Title: Effect of ethanol perturbation on viscosity and permeability of an inner membrane in *Bacillus subtilis* spores

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Abstract

In this work, we investigated how a combination of ethanol and high temperature (70 °C), affect the properties of the inner membrane of *Bacillus subtilis* spores. We observed membrane permeabilization for ethanol concentrations ≥ 50%, as indicated by the staining of the spores' DNA by the cell impermeable dye Propidium Iodide. The loss of membrane integrity was also confirmed by a decrease in the peak corresponding to dipicolinic acid using infrared spectroscopy. Finally, the spore refractivity (as measured by phase contrast microscopy) was decreased after the ethanol-heat treatment, suggesting a partial rehydration of the protoplast. Previously we have used fluorescent lifetime imaging microscopy (FLIM) combined with the fluorescent molecular rotor Bodipy-C_{12} to study the microscopic viscosity in the inner membrane of *Bacillus subtilis* spores, and showed that at normal conditions it is characterized by a very high viscosity. Here we demonstrate that the ethanol/high temperature treatment led to a decrease of the viscosity of the inner membrane, from 1000 cP to 860 cP for wild type spores at 50% of ethanol. Altogether, our present work confirms the deleterious effect of ethanol on the structure of *Bacillus subtilis* spores, as well as demonstrates the ability of FLIM - Bodipy-C_{12} to measure changes in the microviscosity of the spores upon perturbation.

Keywords: *Bacillus subtilis* spores, ethanol perturbation, inner membrane, permeabilization, Fluorescence Lifetime Imaging (FLIM), microviscosity decrease.

1. Introduction

Some bacteria, when faced with unfavorable conditions, such as starvation of nutrients, can undergo a special and energy consuming process termed sporulation [1,2]. Spores formed in such a way are an extremely resistant microbial form with unique properties. Bacterial spores differ from vegetative cells by their shape and structure and their enzymatic pool and are characterized by a strong resistance to physical and chemical agents which allow spores to survive in extreme conditions of pH, temperature (e.g. heat shock up to 140°C) or high pressure (up to 1 GPa) [3,4]. Spores are also able to germinate after very long periods of dormancy [5].

There are several known factors that might be responsible for the spores' resistance to various types of stress. For example, the low level of hydration of the spores' core is known to act in resistance to wet heat and to γ and UV radiation [6,7]; a high level of dipicolinic acid (DPA), located in the core is thought to have a role in resistance to wet heat or to hydrogen peroxide [7,8]; and the spores' DNA protection by the small acid - soluble proteins (SASPs) plays a role in protection against wet and dry heat, UV radiation or chemicals such as hydrogen peroxide [6,9–11]. However, the composition of spores is not the only factor responsible for its extreme resistance, and the structure itself is thought to play a role in protection from chemicals and physical stress [12]. For example, a weak permeability of the inner membrane might prevent or slow down the entry of chemical agents deleterious to DNA [13] and the proteinaceous coat should protect spores from digestion by bacterivores as well as against some chemicals, such as iodine or oxidative agents [14–16].

Bacterial spores are resistant to numerous decontamination processes and can be responsible for various diseases or food spoilage and alteration, if they are not fully inactivated. Alcohols are chemicals that are often efficiently used for disinfection, most often at a concentration of 70% in an aqueous solution [17]. Such solutions are known to be efficient against a range of

microorganisms such as vegetative cells, viruses, and moulds [18], however, are not sporicidal. Indeed, ethanol at 90% was shown to permanently inactivate spores but a long period of contact was needed (at least 2 months, depending on the species) [19]. At low concentrations, alcohols can prevent spores' germination but in a reversible way [20]. At the same time ethanol was identified as one of the few chemicals that significantly affects spore's permeabilization, while preserving the structural integrity of the spore. It is also known that 70% ethanol can inactivate bacterial spores if combined with elevated temperatures, which together lead to permeabilization [21]. Indeed it was hypothesised that one of the major spore's permeability barriers, the inner membrane, was affected by ethanol [21,22]. However, the direct evidence for the mechanistic details of the permeabilization process and the effect of permeabilization on the structure of the inner spore's membrane is not as yet available.

We recently established a method to study the microviscosity of the spore's inner membrane, utilising fluorescence lifetime imaging microscopy (FLIM) of a viscosity sensitive fluorophore termed 'molecular rotor' [23]. The hydrophobic dye Bodipy-C_{12} showed a well characterized response of its fluorescence lifetime to viscosity [24]. Additionally, it was shown to incorporate in lipid-rich structures and allowed the viscosity measurements in a range of membrane-like structures, including lipid monolayers [25], model bilayers [26,27] and live cells [28–30] in a temperature-independent manner [31]. We have recently utilized this method to non-invasively determine the viscosity of the membranous regions of *Bacillus subtilis* bacterial spores [23]. The relevant findings of this study are summarised in Fig. 1. It is clear that in wild type spores two types of environment were observed: characterized by a short lifetime (assigned to the proteinaceous coat) and a long lifetime of ca. 4 ns which was assigned to the outer and inner membranes. The outer membrane is functional during the development of the forespore but not in the dormant spore [32]. It has been previously shown using *Bacillus megaterium* spores that the outer membrane has a different lipid and protein

composition from the inner membrane [33] and this is consistent with our lower viscosity value obtained for this membrane in our earlier work [23]. It has been shown that the outer membrane appears to lose its integrity and might not be an important barrier to permeability [7]. In mutant *cotE gerE* spores that lack the coat only one environment was observed, with 4 ns lifetime, and assigned to the inner spore's membrane, Fig. 1. This lifetime value corresponds to an extremely high viscosity exceeding 1000 cP, and indicates that this lipid bilayer is likely present in a gel phase. In the current work we propose to extend our new method to the studies of modifications of the spores' inner membrane caused by ethanol treatment. We study the combined effect of several ethanol concentrations (30, 50 and 70%) and raised temperature (70°C) on *Bacillus subtilis* spores. The FLIM studies are complemented by permeability assays as well as infrared spectroscopy, to assess the health and structural features of the spores after each type of treatment.

2. Material and methods:

2.1 Spores production

The *Bacillus subtilis* strains used in this work were either the wild type PS533 or the coat deficient strain PS4150 in which most of the *cotE* and *gerE* coding sequences are deleted [15]. PS533 and PS4150 (both from the Department of Molecular, Microbial, and Structural Biology, University of Connecticut Health Center, USA) are the derivatives of isogenic strain PS832, a prototrophic derivative of a strain 168. PS533 carries a plasmid pUB110 encoding resistance to kanamycin [34]. Spores were prepared at 37°C in 2 x Schaeffer's-glucose liquid medium. Pre-warmed medium was inoculated with the preculture at a ratio of 1:10 in a baffled flask. When sufficient sporulation was achieved, spores were harvested, washed 4 times with cold distilled water and stocked at 4°C until further use. Purification of spores was performed by washing with water for several days as previously described [1]. 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 Histodenz purification has been done as follows. After harvesting, spores pellets were suspended in 100 to 200 µl of 20 % Histodenz then layered on 1 to 2mL of a 50 % Histodenz solution and centrifuged for 45 min at 14 000 ***g.*** The centrifugations pellets were then extensively washed to remove Histodenz and spores were stocked at 4°C [35].

2.2 Ethanol perturbations and viability measurement:

Spores at an OD_{600nm} of ~1 were treated for 1h at 70°C with ethanol aqueous mixtures containing 30, 50 or 70 % ethanol. Immediately following this perturbation, the ethanol solutions containing spores were diluted 5-fold with cold distilled water (4 °C) and kept on ice for 5 min before use. Cold spores suspensions obtained this way were directly used for decimal dilution and viability assessments by Colony Forming Unit (CFU) method. Numeration was done after culturing the spores overnight on BCP (Dextrose Tryptone agar, BIOKAR Diagnostics, Allone, Beauvais Cedex, France) plates kept at 37 °C. Each perturbation has been done at least in triplicate on two independent spore productions.

2.3 Permeability assessment (Propidium Iodide staining):

The % of permeabilized spores was estimated as follows. Spores' suspensions obtained after ethanol treatment was washed 3 times with distilled water and centrifuged for 15 min at 4000 *g.* The washed spores were incubated with Propidium Iodide (PI, Sigma Aldrich 4µg/ml in water) for 15 min and counted using confocal microscopy. The permeabilized spores displayed red fluorescence of PI from the protoplast. For each treatment or control, at least 100 spores were observed.

2.4 Staining with Bodipy C_{12}

The molecular rotor Bodipy-C_{12} was synthesized as previously described [29]. Spores were stained during sporulation as previously described [23]. Briefly, 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.

Alternatively, to investigate modifications due to ethanol treatment, Bodipy-C_{12} was added at a concentration of 0.43 µM on control or treated suspensions of spores washed as described previously. Spores were left in contact with the probe for *ca* 3 hours. The spores showing inner staining (corresponding to the inner membrane) were counted as a percentage and FLIM images were recorded in order to determine the viscosity of the inner membrane.

2.5 Microscopy and FLIM analysis:

The transmission and fluorescence images of stained spores were collected 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). An argon laser at 488nm has been used for excitation and emission was collected at 550-700 nm for PI and 500-600 nm for Bodipy-C_{12}.

The FLIM experiments were performed as described previously [23]. Briefly, an aliquot of spore's 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 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 256x256 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 128 ps/channel). FLIM images were analyzed using TRI2 software version 2.4.4.1 (Gray Institute, Oxford [36]). In order to obtain about 1000 counts in the pixel peak maxima, circular binning of 4 was used for analysis of the different wild type spore samples, whereas a circular binning of 2 or 3 was used for *cotE gerE* spores. We also used a signal threshold between 10-20% and 100% and mask tools to avoid noise and collecting signal from weakly stained internal parts of spores (the spore's core). Wild type spores were analyzed using a bi-exponential fitting (Marquardt algorithm) for each pixel of the decay curves whereas *cotE gerE* spores were analyzed with a monoexponential fitting. To study ethanol perturbations, only spores showing deep inner staining were taken into account. 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.

To investigate perturbations suffered by inner membrane upon ethanol exposure, only the long lifetime, τ_1, was considered in the case of wild type spores. The lifetime histograms were analyzed using Origin software (Origin Pro 8, Origin Lab). The "Fit multiple peaks" tool was used to determine the position of peaks' maxima as well as the area and the FWHM of the curve. FWHM/2 served to determine the average error bar (the spread) of each lifetime whereas the area under the curve was used to obtain the relative proportion of each peak.

Viscosity values were obtained from lifetime measurements of Bodipy-C_{12} using the following equation [28] :

$$\log \tau = -0.75614 + \log \eta * 0.45691 \qquad (1)$$

where τ_f is the lifetime of Bodipy-C_{12} in ns 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's environment. Statistical analysis have been performed using the software Sigma-plot in order to compare the lifetime values obtained with different perturbations.

2.6 Infrared spectroscopy:

Fourier transform infrared experiments were performed on a IFS Vector 22 spectrometer (Bruker, Germany) in attenuated total reflectance (ATR) mode. A drop of untreated or treated spores (10 µl) at an OD_{600nm} of about 10 was placed on the ZnSe crystal and dried under nitrogen flux for 5-10 min in order to obtain a homogeneous film. Spectra were obtained by recording 10 scans at a resolution of 2 cm^{-1} at room temperature. Prior to each measurement a background (on air) was acquired to subtract the noise. Spectra were acquired and analyzed using the OPUS software (Bruker, Germany).. Using OPUS software, spectra were baseline corrected and normalized. Then, peak position and relative intensities were obtained using the peak picking tool using the 2^{nd} derivatives method. The β/α ratio of proteins were then obtained by dividing the intensity of the peak at 1615-1618 cm^{-1} for control or 1627-1628 for treated samples (for β structure) by the peak intensity at 1655-1660 cm^{-1} (for α structure).

3. Results:

3.1 Inactivation and permeabilization of spores by ethanol treatment.

Viability assessment and IP staining

The treatment of spores with 30, 50 and 70% of aqueous ethanol solutions at 70°C for 1h was used to perturb the spores. Following this treatment we attempted to identify the structures targeted by ethanol. The percentage of spores' survival was measured by the CFU method and compared to the percentage of permeabilised spores, as measured by propidium iodide (PI)

assay, Fig. 2a. Our results indicate that the ethanol treatment at 30% resulted in small amount of inactivation since it was associated with 36% of spores' survival (0.4 log of inactivation) for wild type spores and to 45 % (0.3 log of inactivation) for coatless *cotE gerE* spores. On the contrary, treatment with 50 and 70 % ethanol resulted in high levels of spore killing with 1.3 log and 2.7 log of destruction (wild type spores) or 1.1 and 2.1 log of destruction (*cotE gerE* spores), respectively. We note that at a temperature of 70°C, a 70% ethanol treatment for 1 hour induces higher inactivation of wild type spores than a 2 hour treatment at 65 °C, for which a 1.7 log of destruction was previously reported [21]. Thus, an increase of 5 °C in the treatment temperature appears to be sufficient to improve the inactivation, even for shorter treatment times.

cotE gerE spores showed a slightly higher level of resistance than wild type spores. This apparent higher resistance of mutant spores is not understood at present, however, this finding suggests that the coat does not play any significant role in protecting against ethanol as was already previously observed for decoated wild type spores [21].

PI is a nucleic acid fluorescent stain known to permeate cells with membranes that lost their integrity. In our experiments, Fig. 2a, inactivation of spores and the loss of membrane integrity as detected by PI appeared to correlate well for each ethanol concentration used, with the percentage of spores' survival (CFU) and impermeable cells (PI) being close. From this data we can assume that inactivation of a spore corresponds to increased permeability of a spore's membrane (at least to PI). These results offer further evidence that the inactivation by ethanol is related in part to the loss of the integrity of a spore's membrane as previously hypothesized [21,22].

DPA loss followed by infrared spectroscopy

We used also ATR FTIR spectroscopy to investigate chemical changes within the spore's core upon ethanol treatment. Dipicolinic acid or DPA is an important compound located within the

spore's core, that is thought to be responsible in part for spore's resistance to external chemical stress. Here we have utilized infrared spectroscopy to follow the concentration changes of DPA in wild type spores upon treatment with ethanol. DPA can be visualized in the infrared by its vibration at 1279 cm^{-1} , the vibration at about 1240 cm^{-1} can be attributed to the antisymmetric stretching vibration of a phosphate group PO_2^- [37,38]. As can be seen from Fig. 3a, ethanol treatment is associated with a loss in DPA content, and the loss is more pronounced upon increase in ethanol concentration from 30 to 70%. Similarly, the intensity of a shoulder band at 1570 cm^{-1}, (data not shown), also associated with the DPA structure [39–41] shows steady decrease upon ethanol treatment. A similar loss of a DPA vibration in the infrared spectrum has also been observed for the treatment of *cotE gerE* spores with 70% ethanol (data not shown).

Loss of refractivity

Modifications in spore content have also been investigated by the loss of refractivity as observed by phase contrast microscopy (Fig. 3b and c). Dormant spores are known to appear phase bright, whereas fully germinated spores are associated with a phase dark state [42]. For germinated spores, the loss of refractivity is associated with full DPA release and protoplast hydration [43]. It could be seen that an increase in ethanol concentration is associated with an increase of the number of wild type and *cotE gerE* spores that appear phase grey (Fig. 3b). According to our data, with 30% of ethanol, ~72 % of spores appeared phase bright for wild type spores (52 % for *cotE gerE* spores) whereas treatment with 70% ethanol led to ~38 % spores appearing phase bright (20 % for *cotE gerE* spores). *cotE gerE* spores appeared to show a more pronounced protoplast hydration since the occurrence of phase grey spores was higher than for wild type spores (Fig. 3b). It is clear from our data that under experimental conditions used here the hydration was not complete since spores were phase-grey rather than phase dark. Thus, these results also suggest a significant change in the permeability of the

inner membrane, the main permeability barrier, especially for concentrations of 50 and 70 % of ethanol. Out of spores that conserved their integrity, the % of spores appearing phase bright is higher than the % of viable spore as measured by PI permeability/CFU (Fig. 2a). This is likely due to the presence of inactivated spores for which permeability barrier is not significantly modified.

3.2 Proteins modifications of spores monitored with infrared spectroscopy.

We also recorded changes upon ethanol treatment in the infrared spectra of whole wild type spores in the spectral region associated with protein secondary structure. As can be seen, 70% ethanol treatment leads to a significant modification of the Amide I protein band, Fig. 4a. Firstly, a shift of the peak corresponding to the alpha helical structure from 1655 to 1658 cm^{-1} is apparent. Secondly, the spectrum shows an emergence of a peak at 1628 cm^{-1} that corresponds to a beta sheet structure. On average, the "β/α" ratio is about 0.2 in untreated wild type spores which shifts to 2.4 upon 70% ethanol treatment. We hypothesised that these modifications are the results of a change in the secondary structure of proteins due to denaturation, and are similar to what was previously observed for autoclaved spores [41].

Coatless *cotE gerE* spores treated with 70% ethanol were also investigated by infrared spectroscopy (Fig. 4b). In this case, almost no changes to the infrared spectra were observed upon treatment, with a very small shift of the peak corresponding to alpha helical structure from 1655 to 1656 cm^{-1} and nearly no beta sheet structure apparent after the treatment. A β/α ratio changed from 0.1 to 0.2 for untreated and treated spores, respectively. This data indicates that the main protein modifications brought about by ethanol treatment occur in the spore's coat. We note that the spore's coat does not appear to play a role in spore resistance to ethanol perturbation, as can be seen comparing *cotE gerE* and wild type spores' resistance to ethanol (Fig. 2a) and as was already documented by others [21].

3.3 Viscosity of the inner membrane:

We have shown above that the inner membrane, known to be an important permeability barrier, is likely to be affected by the ethanol treatment. In order to investigate more precisely the modifications suffered by this membrane, we have used a microscopic technique for measuring membrane microviscosity that we have previously used for determining the viscosity in the inner membrane of *Bacillus subtilis* spores [23]. In these experiments fluorescence lifetime imaging (FLIM) was combined with a viscosity-sensitive fluorophore termed molecular rotor that was incorporated in a membrane of interest. The fluorescence lifetime of this probe can then be determined and directly correlated to the viscosity of the membrane domains, thus creating spatially resolved images of microscopic viscosity. We have used this technique to measure the inner membrane viscosity of wild type and *cotE gerE Bacillus subtilis* spores and shown its potential to follow changes in membrane properties during germination [23].

Staining of spores by Bodipy-C_{12}

In order to measure microscopic viscosity of spores, the molecular rotor Bodipy-C_{12} must be delivered to the inner membrane. Staining of spores with Bodipy-C_{12} was performed after ethanol treatment in order to ensure that the molecular rotor itself remained functional and was not affected by ethanol treatment at a high temperature. Confocal microscopy (Fig. 5) showed that untreated wild type or *cotE gerE* spores are not permeable to the hydrophobic probe Bodipy-C_{12}. Wild type spores show only a peripheral staining (Fig. 5b), with only about 2 % showing deeper staining, contrary to wild type spores stained during sporulation (Fig. 5a, 100%). At the same time, *cotE gerE* spores showed no staining with Bodipy-C_{12} when stained after sporulation (Fig. 5b, overlay of transmission and fluorescence images, the lack of staining is apparent from the lack of green colour around individual spores). The lack of

fluorescence for *cotE gerE* points at the fact that the coat and possibly the outer membrane are involved in peripheral fluorescent staining for the wild type spores observed in Fig. 5b.

However, as shown previously (Fig. 2) ethanol treatment leads to an alteration in spore's structure and permeability, *e.g.* PI was able to stain DNA after the treatment. In a similar way, the hydrophobic probe Bodipy-C_{12}, seems to incorporate into deeper structures within the pore following the enthanol treatment. Figure S1 (Supplementary material) shows the Image J analysis of the spores' cross sections that allowed us to unequivocally distinguish between deep and peripherally stained spores. The deep stained spores (likely showing the inner membrane staining) are shown in Fig. 5c, d and e. However, differences were observed depending on the treatment severity and the strain used. After a treatment at a concentration of ethanol of 30%, a deeper structure staining (probably the inner membrane) could be visualised by the molecular rotor (Fig. 5c), with about 47 % and 63 % of wild type and *cotE gerE* spores being deep-stained, respectively. These values were slightly higher than a number of spores stained with PI under identical conditions (about 37 % and 40 %, respectively), Fig. 2b. This difference is not surprising since PI and Bodipy-C_{12} dyes stain completely different structures (DNA and the lipid membrane, respectively). This intermediate value of staining obtained with 30% of ethanol is most likely due to the fact that some spores are not affected by the ethanol perturbation and therefore not permeabilised (as previously seen with PI).

The concentration of ethanol of 50% gives an optimal Bodipy-C_{12} uptake in wild type spores, Fig. 5d. At these conditions about 46 % of spores show deep staining. In the case of *cotE gerE* at 50 and 70% ethanol almost all of the spores were stained with Bodipy-C_{12} (97%).

However, 70% of ethanol (Fig. 5e) led to a low percentage of wild type spores being stained with Bodipy-C_{12} (3.6%), showing mainly a peripheral staining in a similar manner to untreated spores (Fig. 5b). *cotE gerE* spores were well stained with Bodipy-C_{12} at these conditions (~ 97.1 %). Thus, it seems that for wild type spores the inner membrane cannot be

stained at this ethanol percentage. Given the data above we hypothesize that for ethanol concentration above 50% the membrane of wild type spores becomes too damaged to retain Bodipy-C_{12} efficiently thus no deep-staining was observed. Moreover, *cotE gerE* spores are consistently more resilient to this treatment, Figure 2a. It is possible that this resilience means that their inner membrane is less damaged and more stainable with Bodipy-C_{12} compared to wild type spores.

Fluorescence lifetime measurements.

Following successful staining of ethanol treated spores with Bodipy-C_{12}, we have recorded fluorescence lifetime (FLIM) maps of wild type and *cotE gerE* spores in order to determine the viscosity of their inner membranes as a function of perturbation with ethanol. Only the deep-stained spores (showing the inner-membrane staining) were considered and analyzed for viscosity, unless otherwise indicated.

We have initially considered the viscosity of the membrane within coatless *cotE gerE* spores. These spores exhibit monoexponential lifetime of Bodipy-C_{12}, that was previously assigned to the inner membrane of the spore [23], and hence the data should be straightforward to interpret. Also, a good percentage of deeply permeabilised spores was detected at all ethanol concentrations, from 63% deep staining (at 30% ethanol) to 97% deep staining (at 70% ethanol), Fig. 5.

The fluorescence lifetimes of Bodipy-C_{12} recorded from ethanol-untreated *cotE gerE* spores (that were stained during sporulation) gives a Gaussian shape distribution with the peak at 3.8 ns (Fig. 6c), consistent with our previous study [23]. Upon ethanol treatment, the fluorescence decays remain monoexponential, however, the peak of a lifetime histogram shifts to lower values, which corresponds to lower viscosities of the membrane environment. This can also be seen as a colour change in FLIM images, Fig. 6b, which show a clear shift from

yellow/orange to blue/green colour upon ethanol treatment. For all conditions of ethanol treatments (from 30-70%) the characteristic lifetime (and the distribution of lifetimes) remains roughly the same at (3.0 ± 0.2) ns, which is significantly shorter (see statistical analysis in Supplementary material) than the (3.8 ± 0.3) ns observed for unperturbed spores. Thus we can assume that inactivated spores have a much less viscous inner membrane and an increase in ethanol percentage above 30% during the treatment does not change the viscosity of the inner membrane of the treated spores.

The FLIM data analysis was somewhat more complex for wild type spores, Fig. 7. We have previously demonstrated that untreated spores show a biexponential fluorescence decay [23], with long lifetime τ_1 assigned to both the inner and the outer membranes, while much shorter τ_2 (that was absent in *cotE gerE* spores) assigned to the coat. We will only consider τ_1 in the present work.

Consistent with our previous study [23] the histogram for τ_1 in wild type spores showed two peaks, assigned to the inner (~ 4.2 ns) and outer (~3.3 ns) membranes. The two peaks in the τ_1 histograms were conserved even upon ethanol treatment, Fig. 7c, except for the histogram recorded for 70% ethanol treatment conditions. Here it is important to note that for 0-50% ethanol treatment conditions FLIM images were recorded only for spores that showed deep inner staining. For 70% ethanol treatment of wild type spores there were almost no deep-stained spores observed (Fig. 5) and therefore only peripherally stained spores were analyzed. Consequently, the spore sample treated with 70% ethanol showed a single τ_1 histogram peak in FLIM, at ca. 3.2 ns that most likely corresponded to Bodipy-C_{12} residing in an outer membrane of treated wild type spores. We can consider that the lifetime value obtained from the 70% ethanol treated sample corresponds to the outer membrane staining only. Thus the 70% ethanol sample provides the viscosity value of the outer membrane and this assignment is fully consistent with our analysis.

We have recorded FLIM images and plotted τ_1 histograms for wild type spores treated with 0-70% ethanol, Fig. 7c. From the histogram data, it is easy to follow the evolution of both peaks of τ_1 as well as the relative contribution of each peak (measured as the area under each fitted curve), Fig. 7d. It is clear that following an increase of percentage of ethanol, a decrease in the mean τ_1 value was observed. This shift was accompanied by a colour change in FLIM images (Fig. 7b), with blue colour becoming very prominent on the outside of the spore. The analysis of the individual peaks (using multi-peak Gaussian fitting of the histograms), also revealed that the 'inner membrane' contribution to τ_1 value changed from 4.2 ± 0.5 ns (untreated) to 4.1 ± 0.5 ns (30% ethanol) to 3.8 ± 0.5 ns (50% ethanol). The difference between untreated and 50% ethanol was shown to be significant (see statistical analysis in the Supplementary material). The inner membrane contribution decreased steadily, from 84% (untreated) to 72 and 62% (for 30 and 50% ethanol) and completely disappeared at 70% ethanol, where we believe only the outer membrane was stained by Bodipy-C_{12}.

The lower τ_1 peak value that was thought to be associated with the outer-membrane remained almost unchanged (see statistical analysis in the Supplementary material) for all ethanol treatments (3.3 ± 0.2 ns for 0 and 30% ethanol, 3.0 ± 0.3 ns for 50 % ethanol and 3.2 ± 0.5 for 70% ethanol). Upon ethanol perturbation we observed the most significant changes in the lifetime value associated with the inner membrane staining. A small change in the lifetime associated with the outer membrane staining can also be present, Figure 7. However, the peak attributed to the outer membrane appears as a shoulder on the lifetime histogram thus it is more difficult to determine this value precisely and according to out statistical analysis (Supplementary material) the differences are not significant.

Thus, our results suggest that inner membrane viscosity was reduced upon ethanol treatment, for both wild-type and *cotE gerE* spores. The outer membrane staining was observed only for wild type spores (consistent with [23]) and its viscosity remained largely unchanged upon

ethanol treatment, however, the contribution from Bodipy-C_{12} fluorescence coming from the outer membrane increased dramatically with increasing ethanol content of the treatment. This might indicate that upon disruption of the inner membrane environment, the molecular rotor was dislodged from its original preferred location in the inner membrane and was consequently relocated to another hydrophobic lipid structure, the outer membrane. The outer membrane might have remained largely intact and was still suitable for Bodipy-C_{12} incorporation, even upon ethanol treatment.

4. Discussion

In this work we have observed significant changes in *Bacillus subtilis* survival and permeability to chemicals upon treatment with ethanol at high temperature. In order to understand the role of various spore's structures in the ethanol-induced modifications we have employed infrared spectroscopy to monitor the changes to DPA and protein content of the spore. We have also utilized fluorescence lifetime imaging using molecular rotors in order to quantitatively investigate the microscopic structure of lipid membranes within the spores. We have compared the above results between wild type spores and *cotE gerE* spores that lack the proteinaceous coat.

The comparison between wild type and *cotE gerE* spores using infrared spectroscopy demonstrated that ethanol significantly changes the secondary structure of proteins in the spore's coat. This observation is not surprising since it was previously observed that high ethanol concentration (80% aqueous ethanol) increases the observed percentage of beta sheet structure in model proteins from gastric mucosa (e.g. lysozyme) [44,45]. This increase has been attributed to protein aggregation [46,47]. It was previously reported that bactericidal activity of alcohols is likely due to their ability to denature proteins [48]. However, our data

(Fig. 2) that is consistent with existing literature [21] clearly demonstrated that the lack of protein coat does not lead to a decrease in spores' survival or, indeed, to an increased penetrability. Thus the coat can be discarded as a major factor in spore's resistance to ethanol at high temperatures.

We have next considered the effect of ethanol treatment on the permeability of various membranes in *Bacillus subtilis.* This was probed in several ways, namely, (i) by detecting the staining of the spore's DNA with Propidium Iodide, that is only feasible when the membranes lose their integrity; (ii) by detecting the depletion in DPA content using infrared spectroscopy, since this is only possible when the membrane is too porous to retain DPA or if ethanol treatment modifies or stimulates a DPA release channel [49] and (iii) by detecting the loss in spore's refractivity which is associated with water uptake by the core. In all cases we found that both wild type and *cotE gerE* spores exhibit evidence for increased membrane porosity with increased percentage of ethanol during treatment at high temperature.

Finally we have measured the microscopic viscosity of the membranes of *Bacillus subtilis* using fluorescence lifetime imaging of a viscosity-sensitive fluorophore termed molecular rotor that was able to provide a direct measure of rigidity in their immediate environment. Ethanol is known to induce membrane perturbations of microorganisms [50]. Previous work has shown that ethanol incorporation is associated with a decrease in lipid order of model membranes and could affect the permeability of membranes to ions and fluorescent dyes as well as other diffusing species [51–53], consistent with our observations above. Our imaging results using molecular rotors indicate that ethanol treatment is associated with general decrease in viscosity of the inner membrane of *Bacillus subtilis*.

It is important to note that based on our data for wild type spores, both the inner and the outer membrane of the spore can be stained by the rotor, and they show distinct viscosities of ca

1000 and 600 cP, Fig. 8. These numbers were converted using equation (1) from the detected fluorescence lifetimes of 4.2 and 3.3 ns. Ethanol treatment evidently decreased the viscosity of the inner membrane to 840 cP (3.8 ns) for 50 % ethanol, while leaving the viscosity of the outer membrane unchanged (Fig. 8).

In coatless *cotE gerE* spores only the inner membrane staining was observed, with viscosity of 860 cP (3.8 ns lifetime). Similarly to the wild type spores, 30 and 50% ethanol treatment significantly decreases viscosity of *cotE gerE* to about 500 cP (3 ns lifetime), Fig. 8. It should be noted that this value is very close to what was previously measured for the membranes of germinated *cotE gerE* spores [23], suggesting that these membranes have similar rigidity/permeability.

The effect of ethanol on membranes could be rationalized on the basis of its effect on water activity [54]. Water plays an extremely important role in the organization of the membrane bilayer and ethanol acts as a depressor of water activity. Slater *et al*., have shown that ethanol disrupts the role of the intra-membrane water and its inclusion leads to a decrease in a bilayer transition temperature [55].

The inner membrane of bacterial spores has been suggested to be in part in a gel state [23,56,57]. Furthermore, according to our previous data, the inner membranes were characterized by an extremely high viscosity of ~1000 cP [23]. In this work, we observed that ethanol treatment induces a significant decrease in the inner membrane viscosity to 860 cP, which is still significantly higher than the viscosity of a vegetative cell, 500 cP [23]. A number of studies on phosphocholine membranes have shown that ethanol can induce a transition from a gel state to an interdigitated gel state that is associated with altered membrane properties [58–60]. Of biological significance is the thickness decrease associated with the appearance of the interdigitation that may impact membrane functions significantly

[60], and this factor can explain the increase in permeability observed in the present work. Moreover, Zeng and Chong have observed a decrease in the partition coefficient into membranes of some fluorescent probes (e.g. Prodan and Acdan) when ethanol-induced interdigitation takes place [59]. This observation is consistent with our data and indicates that it is possible that upon 70 % ethanol treatment the inner membrane of wild type spore cannot be stained by Bodipy-C_{12} due to significantly increased interdigitation. Alternatively, it has been previously suggested that ethanol at high concentrations could cause significant protein aggregation on the spore's surface thus preventing deep ethanol penetration [61]. It is thus possible that the treatment conditions used in our experiment (70% of ethanol, 70 °C) caused significant protein aggregation on the spore surface, thus creating a barrier preventing the rotor's insertion deep into the spores.

5. Conclusions

In this work we have used a variety of techniques to investigate the structure of *Bacillus subtilis* spores following perturbation with ethanol at high temperature. Our combined spectroscopic data on permeability and survival of the spores indicates that high percentages of ethanol (*i.e.* $\geq$ 50 %) when used at high temperature lead to a significant permeabilisation of the inner membrane. In order to quantitatively assess the permeability and rigidity of the inner membrane of treated spores we have utilized FLIM microscopy in combination with viscosity-sensitive membrane-bound molecular rotor Bodipy-C_{12} that was able to provide a measure of microviscosity change in the membrane upon perturbation. Consequently, we have detected a significant decrease in viscosity of the spore's inner membrane, while the outer membrane viscosity appeared unchanged upon perturbation. The current work shows the potential of our new method to follow the effect of other types of perturbations (i.e. with high pressure, temperature or chemicals) on the membrane viscosity of bacterial spores.

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Figure Legends:

Figure 1: Comparison of fluorescence lifetimes observed for Bodipy-C_{12} in wild type PS533 and mutant *cotE gerE* spores. The lifetime components τ_1 and τ_2 in PS533 correspond to the signal from the membranes and the coat, respectively. In *cotE gerE* spores the decay is monoexponential and the lifetime τ corresponds to the inner membrane. According to [23].

Figure 2: Effect of different concentrations of ethanol administered for 1h at 70 °C on survival and permeabilization of wild type and mutant spores. (a) Survival in % determined by either the CFU method or Propidium Iodide (PI) staining; the key to symbols is shown in the insert. (b) Confocal microscopy images of wild type and *cotE gerE* spores treated with ethanol at different concentrations and stained with PI. Excitation: 488 nm. Emission: 550-700 nm. Red arrows show selected spores with a protoplast stained by PI. White arrows show selected unpermeabilised spores.

Figure 3: Effect of different concentrations of ethanol administered for 1h at 70 °C on dipicolinic acid (DPA) content of wild type spores and refractivity of wild type and mutant

spores. (a) Infrared spectra of DPA in wild type spores as a function of ethanol concentration (shown as insert). The 1279 cm^{-1} band corresponds to the asymmetric stretching vibration of carboxylate COO assigned to DPA [39,40] (b) Percentage of phase bright spores as a function of ethanol concentration for wild type and mutant spores. (c) Phase contrast microscopy images (recorded at 40x magnification) of wild type and *cotE gerE* spores as a function of ethanol concentration. White and black arrows indicate phase bright and phase grey spores respectively.

Figure 4: The effect of ethanol treatment (70% ethanol for 1h at 70 °C) on the secondary structure of proteins in spores as detected by infrared spectroscopy: (a) wild type spores, (b) *cotE gerE* spores.

Figure 5: Typical fluorescence images of wild type and mutant spores stained with Bodipy-C_{12} before and after ethanol treatment. (a) Spores stained during sporulation, 0% ethanol. (b) Spores stained after sporulation, 0% ethanol; note that for mutant spores no staining is visible. Thus, , image b is an overlay image of transmission and fluorescent image .(c, d, e) Spores treated with 30% (c), 50% (d) or 70% (e) of ethanol at 70 °C during 1 hour and stained with Bodipy-C_{12} after the ethanol treatment. Percentages given below each figure correspond to % of spores with visible inner staining. Images were obtained by confocal microscopy using 488 nm excitation, 500-570 nm emission detection. Each image has a resolution of 0.3 µm/px (512*512 px images) except 0.2µm for image a of *cotE gerE* spores.

Figure 6: The changes in fluorescence lifetimes of membrane-localised Bodipy-C_{12} in *cotE gerE* spores upon ethanol treatment. (a) Fluorescence confocal and (b) lifetime images of

Bodipy-C_{12} as a function of increasing ethanol concentration, when treated for 1h at 70°C; (c) corresponding lifetime histograms. The lifetime of Bodipy-C_{12} in *cotE gerE* is monoexponential and each histogram can be fitted with a single Gaussian peak.

Figure 7: The changes in fluorescence lifetimes of membrane-localised Bodipy-C_{12} in wild type spores upon ethanol treatment. (a) Fluorescence confocal and (b) lifetime images of τ_1 of Bodipy-C_{12} as a function of increasing ethanol concentration, when treated for 1h at 70°C ; (c) corresponding lifetime histograms. Each histogram was fitted with a minimal number of Gaussian peaks. (d) Peak τ_1 values for each peak. Error bars in τ_1, corresponding to FWHM/2 of each peak, are shown as boxes in Δy; while the relative proportion of each peak (measured as a fraction of the overall area under peaks) is represented by the width of each box in Δx.

Figure 8: The summary of changes in the viscosity of membranes as measured with Bodipy-C_{12} upon ethanol treatment of either the wild types spores (in blue) or mutant spores (in red) plotted as a function of increasing ethanol percentage. Two lifetime contributions for wild types spores are assigned individual viscosities.