Electrochemical Mechanics of Nanometer-Scaled Structural Layers of Bacterial Spores

S. Kazakov and H. Hosein

Department of Chemistry and Physical Sciences, Pace University
861 Bedford Road, Pleasantville, NY, USA, skazakov@pace.edu

ABSTRACT

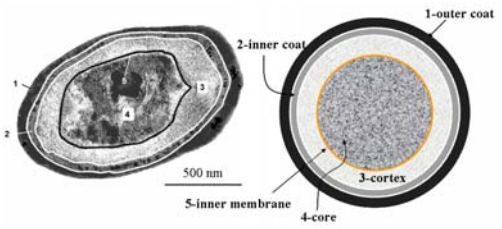
The early events in the nutrient and non-nutrient germination of *Bacillus subtilis* spores are considered in combination with the properties of nanometer-scaled spore structural layers. All structural, not metabolically mediated, transformations at the early steps of germination (cortex contraction/swelling, inner membrane ionic permeability, core water uptake, Ca-DPA release, and activation of cortex degradation) are incorporated into a unified model termed as "electrochemical mechanics" of bacterial spores. The model accounts for a common feature fastening the events involved: they are ion-sensitive.

***Keywords*:** bacterial spores, nanolayers, structure, transformation, ion-sensitivity, model.

1 INTRODUCTION

A bacterial spore is a single cell with the genetic information enclosed in the spore core surrounded by two types of walls: an inner peptidoglycan wall, the spore cortex, and an outer protein wall, the spore coats (Figure 1, [1]). Being the most environmentally resistant organisms known in nature, dormant spores can withstand extreme heating, freezing, drying, extremes of acidity and alkalinity, UV radiation, several chemical and enzymatic treatments, etc. Now, it comes to understanding that germination, the process resulting in breaking of dormancy and loss of spore-specific properties, lies behind the spore resistivity and its deleterious effects [2-4]. In terms of balancing dormancy and germination, a bacterial spore is said to be a naturally occurred, submicrometer-scaled, multilayered exquisite biosensor [5]. However, the origin of spore sensitivity to environmental conditions is not well understood [6,7].

A model of the structural changes on the way to germination triggering is in demand to fill the gap in understanding of (i) the cortex contribution to controlling the water content of the spore core, (ii) molecular mechanism governing the inner membrane permeability for water intake and Ca^{2+} and dipicolinic acid (DPA) release from the core; (iii) physical states of water and Ca^{2+}/DPA within the protoplast: (iv) the mechanism of lytic activity modulation and how the enzymatic activity is restricted to the proper moment in germination, (v) how molecules participating in the physiological (involving germinant receptor) and non-nutrient germination (without a requirement for germinant receptor function) interact with the cortex, membrane, and core to initiate the cascade of structural changes leading towards irreversible degradation of cortex.



$l_{outer\ coat}$ = 69 nm $l_{innet\ coat}$ = 50 nm
l_{cortex} = 132 nm r_{core} = 380 nm
r_{total} = 631 nm

Figure 1: Contours of *B. subtilis* spore compartments adopted from the electron micrograph in [1] (left) and equivalent nanometer-scaled multilayered structure (right).

There is a number of problems, which make it difficult to develop such a model: (i) many pieces of knowledge have come from disparate species of bacterial spores, (ii) bacterial spores are single cells of multilayered structure where each layer affects the others (Figure 1), (iii) "biological" environment is multiparametric (different ions, concentration of spores, temperature, conditions of activation, nutrients, etc.), and (iv) germination triggering by itself is a multistep process.

Our approach is based on the idea that all structural transformations within spores are ion- and temperature sensitive, especially, in terms of the spore cortex volume changes and the inner membrane permeability.

2 ON THE WAY TO GERMINATION

Nutrient germination. Spore germination is normally initiated or triggered by specific nutrients or mixtures of nutrients (e.g., L-alanine or a mixture of D-glucose, D-fructose, L-asparagine, and potassium ions (GFAK) for *B. subtilis*). There is a micro-lag phase after nutrient addition, when no response is observed. Then a rapid loss of refractility (Figure 2a) occurs, followed by a second phase with a reduced rate of further refractility loss.

A rather sketchy scheme for germination triggering in *B. subtilis* spores is given in Figure 2b. It is currently believed that spores recognize nutrient through receptor proteins, known as ger-receptors. Genetic studies have led

to a hypothesis that those proteins are encoded by three operons *gerA*, *gerB*, and *gerK* [8,9]. However, the receptor hypothesis remains untested, as the proteins are unmanageable *in vitro* [9]. Ger-receptors can be activated by heat shock (R → R*). Triggering of a ger-receptor via its interaction with a specific nutrient (R* → R**) somehow changes permeability in the spore's inner membrane. The drop of refractility coincides with the release of DPA and Ca^{2+} [10]. This fact suggests that water uptake into the core [8,11] can be a cause of Ca^{2+} and DPA release. An efflux of monovalent cations (H^+, Na^+, and K^+) occurring shortly before the release of Ca^{2+}DPA may mean that Ca^{2+} displaces these cations from the spore cortex. One can hypothesize that a specific structure (receptor?) may exist to collect the signal of ger-receptor(s)/germinant binding in order to regulate the inner membrane permeability. Genetic analysis suggests [12] that GerD proteins located in the inner membrane could play this role. There is also an idea that perhaps the other proteins encoded by the *spoVA* operon can form channels in the spore inner membrane to release DPA from the core [13].

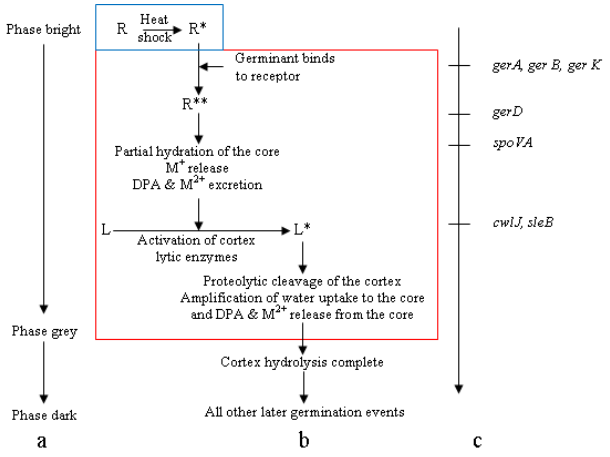


Figure 2: The sequence of germination triggering events in *B. subtilis* spores: (a) as observed under phase contrast microscope, (b) major morphological and chemical changes suggested from literature data, (c) the genetic operons which products might contribute at the early stages of germination.

Interestingly, the maximum amount of Ca^{2+}DPA is released before transfer to a complete phase dark spore [10]. This may mean that further hydration of the core is followed by the Ca^{2+} and DPA release. For now, it is clear that the activation of one or both lytic enzymes, named as CwlJ and SleB in *B. subtilis* spores, occurs after Ca^{2+} and DPA release into the cortex, whereas the hydrolysis of the spore cortical peptidoglycan precedes the further hydration and expansion of the spore core [12].

The conversion of a proenzyme (L) to an active heat-sensitive cortex-lytic enzyme (L*) is shown in Figure 2b as followed by cortex hydrolysis. Most likely, the steps R* → R** and L → L* define the extent of spores commitment to germination, whereas heat activation defines the initial population of spores involved into the germination. One can define the spore dormant and pre-germination states as blue and red boxes, respectively, shown in Figure 2b. The genetic operons, which products might contribute at the early stages of germination are summarized in Figure 2c.

Non-nutrient germination. Spores can be also germinated by a variety of chemical and physical treatments. For example, addition of exogenous Ca^{2+}DPA appears to activate CwlJ, one of the two cortex lytic enzymes, which catalyzes cortex hydrolysis [14]. Herein, the consequent release of endogenous Ca^{2+} and DPA amplifies the process. It looks like no ger-receptors are involved in this pathway of germination triggering. Since mutants lacking all ger-receptors or the cortex lytic enzymes were germinated well by dodecylamine [14,15], it was suggested that cationic surfactants are able to activate Ca^{2+} and DPA release directly without requiring the nutrient receptors. Recent report on germination triggering by the cell wall peptidoglycan fragments [16] gives an alternative and very different chemical mechanism without involving nutrient ger-receptors.

Pressures of ~100-300 MPa can activate the nutrient receptors in *B. subtilis* (mostly GerA and to lesser extends GerB and GerK) [17], whereas stimulation of germination by pressures > 450 MPa does not require ger-receptors [18]. The mechanical damage of spores (abrasion) seems to result in activation of the cortex lytic enzymes in *B subtilis*, CwlJ and SleB, and degradation of the cortex matrix [19].

3 ION-SENSITIVITY OF THE SPORE STRUCTURAL COMPONENTS

Ionic conditions and ionic sensitivity of structural compartments should be essential in their ability to regulate the state of spores. Further understanding of the spore way from dormancy to germination triggering should be focused on coupling of all structural transformations through their ionic sensitivity, i.e. on how ion-sensitive changes in one event vary the ionic conditions for the changes in the others. The significance and role of ionic exchange are supported by the following observations.

A spore coat is layered, coarsely heteroporous, wet, and permeable for low molecular weight molecules [1]. So long the physiological role of the coats is a spore protection from mechanical and harmful chemicals, one can suppose that the spore structural transformations are mostly associated with chemically (covalently) cross-linked network of cortex and physically (ionically) cross-linked network of core interfaced through the inner membrane.

Cross-linked cortex peptidoglycan. The cortical peptidoglycan consists of alternating glycan chains of β–1,4 linked *N*–acetylglucosamine (NAG) and *N*–acetylmuramic acid (NAM) residues. About 47% of the muramic acid residues are present as the spore-specific muramic D-lactam (MAL) [20]. We have estimated the number of carboxyl groups associated with amino acids fixed on the

peptidoglycan network to be of ~10^7 to 10^8 per spore [unpublished data]. Thus, a spore cortex – a peptidoglycan cross-linked polymer – has a negative net charge and a high content of mobile ions; a low degree of cross-linking allows the spore cortex to change the volume in response to varied water and ion content, like ion-sensitive hydrogels do [5].

Nakatani et al. [21] reported an 80% volume decrease of the spore cortex material isolated from *Bacillus megaterium* in the presence of divalent cations. This may mean that Ca^{2+} excreted along with DPA from the core during early steps of germination can cause a collapse of the cortical peptidoglycan. There is a basis to suggest [22] that Ca^{2+} and DPA release occurs prior to the onset of cortex hydrolysis.

Cortex lytic enzymes. Genetic analysis has yielded two enzymes, SleB and CwlJ, involved in cortex degradation of *B. subtilis* spores. Paidhungat et al. [14] hypothesized that SleB acts only on the cortex when it is deformed by CaDPA excreted from the core, whereas CwlJ can be activated by CaDPA directly. The mechanism of inhibition of these enzymes until the proper moment is unknown. Nevertheless, since spores germinate in response to exogenous CaDPA, one could speculate that both DPA and Ca^{2+}-ions interact with the cortex peptidoglycan to stimulate its collapse and thereby activate the cortex lytic enzymes either on nutrient or non-nutrient pathways of germination triggering.

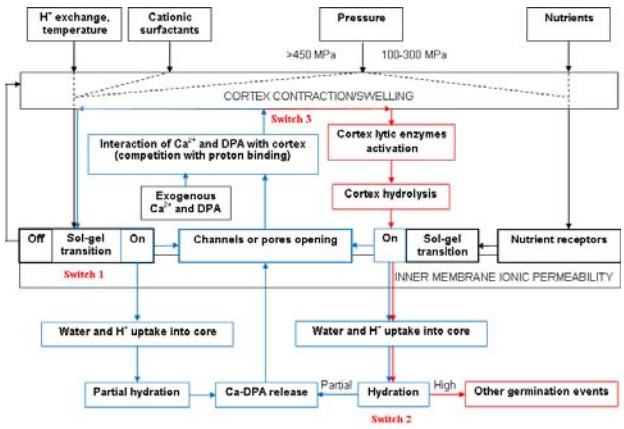
The spore core. A core of the dormant spore also exists as a high-polymer matrix in which cross-linking between macromolecules occurs through stable but reversible bonding [1,20,23,24]. The spore cortex has been thought [25] to force dehydration by pressurizing the core in the dormant state. Ion permeability of the spore core (inner membrane) may play especially significant role in spore germination. The coincidence of Ca^{2+} and DPA release with water entry in the spore core supposes that there should be a switch of the inner membrane permeability allowing water influx and Ca^{2+}DPA efflux. An enormous amount of DPA (5 – 15% of dry weight of the spore [24,26]) is located in the spore core. But the physical state of Ca and DPA within the core is not known. Taking into account the fact that calcium dipicolinate can exist as a trihydrate [27], we compared the percentage of water in CaDPA·$3H_2O$ (20% of dry weight) and the lower value of water percentage in the core of a wet spore (28%) to propose that within the dormant spore core, Ca and DPA are structured into layers ionically cross-linked into a 3D-network, $(–Ca–DPA–Ca–DPA–Ca–)_n$.

Ionic content of spores and external ionic conditions. There is a pool of experimental data indicating that the content of small ions is important in the spore dormancy and its germination triggering [4,24]. In particular, it was found that the higher is the relative amount of divalent ions with respect to monovalent ones, the higher is the resistance (germinability is lower). For example, thermophiles produce the most heat-resistant spores than do mesophiles, while psychrophiles produce the least resistant spores.

The influence of environmental pH on activation of germination, germinant binding, commitment to germinate, and probability of germination triggering has been proven experimentally [28]. In particular, it has been observed [29] that the relative swelling of spores during germination correlates with pH conditions. Recently, it has been found [5] that equilibrium concentrations of free protons outside the spores and free and bound protons inside the spores are redistributed when concentration of spores is changed. Moreover, studying the kinetics of H^+ consumption by dormant spores we predicted the localization of a great majority of carboxyl groups within the cortex [unpublished data].

4 THE MODEL OF COUPLED STRUCTURAL TRANSFORMATIONS

Incorporation of all observations listed above into one model is a challenge. As a starting point, we propose a model outlining electromechanical events in the dormant and pre-germination states of *B. subtilis* spores (Scheme 1). The model distinguishes three coupled cycles (marked in black, blue, and red). The first two cycles (black and blue) are thought to be reversible, the red one leads to irreversible steps toward germination. The switch from one cycle to another is controlled at three levels: permeability of the inner membrane (switch 1), core hydration (switch 2), and activation of cortex lytic enzymes (switch 3). The model takes into account the essential parts of the germination apparatus, like germination receptors and channels or pores in the inner membrane regulating the release of Ca^{2+} and DPA from the spore core. Interactions of different (nutrient and non-nutrient) germinants with the cortical peptidoglycan before binding to ger-receptors and/or before causing Ca^{2+} and DPA release from the core are considered. The model presumes that the degree of swelling of the cortex peptidoglycan can modulate the activity of the cortex lytic enzymes.



Scheme 1: Model of electrochemical mechanics in bacterial spores outlining ion-sensitive structural events in the germination triggering by different germinants. Dashed lines indicate the effect of different factors on physical dimensions of the cortex.

The following ideas are addressed in this model: (i) equilibrium volume of the cortex is defined by the charge on the peptidoglycan chains, associated counterions, and distribution of mobile ions between the spore layers and external solution; (ii) in turn, a change in the physical dimensions of the cortex causes an immediate ion exchange to balance the ion concentration difference and the mechanical elasticity in each spore structural layer; (iii) since the permeability of the inner membrane is strongly dependent on ionic conditions, temperature, and surface pressure, ion-sensitive volume change in the cortex is the first candidate to modulate (catalyze or inhibit) the passive permeability of the spore inner membrane to water, small electrolytes, and non-electrolytes; (iv) water and ion uptake by the core determines the degree of core hydration, which in turn controls the amount of Ca^{2+} and DPA released from the core to the cortex; (vi) Ca^{2+} and DPA excreted from the core affect the cortex dimensions; (vii) at a certain (unknown) degree of cortex swelling/shrinking, cortex lytic enzymes are activated to trigger the cortex degradation.

5 CONCLUSIONS

The bacterial spore is the other marvelous example of nature's nanotechnology: it works as a small exquisite biosensor constructed from tiny environmentally responsive layers to balance dormancy and germination by regulating ion exchange between external parts and the spore core.

The question is: Could we copy some of the mechanisms to our advantage? The proposed sensory mechanisms of the cortex-membrane-core interactions in the course of germination triggering can be a basis for designing novel biosensors, drug/gene delivery, and control release systems using combined hydrogel/lipid bilayer sensing elements *(new generation of nanobioanalytical systems*). The model of electrochemical mechanics of bacterial spores can be used for development of protocols for disinfection, sterilization, preservation, and therapeutic treatments, as well as for discrimination among species of *Bacillus* genus (*crucial principles of spore detection and identification, biodefense*).

This work was financially supported by Pace University (Dyson College of Arts and Sciences, 2009/10 Summer and 2009/10 Scholarly Research Funds).

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