Protons' Binding within Nanometer Scaled Compartments of Natural Ionic Reservoirs: *Bacillus subtilis* Spores

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ABSTRACT

The *Bacillus* spores are considered as dynamic natural ionic reservoirs. By time-variation of pH in the exterior to *Bacillus subtilis* spores suspended in aqueous medium, it was shown that (i) dormant spores can release and consume protons in response to the changes in temperature from 25 to 37°C, and (ii) the proton exchange correlates with variations in the average spore size. The gigantic ionic capacity ($N \sim 10^{10}$ per each spore) has been estimated from the potentiometric titration curves for the spores and a Scatchard-like approach to equilibrium binding of protons to ionizable groups within spores. Kinetics of proton uptake by dormant *Bacillus subtilis* spores corresponds to a multi-step process featuring nm-sized multi-layers of the exosporium-coats-cortex-inner membrane-core structure of spores. The results are of great potential for understanding the spore's alert mechanism to monitor the environment for favorable conditions and for future designing of nature-inspired bionanomaterials and sensors.

Keywords: *Bacillus* spores, natural ionic reservoirs, spore proton capacity, kinetics of pH-equilibration.

1 INTRODUCTION

The dormant (metabolically inactive) spores of bacteria of *Bacillus* genus are the most environmentally resistant organisms known in Nature. Nonetheless, they permanently monitor the external environment in order to trigger germination in the presence of specific germinants [1]. The spore "life" can be considered as the never-ending competition between the alert sensory mechanism and spore's stabilization. In this context, the spores *per se* are dynamic systems with properties varying in time [2]. The experimental data [3] show that germinating spores can exchange ions and other compounds (e.g. depicolinic acid, DPA) with the external solution. Recent experiments [4] support the concept of a dynamic spore by showing that dormant spores are not entirely static: they swell and shrink in response to a increase and decrease in relative humidity. Presumably, the spore dynamic alert system is based on physico-chemical properties of the multi-layered structure of spores which can change with time. The multi-compartmental structure of *Bacillus* spores and known mechanisms of spore resistance and dormancy support the hypothesis that spore is a natural ionic reservoir with an ability to accumulate and release ions in response to environmental conditions. There are at least two of the spore integuments, cortex and core, which can be considered as interacting ion-sensitive polymer networks with different mobility of small ions. Since the physical dimensions of spore compartments are of nm scale, the kinetics of proton exchange between the spores and surroundings can be examined by the time-resolved micro-potentiometry technique developed in our recent study of pH equilibration in suspensions of micro- and nanogels (hydrogel spheres of nanometer diameter) [5].

The main objectives of the present work are: (i) to demonstrate that proton exchange between dormant spores and the surrounding medium exists and correlates with variations in the average spore size when temperature changes from 25 to 37°C; (ii) to estimate the quantitative characteristics of spore ionic reservoir (number of proton binding sites per spore and apparent binding constants); and (iii) to examine the kinetics of proton accumulation and release by *Bacillus subtilis* spores.

2 EXPERIMENTAL SECTION

2.1 Spore Treatments

The *Bacillus subtilis* strain (1A700) used in this study was available from the culture collection of Department of Microbiology at University of Alabama (Birmingham, AL). For spore purification, the method of lysozyme treatment and salt-detergent washes was used [6]. The purified spores were washed three times and finally stored in deionized water at 4°C. The stock suspension of *Bacillus subtilis* spores was tested to have the initial concentration of 1.18×10^9 spores/mL and pH~8.8.

2.2 pH Measurements

A MI-415 micro-combination electrode (Microelectrodes Inc., Bedford, NH) was used for pH measurements.

Potentiometric Titration of Water and Spores. The aqueous suspension of spores was taken as a starting point for titration. Milli-Q water with initial pH ~ 5.85 was used for the reference titration. The sample was stirred by a stirring rod (1.5×2×5 mm) at ~70 rpm during titration. The

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initial volume of pure water or spores' suspension placed in the well was always 1.0 mL. The pH was adjusted with 0.1 M HCl or 0.1 M NaOH.

Time-Resolved pH-measurements. The apparatus for pH real-time measurements provided the injection of acid or base into the inner volume without perturbations of the electrode. A Vernier Logger Pro (version 2.3) software was used for data acquisition, analysis and storage in real time.

Temperature related pH-measurements. To vary temperature, a vial with 1 mL of a spore suspension (5.9×10^9 sp/mL) was placed into a double-walled beaker operating as water thermostat. The head cover of the beaker had two inserts for the microelectrode and temperature sensor so that pH and temperature were measured simultaneously in real time. The quantitative kinetic measurements at 37°C were carried out as follows: a micro-combination electrode was calibrated; a vial with 1.0 mL of a suspension of a known amount of spores was placed into the double-walled beaker with temperature stabilized at 25°C; the signal recording was started and continued until temperature and pH were reasonably stabilized; at this point the temperature was settled at 37°C without stopping the temperature and pH recording for the next 60-100 min. The data on temperature and pH were collected with a sample time of 0.1 s.

2.3 Instrumentation

Suspensions of spores were observed in bright field using a Digital Optical Microscope DC3-163-PH with Integrated Imaging System (Microscope World, CA). The microscope was equipped with a CCD camera.

Dynamic Light Scattering (DLS) measurements of the *Bacillus subtilis* spore sizes were carried out on a "PhotoCor Complex" Photon Correlation Spectrometer (PhotoCor Instruments Inc., MD) utilizing a 20 mW He-Ne laser as a light source. Measurements were performed at 23°C and 37°C. The measurements were done at the constant scattering angle of 90°. Particle size distribution analysis with an automatic or visually assisted choice of the regularization level was carried out using the DynaLS (v.2) software program supplied by Alango Ltd., Israel. Measurements of the average size were performed every 1 min over a course of 120 minutes.

3 RESULTS AND DISCUSSIONS

3.1 Spores Ionic Activity and Average Size Variations with Temperature Changes

To examine the ability of *Bacillus subtilis* spores to release or consume protons, we exposed the spores to the temperature changes from room temperature (25°C) to that favorable for germination (37°C), and to the long-term exposition of spores at 37°C. The time-course of pH changes for a spore suspension (Figure 1, curve 1) exhibits the explicit pH-peak, which coincides with the maximum rate of temperature changes. The peak indicates the intake of protons by spores followed by their release back when temperature reaches 37°C. Those variations in pH are not associated with the effect of temperature on a pH electrode: the temperature-related changes in pH were measured under similar conditions for a plain buffer with no spores (curve 2). However, the variations in pH for a suspension of ion-sensitive microgels [5] were surprisingly similar to the variations in the spore suspension (curve 3) indicating that it is the ionizable groups that are responsible for these pH changes with temperature.

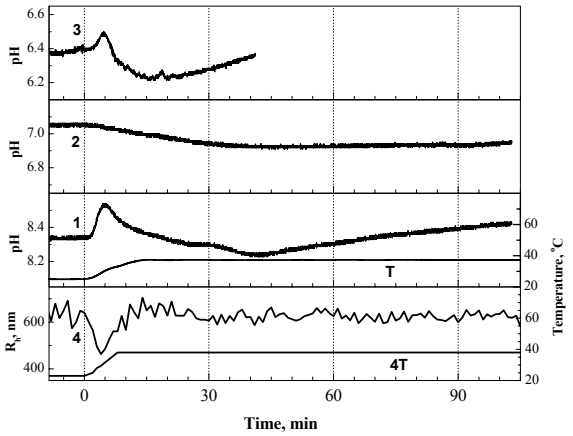

Figure 1: The course of pH changes with the temperature jump from 25 to 37°C for spore suspension (curve 1, $C_{sp} = 5.9\times10^8$ mL^{-1}), a buffer with no spores added (curve 2, pH 7), and a 25% hydrogel aqueous suspension (curve 3, PNIPA-VI [5]). Curve 4 is the time course of the average hydrodynamic radius of *Bacillus subtilis* spores in the suspension. The time courses of temperature changes are shown by curves T and 4T (right Y-axis).

The results of DLS study of the average hydrodynamic radius, R_h, for the spore suspension are depicted in Figure 1 (curve 4). The most striking observation is that the minimum of R_h was found during the temperature jump from 25 to 37°C. This spike show that spores first shrink in response to the temperature increase and then restore their size when temperature reaches 37°C. No temperature-related changes in R_h were found for a suspension of polystyrene latex of 1 μm diameter (data not shown).

3.2 pH of Spore Aqueous Suspensions

In the first experiment, the known amounts of the spores were successively injected into the vial with water of pH 5.85. The pH was measured after ~2 hours of equilibration. As Figure 2a shows, the addition of spores increases the suspension pH value, as though protons from the external solution penetrate into the spores and bind inside. The experiment further indicates the spores' ability to accumulate hydrogen ions, i.e. to act as ionic reservoirs.

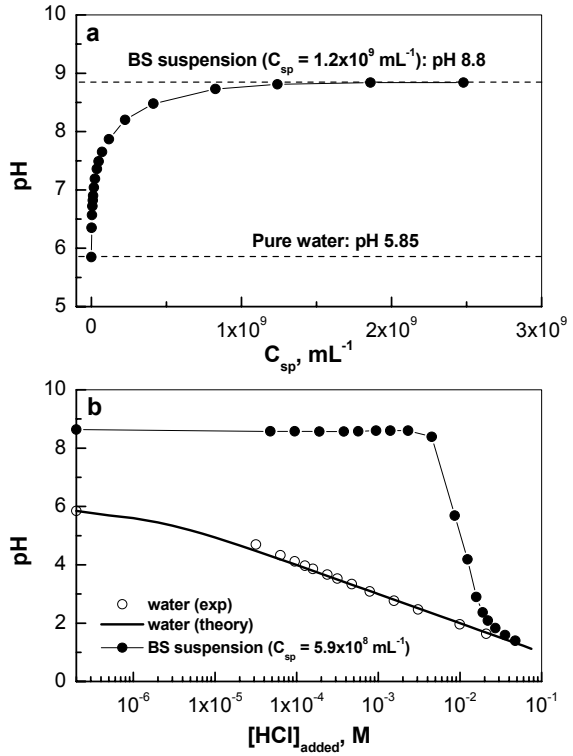

Figure 2: (a) Variation in equilibrium pH with concentration of *Bacillus subtilis* spores injected into distilled water. (b) Changes in pH during titration of pure water (open circles) and *Bacillus subtilis* spores (solid circles). Solid line is a calculated titration curve for water.

In the second experiment, the known amount of acid (protons) was added to the suspension of a given concentration of the spores. In Figure 2b, the titration curve for the spore suspension is compared with that for pure water. If there had been no consumption of H^+ by spores, their titration curve would look like the titration curve of pure water. The comparison clearly indicates that the significant amount of protons is consumed by spores. Most likely, there is a plenty of charged sites (ionizable groups) which bind this amount of H^+ within each spore.

3.3 Spore's Proton Capacity

The number of binding sites for H^+ per each spore (N) is related to the concentration of protons bound to ionizable groups within the spores ($[H^+]_{bound}$).

Using the approach similar to Scatchard [7], we found the relation between the changes in number of bound protons ($\Delta[H^+]_{bound} = [H^+]_{bound} - [H^+]^0_{bound}$) and the changes in free proton concentration outside the spores ($\Delta[H^+]_{out} = [H^+]_{out} - [H^+]^0_{out}$) and the concentration of proton added ($[H^+]_{add}$). The existence of two types of binding sites within spores was assumed to fit all data on either dilution or titration presented in Figure 2, a and b into the two-hyperbola equation in terms of $N_A\Delta[H^+]_{bound}/[Sp]$ versus $\Delta[H^+]_{out}$:

$$\frac{N_A\Delta[H^+]_{bound}}{[Sp]} = \frac{A_1\Delta[H^+]_{out}}{A_2 + \Delta[H^+]_{out}} + \frac{A_3\Delta[H^+]_{out}}{A_4 + \Delta[H^+]_{out}}, \qquad (1)$$

where the first term represents one type of binding sites, and the second term is for the other type of binding sites, A_1 and A_2 are related to N_1 and K_1 whereas A_3 and A_4 are related to N_2 and K_2:

$$A_1 = \frac{N_1}{K_1 A_2},\ A_2 = \frac{1 + K_1[H^+]^0_{out}}{K_1},\ A_3 = \frac{N_2}{K_2 A_4},\ A_4 = \frac{1 + K_2[H^+]^0_{out}}{K_2}.$$

It was found that the number of binding sites per spore ($N_1 = 1.1\times10^{10}$) and the overall equilibrium constant ($K_1 = 5\times10^4\ M^{-1}$) are the same for any process of spore protonation (dilution or titration). For the other type of binding sites, the number of sites per spore ($N_2 = 0.8\times10^{10}$) remains constant, whereas the equilibrium coefficient (K_2) can vary up to the extreme binding when K_2 becomes infinite indicating the extreme avidity of spores to protons.

3.4 pH-Equilibration in Spore Suspension

Since the concentration of protons even in pure water (pH 5.85) is higher than in the initial suspensions of spores (pH 8.8), in the course of a simple injection of a known amount of spores into water one can expect the following possible mechanisms to affect the proton concentration in the solution external to the spore: fast binding of ions to the immediate surface of each spore, a successive diffusion of bound ions into the next interior layer of a spore, and occupation of the newly formed vacancies on the surface by new ions from the exterior.

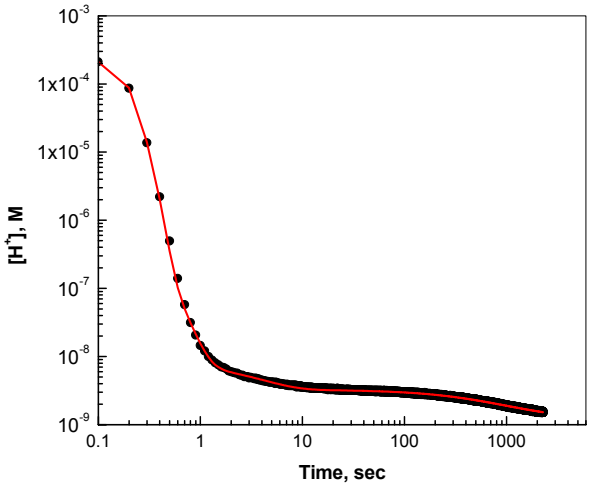

Figure 3: Time course of [H^+]-equilibration after the addition of 5.9×10^8 spores into water with the initial pH of 3.67: points are the experimental data; red line is the fitting curve.

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The initial concentration of water was adjusted to pH 3.67. The kinetics of pH-equilibration in this water sample after injection of spores is shown in Figure 3. The kinetics of proton penetration into the interior of spores can be represented as a set of n monomolecular reversible reactions

$$X_0 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} X_1 \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \ldots \underset{k_{-n}}{\overset{k_n}{\rightleftarrows}} X_n \quad , \qquad (2)$$

where $X_0 = [H^+]_{out}$ is the external concentration of free protons, X_i is the concentration of protons bound in a distinct layer of the spore structure, k_i and k_{-i} are the rate constants for the forward and reversible binding reactions, respectively.

It is known from chemical kinetics [8] that the solution of the system of n linear differential equations of the first order for this scheme is the superposition of n exponential functions

$$X_i = X_i^{eq} + \sum_{j=1}^{n} A_{ji} e^{-t/\tau_j} \qquad (3)$$

where X_i^{eq} is the equilibrium concentration of protons, A_{ji} are the factors which contain the rate constants and depend on the initial conditions, τ_j are the characteristic times of the processes and are the functions of all rate constants k_i, k_{-i}. It is essential to note that the number of exponentially decaying functions corresponds to the number of reversible processes involved into equilibration! It was found that minimum six exponential functions were necessary to fit the data shown in Figure 3 into (3) (Table 1).

4 CONCLUSIONS

The similarity in kinetics of proton exchange and size change of *Bacillus subtilis* spores in response to changes in temperature not only confirms the general statement that dormant spores are the dynamic ionic reservoirs, but also demonstrates that swelling and shrinking may accompany a spore response to acute changes in external conditions. The spore ionic reservoir has been quantitatively characterized in terms of the number of binding sites for protons per spore (N~10^{10}) and the apparent binding constant (K~10^5 M^{-1}). The proton transport in and out of *Bacillus subtilis* spores was measured and modeled as a sequence of six processes with the distinct characteristic times which can be associated, most likely, with the multi-layered structure of spores: exosporium/coats/cortex/inner membrane/core. Strikingly, the characteristic times differ by the order of magnitude. This fact allows one to state that the transition from any "upper" layer to the "deeper" one is rate-limiting. As a result, after fast binding of protons to the immediate surface of a spore, a flux of protons in the opposite direction noticeably contributes to the overall kinetics because of a ~10-fold slower successive diffusion of ions into the next interior layer of a spore.

Table 1: Fitting parameters and possible interpretation of six kinetically distinct steps describing $[H^+]$-equilibration after the addition of 5.9×10^8 spores into water with the initial pH of 3.67 as depicted in Figure 3.

$[H^+]^{eq}$	1.36×10^{-9}	***Equilibrium concentration***
A_1, M	7.99×10^{-3}	***Fast binding to the surface***
τ_1, sec	**0.05**	
A_2, M	-1.62×10^{-2}	***Fast release to exterior***
τ_2, sec	**0.03**	
A_3, M	1.34×10^{-6}	***Penetration into coats***
τ_3, sec	**0.2**	
A_4, M	5.0×10^{-9}	***Transition through outer membrane: coat/cortex***
τ_4, sec	**3**	
A_5, M	7.0×10^{-10}	***Penetration into cortex***
τ_5, sec	**322**	
A_6, M	1.2×10^{-9}	***Transition through the inner membrane: cortex/core***
τ_6, sec	**1164**	

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