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Bacillus cereus spore and cell proteome dynamics

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Chapter 1

General Introduction

1.1. Introduction

1.1.1. *Bacillus cereus*

Bacillus cereus was first isolated from air in 1887 [1]. However, it wasn't speculated to be an agent for food poisoning and considered to be a pathogen until many years later [2]. One of the earliest records was reported in 1906 when a hospital outbreak happened in 300 patients and staff with symptoms of acute gastroenteritis shortly after dinner [3]. Later, it was found from dinner remnants that the food was contaminated with an aerobic sporeforming bacterium, which most probably was a strain of *B. cereus*. In the following several years, researchers were starting to realize *Bacillus* contaminated food stored at improper temperatures is the most probable reason for emetic and diarrhea when ingested.

B. cereus belongs to a bacterial group known as *B. cereus sensu lato*, which includes the species *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis*, *Bacillus cytotoxicus* [4], *Bacillus toyonensis* [5] and *Bacillus wiedmannii* [6], as well as several newly described members identified through more recent genetic taxonomic analyses, such as *Bacillus gaemokensis*, *Bacillus manliponensis* and *Bacillus bingmayongensis* [7]. All the group members are Gram-positive facultative anaerobic spore-forming bacteria found in diverse environments. These bacteria have a high level of genetic similarity [5,10,11], but with large variation in morphological and physiological features as well as clinical manifestations.

As shown in Figure 1.1, the clinical manifestations caused by *B. cereus* include gastroenteritis, endophthalmitis and respiratory tract infections [8]. A recently report described a patient who survived a case of *B. cereus* meningitis/brain abscess in severe neutropenia, presenting as immune reconstitution syndrome [9]. The infections associated with *B. cereus* are mainly related to foodborne outbreaks of gastroenteritis [8]. The foodborne illness is caused by three secreted toxins together with cereulide. The three secreted toxins are: hemolysin BL (HBL), nonhemolytic enterotoxin (NHE) and cytotoxin K (CytK). These toxins are produced and secreted during the exponential phase of vegetative cell growth and are regulated by PlcR [10]. Cereulide is a small, highly heat- and acid-resistant depsipeptide toxin, and is a major concern for the food industry. The cereulide toxin will most likely not be destroyed or inactivated during food processing, even though the bacteria themselves might be destroyed by these processes [11]. The *B. cereus* ATCC 14579 strain studied in this thesis, does not produce the emetic toxin cereulide [12]. This introduction is concerned with a general

introduction on *B. cereus* and its spores. Readers can consult a review [13] on clinical and research findings of mechanisms related to *B. cereus* endophthalmitis in greater depth [13]. Descriptions of more cases caused by *B. cereus* from 1906 to 2019 can also be found in additional reviews [14, 15]. Notably, efforts have been made by worldwide research focusing on developing methods of detection and profiling biomarkers to try to prevent future outbreaks. More details regarding toxins produced by *B. cereus* and detection methods can be found in previous reviews [8, 16].

1.1.1.1. Spore structure and composition

Spores facilitate pathogenesis via their remarkable resistance to killing factors and adhesion to either human epithelial cells [17, 18] or surface of stainless steel [19]. Spores have served as textbook examples of structures from organisms with long-term survival, whilst maintaining an extraordinary ability to rapidly germinate and return to growth once the environment is favorable.

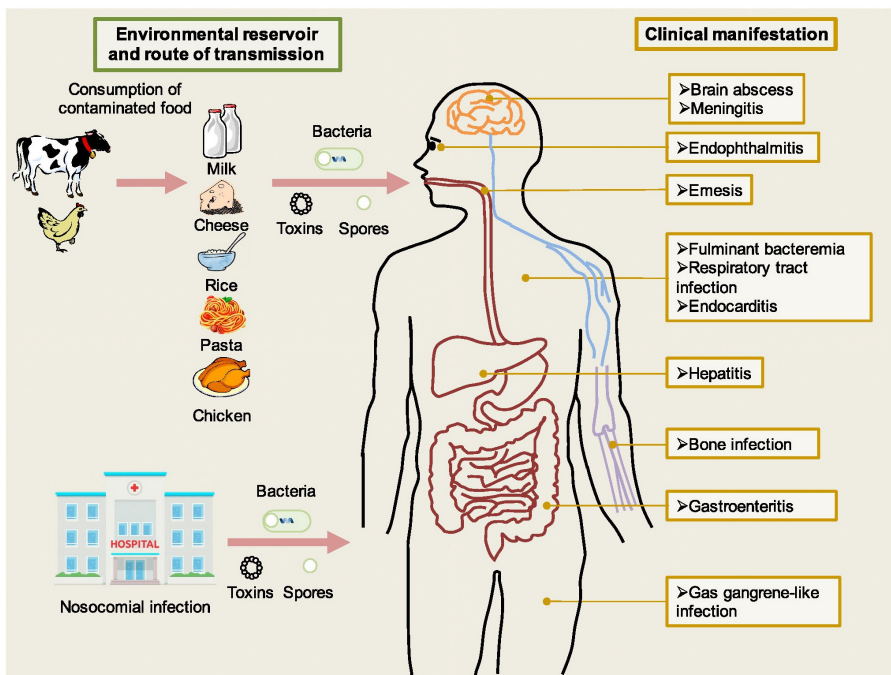


Figure 1.1. Environmental reservoirs, transmission and clinical manifestations of *B. cereus*. *B. cereus* can be transmitted to humans via the oral-gastric route by the consumption of contaminated food or via nosocomial transmission due to the formation of biofilms on items such as catheters and bedsheets. Adapted from [8].

Spores can remain viable for long periods, the upper limit of which is

unknown [20], although some reports place the limit in the millions of years [21, 22]. For example, it was reported that viable spores of a *Bacillus sphaericus* strain preserved in the gut of a bee fossilized in Dominican amber for an estimated 25 to 40 million years could be recovered [23]. Whether such long-term survival is true or not is unclear, but clearly formation of spores enables the survival of these organisms through harsh environmental conditions that can kill vegetative cells. Indeed, the spores are extremely resistant to high temperature, freezing, desiccation, γ -ray and ultraviolet radiation and many toxic chemicals [24, 25]. One of the factors involved in spore resistance is the spore structure as shown in Figure 1.2a.

The innermost layer of the *B. cereus* spore is the core, where spore DNA, RNA and enzymes are stored. The water content in the spore core is low with 25-50% of wet weight, and dipicolinic acid (DPA) chelated to divalent cations,

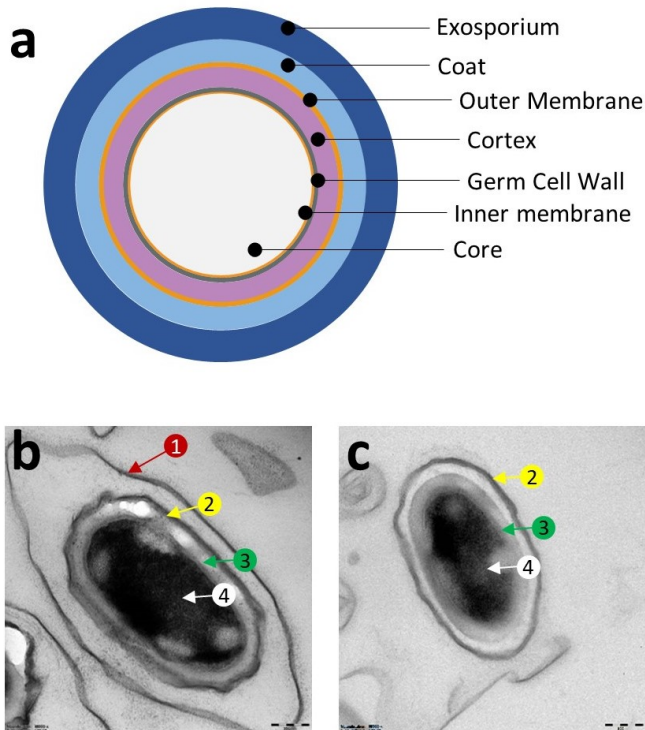


Figure 1.2. Spore structure. (a) The cartoon of a typical *B. cereus* spore. (b) A transmission electron micrograph of a *B. cereus* wild type spore. (c) A transmission electron micrograph of a *B. cereus* CotE mutant spore. The spore exosporium (red arrow), coat (yellow arrows), cortex (green arrows) and core (white arrows) layers are pointed out.

usually Ca^{2+} , constitute $\sim 25\%$ of core dry weight. DNA in the spore core is

saturated with α/β -type small, acid soluble proteins (SASPs) which protects DNA from chemicals, radiation and enzymes. The α/β -type SASPs are degraded soon after the initiation of germination and provide amino acids for protein synthesis. There is also a γ -SASP, which is the most abundant SASP in spores of several species but not well conserved across species. The only function of γ -SASP seems to be a reservoir of amino acids for protein synthesis during spore germination and outgrowth [26]. The core's low water content, high level of CaDPA and saturation of DNA with α/β -type SASPs contribute greatly to spore resistance properties [38].

Surrounding the spore core is the inner membrane (IM). Lipids in the IM are likely somewhat compressed, as lipid probes in the IM are largely immobile which may be assisted by the Ca^{2+} in CaDPA interacting with head groups of phospholipids [27, 28]. Notably, the IM has low permeability to small molecules, including water, and low IM permeability appears likely to be important in spore resistance to chemicals that can damage spore DNA or core proteins [28,34,72]. During spore germination and outgrowth, the IM area can expand ~ 1.3 -fold without ATP and new membrane synthesis during germination. This extra membrane material likely comes from vesicles beneath the IM in dormant spores which appear to coalesce with the IM as germination is completed [29]. Germinant receptors (GRs) that respond to physiological germinants in initiating germination are also located in the IM. The heterogeneity of GR-dependent germination may be due to the heterogeneous levels of GRs. Indeed, increases in the levels of several GRs in *B. subtilis* spores can markedly increase GR-dependent germination [30]. Sublethal heat treatment, or heat activation can also increase GR-dependent germination rates. The mechanism of heat activation is unknown, but has been suggested to cause some conformation change(s) in GR protein [31]. *B. cereus* has seven operons encoding GRs: GerR, GerQ, GerL, GerI, GerK, GerS and GerG [32, 33]. GerR is the most important one, and is crucial in almost all amino acid-induced germination except by L-glutamine which is mediated by GerG. Mutations of GerK, GerL and GerS do not affect germination. In general, GRs are composed of three subunits A, B and C. A and B subunits are integral membrane proteins and the C subunit is a lipoprotein [34]. The A subunit contains around 5 transmembrane spanning regions [34], but it is unclear whether this subunit harbors a germinant binding site. The predicted structure of the B subunit consists of 10 membrane spanning regions and a possible germinant binding site [35]. These three subunits function together, probably in a complex [34]. Multiple GRs can coalesce in the IM to form a "germinosome" which is organized by the GerD protein. Without GerD, the GRs are dispersed around the spore IM

and spore germination is considerably slowed down [34]. Presumably, by forming a germinosome, GR clustering facilitates integration of stimuli from different GRs and simultaneously the transduction of signals. The “germinosome” was seen initially in *B. subtilis* spores but has also recently been seen in *B. cereus* spores [36].

Outside the inner membrane is the germ cell wall. This layer is composed of peptidoglycan (PG) with an identical structure to the PG in the vegetative cell wall. After germination, it becomes the cell wall during spore outgrowth. Underneath the germ cell wall is the spore cortex PG layer which is required for the maintenance of the relatively dehydrated spore core [37]. In the cortex PG every second muramic acid residue is converted to a muramic- δ -lactam, which is not found in germ cell wall PG [38]. The cortex lytic enzymes SleB and CwlJ which hydrolyze cortex PG in germination can only hydrolyze PG with muramic- δ -lactam, thus leaving the germ wall intact. Synthesized in the mother cell during sporulation, CwlJ is located in the spore coat layer together with GerQ (YwdL) and is activated by CaDPA. SleB, synthesized in the forespore, is located in both the IM and coat layer together with YpeB, which is essential for SleB localization [39]. The activation of SleB may be stimulated by proteolysis of YpeB by HtrC [40].

Around the cortex is the spore outer membrane, the function of which is poorly understood. The outer membrane is surrounded by the spore coat layer, which consists of two layers, the inner coat and outer coat. The main components of the coat are proteins, which account for about 30% of the total spore protein content [37]. The key morphogenetic factors are SpoIVA and SafA in the inner coat near the outer membrane, while CotE is necessary for the assembly of the outer coat. Mutation of *cotE* in *B. subtilis* eliminates the outer coat and exposes the inner coat [37, 41]. The functions of the coat are to: 1) contribute to resistance to chemicals and lysozyme; 2) protect the cortex layer; and 3) interact with germinants modulating germination.

The outermost layer of a *B. cereus* spore is the exosporium, which is a balloon-like layer which is loosely attached to the spore coat. CotE is necessary for the complete formation of exosporium [42] and mutation of *cotE* in *B. cereus* eliminates the exosporium (Figure 1.2c) [43]. The exosporium consists of a basal layer and a hair-like nap [44–46]. The length of the hair-like nap can discriminate the strains within the *B. cereus* group. The exosporium provides a hydrophobic surface enhancing adhesion. Some enzymes are also located in this layer, such as alanine racemase (Alr) modulating germination by degrading low levels of germinants. The spore coat and exosporium layer are flexible and respond to environmental conditions, such as humidity by changing spore size and some coat structure

[37].

1.1.1.2. Sporulation

The environmental factors that trigger sporulation include nutrient depletion and/or population density [47, 48]. Spores are formed within a mother cell through a pathway, which is conserved among spore-forming bacteria [49]. Sporulation begins with an asymmetric cell division that separates a sporangium into two compartments, the mother cell and forespore. In the following stage, the mother cell engulfs the forespore, which separates the forespore from the extracellular environment. Once the sporulation is completed, the mother cell will lyse and release the free spores. The master transcriptional regulator Spo0A governs the differential activation of much of the genetic program driving sporulation. The phosphorylation of Spo0A initiates the asymmetric division and controls many regulators including multiple sporulation specific RNA polymerase sigma factors which direct expression of different groups of sporulation-specific genes [20,49].

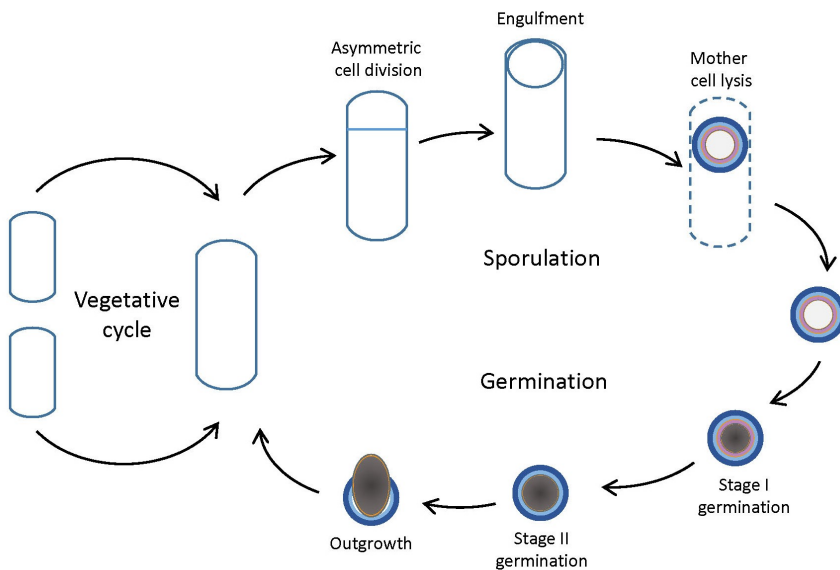


Figure 1.3. Sporulation, and spore germination and outgrowth.

1.1.1.3. Germination and Outgrowth

Spore germination is defined as the time from the addition of germinants to when the core water content rises to $\sim 80\%$ of wet weight. Germination is of interest both because of the fascinating simple differentiation and because spore resistance is lost in germination allowing for a germination to

eliminate strategy [50]. While all the molecular details of signal transduction in spore germination are not fully clear, most proteins involved in the process have been identified [34, 51, 52]. Current models of germination are constructed based on the current available information, most of which is from studies on *B. subtilis* spore germination.

Spores growing into vegetative cells undergo two visible stages under microscopic study. The first is defined as germination referring to a transition from phase bright to phase dark. The following stage which generates vegetative cells is defined as outgrowth. Germination can be triggered in five ways: 1) physiological germinants, often nutrients [32]; 2) dodecylamine [27]; 3) exogenous CaDPA [31]; 4) peptidoglycan fragments [53]; and 5) high hydrostatic pressure (HHP) treatment [54]. Nutrient induced germination is the most common in nature. Usual nutrient germinants for *B. cereus* are amino acids, such as L-alanine, and purines such as inosine, and nutrient germination has several main steps (Figure 1.4). 1) One or several GRs sense nutrient germinants (although note that metabolism of these germinants is not how they trigger germination). There is some evidence that GerP proteins in the spore coat facilitate germinant permeation through spores' outer layers [55, 56]. GR-dependent germination of dormant spores can be activated by sublethal heat treatment. 2) Commitment; Commitment is an irreversible step. Once committed, spores germinate even if germinants are removed. 3) Stage I of germination; the spore core releases monovalent cations, such as H⁺, K⁺ and Na⁺. The transporters involved in this ion flux are unknown. CaDPA will then be rapidly released via SpoVA protein channels located in the spore IM. After this stage, the wet-heat resistance of spores falls significantly as core water content increases ~ 30%. 4) Stage II of germination. The spore cortex is degraded by cortex lytic enzymes, leading to an increase in core water content to ~ 80% and an expansion of the spore core which allows mobility of proteins in the core and lipids in the IM. The resistance of the fully germinated spore is now similar to that of a vegetative cell. Once all germination steps are finished, the spore enters into outgrowth. During this process, the spore coat layer will be broken down and/or shed, and metabolism and synthesis of macromolecules resume.

1.1.1.4. Tools to study spore germination

Along with technology development, “omics” tools, such as transcriptomics and proteomics, are gradually showing their power in helping to understand spore germination and outgrowth as follows. 1) Examination of gene expression during *B. subtilis* spore germination and outgrowth [57], has

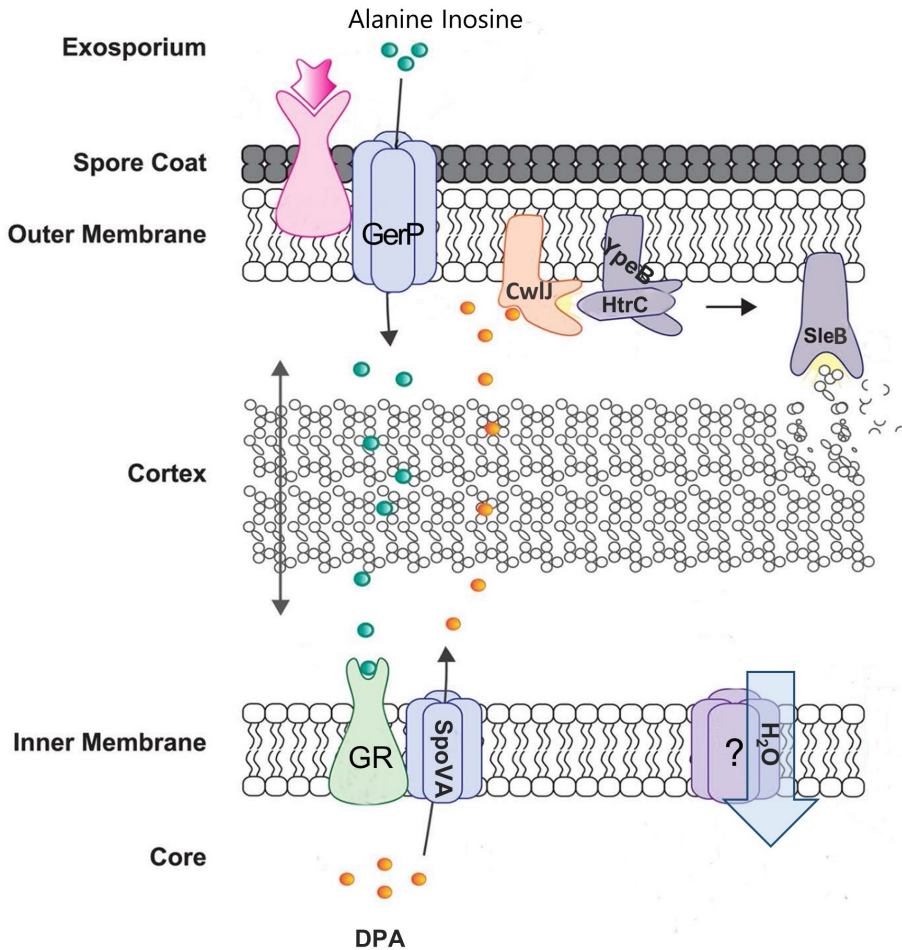


Figure 1.4. Model for germination of a *Bacillus* spore triggered by the germinants alanine and inosine.

revealed key transcriptional events. 2) Transposon sequencing technology has been used in identification of genes with new roles during *B. subtilis* germination [58]. 3) Analysis of differential gene expression has been conducted to explore the effects of heat activation with or without germinant L-alanine on *B. cereus* spores. The latter result show that the *gerA* operon, which encodes the GerA GR and responds to L- alanine is upregulated after heat activation with L-alanine and the genes involved in transporting L-alanine are also upregulated [59]. 4) Proteome and transcriptome analysis have been carried out on *B. subtilis* spores during germination and outgrowth [60] to achieve a comprehensive overview of the molecular

events during these developmental periods. The application of proteomics in spore research is discussed in detail in the next section.

1.1.2. Proteomics studies of spores

Proteins dictate spore structure, protect spores from chemicals and enzymes, assist the interactions between spore and environment and catalyze reactions to support germination and subsequent development. The total proteins are defined as the proteome (encoded by the genome) and the global analysis of proteins is defined as proteomics. Compared with genomic (DNA) and transcriptomic (RNA) sequencing, proteomics is far more complex due to the many proteoforms that far outnumber the genes in a genome. The complexity comes from splicing, post-translational modifications (PTMs), protein-protein interactions (PPIs), single-nucleotide polymorphisms (SNPs) and so on [61]. PTMs include phosphorylation, glycosylation, methylation and acetylation [62]. They are prevalent in expanding complexity exponentially. PPIs contain protein complexes that are formed by multiple proteins that interact with each other and other complexes formed by interactions between proteins with other molecules, such as DNA, RNA and lipids or other co-factors. Such PPIs need to be studied by various techniques including affinity purification (AP)-MS.

Proteomics currently relies on bottom-up mass spectrometry (MS) for protein analysis (Figure 1.5), which involves sample extraction, protein digestion, MS analysis and database searching. Samples are analyzed by liquid chromatography coupled to mass spectrometry (LC-MS). Peptides are fragmented by collision-induced dissociation (CID) and fragment spectra acquired through tandem mass spectrometry (MS/MS).

The first step in a proteomics experiment is sample preparation. Proteins will be digested into peptides, usually with trypsin. Samples can be pre-fractionated depending on the proteomic depth that needs to be acquired. To reduce complexity and improve sensitivity, popular methods include ion-exchange chromatography and hydrophilic interaction liquid chromatography (HILIC). The “one-pot” method is specifically developed for spore samples, which contain complex and insoluble spore proteins. Coupled with ZIC (zwitterionic)-HILIC, over 1000 spore proteins can be identified with high confidence in all spore layers [63]. To enrich specific peptides, like peptides containing PTMs, methods include using affinity resins and specific antibodies. For example, the Ser/Thr/Tyr phosphoproteome has been enriched by TiO₂ beads in germinating *B. subtilis* spores [64], revealing an integral role of phosphorylation events in facilitating spore revival. Later using similar enrichment methods, the

arginine phosphoproteome was studied in *B. subtilis* spores during

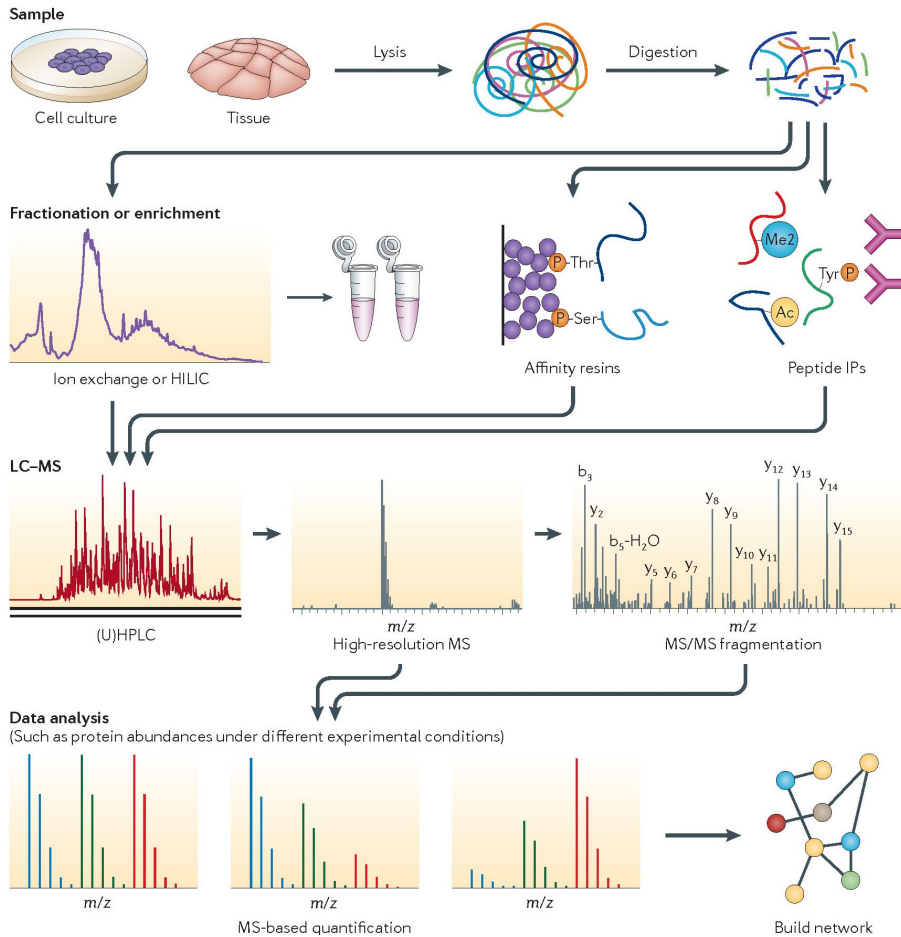


Figure 1.5. Mass spectrometry-based universal proteomics workflow. Adapted from [66].

germination [65], showing that an arginine phosphatase YwIE is crucial for spore germination.

The widely used quantification methods are label-free quantification or stable isotope labeling (label-based) quantification. Label-free quantification utilizes spectral counting and/or signal intensity to quantify detected peptides [66]. The advantages of label-free quantification are robustness, precision, simplicity and economic when done well and processing many replicates. Stable isotope labeling needs extra steps for introduction of labels. This method including metabolic labeling, such as SILAC, ¹⁵N labeling, dimethyl labeling, and ¹⁸O labeling. SILAC is systematically applied using a lysine auxotrophic strain of *B. subtilis* spores

[67]. The ^{15}N metabolic labeling has been applied on *B. subtilis* spore germination and outgrowth [60] and sporulation [68] to monitor protein dynamics. BONCAT is used in *B. subtilis* spore germination to determine the newly synthesized proteins [70]. Newly synthesized proteins are enriched by an incorporated azide-bearing artificial amino acid azidohomoalanine (AHA) tag, which is a substitute for methionine. Chemical/enzymatic labeling methods introduce isobaric chemical labels to quantify proteins or peptides based on MS/MS reporter ions, including ICAT, iTRAQ and tandem mass tag (TMT). The iTRAQ approach was used on identification of *B. subtilis* spore proteins responding to thermosonication [73] and to study the early events during *B. anthracis* spore germination [74]. Protein quantification can also be achieved by adding stable isotope-labeled internal molecules as analogues to analyze molecules. One method using an internal standard is the quantification concatamer (QconCAT). Recently, the QconCAT approach was successfully applied for the absolute quantification of proteins from the *B. cereus* spore coat layer [69].

MS acquisition modes include data-dependent acquisition (DDA), targeted data acquisition and data-independent acquisition (DIA). Targeted MS data acquisition is gaining popularity for reproducibly looking at selected proteins. The most used scan modes are selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) on low resolution instruments as well as parallel reaction monitoring (PRM) on high resolution instruments (often from DIA acquisitions). SRM is applied to find highly specific biomarkers to *B. anthracis* spores. MRM combined with immunocapture is also used to sensitively detect *B. anthracis* spores [71].

All the above shows the successful applications of proteomics in spore research. The potential utilization of new proteomics tools, such as single-cell proteomics, on spores will be discussed in Chapter 6.

1.2. Outline of thesis

To solve the problems caused by *B. cereus* spores in the food chain, mild strategies are proposed around “germination-elimination”: either to prevent germination and toxin production or to germinate all spores and kill them. Understanding spore germination and outgrowth is a pertinent question in this field. Currently, *B. subtilis* is the most well-studied spore-forming organism. Based on studies in *B. subtilis*, the germination and outgrowth model is established. However, germination varies between species, strains and environments. Detailed germination and outgrowth mechanisms of *B. cereus* spores are unclear. Chapter 1 elaborates on spore structure, formation and germination and outgrowth processes. A general introduction

about proteomics and its applications on spores is also presented.

To get a deeper understanding of the spores' germination process and provide fundamental knowledge for the strategies in the food industry, proteomics experiments are designed and conducted on *B. cereus* spores in this thesis. The protein composition of the spore inner membrane is first studied, since the spore inner membrane is an important layer for germination. Proteins known to be essential in spore germination, such as GR, SpoVA and GerD, are located in the inner membrane. Chapter 2 describes the comparison of the spore inner membrane and vegetative cell membrane proteome coupled with membrane fraction enrichment methods adapted from the methods used for *B. subtilis* spore inner membrane enrichment, trying to figure out the proteins that are important and unique in the spore inner membrane. The results showed the differences between spores and cells are in metabolism, virulence and so on. In the following, Chapter 3 contains the analysis of the protein dynamics during germination and outgrowth of spores coupled with co-expression network analysis, exploring whether the germination of *B. cereus* fits the current germination model and investigating the *B. cereus* germination pattern. Besides investigating spore germination, we also study the effects of intracellular perturbations on the spore proteome of *B. cereus*. Previously, to visualize germinosomes in *B. cereus* spores, strains containing GR-fluorescent fusion proteins were constructed [36]. In Chapter 4, we examine and compare the spore proteome of the GR fusion protein mutants with wild type and demonstrate the extent that plasmid based genetic tools influence the composition of spores. Even though the "one-pot" method is already successfully used chapter 3 and 4, we explore the possibility of the application of another sample preparation method using a different detergent based (SDS) protein extraction on spore samples. Chapter 5 describes the successful application of the single-pot, and solid phase-enhanced sample-preparation (SP3) method on spore and vegetative samples. Chapter 6 summarizes the main results of this thesis and discusses future directions of the research and potential applications of new emerging technologies in this field.

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