*Bacillus* Spore Germination: Knowns, Unknowns And What We Need To Learn

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**Summary**

How might a microbial cell that is entirely metabolically dormant – and which has the ability to remain so for extended periods of time – irreversibly commit itself to resuming vegetative growth within seconds of being exposed to certain amino acids or sugars? That this process takes place in the absence of any detectable ATP or *de novo* protein synthesis, and relies upon a pre-formed apparatus that is immobilised, respectively, in a semi-crystalline membrane or multi-layered proteinaceous coat, only exacerbates the challenge facing spores of Bacillales species when stimulated to germinate. Whereas the process by which spores are formed in response to nutrient starvation – sporulation – involves the orchestrated interplay between hundreds of distinct proteins, the process by which spores return to life – germination – is a much simpler affair, requiring a handful of receptor and channel proteins complemented with specialized peptidoglycan lysins. Despite this relative simplicity, and research effort spanning many decades, comprehensive understanding of key molecular and biochemical details and, in particular signal transduction mechanisms associated with spore germination, has remained elusive. In this review we provide an up to date overview of the field while identifying what we consider to be the key gaps in knowledge associated with germination of Bacillales spores, suggesting also technical approaches that may provide fresh insight to this unique biological process.

**1. Introduction**

A number of species of bacteria in the Firmicutes phylum can form intracellular spores when the environment is no longer conducive to further growth [1, 2]. These spores are ultimately released upon lysis of the mother cell in which they develop in the sporulation process. The free spores are metabolically dormant and resistant to all manner of harsh treatments such as high temperatures, desiccation, radiation and a host of chemical agents including antibiotics, and can most likely survive for many years in the environment, as they certainly can in controlled conditions [1, 3, 4]. However, there may be limits on spore longevity due to aspartate isomerization in spore proteins [5]. Many factors are involved in spore’s high resistance and survival. Among the most important ones are spores’ structure (Fig. 1), in particular the: i) outer coat layers that protect more inner layers against chemicals and enzymes; ii) spore cortex peptidoglycan (PG) layer that is outside the germ cell wall PG and participates somehow in establishing and maintaining the core’s low water content, as low as 25% of wet wt in some species; iii) low permeability of the inner membrane (IM), in which lipids have very low mobility; iv) high level, ~ 25% of core dry wt, of a 1:1 chelate of dipicolinic acid (DPA) with divalent cations, predominantly Ca2+ (CaDPA); and v) saturation of spore DNA with novel low molecular weight (mol wt) protective proteins. Importantly, growing and/or stationary cells derived from spores of many species can secrete enzymes and/or toxins that can result in food spoilage or poisoning, as well as some serious human diseases or intoxications including botulism, gas gangrene, tetanus, severe and often fatal diarrhea or anthrax [1].

Because of all these spore properties, there is much interest from the food, health care and defense industries in how to safely and rapidly eradicate spores. While spore eradication is difficult because of their extreme resistance, when spores germinate in order to return to vegetative growth, their resistance properties are lost. Consequently, germinated spores are as easy to kill as are growing cells, including by antibiotics [1, 6-8]. Thus, there is the potential utility of a “germinate to eradicate” strategy for spore decontamination, and therefore much applied interest in how to trigger spore germination. In addition, spore germination is a fascinating biological process, with some features that have few parallels in other organisms. Due to much work over the past ~ 50 years, many of the details of the spore germination process have been determined, but there remains much we do not know, including many details of the signal transduction processes involved in germination.

Thus, it is the purpose of this review to examine in detail what we do and do not know about spore germination, and even more importantly, to assess what information is needed to complete our understanding of spore germination and possible ways this information could be obtained. We will focus on spores of species in the order Bacillales, since the general features of spore germination appear identical in all Bacillales spores studied thus far. However, the germination of spores of species in the order Clostridiales differs in significant ways from that of spores of species in the order Bacillales. In addition, even within the order Clostridiales, spores of species in different genera exhibit significant differences in their germination. For details of germination in spores of both phyla, the reader is referred to recent thorough reviews of what is known about the germination of spores of these two orders, including the regulation of the expression of genes encoding germination proteins [9-12]. Note, however, that generally only recent literature references will be cited in this current review, primarily those not cited in the recent reviews cited above.

**2.0 The Process of Spore Germination**

The current model for Bacillales spore germination (Fig. 2, 3) requires three major types of germination-specific components [10, 12]. Initially, in Stage 1, germinant receptors (GRs) in spores’ IM (Fig. 1) respond to physiological germinants, most often low mol wt molecules such as specific L-amino acids or D-sugars. Invariably, overexpression of GRs in spores increases the rate of GR-dependent germination, and variability in GR levels in individual spores in populations is one factor that contributes to the heterogeneity in the rates of germination of individual spores. Once stimulated, GRs are thought to trigger the opening of IM channels leading initially to release of monovalent cations such as H+, K+, and Na+, slow leakage of a small percentage of spores’ CaDPA, and some physical change in the spore IM which is not understood; these and perhaps other unknown events commit spores to going through germination. However, what commitment is in terms of molecular events, and the identity of the channel protein(s) involved in the monovalent cation release are unknown. Soon after the monovalent cation release, very rapid CaDPA release is triggered by opening of an IM channel for CaDPA composed of multiple SpoVA proteins, one of which one specifically binds CaDPA, and another is a mechanosensitive membrane channel protein. Opening of this SpoVA channel then leads to all CaDPA release in a few min from individual spores, and the released CaDPA is replaced by water, raising *Bacillus subtilis* core water content from 35% to 45% of wet wt. CaDPA release then, either directly or indirectly, triggers the entry into Stage 2 of germination by activation of the two redundant cortex-lytic enzymes (CLEs) that degrade spores’ large PG cortex, but the germ cell wall PG is not a CLE substrate. Cortex PG hydrolysis results in an ~ 2-fold expansion of the spore core via further water uptake, and fusion of sub-IM membrane material with the IM itself increases the overall IM surface area ~ 1.3-fold without the need for new membrane synthesis [13]. Hydrolysis of cortex PG and full core expansion takes 10-15 min after CaDPA release, and during this period the spore core water content slowly increases from 45% to 80% [10, 14]. This results in a fully germinated spore, which allows resumption of metabolism and macromolecular biosynthesis. However, since hydration of the spore core to 80% of wet wt increases only slowly, as noted above, there could certainly be a return of some enzymatic activities in the germinating spore core before full core hydration is achieved.

**2.1 Germinant Receptors (GRs)**

Work from multiple labs over the past 40+ years strongly suggests that in *Bacillus* spores, physiological germinants such as specific amino acids and sugars, alone or in combinations, are recognized by a complex of GR proteins in spores’ IM, and this germinant recognition sets in motion the spore germination cascade (Fig. 2, 3) [10, 12]. Spores generally have multiple GRs, each with different and/or overlapping germinant specificity, and different GRs often cooperate to trigger spore germination [10, 15]. The levels of the GRs in spores are heterogeneous, and this latter heterogeneity is likely to contribute to the heterogeneous rates of germination of individual spores in populations in response to physiological germinants. Heterogeneity in germinative rates between individual spores within a population has also been attributed to the timing of spore formation during culture, with ‘early forming’ spores germinating more rapidly than ‘late forming’ spores when exposed to appropriate germinants. The molecular basis for this discrepancy in germinative responses between sub-sets of spores within populations has not been established but has been suggested as reflecting differences in the ‘quality’ of spores formed at different periods of culture [16]. GRs are composed of A, B and C subunits in a 1:1:1 stoichiometry, genes for which are often, but not always expressed as an operon. The C-subunit protein is an IM anchored lipoprotein and the A and B subunits are integral IM proteins; all these subunits have domains on the outer IM surface, and the A and B subunits also likely have domains in the spore core. There is also a very hydrophobic D subunit, encoded in some GR operons [8, 17], which is presumably also buried in the IM. Although not all GRs have this D subunit, its loss decreases the function of GRs with which it is likely associated. A recent screen has also identified some additional genes that affect *B. subtilis* GR-dependent spore germination [18], but these new genes’ mechanism of action is not known.

The subunits of a single GR likely form a complex in the IM, and generally an individual GR’s subunits show minimal exchange between different GRs, although this has been seen in a few cases [10, 19]. Germinant binding is likely to the A subunit and perhaps also to the B subunit, although could be between the A and B subunits [10, 20]. High resolution structures of large fragments of one C subunit and one A subunit have been determined and the C subunit structure is unique (Fig. 4a), while the A subunit structure (Fig. 4b) shares structural homology to the ligand binding domain of bacterial ABC transporters. However, there is minimal structural information about the B proteins, nor about the full length A and C subunits, although modeling work for the GerAB protein is in progress, which may give some important new insight (S. Brul, personal communication). The IM lipoprotein GerD is also important in GR-dependent germination, as *gerD* spores have >10-fold decreases in these germination rates. A high-resolution structure of most of a GerD protein has been determined (Fig. 4c), but it shows no homology to structures of proteins of known function. Notably, in spores of *B. subtilis*, *Bacillus cereus* and *Bacillus megaterium* all GRs are present in the IM in a large complex termed the germinosome that also includes the highly conserved GerD protein which is essential for germinosome assembly [10, 21, 22]. There are primarily1-2 germinosomes per spore in all three species examined, although a few spores may have none.

There are a number of major questions about GRs and GR function that have not been answered, many of fundamental importance to a full understanding of the whole process of germination. These include the following.

1) What is the structure of a GR with all 3 or 4 subunits? Knowledge of a high-resolution structure of a GR, presumably assembled in a membrane, might give some idea of what a GR could do. In particular, might one or more GRs assemble to facilitate channel formation in the IM, allowing for the monovalent ions to be released when spores become committed to germinate upon GR activation? Use of techniques such as cryo-electron microscopy (cryoEM) or X-ray crystallography of GRs in nanodiscs might be a productive way to attack this problem. However, this will require production of complete GR proteins in significant amounts, and to date this has proven to be challenging. Perhaps *in vitro* translation might be used to overcome this challenge.

2) Do GRs actually bind the physiologically relevant germinants, and if so, which subunit or subunits is/are involved in germinant binding? As noted above, this seems likely to be on the A and/or B subunits but this has not yet been rigorously proven. Again, generation of high levels of these proteins, both of which have multiple membrane spanning regions will be crucial in doing this work. If this can be done for complete GR subunits or GRs themselves which respond to well characterized single or multiple different germinants, then determining which subunit(s) binds the germinant(s) should be possible using techniques such as Surface Plasmon Resonance (SPR), Nuclear Magnetic Resonance Spectroscopy (NMR) or X-ray crystallography, as well as appropriate site directed mutagenesis of likely key residues in putative binding sites.

3) What do GRs do upon germinant binding? It is clear that germinants trigger GRs to set the germination cascade in motion. But what exactly do the “triggered” GRs do? Is there a conformational change in GRs that translates a signal across the IM or to other germination proteins? Indeed, there is some very recent evidence for such a change (see below). How does germinant-GR recognition lead to monovalent cation release and then rapid CaDPA release, the latter via the SpoVA IM channel? While there are no answers at present to these questions, the answers are crucial to understanding the signal transduction pathway in germination. In particular what is “commitment” to germinate on the molecular or spore structural level and how does germinant-GR “interaction” trigger downstream germination events? Attacking these problems may again require experiments *in vitro* with a GR in an artificial liposome, and perhaps even including SpoVA channels.

4) What is the exact structure of the germinosome, and how does this structural arrangement influence spore germination? It is known that spores lacking GerD, and thus a germinosome, do germinate with GR-dependent germinants, but > 10-fold slower than spores with a germinosome [10]. Is there some cooperative interaction between GRs in a germinosome that is not possible when the GRs are spread throughout the IM? Are other germination proteins in close contact with the germinosome, in particular SpoVA proteins? This latter type of interaction was suggested by work a number of years ago but has by no means been proven [23]. Tackling this problem might require assembling a germinosome *in vitro*, a significant undertaking. Notably, recent work has indicated that one or more germinosome proteins in sporesundergo some changes in state soon after germinant addition [24, 25], but the nature of this change is not known.

5) How does the known relative immobility of lipids in the IM [26] affect the function of proteins, including GRs, in or associated with the IM? How IM lipid immobility is achieved in sporulation is also not clear, but very recent work [27] indicates that this requires both the spore coat and core CaDPA.

6) What is the precise function of the likely ion channel proteins that have been identified as important in GR germination of spores of some Bacillales species [10] and with some germinants? The fact that this has not been seen with spores of all Bacillales species indicates that such proteins are not universally essential germination proteins, but learning how such channel proteins function in germination might give new insight into the germination process.

7) How does spore heat activation increase the rate of GR-dependent spore germination? It has been known for years that a sublethal heat treatment increases the rate of GR-dependent germination, a phenomenon called heat activation. Since different GRs exhibit different responses to heat activation, this treatment seems likely to be affecting GRs [10, 28]. Does this treatment alter the state of GRs in spores and could this be probed with appropriate fluorescently labeled GRs in spores? Studying heat activation *in vitro* might be quite difficult, since spore proteins in the IM or the core are generally well protected against heat by the low spore core water content, and it would be difficult, although perhaps not impossible, to mimic this protein heat resistance *in vitro*.

8) Why don’t GRs trigger spore germination in sporulating cells? It is known that a small percentage of developing *B. subtilis* spores germinate prematurely in the sporulating cell, with this germination triggered by the most abundant GR, GerA [29], and when GerA is overexpressed more than ~ 3-fold, almost all developing spores germinate prematurely [30]. In addition, spores with normal GR levels that do not accumulate CaDPA germinate spontaneously both during and after sporulation. The precise factors that maintain GRs in an inactive state during sporulation and during spore dormancy are not known, although it has been suggested that GerA’s actions to trigger premature germination may be a form of quality control for the spores produced [29].

9) How do GR-dependent germinants such as amino acids and sugars get to the IM where GRs are located? A number of labs have shown that normal rates of GR-dependent germination of spores of multiple *Bacillus* species are dependent on a group of GerP proteins, which are likely located in the spore coat [10, 31]. However, the precise mechanism whereby the GerP proteins facilitate germinant access to GRs is not clear. Notably, the GerP proteins do not appear to be essential for normal access of several non-GR-dependent germinants, including CaDPA and dodecylamine (see below).

10) What is the molecular mechanism(s) of the apparent memory of prior germinant exposure of *Bacillus* spores? An initial pulse of a variety of either GR-dependent or GR-independent (see below) germinants to spores of several *Bacillus* species is followed by a heightened response to a second pulse, as if there was some memory of the initial pulse, although this “memory” decayed rather rapidly [10, 32]. Available data suggest the memory is stored in some activated protein state, most likely one of the SpoVA proteins involved in CaDPA release in spore germination, but the latter suggestion has not been proven.

**2.2 Spore Germination by Additional Agents, Most of Which are GR-Independent**

In addition to the sugar and amino acid germinants that trigger germination via GRs, spores are also germinated by other specific agents. Importantly, use of one of these agents has led to significant applied advances, as well as deeper understanding of the process of spore germination [10, 33]. This other agent is high hydrostatic pressure (HHP) which can trigger spore germination via GRs at HHPs of 150-400 MegaPascals (MPa) at 37°C or via the SpoVA channel for CaDPA at > 500 MPa at 50°C. HHP treatments carried out at even higher pressures (600-700 MPa) and with initial temperatures of ~ 90°C, at which adiabatic heating can give sterilizing temperatures of > 121°C, has proven useful in the preparation of some shelf-stable foods and may hold the promise of sterile foods in the future. While it is clear that spore germination is triggered by HHP via either GRs or the CaDPA channel, it is not clear if it is the proteins that are directly affected by HHP, or whether HHP has effects on the spore IM, and these in turn affect IM-embedded proteins. The likelihood of the latter possibility being true is strengthened by recent work on spore germination and killing by the chemical dodecylamine [27]. The latter compound was shown to germinate spores of a few species > 60 years ago, and the list of species with dodecylamine-susceptible spores has grown since then, and includes those of both Bacillales and Clostridiales [10]. A number of previous studies have suggested that it is the CaDPA channel that is opened by dodecylamine and this is followed by killing of the CaDPA-less or germinated spore. However, recent work indicates that *B. subtilis* spores that lack the SpoVA protein CaDPA channel are killed by dodecylamine at the same rate as wild-type spores [27]. Thus, perhaps dodecylamine exerts its direct effects on the IM, and this then leads to effects on the CaDPA channel. This is clearly a matter for further research on the mechanism(s) of HHP’s effects on spores as well.

Another non-physiological germinant is exogenous CaDPA itself [10], and this agent works best with spores that have the CLE CwlJ, and it appears likely that CaDPA activates this CLE. However, to date there is no definitive proof that the effect of CaDPA is directly on CwlJ, and not perhaps on the cortex PG substrate. This topic is discussed further below.

An additional germination mechanism that does not require GRs has been called “spontaneous” germination. This was first definitively identified with *B. subtilis* spores lacking genes for all known GRs, as a low percentage of these spores germinated at a relatively constant rate at 37°C, ~ 0.04%/day over at least eight days, and this did not require a complex mix of potential nutrient germinants [34]. This spontaneous germination was studied further a few years ago [35], and its frequency appears to be under the control of the GerE transcription factor that modulates expression of many genes expressed late in the sporulating mother cell. Levels of GerE in individual sporulating cells were also found to vary widely, and GerE levels varied inversely with the rates of spontaneous spore germination; *gerE* null spores also had a greatly increased rate of spontaneous germination. Given that GerE modulates the expression of many spore coat genes, it was suggested that spore coat differences are responsible for different rates of spores’ spontaneous germination. Indeed, spores lacking the gene encoding the CotE protein important for spore coat assembly exhibited elevated spontaneous germination. There is also other work indicating that spore coat structure can affect rates of GR-dependent germination [36]. However, the mechanism whereby the coat modulates spontaneous spore germination is not known.

Finally, there are muropeptides from growing cells that were initially reported ~ 10 years ago to trigger *B. subtilis* spore germination by activating the protein kinase PrkC ([37] (also see [38]). While the data implicating PrkC as having a role in spore germination were compelling, an essential role for a kinase activity in triggering spore germination is at odds with the absence of detectable ATP in spores’ until well after germination has been triggered, and that germination of at least *B. megaterium* spores is unaffected by blocking ATP accumulation early in germination [26, 39]. Unfortunately, there has been minimal study of the role of PrkC in spore germination in the past 10 years, and reinvestigation of this germination mechanism is certainly warranted.

**2.3 SpoVA Channel for CaDPA**

The long-established observation that significant quantities of CaDPA are released from the spore core during germination led, naturally, to the hypothesis that a specific channel is involved in this CaDPA release. Experimental evidence to substantiate this idea has accumulated over the past 20 years or so, where, despite garnering less research interest than other components of the germination apparatus, the degree of structural and mechanistic insight that has been achieved is arguably on a par with that of the GRs [10]. The channel for DPA movement across the spore IM – in the form of a 1:1 chelate with Ca2+ ions – is almost certainly formed from the SpoVA proteins, since strains with mutations in this locus retain the ability to synthesize DPA but cannot incorporate it into the developing forespore [40]. In addition, mutations in a few *spoVA* genes give rise to defects in CaDPA release in spore germination. Notably, the ability to form stable CaDPA-less spores requires additional stabilizing mutations, namely deletion of GR genes or the *sleB* gene that encodes the major CLE involved in spore germination [40]. As befits their Spo designation, the SpoVA proteins differ from other major components of the germination apparatus in that they are involved in both sporulation and germination, as they transport CaDPA into the spore core during sporulation and then facilitate its rapid release during germination. Additionally, there is a distinct transporter, SpoVV, which moves DPA from the mother cell cytoplasm across the outer forespore membrane during sporulation [41]. However, it is not clear that this transporter plays a role in spore germination, as whether the outer forespore membrane is a permeability barrier in free spores has not been established.

The *spoVA* operon encodes 7 proteins in most *Bacillus* species, designated as SpoVAA through SpoVAF; SpoVAE was belatedly sub-divided into distinct SpoVAEa and SpoVAEb proteins. In contrast to the germinosome-localised GR and GerD proteins, SpoVA proteins appear spread throughout the entire spore IM, with an abundance, based on SpoVAD, that is 2 to 3-fold higher than the total numbers of GRs, at least in *B. subtilis* [42]. Fairly limited data have been presented to date indicating that at least some of the SpoVA proteins physically interact with some GR protein subunits [10, 23], although this is an area that merits further attention, perhaps using super-resolution microscopy techniques. Most of the SpoVA channel components are integral membrane proteins with multiple membrane-spanning domains, although SpoVAD and SpoVAEa are soluble globular proteins that are somehow anchored to the outer surface of the spore IM. A crystal structure for the SpoVAD protein has been determined (Fig. 4d), revealing that the protein adopts a thiolase-type fold that is common to many CoA-dependent biosynthetic enzymes. However, structural differences between the active sites of the thiolase enzymes and the analogous region of SpoVAD indicate that the latter is not associated with catalytic activity. Instead, combined *in silico* molecular docking, SPR and molecular genetic analyses have shown that SpoVAD both binds and is essential to the transport of CaDPA into developing spores [43]. However, whether the protein also has a role in CaDPA movement across the IM during germination is not clear. In contrast, the second of the globular components of the SpoVA channel, SpoVAEa, is not essential for CaDPA uptake into the developing spore but appears instead to have a role in CaDPA release during GR-triggered germination [44].

Whereas mechanistic details of the SpoVAEa protein’s role in CaDPA release have not been elucidated, a significant breakthrough in the entire field of spore germination concerned the identification of mechanosensitive properties associated with the SpoVAC protein. The observation that SpoVAC conferred protection against hypo-osmotic stress when expressed heterologously in *Escherichia coli*, coupled with patch clamp experiments conducted with SpoVAC reconstituted in synthetic lipid vesicles, provided compelling biochemical and biophysical evidence that the SpoVAC protein has channel like properties that are gated by membrane tension [45]. These mechanistic insights are consistent with early events in spore germination, whether triggered via the GRs or by the activity of exogenous enzymes such as lysozyme (or indeed, CLE activity in *C. difficile* and related species [46, 47]), all of which result in the influx of water to the spore core. Equally, this model for germination dictates that some entry of water to the core must precede (at least bulk) CaDPA release in order to induce hypo-osmotic stress across the IM, raising the as-yet-unanswered question as to how water enters the spore core.

If, as indicated above, SpoVAD is responsible for binding CaDPA at the mother-cell proximal side of the CaDPA channel, and SpoVAC individually can form gated pores in the spore IM, what then is the role of the other proteins encoded within the SpoVA operon? The remainder are all predicted to be integral membrane proteins, with the last encoded in the operon, SpoVAF, sharing sequence similarity with GR A-subunit proteins. The significance of this similarity, if any, has not been established, although the recent structure-led determination that the A-subunit of the GR confers a probable germinant binding site raises the possibility that the SpoVAF protein may also be involved in binding a ligand associated in some way with germination. However, in contrast to most of the other components of the CaDPA channel, SpoVAF is not essential for successful sporulation or germination, although its deletion is associated with an extended period of commitment during GR-triggered germination.

How then may we advance our knowledge in this particular area? X-ray crystallography may offer a route to structural insight on the SpoVAEa protein, which is globular, and also to the crucial SpoVAC protein, which is amenable to overexpression in *E. coli*, although crystallization is likely to be challenging owing to SpoVAC’s integral membrane status. The remaining SpoVA components are also integral membrane proteins, which various labs have reported as being recalcitrant to expression in standard bacterial expression systems. One option might be to simplify the task and focus on the minimal requirements for a functional CaDPA channel. The genomes of many Clostridiales, for example, appear to encode only the C, D and Eb proteins of the SpoVA operon, indicating that a functional channel in these species may comprise only these subunits. Co-expression of these three proteins in an appropriate system – perhaps cell-free – with subsequent reconstitution in lipid or detergent micelles may offer a route to structural determination of an intact channel via cryoEM or X-ray crystallography.

**2.4 Cortex Lytic Enzymes (CLEs)**

Spore germination, as indicated above, can be characterized broadly into two stages. Stage 1 comprises GR and channel protein mediated events associated with germinant interaction and flux of ions and small molecules to and from the spore core and across the IM. In contrast, Stage 2 events are principally enzymatic in nature, being associated with the depolymerization of the PG cortex which is crucial to the establishment and maintenance of spore dormancy. Dissolution of the restrictive cortex permits hydration of the now germinated spore core to levels that are commensurate with the resumption of metabolic activity, essentially marking the completion of germination. Note - the question as to where and how coat depolymerization fits into models of germination is a grey area, although it appears from electron micrographs to be initiated at or around the time of cortex degradation [48]; whether this is an enzymatic or purely physico-mechanical process has not been established and remains an objective for future investigation.

In spores of *Bacillus* species cortex depolymerization is conducted by two semi-redundant PG lysins, the CLEs SleB and CwlJ, and spores of some species have multiple CwlJ homologs [10, 49]. These enzymes only hydrolyze PG containing the cortex PG-specific modification, muramic acid--lactam, and thus germ cell wall PG is not attacked. SleB is a lytic transglycosylase, but CwlJ cleavage specificity has not been determined. A number of other CLEs also participate in cortex degradation, including SleL (also known as YaaH), for which high resolution crystal structures have been determined [50] (Fig. 4e), and YdhD. However, these proteins appear to have only a supporting role, cleaving PG fragments generated by SleB and CwlJ, in order to facilitate muropeptide passage through the coat to the environment. In addition, several other CLEs facilitate some PG cortex hydrolysis in *B. megaterium* spores lacking CwlJ and SleB [51].

Despite the accumulation of an extensive literature on CLE structure and function over the past 30 or so years, some major questions relating to these enzymes’ activation during spore germination remain unanswered. We know that SleB and CwlJ are present in the dormant spore in a mature form, in contrast to the situation in spores of some Clostridiales species, where the major CLE, SleC, is present as an inactive pro-enzyme [9-11]. How this functional but inactive CLE state is achieved in Bacillales spores and maintained over potentially extended periods of time is, however, not clear. What signals or structural changes in the spore facilitate the rapid transition to the active forms of the respective enzymes? Hypotheses concerning these questions differ for SleB and CwlJ. The former is a modular enzyme with distinct N-terminal substrate-binding and C-terminal catalytic domains. Crystal structures for both domains from *B. cereus* SleB are available [10, 52] (Fig. 4f), and have helped to identify residues that are crucial for substrate binding and lytic transglycosylase catalytic activity. However, crystal structures deposited thus far, apo-forms of the *B. cereus* [53] and *B. anthracis* [54] enzymes, have not given significant structural insight into SleB’s behavior in dormant and germinating spores. Structures of ligand-bound forms of the enzyme would be beneficial in this regard, perhaps revealing precise mechanisms for cortical PG recognition and conformational changes that may occur during activation of the enzyme. There are no obvious technical barriers to progress in this area, and progress on this topic could be beneficial from an applied perspective, as it might facilitate structure-led design of inhibitors of CLE activity. In terms of SleB activation, early work conducted with *B. megaterium* spores indicated that the enzyme (then known as GSLE) might be processed proteolytically to an active form during spore germination [55]. We know now that this is not the case, and that Western Blot data presented earlier probably represented a transition from dimeric to monomeric forms of SleB during germination, although similar changes to the oligomeric status of SleB are not observed in spores of other species [56].

An alternative idea for modulation of SleB activity has also been postulated which is that mechanical changes in the cortical PG may occur during spore germination, which somehow triggers or allows SleB activity [10]. The hypothesis that only cortical PG under appropriate mechanical stress can act as a substrate for SleB is supported by observations with engineered CaDPA-less spores, which only complete sporulation to release mature (albeit relatively highly hydrated) spores when the *sleB* gene has been deleted [40]. However, this hypothesis has by no means been proven since it is challenging from a technical perspective to measure or visualize changes in the mechanical properties of spore PG. Interestingly, SleB is active against spores in which the coat has been removed chemically or compromised by mutations, as well as against purified sacculi. Presumably then, if the ‘stress’ hypothesis noted above is correct, cortical PG in these substrates must share some structural/mechanical features with PG in germinating spores. Notably, recent work [27] indicates that the spore coat likely does exert some compression on more inner spore layers, which lends further support to this stress model.

The observation that the SleB locus always encodes a second protein, YpeB, the C-terminal domain of which shares a fold common to a class of metalloprotease inhibitors [10, 57, 58] (Fig. 4g), led to a second hypothesis concerning SleB (in)activation, namely that YpeB and SleB interact to mediate SleB function in the spore. Subsequent disruption of this interaction, facilitated perhaps by the proteolytic cleavage of YpeB that is observed during germination, results in SleB activity. This idea is substantiated by the observation that both proteins are dependent on the expression of the other for localization within the spore, but these findings simply shift the question to: how might the protease be activated during germination? Unfortunately, numerous attempts to detect direct interactions between SleB and YpeB have failed to provide evidence for such an interaction. Similarly, deletion of the HtrC protease primarily responsible for cleaving YpeB during germination has little effect on SleB activity *in vivo*, although it seems that other proteases may be able to compensate for the loss of HtrC [59]. There is also one report that the *B. subtilis* lipoproteins YlaJ and YhcN are involved in modulating SleB activity [60].

As noted above, it is not yet clear how SleB is activated during germination. Clarification of the precise location of SleB within the spore may be useful in this regard. Transcription of *sleB* takes place in both the developing forespore and in the mother cell [10]. Furthermore, SleB has been located on the IM outer surface and to the inner coat region, with both locations raising further questions including: i) how is SleB tethered to the IM in the absence of any notable membrane anchoring sequence; ii) does the IM associated protein represent protein that has failed to migrate to the coat during sporulation and is therefore functionally redundant; iii) if functionally important, how does IM-associated SleB negotiate the germ cell wall layer of PG to reach its cortical substrate; iv) how does coat-localized SleB negotiate the outer membrane, assuming this structure presents a barrier at all, to reach the cortex during germination; and v) what is YpeB’s role, if any, in these putative SleB migrations within the spore? IM and coat locations for SleB, if correct, may account for its inactivity during dormancy (i.e. by physical separation from its substrate), but not for its activation during germination. However, progress in understanding SleB activity is undoubtedly less daunting from a technical perspective than gaining insight at the molecular level to proteins and events associated with Stage 1 of germination. We suspect that time resolved super resolution microscopy measurements, for example, allied with structural insights obtained from X-ray crystallography, should prove useful in moving this area forward.

In contrast to SleB, the mechanism of germination signal transduction associated with CwlJ, the second major CLE, appears to be clearer, although perhaps only superficially. CwlJ, like SleB, relies upon a helper protein – GerQ in this case – for its localization within the spore. A substantive amount of experimental evidence places CwlJ within the innermost regions of the spore coat [31], so although the mature protein is present in the spore, it appears to be separated physically from its cortical substrate. Biochemical and structural knowledge of CwlJ lags behind that of SleB, in part because analysis of muropeptides released during germination have thus far failed to reveal its hydrolytic bond specificity. Similarly, heterologous expression of CwlJ from spores of various species typically results in insoluble aggregates of recombinant protein that are not conducive to biochemical or structural analysis. However, sequence analyses indicate that CwlJ comprises a single domain that is homologous to SleB’s catalytic domain. Homology modelling coupled with site-directed mutagenesis experiments identified a single catalytic glutamate residue that supports the idea that CwlJ, like SleB, has lytic transglycosylase activity, although a recombinant version of the *B. anthracis* enzyme was proposed to have N-acetylmuramoyl-L-alanine amidase activity [10]. Further work is warranted to clarify this ambiguity.

Significantly, as indicated above, CwlJ is essential for germination triggered by incubating spores in highly concentrated (>50 mM) CaDPA solutions. Hence, it seems probable that CaDPA efflux from the spore core during Stage 1 of germination represents a simple signal transduction pathway associated with activation of the enzyme. However, questions remain, in particular, does CaDPA interact directly with CwlJ, perhaps triggering a conformational change and enzymatic activation? In the absence of biochemical experiments conducted with purified CwlJ or high-resolution structural information there has been no way to investigate this possibility. Alternatively, perhaps CaDPA somehow increases the plasticity of the inner coat, or stresses the structure of the cortex giving CwlJ the opportunity to gain access to, or recognize its substrate. It is also possible that activation of CwlJ by CaDPA is mediated by an as yet unidentified coat protein. Indeed, recent work with *B. subtilis* spores has shown that the SwsB protein, about which little is known, is required for efficient PG hydrolysis by CwlJ [61]. Regardless, the requirement for high concentrations of exogenous CaDPA to induce germination indicates that the affinity of the target protein, if indeed it is a protein, is low, which may hinder SPR, NMR or X-ray crystallographic attempts at examining the molecular basis of CaDPA induced germination. Similarly, the poor solubility of CwlJ means that a highly engineered variant of the protein may be required for structural and biochemical work, further complicating progress in this area. Regardless, CLEs and their activation represent a potential Achilles heel in spore defense mechanisms, making them attractive targets for ‘germinate to eradicate’ decontamination strategies, arguably more so than the frequently recalcitrant components of the Stage 1 germination apparatus. Similarly, current gaps in knowledge of CLE behavior in the spore represent major omissions in current models of spore germination. Accordingly, further work is warranted from both basic cellular biology and applied perspectives.

**2.5 Additional Stage 2 Germination Events**

Another event in Stage 2 of germination which needs attention is the precise mechanism whereby the spores’ IM bounded volume increases ~ 2-fold to complete spore germination. This increase in IM bounded volume requires an increase in IM surface area of ~ 1.3-fold and takes place in the absence of ATP, and thus in the absence of new phospholipid synthesis. Recent work has shown that the additional membrane needed to provide the increased IM surface area comes from membrane material beneath the IM, which are likely vesicles [13], and recent work indicates these vesicles are most likely continuous with the IM [27]. This extra membrane material disappears as the IM bounded volume increases, but how this membrane fusion event takes place is not known, nor if it requires direct metabolic energy, which as noted above is minimal at this time in germination. Alternatively, the fusion event may become favored when cortex degradation takes place. Given the many well-studied membrane fusion events that take place in biology, it seems likely that one or more spore proteins are involved in the membrane fusion in spore germination and the presumed extrusion of membrane from the developing spore IM in sporulation, and this could again be an interesting area for further research.

Finally, there is the question of the need for protein synthesis during spore germination, as has been suggested in several publications [62, 63]. It seems very unlikely that any essential protein synthesis in germination can be from mRNA in the dormant spore, given that > 99% of all *B. subtilis* spore mRNAs, including all abundant ones, can be lost with no notable effects on spore germination [14, 64]. In addition, several studies have shown that dormant spores have little if any ATP, even during incubation at physiological temperatures [10, 65, 66], and a recent study found there was no significant protein synthesis in germinating *B. subtilis* spores until after germination was complete [67]. Similarly, abolishing > 95% of ATP production does not block germination, at least of *B. megaterium* spores [26, 39]. However, it is certainly possible that enzyme activity could return in spores before germination is complete, as when cortex PG hydrolysis is triggered, it can take 10-15 min for an individual *B. subtilis* spore core’s water content to rise from the 45% wet wt in a CaDPA-less spore at the end of Stage 1 to the 80% of wet wt in a fully germinated spore [14]. Thus, it would be of interest to examine the return of enzymatic activity, in particular metabolism, in the spore core during germination as a function of core water content. However, the heterogeneity in the germination of spore populations could make this analysis difficult, as it precludes population-based measurements. Using spores that germinate rapidly, either *B. subtilis* spores with elevated GR levels or *B. megaterium* spores, might make this feasible, as would examination of the germination of individual spores. Interestingly, there are several recent reports [68, 69] that changes in spores’ phosphoproteome significantly influences spore germination, including by specific removal of phosphates on arginine residues in several proteins.

**2.6 A brief overview of Clostridiales spore germination**

While this review purposely focuses on germination of *Bacillus* spores it is worth drawing attention to some of the key similarities and differences in germinant sensing and signal transduction pathways that exist between Bacillales and Clostridiales spores, including species and group-specific variances within the latter order. Much of the focus of attention centers on the role of subtilisin-like proteases of the Csp family, which are present within spores of several significant pathogenic Clostridiales species. In *Clostridium perfringens*, for example, cortex hydrolysis is initiated by the activity of three distinct Csp proteins (A, B and C), which are somehow stimulated to self-cleave their respective pro-domains to confer catalytically active forms upon germinant-mediated activation of GerA-type GRs. The activated Csp proteases then cleave the inactive pro-form of SleC, the major CLE in this and several other species of Clostridiales, to produce a catalytically active peptidoglycan lysin. Notably, in contrast to the sequence of events in Bacillales spores, it is only *after* cortex degradation that CaDPA efflux from the spore core takes place [10, 11].

The involvement of Csp family proteins in Clostridiales spore germination is taken to another level altogether by the major nosocomial pathogen *Clostridioides difficile*. Its genome encodes three orthologues of these proteins – two of which, CspA and CspC, are considered to be pseudoproteases by virtue of amino acid substitutions within the highly conserved catalytic triad that is present in Csp-type proteins with proteolytic activity. In a major deviation from the Bacillales, the CspC protein, which is localised to the inner coat of the spore, appears to act as the germinant receptor in spores of this species, the genomes of which lack recognizable orthologues of the GerA-type GRs. However, GerA-type GRs are present in spores of many Clostridiales, and have been implicated in triggering spore germination. The germinant recognition profile of *C. difficile* spore is also distinctive, with cholate bile salts acting as primary germinants, and glycine and a handful of other amino acids plus calcium ions providing added stimulus to the germinant signal. Despite intensive study, and significant progress in some regards, precise details concerning ligand binding sites and signaling pathways remain sketchy in *C. difficile*. It has been established, however, that cholate-mediated CspC activation – whatever that may entail – stimulates auto-processing of pro-CspB, which in turn proteolytically activates pro-SleC, resulting in cortex hydrolysis and subsequent release of CaDPA from the spore core [9, 11]. Again, in contrast to the Bacillales, cortex degradation precedes CaDPA efflux form the spore core, albeit via a different pathway to that observed in *C. perfringens*.

Other Clostridiales employ either the SleB/CwlJ or Csp/SleC systems to effect cortex degradation, the division being defined at the group level in *Clostridium botulinum* spores. This species relies upon GerA-type GRs to initiate the germination process, although like all Clostridiales, it lacks an orthologue of the GerD protein. Hence it is not clear whether GRs cluster in germinosome type structures within the Clostridiales in order to facilitate synergistic GR interactions.

**3.0 Conclusions and Looking Ahead**

Nearly 40 years have elapsed since the presence of a GR required specifically for triggering spore germination in response to cognate molecular germinants was first proposed [70]. Similarly, the first detailed biochemical characterization of a CLE from *Bacillus* spores took place more than three decades ago [71]. Significant strides have been made in the time since in establishing the proteinaceous components of the germination apparatus and their location within the spore, both of which we now know are shared essentially across the Bacillales. Time resolved changes that occur to the spore ultrastructure, proteome and chemical composition during germination have also been recorded with ever increasing precision and detail. Astonishingly, however, as detailed above, we still do not know the precise function of GRs, nor how they operate at the molecular level. Neither do we know with any certainty how CLEs are functional only during spore germination. The chain of signal transduction mechanisms between the GRs and the CLEs also remain shrouded in uncertainty. Insight in all three areas, probably in the order presented, arguably represent the greatest scientific prizes that remain in the field. All three additionally offer scope for significant applied interventions. The fact that these fundamental gaps in our knowledge remain reflects, likely, the intrinsic complexity and technical challenges that are associated with working with spores and their constituent proteins. Throw in a unique cellular biology, and collectively this is what makes the subject endlessly fascinating and attractive to a new generation of researchers equipped with new investigative tools.

We are fortunate that the past decade or so has enabled – at last – a glimpse into what the components of the germination apparatus actually look like, with significant fragments of the GR structure, full length CLEs, and various accessory proteins – GerD, SpoVAD, YpeB - being revealed at high resolution (Fig. 4). Equally, although important and undoubtedly challenging from a technical perspective, these structural insights have all been achieved using conventional X-ray crystallographic approaches that arguably could have been delivered at any point over the past number of decades. Evidently, as with all matters scientific, progress is only made when the time is right, in particular when researchers from different disciplines use their unique expertise to address a challenge. However, with this in mind, a complete picture of a GR or a CaDPA channel will almost certainly require the introduction of more recent technological advances, with cryoEM offering perhaps the most plausible route to visualizing the membrane-associated germination complexes at atomic resolution. This can only be attempted, of course, if perhaps the greatest bottleneck to progress in the field can be circumvented, i.e. obtaining correctly-folded protein in quantities that are commensurate with structural analysis. The protein abundance problem applies to both GRs and CLEs (in particular CwlJ), and also to at least some of the SpoVA proteins. Additionally, a number of proteins that are indirectly involved in spore germination – the GerP proteins, for example – have also proven to be recalcitrant to heterologous expression, hindering biochemical and structural analyses. Progress in this area, perhaps taking advantage of developments in cell-free systems or re-visiting the idea of purifying proteins directly from spores, will be essential to alleviate the structural and biochemical bottlenecks in the field. In the meantime, lower resolution approaches, including direct and quantitative super-resolution microscopy, and advanced electron tomography techniques, are being put to good use for *in vivo* imaging of the spore ultrastructure during both dormancy and germination.

Equally, we should acknowledge also that much of the structural and high-resolution imaging work that we anticipate will take place over the next number of years to develop our understanding of spore germination in the Bacillales is based upon the outputs of detailed molecular genetic analyses that took place over the preceding 20 or so years. Surprisingly, after seemingly having identified most if not all of the components of the germination apparatus, genetic screens are still occasionally identifying new genes and their products that appear to have a role in spore germination. The field still presents new and exciting challenges. With the incorporation of new proteins and fresh insight into existing models of germination, coupled with the introduction of new technologies and multifaceted approaches, hopefully new findings will shed light on this most recalcitrant of biological processes.

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**6.0 Figure Legends**

Figure 1. Schematic of a dormant *B. subtilis* spore. Spores of all Bacillales share a common ultra-structure although some species have a loose-fitting exosporium in place of the crust. GR and CaDPA channel proteins are localised to the inner membrane (IM). CLEs are localised to the inner spore coat although the major lysin, SleB, is also found in the IM.

Figure 2. Signal transduction during physiological germination of spores of *Bacillales* (adapted from [10]). Germination starts when germinant molecules interact with GRs, which results in spores becoming committed irreversibly to germinate. The release of monovalent cations from the spore core represents the earliest detectable biochemical event during germination followed by the slow release of CaDPA via SpoVA channels in the IM. CaDPA may in turn activate the coat-localised cortex-lytic enzyme CwlJ, the activity of which subsequently facilitates rapid release of bulk CaDPA from the core. Increased hydration of the core presumably starts during monovalent ion or CaDPA release, although complete hydration only occurs after the cortex has been degraded by the combined activity of CwlJ and SleB. Cortex degradation is followed by a series of late germination events that lead ultimately to the resumption of metabolism and outgrowth of a new vegetative cell.

Figure 3. Key Bacillales germination proteins and their location in spores. GRs comprise three (sometimes four) distinct protein subunits that interact to form functional receptor complexes. The A- and B-subunits are integral inner membrane (IM) proteins although the A-subunit has a large globular domain that may provide a germinant binding site. The CaDPA channel is composed of the SpoVA proteins, which are also localised to the IM. This channel is composed of seven distinct proteins in *B. subtilis*, and in most other Bacillales, including mechanosensitive SpoVAC and the globular SpoVAD subunit which has a probable binding site for CaDPA. For clarity, only the C, D and Eb proteins are depicted although bioinformatic analyses indicate that these subunits encompass the minimal components required for a functional CaDPA channel in the anaerobic spore-forming Clostridiales. The major CLEs, SleB and CwlJ, are localised to the coat, where they may physically be separated from their cortex substrate by the outer membrane. SleB is also observed in the IM, together with the accessory protein YpeB, which has an undefined role in stabilising SleB in the dormant spore.

Figure 4a-g. Crystal structures of Bacillales spore germination proteins. (a) C-subunit of the *B. subtilis* GerB GR (PDB identifier: 3N54); (b) N-terminal domain of the *B. megaterium* GR subunit GerK3A (PDB identifier: 6O59); (c) *Geobacillus stearothermophilus* GerD protein, represented as a trimer (PDB identifier: 4O8W); (d) *B. subtilis* SpoVAD (PDB identifier: 3LM6); (e) *B. cereus* SleL (PDB identifier: 4S3J); (f) C-terminal domain of *B. cereus* SleB (PDB identifier: 4F55); and (g) C-terminal domain of *B. megaterium* YpeB (PDB identifier: 5BO1). Proteins are represented from amino termini (blue) through to carboxy termini (red).

Figure 1

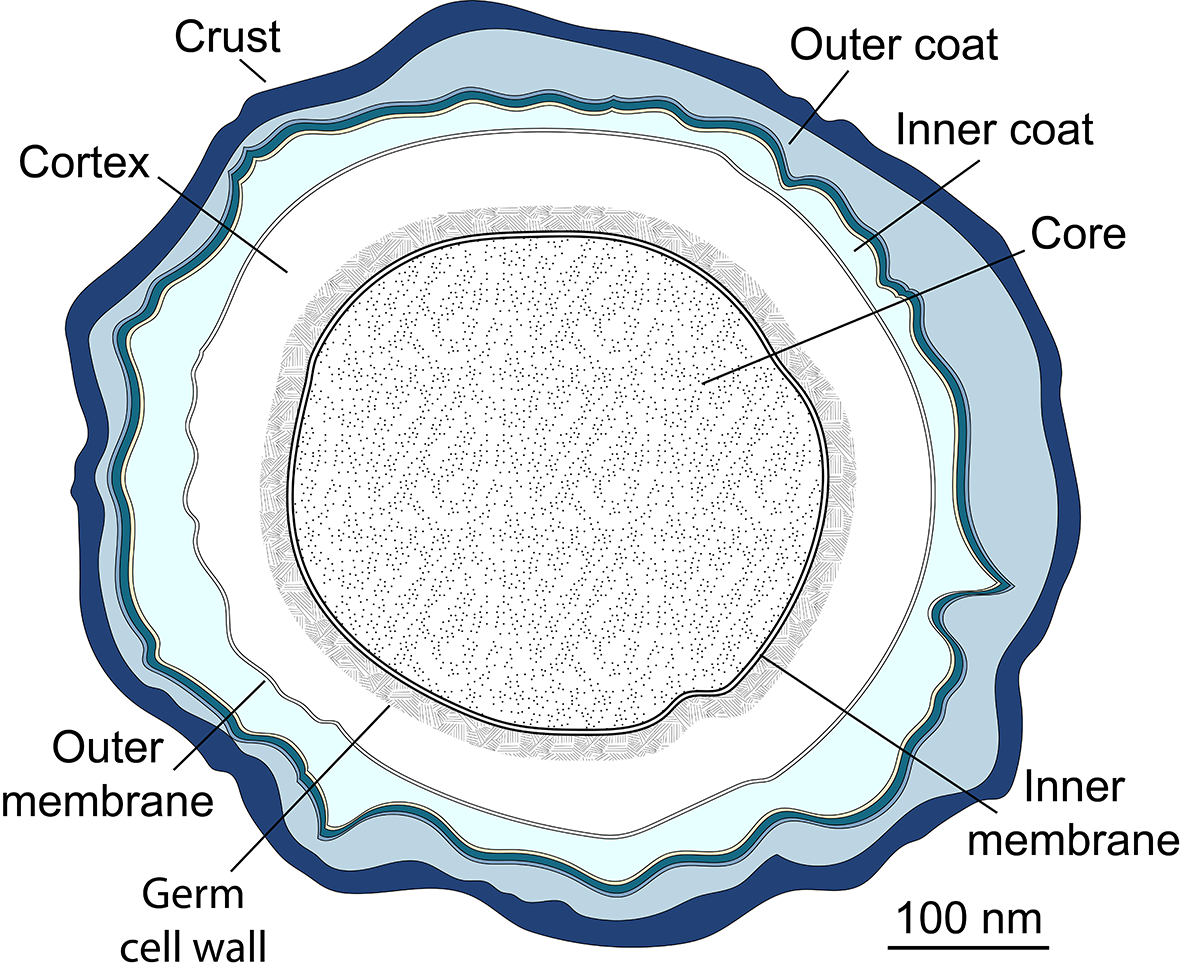


Figure 2

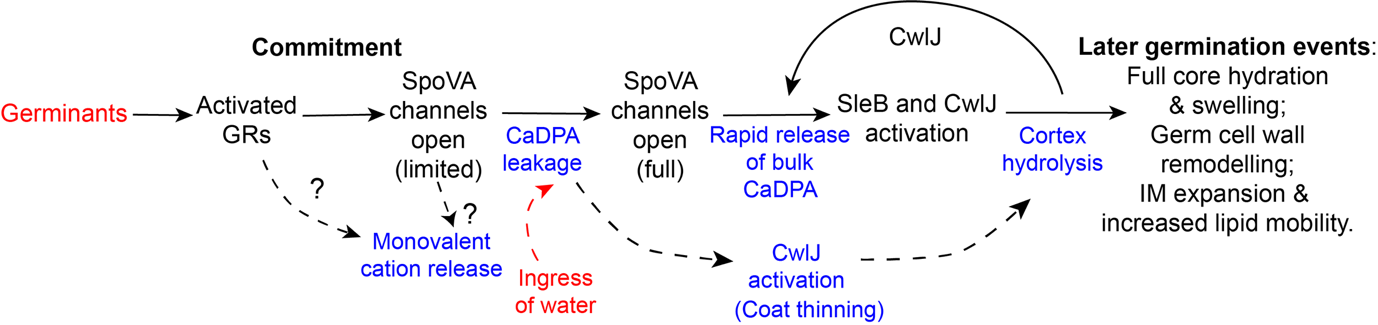


Figure 3

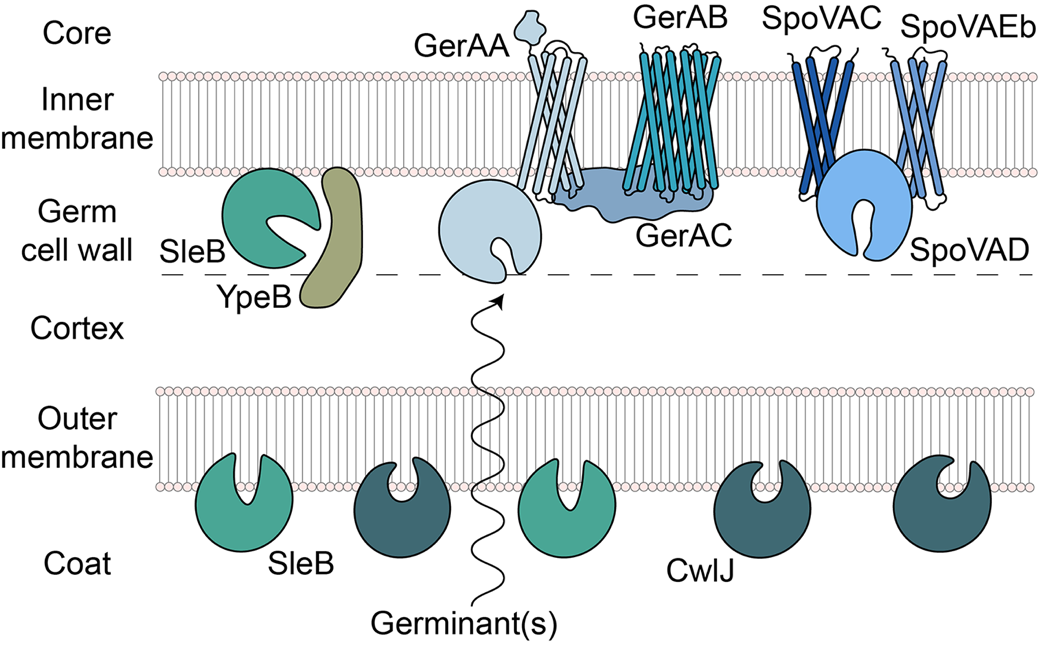


Figure 4

