores, can be stained by DNA fluorescent dyes such as SYTO-9, since spores lose their structural integrity upon germination, which allows for access of the dye into the spore core and subsequent binding to DNA [23-26].

In the current study, we applied FCM combined with SYTO-9 fluorescent dye staining of germinated spores to evaluate germination responses of 15 whole genome sequenced *B. cereus* food isolates and of two well studied sequenced laboratory strains *B. cereus* ATCC 14579 and ATCC 10987 [27, 28], and correlated germination responses to Ger clusters in the corresponding genomes. The approach used provided information on the genetic diversity in GRs and corresponding sub-clusters encoded by *B. cereus* strains as well as their germination behaviour and possible associations with GRs.

Materials and Methods

Strains used in study

Two laboratory strains, *B. cereus* ATCC 14579 and *B. cereus* ATCC 10987, were obtained from the American Type Culture Collection (ATCC), and culture collection of the Laboratory of Food Microbiology, respectively. In addition, we used 15 sequenced *B. cereus* strains [29, 30] isolated from food products and food processing environment

(Table 2.1). Strain B4117 (LJKG00000000.1) was initially included as a *B. cereus* strain but was recently re-classified by NCBI as *Bacillus mycoides* based on criteria of Average Nucleotide Identity (ANI typing; [31]). Strains were cultured in Bacto Brain Heart Infusion broth (BHI; Beckton Dickinson, France) at 30°C with aeration at 200 rpm.

Table 2.1. *B. cereus* strains and reference genomes used in the study.

Strain	Strain used for*	Isolation source /origin	Phylogenetic grouping#	Assembly / WGS code or UID
Bacillus_cereus_B4078	E, G	Food, unknown	III A	LCYJ00000000.1
Bacillus_cereus_B4080	E, G	Dried onion	IV	LCYK00000000.1
Bacillus_cereus_B4082	E, G	Asparagus ham sauce	II	LJKA00000000
Bacillus_cereus_B4084	E, G	Indian rice dish	IV	LJKC00000000
Bacillus_cereus_B4085	E, G	Asparagus soup	III A	LJKD00000000
Bacillus_cereus_B4086	E, G	Boiled rice	III A	LCYL00000000.1
Bacillus_cereus_B4087	E, G	Pea soup	III B	LCYM00000000.1
Bacillus_cereus_B4088	E, G	Dressing	II	LJKE00000000
Bacillus_cereus_B4116	E, G	White sauce	III A	LJKF00000000
Bacillus_cereus_B4117 (B. mycoides)	E, G	Commerical pasteurised milk	VI	LJKG00000000
Bacillus_cereus_B4118	E, G	Ice cream	IV	LJKH00000000
Bacillus_cereus_B4147	E, G	Cereals, pasta and pastries	II	LCYN00000000.1
Bacillus_cereus_B4153	E, G	Dairy products	III A	LCYO00000000.1
Bacillus_cereus_B4155	E, G	Beef salad	IV	LJKJ00000000
Bacillus_cereus_B4158	E, G	Vegetables	IV	LCYP01000000
Bacillus_cereus_ATCC_10987	E, G	Cheese spoilage	III A	uid57673
Bacillus_cereus_ATCC_14579	E, G	Unknown; Type Strain	IV	uid57975
Bacillus_cereus_B4077	G	Chilled dessert	II	LCYI00000000.1
Bacillus_cereus_B4079	G	Canned chocolate beverage	III A	LJIT00000000
Bacillus_cereus_B4081	G	Provolone sauce	IV	LJJZ00000000
Bacillus_cereus_B4083	G	Torteloni con fughi	VI	LJKB00000000
Bacillus_cereus_B4120	G	Water	IV	LJKI00000000
Bacillus_cereus_AH187	G	Vomit; emetic outbreak	III A	uid58753
Bacillus_cereus_E33L	G	Dead zebra isolate (ZK)	III B	uid58103
Bacillus_subtilis_168	G	Unknown; Type Strain	NT	uid57675
Bacillus_weihenstephanensis_KBAB4	G	Soil isolate	VI	uid58315
Bacillus_anthracis_Ames	G	Texas, Cow; plasmids cured	III B	uid57909
Bacillus_thuringiensis_Al_Hakam	G	Suspected bioweapons facility	III B	uid58795
Bacillus_thuringiensis_serovar_konkukian_97_27	G	Severe human tissue necrosis	III B	uid58089

*Strain used for experiments (E) and/or genome comparisons (G)
#Based on Guinebretière et al. [32], data from Warda et al. [33][Chapter 6]. IIIA and IIIB refer to division within group III based on core genome tree (Figure 6.1); NT Not Tested

Sporulation conditions

Spores were prepared on a nutrient-rich, chemically defined sporulation medium, designated MSM medium, previously described [34]. Ten ml of sporulation media was inoculated with 100 µl of an overnight-grown pre-culture in 100 ml flasks and incubated at 30°C with aeration at 200 rpm. When the mid-exponential growth phase was reached (corresponding to an optical density of 0.5 measured at 600 nm (OD_{600} ~ 0.5; Novaspek II,

Pharmacia Biotek, United Kingdom)), 200 µl culture was spread on MSM plates (solidified with 1.5% agarose) and incubated at 30°C within plastic bags to prevent drying. After seven days, chilled phosphate buffer (100 mM, pH 7.4) containing 0.1% Tween80 was added to the plates and spores were scrapped off the surface and harvested by 15 min centrifugation at 5,000 rpm at 4°C (5804R, Eppendorf, Germany). Spores were washed in decreasing concentrations of Tween80 and prepared for use as described previously [35]. A single spore crop per strain was used for all the experiments.

Germination assay

Fifty µl of spore suspension containing approximately 10^8-10^9 spores/ml in phosphate buffer (100 mM, pH 7.4) containing 0.01% Tween80 (further referred as suspension buffer) was exposed to germinants by mixing with 50 µl of concentrated stock solution of either individual or mixed germinants. Final germinant concentrations used were 20 mM for valine (Val), isoleucine (Ile), glutamine (Glu), glycine (Gly), cysteine (Cys), and inosine (Ino), 100 mM for L-alanine (Ala) and for the mixture of Ala and Ino (AlaIno) final concentrations were 10 mM Ala and 2 mM Ino. Amino acid stock solutions were prepared in distilled water and filter sterilised. Control experiments were performed using the complex media BHI and TSB (Trypthon Soy Broth, Beckton Dickinson, France) and HEPES buffer (25 mM, pH 7.4). Spores were exposed to germinants for 30 min at room temperature, followed by a centrifugation step for 1 min at 13,000 x g to remove the germinant. The resulting spore pellet was resuspended in 100 µl water containing 1 µM SYTO-9 (Invitrogen, The Netherlands) and incubated in the dark for 10 min at room temperature to stain permeabilised spores. Next, the unbound dye was removed by centrifugation and the resulting spores were resuspended in HEPES buffer to obtain approximately 1,000 events per second after loading to FACSAria III flow cytometer (BD, USA). Each experimental run included control spores that were not exposed to germinants. For each sample 10,000 events were evaluated.

For heat activation, spores were heated for 10 min at 80°C in a thermal cycler (Verity, Applied Biosystems, Singapore).

Data analysis

Flow cytometer data were analysed using the Data Analysis Software FlowJo (vX.0.7, LCC, USA), Figure S1 represents the flow chart for data analysis. Approach using SYTO-9 staining and FCM for germination evaluation has been previously validated by Cronin and co-workers [23]. Scatterplots of Forward Scatter Area (FSC-A) versus a green fluorescence intensity (FITC-A) were used to exclude atypical data sets indicating presence of spore clumps or strain specific fluorescence i.e. high auto-fluorescence due to presence of DNA on the spore surface. Events with fluorescence intensity lower than the spore auto-fluorescence (control sample, FITC-A < 10^1) and higher than FITC-A > 10^5 were excluded from the analysis.

For each strain individually, a maximum fluorescence of dormant spores was determined based on the cut off between dormant (unstained) and germinating (stained) spores in control samples (not exposed to germinant) and samples exposed to germinant. The control samples showed dormancy between 81.8 and 99.4%, while microscopic observations prior to the experiment showed a minimum of 95% dormancy. Spores with higher fluorescence than maximum value for dormant spores were considered stained/germinated. Next, a percentage of germinated spores in the population was calculated as percentage of stained spores among all events considered (unstained and stained).

Per strain the average percentage of germinated spores in the control samples and its standard deviations were calculated based on three independent measurements, with and without heat activation step. Three categories of germination performance were defined: spores with < 15 % germination (poor germination), 15 – 50% (intermediate germination), and > 50% (good germination).

Free amino acid analysis

The free amino acid content in BHI and TSB (same lot as used in germination experiments) was determined using liquid chromatography as described previously [36].

Genome mining

To investigate whether presence of GR operons correlated with experimentally tested germinant-induced spore germination, genomes were mined as described previously [33]. The analysis included 20 newly sequenced *B. cereus* food isolates [29, 30], nine *B. cereus* group strains with publically available genomes sequences and with experimentally determined germination responses [4, 34, 37]. The used strains and their isolation sources are listed in Table 2.1. To enhance genome comparisons additional genomes previously used for *B. cereus* genome comparison were used [33]. To improve the comperative analysis, all genoes were (re)annotated using RAST [38], and for the resulting annotated genomes Orthologous Groups (OGs; i.e. genes that are descended from the same gene in the last common ancestor of the strains studied, putatively sharing similar functionality) were defined using Ortho-MCL [39]. The OGs containing sequences of known germination receptor subunits were extracted from the dataset, and additional germination receptor subunits were identified manually by keyword searches and inspection of genome context. MSA (multiple sequence alignment) files were made with MUSCLE [40] aligning the protein sequences within specific OGs to facilitate identification of pseudogenes (encoding incomplete proteins) and manual correction of inaccurate auto-annotation using Artemis [41] and Jalview [42]. Next, phylogenetic trees of individual GR subunit A, B or C based on the aligned amino acid sequences of the 29 *B. cereus* group strains and *B. subtilis* 168 were constructed using Clustal X [43] and visualized using LOFT [44] (see Figures S2-S4). When subunits A, B and C of a given type of GRs clustered consistently

in one of the 11 clusters (and their sub-clusters), the candidate GR was assigned the GR name/letter of the well-studied strain, preferentially *B. cereus* ATCC 14579 (Table 2.3). Newly identified and known GR operons are summarized in Table 2.4 using the updated GR naming system (Table 2.3).

Results and Discussion

Nutrient-induced germination of *B. cereus* spores

Germination behaviour of heat activated spores from *B. cereus* food isolates and laboratory strains was evaluated at single spore level for 20 conditions representing saturating concentrations of single amino acids and conditions simulating mixtures of germinants that can be found in food matrixes (Table 2.2).

With the exception of strain B4153, heat activated spores of all strains showed good germination in response to AlaIno. BHI and TSB supported germination for most strains. However, a subset of strains of phylogenetic group IIIA (B4085, B4116, B4153, B4086) showed poor germination in tested media (BHI and TSB) and in response to individual amino acids. Also non-heat activated spores of these strains show poor germination response to AlaIno, next to B4080 and B4087 (Table S2).

The data presented in Table 2.2, clearly show diversity in sensitivity and responsiveness to single amino acids and complex media. We previously showed that strains used in this study are representative for the diversity found amongst *B. cereus* strains with respect to carbohydrate utilisation and capacities to occupy different environmental niches (soil, food products, and intestinal tract) [33]. The poor germinating group IIIA strains (B4085, B4086, B4116 and B4153) were previously shown to lack specific carbohydrate utilisation, lacking specific carbohydrate utilisation clusters (starch, glycogen, aryl beta-glucosides; salicin, arbutin and esculin), suggesting a reduced capacity to utilise plant-associated carbohydrates for growth (see Chapter 6, Figure 6.2) [33]. Since *B. cereus* subgroup IIIA representatives contain host-associated carbohydrate utilisation gene clusters [33] and a subset of unique Ger (sub)clusters, additional studies using host-derived compounds as germinants may provide further insights in their germination efficacy.

Notably, all *B. cereus* strains tested carry *spoVA*^{1} and *spoVA*^{2} operons [16]. Interestingly, only two members of the poor germinating group IIIA, strains, B4085 and B4116, carry a *spoVA*^{2mob} operon which occurrence was recently shown to correlate with slow germination and increased heat resistance of *B. subtilis* spores [16, 17].

Without heat activation exposure to Ala resulted in very efficient germination of spores of strains B4078 and B4088, and less efficient germination of spores of strains B4155 and B4158. A study by Broussolle et al. [45], showed significant germination (measured by OD_{600} drop) of

spores of laboratory strains, i.e. *B. cereus* ATCC 14579, upon exposure to 1 mM Ala, while wild *B. cereus* isolates typically required concentrations above 1 mM and for some even 200 mM did not result in maximal OD_{600} drop [45]. When the maximum germination rate was reached, further increase in germinant concentration did not improve germination. Both the maximum germination rate and the minimal concentration to reach this were shown to be strain and germinant dependent [45]. Notably, the L-alanine-induced germination response may be affected by the presence of alanine racemase (encoded by the *alr* gene) that is able to convert germination stimulating L-alanine into germination inhibiting D-alanine that competitively binds to L-alanine GRs [46, 47]. Differences in L-alanine response (and in the combination with inosine) between strains may be due to differences in Alr activity in the spore cortex.

Table 2.2. Germination of heat activated *B. cereus* spores of 15 food isolates and two laboratory strains exposed to either single amino acids, their mixtures or complex media. Spores showing <15% germination are indicated in red, 15 – 50% germination in orange, and >50% germination is indicated in green.

Phylogenetic group	Strain	Heat activated (10 min at 80°C)										
		AlaIno	BHI	TSB	Ino	Ala	Cys	Glu	Gly	Val	Iso	Av. Blank
II	B4088	98.6	85.5	97.7	76.6	95.6	95.1	14.0	51.8	22.8	19.2	17.1
	B4082	97.5	99.2	97.9	96.0	81.0	56.8	72.4	1.7	6.9	3.0	2.5
	B4147	95.2	97.8	27.6	88.6	90.1	42.5	2.6	1.1	56.5	43.0	1.1
III A	B4085	81.8	13.9	4.1	33.3	1.2	3.8	2.2	3.4	2.2	2.0	1.5
	B4116	71.2	5.0	8.3	37.3	3.5	11.2	9.5	12.1	12.0	8.5	7.9
	B4153	22.2	3.8	4.4	13.2	4.8	4.4	3.4	3.7	4.4	4.1	6.9
	B4086	83.7	50.9	46.5	43.7	9.0	13.7	37.4	10.7	11.5	11.1	11.5
	B4078	98.8	99.8	98.9	99.3	99.5	83.6	32.5	59.6	49.0	34.4	14.7
	ATCC 10987	96.5	95.6	98.4	96.9	97.6	98.5	95.7	77.7	97.1	98.4	36.8
III B	B4087	95.3	89.7	82.3	85.9	51.7	62.6	61.8	63.3	64.3	70.2	54.3
IV	B4084	97.4	99.6	98.9	97.3	94.0	60.5	41.2	3.0	7.4	2.9	4.8
	B4080	98.4	98.7	98.4	14.9	74.7	25.6	13.8	2.9	4.0	2.7	1.4
	B4118	92.4	92.0	96.6	55.5	40.6	15.6	5.6	0.9	5.1	2.8	2.4
	B4155	98.3	98.7	75.5	92.9	95.4	90.0	15.2	50.3	6.3	4.9	3.5
	ATCC 14579	93.0	95.9	98.6	92.6	93.2	94.6	84.3	58.5	90.4	84.1	56.5
	B4158	78.1	80.8	80.1	72.7	78.7	0.0	0.1	0.1	0.1	0.0	6.7
VI	B4117	97.3	95.2	97.4	96.6	87.1	18.1	13.6	16.3	16.1	18.1	16.2

Cysteine has been reported as potent germinant in *B. cereus* strains [5, 48], and significant Cys-induced germination was indeed observed for heat-activated spores of the majority of the strains tested. Despite the heat activation step, spores of seven strains (B4085, B4086, B4116, B4117, B4118, B4153 and B4158) stayed insensitive or responded only mildly to one of six individual amino acids Val, Cys, Iso, Gly, and Glu. All other strains tested showed enhanced spore germination in response to two or more amino acids tested. The applied 20 or 100 mM concentrations of individual amino acids (Ala, Val, Glu, Iso, Cys, Gly) and Ino used in this study are unlikely to be encountered in the environment,

however, they allow fast germination responses. The poorest germination was observed in response to Val and Iso, suggesting that the broader composition of free amino acids in BHI and TSB (Table S1) most likely resulted in a cumulative germination response.

Linking presence of specific GRs to spore germination (genotype to phenotype)

GRs are responsible for recognition and binding of the nutrient germinants, however the exact mechanism in which GRs subunits interact and proceed upon germinant binding to the downstream germination pathway remains to be elucidated. Recognising the fact that annotation of GR's is not a trivial issue and is not consistent across species as well as within the species [4, 11], we present an overview of known GRs and their putative germinant specificity for nine sequenced and well-studied *B. cereus* group reference strains in Table 2.3.

To compare and assign GRs present in the food isolates to the GRs present in well-studied strains, phylogenetic trees were composed for each of the GR's A, B, C subunits of the reference strains and *B. cereus* food isolates (Figures S2-S4). Based on these trees, we have identified consistent clustering of corresponding subunits A, B and C of given type of GRs, illustrating their coevolution as has been observed previously [2, 11]. The clusters were named, when possible after the GR name/letter of the type *B. cereus* strain, *B. cereus* ATCC 14579 (Table 2.3). The GRs of 29 strains could be allocated to 11 main clusters and their sub-clusters (Table 2.4).

B. cereus strains typically harbour a relatively high number of GRs as compared to other spore forming species. Until now, *B. cereus* strains were believed to encompass a core group of five GRs, namely, GerR, GerL, GerK, GerS and GerI, plus a selection of five additional GRs [4, 37]. Indeed, analysis of 29 genomes revealed that all strains contain GerK, GerI, GerL, GerR and GerS clusters, albeit that differentiation can be made between sub-clusters of GerR ($GerR_I$ and $GerR_{II}$) and GerS ($GerS_I$, $GerS_{II}$ and $GerS_{III}$), and that some strains contain one or more conceivably inactive pseudogenes (Table 2.4). The great majority of pseudogenes and/or variants in GerK and GerL are found in strains belonging to phylogenetic group IIIA composed mainly of poorly germinating strains. In fact, both GerK and GerL are pseudogenes and/or variants in B4086 and B4153, the two poorly germinating strains (Table S2-S3). At the same time two other strains B4085 and B4116 also belonging to the phylogenetic group IIIA and producing poorly germinating spores (Table S2) carried a variant of the cluster L (subunit C) in a combination with the $GerX_{III}$. The combination of those two GRs that are possibly non-functional variants could have a negative effect on the germination ability. Both, variant of GerL and $GerX_{III}$ are also present in the *B. cereus* AH187 that was not included in the current germination assays, but spores of this strain were indeed previously shown to germinate poorly in response to amino acids or food [37].

Interestingly, GerR, which is a dominant GR in *B. cereus* ATCC 14579 that responds to most amino acids and to model foods [5, 6], belongs to sub-cluster $GerR_I$. Differentiation between sub-cluster $GerR_I$ and $GerR_{II}$ might also reflect functional differences between the two sub-clusters. In fact, comparative analysis of GerY of *B. anthracis* with unknown function [8] located this Ger receptor in cluster R_{II} although its subunit C is encoded by a pseudogene and might be non-functional version of the GR variant (Table 2.4). Moreover, subunits C of $GerR_I$ of strains B4086 and B4153, which produce poorly germinating spores, are pseudogenes. Complementation experiments could provide insights in the role of *gerR* in spore germination of other strains. Despite the differences in GR presence/absence, the organisation of the GR operons was conserved within the clusters with dominating ABC order, three clusters (R, F and Z) followed an ACB organisation, while cluster X_I and X_{II} revealed a BAC organization, and cluster X_{III} a unique ACC organisation.

In six of the *B. cereus* food isolates and *B. cereus* AH187, a tricistronic *ger* operon composed of one truncated subunit A and two subunits C was found, creating cluster X_{III}. In *B. subtilis*, the incorporation of subunit C of GerA into the membrane was shown to depend on subunit A [51] and B [52]. This suggest that either a different mechanism to form stable receptor assembly (e.g. interaction with subunits of other GR in germinosome) is in place in *B. cereus* or the GR could be not functional. Furthermore, subunit B was previously suggested to play a role in nutrient recognition and specificity of the germination response in *B. megaterium* [53, 54]. However, the assembly and function of GRs within cluster X_{III} remains to be elucidated.

Spores of strain B4117 (*B. mycoides*) germinated well, even without heat activation in response to AlaIno and Ino alone. Strain B4117 encodes two unique (among tested strains) GRs representing cluster Q_{II} and cluster S_{III}. GerQ is known to be involved in Ino induced germination in *B. cereus* [5], and it could be speculated that $GerQ_{II}$ version present in B4117 may be more responsive than more commonly encountered $GerQ_I$. Besides B4117, only *B. weihenstephanensis* KBAB4 carries a GR belonging to cluster S_{III}. In fact, both strains also carry GRs from cluster S_{II} that has been previously referred to as GerS2 [37], while most of the GRs within cluster S belonged to cluster S_I. Notably, van der Voort [37] previously showed that germination of *B. weihenstephanensis* KBAB4 spores with combinations of selected amino acids and inosine was far more efficient than with spores from tested *B. cereus* ATCC 14579 and ATCC 10987 strains.

Our study identified additional putative GRs, a group of GRs creating cluster W including a presumptive *gerT* of *B. cereus* AH187 [37], and a putative GR found only in B4079 composing one-item cluster Z. Those putative GRs share the tricistronic architecture and homology with known GRs however their functionality would require testing of directed deletion mutants.

Table 2.3. GRs that are present in nine selected reference strains.

Cluster / Sub-cluster (this study)		Putative Germinant*	Organism	GR name NCBS			Cluster (Ross2010)	GR name (literature)	Ref.
				subunit A	subunit B	subunit C			
Cluster K		Ba: Pro, Met	B. cereus ATCC14579	gerKA	gerKB	gerKC	S2	gerK	[5]
			B. cereus ATCC10987	gerKA, pseudo	gerKB	gerKC			
			B. cereus E33L	gerKA	gerKB	gerKC			
			B. cereus AH187	gerKA	gerKB, pseudo	gerKC			
			B. thuringiensis serovar konkukian 97_27	gerKA	gerKB	gerKC			
			B. thuringiensis Al Hakam	gerKA	gerKB	gerKC			
			B. weihenstephanensis KBAB4	SGP	SGP	SGP		gerK	[37]
			B. anthracis Ames	gerKA	gerKB	gerKC			
Cluster L		Bc: Caco-2 Ba: Ser, Val	B. cereus ATCC14579	gerLA, pseudo	gerLB	gerLC	L	gerL	[49]
			B. cereus ATCC10987	gerLA	gerLB	gerLC			
			B. cereus E33L	gerLA	gerLB	gerLC			
			B. cereus AH187	gerLA	gerLB	gerLC			
			B. thuringiensis serovar konkukian 97_27	gerLA	gerLB	gerLC			
			B. thuringiensis Al Hakam	gerLA	gerLB	gerLC			
			B. weihenstephanensis KBAB4	SGP	SGP	SGP		gerL	[37]
			B. anthracis Ames	gerLA	gerLB	gerLC			
Cluster G	G_I	L-glutamine mediated germination, L-glutamine + inosine	B. cereus ATCC14579	pseudo	gerBB	gerBC	A2	gerG	[5]
			B. cereus E33L	gerAA	gerBB	gerBC			
			B. cereus AH187	gerAA	SGP	gerAC		gerG	[37]
			B. thuringiensis serovar konkukian 97_27	SGP	SGP	gerAC			
			B. anthracis Ames	gerAA	pseudo	pseudo			
Cluster S	S_I	Ba: aromatic AA	B. cereus ATCC14579	gerSA	gerSB	gerSC	S	gerS	[5]
			B. cereus ATCC10987	pseudo	gerSB	gerSC			
			B. cereus E33L	gerKA	gerIB	gerQC			
			B. cereus AH187	gerSA	gerSB	gerSC			
			B. thuringiensis serovar konkukian 97_27	gerKA	gerIB	gerQC			
			B. thuringiensis Al Hakam	gerKA	gerSB	gerQC			
			B. anthracis Ames	gerSA	gerSB	gerSC			
	S_{II}		B. weihenstephanensis KBAB4	SGP	SGP	SGP	S	gerS2	[37]
	S_{III}		B. weihenstephanensis KBAB4	SGP	SGP	SGP	S	gerS	[37]
Cluster X	X_I	virulence, macrophage	B. cereus ATCC10987	gerXA	gerXB	gerXC	X	gerX	[37]
	X_{III}		B. cereus AH187	SGP	ND	SGP XC SGP LC	X	gerX	[37]

Table 2.3. Continued

Cluster / Sub-cluster (this study)		Putative Germinant*	Organism	GR name NCBS			Cluster (Ross2010)	GR name (literature)	Ref.
				subunit A	subunit B	subunit C			
Cluster R	R_I	Bc: alanine, adenosine, inosine, all AA except L-glutamine, food	B. cereus E33L	gerA	gerB	gerAC	Y	gerR	[37]
			B. thuringiensis serovar konkukian 97_27	gerA	SGP	gerAC			
			B. weihenstephanensis KBAB4	gerA	SGP	SGP			
			B. anthracis Ames	gerYA	pseudo	gerYC			
	R_{II}		B. cereus ATCC14579	garAa	gerB	gerC	Y	gerR	[5, 6]
			B. cereus ATCC10987	gerYa	SGP	gerYC, pseudo			
			B. cereus AH187	SGP	SGP	gerAC			
			B. thuringiensis Al Hakam	SGP	SGP	gerAC			
Cluster Q	Q_I	inosine	B. cereus ATCC14579	gerQA	gerQB	gerQC	Q	gerQ	[5]
			B. thuringiensis serovar konkukian 97_27	gerIA	gerIB	gerIC			
			B. thuringiensis Al Hakam	gerIA	gerIB	gerIC			
Cluster I		inosine, L-phenylalanien + inosine, , L-glutamine + inosine, purine riboside, aromatic amino acid, Caco-2	B. cereus ATCC14579	gerIA	gerIB	gerIC	I	gerI	[5, 49]
			B. cereus ATCC10987	gerHA	gerHB	gerHC			
			B. cereus E33L	gerHA	gerHB	gerHC			
			B. cereus AH187	gerHA	gerHB	gerHC			
			B. thuringiensis serovar konkukian 97_27	gerHA	gerHB / SGP IB	gerHC			
			B. thuringiensis Al Hakam	gerH	gerHB	gerHC			
			B. weihenstephanensis KBAB4	SGP	SGP	gerAC		gerI	[37]
			B. anthracis Ames	gerHA	gerHB	gerHC			
Cluster F	F_{II}	unknown	B. cereus AH187	gerKA/AC	hypothetical	gerAC			
Cluster W		unknown	B. cereus AH187	SGP	SGP	SGP		gerT	[37]
NOT CLUSTERING		unknown	B. subtilis 168	yndD	yndE	yndF	A2	yndDEF	[1]
		L-alanine, L-valine	B. subtilis 168	gerAA	gerAB	gerAC	A	gerA	[50]
		AGFK (in combination with gerK), L-asparagine, many AA	B. subtilis 168	gerBA	gerBB	gerBC	B	gerB	[50]
		unknown	B. cereus E33L	gerKA	extra subunit B gerKB	gerKC	N/C		
		glucose as cogerminant	B. subtilis 168	gerKA	gerKB	gerKC	K	gerK	[19]
		unknown	B. subtilis 168	yfkQ	yfkT	yfkR	N/C	yfkQTR	[1]

* Ba: *B. anthracis*, Bc: *B.cereus*
N/C not clustering; SGP spore germination protein
pseudo: pseudogene

Table 2.4. Assignment of GRs of selected strains to the clusters identified based on phylogenetic trees of subunits A, B, and C. Colour coding: green, presence of GR belonging to given cluster; white, absence of GR belonging to given cluster; and grey, at least one of the subunits is a pseudogene (P) or a variant (V).

Phylogenetic group	Strain NIZO / ATCC code	Cluster K	Cluster I	Cluster L	Cluster R		Cluster S			Cluster G		Cluster Q		Cluster X			Cluster F		Cluster W	Cluster Z	NON CLUSTERING
					R_I	R_{II}	S_I	S_{II}	S_{III}	G_I	G_{II}	Q_I	Q_{II}	X_I	X_{II}	$X_{III\ (CCA)}$	F_I	F_{II}			
II	B4088	B - P														NtgerA			B - P		
	B4082																				
	B4077						B - P														
	B4147																				
III A	B4079			C - P												NtgerA			B - P		
	Bc AH187	B - P		C - V/P												NtgerA					
	B4085			C - V/P												NtgerA					
	B4116			C - V/P												NtgerA					
	B4153	B - P		C - V/P	C - P																
	B4086	C - P		C - P	C - P																
	B4078			B - P C - V/P																	
	ATCC10987	A - P					A - P														
III B	Ba Ames					B - P				A/B/C - P											
	Bt serovar konkukian 97_27																				
	Bc E33L																				4 component GR
	B4087																				
	Bt Al Hakam																				
IV	B4084																				
	B4080																				
	B4081																				
	B4118																				
	B4120															NtgerA			B - P		
	B4155															NtgerA			B - P		
	ATCC14579			A - P						A - P											
	B4158						B - P														
VI	B4083							*													
	B4117																				
	Bw KBAB4																				

NtgerA: N-terminal truncated gerA, Bt: *B. thuringiensis,* Bc: *B. cereus,* Ba: *B. anthracis,* Bw: *B. weihenstephanensis*

*: subunit A ok, subunits B and C fragmented (poor assembly)

Interestingly, the presence/absence of GRs seems to be related to the phylogenetic clustering, we reported previously [33]. All strains encoding GRs belonging to cluster G_{II} represented phylogenetic group II (Table 2.3). This group is also characterised by high prevalence (75%) of GRs from clusters S_{II} and Q_{I}. A phylogenetic tree based on core genes of tested strains (reported in Warda et al. [33]) and phenotypic differences between the strains of group III suggest its division into two sub-groups IIIA and IIIB. In fact, the majority of strains belonging to group IIIA (and only them) encode GRs from cluster F_{II}. The high prevalence of F_{II} might be compensating for lack of GRs from cluster Q and high numbers of pseudogenes within clusters K and L. Based on the encoded GRs, experimentally tested strains within group IIIA seem more comparable to strains within group IV than IIIB (Table S3), possibly suggesting similar niche requirements. This may also be reflected by encoded carbohydrate utilisation genes that show similarities for members within those groups. All the strains within group IIIB encode GRs from clusters K, I, L and S_{I}. GRs from all the clusters can be found only in strain belonging to the group IV, indicating diversity of this group, with half of the strains encoding GRs from cluster F_{I} (not found in other strains tested). Finally, strains belonging to group VI often encode alternative GRs, in fact GRs from clusters S_{III} and Q_{II} are present only in this group while R_{II} and X_{I} are only found in one other group. Moreover, strains representing group VI do not encode GRs belonging to cluster G nor cluster F (Figure 2.3).

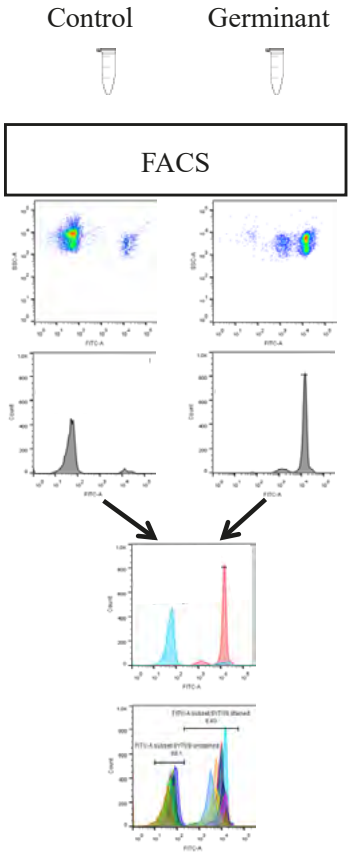
Our study further supports a high degree of diversity in GRs and nutrient induced germination in spores of different strains of *B. cereus*, generating leads for further studies. However, despite the different germination conditions tested, we could not directly link the presence (or absence) of given GRs to the germination responses such that germination behaviour could be predicted. This may be due to the requirement for different types or concentrations of certain germinants, or combinations of germinants. A number of factors that can affect the GR-dependent germination have been previously discussed [10, 18, 55] including accessibility and the number of GRs or the downstream germination mechanisms. Moreover, genome based studies may be affected by the quality of draft genomes i.e. contigs length, presence of GR's subunit on short contigs (i.e. subunits C) and/or location of GRs or its subunits at the contigs edge (i.e. cluster X_{III}). Nevertheless, despite those limitations, the approach presented in the current study allows for inclusion of rapidly increasing numbers of draft genomes.

Previous studies could not link the diversity in spore germination responses with the presence and/or similarity of the GRs, most likely due to the large number of factors affecting GR-dependent germination [56]. Similarly, Krawczyk et al. [57], found no correlation between poor germination of *B. subtilis* spores with Ala and sequences of their GerA subunits. However, the authors did show that "modest germination" with AGFK correlated with the presence of several common amino acid substitutions in subunits of GerB and GerK [57]. Similarly, diverse germination responses to Ala of

spores of 46 *Bacillus licheniformis* strains, which is a close relative of *B. subtilis*, could not be linked to the clusters formed by their *gerAB* - *gerAC* sequences [58]. Nevertheless, complementation of a *gerAA* disruption mutant with *gerA* operons of slow- and fast-germinating *B. licheniformis* revealed that differences in *gerA* family operons are partly responsible for the differences in germination efficiency in response to Ala [58]. The greater diversity and complexity of GRs and germination responses among *B. cereus* strains and *B. cereus* group strains compared to for example *B. subtilis*, creates a challenge for future studies.

In conclusion, our comparative genotyping and phenotyping approach showed four *B. cereus* strains, B4085, B4086, B4116 and B4153, with poor germination responses, to cluster in phylogenetic group IIIA. These IIIA group strains contain either pseudogenes or variants of subunit C in their GerL cluster combined with pseudogenes in the GerK cluster and sub-cluster $GerR_1$ (B4086, B4153), or combined with the presence of a $SpoVA^{2mob}$ transposon (B4085, B4116), that induced heat resistance with concomitant reduced germination response in *B. subtilis* spores [16, 17]. The approach used has provided information on the genetic diversity in GRs and corresponding sub-clusters encoded by *B. cereus* strains as well as their germination behaviour and possible associations with GRs, and provides a basis for further extension of the knowledge on the role of GRs in *B. cereus* (group member) ecology and transmission to the host.

Supporting Information



1. Sample preparation (germination and staining)

2. Loading samples in FACS
3. Recording 10.000 events per sample

4. Visual inspection of FITC-A vs SSC-A scatters

5. Transforming scatter plots into FITC-A signal intensity histograms

6. Overlapping samples FITC-A histograms

7. Overlapping FITC-A histograms of all samples

8. Gating SYTO-9 unstained and stained populations

9. Extracting event number per gate
10. Calculating % of germination

Figure S1. Schematic representation of data analysis from germination assay.

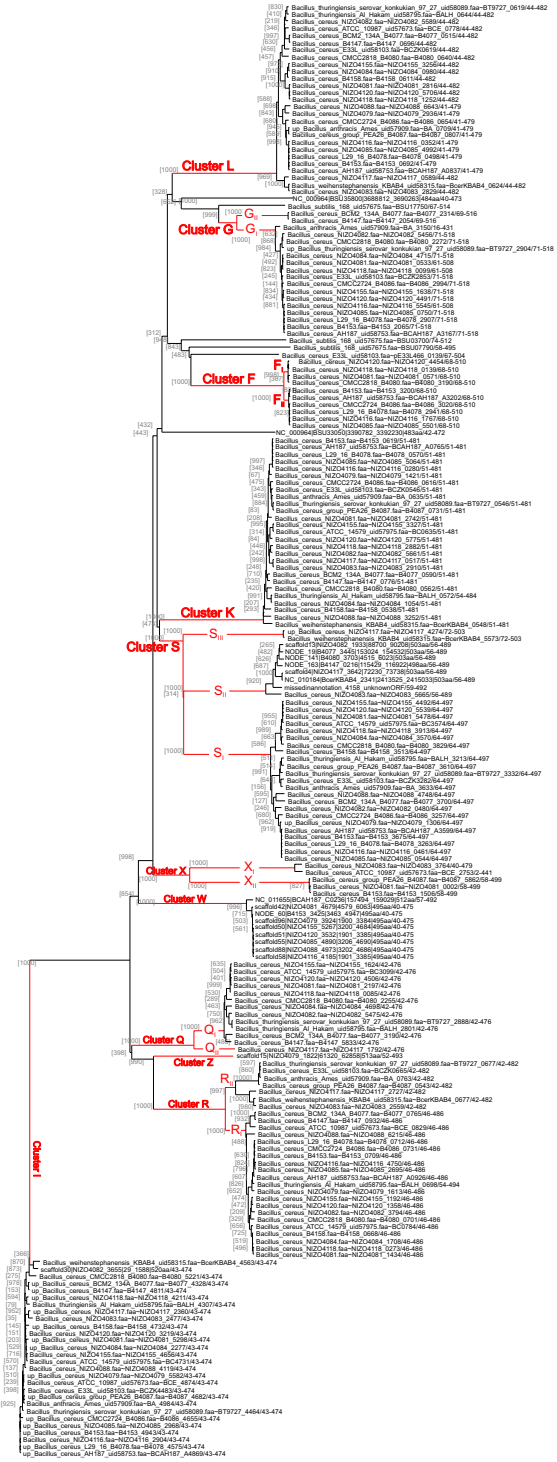


Figure S2. Neighbour-joining clustering of the A subunit protein of the GR.

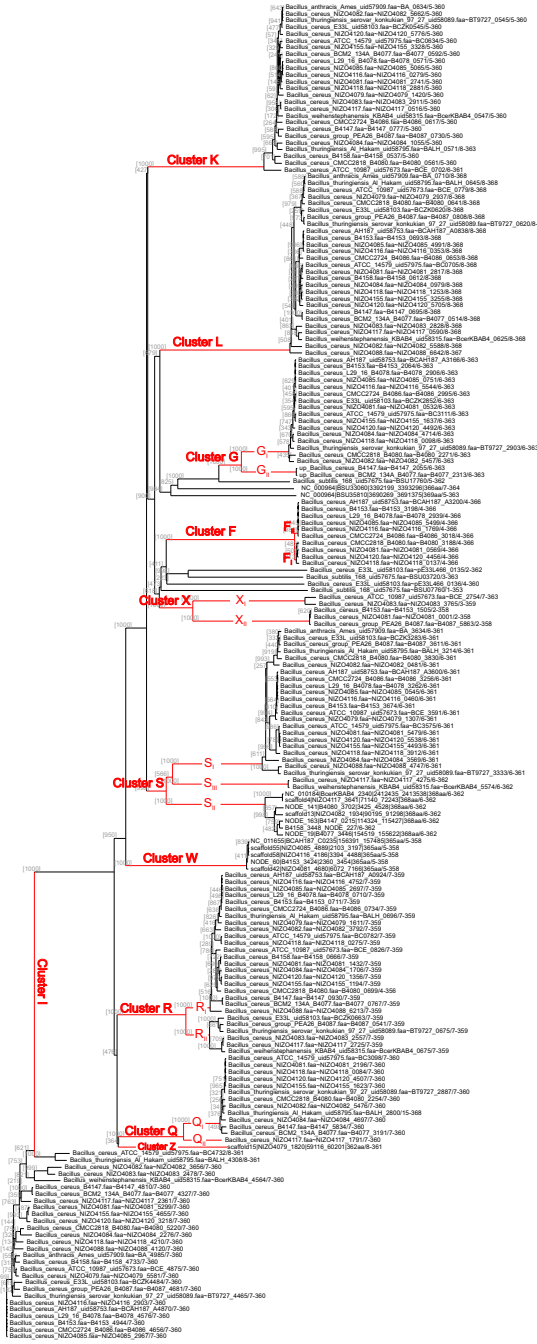


Figure S3. Neighbour-joining clustering of the B subunit protein of the GR.

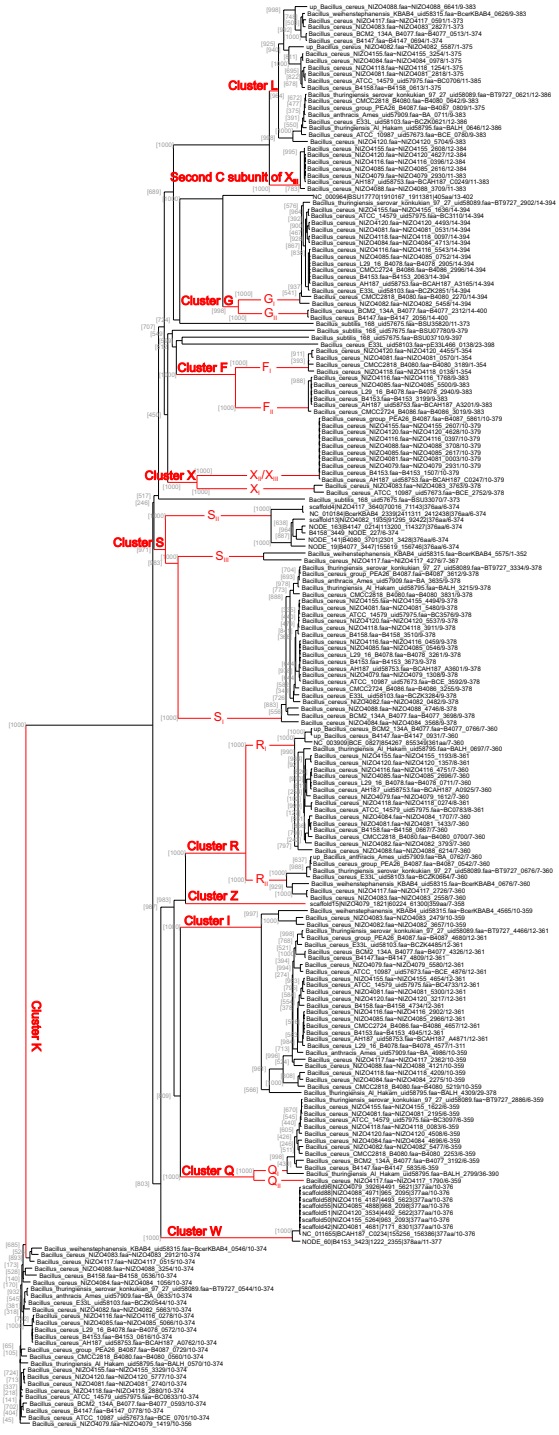


Figure S4. Neighbour-joining clustering of the C subunit protein of the GR.

Table S1. Free amino acid composition of BHI and TSB used in this study. The composition of reach medium varies according to its production lot/batch.

Free amino acid	Average BHI [mmol/l]	SD BHI	Average TSB [mmol/l]	SD TSB
Alanine	**5.80**	**0.11**	**1.03**	**0.00**
Arginine	2.85	0.06	3.07	0.00
Asparagine	1.54	0.02	0.38	0.00
Aspartic Acid	2.32	0.04	0.44	0.00
Glutamic Acid	5.37	0.09	0.86	0.01
Glycine	**2.13**	**0.04**	**0.49**	**0.01**
Histidine	0.72	0.02	0.37	0.01
Isoleucine	**2.72**	**0.04**	**0.96**	**0.01**
Leucine	8.07	0.14	5.36	0.01
Lysine	5.40	0.10	5.33	0.00
Methionine	1.25	0.02	0.83	0.02
Phenylalanine	3.63	0.06	2.53	0.00
Serine	2.73	0.08	0.80	0.00
Threonine	2.30	0.04	0.67	0.01
Tyrosine	0.98	0.01	0.86	0.01
Valine	**3.89**	**0.08**	**1.39**	**0.01**
γ-Aminobutyric Acid	0.35	0.00	<0.13	
Citrulline	0.15	0.00	N.D.	
Proline	1.24	0.03	N.D.	
Ornithine	0.49	0.01	N.D.	
Cysteine	**N.D.**		**N.D.**	
Cystine	N.D.		N.D.	
Glutamine	**N.D.**		**N.D.**	

ND Not Detected

Table S2. Germination % of non-heat activated and heat activated *B. cereus* spores of 15 industrial isolates and two lab strains exposed to either single amino acids, their mixtures or complex media. Colour coding indicate spores with <15% germination (red), 15 – 50% germination (orange), and >50% germination (green).

Phylogenetic group	Strain	Non-heat activated											Heat activated (10 min at 80°C)										
		AlaIno	BHI	TSB	Ino	Ala	Cys	Glu	Gly	Val	Iso	Av. Blank	AlaIno	BHI	TSB	Ino	Ala	Cys	Glu	Gly	Val	Iso	Av. Blank
II	B4088	96.9	47.2	81.3	22.1	96.7	83.3	23.6	46.2	21.1	18.5	18.2	98.6	85.5	97.7	76.6	95.6	95.1	14.0	51.8	22.8	19.2	17.1
	B4082	86.6	43.5	2.1	0.6	1.0	0.4	0.4	0.3	0.5	0.3	1.1	97.5	99.2	97.9	96.0	81.0	56.8	72.4	1.7	6.9	3.0	2.5
	B4147	97.8	11.7	1.6	31.8	6.6	2.3	1.9	2.1	2.1	1.8	2.6	95.2	97.8	27.6	88.6	90.1	42.5	2.6	1.1	56.5	43.0	1.1
III A	B4085	9.5	0.5	2.1	0.7	1.0	1.8	1.0	2.6	1.2	1.0	1.2	81.8	13.9	4.1	33.3	1.2	3.8	2.2	3.4	2.2	2.0	1.5
	B4116	2.3	1.3	4.3	1.4	1.6	4.4	4.2	3.2	3.5	2.8	3.2	71.2	5.0	8.3	37.3	3.5	11.2	9.5	12.1	12.0	8.5	7.9
	B4153	3.5	1.4	1.8	1.8	1.7	1.8	2.0	1.6	1.8	1.8	2.3	22.2	3.8	4.4	13.2	4.8	4.4	3.4	3.7	4.4	4.1	6.9
	B4086	6.2	4.8	8.4	5.1	5.1	5.5	8.9	5.0	4.8	4.7	5.4	83.7	50.9	46.5	43.7	9.0	13.7	37.4	10.7	11.5	11.1	11.5
	B4078	97.8	96.4	72.5	9.1	95.8	72.5	20.9	61.4	9.2	9.9	10.6	98.8	99.8	98.9	99.3	99.5	83.6	32.5	59.6	49.0	34.4	14.7
	ATCC 10987	47.8	24.7	21.8	11.5	20.1	20.3	14.9	16.1	15.9	17.3	13.3	96.5	95.6	98.4	96.9	97.6	98.5	95.7	77.7	97.1	98.4	36.8
III B	B4087	11.2	7.7	15.2	10.2	13.7	34.6	31.5	32.8	27.5	23.7	17.6	95.3	89.7	82.3	85.9	51.7	62.6	61.8	63.3	64.3	70.2	54.3
IV	B4084	96.5	84.3	39.4	42.6	19.5	2.2	4.6	1.9	3.0	2.1	3.9	97.4	99.6	98.9	97.3	94.0	60.5	41.2	3.0	7.4	2.9	4.8
	B4080	4.7	18.6	5.1	0.4	17.6	1.1	0.8	0.3	0.4	0.4	0.6	98.4	98.7	98.4	14.9	74.7	25.6	13.8	2.9	4.0	2.7	1.4
	B4118	78.8	1.0	5.3	2.0	1.9	2.0	1.8	2.7	2.0	2.8	3.1	92.4	92.0	96.6	55.5	40.6	15.6	5.6	0.9	5.1	2.8	2.4
	B4155	96.6	17.0	3.7	5.1	49.4	18.7	4.8	4.0	4.9	4.5	4.3	98.3	98.7	75.5	92.9	95.4	90.0	15.2	50.3	6.3	4.9	3.5
	ATCC 14579	85.9	20.9	34.0	11.0	12.0	29.9	32.6	44.7	41.3	30.9	17.6	93.0	95.9	98.6	92.6	93.2	94.6	84.3	58.5	90.4	84.1	56.5
	B4158	77.6	58.5	25.5	6.9	40.4	10.8	6.3	6.8	6.6	5.1	7.1	78.1	80.8	80.1	72.7	78.7	0.0	0.1	0.1	0.1	0.0	6.7
VI	B4117	98.0	95.6	99.0	94.7	10.7	6.6	8.2	5.7	8.1	7.6	8.1	97.3	95.2	97.4	96.6	87.1	18.1	13.6	16.3	16.1	18.1	16.2

Table S3. Assignment of GRs of experimentally tested strains to the clusters identified based on phylogenetic trees of subunits A, B, and C. Colour coding: green, presence of GR belonging to given cluster; white, absence of GR belonging to given cluster; and grey, at least one of the subunits is a pseudogene (P) or variant (V).

Phylogenetic group	Strain NIZO / ATCC code	Cluster K	Cluster I	Cluster L	Cluster R		Cluster S			Cluster G		Cluster Q		Cluster X			Cluster F		Cluster W	Cluster Z	NON CLUSTERING
					R_I	R_{II}	S_I	S_{II}	S_{III}	G_I	G_{II}	Q_I	Q_{II}	X_I	X_{II}	$X_{III\ (CCA)}$	F_I	F_{II}			
II	B4088	B - P														NtgerA			B - P		
	B4082																				
	B4147																				
III A	B4085			C-V/P												NtgerA					
	B4116			C-V/P												NtgerA					
	B4153	B - P		C-V/P	C - P																
	B4086	C - P		C - P	C - P																
	B4078			B - P C-V/P																	
	ATCC10987	A - P					A - P														
III B	B4087																				
IV	B4084																				
	B4080																				
	B4118																				
	B4155															NtgerA			B - P		
	ATCC14579			A - P						A - P											
	B4158						B - P														
VI	B4117																				

NtgerA: N-terminal truncated gerA

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Chapter 3

Influence of food matrix on outgrowth heterogeneity of heat damaged *Bacillus cereus* spores

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Abstract

Spoilage of heat treated foods can be caused by the presence of surviving spore formers. It is virtually impossible to prevent contamination at the primary production level as spores are ubiquitous present in the environment and can contaminate raw products. As a result, spore inactivation treatments are widely used by food producing industries to reduce the microbial spore loads. However, consumers prefer mildly processed products that have less impact on its quality and this trend steers industry towards milder preservation treatments. Such treatments may result in damaged instead of inactivated spores, and these spores may germinate, repair, and grow out, possibly leading to quality and safety issues. The ability to repair and grow out is influenced by the properties of the food matrix. In the current communication we studied the outgrowth from heat damaged *Bacillus cereus* ATCC 14579 spores on Anopore membrane, which allowed to follow outgrowth heterogeneity of individual spores on broccoli and rice-based media as well as standard and mildly acidified (pH 5.5) meat-based BHI. Rice, broccoli and BHI pH 5.5 media resulted in delayed outgrowth from untreated spores, and increased heterogeneity compared to BHI pH 7.4, with the most pronounced effect in rice media. Exposure to wet heat for 1 min at 95°C caused 2 log inactivation and approximately 95% of the spores in the surviving fraction were damaged resulting in substantial delay in outgrowth based on the time required to reach a maximum microcolony size of 256 cells. The delay was most pronounced for heat treated spores on broccoli medium followed by spores on rice media (both untreated and treated). Interestingly, the increase in outgrowth heterogeneity of heat treated spores on BHI pH 7.4 was more pronounced than on rice, broccoli and BHI pH 5.5 conceivably reflecting that conditions in BHI pH 7.4 better support spore damage repair. This study compares effects of three main factors, namely heat treatment, pH of BHI and effect of food matrix highlighting the impact of different (model) food recovery media on outgrowth efficiency and heterogeneity of non-heat treated and heat damaged *B. cereus* spores.

Introduction

Bacterial spores are widely present in the environment and are often identified as a source of contamination in the food industry. Highly resistant dormant spores that survived processing treatments can be present in final products and may germinate and grow out leading to food-borne illness or (premature) spoilage. The resistance of spores is lost when germination is initiated under nutrient rich conditions or when spores are exposed to specific physical or chemical triggers [1, 2]. However, spores may remain dormant for years until the initiation of germination, a phenomenon that makes the eradication and control of spores difficult for food producing industries.

The control of spores is further complicated by the tendency to use less intense preservation and processing strategies such as the use of milder heat treatments in combination with secondary mild preservation hurdles. However, reduction of the heat treatment intensity may lead to a subpopulation of spores that are sublethally damaged rather than inactivated. Such spores may eventually grow out after repair of the damage. This phenomenon conceivably contributes to increased heterogeneity in the population resulting in less accurate prediction of spore outgrowth behaviour. Knowledge on the behaviour of individual spores and its variability could assist in more accurate prediction of spore behaviour for shelf life prediction and refinement of risk assessments.

Spore germination is a relatively fast process that can be triggered by the presence of nutrients including sugars, single amino acids or combinations thereof [1-5]. Non-nutrient germination triggers have been described including chemical components such as pyridine-2,6-dicarboxylic acid chelated with calcium and the cationic surfactant dodecylamine. In addition, lytic enzymes such as lysozyme and high hydrostatic pressure treatments may trigger germination [6].

The sequential events in germination and outgrowth can be highly heterogeneous, as the precise fine tuning of the process is related to several intrinsic spore characteristics that may differ between individual spores depending on the sporulation conditions [7]. Individual spores may vary in sensitivity to heat and other processing treatments [8] and in superdormancy [9]. Also the food matrix composition may have considerable impact on the germination and outgrowth efficiency of spores. Commonly applied preservation strategies used by food industry to control spores (and vegetative cells) include the reduction of the water activity by using elevated concentrations of salt or sugar, or lowering of pH with organic acids [10], which might affect heat resistance [11-13]. In addition, stress conditions encountered during spore dormancy such as heat treatment, UV and disinfectant treatment can cause sublethal damage to spores that may increase variability in spore behaviour, especially in combination with non-optimal outgrowth conditions such as low pH, presence of salt or other inhibitory compounds that originate from food. As a result, spore germination and outgrowth can be significantly affected.

The spore former *Bacillus cereus* has been associated with food spoilage [14] and food-borne disease [15]. Depending on the type of toxin produced, *B. cereus* can cause two distinct syndromes. The emetic syndrome is caused by ingestion of the preformed heat stable toxin cereulide whereas the diarrheic syndrome is caused by enterotoxins secreted by vegetative cells present in the small intestine. Symptoms are usually mild and self-limiting, but in rare instances they can lead to life-threatening situations [15-19]. Effective heat preservation strategies are required to prevent *B. cereus* proliferation. A number of studies focussed on germination and/or outgrowth heterogeneity after heat treatment but the conditions used were relatively mild that either lead to heat activation or to marginal inactivation such as 10 min at 90°C (*B. cereus*), 10 min at 95°C (*B. subtilis*) or 20 s at 80°C (*Clostridium botulinum*) and laboratory media of optimal compositions were used to monitor outgrowth [20-22]. In this study, we focus on conditions that result in a 1.5-2 log inactivation and a severe heat damage in the surviving spores. Outgrowth capacity and heterogeneity of individual non-heated and heat damaged *B. cereus* ATCC 14579 spores was subsequently assessed using the Anopore approach described previously [22-24] with meat-based BHI, and food-matrices based on rice and broccoli, to mimic conditions that may be encountered in food processing.

Materials and Methods

Strain and sporulation conditions

B. cereus ATCC 14579 obtained from the American Type Culture Collection (ATCC) was cultured in Brain Heart Infusion broth (BHI; Beckton Dickinson, Le Point de Claix, France) at 30°C with aeration at 200 rpm. Spores were prepared in a nutrient-rich, chemically defined sporulation medium designated MSM medium that was described previously [25]. One ml of an overnight-grown pre-culture was used to inoculate 100 ml of sporulation media in 500 ml flasks and incubated at 30°C with aeration at 200 rpm. Sporulation was monitored during 2-3 days by phase contrast microscopy until release of over 99% of spores from the mother cell. Spores were harvested by 15 min centrifugation at 5,000 rpm at 4°C (5804R, Eppendorf, Germany) and washed with chilled phosphate buffer (100 mM, pH 7.4) containing 0.1% Tween80 to prevent spore clumping. Spores were washed twice a day over a period of 2 weeks with phosphate buffer with gradually decreasing Tween80 concentration until a final concentration of 0.01% (further referred as suspension buffer). Spores cleared from vegetative cells and debris were stored at 4°C and used for a maximum of six months.

Heat treatment

Hundred twenty µl of a spore suspension containing approximately 10^8 spores/ml in suspension buffer were transferred into capillary tubes (Micropipettes 200 µl max,

Blaubrand intraMARK, Germany) and heat-sealed at both ends. The capillary tubes were placed in an oil bath (Julabo MC-12, Germany) set at 95˚C for 1 min, followed by immediate cooling in ice-cold water. The heat treated spore suspension was recovered from the capillary tubes and decimally diluted in suspension buffer. Fifty µl of appropriate dilutions were plated in duplicate on BHI pH 7.4 and BHI pH 7.4 supplemented with 5.5% salt as previously used to estimate the degree of damage by Cazemier et al. [26]. Plates were incubated at 30˚C and colonies were counted after 24, 48 h and a week (no increase in colony counts after a week). For the surviving population, the percentage of damaged spores was calculated by the following formula:

$$\%\,Damaged\ spores = \frac{(\text{Number of cfu's BHI}) - (\text{Number of cfu's BHI5.5\% NaCl})}{(\text{Number of cfu's BHI})} * 100$$

Food based media used in this study

Rice based media were prepared by boiling ready-to-cook pouches filled with 125 g rice produced by the manufacturer (Lassie B.V, The Netherlands) in demineralised water (5:32 w/v) for 45 min. The rice bags were removed and 1.5% (w/v) Bacteriological Agar was added and boiled twice to dissolve the agar before pouring into petri dishes. The final pH of the prepared rice medium was 7.2. Broccoli based media were prepared by mixing sterile 5% (w/v) agar solution with Olvarit broccoli baby food (4 months baby food, (60% broccoli, 17% rice, 17% water, 6% apple juice)) (Nutricia, The Netherlands) puree to reach a final concentration of 1.5% (w/v) agar. The final pH of the prepared broccoli medium was 5.8. BHI acidified with HCl to pH 5.5 was selected to simulate mild acid stress. The water activity of the three media ranged from 0.993 - 0.995 and was comparable to the water activity of BHI pH 7.4, while addition of 5.5% salt to BHI pH 7.4 resulted in a drop to 0.958. All media were prepared one day before the experiments.

Anopore

Anopore strips (8 by 36 mm by 60 µm, 0.2-µm-diameter pore size, pore density of up to 50%; Whatman, the Netherlands) were prepared as described previously [23, 24] and were placed on agar based media. Both heat treated and untreated spores were diluted in suspension buffer, and were spotted on the Anopore strips and incubated at 30˚C. At regular sampling times (30 min after spotting, followed by 30 or 60 min intervals after outgrowth), individual Anopore strips were transferred onto an agarose pad consisting of a microscope slide covered with a 1-mm-thick film of 1% (w/v) solidified low-melting-point agarose (Invitrogen, The Netherlands) dissolved in demineralized water containing 1 µM of the fluorescent reporter dye SYTO-9 (Invitrogen, The Netherlands). Following 10 min staining in the dark, the Anopore strip was transferred for 10 min to an agarose pad without SYTO-9 dye, to reduce background signals and placed under a fluorescence

microscope as described previously [22] and routinely for each time point 100 to 1,000 events (spores or microcolonies) were imaged. For comparative purposes time points 30 min, 4.5, 5.5 and 6.5 h for untreated spores are presented, with an additional time point of 3.5 h for BHI pH 7.4 presented in Figure A.2. For heat treated spores, the number of images was adjusted such that 100 microcolonies could be processed. Image analysis and quantification of heterogeneity was performed as previously described [22-24]. Briefly, the distribution of the microcolony area per imaging time point for each experimental condition was calculated in Microsoft Excel and the observed frequency distributions were presented in histograms. For the visualisation purposes the number of cells per microcolony was determined by dividing the microcolony area (excluding the intercellular area) by average size of a single cell. All areas that were smaller than that of one cell were grouped together into one bin (represented by the letter "S" in the figures), representing both germinated (phase dark), swollen and outgrowing spores. The impact of recovery media and/or heat treatment on heterogeneity was quantified by calculating variances for the time points approaching the maximal microcolony size (256 cells per microcolony) and was based on $\log_2$ values of area of each microcolony. Additionally, a non-parametric Levene's test was used to verify the equality of variances in the samples ($p>0.05$) [27, 28].

Vegetative cells on Anopore

To evaluate the impact of the food based media on the microcolony formation starting from vegetative cells, 30 µl of an overnight-grown pre-culture was used to inoculate 100 ml Erlenmeyer flasks containing 20 ml of fresh BHI pH 7.4 broth and were incubated in a shaking water bath at 30°C for approximately 3 h, until reaching an optical density (measured at 600 nm) of approximately 0.5 ($OD_{600} \sim 0.5$; Novaspek II, Pharmacia Biotek, United Kingdom), which corresponds to cells in mid-exponential growth phase. At this point the culture was diluted in peptone physiological salt solution (PPS, 1 g/l neutralized bacteriological peptone [Oxoid, England] and 8.5 g/l NaCl). Appropriately diluted samples were spotted onto Anopore strips, and the development of microcolonies was monitored as described above.

Results

Effect of food media on spore outgrowth

Four different media representing a meat based (BHI pH 7.4), vegetable based (broccoli) and rice based matrix were selected to investigate the influence of matrix composition on spore outgrowth behaviour. To verify whether these media supported growth from *B. cereus* spores, non-heated *B. cereus* ATCC 14579 spores were plated in an initial screening

on either BHI pH 7.4, BHI pH 7.4 supplemented with 5.5% NaCl, rice, broccoli and BHI pH 5.5 agar based media to allow germination and outgrowth. All media supported germination and outgrowth from untreated *B. cereus* spores leading to final colony counts that were rather comparable to those on BHI pH 7.4 media (Figure 3.1). To evaluate the effect of different (food based) media on outgrowth from unstressed spores in more detail, diluted spore suspensions were inoculated on Anopore strips placed on the four selected media to allow germination and outgrowth. The formation of microcolonies for each condition was visualised by SYTO-9 staining and capturing of fluorescent pictures (Figure 3.2A). On BHI pH 7.4 medium, outgrowing spores formed characteristic spherical-shaped single layered microcolonies, while food based media led to the development of different morphological structures (Figure 3.2A). On rice medium, cells were elongated and formed non-spherical microcolonies resembling structured spaghetti threads. Such structures are typical for stressed cells and were reported previously for *B. cereus* ATCC 14579 vegetative cells grown in the presence of high salt concentrations [24], however, the colony morphology on rice could not be attributed to osmotic stress since the water activity was not affected. Similar observations were made for mid-exponential (Figure A.1.) and stationary phase cells (data not shown) cultured on Anopore strips and this suggests that the observed microcolony morphology on rice media is related to vegetative cell growth and not specific for spore outgrowth.

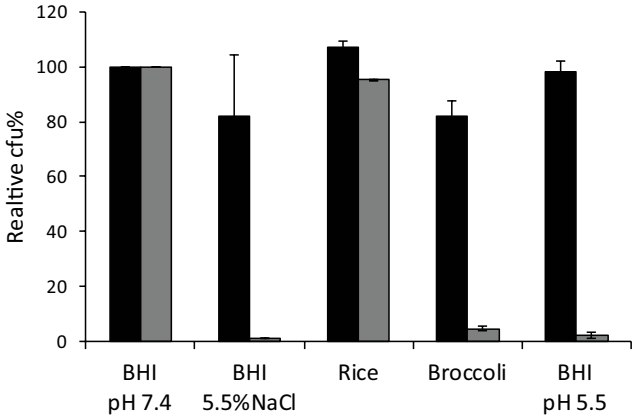

Figure 3.1. The relative impact of (food based) media on colony formation of non-heat treated (black bars) and heat treated (grey bars) *B. cereus* ATCC 14579 spores. Spores heat treated (one min at 95°C) or non-heated were plated on BHI pH 7.4, BHI pH 7.4 supplemented with 5.5% salt, BHI pH 5.5 or broccoli-based and rice-based media. The number of colonies formed by untreated or heat treated spores on BHI pH 7.4 was set as 100%, and all the other percentages for untreated or heat treated spores respectively are relative to these percentages (100% of heat treated spores represents counts on BHI pH 7.4 meaning a 2 log reduction from the 100% unheated spores). Results represent the averages of at least two repetitions.

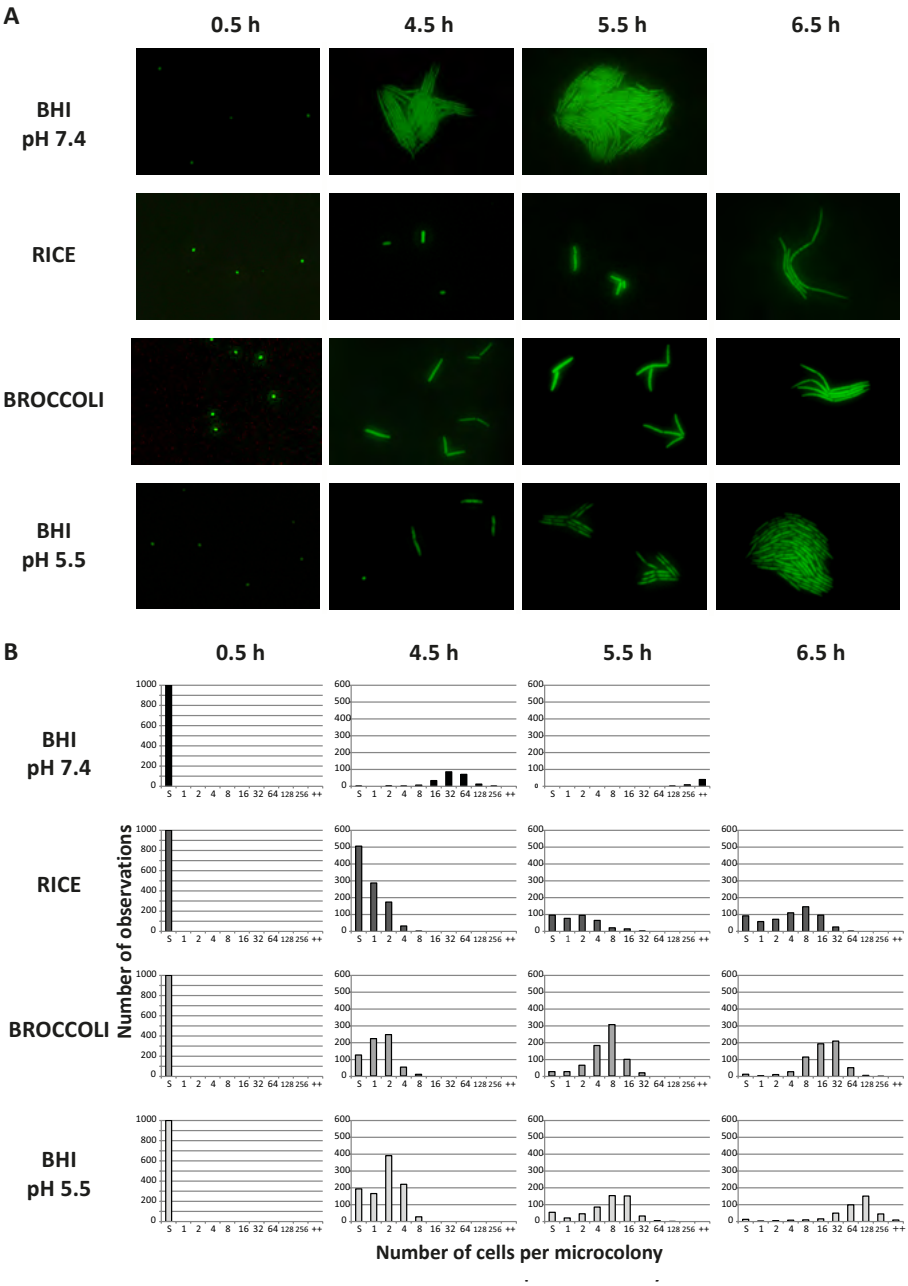

Figure 3.2. The effect of (food based) media on morphology and microcolony formation (A) and frequency distributions of microcolony size (B) on *B. cereus* ATCC 14579 spores. Dormant spores were placed on Anopore strips set on BHI pH 7.4 (black bars), rice (dark grey bars), broccoli (grey bars) or BHI pH 5.5 (light grey bars) plates. Samples were visualised after 30 min, 4.5, 5.5 and 6.5 h. The numbers of each bin on the x-axis represent number of cells per microcolony, "S" indicates germinated (phase dark) spores stained with SYTO-9 as well as outgrowing spores until reaching size of one vegetative cell. Distributions represent actual number of visualised individual spores/microcolonies per condition.

Figure 3.2B represents the microcolony size distribution of untreated *B. cereus* ATCC 14579 spores in time for the different matrices. Within 30 min, the untreated spores germinated which coincides with fluorescent staining of the phase dark spores (represented by the "S" bin in the Figure). The microcolony formation on BHI pH 7.4 displayed a homogenous pattern resulting in outgrowth from all germinated (stained) spores and reaching a maximal microcolony size (256 cells per microcolony) within 5.5 h (Figure 3.2B). The rice and broccoli media readily supported germination but the initial formation of microcolonies (time to reach the first bin) appeared to be delayed for 2 h (broccoli) and 2.5 h (rice) compared to BHI pH 7.4 (data not shown). Additionally, untreated spores on BHI pH 7.4 reached the maximal microcolony size fastest followed by BHI pH 5.5, broccoli and rice. Moreover, on food based media the spore outgrowth displayed a more heterogeneous microcolony size distribution, in particular on rice media as indicated by an about 5 times increase of variance compared to BHI pH 7.4 (Table 3.1). On BHI pH 5.5, germination and outgrowth from spores was delayed leading to a smaller though significant increase in heterogeneity compared to BHI pH 7.4. Reaching maximal microcolony size was slightly delayed for untreated spores on the broccoli media (of comparable pH) compared to BHI pH 5.5, while it was most retarded for untreated spores on rice media. Remarkably, even after 6.5 h incubation, a fraction of the untreated spores on rice, broccoli and BHI pH 5.5 remained in the germinated phase and did not grow out to the vegetative state.

Table 3.1. Variances of frequency distributions approaching maximum microcolony size of 256 cells for non-heated and heat treated *B. cereus* ATCC 14579 spores recovering on BHI pH 7.4, rice, broccoli and BHI pH5.5 based on log2 values of area of each microcolony. Variances are given for different time points at which fastest growing microcolony approached 256 cells.

	Variance non-heat treated	Variance heat treated
BHI pH 7.4	1.29	3.75
Rice	6.70	7.26
Broccoli	2.19	3.02
BHI pH 5.5	4.74	3.72

Effect of heat treatment on microcolony formation (on food media)

Wet heat treatment of dormant *B. cereus* ATCC 14579 spores for 1 min at 95°C led to 2 (for BHI pH 7.4 and rice media) and 3 log (for BHI pH 5.5 and broccoli media) reduction in viable counts (Figure 3.1). Such reduction in recovery compared to untreated spores, indicates that only a small fraction of the spore population could germinate and grow out. The surviving population of this treatment consisted of approximately 95% damaged spores. However, we noticed that the majority of heat treated spores were stained by SYTO-9. The SYTO-9 is known to stain germinated spores only since it cannot penetrate dormant spores [29]. Our data suggest that the heat treatment permeabilized the spores

and allowed penetration of SYTO-9. The inactivated spores are not able to germinate and grow out, and thus, do not contribute to heterogeneity in outgrowth. In practice, the small spore population surviving a (heat) treatment may grow out and this spores are therefore of relevance for product shelf life and safety. Emphasis was therefore in this study on microcolony formation and development of the small surviving fraction on different media (Figure 3.3).

As observed for the untreated spores, formation of microcolonies of heat treated spores was the fastest on BHI pH 7.4. To focus on the outgrowth from heat treated spores we compared the time to outgrowth (a time to reach average size of one cell). In this way we showed that outgrowth of heated spores varied in different media and was least supported by broccoli media, showing a strong delay in microcolony development (Figure 3.3), while BHI pH 5.5 and rice based media were in the middle range. Similarly, the time to reach the maximum microcolony size, a measure for cell division, varied depending on the type of recovery medium. Microcolonies on BHI pH 7.4 reached maximum microcolony size within 6 h, while on BHI pH 5.5 this took approximately 6.5 h, followed by rice media (7 - 7.5 h) and finally at least 8.5 h for broccoli media. After heat treatment, the outgrowth heterogeneity was increased for most media compared to non-heated spores, this was most pronounced for BHI pH 7.4 where heat treatment widened the distribution from 5 to 10 bins, resulting in an almost three times increase in variance compared to non-heated spores on BHI pH 7.4 (Table 3.1). Remarkably, increase in outgrowth heterogeneity of heat treated spores was not significant on rice, but it was significant although less pronounced on broccoli and BHI pH 5.5 compared to BHI pH 7.4, conceivably because those media already led to a more heterogeneous outgrowth from untreated spores, especially in case of BHI pH 5.5, where a negatively skewed distribution was observed.

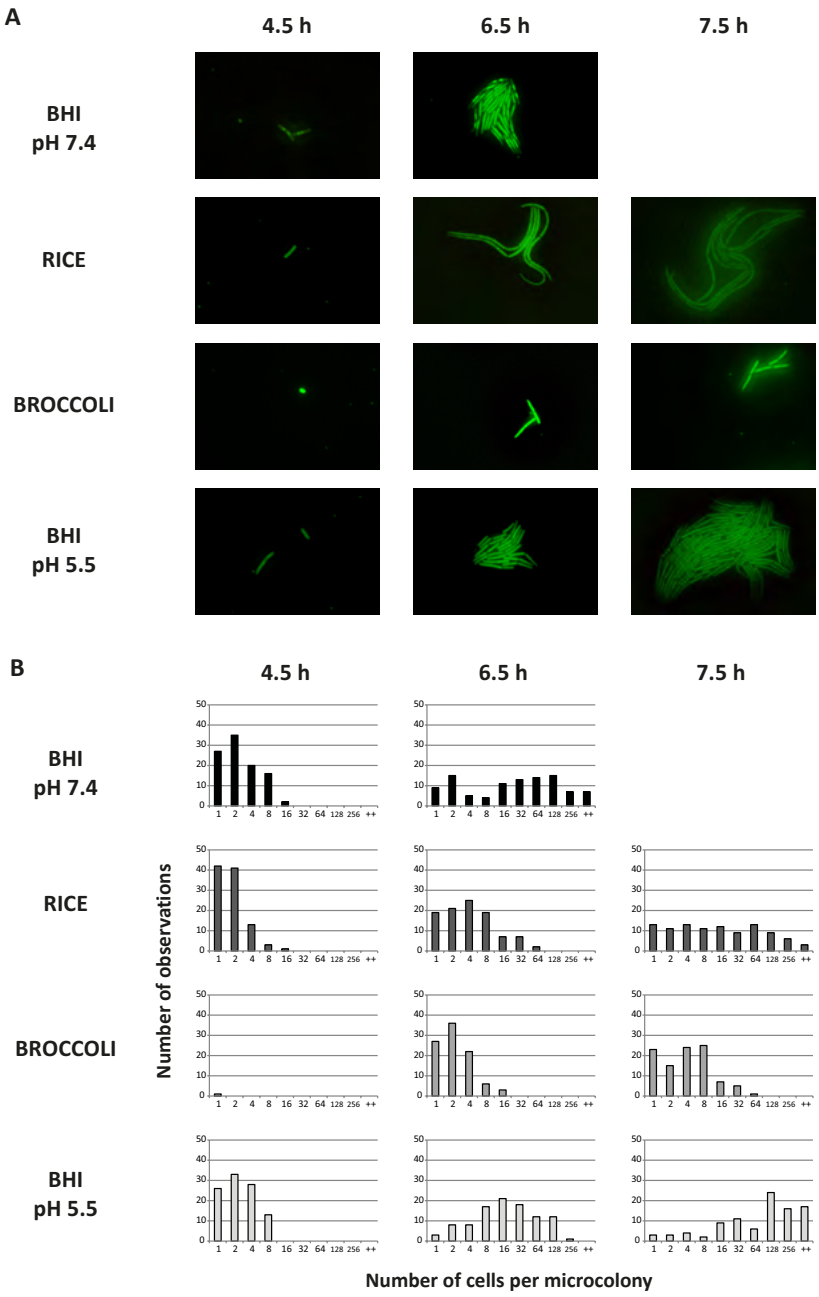

Figure 3.3. The effect of heat treatment and (food based) media on morphology and microcolony formation (A) and frequency distributions of microcolony size (B) on *B. cereus* ATCC 14579 spores. Spores heat treated for one min at 95°C were placed on Anopore strips positioned on BHI pH 7.4 (black bars), rice (dark grey bars), broccoli (grey bars) or BHI pH 5.5 (light grey bars) plates. Samples were visualised after 4.5, 6.5 and 7.5 h. The numbers of each bin on the x-axis represent the number of cells per microcolony. Distributions represent actual number of visualised individual microcolonies per condition.

Discussion

The impact of matrix composition on spore survival has been mostly studied using plate counting methods [30-34], however, these methods do not provide information on heterogeneity during the initial outgrowth phase of single spores to microcolonies. Outgrowth from spores requires conditions that support initiation of spore germination as well as supporting outgrowth to vegetative cells. Thus, varying levels of individual matrix compounds can affect both the process of germination and outgrowth and may result in a heterogenic response of the population. In the present study, we used an Anopore approach [22-24], which allowed to assess the influence of four different food based media on germination and outgrowth from untreated and heat treated *B. cereus* spores.

We showed that rice, broccoli and BHI pH 5.5 facilitated spore germination but also growth of vegetative cells of *B. cereus* ATCC 14579 and the number of spores that formed a colony on the food media was comparable to BHI pH 7.4. A limited number of studies investigated the impact of food matrix on spore outgrowth. It was previously shown that vegetable based media (broccoli, potato, and courgette) readily facilitate vegetative growth of a number of *B. cereus* strains which was not different from laboratory media when cultivated at optimal growth temperature [30]. However, other studies report an effect of media composition on spore outgrowth, for example *B. cereus* spores showed 1 log higher counts on nutrient agar (pH 5.2) compared to nutrient agar supplemented with carrot extract (pH 5.2) [11], while in courgette puree an extended lag phase and reduced growth compared to J-broth (laboratory medium) was observed [31]. These observations are in line with our finding that on broccoli media (pH 5.8) and rice media the time required for colony formation was extended compared to BHI pH 5.5 and BHI pH 7.4 respectively, indicating that vegetables may contain additional factors delaying germination and/or outgrowth or contain suboptimal concentrations of required components.

In a previous study, meat bouillon and rice water were shown to initiate germination albeit less efficiently compared to the addition of individual germinants alanine and/or inosine at mM level [35]. However, the germination efficiency cannot always predict outgrowth efficiency, as studies showed that the first spores to germinate are not necessarily the first ones to grow out [8, 36]. Although the food media supported germination and outgrowth, colony size and morphology of the microcolonies was different from those observed on BHI pH 7.4 media. Such morphological changes were previously observed for salt-stressed *B. cereus* ATCC 14579 microcolonies [24], and suggest stressful conditions conceivably linked to suboptimal nutrient composition and/or presence of inhibitory components. The outgrowth from untreated *B. cereus* spores showed a delay on rice and broccoli media compared to BHI pH 7.4, although the delay on broccoli medium was less pronounced compared to BHI pH 5.5. Besides the delay, also the heterogeneity in outgrowth from untreated *B. cereus* ATCC 14579 spores was increased when subjected to low pH (BHI pH

5.5) and/or broccoli and rice media, with the largest heterogeneity in the latter condition. Notably, previous studies on performance of individual *B. cereus* ATCC 14579 spores also showed increased outgrowth heterogeneity in BHI pH 5.5 compared to BHI pH 7 [22, 36].

The heterogeneity within a population may be increased by the presence of damaged spores resulting from severe heat treatment. Increasing intensity of wet heat treatment of *B. cereus* spores was previously shown to be proportional to the degree of damage as quantified by selective (PEMBA) and non-selective (NA) plating [37]. Moreover, the lag time of *B. cereus* spores was shown to be related to duration and intensity of heat treatment and/or decrease of pH during heating [38]. Damaged spores form a challenge for food producing industries as they can show delayed responses and therefore may be overlooked in commonly used (shelf life/challenge) testing methods. However, those spores are still capable of germination and outgrowth albeit delayed and more heterogeneously and therefore behaviour of damaged spores cannot easily be predicted. Under favouring conditions spores may undergo repair processes that conceivably take place between germination (and resumption of metabolic activity) and first doubling times [8, 39]. For this reason, the Anopore technique is highly suitable since it specifically allows analysis of initiation of outgrowth at single spore level, assessment of first doubling times and microcolony development up to 256 cells on different (food) media. The heterogeneity in outgrowth from *B. cereus* ATCC 14579 spores was considerably higher after a 1 min heat treatment at 95°C, in particular a wider distribution of the microcolony sizes was observed for BHI pH 7.4 and variance increased almost 3 fold. The impact of different food-like matrices on survival during the heating of spores was investigated previously for several media including beef, poultry, milk and cream [1, 12, 40-42], however these studies did not include recovery of individual spore or effect of recovery conditions.

Depending on the applied intensity, heat treatment can affect spores in multiple ways. Moderate heat conditions, typically between 65 and 75°C [1, 3, 4, 9] for *B. cereus* spores, activate its germination. More intense heating conditions may either inactivate or damage spores. Within a heat treated spore population, individual spores may be affected differently. A number of authors focused on germination and outgrowth heterogeneity of heat treated *C. botulinum* spores (treatment of 20 s at 80°C (1D inactivation)) leading to induced heterogeneity in time required to germinate, increased variability in time to outgrowth, and an increased lag time in laboratory media [21]. It has been shown previously that the heat treatment intensity (time and temperature combination) has a (proportional) impact on germination and outgrowth behaviour of spores [8, 43]. In these studies on heterogeneity in spore germination and outgrowth relatively mild heat treatments in the range of 70 to 90°C aiming for heat activation and/or reducing heterogeneous behaviour in the *B. subtilis* [44] and *C. botulinum* [8] spore population were applied. Mild heat treatment (10 min at 70°C) resulted in the acceleration of spore germination for *B. cereus* ATCC 14579 at pH 7 and 5.5 but did not affect outgrowth and its heterogeneity [36] but

at higher temperatures (10 min at 90°C), van Melis et al. [22] showed a delayed and more heterogeneous outgrowth from *B. cereus* ATCC 14579 spores at the lower pH. In both cases, the presence of secondary stresses (pH, sorbic acid, salt) resulted in outgrowth delay and/or heterogeneity within spore populations. The heterogeneity in lab media (van Melis et al. [22] and this study) as well as food media (this study), as expressed by the variance introduced by secondary stress or by heat treatment was of the same magnitude. Similarly Aguirre et al. [42] showed a correlation between heat intensity and estimated heterogeneity as well as lag phase in outgrowth from *B. cereus* ATCC 10876 spores in TSB and milk.

We showed that in more propitious recovery conditions, the heat treatment displayed a larger influence on *B. cereus* ATCC 14579 spore behaviour than the type of recovery media. For example, heat treatment caused delayed outgrowth on BHI pH 7.4, broccoli, and BHI pH 5.5. Interestingly, the effect of heat treatment on population heterogeneity was most pronounced on BHI pH 7.4 (optimal recovery conditions). Similar findings were reported for *C. botulinum* spores where heat treatment displayed a larger effect on delay and heterogeneity of the germination and outgrowth process in comparison to either sporulation conditions or outgrowth in the presence of salt, suggesting that the heat treatment has more impact on spore outgrowth than recovery conditions [8]. In the present study with *B. cereus* ATCC 14579 spores, both unheated and heated spore populations showed a highly heterogeneous behaviour on rice based media, with comparable variance, and no additional delay in formation of fastest developing microcolony due to heat treatment. Heat treatment does not affect heterogeneity on rice media possibly because this media evokes a heterogeneous outgrowth from untreated spores already, possibly due to nutrient limitation, presence of inhibitors. In conclusion, the impact of heat treatment on the heterogeneity and recovery is influenced by the tested media composition, but in media that are not optimal for homogeneous germination and outgrowth the influence of heat on heterogeneity appears less pronounced.

The data presented in this communication focussed on severe heat treatment leading to substantial spore inactivation, that may be encountered during food processing and show the relevance of recovery conditions in studies on outgrowth from heat treated spores. Moreover, the recovery of spores after exposure to other types of stresses, such as disinfectants, would be of interest for industry. Future studies will be extended including next to *B. cereus* ATCC 14579, also industrial isolates and other spore formers. The reported findings are of relevance to food producers as outgrowth from a limited number of sublethally damaged spores can eventually be responsible for spoilage or illness. Particularly in food industry where one surviving/damaged spore per package can cause spoilage, and substantial economic losses especially in the context of mass (bulk) production.

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Supporting Information

Supplementary Figures A.1 and A.2 can be found in the online version of the article.

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Chapter 4

Identification of CdnL, a putative transcriptional regulator involved in repair and outgrowth of heat damaged *Bacillus cereus* spores

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Abstract

Spores are widely present in the environment and are common contaminants in the food chain, creating a challenge for food industry. Nowadays, heat treatments conventionally applied in food processing may become milder to comply with consumer desire for products with higher sensory and nutritional values. Consequently, subpopulations of spores may emerge that are sublethally damaged rather than inactivated. Such spores may germinate, repair damage, and eventually grow out leading to uncontrolled spoilage and safety issues. To gain insight into both the behaviour of damaged *Bacillus cereus* spores, and the process of damage repair, we assessed the germination and outgrowth performance using OD_{595} measurements and microscopy combined with genome-wide transcription analysis of untreated and heat treated spores. The first two methods showed delayed germination and outgrowth of heat damaged *B. cereus* ATCC 14579 spores. A subset of genes uniquely expressed in heat treated spores was identified with putative roles in the outgrowth of damaged spores, including *cdnL* (BC4714) encoding the putative transcriptional regulator CdnL. Next, a *B. cereus* ATCC 14579 *cdnL* (BC4714) deletion mutant was constructed and assessment of outgrowth from heat treated spores under food relevant conditions showed increased damage compared to wild type spores. The approach used in this study allows for identification of candidate genes involved in spore damage repair. Further identification of cellular parameters and characterisation of the molecular processes contributing to spore damage repair may provide leads for better control of spore outgrowth in foods.

Introduction

Spore forming bacteria are commonly present in the environment and difficult to eradicate because they produce highly resistant spores that may remain dormant for years until germination. The high resistance towards a diverse range of stresses make spores an important target for food industry processes aimed to produce safe, ambient stable products. Dormant spores that can be present on raw material or ingredients and survive the heat processing treatments may eventually germinate and grow out, leading to food-borne illness upon consumption of those food products or result in product spoilage. The current practice of industry is to use intense heating regimes to minimize the risk of surviving spores but consumers prefer milder processes which have less effect on sensory and nutritional values of products.

A tendency to use milder heat treatments increases the risk of spores surviving the process and may lead to a subpopulation of spores that are sublethally damaged rather than inactivated. Sublethally damaged spores may still have the capacity to grow out if conditions allow for repair of the damage. Repair of spore damage is conceivably taking place between germination and outgrowth [1], however the processes involved in damage repair have not been studied extensively. A number of factors have been hypothesised to be involved in spore damage repair. Firstly, dormant spores may be equipped with transcripts resulting from late sporulation processes that on the one hand could support early repair of damage accumulated during dormancy, or alternatively, could serve as a reservoir of nucleotides in the germination process [1-5]. Secondly, spore damage repair may involve known repair systems for DNA damage such as AP endonucleases (Nfo and ExoA) or nucleotide excision repair enzymes (UvrA) described for *Bacillus subtilis* [1, 6-9]. Spore DNA damage may accumulate during dormancy and (sub)lethal processing treatments with subsequent outgrowth requiring the activation of DNA repair systems. Despite the possible impact of spore damage repair on subsequent spore outgrowth and associated food quality and safety issues, the frequency and underlying mechanisms of this phenomenon have gained limited attention up to now [10].

The events associated with spore germination appear to occur via a tightly controlled spore outgrowth program [1, 5]. Transcriptomic approaches have been performed to understand the processes and genes involved in the wake up of dormant spores and resumption of metabolic activity for the Bacillus genus [1, 5] and in Clostridia [2-4]. A common finding among those studies is that mRNA levels of the majority of genes on the chromosome increase rapidly during the initial germination processes showing a highly dynamic expression pattern. Transcriptome analyses of spore germination and outgrowth performed so far, predominantly involved the use of optimal conditions in nutrient-rich media at neutral pH values. Few studies, including van Melis et al. [5] for *B. cereus* spores, focus on gene expression during germination and outgrowth under less favourable conditions such as presence of the preservative sorbic acid in mildly acidic

conditions. Nevertheless, suboptimal conditions are typically encountered in practice in processed foods, for example when spores are damaged upon exposure to heat, and their fate is influenced by matrix composition, temperature and/or pH.

In this study, we focus on germination and outgrowth of heat damaged spores of *B. cereus*, a microorganism that has been associated with food spoilage [11] and food-borne disease [12]. *B. cereus* associated diseases are usually mild and self-limiting but in rare instances fatal outcomes have been reported [12-17]. The vegetative cells of *B. cereus* can cause disease either by the production of a heat-stable toxin (cereulide) in food before ingestion resulting in emetic syndrome or by secretion of enterotoxins in the small intestine, causing the diarrheic syndrome. We assessed the germination and outgrowth performance of untreated and heat damaged *B. cereus* spores using optical density measurements and microscopy analysis at selected time points. Transcriptome profiling was used to identify genes and putative molecular mechanisms involved in the repair and recovery of heat damaged spores. To validate this approach, one candidate gene with a potential role in recovery and repair of outgrowing damaged spores was selected for mutant construction and subsequent phenotype analysis. The resulting targeted deletion mutant, *ΔcdnL* (BC4714), showed a higher fraction of severely damaged spores compared to wild type. The work presents the feasibility of the applied approach for identification of novel cellular parameters involved in repair and recovery of heat damaged spores.

Materials and Methods

Strain and sporulation conditions

B. cereus ATCC 14579 was obtained from the American Type Culture Collection (ATCC) and routinely cultured in Bacto Brain Heart Infusion broth (BHI; standard BHI media contains 0.5% NaCl; Beckton Dickinson, Le Point de Claix, France) at 30°C with aeration at 200 rpm. Spores were prepared in a nutrient-rich, chemically defined sporulation medium (MSM medium) described previously [18]. The sporulation process and handling of resulting spores were performed as described previously [19], briefly one ml of an overnight-grown pre-culture was used to inoculate 100 ml of MSM media in 500 ml flasks and incubated at 30°C with aeration at 200 rpm. Sporulation was monitored over 2-3 days by phase contrast microscopy until over 99% of the spores were released from the mother cell. Spores were then harvested by centrifugation at 5,000 rpm at 4°C (5804R, Eppendorf, Germany) for 15 min and washed with chilled phosphate buffer (100 mM, pH 7.4) containing 0.1% Tween80 to prevent spore clumping. Spores were washed twice a day for 2 weeks with a phosphate buffer that was gradually decreased in Tween80

concentration until a final concentration of 0.01% (further referred as suspension buffer). Spore suspensions free of vegetative cells and debris were stored at 4°C and used within six months.

Heat treatment

A 120 µl aliquot of the spore suspension containing approximately $1X10^8$ spores/ml in suspension buffer was transferred into capillary tubes (Micropipettes 200µl max, Blaubrand intraMARK, Germany) and heat-sealed at both ends. The capillary tubes were placed either on ice or in a 95˚C oil bath (Julabo MC-12, Germany) for 1 min and immediately cooled in ice-cold water. The heat treated spore suspension was recovered from the capillary tubes and directly decimally diluted in suspension buffer. For several spore formers, including *B. cereus*, *B. subtilis*, *Bacillus stearothermophilus*, *Clostridium* spp addition of stressful substances such as sodium chloride has been used to assess spore damage, as sublethally injured spores appear to have an increased sensitivity to those substances [20-24]. 5.5% NaCl was added to BHI, resulting in 6% final concentration, which did not affect outgrowth of untreated spores as indicated by Cazemier et al. [23] and own data ([19], S4 Table). A dose dependent sensitivity of damaged spores towards salt can be used to differentiate between severely and mildly damaged spores. Based on preliminary experiments we selected supplementation with 1.5% NaCl as an intermediate cut off providing sufficient resolution to evaluate different degrees of damage for the heat treated spores. In short, 50 µl of serially diluted samples were plated in duplicate on BHI plates and BHI plates supplemented with 1.5% and 5.5% salt followed by incubation up to seven days at 30˚C. To evaluate possible delay in colony formation, colonies were counted after 1, 2 and 7 days (further extension did not affect colony counts). Obtained colony forming units (cfu's) were used to calculate the total damage as reported previously [19] (see formula below). Fractions of mildly and severely damaged spores were calculated using the following formulas:

$$\%\,Total\,damage = \frac{\left(\text{Number of cfu's BHI}\right) - \left(\text{Number of cfu's BHI5.5\% NaCl}\right)}{\left(\text{Number of cfu's BHI}\right)} * 100$$

$$\%\,Mild\,damage = \frac{\left(\text{Number of cfu's BHI1.5\% NaCl}\right) - \left(\text{Number of cfu's BHI5.5\% NaCl}\right)}{\left(\text{Number of cfu's BHI}\right)} * 100$$

$$\%\,Severe\,damage = \frac{\left(\text{Number of cfu's BHI}\right) - \left(\text{Number of cfu's BHI1.5\% NaCl}\right)}{\left(\text{Number of cfu's BHI}\right)} * 100$$

OD_{595} measurement and microscopy

To initiate spore germination and outgrowth, 180 µl of 1.1 times concentrated BHI was added to the wells of a 96 – well plate containing either 20 µl of untreated or heat treated spore suspension containing approximately $1X10^8$ spores/ml resulting in a final concentration of $1X10^7$ spores/ml. Plates were immediately transferred to a plate reader (Tecan Infinite F200 Pro, Austria) for incubation at 30˚C at approximately 200 rpm. OD_{595} was measured every 10 min and read outs were used to calculate the relative change in OD_{595}. Duplicate plates were incubated at 30˚C at approximately 200 rpm (IKA KS 250, Germany), allowing for microscopic observations and imaging at regular intervals.

Sampling for microarray and qPCR experiments

Samples for RNA isolation were gathered from germinating and outgrowing spores in BHI after 10 (t10), 20 (t20), 30 (t30) and 50 (t50) min for untreated spores and 50 (t50), 90 (t90), 120 (t120) and 150 (t150) min for heat treated spores. The time points were selected based on microscopic observations and the relative change in OD_{595}. Eight hundred µl of concentrated spore suspension containing approximately $1X10^{10}$ spores/ml was injected into either pre-heated (95˚C; oil bath) or ice cold glass tubes filled with 5 ml suspension buffer. After 1 min, the content of the tube was cooled by mixing with 40 ml of ice cold suspension buffer, followed by centrifugation (5804R, Eppendorf, Germany) for 5 min at 5,000 rpm at 4°C and resuspension in 8 ml of suspension buffer. Two ml of this spore solution was used to inoculate four flasks with 18 ml of 1.1x BHI reaching a final concentration of $1X10^8$ spores/ml. Flasks were then incubated at 30°C with aeration at 200 rpm. At each sampling point, the content of one flask was rapidly pelleted by centrifugation (5804R, Eppendorf, Germany) at maximum speed at 4°C for 30 s and the resulting pellet was resuspended in 1 ml TRI-reagent (Applied Biosystems, United Kingdom) and rapidly frozen in liquid nitrogen. The frozen samples were stored at −80°C until RNA extraction. Sampling was performed from two independent experiments. Samples were collected for microarray analysis from the first spore batch and to support the specific expression of genes in heat treated spores the samples for qPCR experiments were collected from a second spore batch that was prepared independently.

RNA isolation for microarray and qPCR experiments

RNA was extracted from the samples in TRI-reagent by defrosting on ice followed by mechanically disrupting the spores by exposing them to 6 rounds of 45 s of bead beating (FastPrep-24, MP Biomedicals, Germany) at maximal settings in the presence of Lysing Matrix B beads (MP Biomedicals, Germany). A direct-zol RNA MiniPrep kit (Zymo Research, United States) was used according to the manufacturer's instructions for on column RNA purification. Residual chromosomal DNA was removed from the samples by a 30 min off column treatment using the TURBO DNA-free Kit (Ambion, United Kingdom). The resulting

total RNA was subsequently cleaned with RNeasy Mini Kit (Qiagen, Germany). The RNA quantity and quality were checked by UV spectroscopy (Biophotometer, Eppendorf, Germany) and by analysis on a RNA 6000 Nano chip (Agilent, United States). Examples of RNA profiles are shown in S1 Figure. The RNA samples were stored in 70% ethanol with 83 mM sodium acetate buffer (pH5.2) at −80°C.

cDNA synthesis, labelling and microarray hybridization and design

Fluorescently-labelled cDNA was prepared from the extracted RNA following an indirect labelling approach with amino-allyl-labelled dUTP (Ambion, United Kingdom) and Superscript III (Invitrogen, The Netherlands) as described previously [25]. Two hundred ng of appropriately Cy3 and Cy5 -labelled cDNA was used for each sample hybridization. For each time point, independent biological duplicates were used in combination with a dye-swap approach. Array hybridisation followed a loop design (S2 Figure). Hybridization and removal of the unbound cDNA was performed as described previously [5]. The microarrays used in this study were custom-made *B. cereus* microarrays (8 × 15 K, Agilent, GEO accession number GPL9493, 3rd design) based on *B. cereus* ATCC 14579 genome sequence (NCBI accession number NC_0044722).

Microarray scanning and data analysis

The microarray slides were scanned using an Agilent microarray scanner (G2565BA), and the raw data were extracted using Agilent's Feature Extraction software (version 10.7.3.1). Microarrays were normalized using the approach reported for germinating spores involving the creation of so called synthetic microarrays [5]. Namely, the background-corrected, raw signals (Cy3 and Cy5 channel) of all arrays were hierarchically clustered (Pearson correlation, complete linkage) using Genemaths XT (version 1.6.1, Applied Maths, Belgium). New synthetic arrays (S1 Table) were defined from sample pairs showing the highest similarity in the clustering. In a next step, the synthetic microarrays were normalized (Lowess normalisation), based on normalised values three experimental samples were excluded (see S1 Table). Gene expression levels were calculated relative to that of untreated samples after 10 min (t10). This approach was chosen to exclude genes massively expressed as part of the germination programme (as observed by Melis et al. [5]) and focus on genes specifically expressed in heat treated spores in the phase after germination and before outgrowth. Genes were included for further analysis when the following criteria were met: log2 values should be higher than 1 or lower than -1, with a false discovery rate (FDR) smaller than 0.05 in at least one time point.

To select for candidate genes uniquely expressed in heat damaged spores (and potentially involved in heat damage repair) the following three criteria should be met: i. upregulation (log2 above 1; FDR < 0.05) at all-time points relative to t10 for heat treated spores, ii. downregulation (log2 below -1; FDR < 0.05) at all time points relative to t10 for untreated

spores, and iii. confirmation of expression of genes selected after step i and ii, using qPCR (see section 2.8).

Special attention was given to dormant spore transcripts, putative DNA damage repair genes, and novel genes involved in damage repair. Expression of spore specific mRNAs reported for *B. cereus* [5] was analysed for untreated and heat treated spores, and ratios over germinated untreated spores (t10) and heat treated spores (t50) were plotted. Furthermore, genes known to be involved in DNA damage repair in *B. subtilis* were extracted from *Subti*Wiki [26] and supplemented with *B. cereus* ATCC 14579 genes with predicted roles in DNA repair based on their annotation (S2 Table).

Microarray accession number

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [27] and are accessible through GEO Series accession number GSE73043 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73043).

qPCR

RNA was isolated from untreated (t10, t30) and heat treated (t120) germinating and outgrowing spores in BHI as described above. Primers (S3 Table) were designed targeting the 21 selected genes that were specifically expressed in outgrowth of heat damaged spores and five candidate genes for normalisation (BC0257, BC0544, BC1409, BC4471, and BC4743) were selected based on their constant expression levels across the different conditions used on the DNA microarrays. Due to the high number of target genes, two mixes (Mix A and Mix B) were used for first-strand cDNA synthesis using the SuperScript III reverse transcriptase (Invitrogen, United States). Two hundred ng of total RNA, 0.25 μM (final concentration) of each gene-specific reverse primer (Mix A 16 genes, Mix B 15 genes) and 0.5 mM (final concentration) of dNTP in a total volume of 14.5 μl were incubated for 5 min at 65°C and chilled on ice. After addition of 4 μl of First Strand Buffer, 1 μl of DTT (0.1 M) and 1 μl of SuperScript reverse transcriptase III (200 units/μl), the reaction was incubated for 1 h at 55°C and, finally, for 15 min at 70°C. The obtained cDNA was diluted 1:10 before use in real-time PCR.

qPCR was performed in a total volume of 25 μl using a C1000 Touch Thermal cycler (BioRad, CFX 96 Real-Time Systems). A final concentration of 0.2 μM of each primer, 12.5 μl of Power SYBR Green (Applied Biosystems, United Kingdom) and 5 μl of diluted cDNA was used as a template. The optimal annealing temperature was established at 61°C in a pre-test for primer efficiencies. The qPCR program included an initial 10 min polymerase activation step at 95°C followed by 40 cycles of denaturation (15 s at 95°C) and annealing/extension (1 min at 61°C). For each run the melting curve was checked to assure amplification of the correct target. The efficiency of the primer combinations were checked and showed good performance.

For the selection of suitable normalisation genes, two cDNA primer mixes were analysed separately. All genes in each mix were considered as potential normalisation genes and were evaluated according to the criteria defined by GeNorm [28]. Expression within Mix A was normalised with three (V3/4=0.142) highly stable (M<0.5) genes BC0854, BC1409 and BC4471. Expression within Mix B was normalised with five (V5/6=0.137) stable genes BC0257, BC0544, BC3391, BC3991, and BC4471. Normalised values were represented in relation to untreated t10 and fold change in expression was plotted. Candidate repair genes were selected based on upregulation in heat treated spores and downregulation in untreated spores, relative to t10.

Deletion mutant construction

A targeted mutant was constructed for a candidate gene involved in outgrowth of heat damaged spores designated Δ*cdnL* (BC4714), using the temperature-sensitive plasmid pMAD [29]. To this end, a chloramphenicol resistance cassette was amplified from plasmid pNZ124 using primers CM_fwd and CM_rev (S3 Table), and the resulting fragment was digested with restriction enzymes KpnI and XhoI. The 1.3 kb flanking regions of the BC4714 gene were amplified using primers BC4714_up_fwd and BC4714_up_rev for upstream (fragment A) and, BC4714_down_fwd and BC4714_down_rev for downstream flanking regions (fragment B) (S3 Table). The resulting fragments were digested with KpnI for fragment A and with XhoI for fragment B. Fragment A, fragment B and the digested chloramphenicol resistance cassette were ligated overnight at 16°C using T4 DNA ligase. The expected 3.6 kb amplicon encompassing fragment A, B and the cassette was obtained by PCR amplification (with primers BC4714_up_fwd and BC4714_down_rev) directly on the ligation mixture. The resulting purified PCR product and pMAD plasmid were digested with EcoRI and ligated together using T4 DNA ligase. The resulting vector (plasmid pBC002), was transferred into competent *E. coli* TOP10 (Invitrogen) cells and four erythromycin and chloramphenicol resistant clones were selected. The presence of the correct insert was checked by PCR using primer combinations BC4714_up_fwd/BC4714_down_rev, CM_rev/BC4714_down_rev and CM_fwd/BC4714_up_fwd. Plasmid pBC002 was purified using the Maxiprep Kit (Qiagen) and was transferred into electroporated (400Ω, 25 μF, 1.2 kV) *B. cereus* ATCC 14579 cells as described previously [30], followed by plating on Luria Bertani (LB) agar with 5 μg/ml chloramphenicol (Cm5) and 3 μg/ml erythromycin (Ery3). A single colony harbouring plasmid pBC002 was inoculated in LB broth without antibiotics and incubated at 39°C overnight. The culture was diluted 100 fold in fresh LB medium without antibiotics and propagated for 17 generations at 39°C. Appropriate dilutions were plated on LB with Cm5 and incubated overnight at 37°C to obtain single colonies. Resulting colonies were replica plated on LB with Cm5 or Ery3 and incubated at 37°C overnight. Candidates were selected based on chloramphenicol resistance and erythromycin sensitivity resulting from desired double cross over events. Five candidate colonies displaying the desired Cm-resistant and erythromycin-sensitive

phenotype were verified by PCR using primer combinations BC4714_mu_up/Cm_fwd and BC4714_mu_down combined with Cm_rev and resulted in expected fragment lengths of 2.7 and 2.6 kb, respectively. Correct disruption of the gene was further confirmed by DNA sequencing.

Phenotype of a *cdnL* mutant

Spores of the deletion mutant were prepared and heat treated as described in Materials and Methods. Additionally, wild type and deletion mutant spores were exposed for 5 min to oxidative stress using hydrogen peroxide (5%; Merck) or sodium hypochlorite (0.002% active chlorite; Sigma Aldrich). One hundred µl of concentrated disinfectant solution was added to 400 µl of spore suspension containing approximately $1.2X10^8$ spores/ml. After 5 min incubation, 100 µl was transferred to 900 µl of inactivation solution containing respectively catalase solution (500 U/ml; Sigma) or sodium thiosulphate (10 g/L; Merck). This 10 min inactivation step was followed by serial dilution in suspension buffer and plating. Survival and degree of damage of heat, hydrogen peroxide and sodium hypochlorite treated spores were evaluated as described above. Experiments were performed in triplicate at room temperature.

Results and discussion

Impact of heat treatment on germination and outgrowth

The impact of heat treatment on germination and outgrowth of *B. cereus* ATCC 14579 spores was assessed using spores in suspension buffer heated for one min at 95°C. This heat treatment resulted in 2 log inactivation with a significant fraction (>90%) of damaged spores among the survivors (data not shown) which agrees with our previously reported data [19].

Germination and outgrowth of untreated and heat treated spores was monitored by microscopic observation and by the relative change in optical density at 595 nm (OD_{595}) that reflects the transition from phase bright to phase dark spores. For the untreated spores, a rapid drop in OD_{595} was observed within 30 min from the addition of BHI (Figure 4.1A) resulting from the uptake of water and this coincided with the presence of phase dark spores observed by microscopy (Figure 4.1B). After the initial rapid drop, indicating homogeneous germination (Figure 4.1B), the OD_{595} increased corresponding with spore outgrowth.

Compared to untreated spores, heat treated spores showed a delayed drop in OD_{595} as well as slower decrease in OD_{595} corresponding to a delayed germination process (Figure 4.1A). These observations were in line with microscopy observations (Figure 4.1B), and were conceivably caused by the presence of a high number of heat inactivated spores

that became permeabilised and susceptible to water influx as shown previously [19]. The majority of heat treated spores showed a slow peripheral loss of spore brightness, eventually reaching phase grey within the time frame of the experiment (Figure 4.1B). The number of spores showing this behaviour corresponded to the presence of 2 logs of inactivated spores as determined by plate counting. The remaining population completed germination and outgrowth though the whole process was delayed, slower and more heterogeneous compared to untreated spores (Figure 4.1B). Eventually, rapid exponential growth of surviving germinated outgrown spores for both untreated and heat treated spores was observed (Figure 4.1A).

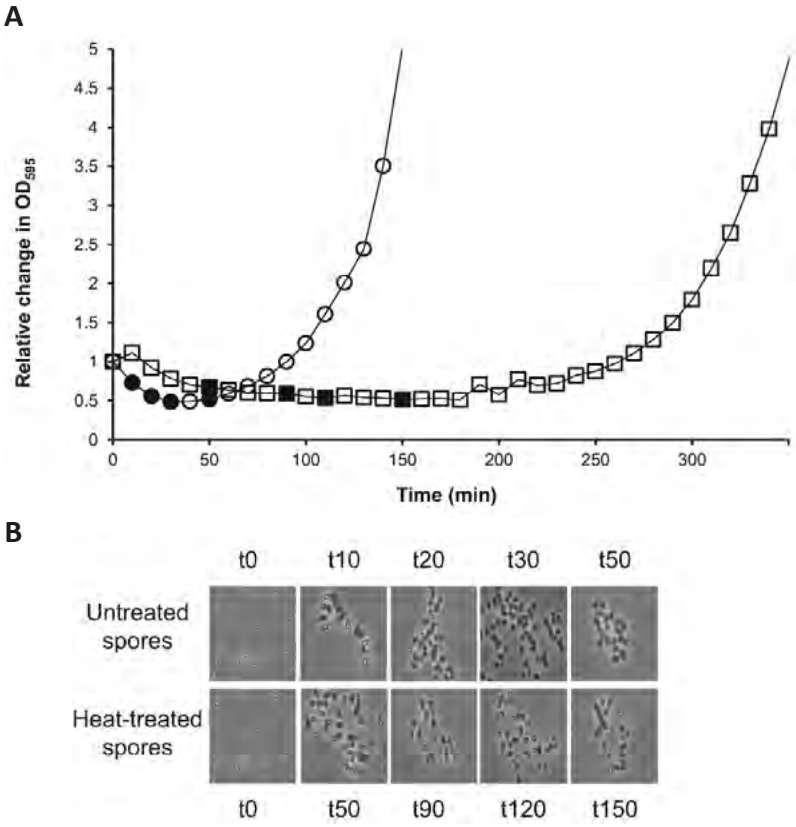

Figure 4.1. Impact of heat treatment on germination and outgrowth of *B. cereus* ATCC 14579 spores. (A) Relative change in OD_{595} for untreated (circles) and heat treated for 1 min at 95°C (squares) dormant spores was monitored in time in BHI broth at 30°C. Closed symbols indicate the sampling points selected for transcriptome analysis. The starting OD_{595} was 0.15-0.2 (B) Microscopy analysis of samples taken before initiation of germination (t0) and at indicated time points (10 up to 150 min) thereafter.

A number of studies have described the effect of heat treatment on germination efficiency and/or outgrowth capacity of dormant spores of Bacilli [31-33] and Clostridia [34]. As expected, the intensity of the heat treatment has a large effect on the behaviour of treated

spores, i.e., milder treatments aiming at spore activation resulted in faster germination and outgrowth, while more intense treatments resulted in inactivation and extended times to outgrowth of surviving spores suggesting spore damage.

The heat damaged spores conceivably activate repair mechanisms leading to recovery and subsequent vegetative growth at growth rates comparable to unstressed populations [34, 35]. It is generally assumed that damage repair takes place between germination (and resumption of metabolic activity) and outgrowth during transition phase (by some authors referred to as ripening time) [34, 36]. Recent findings point to synthesis of a subset of proteins during early germination [37] but the majority of the proteins were synthesized during the transition phase and early outgrowth and therefore we chose to focus on this transition stage. An additional argument is that spores with partly degraded and/or damaged mRNAs displayed an extended transition phase leading to delayed outgrowth [38, 39] which also suggests that repair processes may take place during this stage.

Transcriptome analysis of outgrowth from heat treated spores

A transcriptome analysis was performed for untreated and heat treated spores aiming at identification of genes uniquely expressed during the transition phase and early outgrowth in heat treated spores. Because the timeline of spore germination and outgrowth phases varied between untreated and heat treated spores, we selected time points for microarray sampling based on microscopy and OD_{595} measurements thereby aiming for conditions representing comparable stages in the recovery phase. Based on data presented in Figure 4.1, untreated spores were sampled at time points 10, 20, 30 and 50 min after addition of BHI, and heat treated spores were sampled at time points 50, 90, 120 and 150 min. The time point at 10 min after addition of BHI (t10) was selected as a reference point since a difference in responses was expected in transition phase and not in the germination phase where the majority of the genes are expressed (as observed by Melis et al. [5]). Even with these procedures a high number of genes was (temporarily) affected, with at least 1,000 genes being differently expressed at each time point relative to t10 (data not shown). For heat treated spores, this number was twice as high (data not shown); this increase likely reflects a delay in the germination and outgrowth process but may also include genes specifically expressed to repair damage. Various studies show that spore germination and outgrowth are complex and tightly regulated processes [1, 36, 37]. Dormant spores contain a limited number of transcripts, typically 46 transcripts are present in the dormant spores of *B. cereus* [5] but upon germination 80% of the genomic content of the dormant spore is expressed. Previous studies in *B. subtilis* [1] and *Clostridium difficile* [4] presented gene expression of germinating spores relative to that of mid exponential cells to identify genes specifically expressed in outgrowing spores showing that 27% and 14% of the genes were uniquely expressed at one or more points during outgrowth, respectively. A common finding among all those approaches is that expression of a large proportion of the genome

is initiated during germination and outgrowth, resulting in expression of genes required to resume metabolic activity including transcription, translation and metabolic activities [1, 4, 5].

Spore specific transcripts

Spores contain a relatively small set of transcripts, and for *B. cereus* [5] and *B. subtilis* [1], 46 and 23 transcripts have been identified, respectively. These transcripts, that encode hypothetical proteins and some proteins associated with spore coat composition, are rapidly broken down upon germination [1, 5] suggesting that this phenomenon can be used as a marker for onset of germination. In the current study, 41 of the 46 spore transcripts reported previously for *B. cereus* by van Melis et al. [5] were identified. However, the level of spore transcripts generally decreased rapidly upon germination of untreated spores, while spore transcripts in the heat treated spores decreased at a slower rate (Figure 4.2) being in line with the presumed delayed turnover of the mRNAs in spores arrested in germination [5]. Based on these data, the spore transcripts were not selected for further study.

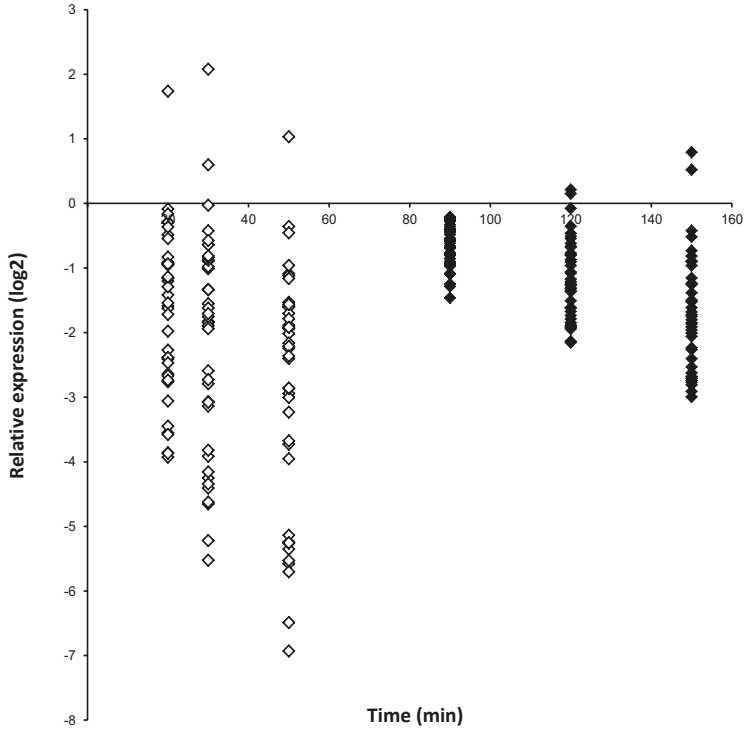

Figure 4.2. Expression profiles (log2 values) of reported spore specific transcripts during germination and outgrowth of untreated (white diamonds) and heat treated (black diamonds) *B. cereus* ATCC 14579 spores. Expression ratio's for untreated spores are relative to untreated germinating control spores at t10, and for heat treated spores relative to heat treated germinating spores at t50.

Expression analysis of putative DNA damage repair genes

Transcriptomes of untreated and heat treated spores were analysed for expression of known DNA repair genes reported in the literature for *B. subtilis* and/or present in *B. cereus* (S2 Table) [1, 9, 40]. During the sporulation process, spores may be equipped with DNA repair enzymes that allow for fast repair upon germination [8]. In addition, genes encoding DNA repair enzymes may be activated during germination and outgrowth of heat damaged spores.

During germination and outgrowth of untreated and heat treated spores, 49 putative DNA repair genes were differently expressed at least at one time point including 33 genes that also showed expression during outgrowth of untreated spores. Previous studies in *B. subtilis* showed significant number of these genes to be differently expressed during spore germination and outgrowth including *addAB*, *urvA*, *mutS*, *nth* and *nfo* [1]. Notably, both untreated and heat treated *B. cereus* ATCC 14579 spores display a similar expression pattern for the DNA repair genes during germination and outgrowth albeit that the response was delayed in the latter case. A recent study in *B. subtilis* shows that DNA repair and outgrowth processes may be aligned to each other mediated by a specific DNA integrity scanning protein (DisA), that was found to delay spore outgrowth until oxidative DNA damage is repaired [7]. Both untreated and heat damaged *B. cereus* spores expressed *disA* during germination (data not shown). Since expression patterns of DNA repair genes did not meet the criteria set in the current study they were not selected for further analysis. Nevertheless, lack of differential expression of known DNA repair genes in outgrowing heat treated spores is in line with previously reported data [10, 41-43] suggesting that wet heat treatment leads mainly to protein damage, in contrast to dry heat treatment that causes DNA damage. However, the exact effect of protein damage and repair processes involved remain to be elucidated.

Novel genes involved in spore damage repair

Transcriptome data were screened for genes specifically upregulated in heat treated spores following the criteria defined in the Materials and Methods section. Expression of 21 genes that met the initial criteria was verified by qPCR using independently prepared spore batches (S3 Figure) to confirm genes specifically expressed in heat treated spores. Using this criterion, 8 genes were selected that displayed the desired expression profile, i.e., gene expressed in heat treated spores while downregulated in untreated spores (see Table 4.1).

The putative functions of candidate genes include transcriptional regulator (4 genes), membrane protein (2 genes), lyase and a hypothetical protein (Table 4.1). Interestingly, only three genes have an orthologue in *B. subtilis* 168 including hypothetical protein BC3921 with an N-acyltransferase motif such as in *ylbP* (BSU15100) and an ArsR family

transcriptional regulator (BC4834) orthologous to SdpR, a transcriptional repressor of the *sdpR-sdpI* operon (BSU33790). The SdpR autorepressor belongs to the ArsR/SmtB family of repressors, whose prototypical member, ArsR, inhibits the transcription of genes involved in resistance to arsenate [44]. Finally, a CarD_CdnL_TRCF family transcriptional regulator (BC4714), with unknown function in *B. cereus* was identified. An orthologue is present in the *B. subtilis* genome, i.e., YdeB (BSU05130), but its role is also unknown. This candidate gene, further referred to as *cdnL* (BC4714), was also upregulated in vegetative cells of *B. cereus* in response to different stresses including cold shock, salt, acid and disinfectants [45]. Therefore, *cdnL* (BC4714) was selected to validate its potential role in spore damage repair. The *ΔcdnL* (BC4714) deletion mutant was constructed and spores were prepared. Wild type and *cdnL* (BC4714) mutant strain spores were heat treated for one min at 95°C resulting in a 2 log inactivation and more than 90% of damaged spores among the surviving fraction for both wild type and *ΔcdnL* (BC4714) spores (Table 4.2). The heat resistance of *ΔcdnL* (BC4714) spores, and the kinetics of spore recovery (S4 Table) were comparable to that of wild type spores. However, plate counting with and without salt supplementation showed significant differences in the fraction of mildly and severely damaged spores, with wild type spores and *ΔcdnL* (BC4714) spores showing approximately 45% and 74% of severely damaged spores, respectively. The increased fraction of severely damaged spores in the mutant point to a role for *cdnL* (BC4714) in recovery of heat damaged *B. cereus* ATCC 14579 spores. Deletion of *cdnL* (BC4714) did not result in increased salt sensitivity of untreated spores (S4 Table), and nor was the growth rate of vegetative cells affected in the presence of salt (data not shown) confirming the role of *cdnL* (BC4714) in recovery of heat damaged *B. cereus* ATCC 14579 spores.

Table 4.1. Array expression ratios (log2 values) of candidate genes displaying specific upregulation during germination and outgrowth of heat treated *B. cereus* ATCC 14579 spores and verified by qPCR. False Discovery Rates below 0.05 are indicated in bold.

Gene	Function	Log2 values over T10 untreated						
		Untreated			Heat treated			
		T20	T30	T50	T50	T90	T120	T150
BC1312	3-hydroxybutyryl-CoA dehydratase	**-1.39**	**-1.34**	**-1.29**	**3.49**	**3.73**	**3.49**	**2.93**
BC3437	cytoplasmic protein	**-1.18**	**-1.34**	**-1.97**	**3.91**	**4.65**	**4.36**	**3.63**
BC3438	PadR family transcriptional regulator	**-1.09**	**-1.25**	**-1.97**	**4.33**	**4.96**	**4.61**	**3.72**
BC3921	hypothetical protein	**-2.31**	**-3.23**	**-3.86**	**1.39**	**1.79**	**1.64**	**1.07**
BC4714	CarD_CdnL_TRCF family transcriptional regulator	**-1.35**	**-1.66**	**-2.59**	**2.60**	**3.20**	**2.76**	**1.91**
BC4834	ArsR family transcriptional regulator	**-1.09**	**-1.29**	**-1.35**	**2.25**	**2.51**	**1.97**	**1.12**
BC5038	MarR family transcriptional regulator	**-1.82**	**-2.21**	**-2.74**	**1.50**	**2.08**	**1.68**	**1.06**
BC5242	membrane protein with C2C2 zinc finger	**-1.09**	**-1.01**	**-1.28**	**1.73**	**1.34**	**1.04**	**1.20**

It is possible that the *cdnL* gene (BC4714) is induced in response to heat damaged proteins however in vegetative *B. cereus* cells, *cdnL* (BC4714) is induced upon exposure to salt and cold stress, and to a lesser extent to acid and oxidative stress, but not in response to heat [45]. The precise function of *cdnL* (BC4714) in those conditions remains to be elucidated. Heat treatment was shown previously to cause secondary oxidative stress in *B. cereus* vegetative cells [46]. To elaborate on the role of *cdnL* (BC4714) in the recovery from stresses other than heat, spores were exposed to an oxidative treatment with hydrogen peroxide (HP) and a combination of oxidative and chloraminating action of sodium hypochlorite (SH). In both cases, deletion of *cdnL* (BC4714) did not result in increased inactivation or altered degrees of spore damage (Table 4.2). This suggests that *cdnL* (BC4714) plays a specific role in repair of heat-induced *B. cereus* spore damage. It cannot be excluded that *cdnL*-deficient spores lack one or more specific proteins that makes them more susceptible to heat damage, however, high upregulation of the *cdnL* gene in heat treated spores supports our finding that its activity is also required during spore outgrowth.

Table 4.2. Surviving and mildly and severely heat damaged spore fractions in *B. cereus* ATCC 14579 and its mutant derivative strain *ΔcdnL* (BC4714) upon exposure to wet heat, hydrogen peroxide and sodium hypochlorite treatments. Averages of three independent experiments are represented.

				Damage in surviving fraction					
		Survival		Total damage		Mildly damaged spores		Severely damaged spores	
Treatment	Strain	%	SD	%	SD	%	SD	%	SD
95°C	WT	8	1	91	2	46	1	45	2
	ΔcdnL (BC4714)	5	0	99	2	25	9	74	9
Hydrogen peroxide	WT	9	2	99	0	17	2	82	3
	ΔcdnL (BC4714)	7	3	99	1	16	11	83	11
Sodium hypochlorite	WT	7	1	98	0	25	6	74	7
	ΔcdnL (BC4714)	11	4	99	0	23	6	76	7

CdnL (BC4714) belongs to CarD_CdnL_TRCF family of bacterial RNA polymerase-binding proteins. A family archetype, CarD, is associated with carotenogenesis and fruiting body formation in *Myxococcus xanthus*, and is one of two prokaryotic examples of high-mobility group A (HMGA) proteins [47-49]. The C-terminus of CarD contains a HMGA domain responsible for DNA binding and the N-terminus contains a transcription repair coupling factor (TRCF) domain that shares a binding site with the RNAP β subunit [50]. A more common member of the bacterial CarD family is represented by the CarD N-terminal like protein (CdnL) that lacks a DNA-binding domain and is also present in *M. xanthus* and *Mycobacterium tuberculosis* where it has an important role in stress resistance [49]. In

particular, the *M. tuberculosis cdnL* gene shows high up-regulation after DNA damaging and starvation treatments, indicating its possible link with damage repair [47, 48, 51, 52]. In *B. cereus* strain ATCC 14579, two paralogs of the *cdnL* gene (BC3648 and BC4714) are present, but both genes share limited similarity. Given differences in regulatory processes between *B. cereus* and *B. subtilis* [25, 45] it is not surprising that YdeB, a *B. subtilis* orthologue of CdnL (BC3648 and BC4714) also shows limited similarity and is believed not be functionally equivalent. Future studies in *B. cereus* will assess the role of the other selected genes in spore damage repair including single and double mutant analysis of the BC3648 (paralogue) and BC4714 genes.

The high number of protection systems and spore structures involved in spore resistance stresses the importance of damage prevention for spore survival. Despite these defence systems, the occurrence of spore damage cannot be prevented and a repertoire of repair mechanisms is required, including genes involved in DNA repair [1, 9, 40]. The common consensus is that damage repair take place during transition phase between germination and outgrowth, however, the processes involved are still largely unknown. The existence of multiple systems acting in parallel is conceivable as we identified here 8 candidate genes with four of these having a possible regulatory role. The relatively mild effect of *cdnL* deletion could be explained by the presence of additional regulators and systems involved in repair.

In conclusion, using transcriptome analysis 8 candidate genes with putative roles in the outgrowth from heat damaged *B. cereus* spores were identified. Comparative analysis of the wild type and a *cdnL* (BC4714) mutant shows that this gene is contributing to heat-induced spore damage repair whereas wild type and mutant spores displayed similar sensitivity to damage caused by oxidative agents indicating that we identified a novel player in heat-induced *B. cereus* spore damage repair. This work provided a new strategy to study and identify putative cellular parameters involved in spore damage repair, applicable not only for heat-induced damage but also for other food relevant conditions. Insights obtained may contribute to development of more efficient strategies to control outgrowth of damaged spores.

Supporting Information

Supplementary Tables S1 – S4 and Figures S1 – S3 can be found in the online version of the article.

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Chapter 5

Recovery of heat treated *Bacillus cereus* spores is affected by matrix composition and factors with putative functions in damage repair

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Abstract

The ability of spores to recover and grow out after food processing is affected by cellular factors and by the outgrowth conditions. In the current communication we studied the recovery and outgrowth of individually sorted spores in BHI and rice broth media and on agar plates using flow cytometry (FCM). We show that recovery of wet heat treated *Bacillus cereus* ATCC 14579 spores is affected by matrix composition with highest recovery in BHI broth or on rice agar plates, compared to BHI agar plates and rice broth. Data show that not only media composition but also its liquid or solid state affect the recovery of heat treated spores. To determine the impact of factors with putative roles in recovery of heat treated spores, specific genes previously shown to be highly expressed in outgrowing heat treated spores were selected for mutant construction. Spores of nine *B. cereus* ATCC 14579 deletion mutants were obtained and their recovery from wet heat treatment was evaluated using BHI and rice broth and agar plates. Deletion mutant spores showed different capacity to recover from heat treatment compared to wild type with the most pronounced effect for a mutant lacking BC5242, a gene encoding a membrane protein with C2C2 zinc finger which resulted in over 95% reduction in recovery compared to the wild type in BHI broth. Notably, similar relative performance of wild type and mutants was observed using the other recovery conditions. We obtained insights on the impact of matrix composition and state on recovery of individually sorted heat treated spores and identified cellular factors with putative roles in this process. These results may provide leads for future developments in design of more efficient combined preservation treatments.

Introduction

An increased demand for food with improved freshness, sensorial and nutritional values has directed food processing towards the use of milder heat treatments that require secondary mild preservation hurdles to assure stability and safety of the products [1]. As a result, these products are challenged by resistant microbial spores, that survive heat and other preservation hurdles used in food processing [2-4]. Reduction of the heat treatment intensity may lead to subpopulations of spores that are sublethally damaged rather than inactivated resulting in increased heterogeneity in the population [5, 6]. Damaged spores retain the capacity to germinate, repair, and eventually grow out leading to spoilage and safety issues [6-9]. Heterogeneity in spore populations can originate from differences in sensitivity of individual spores to inactivating treatments [10] and/or from differences in repair capacity of individual damaged spores. In addition, the presence of superdormant spores may further increase heterogeneity [11], and this conceivably results in less accurate prediction of spore outgrowth behavior.

Wet heat treatment is a common practice in food processing intended to reduce the microbial load of food products. Thermal pasteurization processes aim for inactivation of vegetative cells but are insufficient to kill spores. Sterilization processes aim for spore inactivation but may result in spore damage when target process conditions are not reached or when products contain highly heat resistant spores. The exact mechanism of wet heat killing of the spores and concomitant wet heat damage are not yet fully understood. Wet heat resistance of spores, mainly investigated in *Bacillus subtilis*, is determined by a number of factors including the spore structural components (small acid-soluble proteins (SASP), dipicolinic acid (DPA), metal ions, low core water content) but also the sporulation conditions (temperature, liquid or solid state of medium) affect its resistance [12-14]. Wet heat treatment is thought to kill spores by damaging one or more key spore proteins, however the identity of those proteins remains to be determined [12]. Analysis of single wet heat treated spores of *Clostridium botulinum* [10] and *Bacillus* species [15] revealed a delayed initiation of germination and/or reduced rate of germination, but also the subsequent outgrowth was delayed indicating not only damage to the germination system but also to other spore components affecting outgrowth. The time required for germination and outgrowth of spores was shown to correlate with the wet heat treatment intensity [16]. Heterogeneity in germination and outgrowth of surviving *C. botulinum* and *B. cereus* spore populations is more pronounced in the presence of a secondary mild stress factor such as low pH without and with sorbic acid, and increasing levels of salt [6, 10, 17] or the natural components of food media [6]. In general, damaged spores were shown to be more sensitive to secondary stresses including sodium chloride, pH, sorbic acid compared to undamaged spores [5, 6, 8, 17-21]. Some studies suggest a pre-plating recovery step in optimal (perhaps strain and treatment specific) conditions to allow recovery of injured cells [22] or spores [23]. For

example, a 7 h incubation step of heat treated *Clostridium difficile* spores in BHI broth prior to plating resulted in increased recovery of ethanol resistant fraction (dormant spores) on blood agar [23].

In this study we focus on *B. cereus*, a spore former of concern in processed foods. Its spores are widely present in the environment and are common contaminants in the food chain. *B*. *cereus* has been associated with food spoilage [24] and food-borne disease [25]. The vegetative cells of *B*. *cereus* can cause disease either by secretion of enterotoxins in the small intestine, causing the diarrheic syndrome or by the production of a heat-stable toxin (cereulide) in food before ingestion resulting in an emetic syndrome. *B*. *cereus* associated diseases are usually mild and self-limiting but in rare instances they can lead to fatal outcomes [25-29].

Using a transcriptome approach, we previously identified 21 genes putatively involved in heat damage repair in *B*. *cereus.* For one of these candidate genes, *cdnL* (now referred as *cdnL1*), a role in spore damage repair was further confirmed using a targeted deletion mutant [21]. Here we report on behavior of eight newly and one previously [21] constructed mutant to assess respective putative roles in recovery efficiency of heat treated *B*. *cereus* spores.

To this end, *B*. *cereus* ATCC 14579 wild type and its mutant derivative spores were exposed to a wet heat treatment resulting in over 95% of damaged spores in the surviving fractions. The recovery and outgrowth of spores was followed using flow cytometry (FCM) in combination with single spore sorting and a Most Probable Number (MPN) approach. To quantify the effect of matrix conditions on recovery capacity of wild type and mutant spores BHI and rice media, both in solid and liquid form were included. This approach allows for identification of candidate genes that may contribute to recovery capacity of heat treated *B*. *cereus* spores.

Materials and methods

Strains and sporulation conditions

B. *cereus* ATCC 14579 obtained from the American Type Culture Collection (ATCC), and its mutant derivatives used in this study (Table 5.1) were cultured in Bacto Brain Hart Infusion broth (BHI, Beckton Dickinson) at 30°C with aeration at 200 rpm. A nutrient-rich, chemically defined sporulation medium (MSM medium) described previously [30] was used to obtain spores. Sporulation and spore handling were performed as described previously [6], briefly one ml of an overnight-grown pre-culture was used to inoculate 100 ml of MSM media in 500 ml flasks and incubated at 30°C with aeration at 200 rpm. Sporulation was monitored by phase contrast microscopy until over 99% of the spores were released from the mother cell (typically after 2-3 days). Released spores were

harvested by centrifugation at 5,000 rpm at 4°C (5804R, Eppendorf, Germany) for 15 min and washed with chilled phosphate buffer (100 mM, pH 7.4) containing 0.1% Tween80 to prevent spore clumping. Spores were washed twice a day for 2 weeks with a phosphate buffer that was gradually decreased in Tween80 concentration until a final concentration of 0.01% (further referred as suspension buffer). Spores free of vegetative cells, debris and mother cells residues were stored at 4°C and used within six months. A single spore crop per strain was used for all the experiments.

Table 5.1. Overview of *B. cereus* ATCC 14579 deletion mutants used in this study.

Strain/Genotype	Sorting	Function	Reference
B. cereus ATCC 14579 (wild type)	+		
ΔBC0460	+	Hypothetical protein	this study
ΔBC0690	+	PbsX family transcriptional regulator	this study
ΔBC0852	+	Quaternary ammonium compound-resistance protein/SugE	this study
ΔBC0853	+	Quaternary ammonium compound-resistance protein/SugE	this study
ΔBC1312	-[a]	3-hydroxybutyryl-CoA dehydratase	this study
ΔBC1314	+	PhaQ/PadR family transcriptional regulator	this study
ΔBC3437	-[b]	Cytoplasmic protein	this study
ΔBC3921	-[b]	Hypothetical protein	this study
ΔBC4834	-[a]	ArsR family transcriptional regulator	this study
ΔBC5242	+	Membrane protein with C2C2 zinc finger	this study
Δ*cdnL1* (BC4714), Cm^r	+	CarD_CdnL_TRCF family transcriptional regulator	[21]
Δ*cdnL2* (BC3648)	+	CarD_CdnL_TRCF family transcriptional regulator	this study
Δ*cdnL1* (BC4714), Δ*cdnL2* (BC3648), Cm^r	+		this study

[a] no sporulation, [b] poor spore quality, Cm^r chloramphenicol resistance

Construction of deletion mutants

Deletion mutants (Table 5.1) were constructed using the temperature-sensitive suicide plasmid pAUL-A [31]. Flanking regions of the individual genes were amplified using KAPA HiFi Hotstart ReadyMix (KAPA Biosystems, USA) and the primers UP_enzyme_F/UP_NotI_R and DOWN_NotI_F/DOWN_enzyme_R (Table S1) for upstream and downstream flanking regions, respectively. The resulting fragments were fused in frame via a NotI digestion site introduced with the indicated primers. The resulting plasmid was transferred via electroporation (400 Ω, 25 μF, 1.2 kV, 0.2 cm Gene Pulser Cuvette: BIORAD) in *B. cereus* ATCC 14579 cells, and plated on BHI agar at 30°C with 10 μg/ml erythromycin (E10) to select for the desired transformants. Two erythromycin resistant colonies were selected and grown overnight in BHI at 30°C in the presence of E10. The resulting culture was diluted (1:200) in fresh LB with E10 and grown overnight at 42°C to select for plasmid integration. Selected strains resulting from a single cross-over integration event were

grown overnight in BHI at 30°C to induce double crossover events and subsequently plated and grown at 30°C. Resulting colonies were replica plated on BHI with and without E10 and incubated at 37°C. Colonies sensitive to E10 were selected. PCR analyses (using primers UPFlank_F, DOWNFlank_R, checkINTERNAL_R, check_F, and check_R) (Table S1) and DNA sequencing of erythromycin sensitive colonies confirmed the correct internal in-frame deletion of the gene and lack of other mutations in the targeted region.

A double deletion mutant (Δ*cdnL1*/Δ*cdnL2*) was obtained as described above with the exception that the *cdnL2* knock out plasmid was transformed into a *B. cereus* Δ*cdnL1* (BC4714) mutant strain constructed previously [21] and 5 µg/ml chloramphenicol was included as selective pressure preventing excision of the chloramphenicol resistance cassette that disrupted the *cdnL1*.

Heat treatment

One hundred µl of spore suspension containing approximately 10^8 spores/ml in suspension buffer was transferred, in duplicate, to thin-walled PCR tubes (VWR, The Netherlands). The PCR tubes were kept for 1 min at 4°C followed by a step at 95°C for 45 s and finally cooled for 1 min at 4°C in a thermal cycler (Veriti, Applied Biosystems). The duplicates were pooled and 100 µl of this pooled fraction was used as sample for spore sorting experiments. The same pooled fraction was diluted decimally in suspension buffer and 50 µl samples were used for spore enumeration on BHI plates (in duplicate) and incubated at 30˚C up to 3 days with daily enumeration of resulting colonies. For each strain, from the same spore preparation, at least four independent heating experiments were performed.

Quantification of spore damage

To evaluate the degree of spore damage, the method previously reported by Warda et al. [21] was used. Briefly, 50 µl of decimally diluted heat treated samples were enumerated in duplicate on BHI plates and BHI plates supplemented with 1.5% and 5.5% salt following incubation at 30˚C. To evaluate possible delay in colony formation, colonies were counted after 1, 2 and 7 days (further extension did not affect colony counts). Obtained colony forming units (CFUs) were used to calculate the total damage and fractions of mildly and severely damaged spores as described previously [21] according to the following formulas:

$$\%\,Total\,damage = \frac{(\text{Number of cfu's BHI}) - (\text{Number of cfu's BHI5.5\% NaCl})}{(\text{Number of cfu's BHI})} * 100$$

$$\%\,Mild\,damage = \frac{(\text{Number of cfu's BHI1.5\% NaCl}) - (\text{Number of cfu's BHI5.5\% NaCl})}{(\text{Number of cfu's BHI})} * 100$$

$$\%\,Severe\,damage = \frac{(\text{Number of cfu's BHI}) - (\text{Number of cfu's BHI1.5\% NaCl})}{(\text{Number of cfu's BHI})} * 100$$

Flow cytometry and cell/spore sorting

Flow cytometry was performed with a FACSAriaIII cell sorter (BD Biosciences) using a fiber launched solid state air-cooled laser operating at 488 nm. Only forward scatter (FSC) and side scatter (SSC) functionality was used. The machine was calibrated using standard Cytometer Setup & Tracking beads and Accudrop beads (BD Biosciences). All parameters were measured using logarithmic amplification. During the procedures a 85 micron nozzle (drop driving frequency was ~45 kHz/s) was used with flow rate one and during sorting a maximum event rate of 2,000 events/s was used. Cells and spores were discriminated from electronic noise using both SSC and FSC. Sorting criteria and gating strategy were based on FSC and SSC populations (data collection equals 50,000 events) excluding remaining doublets. In order to achieve high purity and recovery, the "Single Cell" precision mode (Purity mask 32 and Phase mask 16) was used for sorting. Cells or spores were sorted on solid and in liquid media.

Cell sorting

Five µl of an overnight grown culture was diluted in 3 ml of HEPES buffer and loaded into the flow cytometer. Individual vegetative cells of *B. cereus* ATCC 14579 and the mutant derivatives were spotted in duplicate on a single BHI and rice agar plate (according to the scheme in Figure S1C) and incubated at 30°C up to 3 days to confirm that growth was not affected in the deletion mutants.

Spore sorting

One hundred µl of unheated or heat treated spore suspension (containing non-damaged, damaged and dead spores) was diluted in 1.5 ml of HEPES buffer (pH 7.4) in 5 ml polystyrene falcon tube (BD, USA) and loaded into the flow cytometer. For heat treated spores, a series of one, 10 and 100 individual spores were sorted either into wells of 384-well plates (Greiner Bio-One, USA) containing 50 µl of BHI or rice broth or on one of the 52 available locations on standard BHI or rice agar plates. The resulting growth data representing three consecutive decimal dilutions were used as input for the MPN quantification method [32, 33]. For heat treated spores, for each sorting series of one,

10 or 100 spores approximately 754 replicates were performed for liquid media and at least 520 replicates for solid media (Table S2). A single replicate is defined as one well or location on agar plate to which either one, 10 or 100 spores were sorted. For untreated spores, only single spores were spotted on 188 and 104 locations (Table S2) for liquid or solid media, respectively. The resulting plates were incubated at 30°C up to 3 days with daily visual scoring for growth, i.e. colony formation on solid media or appearance of turbidity for liquid media. Wells that were positive for turbidity ranged from OD_{600} 0.2 to 0.3 for rice media (OD_{600} of fresh media 0.16), and in case of BHI values from OD_{600} 0.2 to 0.8 (OD_{600} of fresh media 0.1). The MPN values and their upper and lower limits were calculated using MPN Calculator (http://www.i2workout.com/mcuriale/mpn/).

Model food media used in this study

A rice based medium was prepared according to the method reported previously [6] by boiling ready-to-cook pouches filled with 125 g rice produced by the manufacturer (Lassie B.V, The Netherlands) in demineralized water (5:32 w/v) for 45 min. The rice bags were removed and the remaining liquid was allowed to cool down. The method was modified by addition of a centrifugation step [(AVANTI J-25, Beckman Coulter, USA) for 5 min at 16,000 rpm at 22°C] and filtering of the resulting supernatant (Filter paper, Whatman, England) to remove the big particles and improve the clarity of the solution. Finally, the suspension was pooled and autoclaved. Sterile rice broth was stored in the dark until use. For preparation of rice agar plates, 1.5% (w/v) Bacteriological Agar was added prior to a second autoclaving step. The final pH of rice broth was 6.7, while the pH of rice agar plates was 7.

Results

Impact of matrix on the growth of *B. cereus* spores

The impact of the liquid and solid media composition on the growth of *B. cereus* spores was evaluated using flow cytometry (FCM) in combination with single spore sorting. The single untreated *B. cereus* spores were sorted into four different media namely BHI broth, rice broth, BHI agar plates and rice agar plates. Besides BHI, a rice media was selected as this food matrix was shown previously to support growth from *B. cereus* spores on agar plates [6] and on Anopore strips [6] that resemble growth in broth [34]. All four media allowed outgrowth of 94 up to 99% of the sorted spores within three days (Figure 5.1, Table S2). In rice broth, growth was delayed compared to BHI broth, which was not observed for the corresponding agar media (Figure 5.1). In general, smaller colonies were formed on rice agar plates compared to BHI agar plates for both outgrowing untreated spores (data not shown) and vegetative cells (Figure S1). However, after 3 days, the

percentage of outgrowing spores on rice agar plates reached 99.1% while on BHI agar plates 94.4% was reached (Figure 5.1).

Impact of matrix on the recovery of heat treated *B. cereus* spores

To allow for high throughput heat treatment of spores, spores were treated in thin-wall tubes in a PCR machine. Using this approach, a 45 s holding time at 95°C resulted in approximately 2 log inactivation and 99% of damaged spores in the surviving population of wild type spores (Figure S2). This number is comparable to previous results (91 to above 95%) obtained with capillary tubes in an oil bath [6, 21]. In the surviving population, 13% of spores were mildly damaged, whereas 86% were severely damaged. In previous findings, these numbers were 46% and 45%, for mildly and severely damaged spores, respectively [21]. We previously showed that a *cdnL1* mutant was affected in the ratio between mildly and severely damaged spores [21]. However, the slightly different heating conditions in the high throughput method resulted in a higher fraction of severely damaged spores in the wild type spores. Using shorter holding times, an increased survival was obtained but again a relatively high percentage of severely damaged spores was observed (data not shown), therefore further experiments were performed using a holding time of 45 s.

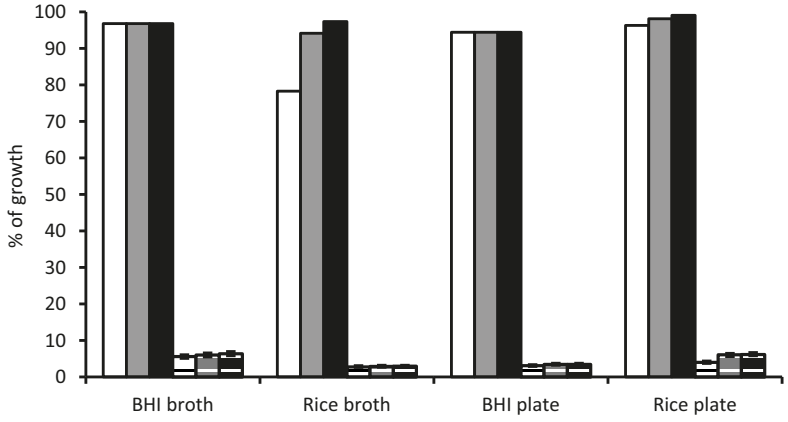

Figure 5.1. Percentage of growth from single untreated spores (plain bars) and percentage of growth from heat treated for 45 s at 95°C spores (pattern bars) of *B. cereus* ATCC 14579 after one (white), two (grey) and three (black) days of incubation in liquid (BHI and rice broth) and on solid (BHI and rice agar plates) media. Values indicated for untreated spores were calculated based on 188 individual spores for recovery in liquid media and 104 individual spores for recovery in solid media. Values for heat treated spores were calculated based on at least 750 wells for each of one, 10 and 100 spores in liquid media and at least 520 locations for each of one, 10 and 100 spores on solid media using the MPN based approach. Error bars for heat treated spores represent the lower and upper limits of the MPN values expressed in percentage.

To evaluate recovery of sorted heat treated spores in a high throughput format, a combination of single spore sorting with MPN method was applied. Sorting of spores in series of one, 10 and 100 of heat treated spores at individual locations (well or spot) increased the resolution of the measurements allowing to observe significant differences within expected 2 log inactivation range. Interestingly, for the heat treated spores a comparable recovery in BHI broth and on rice agar plates was observed while rice broth and BHI agar plate supported recovery of approximately 50% of the surviving spores compared to BHI broth (Figure 5.2). This indicated that not only the composition of the media but also its liquid or solid state has an effect on the recovery of the spores.

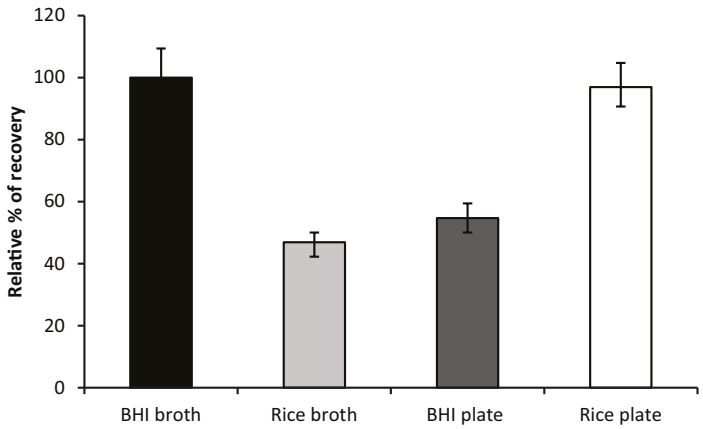

Figure 5.2. Recovery of heat treated *B. cereus* ATCC 14579 spores in BHI broth (black), rice broth (light grey), BHI agar plates (dark grey), and rice agar plates (white) after three days of incubation. Values represent the percentage recovery relative to BHI broth (100% corresponds to 6.4% survival of wild type). Error bars represent the lower and upper limits of the MPN values expressed in percentage relative to BHI broth.

Role of spore damage repair associated genes in recovery of heat treated *B. cereus* spores

Previously, genes expressed during germination and outgrowth of heat treated *B. cereus* spores were studied in a transcriptome study resulting in a set of 21 genes that were highly expressed in heat treated spores relative to the reference time point at 10 min but either temporally or not expressed in untreated spores. Further evaluation with qPCR [21] to confirm the microarray data resulted in selection of 13 target genes that were downregulated in untreated spores and/or upregulated in heat treated spores with expression ratio below minus two or above two. This selection included the eight genes previously shown to be specifically upregulated during germination and outgrowth of heat damaged *B. cereus* spores, namely BC1312, BC3437, BC3438, BC3921, *cdnL1* (BC4714), BC4834, BC5038, and BC5242 [21]. A mutant strain in one of those candidate genes (*cdnL1* (BC4714)), a putative transcriptional regulator, was slightly but significantly affected in

repair and outgrowth of heat treated *B. cereus* spores [21]. A paralogue of *cdnL1*, *cdnL2* (BC3648) is encoded on the *B. cereus* ATCC 14579 genome and it was hypothesized that its gene product masked effects on spore damage recovery in the *cdnL1* deletion mutant. Therefore, a *cdnL2* (BC3648) mutant and a combined *cdnL1/cdnL2* mutant were included in the present study.

Attempts to construct mutants in BC3438 and BC5038 were unsuccessful. Of the 13 successfully constructed mutants (Table 5.1), four displayed various sporulation defects, mutants either did not sporulate (ΔBC4834), displayed an incomplete sporulation process (ΔBC1312) or the resulting spores were not fully released form the mother cell (ΔBC3437 and ΔBC3921). Therefore, these mutants were excluded from further analysis.

Spores of *B. cereus* ATCC 14579 and its mutant derivatives, were exposed to wet heat treatment for 45 s at 95°C. The reduction in survival of deletion mutants ranged from one up to two log with over 95% of surviving spores being damaged. The fractions of mildly and severely damaged spores were comparable to the wild type (Figure S2). The high fraction of damaged spores allows for the assessment of the roles of candidate genes in recovery of heat treated *B. cereus* in different outgrowth conditions i.e. liquid and solid forms of rice and BHI media.

One, 10 and 100 of heat treated spores were sorted either in individual wells of a 384 well plate or onto agar plates resulting in four recovery conditions, namely BHI broth, rice broth, BHI agar plates or rice agar plates. Deletion mutants ΔBC5242 and ΔBC0853 were highly affected reaching only 3.6% and 9.4% recovery in BHI broth compared to that of wild type spores, respectively (Figure 5.3A). Deletion of ΔBC5242 and ΔBC0853 led to the highest reduction in recovery for all tested media (Figure 5.3) suggesting that effects of these genes on recovery and possibly damage repair were media independent. In contrast, deletion of BC0690 resulted in higher recovery compared to the wild type in both BHI broth (50% increase) and on BHI agar plates (150% increase) (Figure 5.3AC). Deletion of *cdnL1* (BC4714) resulted in a recovery in BHI broth comparable to that of the wild type, albeit that time to growth was delayed in BHI broth and to a lesser extent on BHI agar plates (Figure S3, data not shown). Deletion of *cdnL2* (BC3648) resulted in a slight reduction in recovery compared to wild type in BHI broth, while recovery of the *cdnL1/cdnL2* double mutant (ΔBC4714/ΔBC3648) was reduced by approximately 50% in all tested media compared to wild type (Figure 5.3C). BC0852 and BC0460 mutants displayed a comparable reduction in recovery as the *cdnL1/cdnL2* double mutant. Finally, the recovery of ΔBC1314 depended on the recovery media, in rice broth the recovery was comparable to wild type (Figure 5.3B), while in BHI broth deletion led to over 50% reduction in recovery compared to wild type.

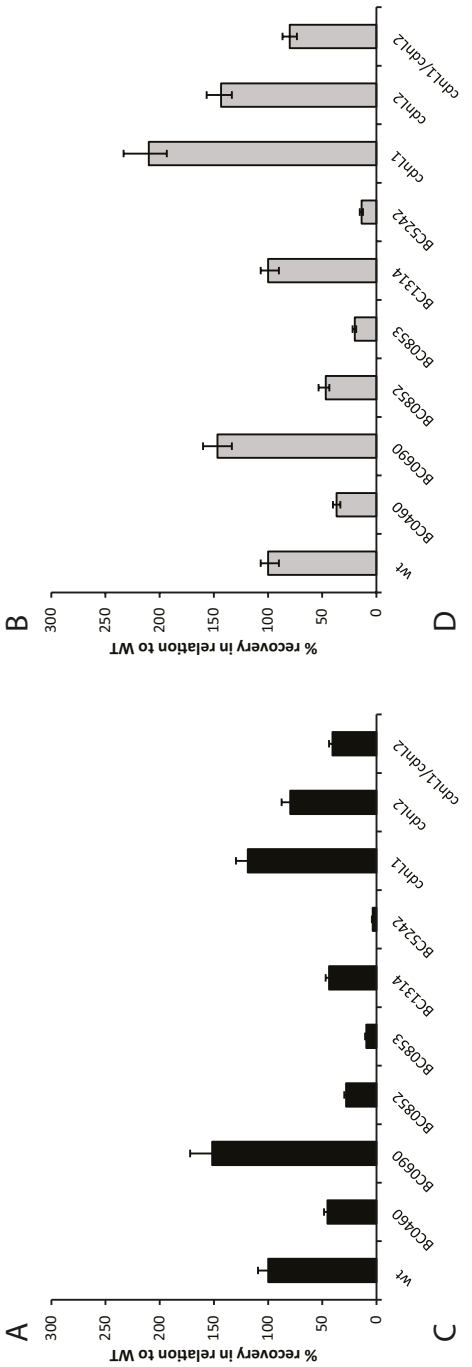

Figure 5.3. Recovery of heat treated *B. cereus* ATCC 14579 spores and its mutant derivatives in (A) BHI broth, (B) rice broth, and on (C) BHI agar plates, and (D) rice agar plates. Values are given in percentage relative to the recovery of wild type in given media. Error bars represent the lower and upper limits of the MPN values expressed in percentage relative to the recovery of wild type in given media.

Recovery of heat treated spores of all but two (ΔBC0460 and ΔBC0690) deletion mutants was higher on rice agar plates, compared to BHI broth (Figure S4). The recovery of those mutant spores improved also in rice broth and on BHI agar plates when compared to relative recovery of the wild type. This suggests that conditions supporting slower growth favor recovery of spores possibly by providing additional time for damage repair.

Discussion

The capacity of spores to repair damage and grow out is not only affected by the processing conditions, but also by spore history and recovery conditions. Although, several studies report on impact of food components on spore survival and cell growth [35-37], mainly plate counting methods that do not allow for analysis of individual spores have been applied. Moreover, the standard plate counting methods are generally not sensitive enough to show changes within the 10-fold range. In practice, product spoilage may result from a single surviving spore and knowledge on behaviour of individual spores can assist in risk evaluation. Here we applied a flow cytometry supported single spore sorting approach in combination with MPN methodology, allowing for evaluation of behaviour of individually sorted spores with high resolution for both untreated as well as heat treated spores.

The 45 s heat treatment at 95°C of *B. cereus* wild type and mutant spores resulted in approximately 2 log inactivation, and above 95% damaged spores in the surviving population, which is comparable to previously reported survival and total damage at this temperature [6, 21]. Limited information is available on the effect of the recovery media on outgrowth of single damaged spores. In the present study, we focused on the effect of media composition, either liquid or solid state, on the combined process of germination, outgrowth and vegetative growth of individually sorted untreated and heat treated *B. cereus* ATCC 14579 wild type and mutant spores. Firstly, we showed for wild type spores that rice broth was least supporting the growth and recovery of heat treated spores while rice agar plates provided comparable recovery as BHI broth, indicating that not only the composition but also the liquid or solid state of media effects the recovery of heat treated spores. Both heat treated and untreated *B. cereus* spores showed similar recovery when plated on BHI and rice agar plates [6]. However, the formation of microcolonies from individual spores on Anopore (a porous membrane allowing nutrient transfer that provides surface for spore/cell growth) conditions, which is more close to conditions in a broth [34] were found different for BHI and rice [6]. More specifically, rice media increased heterogeneity and delayed outgrowth of untreated spores compared to BHI, and also a heat treatment had a limited additional effect on the behavior of surviving spores [6]. Now we show that outgrowth from untreated single spores was slower in rice based media compared to BHI, but final counts for untreated single sorted spores on rice

plates were 99.1% while on BHI plates 94.4%. In line with our previous observations, the time required for colony formation from untreated *B. cereus* spores on rice media was extended compared to BHI, indicating that rice media may contain additional factors delaying germination and/or outgrowth or contain suboptimal concentrations of required components [6].

Comparative analysis of wild type and selected mutants lacking genes with putative roles in damage repair, showed different capacity to recover from heat stress compared to wild type. The most pronounced effect was observed for a deletion mutant, lacking a membrane protein with C2C2 zinc finger (BC5242). This mutation resulted in reduction in recovery down to 3.6% of the wild type recovery in BHI broth. The function of BC5242 is unknown, but orthologues of its gene product can be found in many *B. cereus* group strains though not in *B. subtilis* 168. In eukaryotes, zinc finger containing proteins function in gene transcription, translation, mRNA trafficking, cytoskeleton organization, epithelial development, cell adhesion, protein folding, chromatin remodeling and zinc sensing [38, 39]. In prokaryotes, zinc finger motifs (C4 superfamily) are found in proteins involved in DNA damage recognition i.e. UvrA, Ada, RecR [40], however the diversity in functionality of zinc finger carrying proteins and the zinc finger domains does not allow for prediction of a role for BC5242 in *B. cereus*. Notably, BC5242 was not upregulated in vegetative cells of *B. cereus* ATCC 14579 in response to different stresses including cold, ethanol, some disinfectants and mild acid [41].

BC1314 was found to be highly upregulated during germination and outgrowth of heat damaged *B. cereus* spores [21]. The recovery of ΔBC1314 spores after a heat treatment was decreased with 50% compared to wild type in BHI broth, and on BHI and rice agar plates, albeit less severe for the latter two media, thus suggesting a role of BC1314 in the recovery of heat treated spores. Analysis of the *B. cereus* ATCC 14579 genome sequence suggested that BC1314 (and BC1315) result from a frame-shift mutation in the *phaQ* gene (Figure S5). The *B. cereus phaQ* gene is part of a poly-β-hydroxybutyrate (PHB) synthesis cluster, and PHB was previously shown to be accumulated in cells in the form of granules that serve as a carbon and energy source during the late sporulation process in *B. cereus* [42] and *B. megaterium* [43]. In *B. megaterium*, PHB accumulation involves five genes, namely *phaP* (encoding a phasin protein), *phaQ* (encoding a repressor of *phaP* expression), *phaB* (acetoacetyl-CoA reductase), *phaR* and *phaC* (subunits of PHB synthase) (Figure S5) [44, 45]. Furthermore, in *B. thuringiensis* accumulation of PHB via *phaPQRBC* was shown to be under the control of the sporulation transcription factors sigH and Spo0A [46]. In strains belonging to the *B. cereus* group, orthologues of the *phaPQRBC* system are commonly present, while being absent in *B. subtilis* 168, pointing to a special role for this system in the indicated group.

The *cdnL1/cdnL2* double deletion mutant (ΔBC4714/ΔBC3648), lacking genes encoding both CdnL transcriptional regulators present in *B. cereus* ATCC 14579 showed 60%

reduction in recovery in BHI broth compared to the wild type. Deletion of *cdnL1* (BC4714) was shown previously to increase the fraction of severely damaged spores in the surviving population after a heat treatment of 1 min at 95°C [21]. Since the heat treatments applied in the present study led to a dominant fraction of severely damaged spores already in the wild type, we could not observe the increase in percentage of severely damaged spores in Δ*cdnL1* (BC4714). However, outgrowth from heat treated Δ*cdnL1* spores was delayed in BHI broth compared to the wild type spores, eventually reaching comparable recovery efficiency. The Δ*cdnL2* mutant (BC3648) showed lower recovery compared to Δ*cdnL1* (BC4714), and this was most pronounced in liquid media. Nevertheless, both Δ*cdnL1* and Δ*cdnL2* in media other than BHI broth show improved recovery compared to wild type. It remains to be determined whether the observed increase in recovery of the individual *cdnL* mutants could be explained by cross regulation of the counterpart. Both *cdnL1* and *cdnL2* genes are induced in vegetative cells in response to various environmental stresses, including salt and cold stress, whereas acid and oxidative stress specifically induced expression of *cdnL1* and not *cdnL2* [41]. Our findings suggest partly overlapping functionalities of *cdnL1* and *cdnL2* in recovery and possibly repair of heat damage.

Spores of the ΔBC0690 mutant, lacking a putative PbsX family transcriptional regulator of unknown function, showed higher recovery compared to wild type spores in all tested conditions, with increase of up to 150% on BHI agar plates. Orthologues of BC0690 are commonly found among *B. cereus* group strains, but absent in *B. subtilis* 168, pointing possibly to a unique, but up to now unknown role in heat stress survival in *B. cereus* group members.

Deletion of BC0852 and BC0853, both encoding putative quaternary ammonium compound resistance proteins annotated as *sugE*, resulted in reduction in recovery of spores to 9.4 and 28.1% of the wild type in BHI broth, respectively. Orthologues of BC0852 and BC0853 are present in *B. cereus* group strains, while being absent in *B. subtilis* 168. Besides BC0852 and BC0853, the *B. cereus* ATCC 14579 genome encodes a second orthologues pair of small multidrug resistance proteins (BC4213 and BC4214) orthologues to *ykkC* (BSU13090) and *ykkD* (BSU13100) of *B. subtilis* 168. *ykkC* and *ykkD* are a paired small multidrug resistance (PSMR) members, and their co-expression in *Escherichia coli* led to a multidrug-resistant phenotype [47]. Still, not all PSMR members have demonstrated drug resistance, e.g. *B. subtilis* YvaD/YvaE and YvdR/YvdS, and small multidrug resistance homologues were suggested to be involved in transport of yet unidentified compounds [48].

In the current study, the applied heat treatment resulted in at least 95% of damaged spores in the surviving wild type and deletion mutant spore populations, based on the fact that these spores were not able to grow out on salt supplemented plates (compared to BHI agar plates). At the moment it cannot be excluded that differences in spore recovery in BHI broth are due to lack of one or more specific proteins in spores of tested deletion mutants

that makes them more or less resistant and/or susceptible to heat damage. However, application of rice media and BHI agar plates compared to BHI broth for sorted spores also revealed differences in recovery between media suggesting different requirements for recovery. Particularly deletion of BC0460 or BC0690 resulted in reduced recovery on rice plates while spores of remaining seven deletion mutants showed improved recovery on rice plates compared to BHI broth (Figure S4). As the recovery of the various deletion mutants spores appears matrix dependent, this suggests that mutations conceivably affected different type of damage and/or repair targets as was suggested previously by Adams [49]. Apparently, high numbers of damaged spores were present in the surviving wild type and mutant spore population, but nevertheless, subtle effects of mutations in putative repair genes were noted, resulting in a shift from the fraction of mildly damaged to the fraction of severely damaged spores [21] and in differences in recovery between different media (this study). Still, recovery of heat treated spores is a complex process conceivably involving many different systems, and more studies are required to elucidate the full repertoire of repair systems and the impact of matrix composition and its solid or liquid state on this process.

In conclusion, we have shown that recovery of heat treated *B. cereus* spores is affected by the matrix composition with highest recovery of wild type spores in BHI broth or on rice agar plates, followed by BHI agar plates and rice broth. The comparative analysis of the wild type and newly constructed deletion mutants provided new insights in the putative role of the deleted genes in the recovery of heat treated *B. cereus* spores.

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Supporting Information

Supplementary Tables S1 – S2 and Figures S1 – S5 can be found in the online version of the article.

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Chapter 6

Linking *Bacillus cereus* genotypes and carbohydrate utilisation capacity

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Abstract

We characterised carbohydrate utilisation of 20 newly sequenced *Bacillus cereus* strains isolated from food products and food processing environments and two laboratory strains *B. cereus* ATCC 10987 and *B. cereus* ATCC 14579. Subsequently, genome sequences of these strains were analysed together with 11 additional *B. cereus* reference genomes to provide an overview of the different types of carbohydrate transporters and utilization systems found in *B. cereus* strains. The combined application of API tests, defined growth media experiments and comparative genomics enabled us to link the carbohydrate utilisation capacity of 22 *B. cereus* strains with their genome content and in some cases to the *panC* phylogenetic grouping. A core set of carbohydrates including glucose, fructose, maltose, trehalose, N-acetyl-glucosamine, and ribose could be used by all strains, whereas utilisation of other carbohydrates like xylose, galactose, and lactose, and typical host-derived carbohydrates such as fucose, mannose, N-acetyl-galactosamine and inositol is limited to a subsets of strains. Finally, the roles of selected carbohydrate transporters and utilisation systems in specific niches such as soil, foods and the human host are discussed.

Introduction

Metabolic activity and growth of bacteria requires energy that can be acquired during carbohydrate catabolism. Carbohydrates can be present in nature in complex forms such as polysaccharides including starch, cellulose or glycogen, but also as mono- and disaccharides exemplified by glucose and sucrose. Efficient use of available carbohydrates by microorganisms is determined by the different types of carbohydrate transporters and utilization systems encoded by chromosome- and plasmid-located genes and gene clusters [1, 2]. In addition, expression and regulation of sugar utilisation systems can be affected by environmental conditions such as oxygen availability and temperature, and carbon catabolite repression (CCR) [1, 3, 4].

Systems for carbohydrate uptake and metabolism predicted based on genome analysis can vary significantly between species and strains as shown for *Escherichia coli* [5], *Bacillus subtilis* [6], *Shewanella* spp. [7] and lactic acid bacteria including *Lactobacillus plantarum* [2, 8], *Lactobacillus casei* [9], *Oenococcus oeni* [10] and *Lactococcus lactis* [11]. In gram-positive bacteria, these genes are generally organized in functional gene cassettes or modules, encoding a single or multi-component transporter (1-4 subunits), enzymes for sugar breakdown and a transcriptional regulator, with each cassette (semi)-specific for a certain carbohydrate [2, 12, 13]. A cassette is a functional unit, and can consist of more than one operon. The presence or absence of entire cassettes can be highly variable, and is thought to reflect adaptation to growth on particular sugar substrates in specific niches such as soil and plant rhizosphere, foods and food processing environments, and the human host [1, 13, 14]. Often carbohydrate metabolism cassettes are clustered together on the chromosome, in so-called lifestyle or sugar islands associated with colonisation of specific niches such as dairy environments [8, 11, 13, 15]. The deviating base composition of these genomic islands and cassettes suggests their acquisition via horizontal gene transfer [16]. The increasing availability of microbial genome sequences has stimulated comparative analysis of carbohydrate utilisation capacity, on the one hand to optimize performance of fermentation starter bacteria and probiotics, and on the other hand, to understand pathogen behaviour in the environment and inside the host, enabling development of better preservation and control measurements in food processing and clinical settings [12, 14, 17].

Three main types of carbohydrate transporters can be found in bacteria: ATP-binding cassette (ABC) transporters, permeases and phosphoenol-pyruvate (PEP)-dependent carbohydrate phosphotransferase systems (PTS) that catalyse the transport and phosphorylation of numerous monosaccharides, disaccharides, amino sugars, polyols, and other sugar derivatives. To carry out its catalytic function in sugar transport and phosphorylation, the PTS uses PEP as an energy source and phosphoryl donor. The phosphoryl group of PEP is usually transferred via four distinct proteins (domains)

to the transported sugar bound to the respective membrane component(s) (EIIC and EIID) of the PTS [4]. Notably, the PTS have also numerous regulatory roles for example in carbon and nitrogen metabolism, antibiotic resistance, biofilm formation, toxin production and virulence [4, 18, 19]. Recently sugar acids were shown also to be transported by tripartite ATP-independent periplasmic transporters (TRAPs) [20].

Utilisation of a broad range of carbon and energy resources, either commonly present in the environment or associated with specific niches, can support not only ubiquitous presence but also facilitate transmission and pathogenicity of food-borne pathogens such as *Bacillus cereus* [21]. The ability to thrive in different environments such as soil, food and food processing environments and human gastrointestinal tract is supported by a range of carbohydrate transport and utilisation systems that indirectly facilitate transmission from these environments to the human gastrointestinal tract.

B. cereus is a representative of the *Bacillus cereus sensu lato* group comprising seven closely related species of spore formers that are associated with different environments and contain both non-pathogenic and pathogenic bacteria including *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis*, *Bacillus thuringiensis*, *Bacillus anthracis*, *B. cereus*, and the recently identified *Bacillus cytotoxicus* [22, 23]. *B. cereus sensu lato* group members have been classified into one of seven phylogenetic groups introduced by Guinebrettiere et al. [22], according to which *B. cereus* strains can be found in five of these groups, i.e., group II, III, IV, V and VI, covering a broad range of growth temperatures from 5 to 50°C.

B. cereus is often associated with spices and foods such as rice, cereals and dairy products [21]. Transmission of *B. cereus* is facilitated by the production of highly stress resistant dormant spores that are triggered to germinate in nutrient-rich conditions via an interplay of (combinations of) germinants and a variety of so-called nutrient germinant receptors [24]. Germination and outgrowth of *B. cereus* spores into vegetative cells in foods may lead to food spoilage and safety issues [25, 26]. The vegetative cells of *B. cereus* produce toxins in food before the ingestion or in the small intestine leading usually to mild and self-limiting symptoms but in rare instances it can lead to life-threatening situations [27]. *B. cereus* clinical isolates have been associated with gastrointestinal infections and non-gastrointestinal infections particularly in immune compromised patients or neonates resulting in wound infections, ocular infections and bacteraemia [21, 25-27]. Genome analysis of selected model strains pointed to *B. cereus* specialisation in protein metabolism, suggesting that it has adapted towards a symbiotic or parasitic life cycle [25, 28-30]. *B. cereus* strains carry genes for utilisation of mono- and disaccharides such as glucose and trehalose [28, 31], and polysaccharides such as starch [31]. Moreover performance in selected niches has been associated with transport and metabolism of specific carbohydrates such as glucose-6-phosphate [32].

Despite the putative role of selected carbohydrates in growth and survival in a range of environments, a detailed comparative genotypic and phenotypic analysis that includes multiple genome-sequenced *B. cereus* strains representing the different phylogenetic groups described by Guinebrettiere et al. [22] has not been reported up to now.

The current study provides an overview of the different types of carbohydrate transporters and utilization systems in 20 newly sequenced *B. cereus* food isolates and model *B. cereus* strains ATCC 14579 and ATCC 10987, and links these to metabolic capacity using API tests and/or defined media with selected carbohydrates as carbon and energy sources. Additionally, roles of selected carbohydrate transporters and utilisation systems in specific niches are discussed.

Materials and Methods

Strains used

Twenty *B. cereus* strains previously isolated from food products and food processing environment (NIZO Culture Collection, Ede, the Netherlands) and two reference strains *B. cereus* ATCC 14579 and *B. cereus* ATCC 10987 obtained from the American Type Culture Collection (ATCC) (S1 Table) [33, 34] were streaked from -80°C stock on Bacto Brain Heart Infusion (BHI; Beckton Dickinson, Le Point de Claix, France) agar plates and incubated at 30°C overnight to obtain single colonies. Based on Average Nucleotide Identity (ANI typing; [35]) criteria strain B4117 (LJKG00000000.1), was recently re-classified by NCBI as *B. mycoides*, phenotype information should be included to confirm this.

API growth tests

API 50CH (BioMerieux, France) test was used in combination with API 50CHB/E medium (BioMerieux, France) according to manufacturer's instruction. Shortly, single colonies were used to inoculate API 50CHB/E medium to a turbidity equivalent of 2 McFarland. Test strips were filled with inoculated medium, incubated at 30°C, and checked for media colour change after 24 and 48 h. For each strain, three independent repetitions were performed.

Defined media growth experiments

Ten ml of BHI was inoculated with a single colony and incubated overnight at 30°C with aeration at 200 rpm. One ml of overnight grown culture was pelleted in a table top centrifuge (4,000 rpm, 1 min), washed with Peptone Physiological Salt buffer (PFZ; Tritium Microbiologie, the Netherlands) and resuspended in 1 ml of PFZ. 96-well

plates were filled with 20 µl of 10x diluted washed culture and 180 µl of test media. Plates were then transferred into a plate reader (Tecan Infinite F200 Pro, Austria) for incubation at approximately 200 rpm at 30˚C. OD_{595} was measured every 10 min and read outs were used to score for positive or negative growth. Chemically defined Y1 medium [36] was slightly modified by omission of lactic acid and glucose, and lowering glutamate concentration down to 1 mM. The modified medium was supplemented with one of the following carbon sources (final concentrations in mM): glucose (12.5 mM), sodium gluconate (25 mM), glycerol (50 mM), L-fucose (25 mM), glucose-6-phosphate (25 mM), myo-D-inositol (25 mM), and N-acetyl-D-galactosamine (18 mM). Non-supplemented modified medium was used as control. Three technical replicates were performed for each condition.

Comparative genomics

In total, the genomes of 33 *B. cereus* strains were included in the comparative analysis. Next to the 20 newly sequenced food-spoilage *B. cereus* strains [33, 34] used in the growth experiments, also 13 *B. cereus* genomes including *B. cereus* ATCC 14579 and *B. cereus* ATCC 10987 were obtained from the NCBI-Genbank database (http://www.ncbi.nlm.nih.gov/genome/genomes/157) and compared to each other and to the genome of reference strain *Bacillus subtilis* 168 (S1 Table). All genomes were (re) annotated using RAST [37] to allow a better comparison of all annotations.

Orthologous groups (OGs; i.e. gene families) in the genomes were determined using OrthoMCL [38]. A database (in MS Excel) was built containing aligned information about the location and length of orthologous genes and proteins (i.e. on which contig and base pair position on the assembled genome it is present) of the newly sequenced and published reference genomes. Moreover, for every OG a multiple sequence alignment was made on the amino acid level using Muscle [39] to facilitate identification of pseudogenes (encoding incomplete proteins).

The carbohydrate utilization systems of *B. subtilis* 168 listed in the Subtiwiki database (highly curated) [40] were initially used to search for orthologous systems in the *B. cereus* OG table. Additional genes and systems were found with keyword searches and genome context analysis. Subsequently, selected genes/proteins were manually curated by comparison against sequence databases (using e.g. NCBI-BLASTP; http://blast.ncbi.nlm.nih.gov/), family/domain databases (using e.g. InterPro [41]), enzyme databases (using e.g. Brenda [42]) and pathway databases (using e.g. KEGG [43]). Gene cassettes and gene synteny were visualized using MGcV (Microbial Genome context Viewer [44]). Details of the carbohydrate utilization cassettes in the OG database can be found in supplementary S2 and S3 Tables.

Phylogeny

For the construction of a phylogenetic tree, next to the genomes of 33 *B. cereus* strains and *B. subtilis* 168, additional circular reference genomes of 18 strains from the *B. cereus sensu lato* group were included, i.e. 6 *B. anthracis* strains, 11 *B. thuringiensis* strains and 1 *B. weinenstephanensis* strain. On the basis of an OrthoMCL analysis of all these genomes, all OGs were selected with a single orthologous gene in each of the different genomes. The protein sequences of these core OGs were aligned using Muscle [39] and alignment positions with amino acid differences were selected and stored in a single artificial protein sequence. This protein sequence was used as a basis to generate a whole-genome MLST phylogenetic tree using FastTree [45].

Affiliation of 51 *B. cereus sensu lato* group strains to the phylogenetic groups (I to VII) proposed by Guinebretière et al. [22] was performed by extraction of *panC* gene sequences from the OG group and implementing in the online tool (https://www.tools.symprevius.org/Bcereus/english.php).

Results

Genome statistics and phylogeny

The 33 *B. cereus* genomes were found to contain 1775 core ortholog groups (OGs), i.e., orthologous genes that occur in all genomes only in one copy. A phylogenetic tree was built based on differences in all encoded core proteins derived from 33 *B. cereus* genomes and an additional 18 reference genomes from the *B. cereus sensu lato* group and *B. subtilis* 168 (Figure 6.1). The phylogenetic clustering of the strains is in line with the phylogenetic division of the *B. cereus sensu lato* group members based on the *panC* polymorphism published by Guinebretière [22] and the *B. cereus* strains of our study were found to belong to groups II, III, IV and VI.

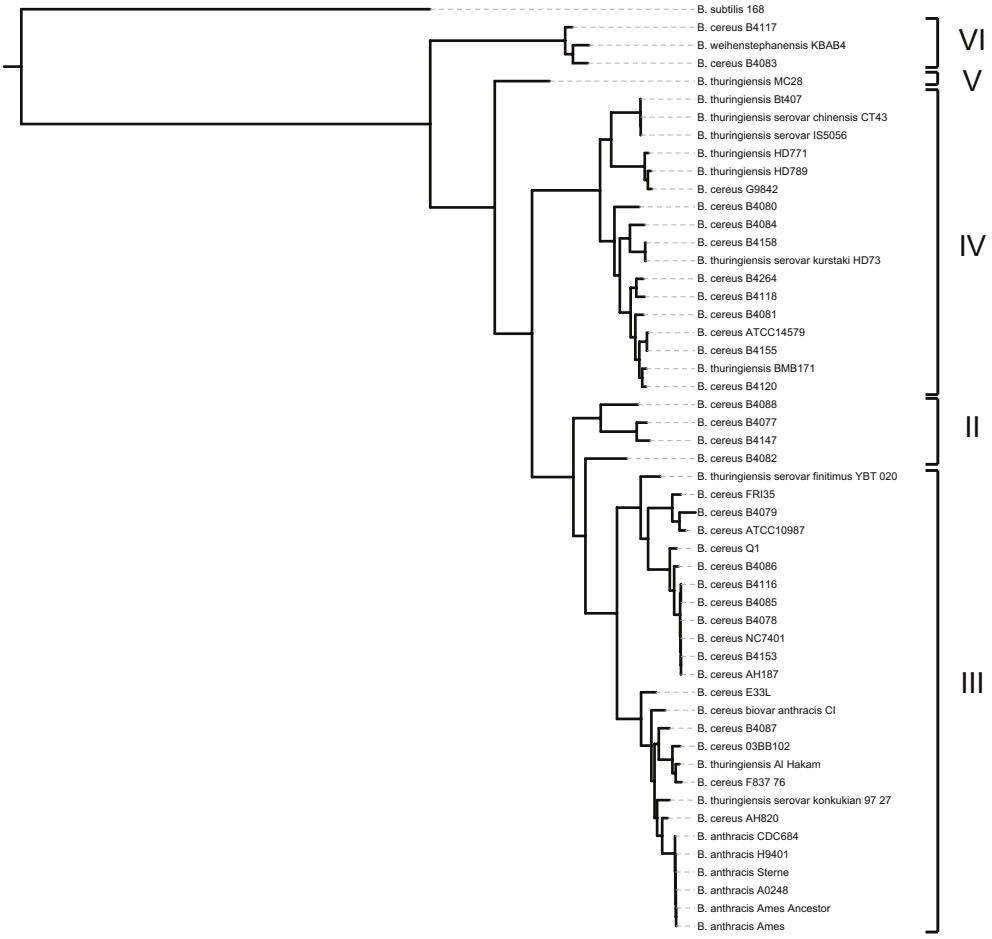

Figure 6.1. A phylogenetic tree based on the core genome of 20 newly sequenced *B. cereus* strains used in this study and 31 previously described strains of the *B. cereus sensu lato* group used for reference purposes; *B. subtilis 168* was used as outgroup. Roman numbers indicate phylogenetic groups defined previously by Guinebretière [22].

Growth experiments

In the API assay, all 22 tested *B. cereus* strains were found to grow on D-glucose, D-fructose, D-maltose, D-trehalose, N-acetylglucosamine and D-ribose, with the majority of the strains growing on sucrose, arbutin, esculin, salicin, and starch/glycogen. Only some of the strains grew on other carbohydrates, such as, lactose, mannose, galactose, cellobiose, and only *B. cereus* ATCC 10987 grew on xylose (Figure 6.2A). Growth studies in aerated defined media showed additional growth on fucose, glycerol, inositol, and gluconate for subsets of strains.

Genome mining

The *B. cereus* genomes were explored for presence of genes and gene cassettes encoding enzymes required for growth on the tested carbohydrates. The carbohydrate utilization systems of *B. subtilis* 168 listed in the Subtiwiki database (highly curated) were used to search for ortholog systems in the *B. cereus* OG table, and additional cassettes were identified by keyword searches and manual annotation. The identified cassettes are shown schematically in Figure 6.3, and the encoded functions are summarized in S2 Table**.** In general, very few pseudogenes were identified, and hence nearly all carbohydrate utilisation systems are predicted to be functional (details in S3 Table). Most of the pseudogenes were found in the *B. cereus* ATCC 14579 genome, but since the corresponding genes are functional based on the experimental data, the non-interrupted fragments of the pseudogenes may still be functional. The various *B. cereus* carbohydrate utilization systems are described below.

Carbohydrates commonly utilised by *B. cereus*

All of the 22 tested *B. cereus* strains grow on glucose (Figure 6.2) corresponding to the presence of a gene cassette encoding a glucose-specific PTS and a BglG family transcriptional anti-terminator (Figure 6.3A, Table A in S2 File). Moreover, all *B. cereus* genomes harbour a cassette encoding a glucose permease GlcU and a glucose 1-dehydrogenase Gdh (Figure 6.3C, Table C in S2 File). In addition, a permease annotated as glucose/mannose:H+ symporter (OG_5022) is present in 5 of the experimentally tested strains and reference strains 03BB102, AH820, and F837_76 (S3 Table).

All *B. cereus* strains carry four gene cassettes, each encoding a PTS transporter, an enzyme for sugar catabolism, and a transcriptional repressor, specific for transport and utilisation of fructose (*fruRKA*), trehalose (*trePAR*), N-acetyl-glucosamine (*nagABR*) and N-acetyl-muramic acid (MurNAc; *murPQR*) (Figure 6.3A, S2 and S3 Tables). Presence of those gene cassettes corresponds with the ability to utilise fructose, trehalose, and N-acetyl-glucosamine by all experimentally validated strains (Figure 6.2).

Additionally, all tested *B. cereus* strains grow on maltose, a disaccharide (Figure 6.2). The two gene cassettes for utilization of maltose present in *B. subtilis* genomes, *malARP* and *yvdEFGHIJKLM*, are absent in the *B. cereus* genomes. However, there is an equivalent, but different maltose/maltodextrin utilization cassette in all selected *B. cereus* genomes, encoding a maltose/maltodextrin ABC transporter MalEFGK, two enzymes for degradation of maltose polymers (i.e. an alpha-glucosidase/oligo-1,6-glucosidase, and a neopullulanase/alpha-amylase), and a maltose operon transcriptional regulator MalR (Figure 6.3B, Table B in S2 File, S3 Table).

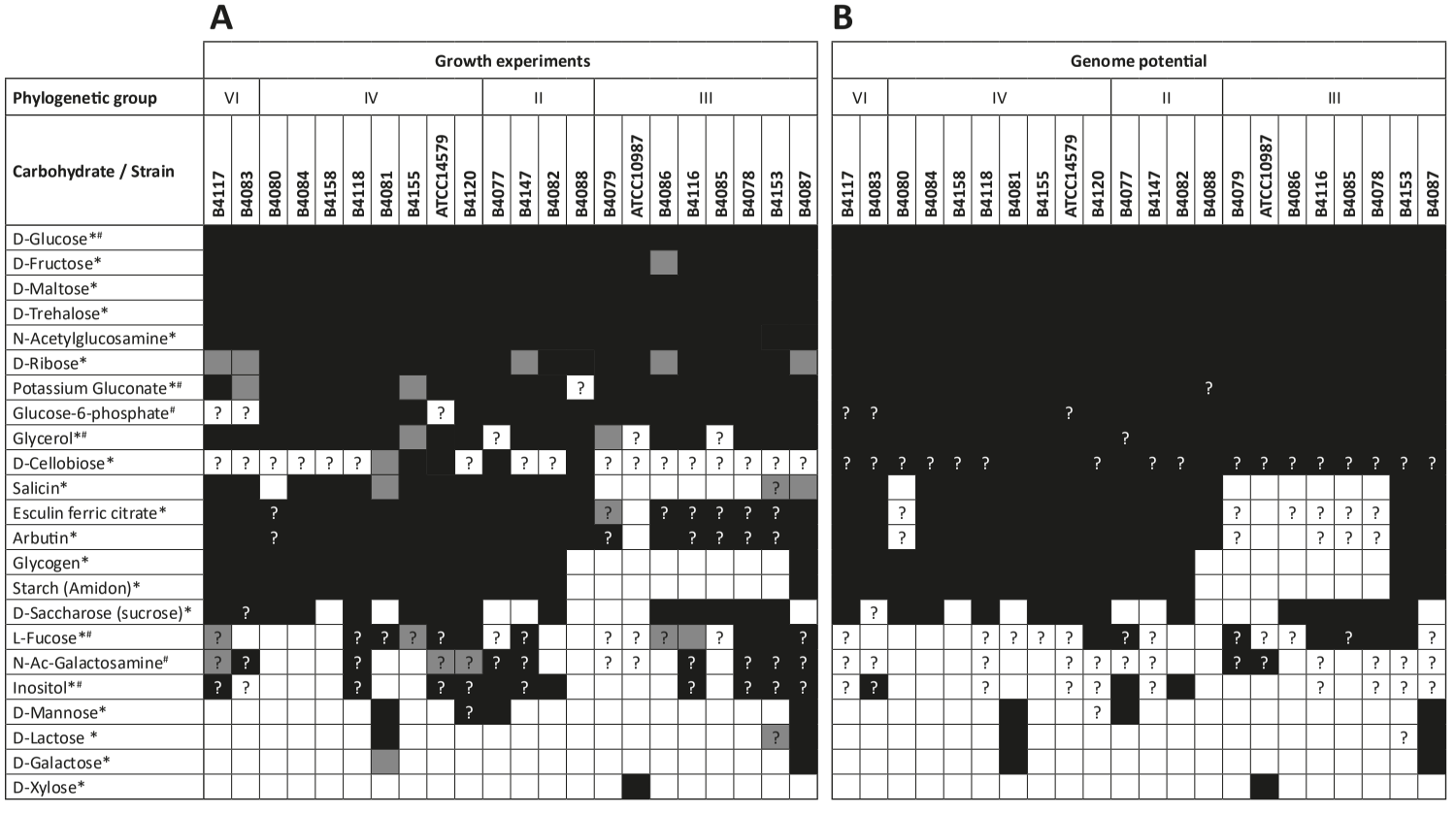

Figure 6.2. *B. cereus* carbohydrate utilisation based on growth experiments and genome potential. (A) *B. cereus* growth on different carbohydrates as determined by an API 50CHB/E test (*) and/or in defined media (#). Black: positive growth in at least two out of three replicate reactions; grey: positive growth in one out of three replicate reactions or doubtful reaction; white; no growth. (B) *B. cereus* genome potential. Black: at least one cassette present; white, no cassettes identified. Question marks indicate discrepancies between predicted and experimentally observed phenotypes.

Footnote: No colour change (hence no growth) observed for all strains in API 50CHB/E on amygdalin, D-adonitol, arabinose, arabitol, D-fucose, D-lyxose, D-mannitol, D-melezitose, D-melibiose, D-raffinose, D-sorbitol, D-tagatose, D-turanose, dulcitol, erythritol, gentiobiose, gluconate, glycerol, inositol, inulin, L-fucose, L-rhamnose, L-sorbose, L-xylose, methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside, methyl-β-D-xylopyranoside, potassium 2-ketogluconate, potassium 5-ketogluconate, and xylitol.

Finally, a complete and intact ribose utilization gene cassette *rbsRKDACB*, encoding an ABC transporter (RbsDACB), a ribokinase RbsK and a ribose operon transcriptional regulator RbsR is present in all *B. cereus* as well as in all considered *B. cereus sensu lato* group genomes and *B. subtilis* 168. All *B. cereus* strains grow on ribose (Figure 6.2), with API tests of five strains being not conclusive. The latter observation may be linked to the in general poor readability of the ribose reaction in the API test.

Soil/plant related carbohydrates

Cellobiose and xylose can function as energy sources and are common components of plant cell walls that due to the plant decay process can be found in soil, while sucrose is a naturally occurring carbohydrate found in many plants.

B. cereus genomes encode four gene cassettes annotated as cellobiose-specific PTS (Figure 6.3A, Table A in S2 File, S3 Table) with three of these found in all selected *B. cereus* strains. The fourth putative cellobiose-specific cassette is present only in strains B4082 and AH820, and consists of a PTS transporter, a 6-phospho-beta-glucosidase and a phosphoglycerate mutase. The conserved presence of the first three PTS cassettes in all *B. cereus* genomes does not correlate with growth in the API test, as only 5 *B. cereus* strains were positive for growth on cellobiose (Figure 6.2). This could be explained by incorrect annotation of these PTS. For example, alternative annotations given in NCBI data base are diacetylchitobiose-specific (= glucosamine units) for the first gene cluster, and lichenan-specific (= complex branched glucose polymer) PTS for the second and third gene clusters. The beta-glucoside PTS (*bglP*, OG_5777) is another candidate for the import of cellobiose (a beta-glucoside), but the *bglP* gene was found only in *B. cereus* strains B4077, B4083, B4088, B4117, E33L and FRI_35, which also does not agree with the API growth results on cellobiose. Thus further work is required to elucidate the roles of these clusters in carbohydrate transport and metabolism.

Notably, the previously identified gene cassette for xylose uptake and utilization [31] was found only in ATCC 10987 (and few other *B. cereus* strains (i.e. FT9, F3162-04) not included in our study) and this was indeed the only strain growing on D-xylose (Figure 6.2). The corresponding cassette encodes a xylose permease XylP, a xylose isomerase XylA, a xylulose kinase XylB, a xylose 1-epimerase XylE and a ROK family transcription regulator XylR (Figure 6.3C, Table C in S2 File, S3 Table). The origin of the cassette is not known, but it replaces gene cassettes for nitrate reductase and molybdenum cofactor biosynthesis [28] which are present in that genomic region in all other *B. cereus* genomes studied here, except strains B4079, B4081 and B4147.

Fourteen *B. cereus* strains were found to grow on sucrose, whereas 8 strains did not grow (Figure 6.2). Mols et al. [31] suggested that growth of *B. cereus* ATCC 14579 on sucrose was linked to a gene cassette composed of a sucrose-specific PTS named SacP, a sucrose-6-phosphate hydrolase SacA, a fructokinase FruC, and a sucrose operon repressor SacR

(BC0773-BC0776). The corresponding 4 genes are also present in *B. subtilis* 168 (but not in a single cassette) and are known to be responsible for growth on sucrose [46]. Indeed, an almost perfect correlation was found between the presence of this sucrose utilization cassette in *B. cereus* strains and their ability to grow on sucrose (13 of 14 strains) (Figure 6.3A, Table A in S2 File). Half of the publicly available *B. cereus* genomes also contain this cassette, suggesting that growth on sucrose is not a rare trait in *B. cereus* (S3 Table).

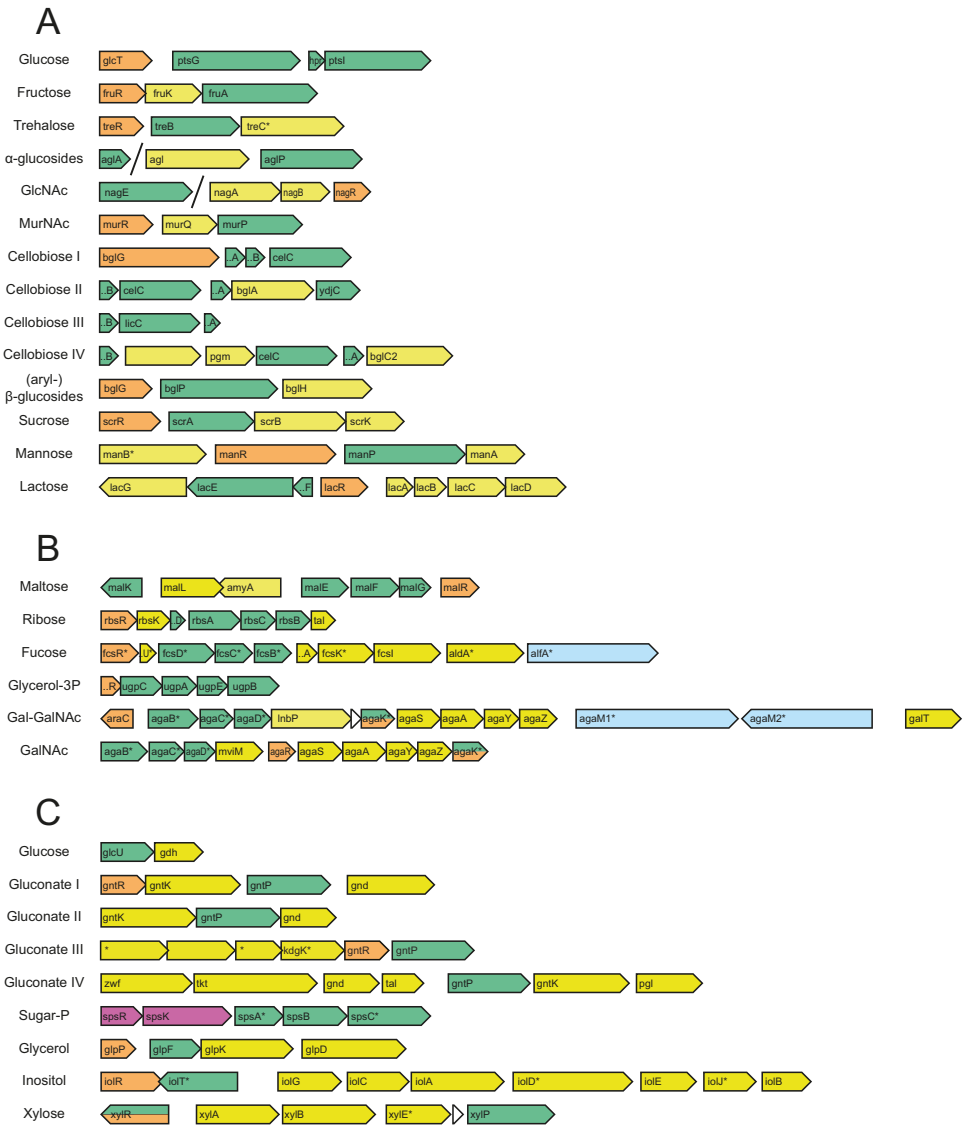

Figure 6.3. Predicted carbohydrate utilization gene cassettes. (A) PTS transporters. (B) ABC transporters. (C) secondary transporters (permeases). Colour coding for encoded proteins: green, transporters; yellow, intracellular enzymes; light blue, extracellular enzymes; orange, regulators; purple, two-component regulator. * genes with newly predicted function.

Food related carbohydrates

Bacteria present in soil are commonly transmitted via raw materials of plant (vegetable) or animal (milk, egg, meat) origin into food products. In general, raw materials and final food products are considered nutritious commodities. However, some groups of products may offer limited carbohydrate supplies, for example staple foods contain mainly starch while dairy products contain mainly lactose.

Starch is a polymer of glucose units, and consists of the linear-chain amylose and the branched-chain amylopectin. An analogue of starch, glycogen, has a similar structure to amylopectin, but more extensively branched and compact than starch. There is a glycogen biosynthesis gene cassette *glgPADCB* present in all *B. cereus* genomes, however 8 of the *B. cereus* strains are not able to utilise glycogen and starch (Figure 6.2). The genome of *B. cereus* ATCC 14579 contains an alpha-amylase gene (*amyS*, BC3482, COG0366) which was proposed to support growth on starch [31]. Alpha-amylase (EC 3.2.1.1) breaks down polysaccharides, ultimately yielding maltotriose and maltose from amylose, or maltose and glucose from amylopectin. The presence of the *amyS* gene (OG_4318) in the *B. cereus* strains correlates exactly with growth on starch or glycogen, indicating that this is the sole gene responsible for degradation of these polymeric substrates (Figure 6.2B, S3 Table). Moreover, this gene is absent in the *B. cereus* genomes available in the public database (strains E33L, FRI_35, NC7401, AH187 and Q1), suggesting that these strains will also not grow on starch/glycogen. The encoded protein AmyS of 513 residues is most likely secreted, since it has a signal peptide and a typical signal peptidase cleavage site (AYA|D). The extracellular degradation of starch or glycogen will lead to release of glucose units which can be accumulated by the maltose and glucose uptake systems and subsequently metabolised as described above.

B. cereus is commonly associated with dairy environments, and therefore we tested for utilisation of lactose, the main carbohydrate present in milk [47]. From our set of isolates, strains B4081 (isolated from Provolone sauce) and B4087 (isolated from pea soup) consistently grow on lactose (Figure 6.2), corresponding exactly with the presence of a lactose utilization gene cassette *lacGEFRABCD* in their genomes (Figure 6.3A). This cassette encodes a lactose PTS (LacEF), a 6-phospho-beta-galactosidase LacG, a lactose operon repressor LacR, a galactose-6P isomerase LacAB, a glucokinase and two enzymes of the tagatose pathway, i.e. tagatose-6-phosphate kinase LacC and tagatose 1,6-bisphosphate aldolase LacD. In both cases, the lactose utilization genes are predicted to be located on a 26-28 kb plasmid, and all encoded proteins are highly similar, suggesting that these 2 strains contain the same lactose plasmid. The 9-kb lactose utilization cassette is located on a 16-kb transposon which is flanked by Tn1546 transposases and resolvases. Parts of this plasmid, but not the lactose cassette, are also present in genomes of other *B. cereus* strains of this study (data not shown). A BLASTP analysis to the NCBI database shows presence and 100% protein sequence identity of this lactose cassette in *B. cereus*

strains m1293, VD102, VD140, NVH 0075-95, and MHI 86 (S4 Table). The next matches to these proteins are found in *Carnobacterium* and various Bacilli, with 55-75% amino acid sequence identity, while the corresponding proteins on *Lactococcus lactis* plasmids have only 45-65% identity.

Notably, only strains B4087 and B4081, though poorly, grow on galactose (Figure 6.2). We could not find any genes or gene cassettes annotated as specific for uptake of galactose in any *B. cereus* genome. Since these are the only two strains that also grow on lactose, they may also be able to utilize free galactose via the lactose PTS, as lactose PTS was previously shown to transport galactose, albeit less efficiently in lactococci [48] and streptococci [49, 50].

Host related carbohydrates

Food-borne human pathogens invading the intestinal tract and/or host cells depend on the availability of simple carbohydrates and/or degradation of complex host-associated carbohydrates/polymers, present for example in mucus. Typical host-derived carbohydrates may include fucose, mannose, N-acetyl-galactosamine and inositol [51-53].

L-fucose is a sugar present in human milk as building block of oligosaccharides, mucins and other glycoconjugates in the intestinal epithelium [54]. None of the strains grew on D-fucose or L-fucose in the API test, but in the defined medium growth experiments with aeration 8 strains showed growth on L-fucose, while 4 strains showed questionable growth (Figure 6.2). Fucose transport via a permease, an ABC transporter or a PTS transporter and subsequent utilization have been described in Gram-negative [55-57] and Gram-positive bacteria [54, 58]. Additionally, extracellular alpha-fucosidases have been characterized from several bacilli [59-61], including *B. cereus* [62]. We identified a cassette comprising 10 genes for fucose utilization (Figure 6.3B, S3 Table), that includes a fucose ABC transporter FcsBCD, in seven of experimentally verified *B. cereus* strains and in genomes of Q1, NC7401, and AH187. This cassette encodes an extracellular alpha-L-fucosidase AlfA (Table B in S2 File), suggesting that fucose can be cleaved from complex oligosaccharides, and then taken up into the cell and metabolized, ultimately to dihydroxyacetone phosphate and lactate, since a lactaldehyde dehydrogenase AldA is also encoded, next to an L-fucose isomerase FcsI, an L-fuculose kinase FcsK, an L-fuculose-6P aldolase FcsA, and a LacI family transcription regulator FcsR. Not all strains caring the complete cassette were able to grow on fucose (Figure 6.2), while some strains lacking this cassette did grow on fucose, indicating that additional genes may be involved in fucose utilization.

Four *B. cereus* strains were found to grow on mannose, i.e strains B4077, B4081, B4087, and B4120 (Figure 6.2). A mannose utilization cassette *manRPA* is present in genomes of three of those tested strains (B4077, B4081 and B4087) and in genomes of *B. cereus* strains 03BB102, F837_76, Q1 and in *B. subtilis* 168. The cassette encodes a mannose-specific PTS transporter ManP, a mannose-6P isomerase ManA and a BglB family transcriptional

antiterminator ManR (S2 Table). The *manRPA* cassette, as well as any other gene cassette annotated as "mannose" utilization, was not found in strain B4120.

A 9-gene cassette *iolRTGCADEJB* for myo-inositol utilization, orthologous to that of *B. subtilis* 168, is present in three of the 22 experimentally verified strains B4077, B4082, B4083 and in genomes of *B. cereus* reference strains 03BB102, AH820, BCM2_134A, CI, E33L (with an additional plasmid-located cassette), F837_76 and FRI_35. None of the strains grew on inositol in the API test, but 11 strains were found to grow in the aerobic growth experiment in defined medium (Figure 6.2) including two strains harbouring the *iol* cassette (i.e. B4077, B4082). Therefore, there must be an additional cassette for inositol utilization.

Additionally, 8 strains were observed to grow on N-acetyl-galactosamine in aerated medium, while 3 strains showed possible growth (Figure 6.2). The annotation of most of the genes in a 10-gene cassette (BCE1899-BCE1908 of strain ATCC 10987), previously suggested to be related to growth on tagatose [31], suggests that the actual substrate could be N-acetyl-galactosamine. The cassette encodes a 3-component ABC transporter (putatively for N-acetylhexosamines; OG_9457, OG_6509, OG_6510), and all enzymes required for degradation of N-acetyl-galactosamine via the tagatose pathway to glyceraldehyde-3P (Table B in S2 File) [63]. However, we find this complete gene cassette only in *B. cereus* strains B4079, ATCC 10987, E33L, and FRI_35 which does not correlate well with observed growth on N-acetyl-galactosamine. Nevertheless, none of the strains grow on tagatose in static conditions (Figure 6.2), therefore it seems unlikely that this gene cluster is related to growth on tagatose. We found another 14-gene cassette presumably for utilization of the disaccharide Gal-(1->3)-beta-GalNAc (galactose linked to N-acetyl-galactosamine) (Table B in S2 File). Gal-GalNAc is bound to proteins in mucins (O-linked to Ser/Thr), and can be released by extracellular endo-alpha-N-acetylgalactosaminidases. This cassette is very similar to the cassette described above for utilization of N-acetyl-galactosamine, but it has 2 additional genes encoding extracellular endo-alpha-N-acetyl-galactosaminidases, and a gene encoding an intracellular 1,3-beta-galactosyl-N-acetylhexosamine phosphorylase which cleaves Gal-(1->3)-beta-GalNAc into alpha-D-galactose-1P and N-acetyl-D-galactosamine. This cassette is found in 8 of 22 experimentally verified *B. cereus* strains and two reference genomes (Q1 and NC7401) (S3 Table), but it could not explain utilisation of Gal-GalNAc nor N-acetyl-galactosamine by strains B4083, B4087, B4118, and B4147.

Recently, a new sugar phosphate uptake system specific for glucose-6-phosphate and fructose-6-phosphate has been described in *B. cereus* [64]. This 5-gene cassette includes a transporter SpsABC and a two-component sensor system SpsKR which responds to extracellular sugar phosphate levels. The *spsA* gene was specifically expressed during oral infection of *Galleria mellonella* [65]. The cassette was found in all the *B. cereus* genomes, and all but three (B4083, B4117, ATCC 14579) experimentally validated strains were observed to grow on glucose-6-phosphate (Figure 6.2).

Other carbohydrates

In *B. cereus* genomes there are several putative gluconate uptake and utilization systems, based on the gluconate permease GntP. The annotation of these systems suggests that the substrate is gluconate, but it could also be glucuronate or galacturonate, or derivatives of these acids. Orthologs of the gluconate utilization gene cassette *gntRKPZ* of *B. subtilis* 168 [66] were found in 8 of 22 experimentally verified strains and in additional 8 genomes (S3 Table). All but one (ATCC 10987) experimentally validated strains belonging to phylogenetic group III carried this cassette encoding a gluconate permease GntP, a gluconate kinase GntK, a 6-phosphogluconate dehydrogenase GntZ, and a gluconate operon repressor GntR. A second cassette (system II), not present in *B. subtilis* or *B. anthracis,* is the same as system I but lacks the regulator, and is present in 16 out of 22 experimentally validated strains, mainly not belonging to phylogenetic group III, and five additional genomes (S3 Table). A third cassette (system III) with a gluconate permease GntP is present in all considered *B. cereus sensu lato* group genomes, but not in *B. subtilis*. This cassette also encodes a GntR family transcription regulator, a 2-keto-3-deoxygluconate kinase, an amidohydrolase, an aldolase and a pyridoxal phosphate-dependent enzyme, suggesting that this cassette may encode utilization of a derivative of gluconate such as D-glucosaminate. Finally, a fourth 7-gene cassette (system IV) is present in all *B.cereus* genomes, and next to the *gntP, gntK* and *gntZ* genes it contains genes encoding 6-phosphogluconolactonase Pgl, glucose-6-phosphate 1-dehydrogenase, transketolase, and transaldolase (Table C in S2 File). However, in a few strains, i.e. *B. cereus* strains B4083, B4088, B4117, and G9842, this cassette lacks both the *gntP* and transketolase genes, suggesting that this cassette represents a breakdown pathway of glucose-6P via gluconate-6P, and not an uptake system for gluconate.

None of the strains grew on potassium gluconate in the API test, but in the growth experiments with aeration most strains showed growth (Figure 6.2) suggesting that metabolism of this carbohydrate requires oxygen. Only strains B4083, B4088, and B4155 showed poor or no growth on gluconate. This phenotype correlates best with the gluconate cassette system IV, and therefore this cassette may represent the main gluconate utilization system under these growth conditions.

Arbutin, esculin and salicin are aryl beta-glucosides (or aromatic beta-glucosides), consisting of a glucose moiety linked to an aromatic ring. The *B. subtilis* 168 genome encompasses 3 genes encoding intracellular (aryl-) phospho-beta-D-glucosidases, i.e. *bglA* (BSU40110), *bglC/yckE* (BSU03410), and *bglH* (BSU39260) [67]. In the *B. cereus* genomes there is no equivalent of *bglA* of *B. subtilis*. The *bglC* gene (OG_4907) of *B. subtilis* 168 has one ortholog in 11 *B. cereus* strains, but it is not part of a sugar utilisation cassette, while a second ortholog of *bglC* is found only in strains B4082 and AH820 as part of a 7-gene cassette for cellobiose utilization. Finally, *B. cereus* strains B4077, B4083, B4088, B4117, E33L and FRI_35 carried orthologues of *B. subtilis* 168 *bglPH* operon encoding

a beta-glucoside-specific PTS system BglP, and an aryl-phospho-beta-D-glucosidase or aryl-6-phospho-beta-glucosidase BglH [68] additionally encoding also a beta-glucoside *bgl* operon antiterminator BglG. Taken together, there are 14 *B. cereus* strains that have 1 or 2 genes encoding an aryl-6-phospho-beta-glucosidase. It is not clear what the transporter is for aryl beta-glucosides in these strains, but it is possibly a more general PTS for beta-glucosides, such as for cellobiose (see above for 3 putative PTS cellobiose utilization cassettes). This occurrence of one or more aryl-6-phospho-beta-glucosidases in 12 *B. cereus* strains correlates almost perfectly with the growth observed on salicin (Figure 6.2). The only discrepancies correspond to uncertainties in the API test. On the other hand, there are 20 strains that grow on arbutin and esculin, suggesting that there may be an additional system in the other strains which is more specific for arbutin and esculin, but not for salicin.

None of the strains grew on glycerol in the API test, but in the growth experiments with aeration all but three (B4077, B4085, and ATCC 10987) strains showed growth (Figure 6.2). A glycerol utilization cassette, equivalent to the *glpPFKD* genes (BSU09270-BSU09300) of *B. subtilis* 168, was found to be present in all *B. cereus* genomes. The cassette encodes a glycerol uptake facilitator protein GlpF, a glycerol kinase GlpK, an aerobic glycerol-3-phosphate dehydrogenase GlpD, and a glycerol uptake operon regulator Glp (Table C in S2 File). Therefore, all strains have the potential to grow on glycerol in aerobic conditions.

A glycerol-phosphate uptake system, based on an ABC transporter UgpBEAC, was found in most of *B. cereus* genomes, but not in strains B4082, B4083, B4088, B4147, 03BB102, and F837_76. Moreover, all *B. cereus* genomes encode a glycerol-3-phosphate permease (OG_511). However, this substrate was not experimentally tested.

Absent sugar utilization systems

There are a few sugar utilization cassettes of *B. subtilis* 168 which did not have ortholog cassettes in any of the *B. cereus* strains. These include the mannitol cassette *mtlAFD* (BSU03981, BSU03982, BSU03900), the sorbitol/glucitol cassette *gutRBP-fruC* (BSU06140-BSU06170), the arabinan/arabinose cassette *abnA-araABDLMNPQ-apfA* (BSU28720-BSU28810), the L-rhamnose cassette *rhaAMBR(EW)* (BSU31180-BSU31220), and the oligomannoside cassette *gmuBACDREFG* (BSU05810-BSU05880). In agreement with this lack of genes, the selected *B. cereus* strains in our study did not grow on mannitol, sorbitol, arabinose or rhamnose (Figure 6.2).

Orthologs of the levan/fructose-specific PTS system *levDEFG* of *B. subtilis* 168 are only found in *B. cereus* strain E33L on a plasmid. However, the cassette in E33L is much larger (12 genes) and may be specific for breakdown of more complex polysaccharides as levan is a fructose polymer (fructan).

Discussion

Equipment of ubiquitous food-borne human pathogens such as *B. cereus* with a broad range of carbohydrate transporter and utilisation systems supports their transmission from soil to host. Comparative analysis of genotypic and phenotypic diversity of carbohydrate utilisation among 20 newly sequenced *B. cereus* food isolates and *B. cereus* ATCC 14579 and ATCC 10987 suggests an adaptative metabolic capacity to different environmental niches represented by carbohydrates such as cellobiose or xylose found in soil, starch or lactose in food and dairy products, and fucose and glucose-6-phosphate found in intestinal tracts of animal and human hosts. In addition to such strain-specific features, all the strains could utilise compounds typically reported to support growth of *B. cereus* such as glucose, fructose, maltose, trehalose, N-acetyl-glucosamine, and ribose [23, 69, 70].

The diversity in sugars utilised by *B. cereus* isolates may correspond to the classification of the isolates to four of the phylogenetic groups identified previously by Guinebretière [22] within the *B. cereus sensu lato* group. For example, strains in groups VI and II show a broader substrate utilisation capacity compared to strains belonging to group III. Members of the latter group, with exception of strain B4087, did not grow on glycogen, starch or salicin. In addition, the genomes of these strains lack aryl-6-phospho-beta-glucosidases involved in the utilisation of two other aromatic glucosidases, esculin and arbutin. Notably, strain B4087 displayed a distinctive growth pattern and genome potential compared to the other members of group III. Within group IV, a sub-branch encompassing strains isolated from vegetables (B4080, B4084, B4158), showed limited substrate utilisation. Interestingly, two strains (B4079 and ATCC 10987) representing a sub-branch within group III showed the least carbohydrate utilisation capacity, and both strains originate from spoilage incidents, namely from a retorted can with chocolate beverage and spoiled cheese, respectively.

Carbohydrate uptake in bacteria is mediated by different transport mechanisms including phosphoenol-pyruvate-dependent phosphotransferase systems (PTS systems), ATP-binding transporters (ABC transporters) and secondary transporters (permeases). We searched for transporters and associated metabolic enzymes in the genomes of newly sequenced *B. cereus* isolates and identified a significant number of new carbohydrate utilisation cassettes. The collective capacity of *B. cereus* strains to utilise carbohydrates is visualised in Figure 6.4. Carbohydrate uptake in bacilli occurs mainly by PTS systems as indicated by identification of 15 PTS cassettes in *B. cereus* genomes (see worksheet PTS systems in S3 File), while ABC transporters and permeases for carbohydrate uptake contribute with 6 and 11 identified systems, respectively (see worksheet ABC-permeases in S3 File).

The possible roles of specific carbohydrate transporter and utilisation systems in transmission of *B*. *cereus* from soil, via food processing environments and food, to the host, is discussed below.

The soil environment is rich in chitin, a constituent of insects exoskeletons and cuticles but also fungal cell walls. Chitin is a 1-4 polymer of N-acetyl-glucosamine and by activity of commonly found chitinases, simple amino sugars can be released and become available for soil bacteria [3]. Amino sugars such as N-acetyl-glucosamine and N-acetyl-muramic acid are also the major components of bacterial peptido-glycan. The presence of *nagEABR* and *murRQP* cassettes in all tested isolates perfectly correlates with the observed growth on these substrates.

Cellobiose is a catabolite of cellulose degradation, and is therefore also present in soil and decaying vegetation. While all the *B*. *cereus* isolates possess three or four cassettes for cellobiose utilisation, only five *B*. *cereus* isolates were found to utilise cellobiose in static conditions, which suggests that activation of cellobiose systems is not achieved in most cases. Conceivably, activation of cellobiose uptake and utilisation systems requires specific conditions that remain to be elucidated. In *Listeria monocytogenes* presence of β-glucosides (cellobiose, salicin but not arbutine) in the environment causes repression of virulence genes via a specific regulatory pathway (involving *bvr* locus) independent from carbon catabolite repression (CCR) mechanism [71, 72] as well as via a CCR mechanism. A specific role for cellobiose in repression of *B. cereus* enterotoxin production has not been reported up to now.

Notably, growth on xylose, another common plant-derived carbohydrate was only observed for *B*. *cereus* ATCC 10987, and this perfectly matched the unique presence of the previously described cassette for xylose utilisation in this strain [28].

Concerning transport and utilisation systems related to food, the capacity to grow on lactose, a common carbohydrate in milk and dairy environment, is restricted to only two of 22 tested *B*. *cereus* strains. This is in line with previous observations, that showed limited ability to utilise lactose among *B*. *cereus* dairy farm isolates, although prevalence increased along the production chain, reaching 20% lactose-positive strains among *B*. *cereus* household milk isolates [47], pointing to the selection and adaptation of the strains in the dairy chain. The two lactose-positive strains in our study contain a lactose utilisation cassette predicted to be on a plasmid. This lactose cassette is also present in genomes of a few other *B*. *cereus* strains not included in this study (S4 Table). Similarity with cassettes present in other bacteria is limited, suggesting that mobility of this *B*. *cereus* specific lactose cassette may be responsible for the increasing prevalence of lactose-positive strains.

Notably, *B*. *cereus* potential to growth on starch was found to depend on the presence of a single gene encoding alpha-amylase, AmyS. This extracellular enzyme degrades starch releasing glucose that can be transported and utilised by one of the glucose uptake systems commonly found in *B*. *cereus* strains.

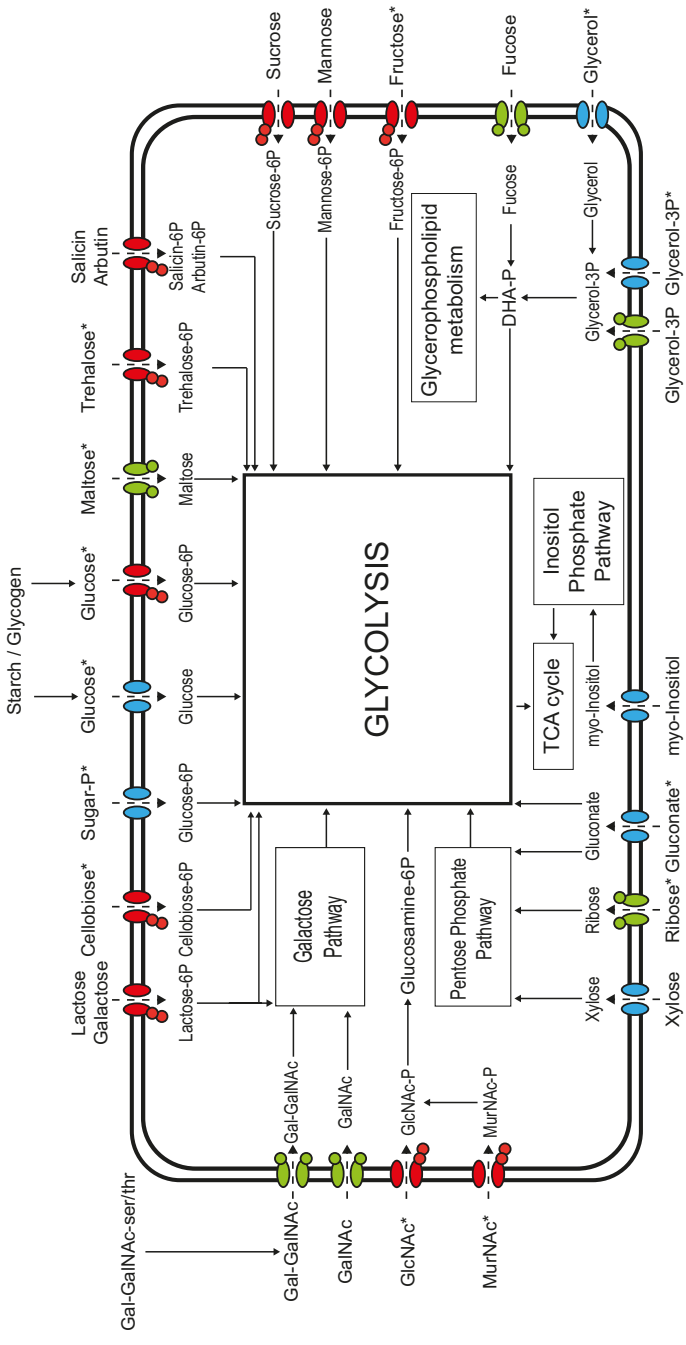

Figure 6.4. Schematic overview of predicted carbohydrate utilization systems in *B. cereus*. For transporters, green indicates a putative ABC transporter; red, a putative PTS transporter; and blue, a putative permease. Star indicates systems present in all 22 strains. Abbreviations used are N-Acetyl-glucosamine (GlcNAc), N-Acetyl-galactosamine (GalNAc), galactose-N-Acetyl-galactosamine (Gal-GalNAc), N-Acetyl-muramic acid (MurNAc), Dihydroxyacetone phosphate (DHA-P).

Concomitant with milk or other food products *B. cereus* can enter the host gastro-intestinal tract and encounter a nutrient-limited and competitive environment. One of the available substrates is fucose, a major component of mucin glycoproteins. Fucose is abundant in the human intestine [73] and is often associated with biologically active molecules such as serum glycoproteins, immunoglobulins, blood group substances, or gastric and submaxillary mucins [61]. Of the 22 tested *B. cereus* strains, seven contained a *fcs* fucose utilisation cassette in their genomes, whereas 12 strains showed growth or poor growth on fucose, suggesting the presence of an additional or alternative system. The Fcs system includes extracellular α-L-fucosidase that may liberate fucose from natural substrates e.g. host mucin, but not always from artificial substrates such as p-nitrophenyl or α-L-fucoside as used in our assay. However, several soil isolated Bacilli are able to utilise both categories of substrates by producing more types of α-L-fucosidases [60-62]. Although fucose and the *fcs* system influence *Streptococcus pneumoniae* virulence [74] and the human gut microbe *Bacteroides thetaiotaomicron* can harvest monomeric fucose from host Fucα1,2-Galβ-containing structures [73], the role of the Fcs system in *B. cereus* performance in the intestine and/or pathogenicity remains to be elucidated.

A disaccharide Gal-GalNAc (galactose linked to N-acetyl-galactosamine) is also bound to proteins in mucins. Prior to uptake and utilisation these mucin-bound sugars can be released by endo-alpha-N-acetylgalactosaminidases which are generally found in the human gut. Notably, 8 of the tested *B. cereus* strains encode this extracellular enzyme. The cassette for Gal-GalNAc utilisation possess also the genes required for utilisation of N-acetyl-galactosamine. Theoretically if GalNAc would be transported into the cell, it could be utilised by those 8 strains. However, that still does not explain growth of 11 of the tested *B. cereus* strains on GalNAc. Interestingly all but one of the strains utilising GalNAc could also use inositol, and the majority of them could use fucose as well suggesting their adaptation to the gut environment.

Finally, 19 of tested *B. cereus* strains were shown to utilise glucose-6-phosphate, while genomes of all the strains carry the conserved sugar-phosphate specific *spsRKABC* cassette [64]. Sugar phosphates can be found in a range of hosts including the perithropic matrix separating food bolus from the mid-gut epithelium cells of insects, possibly facilitating initial multiplication of bacteria in the gut and their persistence, an initial step for host infection [75]. In particular the Sps system was shown to play a role in *B. cereus* sugar phosphate sensing and uptake during growth in the intestinal environment of *Galleria mellonella* larvae [64]. Moreover, glucose-6-phosphate was shown to inhibit expression of *hlyII*, encoding the pore-forming toxin haemolysin II, by activation of HlyIIR in plasmid-curated acrystalliferous *B. thuringiensis* [32].

Besides the effect of available carbohydrates on the expression of virulence factors, that may facilitate the host infection as well as outcompeting competitive flora, carbohydrates were shown to affect biofilm formation both in gram-positive and gram-negative bacteria

[76-78]. Formation of biofilms can facilitate colonisation of environmental niches including soil, food-processing environments and host intestine [79]. Roles of the different carbohydrates in *B. cereus* biofilm formation and virulence remain to be established.

The analysis of complete carbohydrate transporter and utilization cassettes allowed for the prediction of substrate utilization. Particularly for carbohydrate substrates used by only small numbers of strains such as xylose, lactose, mannose, sucrose, starch/glycogen, and salicin, there is an excellent correlation with the presence of the corresponding gene cassettes. Discrepancies between observations and predictions are conceivably due to: i. lack of expression of cassettes in tested conditions, e. g. availability of oxygen, as the number of strains utilising gluconate, L-fucose and inositol was higher in defined, aerated media compared to static API tests; ii. miss-identification of cassettes based on incorrect or incomplete prediction of carbohydrate specificity of transporters; and iii. lack of identification of cassettes e.g. presence of several ABC transporters with unknown specificity and proteins annotated only as "membrane proteins" may function as transporters (e.g. permeases). The latter two points of the genome-mining approach are clearly limitations for the identification of novel sugar utilization pathways which have not been described in literature earlier.

In conclusion, this study provides extensive information on the genetic potential of *B. cereus* strains as well as the corresponding potential to utilise carbohydrates for growth, reflecting strain diversity and the capacity to occupy different niches including soil, food products as well as intestinal tract of insects and mammals.

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Supporting Information

Supplementary Tables S1 – S4 can be found in the online version of the article.

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Chapter 7

General discussion

Bacterial spores are ubiquitously present in the environment and are therefore inevitable contaminants in food and food ingredients, causing a risk of spoilage and/or foodborne illness. Spores can withstand harsh environmental conditions including treatments commonly applied in food processing. Due to their resistance properties, effective preservation treatments are required to inactivate spores, but the desire for milder production processes and reduction of preservatives creates an additional challenge for food industry. Thus, milder processing requires good understanding of spore behaviour to design milder preservation concepts. For research purposes, laboratory conditions are tightly controlled and include a limited number of variables to limit batch to batch variation of produced spores. However, in practice heterogeneity could be much larger, and this illustrates the complexity of spores produced outside the laboratory since environmental sporulation conditions can be highly diverse next to a variety of other factors that may determine spore characteristics. It is well known that sporulation history can affect spore behaviour. Moreover, diversity within a single species or even within a spore population of a single strain can have substantial impact on outgrowth potential and associated spoilage and/or safety risks.

In the thesis project, factors contributing to delayed outgrowth of dormant spores at the germination stage (Chapter 2), the outgrowth stage (Chapter 3), and processes involved in spore damage and repair (Chapters 4 & 5) were identified. Additional aspects with impact on spore germination and outgrowth such as recovery conditions including food matrices (Chapters 3 & 5) and strain specific growth requirements (Chapter 6) were characterized. Figure 7.1 presents an overview of factors investigated that underlie spore performance at various stages of the germination and outgrowth process in this thesis and corresponding thesis chapters. Findings for laboratory *B. cereus* strains were extended to food isolates to address how diversity, both on genotypic as well as phenotypic level, affects behaviour (Chapters 2 & 6).

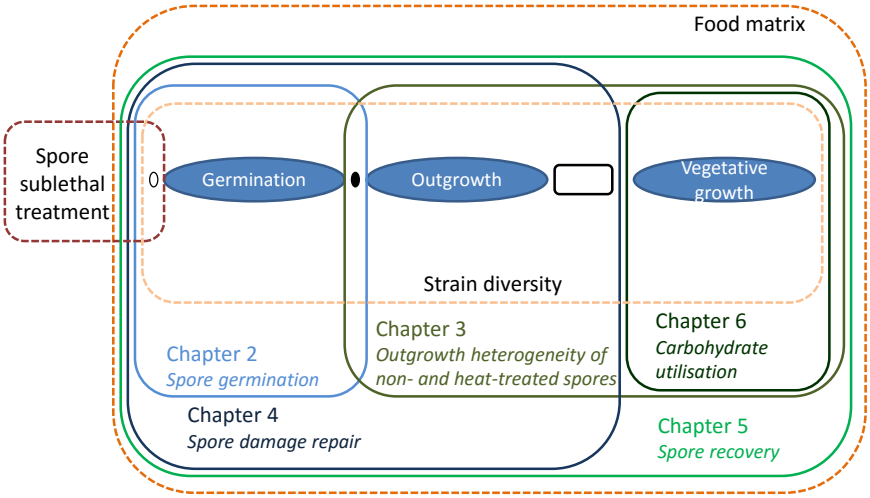

Figure 7.1. Overview of research themes addressed in this thesis.

Genes potentially involved in recovery from spore damage

Thermal treatment is a commonly applied method to inactivate spores in food industry but also at home in the kitchen. Notably, in contrast to other stresses i.e. dry heat, acid, disinfectants and UV treatment, the mechanisms involved in inactivation of spores by wet heat treatment are still poorly understood at the mechanistic level and this requires more research. Wet heat treatment is assumed to cause damage to single or multiple spore proteins, however the identity of the damaged protein(s) is still unknown. Available data in literature suggest that proteins involved in both germination and outgrowth of spores are affected by wet heat treatment [1].

At the start of this thesis project, delayed and/or unpredicted outgrowth of spores was frequently ascribed to the presence of damaged spores [2-5]. Selective plating using sub-optimal recovery conditions, typically achieved by addition of growth inhibiting compounds as for example salt or acid, has been used to quantify the number of damaged spores in surviving populations upon exposure to sublethal treatments i.e. heat and disinfectant [6-10]. In this thesis, salt supplementation was successfully used to search for heat treatment conditions that result in a high fraction of damaged spores (Chapter 2). By using a gradient of salt, supplemented to media used for plating of untreated and heat treated spores, severely and mildly damaged fractions in the spore population could be discriminated (Chapter 4).

Heat treatment conditions were selected that led to a high fraction of damaged spores to study outgrowth behaviour of the damaged spores at the population level. Relative change in OD_{595} during the germination and outgrowth of heat treated spores in combination with

microscopic observations, was used to demonstrate a delay in germination and outgrowth of heat treated spores compared to untreated spores (Chapter 4). To date, it remains a challenge to discriminate outgrowth from heat damaged spores and outgrowth from a small fraction of undamaged spores that survived the heat treatment. The advantages and limitations of available single spore/cell approaches are discussed in a separate section on available tools to study behaviour of damaged spores at single spore level (see below).

To gain insight in molecular mechanisms involved in outgrowth and recovery of heat treated spores, transcriptome profiling was performed during the germination and outgrowth process of heat treated versus untreated *B. cereus* spores. In the approach taken, we focussed on genes specifically expressed during outgrowth of heat damaged spores (Chapter 4). This approach allowed for identification of genes potentially involved in outgrowth of damaged *B. cereus* spores, including a gene encoding CdnL1, a putative CarD_CdnL_TRCF family transcriptional regulator. In the selected heat treatment conditions used, deletion of *cdnL1* resulted in a higher fraction of severely damaged spores compared to the wild type.

A possible role of additional putative damage repair factors was assessed by comparative analysis of damage recovery of wild type and a range of targeted deletion mutants (Chapter 5). In the tested conditions, with the fraction of damaged spores mainly composed of severely damaged spores, mutants lacking *cdnL1* or *cdnL2*, encoding CdnL paralogues (orthologues to YdeB of *B. subtilis*), showed improved or comparable recovery as the wild type, while a double *cdnL1/cdnL2* deletion mutant showed reduced recovery in laboratory as well as food based media, indicating that overlap in functionality between the two paralogues may take place (Chapter 5). The role of the individual *cdnL1* and *cdnL2* genes as well as their interactions and regulons remain to be elucidated. According to the string network [11] based on neighbourhood of homologue genes in other genomes, both *cdnL1* and *cdnL2* can be linked to *ispD*/*ispF* involved in terpenoid metabolism and to BC0105, an orthologue of *yacL* of *B. subtilis*. Indirectly, via BC0105 (or its equivalent *yacL* in *B. subtilis*) both *cdnL1* and *cdnL2* genes (or in case of *B. subtilis cdnL2* orthologue *ydeB*) are associated with *disA*, encoding a DNA integrity scanning protein DisA that was shown to delay outgrowth of *B. subtilis* oxidatively damaged spores until repair of DNA has occurred [12]. *B. cereus* also expresses *disA* upon spore outgrowth from both untreated and heat treated spores, which suggests a role of DisA in DNA repair in this species (Chapter 4). In *B. subtilis yacL* connects (via gene neighborhood and/or co-occurrence) to two more interesting genes, *radA* encoding DNA repair protein RadA that may play a role in the repair of endogenous alkylation damage, and *ctsR*, encoding CtsR transcriptional regulator controlling the expression of the cellular protein quality control genes *clpC*, *clpE* and *clpP*.

Interestingly, the sporulation process was affected in a number of deletion mutants lacking genes with putative role in outgrowth of damaged *B. cereus* spores (Chapter 5). For the BC1312 mutant, deletion led to disruption of the sporulation process and formation of

multiple granules within the cell (data not shown). The constructed deletion in BC1312 was in-frame but polar effects on the BC1311 gene, located 148 bp down-stream, and encoding one of the α/β-type small acid-soluble spore proteins (SASPs), cannot be excluded. The BC1311 gene encodes an orthologue of a minor SASP [13], *sspD* of *B. subtilis*. SASPs are synthesized during late sporulation, and compose up to 20% of total spore core proteins and play an important role in protecting DNA from damage [14]. However, deletion of genes encoding one or more minor α/β-type SASP displayed no obvious phenotypic effect in the *B. subtilis* sporulation [15]. Alternatively, the gene product of BC1312 may be required for utilization of poly-β-hydroxybutyrate (PHB), a compound known to accumulate in the form of granules in bacterial cells, serving as a carbon and energy source during the late sporulation process in *B. cereus* and other species [16]. One could speculate that when PHB synthesis is initiated during sporulation [17], in a strain with a non-functional repressor (BC1314 appears to be a pseudogene) it would result in uncontrolled PHB synthesis. Together with the inability to utilize PHB, i.e. by deletion of BC1312, it may result in disruption of the sporulation process due to lack of energy. During sporulation of the *ΔBC1312* mutant, granule formation was observed which may be linked to excessive accumulation of PHB. Clearly the transcriptome approach used led to identification of genes involved in wide range of processes and their contribution at different stages of spore formers life cycle indicating importance of the newly identified factors. Their roles in recovery of heat treated spores and other physiological processes remains to be determined.

Tools to study behaviour of damaged spores at single spore level

Most studies on spore inactivation use plate counting methods to evaluate the impact of recovery conditions on spore survival and to quantify the number of damaged spores, whereas the relative changes in OD_{595} are mainly used to determine the kinetics of germination and/or outgrowth [9, 18-20]. Despite their usefulness at the population level, both methods are limited by the fact that the outcome represents the average behaviour of the population, and not the behaviour of individual spores. In the past decade, new techniques to follow single spores/cells were developed and resulted in the intensification of studies on single spore germination and to a lesser extent on outgrowth from single spores. Germination and early outgrowth is traditionally followed with microscopy, in particular phase contrast microscopy can be used to follow the gradual shift from dormant phase bright spores into the germinated phase dark spores, the decrease in refraction is associated with uptake of water during the germination process. Figure 7.2 represents tools used in this thesis to study behaviour and processes in heat damaged spores at population and single spore level.

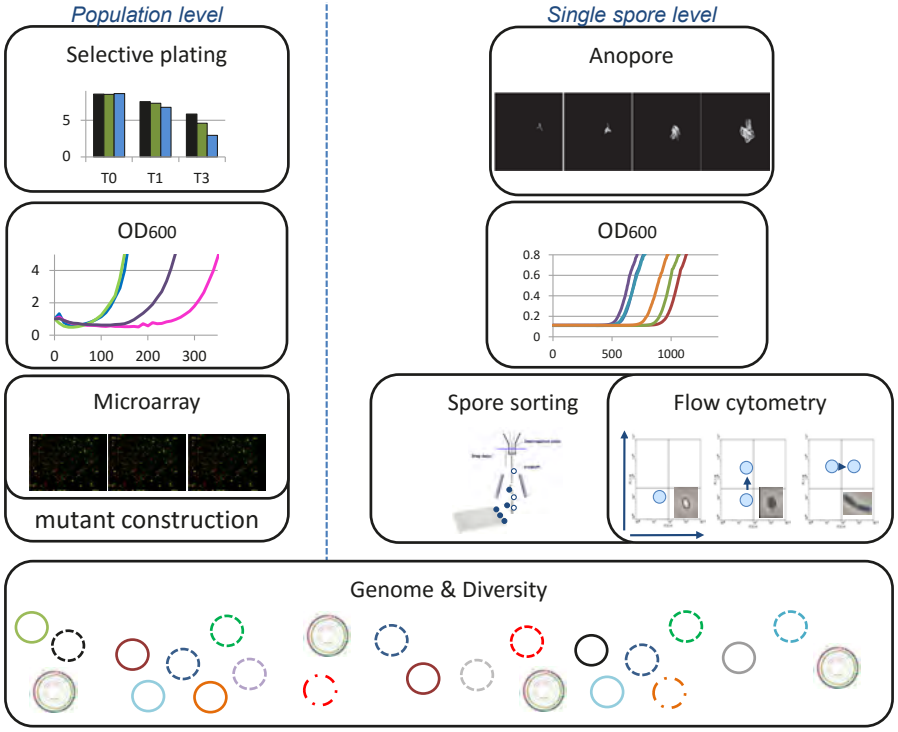

Figure 7.2. Tools to study spore damage at population and single spore level used in this thesis.

Time-lapse phase contrast or fluorescence microscopy is a tool that can be used to study the spore developmental processes in time. In combination with software developed to track individual spores, the behaviour of multiple spores can be simultaneously analysed. Time-lapse approaches are widely used to study germination kinetics, in particular the length of the lag time of multiple individual spores [21-23]. Recently, media of increasing complexity are applied, for example with added tea extracts [24], for evaluation of germination and outgrowth of individual spores. However, the requirement for transparency of the medium remains a limitation of time-laps phase contrast microscopy as well as OD measurements for studies in complex media or food matrixes.

As an alternative, the Anopore technique can be used to monitor outgrowth from single germinated spores until the microcolony stage, typically consisting of up to 256 cells. Anopore has been applied to test heterogeneity in spore outgrowth of heat treated and control *B. cereus* spores in the presence of secondary stresses [25]. In contrast to phase contrast microscopy, it can be used with complex media such as food matrices as media transparency is not required. The Anopore technique was used to study outgrow heterogeneity in complex food based media i.e. rice and broccoli for both untreated and severely heat treated spores (Chapter 3). Still, picture capturing and the data processing remain time consuming activities.

Although useful to study the early outgrowth events, the Anopore approach cannot be used to examine the germination process as dormant and early germination phases cannot be visualised as spores are impermeable to fluorescent probes. Alternative methods to study early germination events include surface enhanced Raman spectroscopy in combination with laser tweezers allowing for measurement of Ca-DPA levels in individual trapped spore and following Ca-DPA release kinetics during germination [26, 27]. More recently, it was demonstrated that the intensity of phase-contrast images is directly proportional to the level of the Ca-DPA inside the spore suggesting that phase-contrast microscopy alone could be used to estimate Ca-DPA levels in individual spores and to measure kinetics of its release [26]. In fact, differential interference contrast (DIC) microscopy and phase-contrast microscopy have been used to monitor simultaneous germination of up to hundreds of individual spores adhered to the microscope cover slip or agarose pads [28-30]. Despite clear developments in this field, it remains a challenge to study germination and outgrowth in non-transparent media and/or complex food matrixes.

Additionally, flow cytometry has been used to evaluate germination and/or outgrowth of individual spores. Flow cytometry in combination with fluorescent probes as for example SYTO-9 that stains DNA, can be used to monitor germination and early stage outgrowth of *B. cereus* spores under sublethal conditions [31]. In contrast to vegetative cells, dormant spores are considered impermeable to membrane-permeant fluorescent dyes such as SYTO-9 [31-34] and this fact was utilised in Chapter 2, where SYTO-9 staining of germinating spores was used to evaluate nutrient induced germination of *B. cereus* laboratory strains and food isolates. This method allowed high trough put testing of germination of spores from 17 strains in response to 20 conditions at single spore level. The speed and accuracy of the method improved the recognition of diversity and heterogeneity of spore germination among *B. cereus* strains.

It is important to realise that severe heat treatments inactivate and permeabilise spores, with concomitant water influx, also facilitate entrance of fluorescent dyes such as SYTO-9 targeting the DNA (Chapters 3 & 4). As a consequence, for severely heat treated spores, DNA binding dyes, even in combination with side/forward scatter, do not permit discrimination between outgrowing spores (representing signal intensity in between dormant and germinated spore signal) and inactivated spores. Further work is required to identify conditions allowing for reliable discrimination of the two fractions, ideally not affecting the downstream germination and/or outgrowth. Under ideal conditions, targeted subpopulations could be identified and separated by spore sorting, followed by further characterisation of their germination and outgrowth behaviour.

Behaviour of spores may be different in food matrices compared to laboratory media [2, 35], due to the presence of germination and/or growth inhibiting compounds or lack of other required compounds for these processes (discussed in more detail in the next section). To investigate the behaviour of individual spores or cells in food relevant

conditions, often a combination of conventional techniques is used at population level i.e. plate counting and measurement of change in OD is used with single spore/cell approaches. The presence of a single cell or spore in a well can be obtained using appropriate dilutions of the suspension [23, 36], but the most efficient approach is offered by flow cytometry in combination with high capacity cell sorting ([37] and Chapter 5). A range of growth related parameters can be obtained and variability between the individual outgrowth curves can be used to determine the outgrowth behaviour of single spores. Additionally, behaviour of single spores can be evaluated on non-transparent media including food matrixes, as spores can be sorted on solid media and evaluated for their colony formation. Such sorting approaches have been mainly used to study mildly heat treated or more commonly heat activated spores in presence or absences of secondary stresses [38].

Advanced methods to assess behaviour of single spores have allowed for direct observation of heterogeneous germination and outgrowth behaviour of spores, contributing to our knowledge on for example effect of different inactivation treatments on Ca-DPA release, commitment to germination or resistance to germination treatments. Despite the clear benefits, available methods have their limitations regarding conditions that can be tested, throughput, or the possibly destructive (i.e. intensive staining) process [33, 34]. Some of the limitations could be overcome by developments in Raman flow cytometry [39] and microfluidics [40, 41].

The growing number of publically available genomes enables to link genomic content of a strain or group of strains to observed phenotypes. Gene-trait-matching approaches aim for correlations between a presence/absence of a gene and considered phenotype. Despite the fact that identified correlations are not equivalent to causative effects, this approach can provide leads for less characterised phenotypes as well as lead to identification of novel players. Such approaches may also be used to identify genes putatively involved in recovery of damaged spores as discussed below.

Impact of the food matrix on germination and outgrowth of damaged spores

Germination and outgrowth of spores, and in particular damaged spores, is largely determined by environmental conditions including the food matrix composition. The effect of food matrix on behaviour of spores has been reported in a number of studies mainly focussing on impact of the heating matrix on spore survival [2, 42, 43]. The effect of food matrix on germination and outgrowth of spores remains largely unknown. In Chapter 3, we show that outgrowth of untreated *B. cereus* spores was affected by food matrix composition, in particular outgrowth heterogeneity was higher in rice based media. In fact, the slower (out)growth of *B. cereus* on rice media compared to laboratory media could be related to in general slower germination of *B. cereus* spores in rice media (data not shown), while

for heat treated spores additional time is available for repair of damaged spores, thereby supporting outgrowth from damaged spores (Chapter 5).

Germination of *B. cereus* spores in response to meat-, soy- and milk protein-based media was evaluated and compared with a selection of free amino acids commonly encountered, though in lower concentrations, in the environment (Chapter 2), demonstrating a higher germination triggering capacity of complex media than single compounds at saturating concentrations. Those observations stress the importance to include media composition as a parameter that can affect the efficiency of germination and outgrowth processes.

Food matrixes are usually rich in carbohydrates and therefore considered to support growth of microorganisms in foods. The ability to utilise different carbohydrates requires specific transporters and utilisation clusters, and such ability can reflect the adaptation to particular environmental conditions e.g. rhizosphere, foods or gastro intestinal (GI) tract. However, certain compounds such as glycerol and inositol can be both linked to the plant or root environment and to the GI tract, and consequently the capacity to metabolize these compounds cannot be directly linked to a specific niche. Additionally, activation of utilisation clusters may also depend on other environmental conditions such as oxygen availability, pH and other conditions as indicated in Chapter 6. Nevertheless, a core set of carbohydrates that could be used by all strains has been identified, whereas utilisation of other carbohydrates like xylose, galactose, and lactose, and host-derived carbohydrates such as fucose, mannose and N-acetyl-galactosamine is limited to subsets of strains. Known genes encoding utilisation of the later ones are carried mainly by strains belonging to phylogenetic group III, to which all emetic *B. cereus* strains initially tested by Guinebretière and co-workers were classified [44]. In fact, carbohydrate utilisation systems and their activity have been linked to the toxicity and virulence of emetic *B. cereus* strains. A complex interplay of fatty acids, C-sources, N-sources, micro- and macro-nutritional environments in combination with global factors such as pH and water availability was suggested to determine the risk of food-borne intoxications, either by stimulating or inhibiting cereulide synthesis by emetic *B. cereus* [45].

Species and strain diversity

For many years, *B. subtilis* has been used as model spore forming organism to investigate sporulation, germination, outgrowth and resistance mechanisms [14, 46-48]. However, not all physiological responses can be extrapolated from model strain to other strains and/or species. The development of genetic tools for other spore forming species and the increasing number of genome data has contributed to better understanding of molecular mechanisms. Not only the differences between *Bacillus* and *Clostridia* species [49, 50], but also within the Bacillus genera, including differences between *B. subtilis* and *B. cereus* become disclosed.

The majority of genes potentially involved in recovery of heat damaged *B. cereus* spores (Chapters 4 & 5), have no orthologues in *B. subtilis*, but are well represented within the *B. cereus* group. The majority of the identified genes in Chapter 5 encode transcription regulators for which the regulon is still unknown and can be highly different between species. As an example, the σ^B regulon members of *B. subtilis*, involved in the general stress response, exceed that of *B. cereus* by a factor four with only eight genes shared between two species [51].

The GRs encoded by *B. cereus* group strains revealed a large diversity (Chapter 2), pointing to possible differences between the phylogenetic groups which in turn may reflect their potential to germinate in different environments. Growth in those environments would imply utilisation of carbohydrates present therein, and indirectly requiring designated transporters and metabolic enzymes. A diverse set of *B. cereus* carbohydrate utilisation systems was described (Chapter 6) reflecting the capacity to obtain energy from multiple sources. Despite the clear assignment of *B. cereus* strains to the phylogenetic groups (I to VII) defined by Guinebretière et al. [52] the phylogenetic tree based on core genomes (Figure 6.1) presented a clear division of group III into two sub-groups, referred as IIIA and IIIB. Not only core genes but also the genes or gene cassettes present in a selection of strains (Table 2.4 and 7.3) indicate the differences between sub-groups IIIA and IIIB.

Next to absence/presence of genes, gene expression is tightly controlled particularly for genes encoding proteins used in specific environments. Not surprisingly, linking of phenotypic and genotypic characteristics is challenging, for example illustrated by the fact that germination response could only be partially linked to GRs encoded by the *B. cereus* genome (Chapter 2). By contrast, carbohydrate utilisation showed good agreement with the presence of genes involved in carbohydrate utilisation (Chapter 6).

Final remarks

The results presented in this thesis have been obtained using a wide range of tools enabling assessment of *B. cereus* spore germination and outgrowth, and provide novel insights in spore behaviour in (model) foods including damage recovery and outgrowth. Furthermore, first time overviews are presented of the distribution and putative functionality of (sub)clusters of GRs and carbohydrate utilisation clusters. Knowledge on spore damage repair, germination and metabolism capacity adds to further understanding of *B. cereus* ecology including niche occupation and transmission capacity and provides leads for future studies. Insights obtained in this study may support the design of novel (combination) treatments to control these notorious spoilage and pathogenic bacteria.

Phylogenetic group	Strain	D-Glucose	D-Fructose	D-Maltose	D-Trehalose	N-Acetylglucosamine	D-Ribose	Potassium Gluconate	Glucose-6-phosphate	Glycerol	D-Cellobiose	Salicin	Esculin ferric citrate	Arbutin	Glycogen	Starch (Amidon)	D-Saccharose (sucrose)	L-Fucose	N-Ac-Galactosamine	Inositol	D-Mannose	D-Lactose	D-Galactose	D-Xylose
II	B4077, B4082, B4088, B4147														75%	75%	25%	25%		50%	25%			
III A	B4078, B4079, B4085, B4086, B4116, B4153, ATCC10987, AH187											13%	13%	13%	13%	13%	75%	75%	25%					13%
III B	Ba Ames, B4087, E33L, Bt Al Hakam, Bt serovar konkukian 97_27																20%	20%	40%	20%	40%	20%	20%	20%
IV	B4080, B4081, B4084, B4118, B4120, B4155, B4158, ATCC14579											88%	88%	88%			75%	13%			13%	13%	13%	
VI	B4083, B4117, Bw KBAB4																33%	33%		33%				

Figure 7.3. Distribution of carbohydrate utilisation according to the phylogenetic grouping. Cassette for utilisation of given carbohydrate present (black) or absent (white) in tested strains belonging to a given group. Present in above 66% (dark grey), 33 - 66% (grey), bellow 33% (light grey) of strains.
Ba: *B. anthracis*, Bt: *B. thuringiensis*, Bw: *B. weihenstephanensis*

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Alicja

About the author

Alicja Katarzyna Warda was born on 5th April 1984 in Warsaw, Poland. Her adventure with food microbiology started during her BSc in Field of Commodity Science at Warsaw University of Applied Sciences (SGGW), Poland and continued during the MSc in the same field. During this period, Alicja focused on food quality and lactic acid bacteria first by reviewing mechanisms of action and use of bacteriocins and later on by investigating the effect of storage conditions on the survivability of the probiotic strain *Lactobacillus casei* in a synbiotic drink. In 2007, thanks to Socrates - Erasmus Exchange Program Alicja came to Wageningen University, the Netherlands, where driven by a new environment she started a second MSc program, this time in Food Safety. Her major thesis was conducted at the Laboratory of Food Microbiology of Wageningen University and focused on the role of alternative sigma factor sigma B in response of *Bacillus cereus* to disinfectants. As part of her MSc program Alicja completed an internship at Unilever's R&D Centre in Vlaardingen, the Netherlands. During this project, she was working on developing assays for detection, identification and quantification of yeast. After graduating the MSc program Alicja started her PhD program entitled "*Bacillus cereus* spore damage recovery and diversity in spore germination and carbohydrate utilisation". The results of this work performed at Wageningen University, in the Laboratory of Food Microbiology and Food & Biobased Research (FBR) are described in this thesis. Currently, Alicja is working as Postdoctoral Researcher at APC Microbiome, University College Cork, Ireland.

List of Publications

Warda A.K., den Besten H.M.W., Sha N., Abee T., Nierop Groot M.N. (2015) Influence of food matrix on outgrowth heterogeneity of heat damaged *Bacillus cereus* spores. International Journal of Food Microbiology, 201: 27-34

Warda A.K., Tempelaars M.H., Boekhorst J., Abee T., Nierop Groot M.N. (2016) Identification of CdnL, a putative transcriptional regulator involved in repair and outgrowth of heat-damaged *Bacillus cereus* spores. PlosONE, 11(2): e0148670

Warda A.K., Siezen R.J., Boekhorst J., Wells-Bennik M.H.J., de Jong A., Kuipers O.P., Nierop Groot M.N., Abee T. (2016) Linking *Bacillus cereus* genotypes and carbohydrate utilization capacity. PlosONE, 11(6): e0156796

Warda A.K., Tempelaars M.H., Abee T., Nierop Groot M.N. (2016) Recovery of heat treated *Bacillus cereus* spores is affected by matrix composition and factors with putative functions in damage repair. Frontiers in Microbiology, 7: 1096

Warda A.K., Xiao Y., Boekhorst J., Wells-Bennik M.H.J., Nierop Groot M.N., Abee T. (2016) Linking germination capacity of genome sequenced *Bacillus cereus* environmental isolates and model strains to germinant receptor (sub)clusters. (submitted for publication)

Wells-Bennik M.H.J., Eijlander R.T., den Besten H.M.W., Berendsen E.M., Warda A.K., Krawczyk A.O., Nierop Groot M.N., Xiao Y., Zwietering M.H., Kuipers O.P., Abee T. (2016) Bacterial spores in food: survival, emergence, and outgrowth. Annual Reviews in Food Science and Technology, 7: 457-82

Overview of completed training activities

Discipline specific activities

Courses

Hands-on bioinformatics, TIFN, Nijmegen (2012)
Genetics and physiology of food associated microorganisms, VLAG, Wageningen (2013)
Management of microbiological hazards in foods, VLAG, Wageningen (2014)
Basic statistic course, PE&RC, Wageningen (2014)

Meetings

SPOILERS2013 Conference, Quimper, France (2013, flash poster presentation)
SILS Seminar: When The Sleepers Wake, Amsterdam, Netherlands (2013)
6th European SPORE Conference, Royal Holloway, United Kingdom (2014, oral & poster presentation)
6th Congress of European Microbiologists, FEMS, Maastricht, Netherlands (2015, poster presentation)

General courses

Presentation skills, WGS, Wageningen (2011)
PhD week, VLAG, Baarlo (2012)
Project & Time Management, WGS, Wageningen (2012)
Effective behaviour in your professional surroundings, WGS, Wageningen (2012)
Voice matters - Voice and presentation skills training, VLAG, Wageningen (2013)
Reviewing a scientific paper, WGS, Wageningen (2014)
Advanced course 'Guide to scientific artwork', Wageningen UR library, Wageningen (2014)
Scientific writing, WGS, Wageningen (2014/15)
Carrere perspectives, VLAG, Wageningen (2015)

Other activities

Preparation of PhD research proposal (2011)
TIFN Project meetings (2011-2015)
Work discussion meetings, Laboratory of Food Microbiology (2011-2015)
PhD study trip Laboratory of Food Microbiology, Japan (2012, organisation)
PhD study trip Laboratory of Food Microbiology, Ireland (2014)

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