Use of a molecular rotor for a better understanding of the hydrophobic structures of bacterial spore

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INTRODUCTION

Spores are a major concern in the food industry as they are responsible of spoilage and food borne disease due to their high resistance to food preservation processes. Their particular and partitioned structure are responsible of extreme resistance to environmental stresses (heat, radiation, dehydration…) and thus explain the difficulty to inactivate them. The inner membrane of spores is particularly interesting: it has particular properties, a high rigidity and a low permeability. However, few things are known about the structure and organization of this membrane. This is especially due to the difficulty to probe the inner layers of spores without disturbance. This work proposes to use fluorescence lifetime imaging microscopy (FLIM) combined with molecular rotor to study the mobility of hydrophobic layers of spores and in particular its deepest.

OBJECTIVES

• Find tools which allow to characterize the spore's structures
• Improve our knowledge on effects of external perturbations on spore's layers and in particular those which play on the permeability.

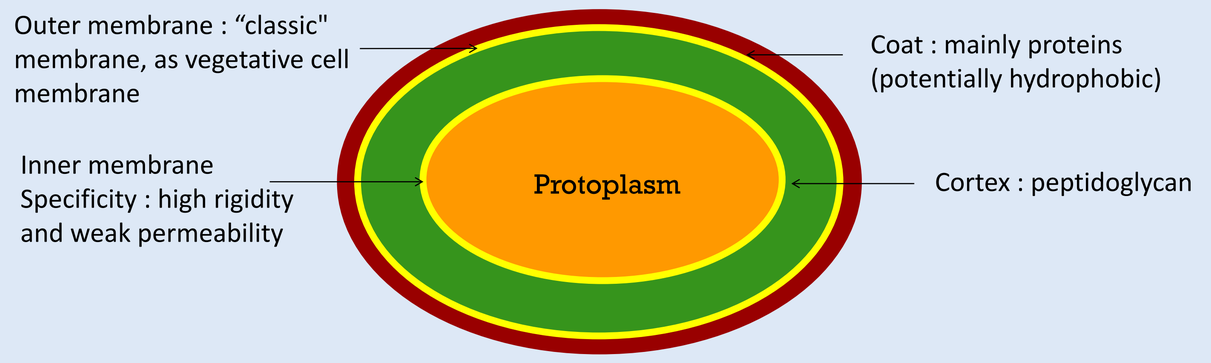
MATERIALS & METHODS

Microorganismes:

•Dormant spores of *Bacillus subtilis 168* produced in DSM medium
•Decoated spores obtained by chemical treatment on stained spores
• Germinated spores obtained by resuspension in LB medium (30min)

Materials:

•Molecular rotor:*meso*-substituted 4,4'-difluoro-4-bora-3a,4a-diaza-s-indacene
• Probe introduced during sporulation process
•Fluorescence Lifetime Imaging Microscopy (FLIM)

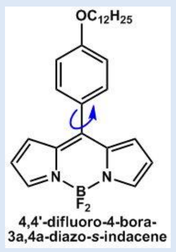


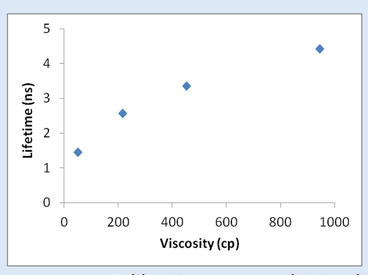
Schematization of the structure of bacterial spore (size is about 1 µm)

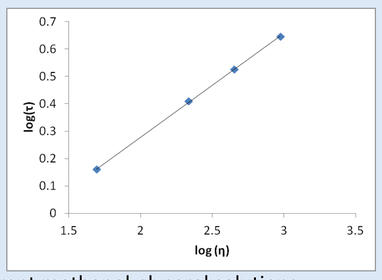
RESULTS & DISCUSSION

1) Use of a molecular rotor into bacterial spores

• **Molecular rotors** →possess a high internal degree of flexibility.
→emission intensity and lifetime (τ) are strongly dependent of the viscosity (η) of their environment.



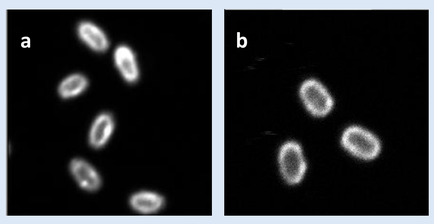




Calibration curves obtained in different methanol-glycerol solutions

➢**Log (τ) is linearly related to log (η) (idem as Kuimova *et al.*, 2008).**
➢**Allow to study a wide range of viscosities (49 to 945 cP)**

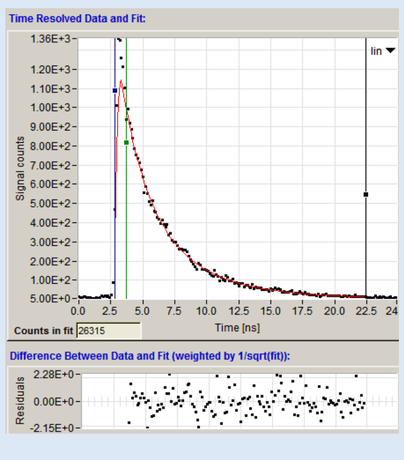
• **Introduction of the molecular rotor during sporulation process**



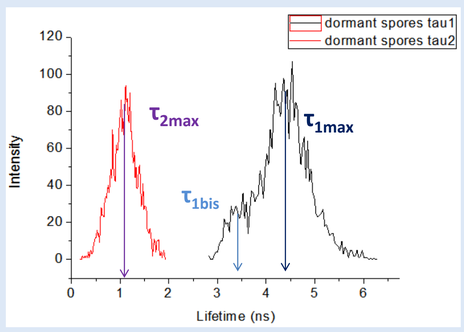
Intensity images of dormant spores stained after (b) or during sporulation process (a)

• Probe added during sporulation process (a) → well incorporated into spores.
• Spores stained after production (b) → probe is not incorporated into deepest layers (probably inner membrane).
• In any case → the protoplast is not stained.

It seems thus possible to use the molecular rotor to probe the viscosities of hydrophobic layers of spores



In dormant spores, a biexponential fitting per pixel is needed to analyze the fluorescence lifetime of the molecular rotor. Two principal components are extracted from the decay curve (long: τ_1 and short: τ_2)

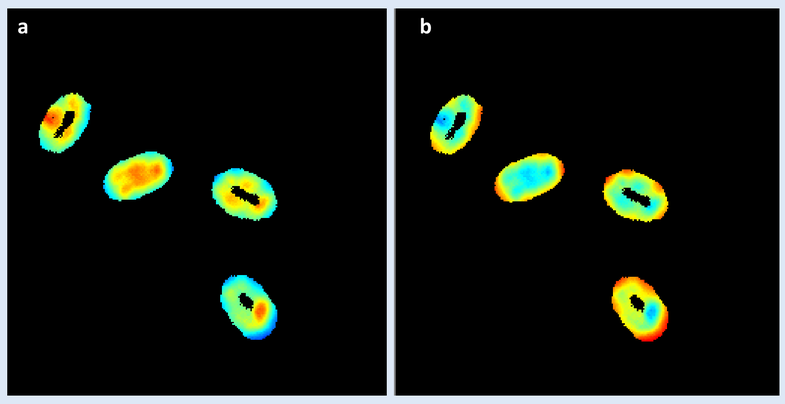


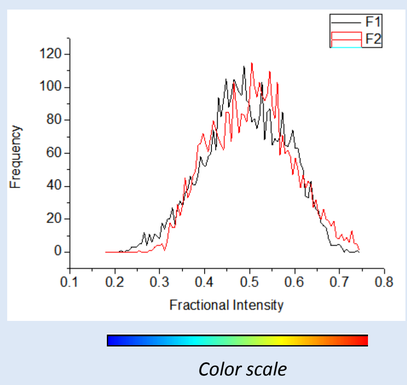
Example of the measure of the lifetimes in spores

τ_1 :τ_{1max} ≈ 4.1 ± 0.5 ns (η_1 ≈ 750 cP) with another contribution τ_{1bis} ≈ 3.4 ns ($\eta_{1\ bis}$ ≈ 457cP)
τ_2 : τ_{2max} ≈ 1.1 ± 0.3 ns (η_2 ≈ 23 cP)
Example: η_{Water} ≈ 1cP

In spores : the molecular rotor measures different viscosities → two lifetimes which correspond to two different environments : which ones?

2) Localization of the different viscosities into spores:





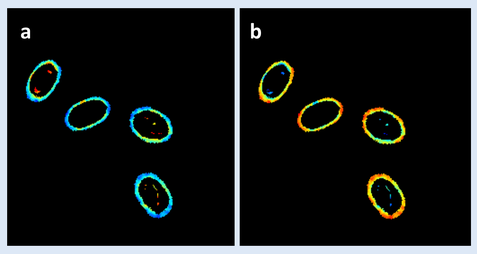
Color scale

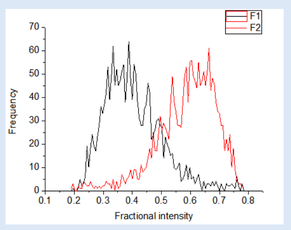
Fractional intensities images of τ_1 (a) and τ_2 (b) measured in dormant spores.

Global fit: τ_1 max /τ_2 max	F_1 (Fractional Intensity τ_1)	F_2 (Fractional Intensity τ_2)
Central	50-70%	30-50%
Edge	25-50%	50-75%

τ_1:high amplitude comes from center
τ_2: high amplitude comes from outer ring

• Use of the threshold to isolate regions :





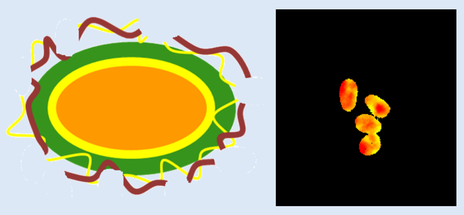
Color scale

Fractional intensities images of τ_1 (a) and τ_2 (b) measured in dormant spores (example of threshold)

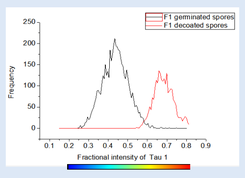
In the outer ring, τ_2 is predominant, whereas in the center, both τ_1 and τ_2 are present with a majority of τ_1. Moreover, τ_{1max} is lower (3.2 ± 0.4ns), close to τ_{1bis} .

outer and inner membrane ? ← **τ_1** **τ_2** → **coat/and or outer membrane ?**

3) Comparison with germinated and decoated spores



Decoated spores
(*most of the coat and outer membrane are removed*)



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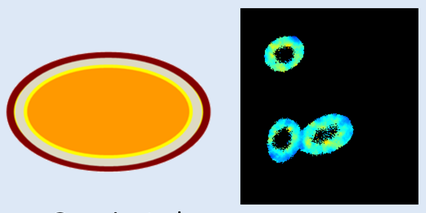

Germinated spores
(*swelling,hydrolysis of the cortex, changes in fluidity of inner membrane*)

τ_1 max ≈ 4.2 ± 0.4 ns η_1 ≈ 798 cP
τ_2 max ≈ 1.4 ± 0.4 ns η_2 ≈ 43 cP

τ_1 max ≈ 3 ± 0.3 ns η_1 ≈ 328 cP
τ_2 max ≈ 0.9 ± 0.2 ns η_2 ≈ 14 cP

Decoated spores: **τ_1 is predominant, with η_1 quite similiar than in dormant spores (τ_{1bis} is no more visible) .** Evolution during germination: **τ_2 is predominant. η_1 ↘ by ≈ 2 folds whereas η_2 is quite similar**

Hypothesis: τ_1 corresponds to the **membranes** → during germination, inner membrane becomes more fluid (decrease in η), outer membrane begins to degrade ? (decrease in F_1). τ_2 corresponds mainly to the **coat**: few evolution of viscosity during germination and decreases in F_2 in decoated spores.

CONCLUSION

➢ A high inner viscosity, maybe due to inner membrane, is measured in dormant spores : **η_1 ≈ 750 cP**
➢ The use of the rotor is appropriate to study the structure of bacterial spore. It is, by example, possible to follow modifications due to germination (↘ of one viscosity)
➢ It represents a powerful tool to follow the inner state of spores and can provide a better understanding of the effects of external perturbations on spore's layers.

PERSPECTIVES

➢ Confirm the attribution of the different lifetimes in using other models : vegetative cells, vesicles…
➢ Follow the effects of environmental stresses on the viscosity of the spore's structures (High pressure, ethanol treatments…)
➢ Use of an hydrophilic rotor to study the state of the core of spores