The mechanisms of bacterial spore germination in *Bacillus subtilis* as a proxy for spore formers in the gut microbiome

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Introduction

Bacillus subtilis forms dormant spores upon nutrient depletion. Spores are abundant in the gut microbiome. Once favorable environmental conditions occur, spores germinates, progresses to outgrowth and resumes growth. Receptors located in the spore's inner membrane can detect the presence of exogenous ligands, such as L-alanine, and trigger the process of spore germination and subsequent outgrowth (*1-3*). The germination of *bacillus* endospores is a strictly regulated process, in which the tricistronic *gerA* is an important operon. Its protein products GerAA, GerAB, and GerAC are located in the cell membrane of *Bacillus subtilis* spores. Upon binding of L-alanine to GerAB, spore germination is activated, mediated by GerAA, GerAB and GerAC (*4-6*). For the PhD project, we will conduct an iterative cycle of state-of-the-art molecular simulation techniques, including path sampling*(9)* and experimental validation using a super resolution phase-contrast microscopy chamber coupled to image analysis with Spore Tracker(*6*). This will allow me to study both experimentally and theoretically the interaction between L-alanine and GerAB and hence elucidate an as yet unresolved molecular basis of the mechanism of bacterial spore germination.

Methods applied in the project

(I) Molecular dynamics simulation

All-atom MD simulations can successfully model protein conformations and their changes with environmental conditions as well as predict binding sites of small molecules to proteins*(7)*. However, one of the crucial problems is that relevant transitions are often rare and occur on the millisecond to second timescale, currently inaccessible for all-atom MD simulations. Such slow transitions are usually related to high free energy barriers separating stable states. To efficiently sample rare events, we will use such as steered MD (SMD) which applies external forces in the simulation, pulling L-alanine out of a predicted binding site to obtain a free energy profile for unbinding*(8)*. Transition path sampling (TPS) focuses on transitions between stable states, and thus bypasses the long waiting times, resulting in ensembles of transition paths, i.e. 'molecular movies' *(9)*. Analysis of these ensembles leads to the characterization of reaction coordinates*(10)*, and hence provides insights in the molecular mechanisms, such as which amino acids of GerAB are involved in the binding with L-alanine and what are conformational changes that may occur upon binding the germinant. Rate constants of activated process transitions between stable states separated by high free energy barriers in a complex environment are effectively computed using the transition interface sampling (TIS) method*(11)*.

(II) Phase-contrast time-lapse and image analysis with the Spore Tracker

The phase-contrast microscope can show the real time status for spore germination of bacteria from the Bacilli or Clostridia class *(12, 13)*. Furthermore, using fluorescence microscopy of the germinant receptors coupled to a Green Fluorescent Protein GFP (or derivative) reporter coupled to phase-contrast microscopy of the samples, we will obtain dynamic information of germination at the single spore / cell level*(14, 15)* **(Figure 1)**. The data shown show germination by revealing spore transitions from phase-bright to phase-dark, indicative for water uptake. The latter is conditional for the resumption of metabolism. By measuring the period between the phase-dark appearance and the first cell division outgrowth is characterized. For the image analysis we use purposely built, Spore Tracker, which can measure the time to

germination, the germination duration and the growth rates from the spores in real time(*6*) **(Figure 2)** Firstly, Spore Tracker detects phase-bright spores and marks them. Then, bright-to-dark transitions of germination can be highlighted by using a second marker to mark the spores in various time frames. Finally, the macro detects the growing cells and records the rate of their appearance. All of the above helps us to study the real time dynamics of spore germination.

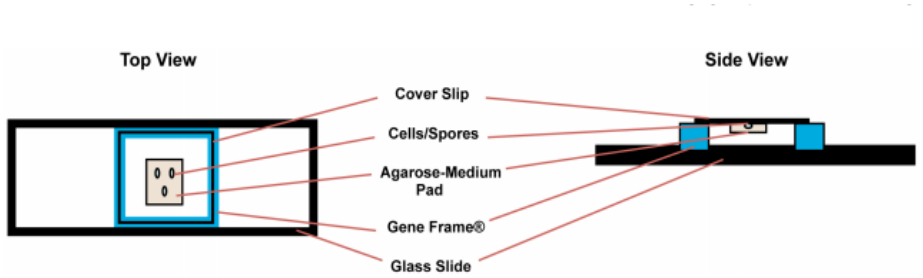

Figure 1. Closed air-containing chamber for live cell imaging

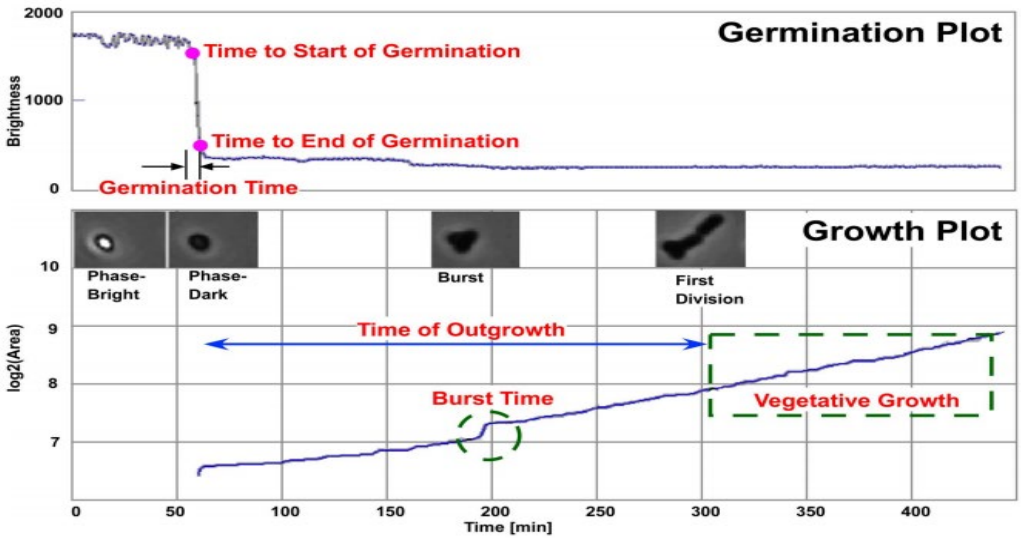

Figure 2. A typical screen shot of the analysis routine used by Spore Tracker (discussed extensively in refs. *6, 28*).

Project goals and strategies

(I) Detect interaction between L-alanine and GerAB

(i) Identify amino acids in GerAB that can bind to L-alanine

Molecular dynamics (MD) simulations are particularly well suited for studying small molecules interacting with proteins, and can generate insights at high spatial and temporal resolution. MD simulations of biomolecular systems can now be extended to the μs time scale and beyond*(16)*, thus near to the gap between time scales that can be modelled and those that are experimentally traceable. In fact, when performing simulations of conformational changes in proteins, one of the crucial problems is that these changes are often rare and occur on the millisecond to second timescale, currently inaccessible for all-atom MD simulations even on today's fastest computers. One way to overcome the time scale challenge involves the use of artificial potentials forcing the system to undergo the process of interest*(17)*, approaches known as free energy methods. Such methods are very useful for the computation of equilibrium constants and thermodynamic properties. In addition, the transition path sampling (TPS) method harvests ensembles of unbiased dynamical trajectories between stable states by importance sampling*(18, 19).* The group of Dr. Vreede in Amsterdam successfully applied the path-sampling method to many activated rare event processes*(20)*, including the light-induced unfolding of a blue-light receptor*(21),*as well as the elucidation of the N and C termini of GerAB in the bacterial spore membrane with respect to the germinant binding pocket. It was observed that both termini were on the same side, facing towards the spore cortex. This grounding work now allows subsequent analysis*(22)* of the path ensembles and leads to unbiased insight in reaction coordinates. Hence, we aim through this approach to unravel the mechanisms of GerAB folding that occur upon conformational changes during L-alanine binding *(23)*. Devised originally for two states*(18, 19)*, TPS has been extended to allow sampling between many states*(24)*. Using TPS, we will investigate the mechanisms underlying

the activation of GerAB upon the binding of alanine. An L-alanine binding pocket to GerAB might be associated with a (putative) water channel that recent Molecular Dynamics modelling revealed (Blinker et al., 2020 manuscript submitted).

(ii) Site-directed mutation of amino acids in GerAB for verification

Of the amino acids that the model will predict to be involved in germinant binding and signal relay we will generate mutant GerAB variants using state of the art molecular biology techniques that the laboratory of prof. Brul (Molecular Biology & Microbial Food Safety) has at its disposition. Spores of the mutant GerAB containing strains will be analyzed with Spore Tracker in single spore germination assays.

(iii) Observing germination status at the cell level to verify the interaction

Firstly, spores (wild type & variant) should be prepared by culturing, collecting, washing and storing. And then different concentration of L-alanine will be added when the cell density increase to certain value, to trigger germination. Finally, the germination phenotype can be characterized from different perspectives: At the population level we will assess the OD of spore suspensions *(26*) and thus have a proxy for spore CaDPA release(*27*). Furthermore as indicated above we will use phase contrast microscopy for single spore analyses in which individual heterogeneity of the various germination and outgrowth phases is assessed. These real time data of the dynamics of the mutant and wild-type spore germination will be compared with Spore Tracker *(6, 28*) which will provide detailed information of differences in the time dependent status of germination between individual mutants and wild-type GerAB *(6)* **(Figure 3)**.

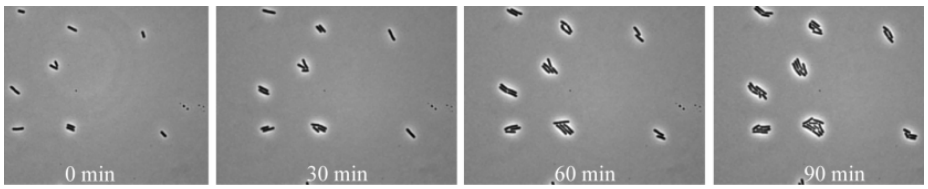

Figure 3. Time to status of germination

(iv) Molecular docking to verify iteratively the experimental results and FRET.

After experimental analyses are done we will perform a second round of docking the GerAB variants with L-alanine to verify the models and refine them. It will be a final challenge to analyze gerAB interactions with domains of the SpoVA channel protein that are presumed to be involved in DPA transport (SpoVAD and SpoVAEa) Key sites of GerAB will be verified by the methods described above. For the interaction studies we will make use of Fluorescence (Förster) Resonance Energy Transfer (FRET). Preliminary experiments in *Bacillus cereus* have shown FRET in for the interaction between gerRB and gerD in *B. cereus* germinosomes (our unpublished results and *29*).

Time plan for the project

Year	Tasks
Year 1	Identify amino acids in GerAB that can bind to L-alanine
Year 2	Site-directed mutagenesis of amino acids in GerAB for verification
Year 3	Observing germination status at the cell level to assess germination protein interactions
Year 4	Molecular docking in an iterative cycle to verify the experimental results; Experimental verification using FRET experiments. Writing of the PhD thesis

Reference

1. Breedijk R.M.P., Wen, J., Krishnaswami, V., Bernas, T., Manders, E.E.M., Setlow, P., Vischer N.O.E. and Brul, S. A live-cell super-resolution technique demonstrated by imaging germinosomes in wild-type bacterial spores. *Sci. Rep.* **2020,** 10, 5312.

2. Christie G. and Setlow, P. Bacillus spore germination: Knowns, unknowns and what we need to learn. Cell Signal. **2020,** 74, 109729.
3. Wen, J., Pasman, R., manders E.E.M., Setlow P. and Brul, S. Visualization of germinosomes and the inner membrane in Bacillus subtilis spores. *J. Vis. Exp.* **2019**, 146.
4. Zuberi, A. R.; Moir, A.; Feavers, I. M., The nucleotide sequence and gene organization of the gerA spore germination operon of Bacillus subtilis 168. *Gene* **1987,** *51*, 1-11.
5. Paidhungat, M.; Setlow, P., Isolation and characterization of mutations in *Bacillus subtilis* that allow spore germination in the novel germinant D-alanine. *Journal of bacteriology* **1999,** *181*, 3341-50.
6. Pandey, R.; Ter Beek, A.; Vischer, N. O.; Smelt, J. P.; Brul, S.; Manders, E. M., Live cell imaging of germination and outgrowth of individual *Bacillus subtilis* spores; the effect of heat stress quantitatively analyzed with SporeTracker. *PloS one* **2013,** *8*, e58972.
7. McCammon, J. A.; Gelin, B. R.; Karplus, M., Dynamics of folded proteins. *Nature* **1977,** *267*, 585-90.
8. Park, S. & Schulten, K. Calculating potentials of mean force from steered molecular dynamics simulations. *J. Chem. Phys.* **2004**, 120, 5946–5961.
9. Dellago, C., Bolhuis, P. G., Csajka, F. S. & Chandler, D. Transition path sampling and the calculation of rate constants. *J. Chem. Phys.* **1998**, 108, 1964–1977.
10. Vreede, J., Juraszek, J. & Bolhuis, P. G. Predicting the reaction coordinates of millisecond light-induced conformational changes in photoactive yellow protein. *PNAS* USA. **2010**, 107, 2397–2402.
11. Van Erp, T. S., Moroni, D. & Bolhuis, P. G. A novel path sampling method for the calculation of rate constants. *J. Chem. Phys*. **2003**, 118, 7762–7774 .
12. Stringer, S. C.; Webb, M. D.; Peck, M. W., Lag time variability in individual spores of Clostridium botulinum. *Food microbiology.* **2011,** *28*, 228-35.
13. Stringer, S. C.; Webb, M. D.; Peck, M. W., Contrasting effects of heat treatment and incubation temperature on germination and outgrowth of individual spores of nonproteolytic Clostridium botulinum bacteria. *Applied and environmental microbiology* **2009,** *75*, 2712-9.
14. Zhang, Y.; Xie, C., Differential-interference-contrast digital in-line holography microscopy based on a single-optical-element. *Optics letters* **2015,** *40*, 5015-8.
15. de Jong, I. G.; Beilharz, K.; Kuipers, O. P.; Veening, J. W., Live Cell Imaging of *Bacillus subtilis* and *Streptococcus pneumoniae* using Automated Time-lapse Microscopy. *Journal of visualized experiments : JoVE,* **2011**.
16. Hertig, S., Latorraca, N.R., Dror, R.O. Revealing atomic-level mechanisms of protein allostery with molecular dynamics simulations. *PLoS Comput. Biol.* **2016**, 12:e1004746
17. Laio A., Parrinello, M. Escaping free-energy minima *Proc. Natl. Acad.* Sci. USA, **2002**, 99:12562.
18. Dellago, C., et al. Transition path sampling and the calculation of rate constants. *J. Chem. Phys.* **1998**, 108:1964.
19. Bolhuis, P.G., et al. Transition path sampling: throwing ropes over rough mountain passes, in the dark *Ann. Rev. Phys. Chem.* **2002**, 53:291 .
20. Bolhuis, P.G. Transition-path sampling of beta-hairpin folding *Proc. Natl. Acad. Sci. USA*, **2003**, 100:12129.

21. Juraszek, J. and Bolhuis, P.G. Sampling the multiple folding mechanisms of Trp-cage in explicit solvent *Proc. Natl. Acad. Sci. USA*, **2006**, 103:15859.
22. Juraszek, J.,Vreede, J.,Bolhuis, P.G. Transition path sampling of protein conformational changes *Chem. Phys.* **2012**, 396:30.
23. van Erp, T.S., Moroni, D. and Bolhuis, P.G. A Novel Path Sampling Method for the Calculation of Rate Constants *J. Chem. Phys.* **2003**, 118:7762 .
24. Vreede, J., Juraszek, J. and Bolhuis, P.G. Predicting the reaction coordinates of millisecond light-induced conformational changes in photoactive yellow protein. *Proc. Nat. Acad. Sci. USA*, **2010**,107:2397 .
25. Cooper, G. R., Moir, A., Amino acid residues in the GerAB protein important in the function and assembly of the alanine spore germination receptor of *Bacillus subtilis* 168. *Journal of bacteriology* **2011,** *193*, 2261-7.
26. Kort, R., O'Brien, A. C., van Stokkum, I. H., Oomes, S. J., Crielaard, W., Hellingwerf, K. J., Brul, S. Assessment of heat resistance of bacterial spores from food product isolates by fluorescence monitoring of dipicolinic acid release. *Applied and environmental microbiology* **2005,** *71*, 3556-64.
27. Setlow, B., Cowan, A. E., Setlow, P. Germination of spores of *Bacillus subtilis* with dodecylamine. *Journal of applied microbiology* **2003,** *95*, 637-48.
28. Omardien, S., ter Beek, A., Vischer, H., Montijn, R., Schuren, F. and Brul, S. Evaluating novel synthetic compounds active against Bacillus subtilis and Bacillus cereus spores using Live Imaging with SporeTrackerX. **2018**, *Sci. Rep.* 8, 9128.
29. Wang, Y., de Boer, R., Vischer, N., van Haasterecht P., Setlow, P. and Brul S. Visualization of germination proteins in putative *Bacillus cereus* germinosomes. **2020**. *Int. J. Mol. Sci.* 21, 5198