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Publication date
2026

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Citation for published version (APA):

Chen, L. (2026). *Molecular dynamics guided analysis of Bacillus subtilis spore germination mechanisms*. [Thesis, fully internal, Universiteit van Amsterdam].

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1 Introduction:

Molecular switch of *Bacillus subtilis* spore germination



1.1 Bacterial spores

Experiencing and adapting to the environment is a constant task for every form of life on earth. As the most numerous and genetically diverse group of living organism, bacteria thrive in nearly every environment. To survive, bacteria must adapt to environmental changes and maintain their homeostasis. This holds true especially when bacteria face challenging growth-limiting physiochemical conditions including changes in pH or temperature, the presence of reactive oxygen species or other toxic chemicals, and the lack of nutrients¹. An important part of the bacterial stress response is to sense and adapt to environmental changes in a timely manner. This can be realized by alteration of gene expression, post-translational protein regulation and/or toxin remediation. Alternatively, some bacteria adopted the ability to form spores, which are a special form of a microbic cells that are metabolically dormant and structurally tough, thereby enabling bacteria to survive in inhospitable environments².

Bacterial spores are an environmentally ubiquitous bacterial cell form. The genus *Bacilli* is known as a soil borne genus, as its members, including *Bacillus subtilis*, *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus megaterium* spores can be commonly found in soil. A notoriously dangerous species in the same group, *Bacillus anthracis* spores, even found in permafrost, caused a *B. anthrax* outbreak in 2016 in Siberia³. In another extreme environment, *Clostridium algariphilum* is a psychrophilic bacterium (i.e. microbes that thrive in temperatures typically below 15 °C, and are predominantly found in polar regions, deep-sea waters, and permafrost)⁴. Its spores are found in frozen brine, an extreme environment due to the low temperature (-9 ~ -11 °C) and high salt concentration (mineralization of 170–300 g/L)⁵. Besides soil, bacterial spores also occur in air and atmosphere. Environmental microbiology field research showed that bacterial spores originating in a sandstorm area north of the Black Sea could be transmitted to Sweden by air⁶. Spore formers are also present in all sorts of natural or artificial aquatic environments, including rivers, endolithic bacterial ecosystems (within the pore space of rocks), the ocean, and artificial water facilities like sewage systems⁷⁻¹⁰.

Bacteria spores also reside inside other organisms. Spore-formers in the *Bacillaceae* family can be found in the gastrointestinal tract (GIT) of insects and animals¹¹. Human faeces can contain up to 10⁴ *Bacillus subtilis* spores per gram¹². Therefore, it is worth mentioning that the resilience of human gut spore formers poses both benefits and threats to human health. The *Bacillaceae* spore formers are human gut colonizers commonly acquired from food or water ingestion. For example, *Bacillus intestinalis* spores were isolated from a patient with intestinal cancer¹³. *Bacillus cereus* is associated with gut disorders including diarrhoea and irritable bowel syndrome (IBD)

¹⁴. On the other hand, the spore forming ability of *B. subtilis* allows it to survive long enough to promote development of gut-associated lymphoid tissues (GALT) ¹⁵. *Bacillus coagulans* also has long been used as a probiotic as it is capable of suppressing the growth of pathogens, stimulating the growth of beneficial bifidobacteria and exhibiting immune-modulating effects. Similarly, spore forming members of the anaerobically growing *Clostridiaceae* family also have positive and negative effects on human health. *Clostridiodes difficile* and *Clostridium perfringens* are well known for their pathogenicity, and *C. difficile*'s spore forming ability even allows it to survive and proliferate after antibiotic treatment ¹⁶. In contrast, members of the *Clostridium leptum* and *Clostridium coccooides* groups have been suggested to be involved in the prevention of inflammatory bowel disease (IBD) ¹⁷.

Apart from their impact on human health, the ability of bacterial spores to survive unfavourable environments also gives them an edge in biotechnology. *Bacillus* spores have been used as a method for vaccine delivery ¹⁸ or enzyme display (anchors enzymes to the surface of whole microbial cells, creating stable and reusable whole-cell biocatalysts for industrial applications)¹⁹. Additionally, *Bacillus thuringiensis* (*Bt*) spores are a well-established bio-pesticide for commercial usage, as *Bt* produces toxins alongside spore formation, which damages the gut of insect larvae upon ingestion²⁰.

1.2 Categories of bacterial spores and the importance of endospores

Bacterial spores occur in several forms, including endospores, exospores, and myxospores. Endospores are formed intracellularly mainly by *Firmicutes*²¹. This phylum consists of a large group of diverse and morphologically complex members. Among them, the species *Bacillus subtilis* is widely used as a model organism to study endospore formation. Another well-known endospore former, *Clostridiodes difficile* is a rather persistent pathogen due to its spore forming ability. Besides Gram-positive spore formers like *Bacilli* and *Clostridia*, Gram-negative spore formers are also found within the *Firmicutes* phylum. *Actonema longum*, which stains Gram negative, belongs to the family *Velillonellaceae* within the *Firmicutes* phylum. In this organism, the outer membrane is formed through the inversion of the inner membrane during sporulation. It has therefore been proposed that the outer membrane of Gram-negative bacteria may have originated via the sporulation process ²². Hence, endospore formers represent valuable systems for bacterial morphologic studies. Exospores are produced by members of the *Streptomyces* genus, belonging to the *Actinobacteria* phylum²³. Exospore formation of *Streptomyces* initiates from outside of the cell, while *Streptomyces* vegetatively grow as multicellular branching filamentous hyphae, and exospores emerge in branching aerial hyphae from the surface of its mother cell colony. Myxospores are a third type of bacterial spores

produced by *Myxococcus xanthus*. In this bacterium, spores form by rearranging the rod-shaped vegetative cell into a spherical spore under stress²⁴.

Being the most predominant spore type, bacterial endospores have gained interest among microbiologists. The formation of endospores (hereafter referred to as “spores”) is termed sporulation. As it follows a complex and highly regulated developmental pathway, it serves as an excellent model to study cellular differentiation and decision making^{25,26}. Following sporulation, the process of a spore losing its dormancy and unique structural organization is termed germination. With spore-formers posing a threat towards human health and their ubiquitous existence, there is much interest from the food, health care and defence industries in how to safely and rapidly remove spores. Importantly, the germinated spores are as easy to remove as normal cells, making “germinate to eradicate” a popular strategy for spore decontamination²⁷. Consequently, spore germination is of high interest to both scientific and industrial fields.

1.3 Spore of the model bacterium *Bacillus subtilis*

In the study of morphological, biochemical and genetics of sporulation and germination, *Bacillus subtilis* has served as a powerful model system. *B. subtilis* is the second most well-studied bacterial model organism behind *E. coli*, and the most well-studied Gram-positive bacterium. The popularity of *B. subtilis* could also be attributed to the ability of it to take up extracellular DNA, making it easy to manipulate genetically²⁸.

In *B. subtilis*, sporulation is triggered by lack of nutrients and high cell density²⁹⁻³¹. The most often occurring mode of sporulation in *B. subtilis* initiates with an asymmetrical cell division resulting in a mother cell and a smaller forespore. This is followed by engulfment of the forespore by the mother cell, resulting in the formation of a pre-spore. The mother cell ultimately undergoes programmed cell death and lysis, releasing the mature spore (Figure 1). The mature spore is a smaller, oval-shaped cell with a dimension of ~1.2-1.5 μm and width of ~0.6-0.8 μm ³². It consists of many protective layers (Figure 2A), with its structure being one of the determining factors in its high resilience. The core of *B. subtilis* spores contains spore DNA saturated with low molecular weight protective proteins, termed small acid soluble proteins (SASP), a high level, ~ 20% of core dry wt, as dipicolinic acid (DPA) chelated 1:1 with divalent cations predominately Ca^{2+} . In addition, the spore core has a water content as low as 25% of wet wt compared to approximately 80% in living cells. The spore inner membrane (IM) surrounds the spore core, and exhibits low permeability to small molecules and low lipid mobility. It contains membrane bound receptor proteins that are responsible for initiation of and continuation of spore germination. Outside of the

IM there is the germ cell wall, the peptidoglycan (PG)-rich cortex, the outer membrane, and the outer coat that protects inner regions from chemicals and enzymes and lastly the crust. This overall structure is shared by all *Bacillales* spores, with only minor differences (e. g. *B. cereus* spore structure includes a loose-fitting exosporium as the outer most layer instead of a crust) (Figure 2B).

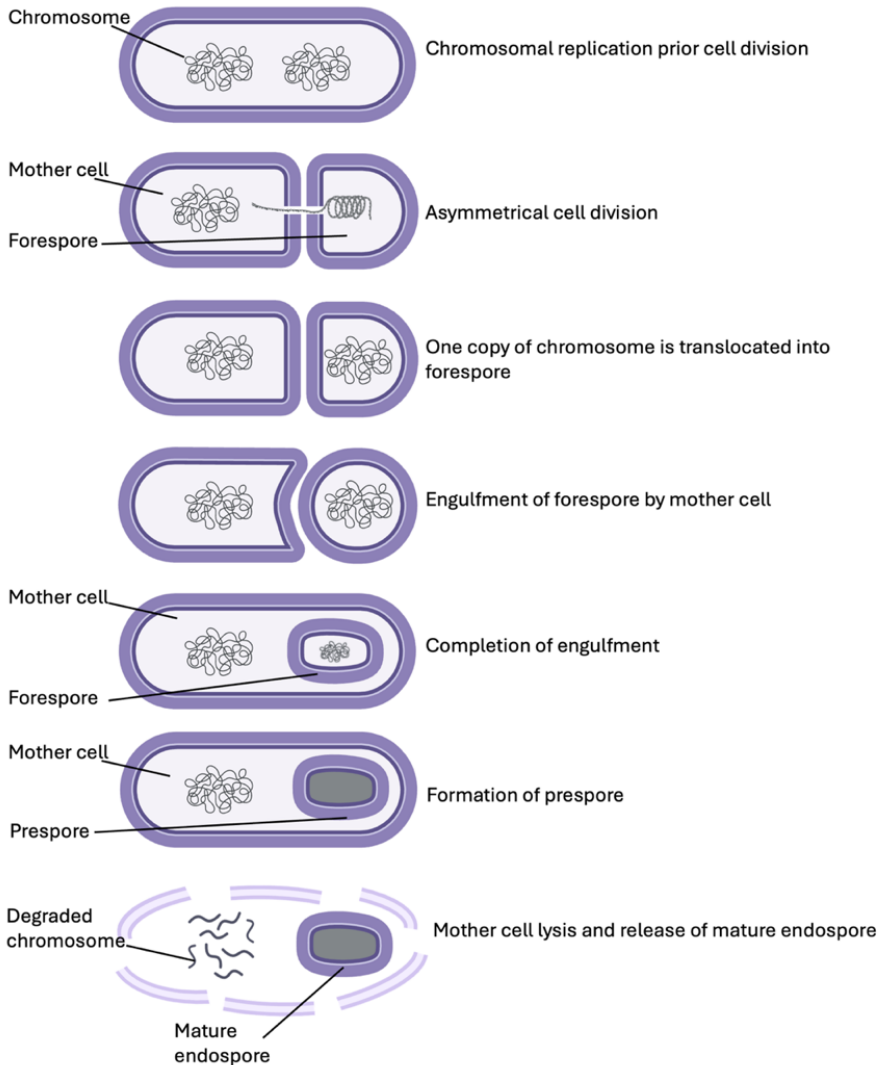


Figure 1. Sporulation of *Bacillus subtilis*, adapted from literature³⁰. Each stage of sporulation is briefly explained with a schematic illustration.

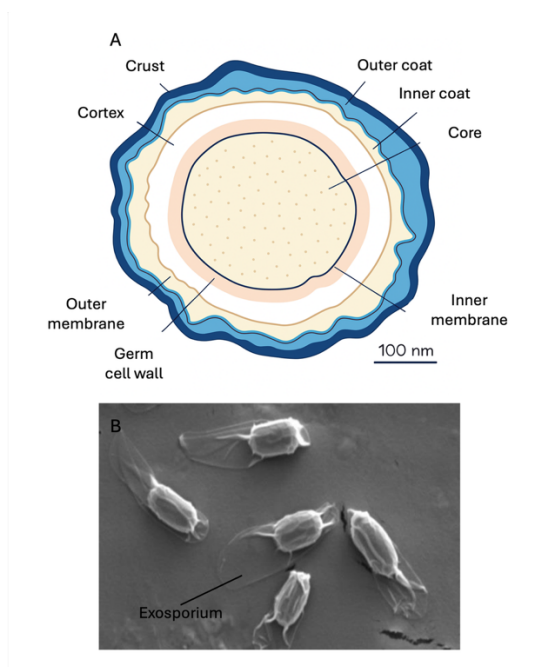


Figure 2. Structure of *Bacillus* spores. (A) Schematic illustration of a *B. subtilis* spore highlighting the structure of each protective layer, figure adapted from literature²⁷. (B) Scanning Electronic Microscopy (SEM) image of *B. cereus* spores with the exosporium prominently visible. Image source: French Agency for Food, Environmental and Occupational Health & Safety (ANSES)³³. © INRA, PIHM.

1.4 Germination in the model bacterium *Bacillus subtilis*

Under certain conditions, *Bacillus subtilis* spores can exit dormancy even in the absence of a functional bioenergetic system³⁴. These conditions include the presence of physiological germinants, which are low molecular weight nutrients such as specific L-amino acids or D-sugars, as well as non-nutrient germinants, including Ca²⁺-dipicolinic acid (CaDPA) and dodecylamine. Germination can also be induced by physical conditions such as moderate to high hydrostatic pressure, or by the action of cortex-lytic enzymes. Once activated by these stimuli, spores rapidly resume metabolism and progress to vegetative growth. These processes are termed as germination and outgrowth.

The physiological germinant-triggered germination process requires the function and cooperation of multiple germination proteins. Initially, when the germinant receptors (GRs) are triggered by germinants, the spore enters germination Stage I. In this stage, the information of germinant binding is passed on within a GR and possibly triggers

the opening of IM GerAA channel that release monovalent cations such as H^+ , K^+ and Na^+ ³⁵, followed by a small amount of leakage of core CaDPA. This process, which is not fully characterized, allows spores to commit to germination, which means the process cannot be reversed. The commitment is followed by bulk CaDPA release through a fully open SpoVA channel. The SpoVA channel opening and CaDPA release takes a few minutes in individual spores. And the CaDPA leakage happens along with ingress of a small amount of water by an unknown mechanism, raising the core water content from ~25% to ~45% wet wt. CaDPA release then triggers spores to enter Stage II of germination, which is the activation of cortex-lytic enzymes (CLEs) responsible for degrading spore cortex PG. Cortex hydrolysis causes spore cores' further expansion to around twice its original size via water uptake, and during this process, the IM can expand up to 1.3-fold of its original size without the need for lipid synthesis. Hydrolysis of cortex PG and full core expansion take 10-15min following the original CaDPA release, and the core water content also slowly rises to 80%. The mechanism of this step, along with the previous step that allows small amounts of water intake during germination, are unknown in *B. subtilis*³⁶. Nevertheless, this step results in a fully germinated spore (Figure 3). Notably, only fully germinated spores are capable of resuming metabolism and macromolecular biosynthesis. Prior to reaching full germination, spores contain only negligible amounts of ATP, and neither the spore core nor the inner membrane provides subcellular environments conducive to oxidative or substrate-level phosphorylation, gene transcription, or protein translation. This indicates that the germination process itself is largely completed without energy generation, energy consumption, or biomolecule synthesis.

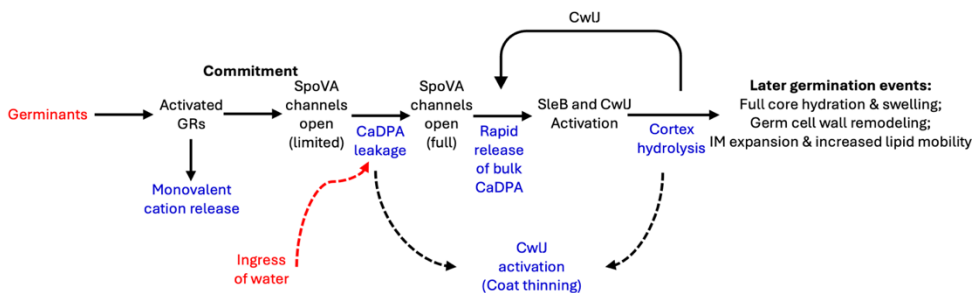


Figure 3. Signal transduction model during germination, figure adapted from literature²⁷.

Spore germination can also be initiated through several alternative mechanisms in addition to nutrient-based germinants. When endogenous CaDPA is released from the spore core during germination, it activates the cortex lytic enzyme CwlJ. Interestingly, exogenous CaDPA can also directly activate CwlJ, bypassing the nutrient-induced GR pathway entirely^{37,38}. Pressure-induced germination operates via two distinct mechanisms. At moderate pressures (100-300 MPa), germination proceeds in a manner similar to nutrient-induced pathways, involving GRs. However,

at higher pressures (500–800 MPa), germination becomes GR-independent and results in the direct release of DPA³⁹. Long-chain alkylamines, such as dodecylamine, can trigger germination at temperatures up to 70 °C. This type of germination occurs also independently of GRs, while spores remain relatively dehydrated and are rapidly killed after germination^{40,41}. Lysozyme-induced germination relies on the enzymatic degradation of the spore cortex peptidoglycan (PG). When the spore coat is removed artificially, lysozyme can access and degrade the cortex PG, leading to the release of CaDPA from the core⁴². Peptidoglycan fragments serve as growth signals from surrounding vegetative cells. Specific mucopeptide fragments—such as disaccharide tri- or tetrapeptides—can induce germination in *B. subtilis* spores by activating the membrane-associated threonine kinase PrkC, independently of germinant receptors⁴³.

1.5 Molecular apparatus in *Bacillus subtilis* spore germination

The molecular apparatus of germination includes germinant receptors (GRs), the SpoVA channel, and cortex lytic enzymes (CLEs), and all are proteins that are responsible for different steps of germination (Figure 4A). It is vital to gain insight to their structure and function.

The GRs respond to physiological germinants, including the three main GRs in *Bacillus subtilis*: GerA, GerB and GerK. Each GR consists of three subunits A, B and C in a 1:1:1 stoichiometry. GerA was intensively studied as model of GRs and it's proven to act like a nutrient-gated ion channel that responds to L-alanine and then releases monovalent cations; it forms a pentameric complex of the GerAA, GerAB and GerAC trimer³⁵ (Figure 4B). This mechanism was proposed and verified via structural prediction and mutagenesis experiments³⁵. GerAB is a transmembrane transceptor belonging to the APC transporter super family that binds substrate and allows further signal transduction without consuming ATP or transporting the substrate molecule across membrane^{44,45}. Notably, a recent study also showed that GerAB may act like a water channel, providing a potential mechanism for spore water intake during germination⁴⁵. GerAA is an integral membrane protein that includes a large soluble N-terminal globular domain⁴⁶. Its transmembrane domain likely pentamerizes into a cation channel and it also includes a potential signal transduction domain that interacts with GerAB to trigger downstream signalling^{35,47}. While GerAC is a membrane anchored lipoprotein, its individual function remains unclear. GerB and GerK GR, although less studied, are known to respond to a mixture of L-asparagine, D-glucose, D-fructose, and potassium ions (AGFK) in a collaboration with each other²⁷. Besides GRs, GerD is also an important member in the Ger family. Although its detailed function remains unclear, fluorescence microscopy has shown that GRs and GerD in *B. subtilis* spores colocalize primarily into a single cluster in dormant spores.

This cluster is termed a “germinosome” and it is located in the spore IM to carry out a rapid and cooperative response to nutrient germinants⁴⁸.

The SpoVA channel is another spore IM complex that is responsible for both importing DPA during sporulation and exporting DPA during germination^{49,50}. This process is carried out through the collaboration of multiple SpoVA subunits: SpoVAC and SpoVAEb form a membrane channel, while SpoVAD binds CaDPA and is essential for its transport into developing spores^{49,51}. Cortex lytic enzymes are responsible for cortex hydrolysis during germination. There are two partially redundant cortex lytic enzymes, CwlJ and SleB^{52,53}. Both are localised to the spore coat, where the spore outer membrane may serve as a physical barrier between them and their cortex substrate. SleB is also observed in the IM, together with the accessory protein YpeB, which has an undefined role in stabilizing SleB in the dormant spore²⁷. Their activation occurs in Stage II of germination, although the mechanism is not fully understood. However, their importance in germination is clear, since a *sleB/cwlJ* double mutant is completely blocked in nutrient-induced germination, although the early-stage germination-associated changes do occur, namely the release of monovalent cations and DPA⁵⁴.

1.6 Towards understanding of GerAB function using Molecular Dynamics simulations and *in vivo* experiments

GerAB functions as the nutrient-sensing subunit that initiates spore germination upon detecting L-alanine, it is responsible for the first step of the molecular cascade of more than one germination pathways. GerAB operates in close coordination with GerAA and GerAC, forming a trimeric-pentameric receptor complex^{35,44,55}. However, much of the current understanding of GerAB's role is based on static structural predictions, which rely heavily on template-based modelling or AlphaFold prediction (since no experimental method is capable of determining GerAB structure). These approaches, while informative, offer a limited view of GerAB's function in dynamic contexts, especially regarding how GerAB may undergo conformational changes during germination. As a result, key questions remain unanswered, such as: How does GerAB activate itself upon sensing L-alanine? What are the conformations and conformational transitions involved in this process? What is the mechanism of the putative GerAB water channel in core water intake during germination? To address these knowledge gaps, this study focuses on moving beyond static models and investigating the dynamic behaviour of GerAB in atomistic resolution.

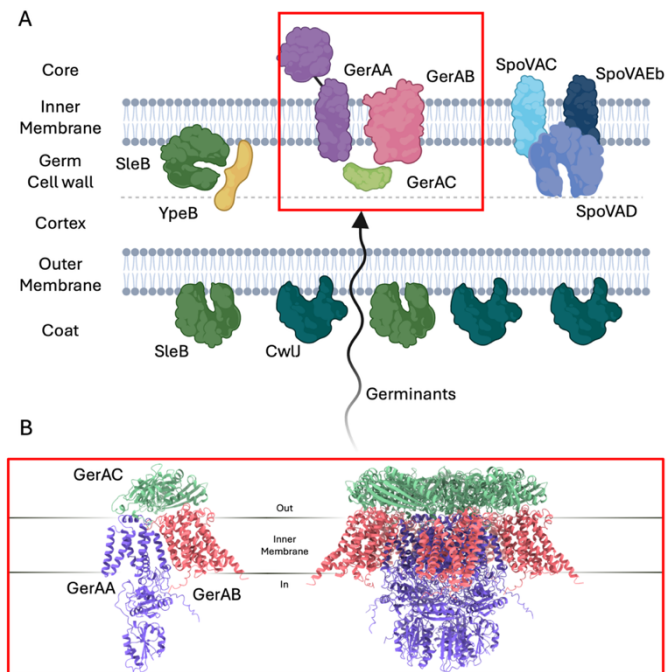


Figure 4. Molecular apparatus involved in *B. subtilis* spore germination. (A) Schematic illustration of key germination proteins and their localization within the spore. The primary germinant receptor, GerA, is located in the inner membrane (IM) and is highlighted with a red rectangle. Proteins are represented in space-filling models, figure updated from literature³⁵. (B) On the left is the predicted structure of the GerA trimeric complex, composed of GerAA (purple), GerAB (pink), and GerAC (green), shown in the context of its position within the spore inner membrane. Right, space-filling model of a GerA complex formed by five trimers (pentamer of trimers), illustrating its organization and integration within the inner membrane³⁵.

Molecular Dynamics simulations provide insights in protein structural dynamics with high resolution in both space and time. With all-atom molecular dynamics, researchers are capable of studying molecular mechanisms and dynamics of biological macromolecules that are otherwise impossible^{56,57}. Recent studies employing MD on protein systems have revealed important insights into conformational transitions⁵⁸, identified novel conformations⁵⁹, calculated free energy of ligand binding⁶⁰, and elucidated mechanisms of small molecule permeation⁶¹. Notably, a recent study employing MD simulations on a sporulation protein SpoIVFB, offered valuable insight in mechanism of protein function, protein interaction and signal transduction during sporulation⁶². In the present study, molecular dynamics (MD) simulations were applied to GerAB to gain a deeper understanding of its functional dynamics. Specifically, insights from the simulations were used to generate hypotheses for experimental validation. To test these hypotheses, multiple experimental methodologies were employed. Using genome editing and live-cell imaging via phase-contrast microscopy, mutant spores were constructed and their germination

behaviour *studied in vivo*. By bridging *in silico* analysis with *in vivo* experimentation, this study provides a more comprehensive and in-depth investigation into the functional mechanisms of the GerAB protein.

1.7 Outline of the study

This study investigates the molecular mechanism of *B. subtilis* spore germination with a focus of investigating molecular details of GerAB using molecular dynamics (MD) simulations, corroborated with mutagenesis and germination assays. Chapter 1 introduces the background and objectives of the research. Chapter 2 describes the research methods in detail. Chapter 3 employs steered Molecular Dynamics (SMD) simulations to explore the preliminary path of water molecules through GerAB and verifies the role of potential blockade residues through experiments. Chapter 4 identifies residues exhibit high contact frequency with passing water using MD simulations and validates their roles with *in vivo* and *in silico* mutagenesis. Chapter 5 explores molecular details of GerAB including gating of water passage, and ligand disassociation with MD and SMD simulations. Chapter 6 describes a collaboration with the University of Connecticut (USA) and the University of Cambridge (UK). It investigates the germination of *Bacillus* spores with Li^+ , including characterizing the germination with experiments and exploring the molecular detail of GerAB-cation interaction with MD simulations. Chapter 7 documents the effort of implementing spore germination in multidisciplinary education for Bachelor students. Finally, Chapter 8 concludes the thesis by discussing the key findings and outlining future challenges.