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# Direct investigation of viscosity of an atypical inner membrane of *Bacillus* spores: A molecular rotor/FLIM study

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ABSTRACT

We utilize the fluorescent molecular rotor Bodipy-$C_{12}$ to investigate the viscoelastic properties of hydrophobic layers of bacterial spores *Bacillus subtilis*. The molecular rotor shows a marked increase in fluorescence lifetime, from 0.3 to 4 ns, upon viscosity increase from 1 to 1500 cP and can be incorporated into the hydrophobic layers within the spores from dormant state through to germination. We use fluorescence lifetime imaging microscopy to visualize the viscosity inside different compartments of the bacterial spore in order to investigate the inner membrane and relate its compaction to the extreme resistance observed during exposure of spores to toxic chemicals. We demonstrate that the bacterial spores possess an inner membrane that is characterized by a very high viscosity, exceeding 1000 cP, where the lipid bilayer is likely in a gel state. We also show that this membrane evolves during germination to reach a viscosity value close to that of a vegetative cell membrane, ca. 600 cP. The present study demonstrates quantitative imaging of the microscopic viscosity in hydrophobic layers of bacterial spores *Bacillus subtilis* and shows the potential for further investigation of spore membranes under environmental stress.



## 1. Introduction

Some bacterial species such as *Clostridium*, *Bacillus*, or *Sporosarcina*, have the capacity to sporulate when placed in an unfavorable environment. These spores are a major concern in the food industry because they are responsible for spoilage and food borne disease due to their high resistance to food preservation processes. Spores resist numerous stress factors efficiently, including extreme heat, starvation, treatment with chemicals and radiation and are able to germinate even after long periods of dormancy [1–4]. This high resistance to environmental stress is due to their particular and partitioned structure [5]. Bacterial spores possess numerous layers, each protecting the inside of the spore from stress: a proteinaceous coat, a cortex made of peptidoglycan, two phospholipid-based membranes and, finally, a protoplast with a low water content where the DNA is located. The two membranes have different location and function: the outer membrane is localized between the coat and the cortex and the inner membrane surrounds the protoplast. The inner membrane is particularly interesting as it is generally defined as the main permeability barrier; however, its viscoelastic properties and its structure are largely unknown. Indeed, its composition is thought to be similar to that of a vegetative cell membrane, with only a slight variation in proportion of different phospholipids present [6]. However, the inner membrane has a set of very particular properties: a low mobility of lipids [7] and a weak permeability to small molecules [8,9]. This membrane is thought to be key in the spore's resistance to toxic chemicals, in particular DNA-damaging agents [10] and it is also thought to play a major role in germination, stemming from the fact that it contains numerous germination receptors [11–13]. However, very little is known about the biophysical state of this membrane and the details of its evolution during germination. Study of the inner membrane is particularly difficult as it is buried deep within the spore and surrounded by multiple layers. It is therefore challenging to study the inner membranes mechanical and biophysical properties by standard biophysical techniques, while excluding the influence of the neighboring layers. In this work we set out to use a new viscosity-sensitive spectroscopic technique that visualizes the viscoelastic properties of different hydrophobic structures within the bacterial spore *Bacillus subtilis*, in particular focusing on the inner membrane.

Viscosity is a key property that influences diffusion and mobility in fluids. In microscopically heterogeneous biological systems, such as biopolymer gels, lipid bilayers or even individual live cells, traditional mechanical methods for probing viscosity are not suitable; they are destructive and require large volumes of material for analysis. Alternatively, several spectroscopic and microscopic approaches have

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been developed, such as fluorescence recovery after photobleaching (FRAP) [14], fluorescence correlation spectroscopy (FCS) [15–17] or single particle tracking [18], however, these methods are typically limited to a single point measurement. Here we use fluorescence lifetime imaging microscopy (FLIM) in conjunction with a viscosity-dependent fluorophore termed "molecular rotor" [19–21] to directly visualize the viscosity of hydrophobic domains of spores.

The molecular rotor meso-phenyl-4,4′-difluoro-4-bora-3a,4adiaza-s-indacene (Bodipy-$C_{12}$) has been used as a probe for microscopic viscosity in a variety of systems, including live cells, sol-gels and lipid mono- and bilayers [19–22]. It was established that for this fluorophore the non-radiative deactivation pathways are activated at low viscosity. In practice this means that both the fluorescence intensity and the lifetime are strongly viscosity dependent. We have previously demonstrated that the fluorescence lifetime is a superior marker for micro-viscosity in heterogeneous systems, since it is not affected by changes in probe concentration or by its distribution [20,21]. Consistent with the modified Förster–Hoffmann equation [23], the fluorescence lifetime ($\tau_f$) of molecular rotors displays the following dependence on viscosity ($\eta$):

$$\log\tau_f = \log\left(\frac{z}{k_r}\right) + \alpha\log\eta \quad (1)$$

where $k_r$ is the radiative decay rate constant, and $z$ and $\alpha$ are constants required to fit the data to the equation.

We have calibrated a lifetime of the Bodipy-$C_{12}$ rotor vs. viscosity in a wide range of viscosities. Fitting the experimental data obtained between 15 and 1500 cP [20] we have demonstrated that for Bodipy-$C_{12}$ in methanol/glycerol mixtures Eq. (1) becomes:

$$\ln\tau_f = 0.5336 \times \ln\eta + 4.5862 \quad (2)$$

where $\tau_f$ is the lifetime of Bodipy-$C_{12}$ in ps and $\eta$ is viscosity in cP.

This expression provides a direct means of converting the lifetime of the Bodipy-$C_{12}$ into the micro-viscosity of the probe environment.

For the applications of Bodipy-$C_{12}$ as a bioviscosity sensor it is essential that its fluorescence lifetime is not affected by other environmental factors such as pH, ionic strength, polarity of the solvent and the presence of the excited state quenchers. It is well known that the spectral characteristics of unmodified Bodipy chromophores, including lifetimes, are largely independent on the solution pH and polarity of the solvent [24,25]. The quenching of Bodipy with protein components has also been shown to be inefficient [24]. Thus we concluded that the major factor affecting the non radiative decay and hence the fluorescence lifetime of Bodipy-$C_{12}$ is viscosity [20].

Given the hydrophobic structure of the Bodipy-$C_{12}$ rotor, in particular its saturated $C_{12}$ hydrocarbon chain, we expect partitioning of the probe in all the hydrophobic domains of the spore, namely the inner and the outer membranes and the protein coat. FLIM provides the means to obtain lifetime information (and hence the microviscosity via Eq. (2)) in every pixel of a diffraction-limited fluorescence image. We performed FLIM imaging of whole dormant or coatless spores, germinated spores and vegetative cells to directly determine the viscosity of internal layers for the first time.

## 2. Materials and methods

### 2.1. Spores and cell culture

The *Bacillus subtilis* strains used in this work were wild type 168 (Bacillus Genetic Stock Center, Department of Biochemistry, The Ohio State University, Columbus, OH 43010, USA) or PS533. We also employed the strain PS4150 in which most of the *cotE* and *gerE* coding sequences are deleted, hence this strain lacks most of its coat [26]. PS533 and PS4150 (both from the Department of Molecular, Microbial, and Structural Biology, University of Connecticut Health Center, USA) are derivatives of isogenic strain PS832, a prototrophic derivative of strain 168. PS533 carries a plasmid pUB110 encoding resistance to kanamycin [27]. We ensured that similar fluorescence lifetime values were obtained in dormant PS533 and strain 168 spores. In the text 168 strain is referred to as wild type spores and PS4150 strain as *cotE gerE*.

Spores were prepared at 37 °C in 2xSchaeffer's-glucose liquid medium. Pre-warmed medium was inoculated with the preculture at a ratio of 1:10 in a baffled flask. After sufficient sporulation was achieved, spores were harvested, washed 4 times with cold distilled water and stored at 4 °C until further use. Purification of spores was performed by washing with water for several days as previously described [28]. If required, the spores were further purified by Histodenz® (Sigma Aldrich) centrifugation and extensively washed to have purity above 95% of phase bright spores.

The molecular rotor Bodipy-$C_{12}$ was synthesized as previously described [21]. The stock solution of Bodipy-$C_{12}$ in DMSO (2.15 mM) was added to the medium containing cells *ca.* 3 hours after inoculation to create the final concentration of the dye of 4.3 μM. Control experiments verified that the presence of the probe had no effects on growth and germination of spores. Fluorescence images were acquired at least 7 days after spore production.

Vegetative cells were grown and stained following the procedure adapted from [7] in Luria Broth (LB) medium (Sigma-Aldrich) until an $OD_{600nm}$ of *ca.* 0.2 was achieved. Bodipy-$C_{12}$ was then added at a final concentration of 4.3 μM and vegetative cells remained in contact with the probe for 2 to 3 hours. The cells were then harvested by centrifugation, washed with free LB medium and re-suspended in physiological buffer for observation.

### 2.2. Spores germination and coat fragments

Prior to germination, spores of *B. subtilis* were heat activated for 30 min in a water bath at 70 °C and then immediately cooled down on ice. For germination, spores were re-suspended at an $OD_{600\ nm}$ of *ca.* 1 in LB rich medium or in 10 mM L-alanine in 25 mM Tris–HCl buffer at 37 °C. 168 (wild type) and PS4150 (*cotE gerE* mutant) were germinated for 45 min and 1 hour, respectively. After this time, spores were harvested by centrifugation, washed with distilled water at 4 °C and then re-suspended in fluorophore-free water, after which the FLIM images were recorded. Coat fragments were identified by transmission and lifetime microscopy following germination of wild type spores for at least 1.5 hours.

### 2.3. Decoated spores preparation

Decoated spores were prepared according to a procedure described in [11]. Spores were suspended at an $OD_{600\ nm}$ of *ca.* 10–15 for 30 min at 70 °C in 1% sodium dodecyl sulfate (SDS), 0.1 M NaOH, 0.1 M NaCl and 0.1 M dithiothreitol in order to extract coat proteins. Thus decoated spores were washed extensively with distilled water (*ca.* 8 times) and stored at 4 °C in PBS. The decoating efficiency was checked by following the decrease in $OD_{600\ nm}$ in the presence of 0.5 mg/ml of lysozyme.

### 2.4. Microscopy and FLIM analysis

In all microscopy experiments an aliquot of spores or vegetative cells sample (10 μl) was placed on a microscope coverslip and covered with a pad of 10% agarose to hold the cells in place and to preserve moisture. The slides were discarded after 30 min of imaging.

The transmission and fluorescence images of stained spores and vegetative cells were captured on a Nikon C1Si Eclipse TE 2000 U confocal microscope and analyzed using the EZ-C1 software 3.50 (Nikon, Japan). Imaging was carried out with a ×100 PlanApo objective