Workshop Report: Modeling the Molecular Mechanism of Bacterial Spore Germination and Elucidating Reasons for Germination Heterogeneity.

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Dr. Lund,

Please accept the following revised manuscript by Indest and others entitled, "Workshop Report: Modeling the Molecular Mechanism of Bacterial Spore Germination and Elucidating Reasons for Germination Heterogeneity ", for consideration of publication in the *Journal of Food Science*.

On February 5 and 6, 2008, a total of 29 individuals from academia, including participants from the United Kingdom and the Netherlands, and the Department of Defense gathered in Key West, Florida for an Army sponsored workshop. Top researchers in the fields of spore biology and computational biology interacted over the course of two days, identifying biological and mathematical data gaps as well as experimental approaches and alternative computational strategies appropriate for modeling spore germination. Outcomes from this workshop are summarized in the attached manuscript.

Sincerely,

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Title: Workshop Report: Modeling the Molecular Mechanism of Bacterial Spore Germination and Elucidating Reasons for Germination Heterogeneity.

Subtitle: Top researchers in the fields of bacterial spore biology and computational biology interacted over the course of two days, identifying biological and mathematical data gaps as well as experimental approaches and computational strategies appropriate for modeling the molecular mechanism of spore germination and elucidating causes of germination heterogeneity.

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On February 5 and 6, 2008, 28 individuals from academia, including participants from the United States, the United Kingdom and the Netherlands, and the Department of Defense gathered in Key West, Florida for an Army sponsored workshop. Top scientists in the fields of *Bacillus* and *Clostridium* spore biology and computational biology interacted over the course of two days to address one of the major remaining questions in bacterial spore biology: **Why is there heterogeneity in the germination rates of individual bacterial spores within a population?** Goals of the workshop were to: 1) attempt to answer the major question posed above using available data; 2) if 1) was not possible, propose further experimental work to obtain data that will allow a definitive answer to this question; and 3) examine available data on the molecular mechanism of spore germination to determine: a) if these data are sufficient to allow generation of a predictive model of the molecular mechanism of spore germination; and b) if a) is not currently possible, determine what additional data would be required to effectively construct such a model. It must be emphasized that the focus of discussions about generation of models of spore germination was on generating a model of the molecular mechanism of spore germination that would have predictive value. There have been a number of models constructed to analyze the kinetics of spore germination and subsequent cell growth, often in food matrices (for examples see Barker et al., 2005; Collado et al., 2006; Smith-Simpson and Schaffner, 2005; Zhao et al., 2003). However, there have been few attempts to construct predictive models of the molecular mechanism of spore germination, and the most notable of these (Woese et al.,

1968) was developed more than 40 years ago when almost nothing was known about components of the spore germination apparatus.

At the beginning of the workshop, three keynote addresses were presented to establish a starting point for discussions. Karl Indest presented an overview of the Army's interest in spore germination; Peter Setlow gave a concise but in-depth summary of the current state of knowledge of spore germination, focusing on spores of *Bacillus* species; and Jeremy Edwards concluded with a summary of the current state of computational modeling of biochemical systems. Keynote addresses were followed by a discussion session where participants were split into breakout groups containing equal numbers of spore biologists and modelers to address specific tasks. The results of the breakout discussions were presented to the group as a whole for discussion. New issues that arose from discussions were addressed again in the breakout groups and the process was repeated.

Relevance of Bacterial Spore Germination

Bacterial spores of *Bacillus* and *Clostridium* species are ubiquitous in the environment, formed from vegetative cells through a process known as sporulation when conditions for growth are unfavorable (Piggot and Hilbert, 2004; Setlow and Johnson, 2007). Spores are metabolically dormant and resistant to a wide range of environmental conditions including heat, radiation, desiccation, pH extremes and toxic chemicals (Setlow, 2006). There are a variety of reasons for the extreme spore resistance, most related to the structure of the spore (Fig. 1),

which is quite different from that of growing cells. Probably the most unusual aspect of spore structure is the extremely low water content of the spore's central region or core. While growing cells have ~80% of their wet wt as water, the core of dormant spores suspended in water may have as little as 25-30% of wet wt as water, while the remainder of the spore has the more normal high water content (Gerhardt and Marquis, 1989). The low core water content is undoubtedly the reason for the high resistance of dormant spores to wet heat, as well as spores' extreme dormancy (Cowan et al., 2003; Gerhardt and marquis, 1989; Setlow, 2006; Setlow and Johnson, 2007). However, despite their dormancy, spores can respond to favorable environmental conditions and rapidly transform into metabolically active cells in a process termed germination followed by outgrowth, and in these processes the spore loses its extreme resistance properties (Moir, 2006; Setlow, 2003; Setlow and Johnson, 2007).

Spore germination has attracted significant interest, at least in part because of the impact spores have historically played in food spoilage and disease (Setlow and Johnson, 2007). Consumer demand for milder, more efficient food sterilization technologies that maintain food product nutritional value and esthetics has provided new opportunities for food spoilage and food-borne disease. Unfortunately, relatively harsh processing conditions are most often necessary to inactivate dormant spores, reflecting the delicate balance between food preservation and safety.

In addition to the threat of food-borne illnesses, spores of specific *Bacillus* and *Clostridium* species are responsible for a number of serious human diseases

including gas gangrene, pseudomembranous colitis, tetanus, botulism, and anthrax (Fischetti et al., 2000; Setlow and Johnson, 2007). Following the 2001 anthrax spore terrorist attacks in the United States, there has been a renewed sense of urgency in development of both treatment and decontamination strategies for spores of *Bacillus anthracis*. Since dormant *B. anthracis* spores can reside for several months within infected mammals including humans (Brookmeyer et al., 2003; Heine et al., 2007; Henderson et al., 1956), the ability to thwart spore germination following exposure could greatly help in preventing morbidity and mortality.

Assessing the threat that spore contaminants pose is a great challenge due in part to the inability to predict germination outcomes. A potential simple strategy towards eliminating the threat posed by spores would be to trigger spore germination and then relatively easily inactivate the less resistant germinated spores or vegetative cells. However, germination of spore populations is invariably heterogeneous and almost certainly always incomplete, as a small percentage of spore populations consist of a very slowly germinating fraction, often called super-dormant spores (Gould, 1969, 1970; Keynan and Evenchick, 1969). To ensure the effectiveness of spore inactivation and further reduce the potential of a residual risk of germination, it is essential to determine reasons for the heterogeneity in germination kinetics between individuals in spore populations as well as the causes of spore super-dormancy, as such knowledge may suggest ways to eliminate this problem. It would also be most helpful to have a good predictive model of the molecular mechanism of bacterial spore

germination, since a number of mechanistic aspects of spore germination are only poorly understood.

Status of Our Understanding of Spore Germination

Many aspects of spore formation are, however, relatively well understood, especially for the model organism *Bacillus subtilis* (Piggot and Hilbert, 2004; Setlow, 2003; Setlow, 2006; Setlow and Johnson, 2007, and references within). The most common stimulus for spore formation in nature is probably starvation. Once formed, the spores are dormant and highly resistant to environmental stresses. What is truly amazing is that they can remain metabolically inactive for years, but can "come to life" (germinate) within minutes if presented with appropriate stimuli termed germinants. In nature it is likely that specific nutrients such as amino acids or sugars serve as germinants, but nutrient metabolism *per se* is not essential for the triggering of spore germination, as the early events of germination are thought to be primarily biophysical (Moir, 2006; Setlow, 2003). Indeed, energy metabolism is not required for germination. For example, recent work (Shah et al., 2008) has found that peptidoglycan fragments released from growing cells of the same or similar strain giving rise to spores can trigger germination. In addition to nutrients, a number of non-nutrients such as cationic surfactants, high pressure, or in some cases lysozyme can also trigger germination, as can high concentrations of a 1:1 chelate of Ca^{2+} and pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA) (Ca-DPA), a major component of the spores' or core (Fig. 1). Non-nutrient germination stimuli are not generally

encountered by spores other than in the laboratory, however, understanding how such agents trigger spore germination may provide insight into the molecular mechanisms involved in spore germination.

Most work to date on the molecular mechanism of spore germination has been with spores of *Bacillus* species, primarily *B. subtilis* (Setlow, 2003), although there has been recent work on the molecular details of the germination of *Clostridium perfringens* spores (Paredes-Sabja et al., 2008a,b, 2009 a,b). Discussion at the workshop focused primarily on the germination of spores of *Bacillus* species. Germination of spores of *Bacillus* species proceeds in two Stages, I and II (Fig. 2), and involves the action of a number of critical components including germinant permeation proteins, germinant receptors, channel proteins and cortex lytic enzymes (CLEs) and perhaps monovalent cation antiporters (Behravan et al., 2000; Heffron et al., 2009; Senior and Moir, 2007; Southworth et al., 2001; Setlow, 2003; Setlow et al., 2009). Permeation proteins facilitate movement of nutrient germinants through the spore's outer layers, most importantly the coats (Fig. 1). Spores then sense nutrient germinants by germinant receptors located in the inner membrane (Hudson et al., 2001; Paidhungat and Setlow, 2001). The germinant receptors are composed of three different subunits, each of which is essential for receptor function, and spores of *Bacillus* species have 3-7 different germinant receptors each of which have different and exquisite nutrient specificity (e.g., L-alanine is recognized whereas D-alanine is not). While some individual germinant receptors can trigger germination when their nutrient ligand binds, other receptors cooperate

somehow in responding to mixtures of nutrients (Atluri et al., 2007; Setlow, 2003). Levels of the germinant receptors appear to be low, averaging tens of molecules per spore (Paidhungat and Setlow 2001), raising the possibility that some individuals in spore populations may have few if any receptors. Strikingly, *B. subtilis* spores that lack all functional germinant receptors germinate extremely poorly with nutrient germinants but exhibit relatively normal germination with non-nutrient germinants, as well as a low level of "spontaneous" germination (Paidhungat and Setlow, 2000; Setlow, 2003). Spores of *Bacillus* species, but not *Clostridium* species, have an additional protein, GerD, which appears to be essential for germination with nutrient germinants (Setlow, 2003; Pelczar et al., 2007), although the precise function of this protein is not known

Within minutes of exposure of spores to nutrient germinants, the core's large depot (15-25% of dry wt) of Ca-DPA is released from most spores along with other small molecules (Setlow, 2003; Setlow et al., 2008). Small molecules released from the core are replaced with water, thus raising the core's water content slightly, although not sufficiently to allow either enzyme action or protein motion (Cowan et al., 2003; Setlow, 2003; Setlow et al., 2009). The timing of these early events in spore germination, in particular Ca-DPA release, is quite heterogeneous between individual spores in populations, as has been shown by a variety of techniques (Chen et al., 2006; Woese et al., 1968), although the causes of this heterogeneity are not known (see below).

Release of Ca-DPA and other small molecules are among the earliest detectable indicators of germination and are thought to involve channel proteins.

While the mechanism of such channels and channel gating are unknown, the Ca-DPA channels may be composed at least in part of the SpoVA proteins. These proteins are expressed just prior to Ca-DPA uptake into the developing spore during sporulation, *spoVA* null mutants do not take up Ca-DPA during sporulation, and spores of temperature sensitive *spoVA* mutants do not release Ca-DPA at non-permissive temperatures (Tovar-Rojo et al., 2000; Vepachedu and Setlow, 2004, 2005). In addition, at least one SpoVA protein is located in the spore's inner membrane and is present at much higher levels than germinant receptor proteins.

Ca-DPA release and concomitant water uptake complete Stage I of germination, and these events trigger the onset of Stage II (Heffron et al., 2009; Setlow 2003; Setlow et al., 2009). The key event in Stage II is hydrolysis of the spore's peptidoglycan (PG) cortex just outside of the spore's germ cell wall (Fig. 1, 2) by CLEs. The structure of the PG in the cortex has several novel features not present in vegetative cell or germ cell wall PG. One of these cortex PG - specific features, muramic acid-δ-lactam, is the recognition determinant for CLEs, thus ensuring that only cortex PG and not germ cell wall PG is degraded during germination, as the germ cell wall becomes the cell wall of the outgrowing spore (Popham, 2002). Spores of *Bacillus* species contain two redundant CLEs, and loss of both almost completely eliminates cortex hydrolysis and prevents completion of germination (Heffron et al., 2009; Setlow, 2003; Setlow et al., 2009). This results in an enormous decrease in apparent spore viability, although spores that lack CLEs can be recovered by addition of exogenous lytic enzymes

under appropriate conditions. The two CLEs in *Bacillus* spores are CwlJ and SleB. CwlJ is activated by Ca-DPA either released from the core or supplied exogenously, but the mechanism of activation of SleB is unknown. Cortex degradation allows an ~2-fold increase in spore core volume as upon removal of the restraining cortex PG the core expands due to further water uptake to ~80% of wet wt, thus completing spore germination. With full core hydration to ~80% wet wt as water, protein mobility in the core resumes and enzyme activity and metabolism followed by macromolecular synthesis begin in the core, as the germinated spore begins the process of outgrowth that ultimately generates a growing cell (Cowan et al., 2003; Setlow, 2003). In addition to resumption of enzyme activity and metabolism, the dormant spore's resistance to environmental stresses is lost either upon completion of Stage II of germination, or very early in outgrowth (Setlow, 2003, 2006). Consequently, fully germinated spores are much easier to kill than the starting dormant spores by a variety of agents.

Spore Germination Biology: Where are the Knowledge Gaps?

A number of knowledge gaps remaining with respect to the molecular mechanism of spore germination were identified and discussed in presentations, general discussion and breakout groups. Since no energy use or metabolism is needed for spore germination, the events in germination appear to be largely biophysical. Germination can be potentiated and made more synchronous by a variety of activation treatments, most usually a sublethal heat treatment (Keynan

and Evenchick, 1969). However, the mechanism of spore activation is not known, although it is reversible. When incubated with nutrient germinants, brief (seconds) exposure to a germinant commits spores to germinate, and this commitment is irreversible, although the mechanism of commitment is not known. There are a number of additional questions about early events in spore germination, and these include: a) what do germinant receptors do upon binding of nutrients; b) is there a signal transduction cascade following germinant-receptor interaction, and what is it; c) how do non-nutrients trigger germination; and d) do germinant receptors directly transport something, do they signal other biomolecules to transport something or do they open channels for Ca-DPA and other small molecules? As noted above, the mechanism of Ca-DPA release and concurrent water uptake in Stage I of germination is unknown but is presumably mediated by selective channels. However, the precise composition of such channels, how they are gated, if and how they interact with germinant receptors, and whether they are selective are not known. Similarly, not all details of the regulation of CLE activity, in particular of SleB are known. Given the many unanswered questions about spore germination, what data are absolutely required to begin developing a usable predictive model of the molecular mechanism of spore germination and what data could be approximated? In the course of the workshop, a large number of experimental approaches were suggested to allow the collection of key data that would facilitate the development of such a model of spore germination as discussed below.

Workshop Discussions

The central question posed to the workshop was: *What causes heterogeneity in germination of individual spores within a population?* Whereas most often approximately 95% or more of bacterial spores in populations commit to germinate within seconds to minutes of exposure to an appropriate germinant, a small proportion (super-dormant spores!) fail to respond for days, weeks or months (Gould, 1969, 1970; Keynan and Evenchick 1969). Following the introductory presentations, discussions followed on what data would be needed to develop a useful model of the molecular mechanism of spore germination, which of these data could be approximated, which will need to be determined experimentally and which might be predicted from early modeling efforts.

A group discussion followed the formal presentations in which multiple factors were identified that could affect heterogeneity of germination including variability in: 1) spore activation, 2) diffusion of germinant ligands into spores, 3) activity of permeation proteins, 4) receptor binding of ligands, 5) receptor numbers, 6) channel protein activation, and 7) CLE activation. Given the central, and largely not understood role for the germinant receptors in spore germination, lively discussion ensued about possible approaches to studying these receptors. Crystal structures of these proteins would be valuable and informative but attempts to express germinant receptor proteins in *Escherichia coli* have not been fruitful due to apparent toxicity (G. Christie, unpublished). Cryo-electron microscopy was put forth as a possible approach but the buried location of the

298 germinant receptors in the inner membrane deep in the spore (Fig. 1) makes that
299 approach unlikely to succeed.
300 Additional talking points included the asymmetric stoichiometry of
301 germinant receptor proteins compared to channel proteins, and the need to focus
302 on comparisons between mutant and wild-type spores and yet how these
303 comparisons might be misleading. Saturation mutagenesis has been used to
304 identify germinant receptor mutants but not channel protein mutants. Potential
305 redundancy or lethality may be masking these latter mutants. Two additional
306 questions arose in response to the break-out-topic question. The first dealt with
307 whether or not the non-germinating fraction (super-dormant fraction) of a given
308 spore population was genetically determined. The suggestion that a fraction
309 enriched for super-dormant spores, when subjected to a round of germination-
310 sporulation-germination, maintained a constant germination ratio indicates that
311 super-dormancy is not genetically determined. However, as a caveat here, it
312 was acknowledged that super-dormancy has been only very poorly studied and
313 that standardized methods for isolating large populations of super-dormant
314 spores have not been reported. Notably, and largely as a result of stimulation
315 provided by this workshop, super-dormant spores of *Bacillus cereus*, *Bacillus*
316 *megaterium*, *B. subtilis* have recently been isolated in relatively pure form and
317 characterized (Ghosh and Setlow, 2009; S. Ghosh and P. Setlow, unpublished).
318 Further studies of these super-dormant spores may well provide more
319 information on reasons for their super-dormancy.

The question was also posed as to whether the presence of superdormant spores could be explained by stochastic Markov processes. In particular, is imperfect sporulation and/or germination the source of germination variation? It was proposed that this could be experimentally tested by genetically and physiologically altering these processes and looking for subsequent changes in germination variation. These discussions concluded on a philosophical note by asking whether there is any obvious advantage to imperfect germination. It was rationalized that it may be beneficial for the spore population as a whole to vary germination rates, since germination of all spores in a population in inappropriate conditions could deplete limited or transient levels of nutrients and lead to death of the whole spore population.

Break-out-group results.

The whole group also broke into three smaller groups each containing both spore biologists and computer modelers for more in-depth discussions and to promote closer interactions between biologists and modelers. Interestingly, upon reassembling the main group for further discussion, many of the same approaches and questions had been identified by the separate groups. The general consensus was that it would be desirable to simplify the system, and that it would be relatively straightforward to experimentally determine whether three of the seven factors noted above proposed to contribute to germination heterogeneity actually do so. Diffusion of ligand could be evaluated by looking at temperature effects on germination kinetics. Ligand diffusion as well as activity

of permeation proteins could be evaluated by removal of the spore coat and subsequent germination studies. Potential heterogeneity of CLE activation could be determined by correlating the release of Ca-DPA with a change in spore core water content (core refractility) mediated by the CLEs. If heterogeneity was not observed in these experiments, factors 4, 5 and 6 (germinant receptor binding, germinant receptor numbers and channel protein activation) would remain as potential mediators of germination heterogeneity.

A number of questions relating to factors 4-6 above were also put forward and discussed. Could the lack of an “adequate” number of germinant receptors be responsible? Is there a germinant receptor-channel protein amplification mechanism in play? Might activation of the spores (e.g., by heat) shorten the lag time before germination? If so, this might suggest that positioning or conformational changes of the germinant receptors, the channel proteins, or both might enhance “activation”. Would changing, for example the temperature regime for pre-activation influence the germination frequency or timing? Could spore core water content be influencing germination? Can germinant concentrations be limited to slow germination or limit the frequency of germination? If so, set up an experiment where 2/3 of all spores germinate immediately, collect those that don’t, wash them and then expose them again to the germinant. Do 2/3 of the remaining spores now germinate immediately? Other questions included: 1) what triggers “spontaneous” germination; and 2) do germinating spores produce molecules that prevent or activate the germination of nearby spores?

366

367 **Computational Modeling of Spore Germination**

368 In order to formulate a mathematical model encompassing the molecular
369 mechanism of spore germination certain basic features of the process need to be
370 known or postulated. What factors determine the kinetics of germinant receptor
371 activation? How is activation of germinant receptors coupled to the activation of
372 channel proteins? The more detailed and quantitative answers that can be
373 obtained to assist in formulation of models, the more detailed the predictions that
374 the resulting model can give. Some specific examples of the kinds of information
375 that would be useful include detailed biophysical and biochemical
376 characterization of the processes coupling ligand binding by germinant receptors
377 to activation of channel proteins, quantitation of the proteomic composition of the
378 spore's inner and outer membranes, including the number of permeation
379 proteins, germinant receptors, and channel proteins, and information about the
380 spatial distribution of germinant receptors in the inner membrane and their
381 orientation relative to channel proteins and other possible relays.

382 Detailed data such as is described above are necessary for developing
383 the predictive models that are the ultimate goal of this effort, but preliminary
384 models based on current information and data may also be highly informative.
385 For example, more than forty years ago, long before the proteins or biochemical
386 steps leading to germination had been characterized, Woese et al. (1968)
387 developed a simplified model of germination kinetics that could explain several
388 observed properties of germinating populations. In this model, which could serve

as a starting point for current efforts to model the molecular mechanism of spore germination, germination was assumed to arise from accumulation of a substance, *P,* whose rate of production was taken to be proportional to the number of activated germination “enzymes”, although the identity of such “enzymes” was not specified. Perhaps these enzymes are equivalent to the germinant receptors, which had not been identified when this model was presented. By assuming that a threshold amount of *P* was required for germination, the time for a spore with *n* active enzymes to germinate was found to be *a/n*, where *a* is a constant. Thus, the model predicts that for small *n*, each step increase in *n* leads to a step decrease in the time to germinate, and the distribution of germination times should exhibit observable jumps, at least if homogeneity arising from other factors does not obscure such jumps. The experimental germination time distribution obtained by Woese et al. (1968) did exhibit these predicted jumps, and by fitting their model to these data they estimated the average number of active germination “enzymes” per spore to be in the range of 9-11, which matches reasonably well with current estimates of ≤ 25 germinant receptors per spore (Paidhungat and Setlow, 2001).

The simple version of the Woese model, which considered only the production of *P,* predicted a finite, relatively short time for all spores to germinate, except for a tiny fraction of the spores in the distribution that would contain no germination enzymes (the germinant receptors?). This could not be easily reconciled even with the limited data that existed at that time, so Woese et al. (1968) extended their initial model by allowing *P* to be degraded at a constant

412 rate, which produces a steady state level of *P* that is proportional to the number
413 of activated germination enzymes (receptors?). If a threshold for *P* to trigger
414 germination is assumed, there will also be a threshold in the number of active
415 germinant receptors required, which divides the population into germinating and
416 non-germinating fractions based on the number of enzymes (germinant
417 receptors?) present in each spore. Based on their data, Woese et al. (1968)
418 estimated the germination threshold was at about 2-3 germination enzymes per
419 spore. They also found that the model could account for the observed increase
420 in the non-germinating fraction at temperatures above the optimal germination
421 temperature if the degradation rate of *P* increased more rapidly as a function of
422 temperature than the production rate of *P.* Interestingly, a similar type of
423 threshold activation model has recently been proposed to account for the broad
424 distribution of delay times observed in the induction of apoptosis through extrinsic
425 signals (Albeck, 2008). In that system, the molecular mechanisms are well-
426 understood and appear to produce a linearly-accumulating signal that is
427 proportional to the initial concentrations of several signaling proteins. Just as in
428 the Woese spore germination model, a molecular “snap action” switching
429 mechanism appears to rapidly produce irreversible commitment to apoptosis
430 within a narrow window of the signal threshold.
431
432 The model proposed by Woese et al. (1968) is thus an excellent starting
433 point for the initial modeling effort because it relates the distribution of
434 germination times and the non-germinating fraction to relative strength of two

terms: a positive signal that is generated by germinant receptor activation and a resistance that is generated by as of yet unknown mechanisms. Of critical importance is the ability of the model to relate changes in the balance of these terms to changes in the kinetics and extent of germination, which allows a direct connection to be made between measurements of these properties and the effects predicted by different hypothetical mechanisms. For example, the model can easily be extended to consider the effect of ligand concentration under non-saturating conditions and different binding / activation modes can be investigated. The previous modeling of Woese et al. (1968) suggests that the germination kinetics may be quite sensitive to different binding models, increasing the likelihood that the correct model can be identified by comparison with the data. Some key questions moving forward are as follows. What is the correct model for binding of ligand to a germinant receptor? How is ligand binding coupled to germinant receptor activation, and how is germinant receptor activation coupled to channel protein activation? Does channel protein activation correspond to the quantity *P* in the Woese model? What factors give rise to the apparent activation threshold for the germination process? Although modeling can help to answer these questions, the form of the model extensions will have to be based on further experiments to fill in the biochemical details. Based on the current state of knowledge, for the foreseeable future mathematical or computational requirements for the spore germination model will not push the limits of available methods or resources.

Future Research Directions

The most promising and doable avenues to determine the mechanism(s) of bacterial spore germination heterogeneity were outlined as follows.

1. Removal of the spore coat followed by germination studies would determine whether ligand diffusion or permeation proteins were involved in germination heterogeneity.
2. Permeation protein involvement in germination heterogeneity could be evaluated directly by using deletion or over expression mutants.
3. Correlation of rates and timing of Ca-DPA release and changes in spore refractility (water content) would indicate whether CLEs are involved in germination heterogeneity.
4. Correlation of over- or under-expression of germinant receptors or changes in activation temperature with changes in germination patterns would indicate whether germinant receptors are involved in germination heterogeneity.
5. Comparing the spontaneous germination pattern of spores of germinant receptor mutants with wild-type spores could also indicate whether or not germinant receptors are involved in germination heterogeneity.
6. Co-culture of multiple spores in small volumes of germinant and statistical analyses of the resulting germination would address potential production of inhibitory substances by germinating spores.

Towards the goal of developing a predictive model of spore germination, tight integration between the experimental and modeling efforts should result in a robust model of the molecular mechanism of spore germination that might be used in developing sterilization/treatment/decontamination strategies aimed at mitigating the threats posed by spores. Such an in depth understanding of spore germination could well allow prevention or alternatively, a controlled initiation, of the germination process.

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610

611 **Figure Legends**

612 Figure 1. Spore structure and location of components of the germination
613 apparatus (adapted from Setlow, 2006). Note that sizes of all layers are not
614 necessarily drawn to scale.

615 Figure 2. Events in spore germination (adapted from Setlow, 2003).

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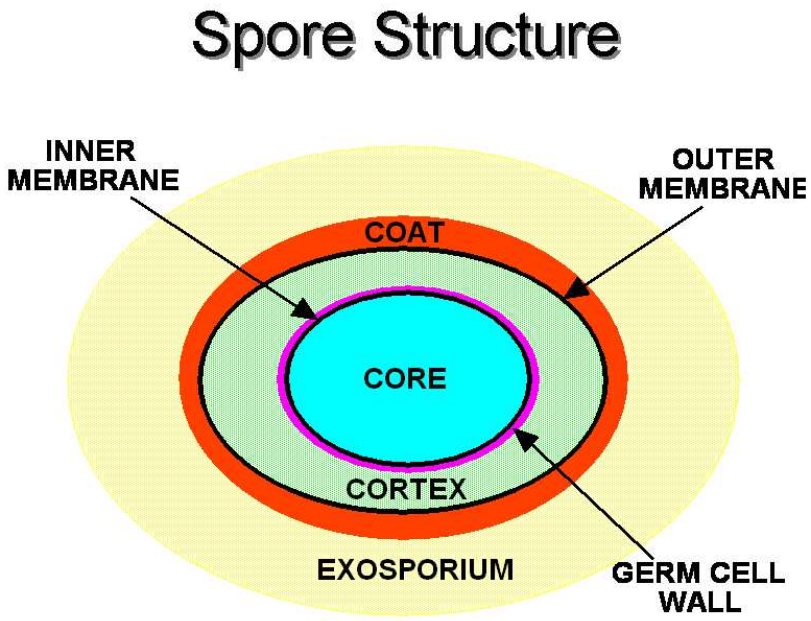

Figure 1. Spore structure and location of components of the germination apparatus (adapted from Setlow, 2006). Note that sizes of all layers are not necessarily drawn to scale
254x190mm (96 x 96 DPI)

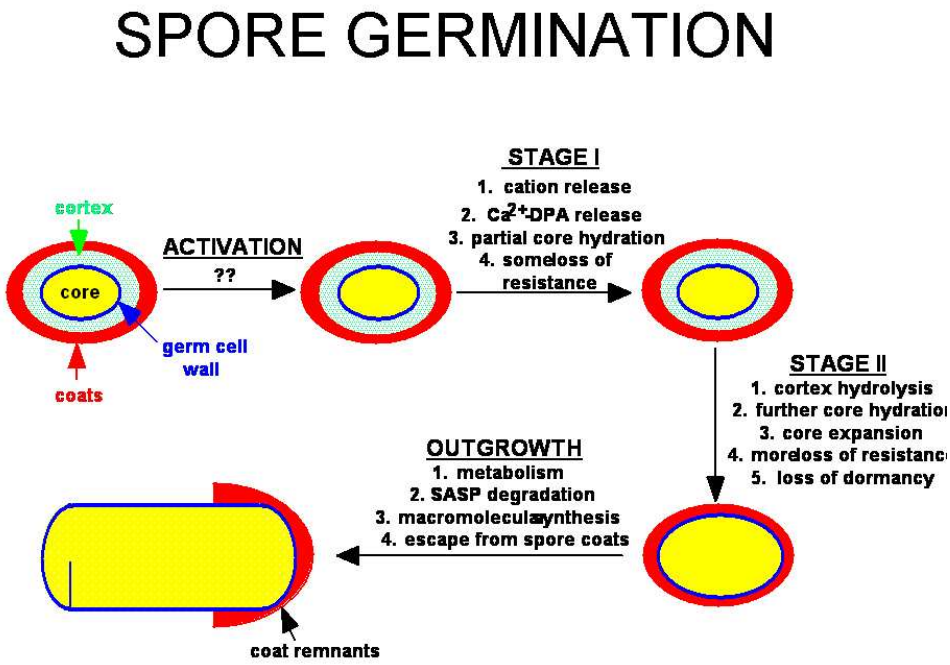

Figure 2. Events in spore germination (adapted from Setlow, 2003).
254x190mm (96 x 96 DPI)