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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 (τ_f) of molecular rotors displays the following dependence on viscosity (η):

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

where k_r is the radiative decay rate constant, and z and α 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 τ_f is the lifetime of Bodipy-C_{12} in ps and η 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

(NA: 1.4, oil, Nikon, Japan) at a scanning speed of one frame per second. The FLIM system (LSM kit, Picoquant) was incorporated into the microscope and consisted of a pulsed diode laser (485 nm, FWHM = 83 ps, 40 MHz) and a double SPAD detector. Images of 256 × 256 pixels were recorded for about 210 s with a pixel dwell of 9.60 μs. TCSPC lifetime recording was performed over 200 temporal channels (final resolution of 0.128 ps/channel). The absence of Bodipy-C_{12} aggregates, which could be detected by a broad emission band at >600 nm was verified for the different samples, to ensure the robustness of lifetime analysis [20,29].

FLIM images were analyzed using TRI2 software version 2.4.4.1 (Gray Institute, Oxford, [30]). In order to obtain between 1000 and 10,000 counts in the pixel peak maxima, circular binning of 4 was used for analysis of the different spore samples, unless otherwise stated. We have also applied a signal threshold between 10–20% and 100% to avoid background noise and signal from weakly stained internal parts of spores (the spore's core). Wild type spores, coat fragments, decoated and germinated spores were analyzed using a bi-exponential fitting (Marquardt algorithm) for each pixel of the decay curves. *cotE gerE* were analyzed by a mono-exponential fitting since the biexponential algorithm did not produce an improvement in χ^2_r (monoexponential fit χ^2_r between 1.1 and 1.2, biexponential fit χ^2_r between 1.0 and 1.1). Vegetative cells were analyzed using a mono-exponential fitting with the Bayesian algorithm, which allows a decay trace with lower pixel counts to be fitted. This procedure helped to avoid excessive binning and a reduction of spatial resolution. For vegetative cells, a circular binning of 2 and a threshold of between 10–20% and 100% was used. In all cases, the instrument response function (IRF) was recorded by detecting the extremely short-lived fluorescence of fluorescein (0.2 mM) diluted in a saturated solution of KI, using the same setup parameters as for the sample. For biexponential decays the amplitudes of each component are represented as 'fractional intensities' (Fi) varying between 0 and 1 (equivalent to 0–100%).

The lifetime and fractional intensity histograms were exported and analyzed using Origin software (Origin Pro 8, Origin Lab). The "Fit multiple peaks" tool was used to determine the maxima and FWHM (full-width half maxima) of the peak representing the average and error bars of each lifetime and fractional intensity value respectively.

3. Results

3.1. Measuring viscosity of dormant spores

Confocal fluorescence images of wild type spores were collected at least 7 days after harvesting (Fig. 1) and showed bright green fluorescence typical of Bodipy-C_{12}. Thus molecular rotor incorporated deeply into spores and was stable. At the same time the fluorescence intensity in the central region of the spore was *ca.* 10% of the outside region, indicating a low level of staining in the spore's core. Apart from the central region, fluorescence appeared uniform across the external layers of spores. It was not possible to distinguish between different structures, at least with the diffraction-limited resolution, and based on the acquired fluorescence intensity images.

The Bodipy-C_{12} fluorophore utilized here is a hydrophobic probe, and we expect it to stain the hydrophobic parts of the spores. Therefore, in dormant spores, we expect the inner and outer membrane and the coat to be stained with Bodipy-C_{12} [31]. We hypothesized that the viscosity-sensitive lifetime of Bodipy-C_{12} would allow us to differentiate these structures based on their respective viscosity/rigidity, in spite of the fact that their spacing was below the resolution of confocal microscopy.

The FLIM image of Bodipy-C_{12} obtained in wild type spores is shown in Fig. 2. The time-resolved fluorescence decay curves in each pixel of the image required biexponential fitting (Fig. 2a). As homogeneous samples, including organic solvents, model lipid bilayers and monolayers, and even cellular membranes, display monoexponential decays [20,22], we concluded that in dormant spores Bodipy-C_{12} was probing at least two environments of different viscosity, in every pixel of the image. This could be expected as the distance between two lipid membranes in the spore and protein core is below the resolution limit, e.g. see Fig. 1c for the TEM image of a dormant spore [32].

A lifetime histogram measured in wild type spores is shown in Fig. 2b: the shorter lifetime τ_2 is 1.1 ± 0.3 ns and the longer lifetime τ_1 is between 3 and 5.5 ns. While the histogram for τ_2 has a Gaussian distribution, the histogram of τ_1 is clearly asymmetric. By fitting the asymmetric histogram in Origin we could identify two separate peaks: 3.4 ± 0.2 ns and 4.2 ± 0.5 ns. In order to assign the observed lifetimes τ_1 and τ_2 to a particular spore layer and to observe how the

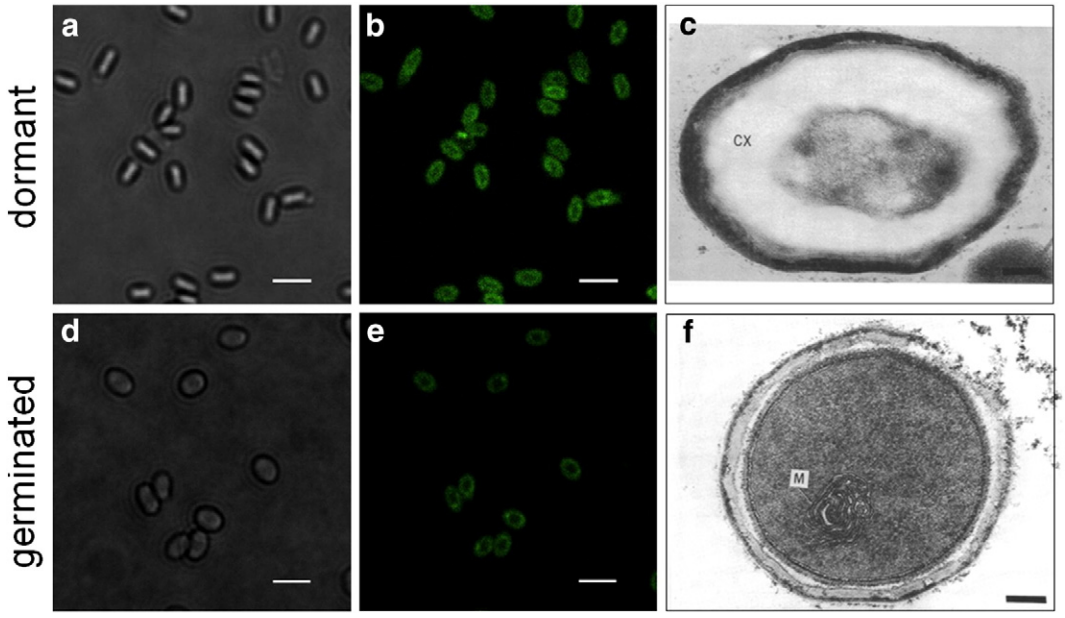

Fig. 1. Transmission (a, d) and fluorescence (b, e) images of dormant and germinated wild type spores stained during sporulation with 4.3 μM solution of Bodipy-C_{12}. Images were obtained by confocal microscopy with 488 nm excitation. TEM images (c, f) of dormant and germinated spores. Scale bars = 2 μm for a, b, d, e; 0.1 μm for c and f. (c) and (f) are reproduced with permission from [32] (original images Fig. 2a and e). Copyright American Society for Microbiology, 2013. Original legends: M, "Mesosome" (artifact); Cx, Cortex.

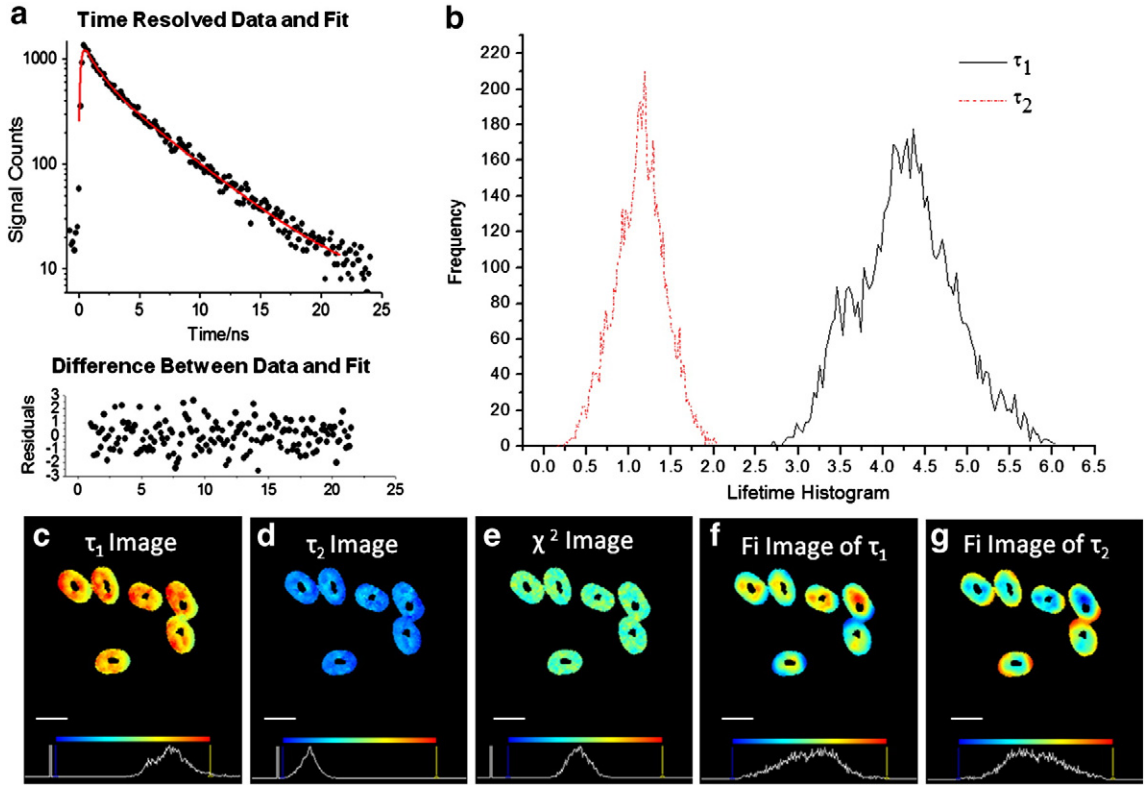

Fig. 2. Fluorescence lifetime image analysis of dormant wild type spores stained with 4.3 μM solution of Bodipy-C_{12}. (a) A typical time-resolved fluorescence decay curve, showing a biexponential decay; difference between data and fit is weighted by 1/sqrt fit. (b) A lifetime histogram of τ_1 and τ_2 and corresponding images of τ_1 (c), τ_2 (d), χ^2 (e) and fractional intensities (Fi) of τ_1 (f) and τ_2 (g). The false-color lifetime scale is from 0.2 to 5.6 ns, χ^2 scale from 0.2 to 2.0 and the fractional intensity scale from 0.2 to 0.8. Scale bar = 1 μm.

viscosity of the layers changed upon spore perturbation, we performed additional experiments and data analysis.

Firstly, to visualize the primary localization of τ_1 and τ_2 in dormant spores, we performed global lifetime analysis of the FLIM image. This protocol assumes that the lifetimes τ_1 and τ_2 are shared between all pixels in the image. Using this algorithm the fractional intensity images of τ_1 and τ_2 can be determined more precisely, as shown in Fig. 2f and g. The false color scheme corresponds to intensities between 20% and 80%. From these images it can be clearly seen that the shorter lifetime τ_2 has the highest intensity (65% — shown by the orange-to-red color in Fig. 2g) in the thin layer on the outside of the spore, and is low on the inside (35% — shown by the green-to-blue color in Fig. 2g). Conversely, the longer lifetime τ_1 has the highest intensity (65%) in the inner part of the spore (Fig. 2f). These lifetime images highlighted a significant degree of spatial offset between the two lifetimes in dormant spores and allowed the separation of different domain contributions by image analysis. It is important to emphasize that no separation of spore domains is possible by standard intensity imaging (*cf.* Fig. 1).

3.2. Separation of domain contributions by image analysis

Using the lifetime image obtained from a global analysis as a guide, we used the masking tool in the TRI2 software to divide each imaged wild type spore into two parts. Firstly, the outer part of the spore was isolated for analysis (Fig. 3b), which consisted of a thin rim of material, characterized by a green-blue color in Fig. 2f. Secondly, applying the information from the TEM image of a dormant spore (Fig. 1c), a separation line can be drawn anywhere within the cortex layer isolating the coat and the outer membrane from the inner membrane. Even though both parts should have some contribution from the cortex, it was anticipated to be only weakly stained by Bodipy-C_{12} due to its hydrophilic nature.

Fig. 3 shows maps of fractional intensities of the inside and outside of the wild type spores. It can be seen that the τ_2 values vary little between the inner and outer parts: 0.9 ± 0.4 ns in the outer part and 1.1 ± 0.4 ns in the central part. These values correspond to an average viscosity of *ca.* 80 cP, according to the Bodipy-C_{12} calibration [Eq. (2)].

At the same time, τ_1 showed significant differences: 3.3 ± 0.5 ns (the outer part) and 4.1 ± 0.5 ns (the inner part) ($p < 0.001$ according to the Mann–Whitney Rank Sum Test, with no equality of the variance). In the external part, the slow decay with τ_1 contributed 42% of the total intensity (blue color), while the fast decay with τ_2 contributed 58% of the total. On the contrary, in the inner part, the fractional intensity of τ_1 was significant in comparison to τ_2 (yellow-red color, 56% of the total decay), as shown in Fig. 3a and c. The partial masking of the spore allowed us to distinguish between the values of the longer lifetime τ_1 in the outer and the inner parts of the spore, 3.3 ns (725 cP) and 4.1 ns (1090 cP), respectively. In fact, the corresponding peaks could also be clearly seen on the τ_1 histogram of the whole spore (Fig. 2b) and this gave us the confidence to conclude that these differences were not an artifact of the fitting and/or masking.

3.3. Coatless spores and germination residues

In order to assist the assignment of viscosity values to a particular domain within the spores, we obtained FLIM images of chemically decoated wild type spores and coat residues formed as a result of a germination process, as well as of mutant spores *cotE gerE*. Persistent fluorescence of Bodipy-C_{12} after the decoating treatment confirms that the probe is firmly incorporated into the inner membrane. Fig. 4 provides the average τ_1 and τ_2 values measured in all these samples.

The decoating process used in our experiments is known to remove a large amount of coat proteins and also a very large part

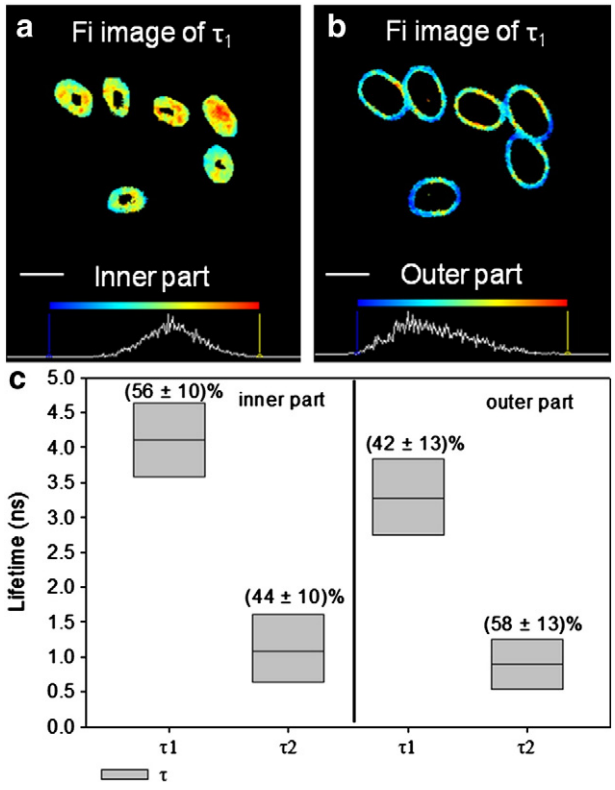

Fig. 3. Analysis of fluorescence lifetime image of dormant wild type spores stained with 4.3 μM solution of Bodipy-C_{12} by spatial masking of the inner and the outer parts of the spore. A circular binning of 2 was used. Fractional intensity (Fi) images obtained in the masked inner (a) and the outer (b) parts of the spore. (c) Average τ_1 and τ_2 values (*y* scale, with error bars shown as boxes) and the corresponding fractional intensities (shown as % in the graph) measured in the inner and the outer parts of the spore. Scale bar = 1 μm.

(if not all) of the outer membrane [33,34]. In the decoated spore sample, the fluorescence decays required fitting with a bi-exponential model. Here, the most significant lifetime contribution was from τ_1 (4.4 ± 0.6 ns) with an average fractional intensity of 66% (Fig. 4c).

Conversely, in the coat residues the dominant lifetime was τ_2 (72% of the total decay) with an average value of 0.7 ± 0.2 ns. This value is close to the dominant lifetime found in the masked outer part in dormant spores (Fig. 3c).

Finally, we imaged *cotE gerE*, the mutant spores known to lack most coat proteins except for a thin layer of insoluble coat material [26]. For *cotE gerE* spores a mono-exponential model was sufficient to fit the decay curves with good χ^2 = 1.1–1.2. The lifetime measured was 3.9 ± 0.2 ns, i.e. close to the τ_1 value of decoated spores and whole dormant spores. No shorter component of *ca.* 1 ns was detected for *cotE gerE*, providing strong evidence that this component originated from the protein coat, which is missing in *cotE gerE*.

These data allowed us to assign Bodipy-C_{12} lifetimes to particular structures within spores. In wild type decoated and *cotE gerE* spores, the hydrophobic structures remaining were the membranes, in particular the inner membrane, apart from a small amount of coat proteins. Thus, the matching and dominant τ_1 lifetimes (Fig. 4a) measured in dormant, decoated and *cotE gerE* spores were attributed to the inner membrane. The average lifetime value of 3.9–4.4 ns corresponds to a viscosity range between 990 and 1220 cP, which is consistent with the weak mobility suggested for this membrane by other workers investigating *Bacillus subtilis or Clostridium* spp. spores [7,35].

The shorter lifetime τ_2 was dominant in the wild type spore coat residues (72%) and in the outer masked part of the whole dormant spore (58%), but was missing in *cotE gerE* spores and had a minimal contribution in wild type decoated spores. On this basis we assign the τ_2 lifetime of Bodipy-C_{12} as residing in the spore coat. Finally, the τ_1 value of 3.4 ± 0.2 ns, detected as a shoulder on a histogram of whole dormant spores (Fig. 2b) and as a 42% contribution in the outer masked part of the spore (Fig. 3) most likely corresponded to the outer spore membrane. This contribution was absent in wild type decoated and *cotE gerE* spores. The viscosity of this membrane (725 cP) was significantly lower than that of the inner membrane.

We note that a shorter lifetime τ_2 (=1.4 ns) was still observed in decoated spores (34% of the total decay), and τ_2 (=1.1 ns) was observed in the inner masked part of the whole dormant spore (44% of the total decay). This lifetime is unlikely to be due to the cortex staining as no such lifetime was detected in the *cotE gerE* sample, which has an intact cortex. The masking process is of course not robust. Likewise, the decoating process is supposed to remove most of the outer membrane and a large amount of coat, however some proteins still remains and this could explain why even the inner part shows some contribution from τ_2.

3.4. Evolution of the spore environments during germination

We also examined how the viscosity of different spore compartments, in particular the inner membrane, changed during germination. For this purpose, stained wild type and *cotE gerE* spores were tested for germination in L-alanine. FLIM measurements were performed after 45 min of germination for wild type and after 1 hour for *cotE gerE* spores. At these germination times, both types of spores were in stage II, as reported previously [13]. This meant that the cortex was already degraded by enzymes and the coat had split open [13,32]. The TEM structure of the germinated wild type spore is shown in Fig. 1f.

In wild type spores, the probe visualized two environments using FLIM before germination, and still detected two fluorescent lifetimes after germination, τ_1 = 3.0 ± 0.4 ns (41%) and τ_2 = 0.9 ± 0.2 ns (59%). The principal component τ_2, assigned to the spore coat, was not modified by germination. On the contrary, the lifetime attributed to the membrane decreased to an average value of 3.0 ± 0.4 ns, equivalent to a viscosity of 610 cP (Fig. 5d). This significant reduction in viscosity is likely due to the shape changes that the inner membrane has to undergo during germination. The membrane is very compact in the dormant state and has to increase its area by *ca* 1.6 fold to follow the increase in volume of the core upon germination [7].

We have also recorded FLIM images of germinated mutant *cotE gerE* spores. For these spores a single fluorescence lifetime was recorded both before (Fig. 5a) and after germination (Fig. 5b). The average lifetime measured in *cotE gerE* spores changed from 3.9 ± 0.2 ns to 2.9 ± 0.1 ns as a result of germination, which corresponds to a viscosity decrease from 990 to 570 cP.

The lifetime values 2.9 and 3.0 ns, measured in germinated *cotE gerE* and wild type spores, respectively, were similar to the monoexponential lifetime measured in the membrane of vegetative cells, 2.7 ± 0.2 ns (Fig. 5c). Thus, during germination, the inner membrane transforms to acquire the viscoelastic properties of a vegetative cells membrane. This is in agreement with previous observation of how the inner membrane modifies during germination [7].

4. Discussion

Viscosity is a vital parameter that determines the rate of diffusion of species in a condensed media. In spite of its importance, very few viscosity values have been reported to date for bacterial cells. In *Escherichia coli*, spin labels inserted in the membrane's vesicles were used to measure viscosity [36], giving a value of 250 cP at 23 °C.

Using single-point FRAP [7] measurements of di-4-ANEPPS, it was previously demonstrated in dormant spores that the lipid mobility of the inner membrane is very low. Indeed, the fluorophore mobile fraction measured in dormant spores was 0.31 compared to 0.75 in

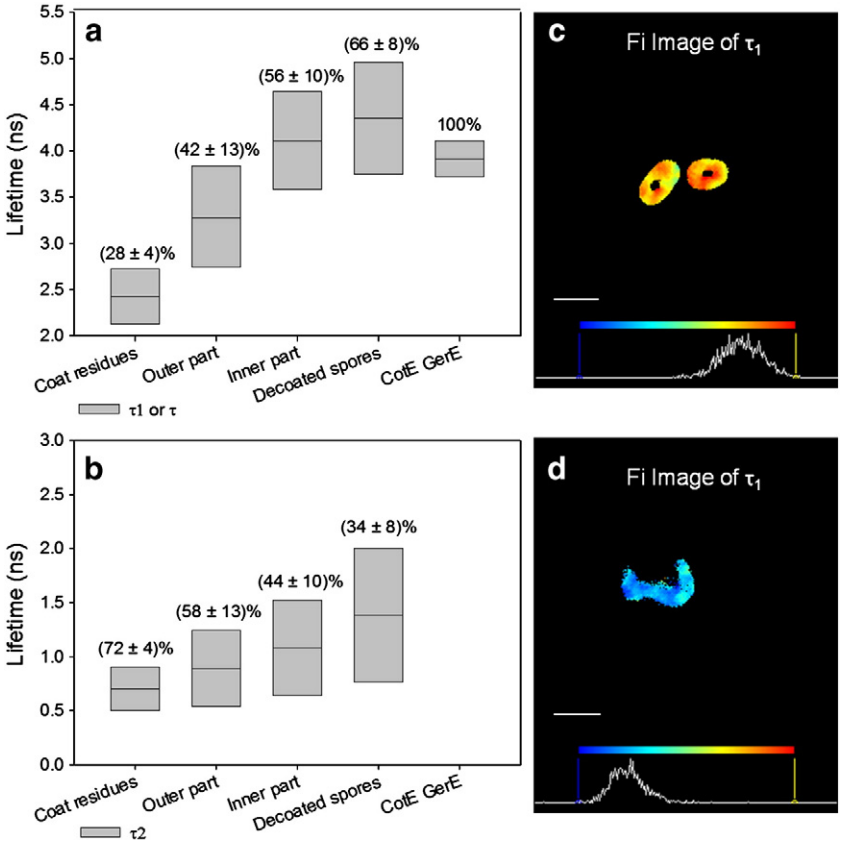

Fig. 4. Comparison of lifetime components obtained in wild type and *cotE gerE* spores stained with 4.3 μM solution of Bodipy-C_{12}. Average lifetimes τ_1 (a) and τ_2 (b) measured in various samples of wild type spores (inner and outer parts, chemically decoated and coat residues) and in *cotE gerE* spores; corresponding fractional intensities are shown as % in the graph. Fractional intensity images of τ_1 for decoated wild type spores (c) and coat residues (d), scale from 0.2 to 0.8. Scale bar = 1 μm.

germinated spores or vegetative cells. Moreover, this work suggested that the inner leaflet of this membrane could be partly in the gel phase. A recent study reported GP values of Laurdan in *Clostridium sporogenes* vegetative cells vs. dormant (or decoated) spores to be 0.31 and 0.77, respectively. These values again suggested a high degree of lipid order in the inner membrane of these spores [35].

Molecular rotors provide a new type of probe for imaging microviscosity in microbiological environments. The rotor used in this study is based on the highly fluorescent Bodipy structure that partitions in the hydrophobic and lipid-rich regions of the spore. We add the probe during sporulation, to ensure that it is well incorporated in the deepest hydrophobic regions of spores and, in particular, its inner membrane [7,37]. Importantly, the use of fluorescence lifetime to quantify viscosity makes it possible to separate and visualize the contributions from different domains inside whole spores. This provides significant advantages compared to intensity-based imaging as well as the single point measurements of diffusion coefficients, such as FRAP and FCS.

In wild type spores, fitting FLIM data required a biexponential model. We attributed the biexponential decays to the fact that Bodipy-C_{12} stains several domains that are below the resolution limit of the confocal microscopy (*ca.* 200 nm) and so every pixel in the image contains information from at least two spore domains. The long Bodipy-C_{12} lifetime of *ca.* 4 ns (1100 cP) was assigned to the inner membrane, thereby confirming its extremely high viscosity.

In wild type spores, the dominant Bodipy-C_{12} lifetime (τ_2) of *ca.* 1 ns (70–80 cP) was assigned to the coat. The coat represents a high fraction of the entire spore volume in dormant spores. According to the literature values the total coat thickness is about 77 nm, while a total spore "radius" is about 577 nm [38]. Approximating the spore as a sphere, the coat volume amounts to about 35% of the total spore volume while the coat proteins represent 50% to 80% of the total spore protein [39]. This could explain why τ_2 (assigned to the coat) was the dominant contribution in the FLIM images of wild type spores. Since the coat is not a homogeneous environment, we were not able to ensure whether 70–80 cP was a real value of solvent-like viscosity inside the protein network, or whether it was due to partial binding of the probe.

Alternatively, it is possible that the short lifetime originates from a small fraction of the Bodipy-C_{12} probe, localized in the very viscous inner membrane itself. We have measured the decays of Bodipy-C_{12} from model lipid bilayers above and below their phase transition temperature. While in the fluid disordered phase (above T_t) the decays were monoexponential, the decay became biexponential when the bilayer was in the gel phase, i.e. for Bodipy-C_{12} embedded in a DPPC bilayer at room temperature 1.5 ± 0.1 ns and 4.5 ± 0.3 ns decays (*ca.* 50:50 contribution) were measured. The values of lifetimes measured in various spores in this study were very similar to the above example, with τ_1 between 3.9 and 4.4 ns. Thus, the data indicate that the inner membrane of the *B. subtilis* spore might be in the gel rather than the fluid phase. The high viscosity values alone (990–1220 cP) are fully consistent with the gel phase hypothesis, which can go some way to explain the inner membrane's rigidity and impermeability to most chemicals.

In *cotE gerE* spores, a viscosity of 990 cP was attributed to the inner membrane. We note that in the literature, the inner membrane of this mutant is described as less impermeable than that of wild type spores [9], which is in agreement with our data.

We have demonstrated that the outer membrane of dormant wild type spores has a viscosity of about 725 cP. This value is slightly higher than measured in the membrane of vegetative cells. It indicates that this membrane is slightly more viscous than a classical membrane, which

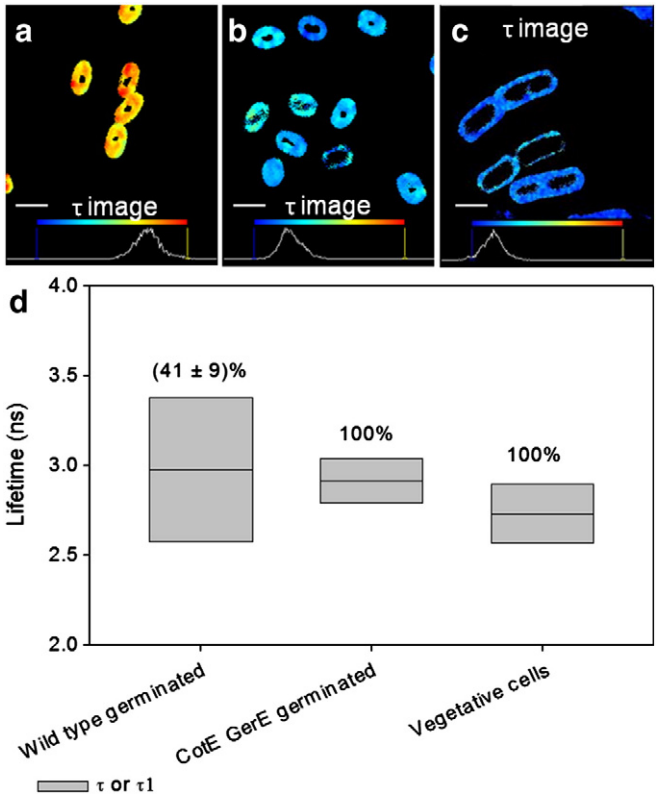

Fig. 5. The changes in lifetime upon germination of *B. subtilis* spores stained with 4.3 μM solution of Bodipy-C_{12}. Fluorescence lifetime images of *cotE gerE* spores before (a) or after 1 hour of germination in 10 mM L-alanine (b) and in vegetative cells (c). The false-color lifetime scale is from 2.3 to 4.65 ns. (d) Fluorescence lifetimes (τ_1 for biexponential decay in wild type spores) measured in various spore samples; Corresponding fractional intensities are shown as % in the graph. Scale bar = 1 μm.

could be due to its localization between the coat and the cortex, adding extra rigidity to the structure. However, the outer membrane is not thought to be a significant permeability barrier [40], which is consistent with the lower viscosity value measured by Bodipy-C_{12}. We were not able to detect a specific lifetime related to the outer membrane in mutant *cotE gerE* spores.

During germination, the inner membrane of the spore becomes the cell membrane of the outgrowing cell [11]. Thus, it loses its special characteristics of low permeability and rigidity and acquires features of the future plasma membrane of vegetative cell. Our FLIM data on germinated spores (stage II) incubated with Bodipy-C_{12} demonstrate that the viscosity of the inner membrane becomes 600 cP and 570 cP in wild type and *cotE gerE* spores respectively, which is a viscosity reduction of approximately a half, in excellent agreement with the 2-fold increase in mobility measured upon germination by FRAP [7].

5. Conclusions

Lifetime-based viscosity determination using molecular rotors is a very promising technique for measuring the rigidity of different layers in spores, where the concentration of a fluorophore introduced during sporulation is very difficult to estimate. FLIM microscopy allows the measurement of microviscosity without any of the problems associated with unknown probe concentration. For bacterial spores, this presented a real advantage when studying the internal layers of whole unmodified spores. Our data demonstrate the power of FLIM imaging using molecular rotors for the non-destructive yet detailed study of bacterial spores in their natural environment. Our future studies will concentrate on establishing how the viscoelastic properties of the hydrophobic domains in bacterial spores respond to environmental stress, such as high pressure and temperature and administration of toxic chemicals which are known to alter membrane properties.

Competing financial interests

The authors declare no competing financial interests.

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