Spore Germination

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ABSTRACT Despite being resistant to a variety of environmental insults, the bacterial endospore can sense the presence of small molecules and respond by germinating, losing the specialized structures of the dormant spore, and resuming active metabolism, before outgrowing into vegetative cells. Our current level of understanding of the spore germination process in bacilli and clostridia is reviewed, with particular emphasis on the germinant receptors characterized in Bacillus subtilis, Bacillus cereus, and Bacillus anthracis. The recent evidence for a local clustering of receptors in a “germinosome” would begin to explain how signals from different receptors could be integrated. The SpoVA proteins, involved in the uptake of Ca\(^{2+}\)-dipicolinic acid into the forespore during sporulation, are also responsible for its release during germination. Lytic enzymes SleB and CwlJ, found in bacilli and some clostridia, hydrolyze the spore cortex: other clostridia use SleC for this purpose. With genome sequencing has come the appreciation that there is considerable diversity in the setting for the germination machinery between bacilli and clostridia.

A PERSPECTIVE ON GERMINATION STUDIES

The specialized structure that maintains the dormancy and resistance properties of endospores provides an opportunity for wide dispersal of spores in the environment, and for survival over long periods under conditions unfavorable for growth. Although dormant and uniquely resistant to environmental insult (1, 206), a spore remains sensitive to changes in its environment. Specific germinants are detected by receptors in the spore inner membrane; this signal is then transduced by mechanisms that are not understood in detail, but result in the activation of proteins that variously allow movement of small molecules across the membrane and deconstruct protective layers, restoring normal hydration and active metabolism (2, 3).

In addition to recent reviews of germination (4, 5), the literature also provides a valuable earlier perspective. The germination behavior of a variety of species, including bacilli (6, 7) and clostridia, was reviewed by Gould (8). Biochemical and physiological changes during germination and outgrowth were examined (3, 9). In the decades following the 1970s, classical and molecular genetics, mainly in Bacillus subtilis, then allowed identification of many of the gene products involved in the germination process (2, 3, 10–12). More recently, genome sequencing has allowed wider comparisons across endosporeformers, and has led to direct testing of the contribution of candidate gene products to the overall germination behavior in other species in which gene knockouts are possible, as reviewed elsewhere (13–18). Multiple receptors in a species would allow effective germination in different environmental conditions, and are commonly found.

Common germinants include amino acids, sugars, purine nucleosides, inorganic salts, or combinations of these molecules (7, 19). Although there is no need for them to be metabolized, or to enter the spore core, they are often described as “nutrient” germinants to distinguish them from “nonnutrient” germinants, such as Ca\(^{2+}\)-dipicolinic acid (CaDPA) or dodecylamine. The amino acid L-alanine and its analogues are frequently effective (6) as sole germinants or in combination with others. Recently, the germinant receptors of B. subtilis have been shown to cluster in a “germinosome,” and
definitive evidence has been obtained for the role of SpoVA proteins in release of CaDPA from the spore core. Other current areas of research include detailed measurements of events at the single spore level, structure/function relationships in germination proteins, both in vitro and in vivo, and an appreciation of the properties of “superdormant” spores within a spore population.

Spore germination can also be induced by other means in a receptor-independent process. These agents include exogenous CaDPA (20), lysozyme (21), decylamine (22), or very high pressure (23). In addition, fragments of vegetative cell peptidoglycan (24, 25) induce spore germination in a protein kinase-dependent manner that is not at all understood.

KINETICS OF GERMINATION

In general, the response of a spore population to a germinant stimulus is heterogeneous. The timing of germination of individual spores was studied by phase-contrast microscopy (26, 27), and two parameters were defined: microlag, the time between the addition of germinant and the start of phase darkening, and microgermination, the time for germination-associated phase darkening to occur, as a result of rehydration of the spore core, which is much more rapid. The heterogeneity of germination was due to the variation in this microlag. Conveniently, the response of spores to germinant can be measured in suspension, where observations such as loss of heat resistance, release of dipicolinic acid (DPA) into the supernatant, and reduction in optical density reflect the aggregated behavior of the population. As a result, the kinetics of such overall changes will reflect the distribution of lag times in the spore population.

Dissection of germination events in large numbers of single spores of wild type and mutants, measured in real time, is now possible, using technologies such as Raman spectroscopy with laser tweezers to follow DPA release from single spores, which is concomitant with the loss of refractility, and differential interference contrast (DIC) microscopy, which can also detect the rehydration that accompanies cortex hydrolysis (28–30). Such approaches have confirmed and extended the earlier work, showing for example that the level of germinant receptors in the spore affects the lag time, but not the 0.5 to 3 min taken to release the bulk of the DPA. This bulk release is slowed in a CwlJ mutant and is faster in a spore with higher-than-normal levels of SpoVA proteins. In some spores, up to 15% of DPA may be released during the “lag” period, suggesting that limited germination-associated changes in the spore may occur well before the point when the spore is committed to germinate.

From a germinating spore population, the spores that are less responsive to germinant and have remained dormant can be separated on a density gradient, and the behavior of this “superdormant” fraction, which is of considerable applied significance, has been examined (31–34). The variation in response appears to be related to heterogeneity in individual spores within a population, in terms of the amount of receptor and water content, for example. A recent review (35) discusses this in more detail from a mathematical perspective, while another review (36) focuses on the consequences of heterogeneity from the applied perspective of food microbiology. Progress through germination and outgrowth of Clostridium botulinum has also been examined at the single-spore level (37, 38) to inform predictive food microbiology.

A Cautionary Reminder

In practical terms, data in the literature only provide a general guide to the behavior of the reader’s favorite spore-forming species or strain: the overall germination rate of the spore suspension can also vary considerably, depending on the conditions of sporulation, such as temperature or medium used, or length of time for maturation after the completion of sporulation, whether in a psychrotrophic Bacillus cereus (39) or the laboratory strain of B. subtilis (40–42). Germination response is often measured at a single pH, temperature, and germinant concentration; this may not be optimal and may underplay the contribution of different receptors to different environmental conditions. Different wild isolates from the same species may behave very differently (43). In addition, the consequences of domestication may need to be considered. While it is important that longer-term subculture or storage of Bacillus strains on non-sporulating media is to be avoided (44, 45), long-term storage on sporulation agar may favor the survival of spores that do not rapidly germinate—some B. subtilis 168 isolates from different laboratories show differences in germination behavior, and even mutations in recognized ger genes (46).

STAGES OF GERMINATION

The change from dormant spore to germinated spore (Fig. 1A and B, respectively) and then to vegetative cell can be rather arbitrarily divided into four main stages: activation, stages I and II of germination, the latter dependent on cortex hydrolysis, and then the last stage,
where a fully hydrated germinated spore resumes metabolism and outgrows. These are discussed in turn, based largely on experiments with the paradigm organism, *B. subtilis*.

**Activation**

Depending on the strain, sporulation conditions, and the extent of washing to remove surface-bound molecules that may potentiate germination, the average spore will respond more rapidly to germinants if the population has first been activated, for example, by exposure to sublethal heating or by extended storage at 4°C (47). A classic analysis of *B. subtilis* (48) showed that temperatures in between these extremes also lead to activation. Spores of *Geobacillus stearothermophilus* may be effectively activated by incubation at 37°C (49, 50).

For laboratory-based study, a brief sublethal heating of *Bacillus* spores accelerates the activation of germination via nutrient receptors, by reducing the micro-

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**EVENTS IN SPORE GERMINATION**

The full molecular details of signal transduction in spore germination are not yet clear, but many of the proteins involved in the process are identified, and reasonable hypotheses can be constructed with the available information (Fig. 2). Most of the evidence is derived from studies with *B. subtilis*, the genetic and biochemical paradigm sporeformer. Large-scale transport or metabolism of germinant is not required for germination (55), and neither energy metabolism nor macromolecular synthesis is required for the earliest stages. However, the assumption that the germination process is essentially a biophysical and degradative one is challenged lag; the degree of activation required depends on the receptor that is stimulated (51). Stimulation by multiple germinants, targeting different receptors, reduces the need for heat activation in the general spore population (33). Sublethal heating improved germination in a *gerP* mutant of *B. cereus* (52), suggesting that activation may also increase the permeability of coat layers. Germination of superdormant spores that are more resistant to wet heat and have a lower water content could also be stimulated by heat activation, but at higher temperatures than the average spore in the population (33). In contrast, spores that lack the spore maturation *spmA* or *spmB* genes have a higher core water content and respond to germinants more quickly than wild type (53, 54). Speculatively, it may be that the tipping point for signal transduction downstream of the receptor is influenced by the local hydration state.
by recent work (56) using dormant spores of B. subtilis permeabilized to antibiotics; late stages of germination (after loss of DPA and heat resistance, but before the complete loss of phase brightness) were then dependent on protein synthesis. How this is to be integrated with other observations of late-germination events must await a more detailed analysis of the properties of such part-germinated spores.

How Does the Germinant Access the Receptor?
First, the germinant must traverse the outer layers of the spore to access nutrient germinant receptors in the inner membrane. The spore coats act as a molecular sieve, excluding enzymes, but small molecules such as germinants ought to be able to penetrate them. In the B. cereus/B. anthracis family, and to a lesser extent B. subtilis, the loss of GerP proteins from the spore coat (52, 57) reduced the effectiveness of nutrient germinants and CaDPA, suggesting that the permeability of spore coats to germinants is reduced if GerP proteins are absent. The coats may also influence germination rates because of the presence of enzymes that can modify or degrade the germinant, such as alanine racemase in spore coats (58, 59), and, along with inosine hydrolase, in the exosporium of the B. anthracis/B. cereus/B. thuringiensis family. As discussed later, some spore cortex lytic enzymes, such as CwlJ, are sequestered at or near the spore coat.

Stage I Germination
The germinant(s) interact with a membrane-located receptor complex, which transduces the stimulus, activating directly or indirectly the membrane-associated changes and hydrolytic reactions that occur in germination. The mechanism of signal transduction is not known, but it is likely to involve some conformational change of receptor protein(s) as a result of germinant binding. That moderate physical pressure induces germination via such receptors (60) may suggest that it also induces conformational change. Germinant receptors are discussed in considerable detail later in this review. Once the receptor has been activated, and the signal transduced, the spore is committed to germinate, and events will proceed even after removal of the germinant, or addition of d-alanine to competitively inhibit further l-alanine binding (61).

Large-scale excretion of monovalent cations, including H⁺, Na⁺, and K⁺, occurs very early (62). The release of H⁺ from the core results in an elevation of core pH, which will be required to activate phosphoglycerate mutase that metabolizes stores of 3-phosphoglycerate, generating ATP during late germination or outgrowth (63). The proteins involved in these ion fluxes are unknown, but, since the number of receptors in the spore is low (see below), it is unlikely to be mediated directly by receptor proteins, although it may be initiated by them. Later in stage I germination, the large store of CaDPA within the spore core is released and is replaced by water. The influx of water acts to partially rehydrate the spore core and, as a result, there is a marked reduction in wet-heat resistance (64). Proteins of the spoVA operon mediate CaDPA release during germination (65), as well as CaDPA uptake from the mother cell compartment during sporulation (66), and roles of individual proteins are being identified (67, 68). Bacilli and clostridial genomes all encode at least a minimal set of SpoVAC, SpoAD, and SpoAE proteins (15, 207), emphasizing the central role of these proteins in endospore biology. A transient increase in spore permeability, resulting in the release of free amino acids (glutamate and arginine) and other small molecules from the core also occurs at about the same time as the release of CaDPA, but its mechanism is not known: the retention of adenine nucleotides and 3-phosphoglycerate reveals that there is selectivity in the excretion of these various molecules (69).

After the early events of germination, the extreme heat resistance of the spore has been lost, but protein mobility in the core (70) and bulk lipid mobility in the inner membrane (71) have not yet increased. Up to this point there is no detectable metabolism and the majority of common high-energy compounds are found in their lower-energy states, such as ADP and AMP, coenzyme A, and NAD (9). Enzyme activation and ATP synthesis do not occur without complete rehydration of the spore core, which requires cortex lysis in stage II.

Stage II Germination
In B. subtilis, cortex hydrolysis is dependent on two, partially redundant, cortex lytic enzymes, CwlJ and SleB (72, 73). In stage II of germination, cortex lytic enzymes are activated, and cleave peptidoglycan containing muramic δ-lactam, leaving the germ cell wall intact. Spores with a mutation in cwlD, or in pdaA, a gene encoding a polysaccharide deacetylase gene, have no muramic δ-lactam, and are defective in nutrient-induced germination, because their cortex is no longer recognized by the lytic enzymes (64, 74). Cortex breakdown is completely blocked in a double sleB cwlJ mutant, although early germination-associated changes, including some release of CaDPA, occur (75).
The SleB/CwJ pair of enzymes are common in bacilli (15), and have been studied in B. subtilis (73, 76, 77); homologues play equivalent roles in Bacillus anthracis (78–80) and Bacillus megaterium (81, 82). Because CwJ can be activated, bypassing the germinant receptors, by addition of endogenous CaDPA (20), it would be activated somehow in nutrient-stimulated germination by the CaDPA released from the spore core. The two cortex lytic enzymes are located differently in the spore. The CwJ protein is synthesized in the mother cell and is localized in the spore by the coat protein YwdL (GerQ) (83). In contrast, SleB is synthesized in the forespore and secreted, and is present in two locations in the mature spore (73)—at the inner membrane and in the outer layers, presumably in association with the coexpressed YpeB protein, which is required for the retention of the SleB protein in the spore (73, 84). The role of YpeB may extend beyond localization (85, 86).

Spore coat depletion, which removes CwJ and the outer component of SleB, does not completely block spore germination, and, therefore, the SleB associated with the inner membrane is sufficient for cortex hydrolysis. How the SleB protein is activated during germination is not yet clear. SleB has a lytic transglycosylase activity and is active on cortex, either intact or fragmented (87). The recently determined 3D structure of the catalytic domain of SleB protein from the B. cereus/ B. anthracis group demonstrates the specificity for cortex peptidoglycan (88, 89) and the active site topology. In contrast, the YaaH (SleL) protein (90) is an N-acetylgalcosaminidase that is active on cortex fragments during germination, but it is not required for the spore to germinate (91).

Cortex lysis leads to full rehydration and expansion of the core; the inner membrane and germ cell wall expand, and the germinated spore regains more normal cellular properties (Fig. 1B). With the rehydration and expansion of the spore core, proteins and lipids in the inner membrane become mobile again. This marks the end of dormancy and the spore’s sensitivity to heat and to water is now similar to that of vegetative cells. Small acid-soluble proteins (SASPs), including those of alpha and beta type that are DNA associated, are degraded following the activation of the specific GSP protease (92), freeing the DNA for transcription and providing a source of amino acids for biosynthesis during outgrowth. DNA repair proteins already present in the spore are now active (93, 94) to repair damage incurred during spore dormancy. ATP is generated from the spore’s store of 3-phosphoglycerate, because phosphoglyceromutase is activated as the pH in the core rises.

**Spore Coat Degradation**

Somehow, the germination signal is transduced to activate partial spore coat breakdown. Although remnants of spore coat remain even after germination, there is significant local degradation, beginning at about the same time as cortex hydrolysis, visible in a series of beautiful electron micrographs of thin sections of germinating and outgrowing spores (95) and in atomic force microscopy images (96). The outer coat thins from the inside, and gaps appear in the inner multilamellar coats. Presumably, local machinery is present in the coat to initiate degradation. Nothing is yet known of the proteases involved, or how they are activated; some spore coat changes are seen in germinating spores of a cwlD mutant that cannot hydrolyze the cortex (A. Moir, unpublished data), so some signal to activate spore coat degradation must result from receptor activation or other early events, independent of cortex hydrolysis or complete core rehydration. A proteomics study of B. anthracis spores from dormancy through germination and outgrowth has noted the reduction in levels of spore proteins during germination, including some coat proteins and a number of uncharacterized small proteins (97).

**Outgrowth**

The resumption of metabolism and de novo synthesis of macromolecules in the cell results in outgrowth of the germinated spore into a vegetative cell. In the first few minutes, energy and intermediates must depend on catabolism of molecules carried over in the dormant spore. Sources of energy and intermediates include stores of 3-phosphoglyceric acid (3-PGA) and amino acids chiefly generated by degradation of the large stores of SASPs. Lipid synthesis resumes early, followed by bulk protein and DNA synthesis. In general, genes required during outgrowth (98, 99) are also required for vegetative growth. A detailed transcriptome analysis, following B. subtilis spores through germination and outgrowth (100), demonstrated a clear pattern of gene expression during outgrowth from earliest times; transcripts encoding transporters, DNA helicases, and DNA repair proteins were all synthesized early. Several late, probably long-lived, forespore sigma G-dependent transcripts were carried through in dormant spores (100). There are other reports of mRNAs in dormant spores in Clostridium novyi (101) and B. subtilis (102). The term “spore revival” has been used to encompass late germination and outgrowth (56); these authors have identified proteins synthesized during this period in B. subtilis, and subsequent analysis of mutants has
revealed additional components important in the process. Most strikingly, the very early translated proteins, translation-associated factors Tig and RpmE, are required for the latest stages of spore germination, and malic enzymes for utilization of endogenous malate in the spore are required for the “ripening” phase, where metabolically active spores have not yet shown significant cell outgrowth (56).

**GERMINANT RECEPTORS FOR NUTRIENT-INDUCED GERMINATION IN B. SUBTILIS**

The GerA receptor of *B. subtilis*, the first to be identified (103), is the paradigm for germination receptor complexes and lends its name to this family of receptors. Expressed at a low level in the forespore during sporulation, the three protein components (GerAA, GerAB, and GerAC) are all membrane associated and function together, probably in a complex. In addition, some receptor operons also encode a small (60 to 85 amino acids) protein that influences (either up or down) the response to the cognate receptor (104).

Five germinant receptor operons are encoded in the genome of *B. subtilis* 168. *B. subtilis* spores with a deletion in either the gerB or gerK operons are defective in their response to a cogerminant mixture of an amino acid plus sugars glucose and fructose, and K⁺ ions (AGFK), but are normal in their response to l-alanine as a single germinant, which is mediated by the GerA receptor. If all three are deleted, only one spore in 10⁴ germinates to form a colony on rich medium (105). Deletion of the other two operons (yndDEF and yfkQRT) has no effect on the ability of spores to germinate in response to nutrients under standard conditions (105). The levels of GerA, GerB, and GerK receptor proteins in *B. subtilis* spores have been estimated in comparison with the more abundant GerD and SpoVAD proteins (106).

**Regulation of Expression of the Receptor Proteins**

Much of the germination machinery, including the germinant receptor subunits, GerD, SpoVA proteins, and SleB-YpeB proteins, is expressed in the developing forespore, directed by sigma G. The SpoVT protein, an AbrB homologue, moderates expression, reducing transcription of gerA and gerB and increasing expression of spoVA and gerD (107). Overexpression of RNA polymerase binding factor YlyA (108) results in down-regulation of germinant receptor proteins, and receptor expression may also be moderated by the protein phosphatase PrpE (109), although its role is not well characterized.

**Receptors Encoded in Genomes of Other Species**

The sequences of hundreds of germinant receptor operons are now available; attempts have been made to classify these in terms of molecular phylogeny (117, 110). Such receptors are encoded in the genomes of almost all *Bacillales* and *Clostridiales* whose genomes have been sequenced to date, with the notable exception of *Clostridium difficile* and closely related species. The majority are organized as tricistronic operons, although there are many variations on this theme, especially in clostridia, where individual genes are common. Several are encoded on plasmids; for example, a p8 toxin plasmid-encoded operon confers on *B. thuringiensis* var. *israelensis* the ability to germinate in the alkaline conditions of the insect midgut (111). The *B. anthracis* gerX operon is encoded in the pathogenicity island on the toxin-encoding plasmid pXO1 (112), and is associated with a transposase and a site-specific recombinase, in an apparent cointegrative transposon (113), while the gerU operon and gerVB gene of *B. megaterium* are also plasmid located (114).

**The Germinosome**

Providing a major advance in our conceptual image of the germination process, recent technically demanding experiments have visualized germinant receptor proteins as green fluorescent protein (GFP) fusions in spores (115), in an apparent cluster named a “germinosome.” The spores used were coat defective (116) so that auto-fluorescing coat proteins did not interfere. Within the limits of the resolution of the microscopy, all three types of subunit of the germinant receptors are present at a single focus on the spore inner membrane, and the different GerA, GerB, and GerK receptors all colocalize in one cluster. This extends the earlier evidence, from Western blotting of proteins after spore fractionation, that germinant receptors are located in the relatively protected environment of the spore’s inner membrane, for GerAA and GerAC (117) and for GerBA (118) in *B. subtilis*. The work also defines a role for the GerD lipoprotein (119–121), which colocalizes in the same cluster with the receptors; without the GerD protein, the receptor proteins are dispersed around the spore inner membrane. The GerD protein that organizes the receptor proteins in the germinosome crystallizes as an α-helical trimeric, rodlike structure (122). The cluster of germinant receptors in a germinosome would
facilitate the integration of stimuli from different receptors (123, 124), and provide a means of signal transduction, either to other proteins in the locality or by creating a sufficient local change in the membrane properties or ionic environment. In contrast, the inner membrane-associated SpoVA protein complex that would represent the next known component in the germination cascade is distributed throughout the membrane (115).

How might a receptor be organized? At least in the paradigm B. subtilis GerA receptor, all three coevolved component proteins are required for function. Indirect but strong evidence that the subunits of an individual receptor interact comes from a study of point mutants; some single-amino-acid changes in the GerAA and GerAB subunits result in a likely failure to assemble a stable receptor, because levels of the GerAC lipoprotein are low or absent in the spore (125, 126). In general, the receptors are functionally distinct, suggesting that the subunits are not typically interchangeable, although interchangeable behavior is seen in B. megaterium QM B1551 for the more closely related GerUB, GerVB, and GerWB subunits. Clustering of receptors could facilitate competitive as well as cooperative interactions (127, 128).

**STRUCTURE/FUNCTION RELATIONSHIPS IN GERMINANT RECEPTOR PROTEINS**

The GerAA and GerAC components have no homologies outside the endosporeformers, whereas those of the GerAB family represent a distinct branch of the APC (amino acid polyamine organocation) superfamily of membrane-associated single-component membrane transporters (129). The homology between members of the component families of receptor subunits suggests that all would have an equivalent domain structure and general topology.

**GerAA Subunit**

The GerAA subunit, ca. 53 kDa, is an integral membrane protein composed of three domains. As yet, no protein or individual domain has been successfully overexpressed for structural studies. Hydropathy profiles have suggested that the central domain might contain five membrane spans (2), but programs that predict likely membrane-spanning helices based on amino acid sequence give very variable results for this protein family. The only experimental data so far have been for the important GerHA receptor protein of B. anthracis, based on an analysis of GFP fusions to internal positions (130). For GerHA, and the A subunits in general, the possible organization suggested would include an N-terminal hydrophilic domain located on the spore core side of the membrane, followed by a large hydrophobic domain containing four membrane-spanning α-helices, then a C-terminal hydrophilic domain that would also be located in the spore core (Fig. 3). Substitutions in various positions in the GerAA protein (126) may be interpreted in the light of this likely topology. For example, the apparent (if somewhat unusual) second transmembrane helix, commencing with a conserved PFPP hinge-like motif, contains several conserved acidic residues and appears to be of particular significance: substitution of a serine for the first proline increases the responsiveness of the GerA receptor to L-alanine and its analogues as germinants; this is also the case in GerBA, where the equivalent GerBA substitution (131) results in this receptor responding to L-alanine or L-asparagine as sole germinant (132), without the need for a contribution from the GerK receptor (132). In GerAA, changing the second proline results in the release of phase dark (i.e., germinated) spores, as though the receptor is permanently activated (126); removing conserved acidic groups from this second transmembrane helix destabilizes the receptor, resulting in little or no receptor complex. Whether the GerAA subunit binds germinant is unclear, but increases in responsiveness would suggest that this region could play a role in germinant binding and/or be subject to conformational changes during germination.

**GerAB Subunit**

The GerAB subunit and its homologues in sporeformers are integral membrane proteins with 10 predicted membrane spans and represent a subunit that is almost certain to bind germinants. The strongest evidence for this is the alteration of germinant specificity in the B. megaterium germinant receptor, depending on which of the GerUB, GerVB, or GerWB subunits is associated with the GerU receptor (114, 133, 134). Also, two mutant alleles in GerAB, with amino acid changes in likely transmembrane helices 1 and 6, reduce the responsiveness of spores to L-alanine by 8-fold and 100-fold, respectively (2, 135). It is not likely that germinant receptors mediate bulk transport of germinant (55), but there are precedents within the APC superfamily of proteins acting as sensors of extracellular substrates (136). Topology predictions and experiments with GFP fusions in the GerHB subunit of B. anthracis both suggest 10 membrane-spanning helices (130). Site-directed mutations in GerAB of B. subtilis (125) and in GerVB of B. megaterium (133, 134) that alter receptor function have been described. The recent determination of 3D
structures of several members of the APC family, including AdiC (137) and ApcT (138), with and without bound ligand, has highlighted the importance of common structural elements and demonstrated major conformational changes. Extended regions in the otherwise helical structure of transmembrane helices 1 and 6 are conserved and of importance to structure and binding of the cognate transported substrate; such extended regions within these helices would also be predicted in GerAB family proteins. Changes in the TM6 region of GerVB, informed by the structural data for these transporters, identified effects on the concentrations of glucose, and sometimes of other germinants, required to stimulate germination (133).
GerAC Protein
The GerAC protein and its homologues are predicted to be hydrophilic, with the exception of the conserved N-terminal prelipoprotein signal sequence. GerAC is synthesized in the forespore, secreted, and covalently attached to lipid, anchoring it to the outer surface of the inner spore membrane. Without this lipomodification, the protein is not retained in the spore (125).

Uniquely among these receptor proteins, a 3D structure has been reported for GerBC (139). The protein consists of three domains, each with a unique fold; there is an extended linker between domains I and II. Sequence alignments suggest that the other members of the family are likely to adopt the same overall structure. Single and multiple alanine substitutions have been introduced into conserved regions, and the extended linker deleted (140). This has defined several residues important for function in germination, where the altered protein is still present in spores at normal levels, whereas changes to other residues (including the linker) result in the reduction or loss of the protein from the spore. An understanding of these observations will have to await structural information on interactions of the C subunit within the complete receptor complex.

Biochemical evidence for the accessibility of germination proteins in decoated dormant spores of *B. subtilis* confirms the location of the majority of the proteins at the outer surface of the inner membrane (141). However, some proteins (GerAA, GerD, SpoVAD) are not fully accessible until germination has occurred, suggesting that there is some reorganization of the proteins or their environment during germination.

GERMINATION RECEPTORS AND THEIR FUNCTION IN *B. CEREUS SENSU LATO*
Apart from *B. subtilis*, the paradigm sporeformer, the next most extensively examined species are members of the *B. cereus/B. anthracis/B. thuringiensis* family, described as *B. cereus sensu lato*. Although named on the basis of their pathogenicity, *B. cereus* and *B. thuringiensis* strains are intermingled in the phylogenetic group, and *B. anthracis* represents a clonal internal lineage (142, 143). The functions of individual germinant receptors have been studied by mutation in *B. anthracis* and in two *B. cereus* strains, ATCC 10876 and ATCC 14579. Strains of the same species may share some, but not all, ger receptor operons, as discussed below.

*B. cereus ATCC10876*
The first germination mutants in *B. cereus* (defining receptor operons *gerI*, *gerQ*, and *gerL*, and the *gerP* operon) were generated by transposon mutagenesis in strain ATCC10876, before genome sequencing in any of this group. The draft genome sequence reveals the additional presence of homologues of the *gerR*, *gerG*, *gerS*, and *gerK* operons of ATCC14579, and also one (ZP_04321160) not found in ATCC14579 but present in many other *B. cereus* and *B. thuringiensis* strains.

Of the three characterized receptors, GerI is necessary for germination in response to inosine as a sole germinant and also has a role in l-alanine germination (144). GerQ, in combination with GerI, is required for germination responses in inosine as a sole germinant, but is not involved in l-alanine germination (145). GerI and GerQ receptors have different spectra of inhibition by nucleoside analogues (146), and germination by inosine alone may also involve release of endogenous alanine (147).

Spores lacking GerI receptor function will still germinate in l-alanine, albeit at a slower rate than the wild-type parent, and another receptor, GerL, is responsible for the major l-alanine germination response in this strain (145). The GerI and GerL receptors function at distinctly different optimal temperatures, pH, and germinant concentrations, extending the range of conditions under which germination can be triggered by l-alanine.

The GerI receptor has several unusual properties. Germination in inosine (but not in l-alanine) is inhibited by K⁺, whereas most germination receptors are stimulated by K⁺ (7). Like its homologue in *B. anthracis*, GerH, the GerA protein has a much longer N-terminal domain than most receptors, containing multiple glutamine-rich repeats (144) of unknown function. Next, all GerI-mediated responses in *B. cereus* ATCC 10876 depend on GerN, an electrogenic Na⁺/H⁺, K⁺ ion antiporter, which is particularly important for inosine germination (148–150). A second GerN-like protein in *B. cereus*, GerT, will substitute for GerN in inosine germination if the stimulus is high enough, i.e., at the highest inosine concentrations, but has a more significant role in outgrowth in high salt or at alkaline pH (150). The function of other germinant receptors in *B. cereus* does not require GerN or GerT, so whatever their role, it is specific to the GerI receptor. An interesting speculation is that they may be required to restore ion or charge balance during the function of this, but not other, receptors. It is likely that other ion transport proteins are responsible for the bulk ion movements that occur early in germination in response to all nutrient germinants.
The earliest report of a GerN-like protein (GrmA) required for germination was in *B. megaterium*, although this was not confirmed for another *B. megaterium* strain (114, 151). A GerN homologue in *Clostridium perfringens*, named GerO, is required for germination (152), but is expressed in the mother cell, unlike the forespore-expressed GerN; it is therefore less likely to be appropriately located to participate in inner membrane events during germination. There is no close GerN homologue in *B. subtilis* (148).

**B. cereus ATCC14579**

Seven gerA operon orthologues were identified in the genome sequence of this type strain. Six of the operons, gerQ, gerL, gerI, gerK, gerS, and gerG, have the same organization as gerA of *B. subtilis* with the individual genes ordered A-B-C. The final homologue, gerR (not to be confused with the identically named late sporulation transcription factor), is ordered gerRA-gerRC-gerRB. Very differently from the ATCC10876 strain, the gerR operon proved to be the most important, because the first screens for germination mutants identified gerR; germination in l-alanine and in inosine (as individual components but not in combination) was defective in a gerR mutant (153). A wider study of mutants with targeted disruptions in each operon individually (154) revealed that the GerR receptor is crucial to germination in all amino acids except l-glutamine, which was mediated by the GerG receptor. GerI and GerQ contributed with GerR to germination in nucleosides, and GerL and GerG were both required for a response to glutamine/inosine combination. Mutations in gerK, gerL, and gerS had no effect on germination, possibly as a result of redundancy in function with other receptors such as the dominant GerR.

**B. anthracis**

Of a clonal lineage, *B. anthracis* has a complement of receptor genes that is common to all isolates so far described. The chromosome encodes four functional germinant receptors, GerH, GerS, GerL, and GerK, and the fifth, GerX, is encoded on the virulence plasmid pXO1; two other chromosomal operons, named gerY and gerA, harbor frameshifts that would lead to loss of function (155), and are closely related to gerR and gerG of *B. cereus* ATCC14579, respectively (17). The GerH proteins (156) are extremely similar to the GerL proteins of *B. cereus*, except that the unusual glutamine-rich repetitive sequence close to the N terminus of GerIA/HA is slightly different in sequence. The GerS and GerL receptors are closely related in sequence to those with the same names in *B. cereus*.

A detailed analysis of germination behavior *in vitro* has been undertaken in the attenuated *B. anthracis* Sterne strain, which lacks pXO2, the plasmid encoding capsule biosynthetic enzymes. Each receptor operon was knocked out individually (157). Unlike the situation in *B. cereus*, inosine is not effective as sole germinant, probably because of the absence of GerQ and the nonfunctionality of GerY/R. In addition, strong germination responses to l-alanine are only seen at high concentrations (≥100 mM). In contrast, when used as cogerminants, either together or separately with other amino acids, 1 mM concentrations of alanine and inosine are effective. In general, the stimulation of at least two receptors by distinct germinants leads to the most rapid germination in *B. anthracis*. The GerH operon is required for response to cogerminant mixes containing inosine. The GerK and GerL receptors contribute individually to germination in high alanine, while also being individually required for the recognition of cogerminants (GerK: methionine and proline; GerL: serine and valine) in inosine-induced germination. GerS is required, in combination with another receptor, for germination in combinations of alanine or inosine with an aromatic amino acid.

No specific germinant could be identified for the pXO1-encoded GerX receptor, and a significant role in *B. anthracis* virulence is not proven, despite its location within the pathogenicity island. Germination of spores with a gerX defect was significantly reduced within phagocytic macrophages, suggesting that gerX may be important for pathogenicity (112), and reduced germination of a gerX mutant in murine macrophages was independently observed (noted in the discussion in reference 158). In contrast, studies of strains lacking GerX, or with GerX as sole remaining receptor, found no change in virulence in comparison with the receptor-complete parent or the complete receptor null, respectively (13). The latter study attempted to define the role of individual receptors in germination *in vitro*, and their contribution to virulence in a mouse model, by fully deleting all the functional receptors, except one. Under the conditions used, the GerH receptor was sufficient for germination in inosine combinations, and GerK and GerL receptors individually allowed germination in high alanine concentrations. Spores of a strain lacking all five functional receptors germinated very poorly indeed, with a colony-forming efficiency of 1 colony per 1,000 spores inoculated on the very rich Brain Heart Infusion agar; retention of any one of the H, K, L, or S receptors would restore this to near wild-type levels. Equally, the deletion of all of these four receptors would
dramatically reduce the virulence in mice, whether in-
oculated intratracheally or subcutaneously. Any one of
the four was sufficient for a fully virulent infection by
the intratracheal route, although time to death might
be extended. In contrast, GerH was the only receptor
required for virulence in subcutaneous infection and for
spore germination in blood (13). An independent trans-
poson mutagenesis study to identify spots that did not
germinate in macrophages gave multiple hits to the gerH
operon (159), suggesting its particular importance.

**B. megaterium**

Unusually, the tricistronic gerU receptor operon on
pMB700 in *B. megaterium* QMB1551 is followed by an
adjacently encoded monocistronic gerVB. The A and C
subunits of the receptor can interact either with the
B subunit or with the chromosomally encoded GerWB
(133). The gerU operon restored the germination re-
sponse to glucose and leucine as single germinants,
and to KBr as a cogerminant, in a plasmidless strain
of *B. megaterium*. Expression from gerVB along with
gerU confers the germination response to proline and
KBr as single germinants, while also enhancing the re-
sponse to glucose and leucine as single germinants.
Introducing a plasmid carrying this gene cluster into
*B. megaterium* KM, a strain that germinates in alanine
(65), allowed germination in all these additional ger-
minants (114).

**GERMINANT RECEPTORS IN CLOSTRIDIA**

Germination in clostridia often requires multiple ger-
minants, and there is an extensive literature over the
years covering germination requirements of different
strains, as, for example, in references 8, 160, 161, and
162. Homologues of many, but not all, of the germa-
nation proteins identified in *B. subtilis* are found in clo-
stridia (15, 18). A review of germinant receptors (14)
considers clostridial examples, and germinant receptors
in *C. botulinum* and *C. sporogenes* have recently been
characterized (163).

A detailed study of *C. perfringens* spore germination
has revealed that there may be some differences in re-
ceptor function from the *Bacillus* paradigm. For exam-
ple, the germinant receptor GerKC-KA can recognize
germinants without the presence of the divergently tran-
scribed GerKB component, although the latter does im-
prove germination (19, 164, 165). There is no GerD
protein in clostridia, so the question of whether recep-
tors cluster together in germinosomes, and, if so, how
this is mediated, remains an open one.

**An Alternative System for Cortex Lysis**

The prevalence of encoded cortex lytic enzymes in
clostridial genomes has been explored (15, 18). Some
*C. botulinum* strains encode SleB, YpeB, and CwI
proteins, as do *C. novyi* and *C. kluyveri* (18); in many
others, including *C. perfringens* and Group II *C. botu-
linum* strains, a different enzyme, SleC, is essential for
cortex lysis. This cortex lytic enzyme was first described
in *C. perfringens* S40, and is expressed with its cognate
protease(s), encoded by the cspA, B, and C genes (166,
167). SleC is processed from a precursor during spore
formation (168) and localized as a proform to the outer
cortex/coat boundary along with the Csp proteins (169),
where it is further activated by processing during ger-
mination; the signal stimulating such processing is not
known. Homologues of SleC and its protease are found
in many clostridia, and SleC proteins have been shown
to be required for germination in other *C. perfringens*
strains (170, 171). An additional lytic enzyme, SleM,
commonly found in *C. perfringens* (167), degrades cor-
tical fragments but is not essential for germination or
outgrowth.

*C. difficile* encodes no germinant receptors at all, yet
responds to a combination of the bile salt taurochro-
late, with glycine and histidine (172–174). The clinical
importance of this species has led to a concentrated
effort to explore its spore biology. Here, the SleC cortex
lytic enzyme is essential for germination, and the ac-
tivation protease Csp proteins appear to have evolved
to contribute more directly to germinant sensing, CspC
providing a bile acid-specific receptor (175), although
nothing is yet known of the mechanism of glycine
sensing (176). Unlike in other clostridia encoding recep-
tors, and where cortex lysis is achieved by downstream
activation of SleC protein (177, 178), the direct activa-
tion of SleC in *C. difficile* results in cortex hydrolysis
preceding DPA release (179).

**THE LIPID ENVIRONMENT
AND GERMINATION**

The changes in membrane properties during spore
germination, including the passage of ions and water,
and the restoration of membrane fluidity, highlight the
importance of the membrane environment around the
germinant receptor. Mutations in the gerF (*lgt*) gene
of *B. subtilis* greatly reduced nutrient-induced germina-
tion responses, while non-nutrient-induced germina-
tion remained like that of wild type (46, 180). The same
phenotype is seen in *B. anthracis* (44). This can be ex-
plained on the basis of its importance to germinant
receptors—the C subunits of germinant receptors, and the GerD protein, are all predicted lipoproteins and dependent for processing on GerF/Lgt. Mutations in the prelipoprotein processing site of GerD (121) or of GerAC (125) resulted in the absence of these proteins from the spore, although the effect on GerBC and GerKC receptors may be less marked (180).

The lipid environment of receptors or their associated proteins may influence germination efficiency. Spores of B. subtilis with only traces of cardiolipin in their membranes germinated poorly in L-alanine and AGFK, as measured by the loss of turbidity (181), although approximately 15 to 20% of the normal level of DPA release was seen in both germinants. In another study, an equivalent reduction in cardiolipin levels had a less dramatic effect, although still slowing germination significantly (182). Both experiments used very high saturating concentrations of germinants, although at different pH values; it might be interesting to test under conditions where the germinant stimulus is less strong, such as with valine rather than alanine as germinant. In another example where alterations in spore lipid properties may influence germination, the spore coat-associated phospholipase LipC (YcsK) influences spore fatty acid levels (183): mutant spores do not germinate efficiently in L-alanine, although there was no defect in AGFK germination. Our lack of understanding of the nonfluid lipid environment of the inner membrane of the dormant spore hampers understanding of these observations.

INFLUENCE OF THE SPORE COAT ON GERMINATION BEHAVIOR

The role of three relevant proteins has already been described: that of the GerP proteins, required for access of germinants through the spore coats, and the cortex lytic enzyme, CwlJ, localized in the spore coat by the GerQ/YwdL protein. Mutations in most of the individual B. subtilis coat genes do not affect germination significantly, unless they have a wider morphogenetic effect on spore coat assembly.

The gerE gene, encoding a transcriptional regulator, activating expression of many late-expressed spore coat protein genes, and repressing others (184), is a classic example. A gerE mutant is blocked after the initial, receptor-dependent, loss of heat resistance (185), and the pleiotropic coat defect could mean that there are several reasons for the poor progress through late germination. One of the genes in the GerE-αK regulon is gerT (previously named yozR), which encodes a spore coat protein whose expression is downregulated by GerE (186), and whose assembly around the spore depends on the morphogenetic protein CotE. Mutants of gerT were impaired at an early stage of germination, responding poorly to nutrient germinants and to non-nutrient CaDPA, a phenotype reminiscent of mutations in the similarly regulated gerP operon that appears to be required for spore coat permeability to germinants (52, 57).

In B. subtilis, a small (28 amino acids) spore coat protein SscA is required for correct assembly of the outer spore coat proteins CotH, G, and B, and sscA (previously named yhzE) mutants germinate more slowly, presumably because of their coat defect (187). A cotH mutant has already been described as defective in germination (188), especially in combination with a defect in the major morphogenetic protein CotE (189), which itself is required for normal germination (190). A cotT mutant, which has an altered inner coat, germinates poorly (191). Despite all these effects, the coat is not required for the earliest receptor-dependent changes in the spore that precede cortex lysis, because spores of a double mutant of cotE and gerE contain only a small amount of residual coat material but still show a relatively normal stage I germination response (116), as do chemically coat-depleted spores. Coat permeability to germinants could influence germination, and the coat region is important to the localization of various cortex lytic enzymes that are sequestered in an inactive form until germination.

At least in a few bacilli spore coats generally contain an alanine racemase activity that is probably important in preventing germination during spore development, and would be responsible for generation of D-alanine, a competitive inhibitor of germination (192). In the B. anthracis/B. cereus/B. thuringiensis group, alanine racemase and inosine hydrolase are both located in the exosporium or between it and the coat (a region known as the interspace), the outermost layer around the spore (193), and may influence germination rates (58, 59, 194). By contrast, the alanine racemase YncD is located in the outer coat in B. subtilis.

GERMINATION IN RESPONSE TO NONNUTRIENT ENVIRONMENTAL CUES

Spores are able to germinate in response to a variety of stimuli, in addition to the common nutrient germinants. These may be chemical (CaDPA), enzymic (lysozyme), or physical (pressure); dodecylamine also causes “germination,” but kills the spore.
**CaDPA-Induced Germination**

Activation of the cortex lytic enzyme CwlJ of *B. subtilis* by endogenous CaDPA released from the core is a later event in receptor-mediated germination (20). However, exogenous CaDPA was found to independently activate CwlJ, entirely bypassing the nutrient-induced germination via the GerA receptor family, and effective in the absence of all such receptors (83, 105). CaDPA is an effective germinant in clostridia as well as bacilli, even though the former frequently do not encode a CwlJ protein. In the case of *C. perfringens* (19), which uses a SleC cortex lytic enzyme, the response to CaDPA requires the GerK nutrient germinant receptor, but it is not yet known whether this is a general mechanism that also applies in other SleC-containing species.

**Pressure-Induced Germination**

Germination in response to pressure has been shown to follow two distinct pathways, differently dependent on the extent of pressure applied to spores. Relatively low pressures (100 to 300 MPa) activate a germination process that resembles that of nutrient-induced germination (195, 196) and depends on germinant receptors (60, 196, 197). In contrast, spores germinating under higher pressures of 500 to 800 MPa do not generate ATP or degrade SASPs and retain their resistances to UV light and H₂O₂ (195), and the process is receptor independent. High pressure seems to cause direct release of DPA, and at least one of the cortex lytic enzymes is required to complete germination (198).

**Dodecylamine-Induced Germination**

Long-chain alkyl amines act as potent germinative agents up to temperatures of 70°C (199). Spores germinating at 37°C in dodecylamine release CaDPA from their core, and their cortex is degraded normally; at 70°C, CaDPA is released, but the cortex is not degraded, presumably because cortex lytic enzymes are inactivated. Dodecylamine-germinated spores have relatively dehydrated cores, and show no metabolism; SASPs are not degraded, and the spores are rapidly killed (22). Germination is independent of germinant receptors, cortex lysis, or the presence of a spore coat; dodecylamine induces germination by a separate mechanism to other nonnutrient stimuli, perhaps via direct membrane effects (22) leading to CaDPA release via the SpoVA proteins (200).

**Lysozyme-Induced Germination**

The spore coat acts as a barrier to exogenous enzymes, but once this has been breached, for example, by chemical decoating procedures, the spore cortex peptidoglycan can be degraded by lysozyme, initiating the release of the core’s CaDPA, and the rehydration of the spore core. In some clostridia, lysozyme-containing agar can be used to improve colony formation from spores (201, 202). In contrast, in *B. subtilis*, lysozyme also degrades the spore’s germ cell wall, meaning that spores germinated in this way are unable to outgrow without osmotic stabilization (64).

**Germination Induced by Peptidoglycan Fragments**

Spores may germinate in response to growth signals from other cells. Disaccharide tri- or tetrapeptide muropeptide fragments from vegetative cells (disaccharide tri- or pentapeptides, with a meso-diaminopimelate at the third position in the peptide side chain) are able to induce germination (25). A membrane-associated serine/threonine kinase PrkC, located in the spore inner membrane and with a triple extracellular PASTA domain able to bind peptidoglycan, is essential for this response to the cognate muropeptides (25). How PrkC functions in germination is not known, but presumably it phosphorylates some important component; germination stimulated by muropeptides, or by bryostatin, which activates PrkC, does not require germinant receptors and is effective in superdormant spores (203). These are effective in *B. cereus* as well as in *B. subtilis* (204); the full range of species that will respond to muropeptides has not yet been established, although PrkC homologues are found in bacilli and clostridia. This family of eukaryotic-like Ser/Thr kinases has a variety of roles in bacteria (24). In *B. subtilis* vegetative cells, PrkC, activated by muropeptides, induces expression of an extracellular muralytic enzyme YocH by an unknown mechanism. This YocH enzyme would then generate muropeptides from peptidoglycan fragments, amplifying its own expression (205). How PrkC functions in *B. subtilis* germination is not known; germination stimulated by muropeptides, or by bryostatin, which activates PrkC, does not require germinant receptors, and is effective in superdormant spores (203).

**OVERVIEW**

Exciting progress has been made in the detailed description of the germination process at the single spore level, and in possible structure/function relationships in nutrient receptors, both at the level of individual protein subunits, and their potential clustering to provide a node for receptor-associated changes, such as local
rehydration and increased ion permeability. There is the beginning of an appreciation of some wider membrane-associated events in germination, notably the likely role of a SpoVA channel in the bulk release of CaDPA from the core across the inner membrane. We understand none of the mechanisms at the molecular level, and there are many important questions to be addressed, including studying candidate proteins that are now clearly defined. With available genome sequences of many sporeformers, and recent technologies for making mutants in a wider variety of bacilli and clostridia, has come the appreciation that there is considerable diversity in the setting for the germination machinery, and in some of the components, that remains largely to be explored.

REFERENCES

Comparison of the properties of Bacillus subtilis spores prepared at different temperatures.


