Visualizing Spore Germination Protein Complexes in Action in Bacterial Endospores; Lessons and Challenges Ahead

Swammerdam Institute for Life Sciences - SILS - University of Amsterdam

Molecular Biology and Microbial Food Safety

Stanley Brul head of laboratory with the input from many PhD students and PETER SETLOW UConn Health

Bacterial spores; the good are inhabitants of our intestines!

LETTER

OPEN

doi:10.1038/nature17645

Culturing of ‘unculturable’ human microbiota reveals novel taxa and extensive sporulation

Hilary P. Browne[1]*, Samuel C. Forster[1,2,3]*, Blessing O. Anonye[1], Nitin Kumar[1], B. Anne Neville[1], Mark D. Stares[1], David Goulding[4] & Trevor D. Lawley[1]

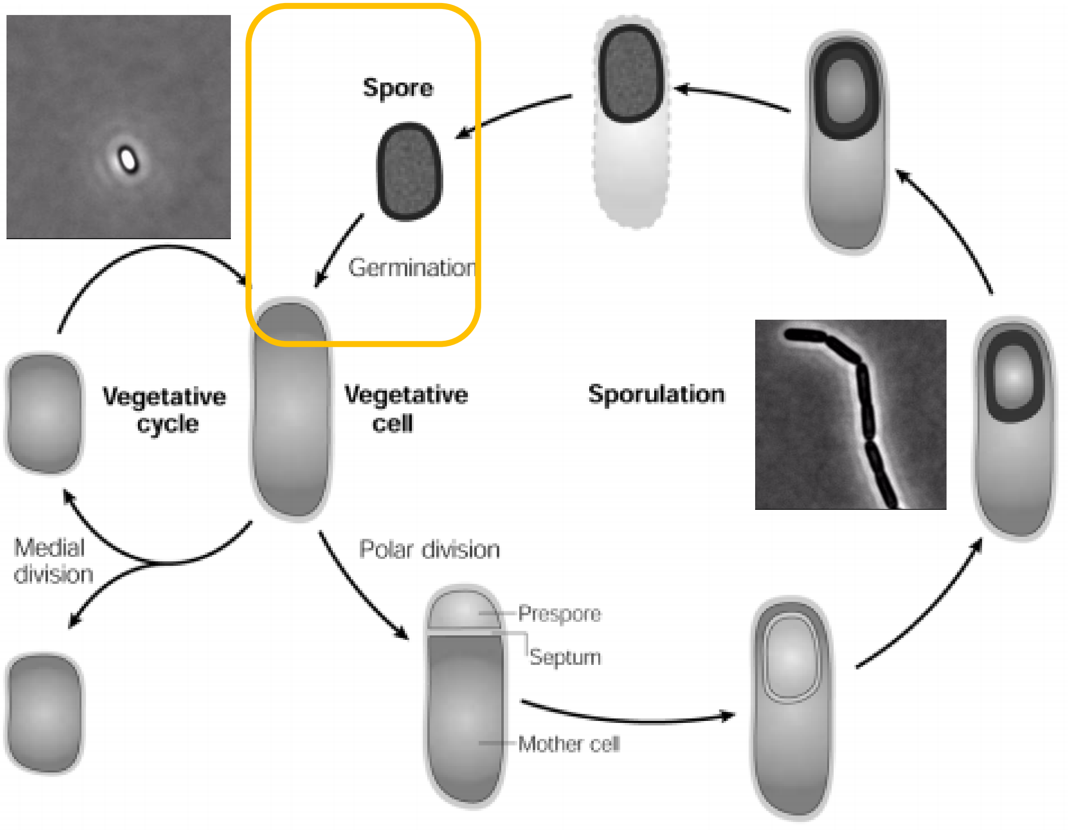
See also the Holomicrobiome Initiative
https://holomicrobioom.nl/en/home-en/

A Holland-based public-private partnership that aims to map, analyze and model the many contacts between microbiota in Water, Soil-Plant, Animals and Humans

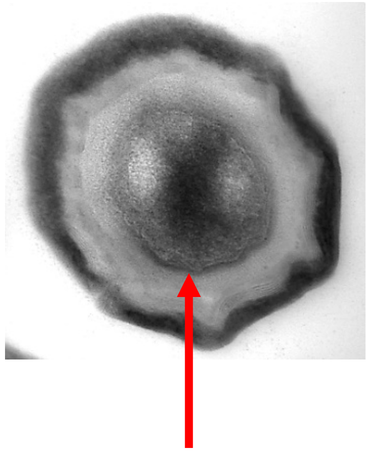
and the Research Priority Area Personal Microbiome Health https://pmh-uva.nl/

Full professor in Microbiome Engineering vacancy open @ SILS-UvA

Main interest in *Bacillus subtilis* and *Bacillus cereus* as a toxigenic spore former that causes food borne disease upon spore germination and outgrowth.



the life cycle of spore-forming bacteria



Inner membrane with GRs

Ynte Piet de Vries's PhD thesis

Germinant **R**eceptor proteins (**GR**s) were initially visualized @ super-resolution in *B. subtilis*

jove Journal of Visualized Experiments www.jove.com

Video Article

Visualization of Germinosomes and the Inner Membrane in *Bacillus subtilis* Spores

Juan Wen[1], Raymond Pasman[1], Erik M.M. Manders*[2,3], Peter Setlow*[4], Stanley Brul*[1]

[1]Molecular Biology and Microbial Food Safety, Swammerdam Institute for Life Sciences, University of Amsterdam

[2]Van Leeuwenhoek Centre for Advanced Microscopy, Swammerdam Institute for Life Sciences, University of Amsterdam

[3]Confocal.nl BV.

[4]Dept. of Molecular Biology and Biophysics, UConn Health

*These authors contributed equally

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SCIENTIFIC REPORTS

natureresearch

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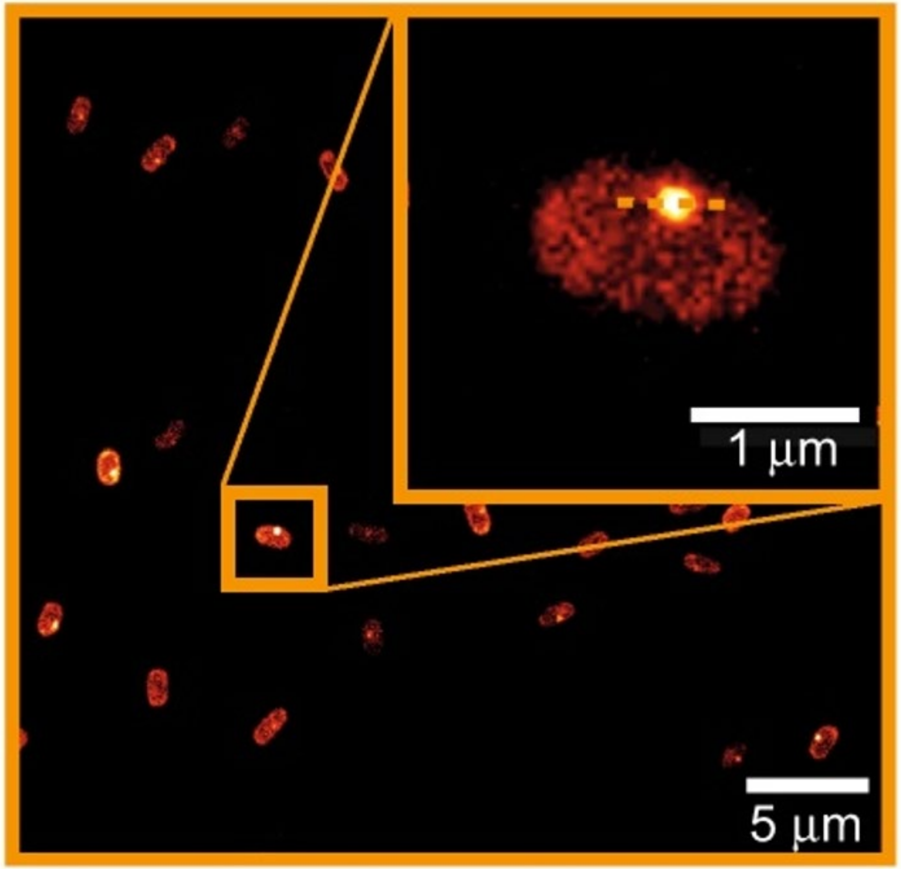
A live-cell super-resolution technique demonstrated by imaging germinosomes in wild-type bacterial spores

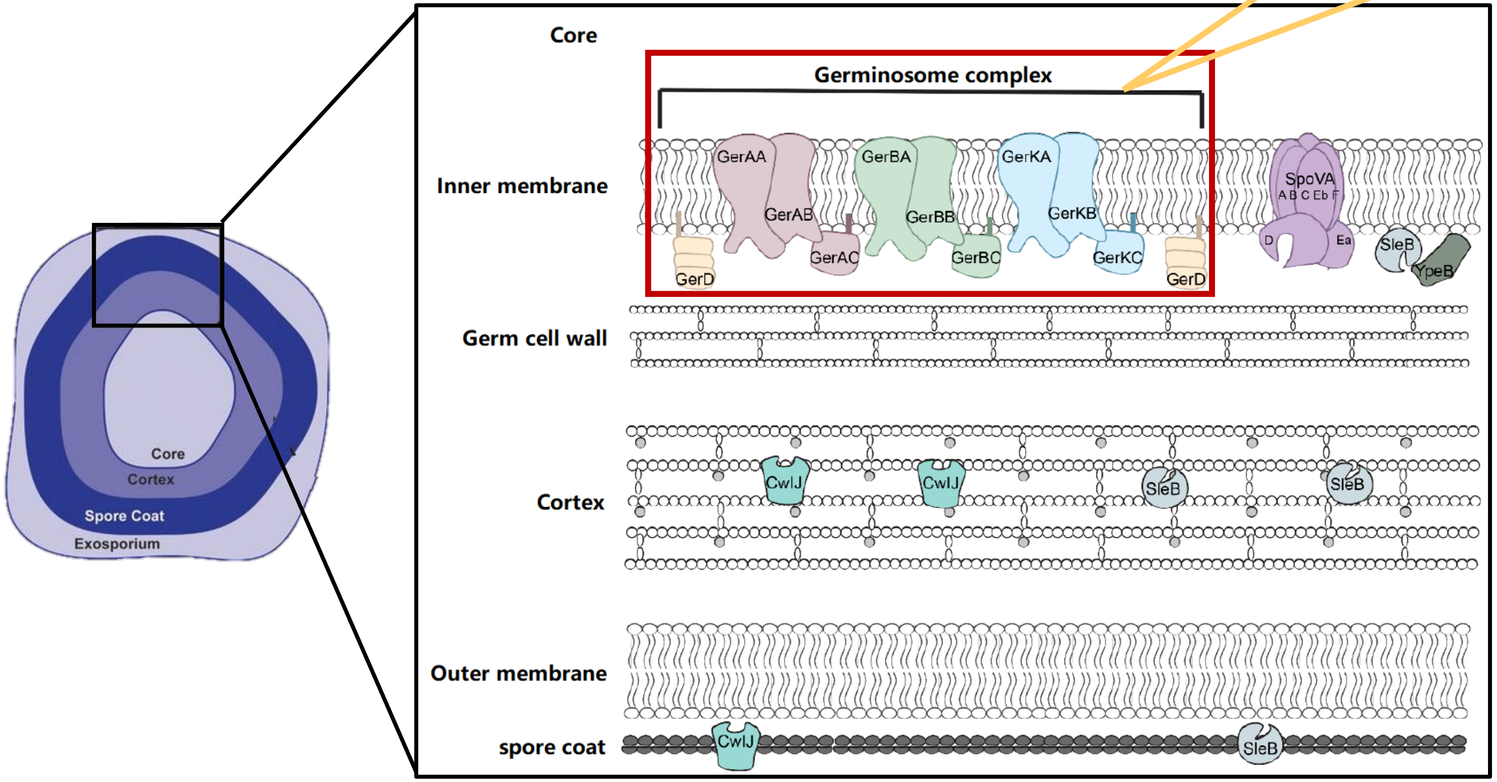
R. M. P. Breedijk[1,6], J. Wen[2,6], V. Krishnaswami[1,6], T. Bernas[3], E. M. M. Manders[1,4], P. Setlow[5], N. O. E. Vischer[2] & S. Brul[2*]

A germinant receptor protein cluster **(germinosome)** as shown by annular rescan confocal microscopy of GerKB-sGFP.



Key germination proteins are clustered in germinosomes in *Bacillus subtilis* spores

How about *B. cereus* spores?



Germinant receptors (GRs) (sense germinants*)

- GerA   A / B / C subunit
- GerB   A / B / C subunit
- **GerK**   A / C / **B** subunit

Germination protein GerD (as a scaffold)

- **mediates** clustering of germination proteins in germinosomes and
- **promotes** the rapid and cooperative GRs response to germinants!

*GR germinants are generally **amino acids**, sugars or purine nucleosides

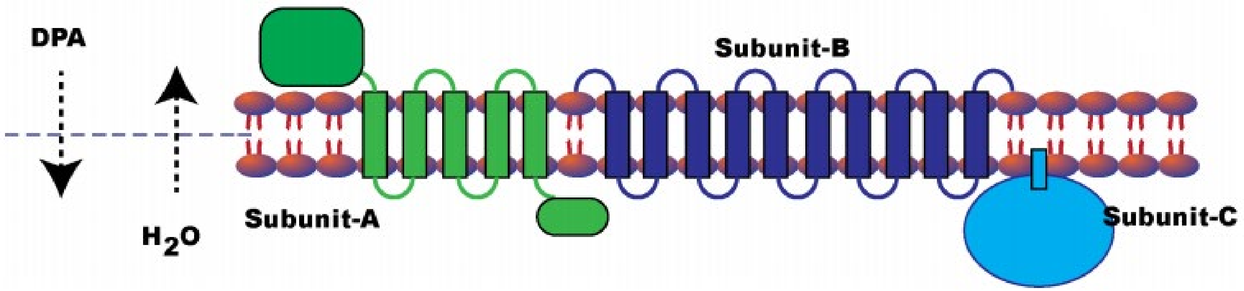
Juan Wen's PhD thesis UvA 2020; Setlow group J Bacteriol. 2015; Mol Biol. 2014.

B. cereus spores have seven different germinant receptors; we focussed on the viualization of the alanine binding tricistronic receptor GerR.

Germinant receptors

- **GerR A / C / B subunit**
- GerK A / B / C subunit
- GerI A / B / C subunit
- GerS A / B / C subunit
- GerQ A / B / C subunit
- GerG ? / B / C subunit
- GerL ? / B / C subunit

[can sense L-alanine as a trigger of germination]



Schematic diagram of the topology of germinant receptor subunits in the inner membrane of Bacillus spores

Germination scaffold protein: GerD

- Compared to GerD of *B. subtilis*: 43% identity in sequence of amino acid

Pandey, 2014 PhD thesis

Research questions (I)

Visualization of germination proteins in putative *Bacillus cereus* germinosomes

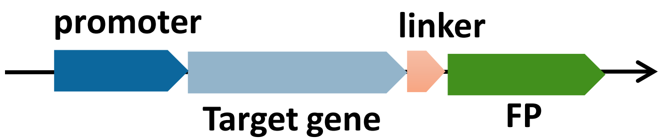
Analysis of the interaction of germinant receptor proteins of the gerR operon and GerD in *Bacillus cereus* spores using FRET

How to visualize GRs and SpoVAEa proteins in *B. cereus* spores ?

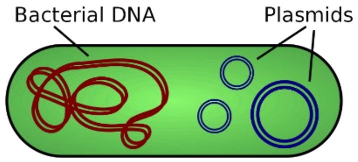
Construction of Recombinant plasmid → **Electroporation and selection** → **sporulation** → **Image Acquisition and Analysis**

Construction of Recombinant plasmid

- ✓ shuttle vector pHT315 (~10 copies/ chromosome)
- ✓ upstream region of target gene acts as promoter
- ✓ fluorescent protein (FP) was fused to C-terminus of target gene
- ✓ flexible linker GSGSGS was used to fuse FP to target gene



Electroporation and selection



2kV, 1mm gap

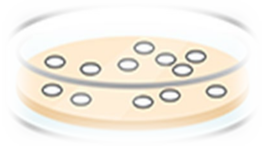


plate with erythromycin

sporulation

- ✓ Medium: Chemically Defined Growth and Sporulation (CDGS)
- ✓ conditions: 30°C, 200rpm, 5 days
- ✓ resistance: 10 μg/ml erythromycin

Image Acquisition and Analysis

- ✓ phase-contrast and widefield fluorescence microscopes
- ✓ ImageJ
- ✓ GraphPad Prism 9.0

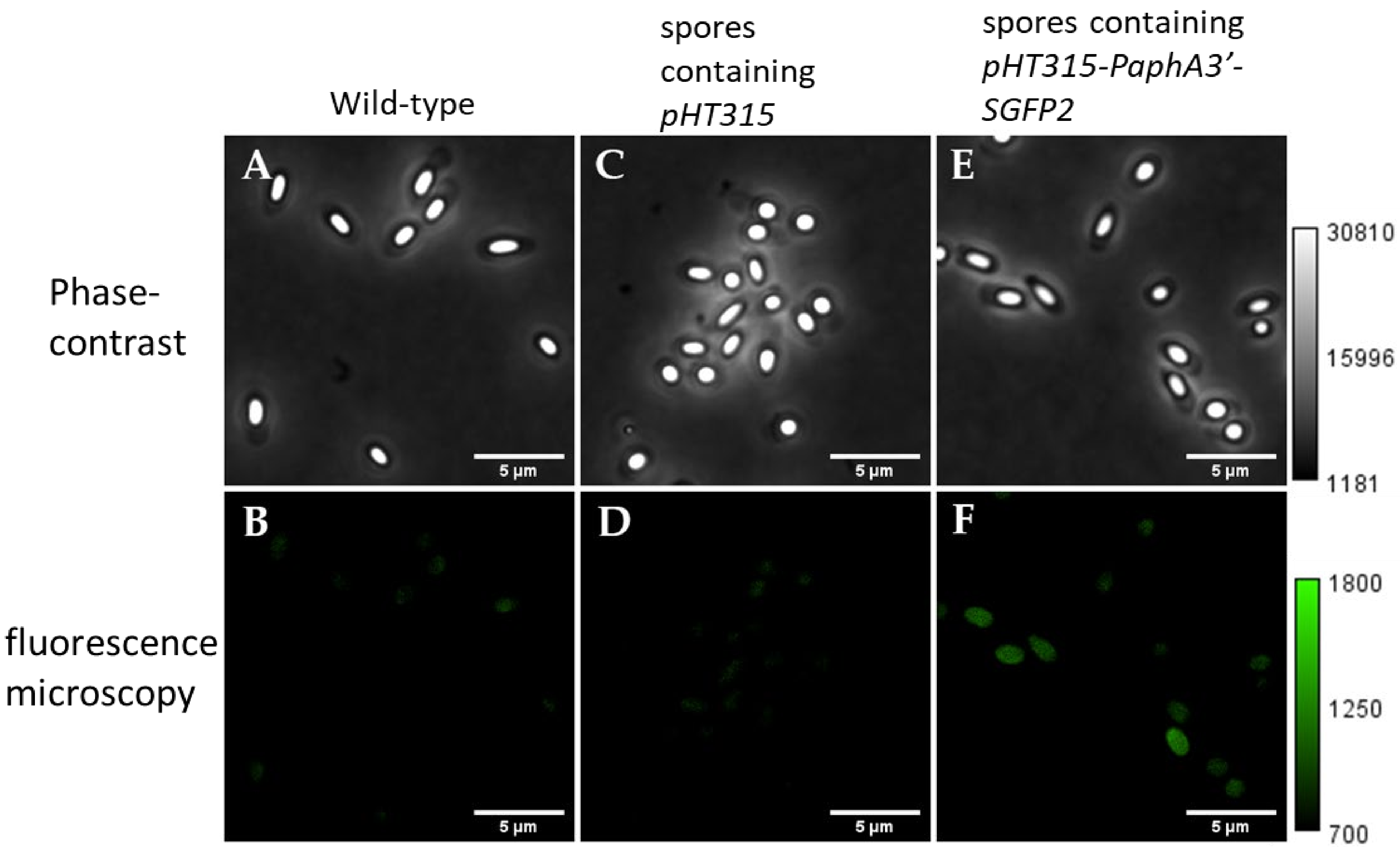
Arantes O and Lereclus D. 1991; Y Wang , et al. Int J Mol Sci. 2021, 22(20).

Research questions (I)

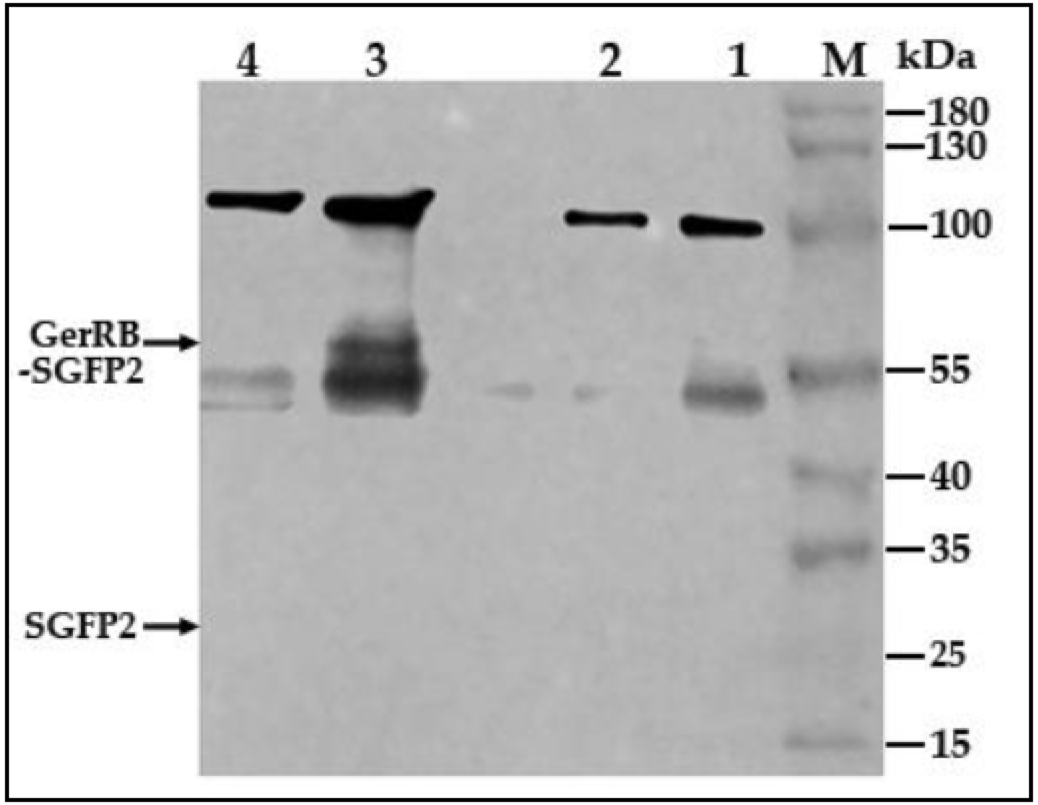
Visualization of germination proteins in putative *Bacillus cereus* germinosomes

Analysis of interaction of germination protein GerR and GerD in *Bacillus cereus* spores using FRET

Visualization of SGFP2 protein expression from *pHT315* in *B. cereus* spores

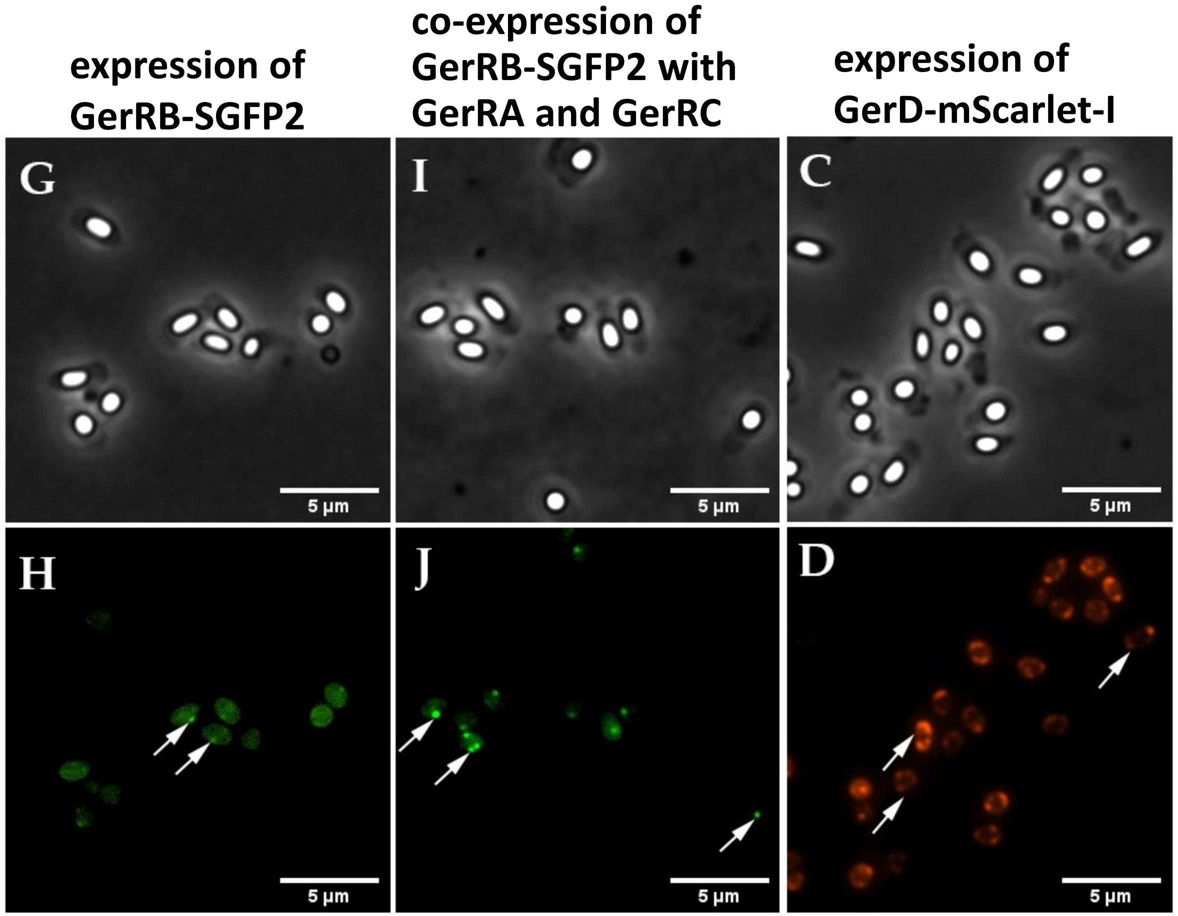


Recombinant spores express fusion protein GerRB-SGFP2 encoded from *pHT315* in *B. cereus* spores



lane 1, wild-type spores;
lane 2, spores carrying pHT315;
lane 3, spores carrying pHT315-PgerR-gerRB-SGFP2;
lane 4, spores carrying pHT315-gerRB-SGFP2;
Lane M, prestained protein ladder

First time visualization of germination proteins in *B. cereus*



These data of visualization suggested that co-expression of GR subunits improves their stability and are the first evidence for the existence of germinosomes in spores of the pathogen *B. cereus*.

Percentage of germinosomes in *B. cereus* spores with GerRB-SGFP2 or GerD-mScarlet-I

- **expression of only GerRB-SGFP2** (*B. cereus* 003)**:**

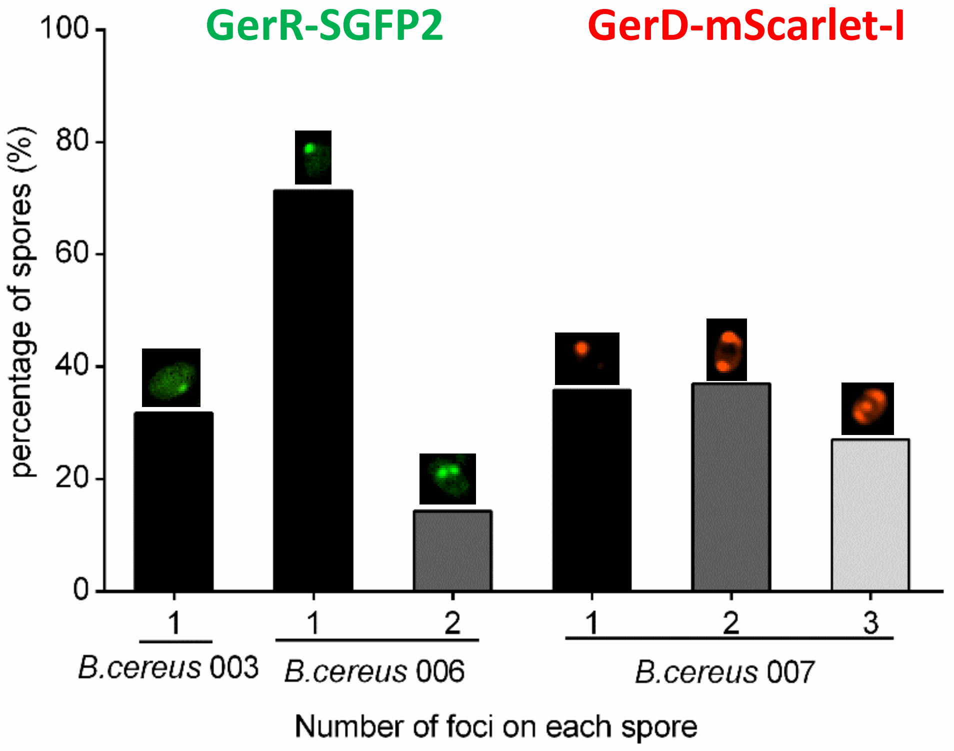
  one germinosome was observed

- **co-expression of GerRA and GerRC** (*B. cereus* 006)**:**

  one and two germinosomes were observed

- **expression of GerD-mScarlet-I** (*B. cereus* 007)**:**

  one, two and three germinosomes were observed



CONCLUSIONS

- High resolution wide-field fluorescence microscopy visualized GerRB-SGFP2 specific bright foci (germinosomes) in ~30% of individual dormant spores if only GerRB-SGFP2 was expressed,
- but in ~85% of spores upon co-expression with GerRA and GerRC. Our data corroborates the notion that co-expression of GR subunits improves their stability.
- All spores displayed bright fluorescent foci upon expression of GerD-mScarlet-i under the control of the *gerD* promoter.

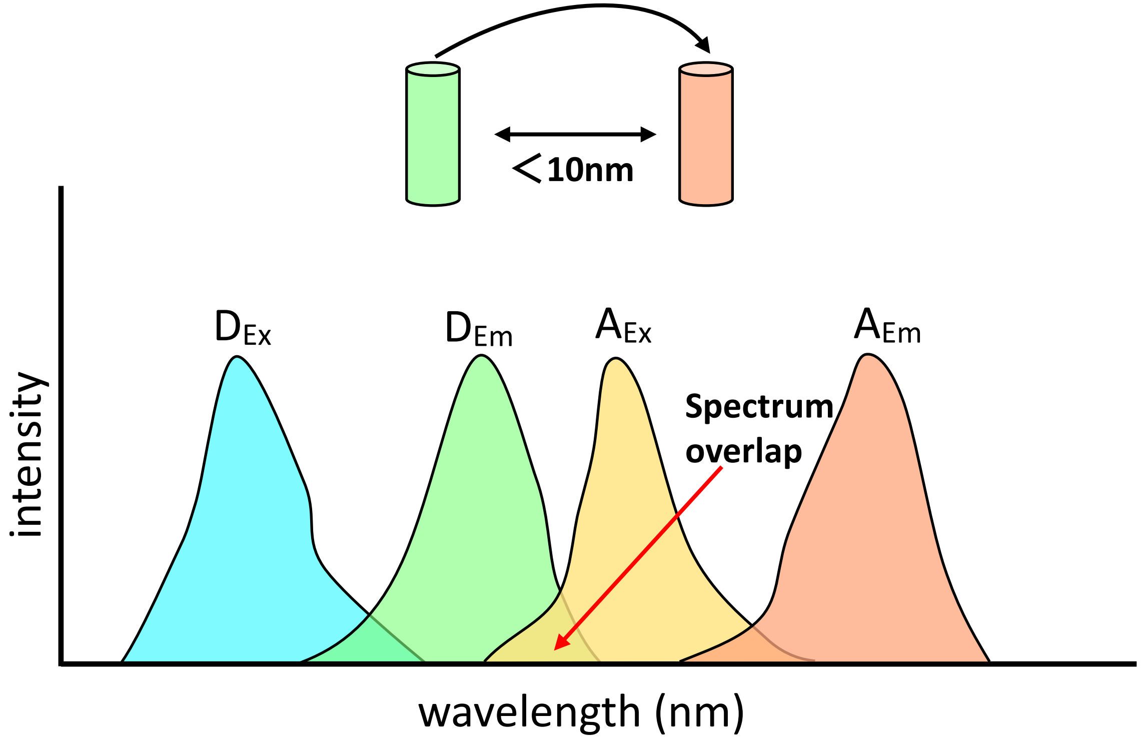
Published: Wang Y, de Boer R, Vischer N, van Haastrecht P, Setlow P, Brul S. Visualization of Germination Proteins in Putative *Bacillus cereus* Germinosomes. Int J Mol Sci. 2020 Jul 22;21(15):5198. doi: 10.3390/ijms21155198.

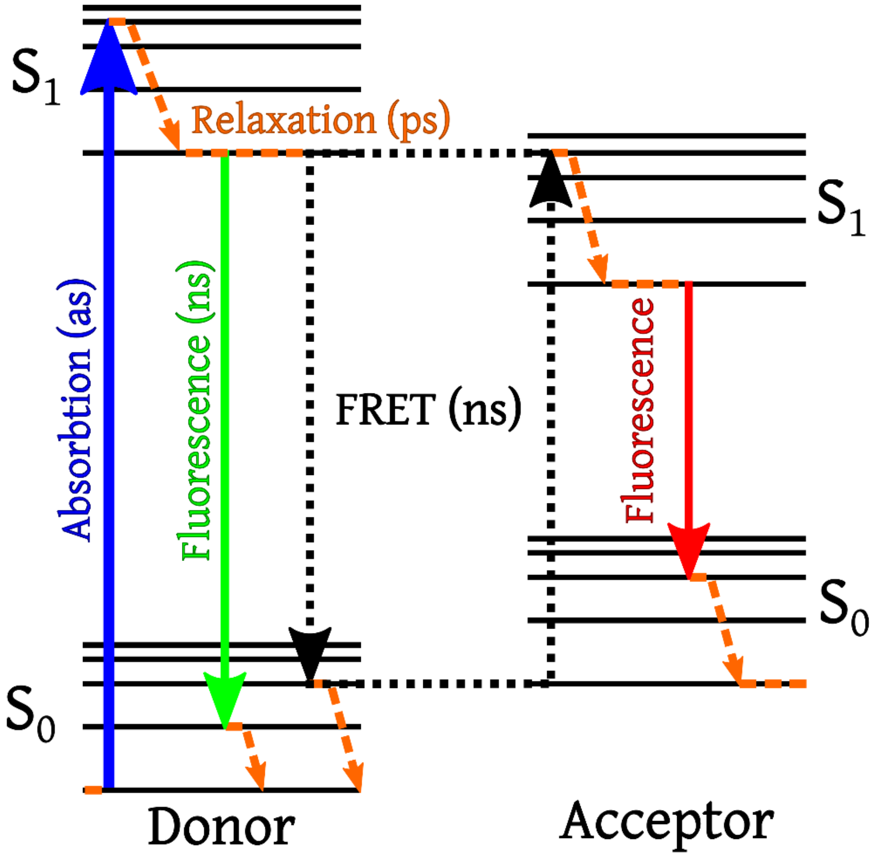
Research questions (I)

Visualization of germination proteins in putative *Bacillus cereus* germinosomes

Analysis of interaction of germination protein GerR and GerD in *Bacillus cereus* spore putative germinosomes using FRET.

Fluorescence Resonance Energy Transfer (FRET) analysis principles





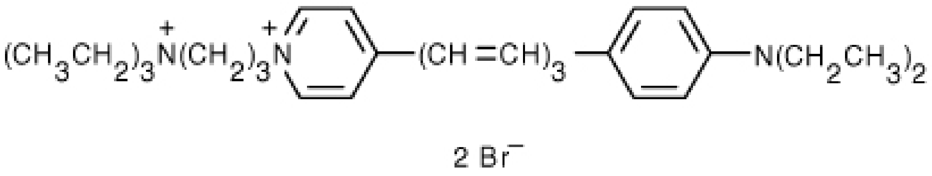
Jablonski diagram illustrating the FRET process. Note that the black dashed line indicates a virtual photon.

B. cereus forespore membrane stained with FM 4-64 indeed harbours GerRB and GerD

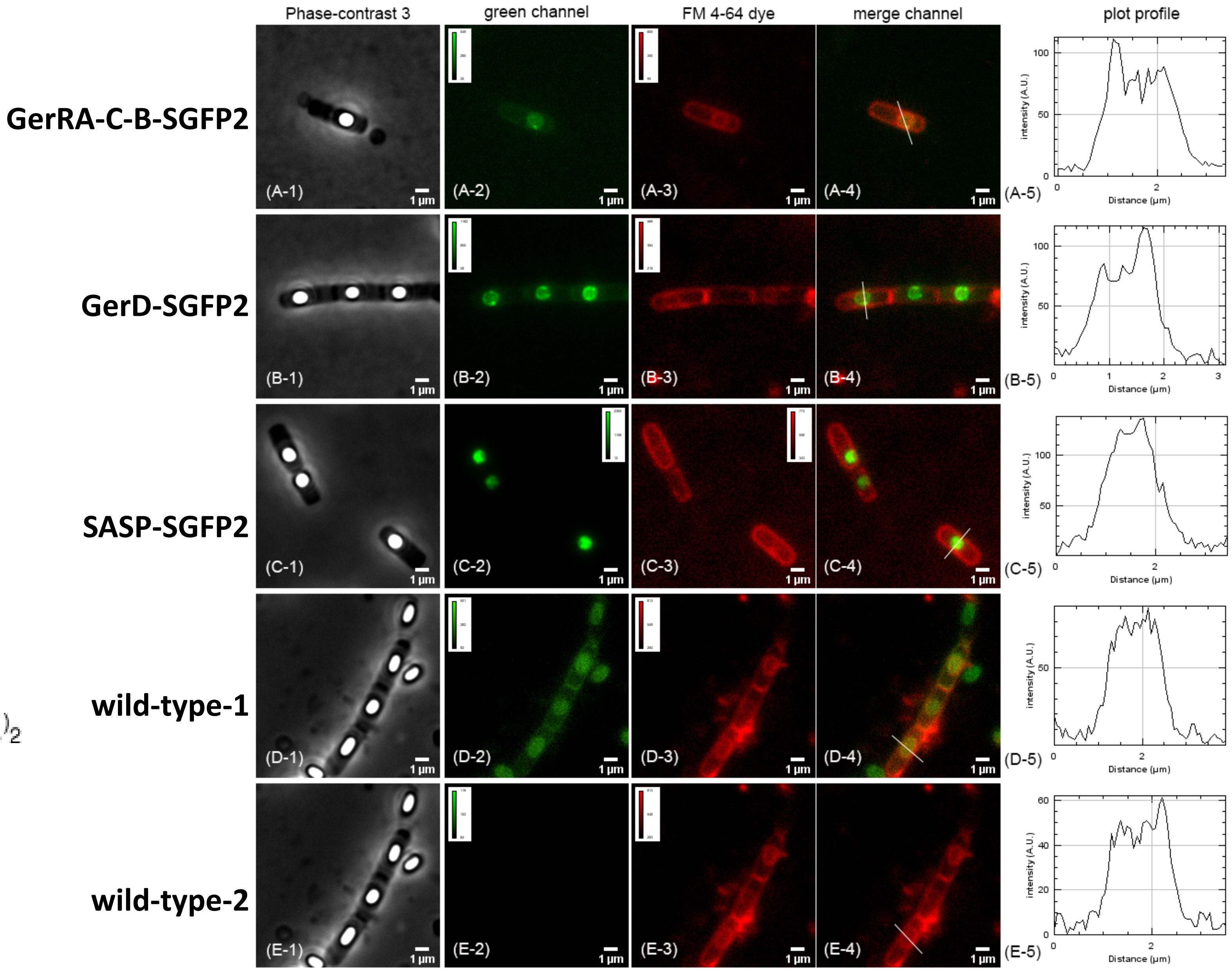
a. FM-4-64 stains the spore membrane during the sporulating process.

b. The germination receptor gerRB and GerD are present in the forespore membrane.

c. SASP are, in contrast, as expected localized in the core of forespore.



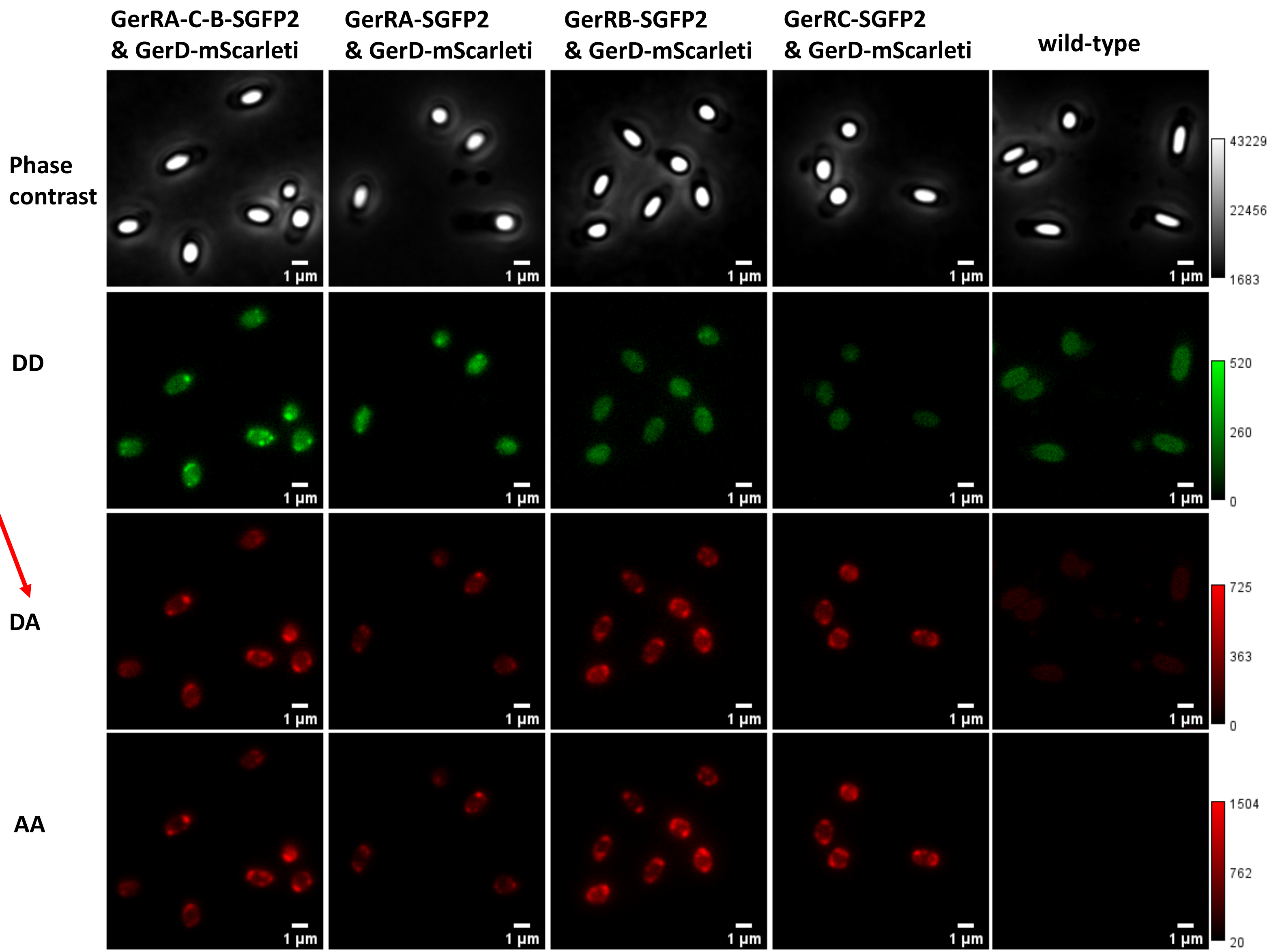
the chemical structure of FM 4-64



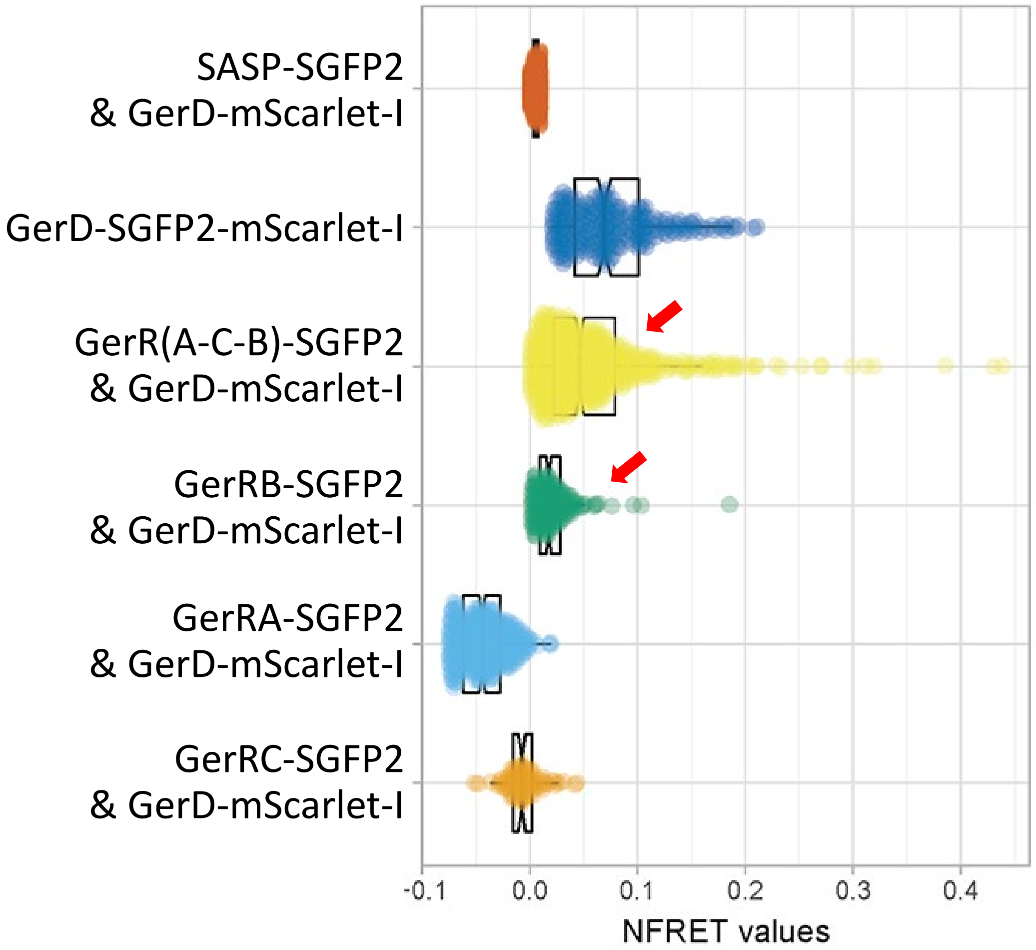
Protein-protein interactions in the inner spore membrane visualized through FRET

Definition

Channels	Excitation	Emission
Donor channel (DD)	Donor	Donor
FRET channel (DA)	Donor	Acceptor
Acceptor channel (AA)	Acceptor	Acceptor



Quantification of the FRET results of interaction between GerR and GerD



FRET efficiency was,

- 0% in biological negative control,
- 8% in biological positive control,
- 6% between GerR and GerD,
- 6% between GerRB and GerD,
- -4% between GerRA and GerD,
- -1% between GerRC and GerD.

Summary: GerD mainly interacts with the GerR B subunit, not with A or C- subunits.

Conclusions:

- There is a close interaction (< 10 nm distance) between GerRB and the GerD protein in *B. cereus* germinosomes.
- The germinant receptor proteins from the GerR operon and GerD were localized in germinosomes in the innermembrane of forespores using reporter proteins and FM 4-64 staining.

Published: Wang Y, Breedijk RMP, Hink MA, Bults L, Vischer NOE, Setlow P and Brul S. Dynamics of germinosome formation and FRET-based analysis of interactions between GerD and germinant receptor subunits in *Bacillus cereus* spores. Int J Mol Sci. 2021 Oct 18;22(20):11230. doi: 10.3390/ijms222011230.

The major *Bacillus* spore germination proteins and their location in the inner membrane

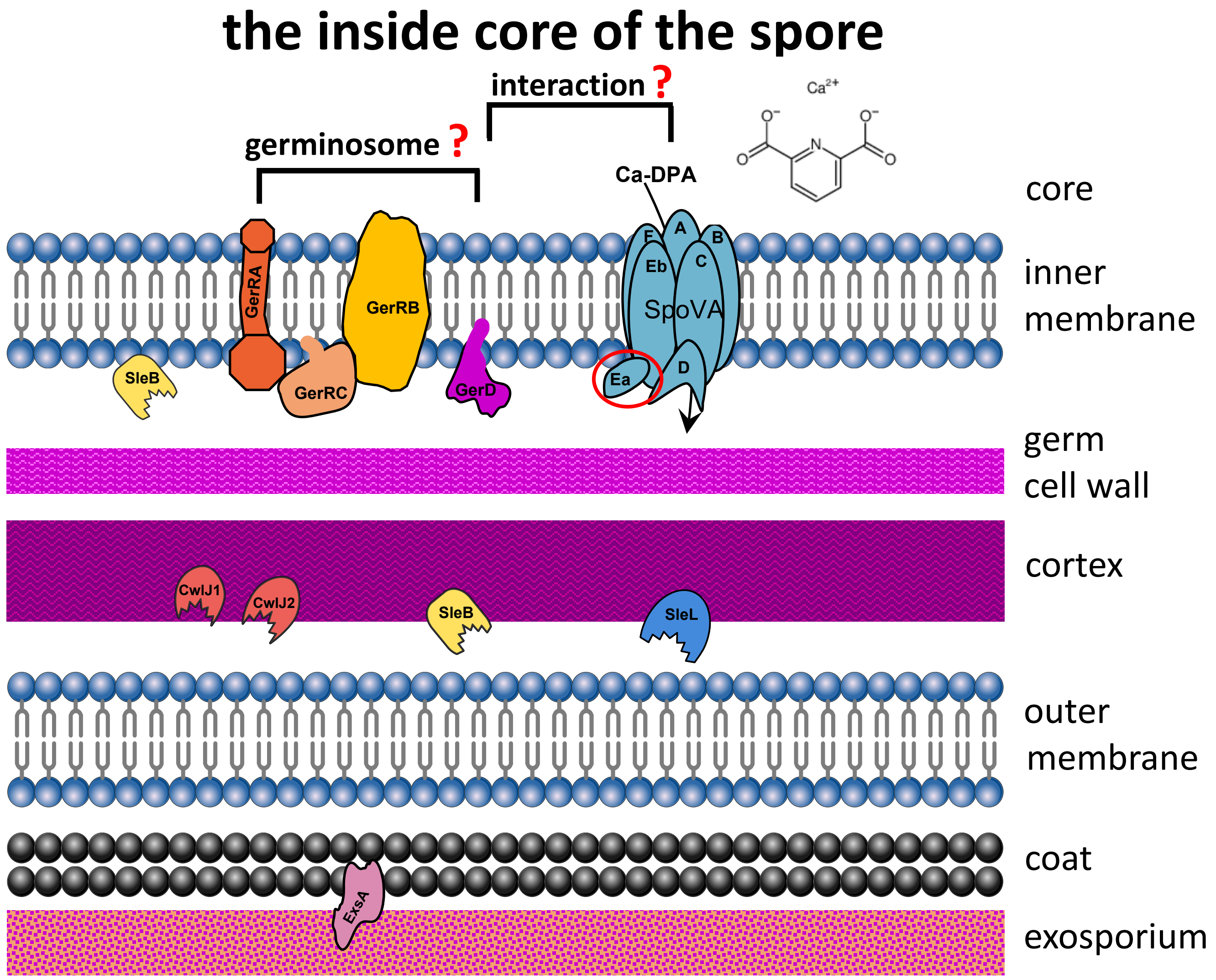
Germinant receptors

- **GerR** **A / C / B subunit**
- GerK A / B / C subunit
- GerI A / B / C subunit
- GerS A / B / C subunit
- GerQ A / B / C subunit
- GerG ? / B / C subunit
- GerL ? / B / C subunit

Germination scaffold protein: GerD

SpoVA (stage V sporulation protein A)

A / B / C / D / Eb / **Ea** / F subunit



Ca-DPA, Ca^{2+} and dipicolinic acid

Research questions (II)

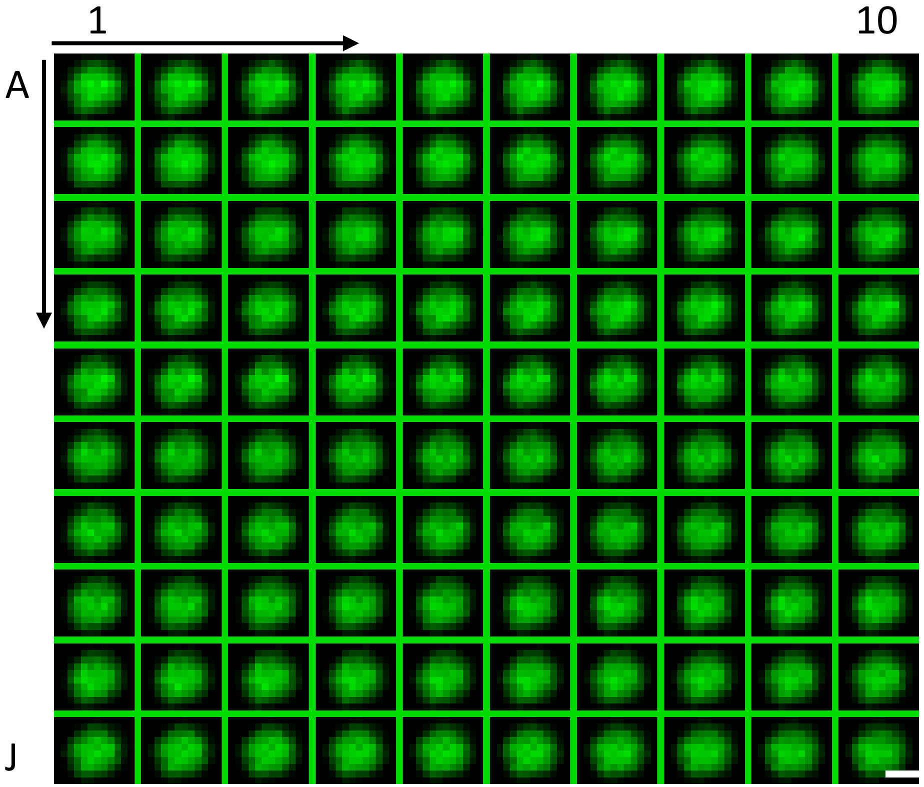
Visualization of SpoVAEa protein dynamics in dormant *B. subtilis* & *B. cereus* spores

Dynamic changes in the germinosome and SpoVAEa during germination of *B. cereus* spores

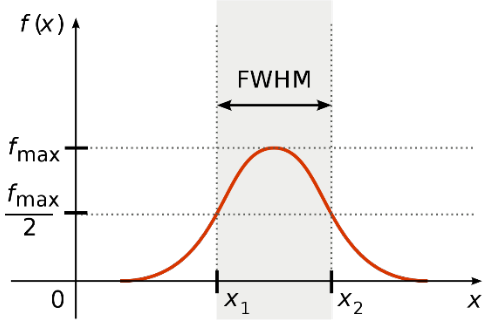
High frequency time-lapse images acquisition and analysis in *B. subtilis* and *B. cereus*

Capture condition: exposure 50ms,

record 100 frames and no delay between each frame



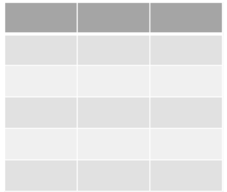
As an example frames of *B. cereus* spore with montage



Full width at half maximum (FWHM)

Measure the FWHM of each frame

with plugin Adrian's FWHM in ImageJ



Calculate the percent change of FWHM

$$(A2 - A1)/A1$$

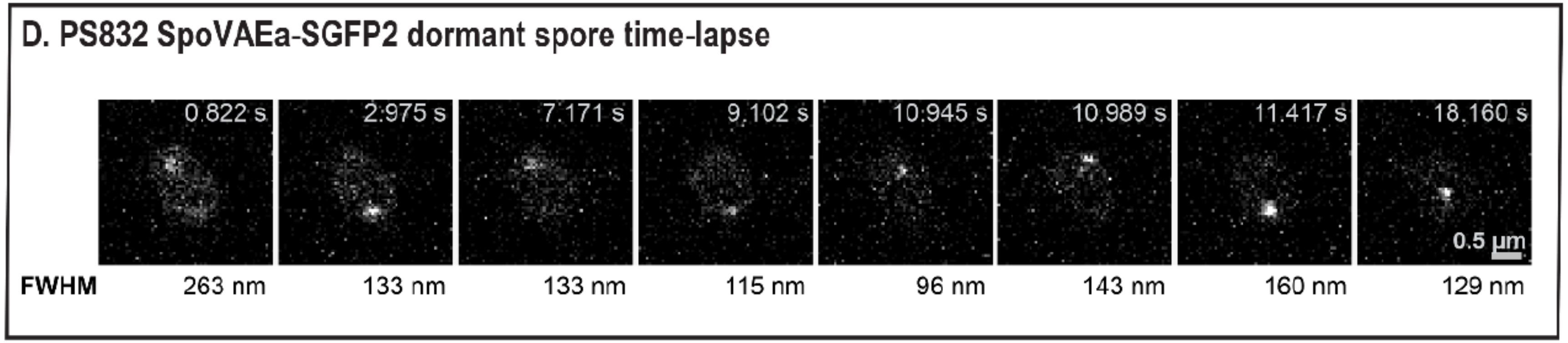
(A3, A4, A5,.............)

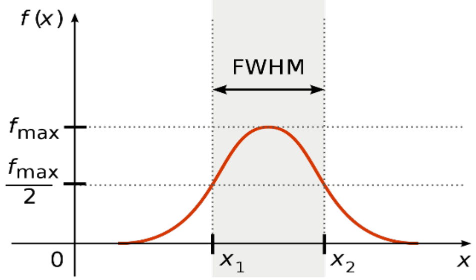
Layout of results

Super-resolution imaging of high frequency movement of SpoVAEa in *B. subtilis* spores

The RCM high frequency time lapse images (22.2 ms/frame) of the SpoVAEa-SGFP2 fusion protein of genomic expression in *B. subtilis* dormant spore.

RCM= Rescan Confocal Microscopy

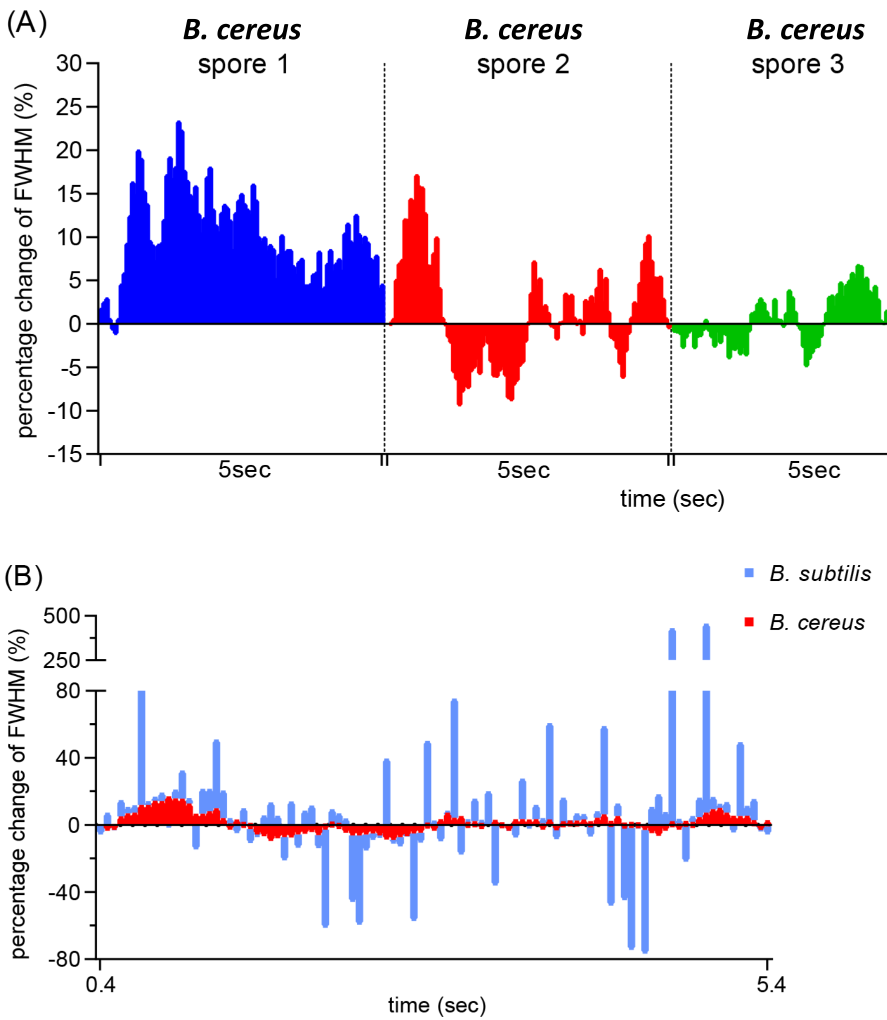




Full width at half maximum (FWHM)

Published: Wen J,Vischer NOE, de Vos AL, Manders EMM, Setlow P and Brul S. Organization and dynamics of the SpoVAEa protein and its surrounding inner membrane lipids, upon germination of *Bacillus subtilis* spores. Scientific Reports(2022) 12:4944
doi.org/10.1038/s41598-022-09147-3

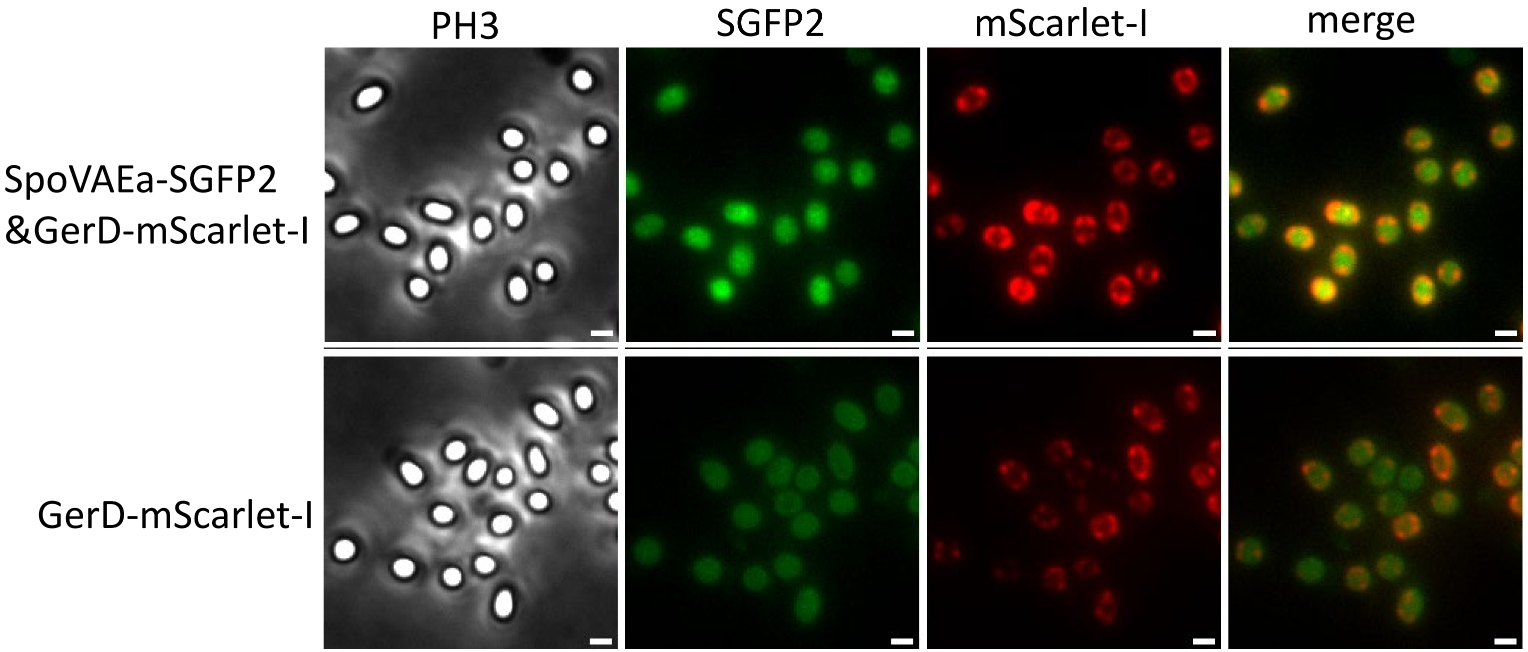
Comparing the mobility of SpoVAEa foci in dormant spore of *B. cereus* and *B. subtilis*

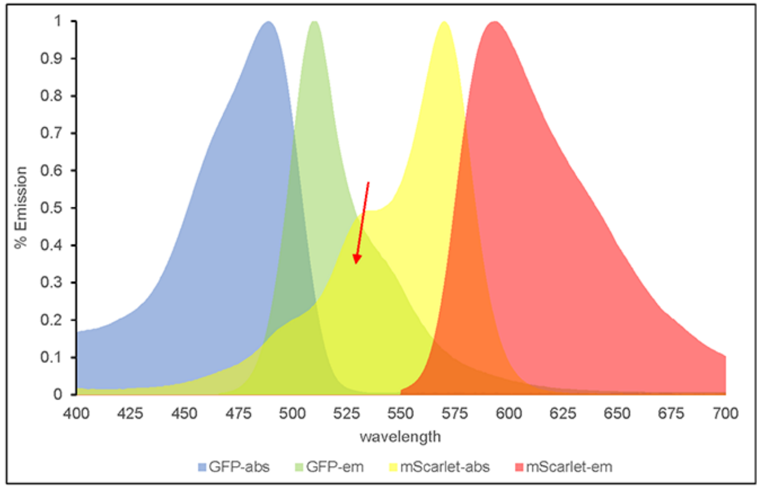


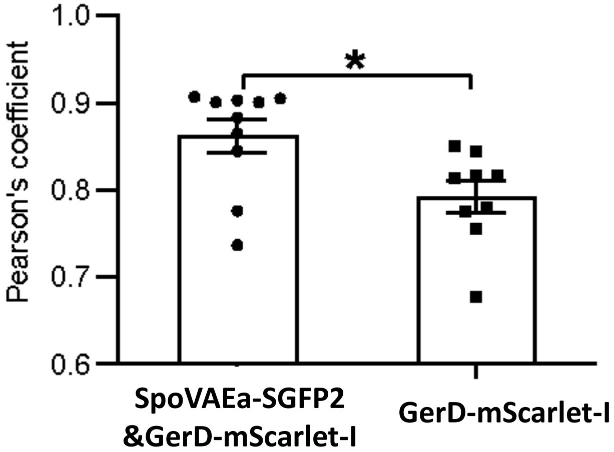
Summary:

- In *B. cereus* (panel A,B) and *B. subtilis* (panel B) the SpoVAEa foci of different spores behave differently.
- SpoVAEa fluorecent foci of *B. subtilis* spores* redistribute at a higher frequency than those of *B. cereus.*
- This difference may be due to the fact that we look at different species with a different protein complement and at genomic expression in *B. subtilis* versus overexpression from a plasmid in *B. cereus*.

Co-localization shown between SpoVAEa and germinosome GerD?



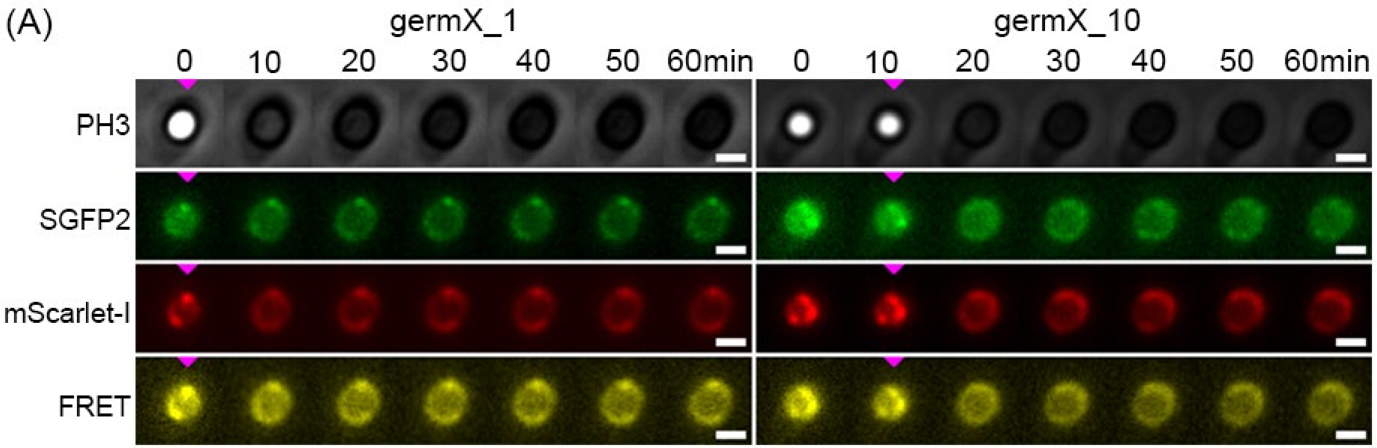


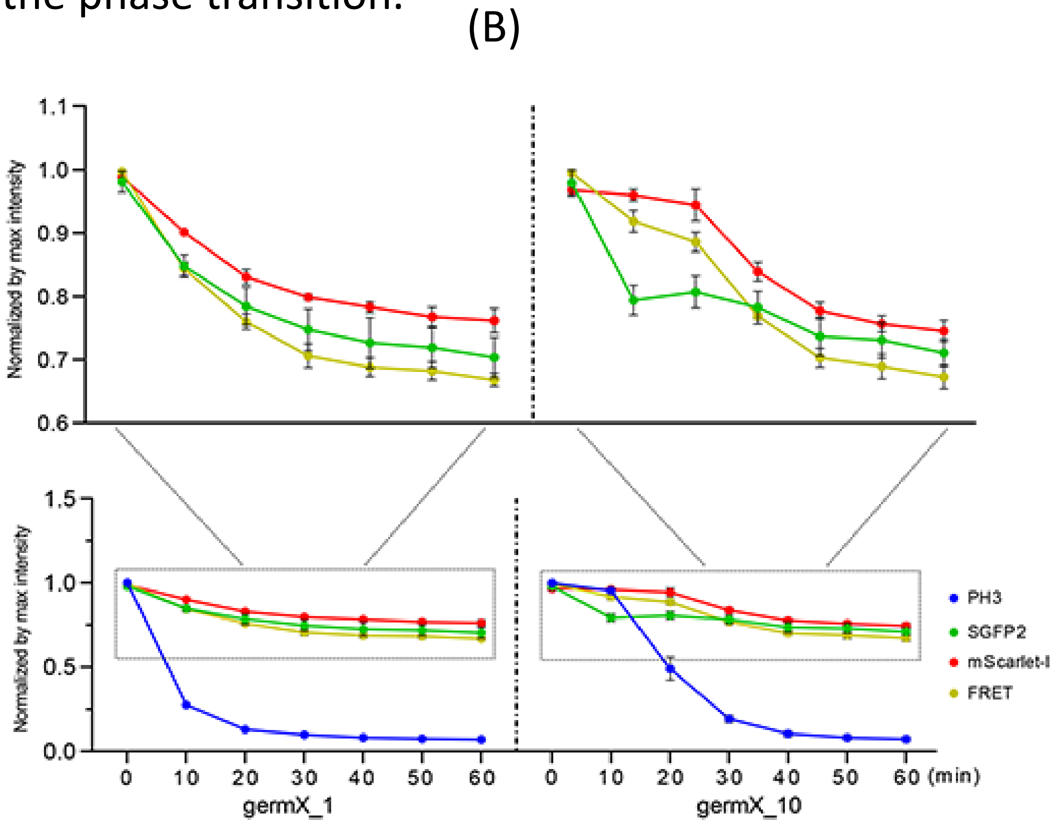


Dynamics of germinant receptor foci FRET and fluorescence upon germination triggering by L-alanine

Germinosome FRET foci GerRB-SGFP2 GerD-mScarlett *in B. cereus* spores

- were lost earlier than loss of SGFP2 foci and mScarlet-I foci,
-were lost to a large extent upon the phase transition,
-but the fluorescent germinosome foci as such persist well beyond the phase transition.



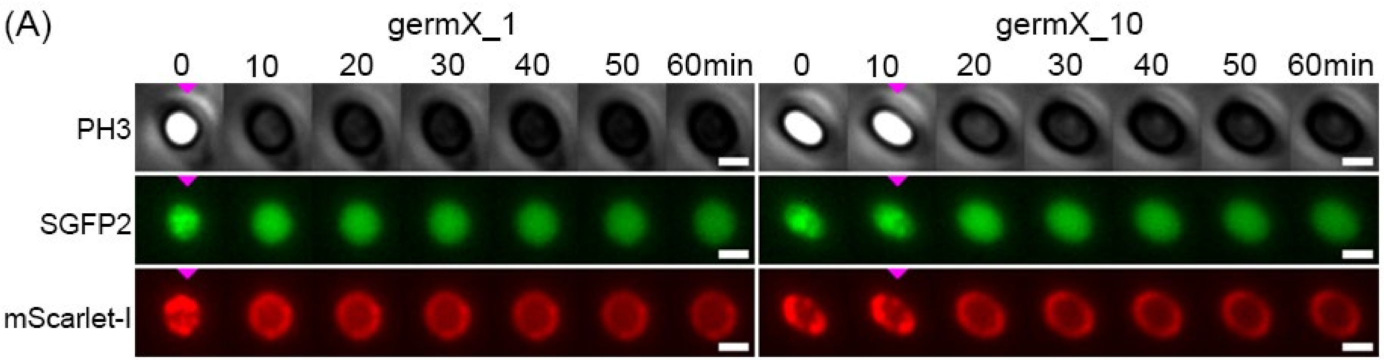


Dynamics of SpoVAEa and GerD proteins upon germination triggered by L-alanine in *B. cereus* spores

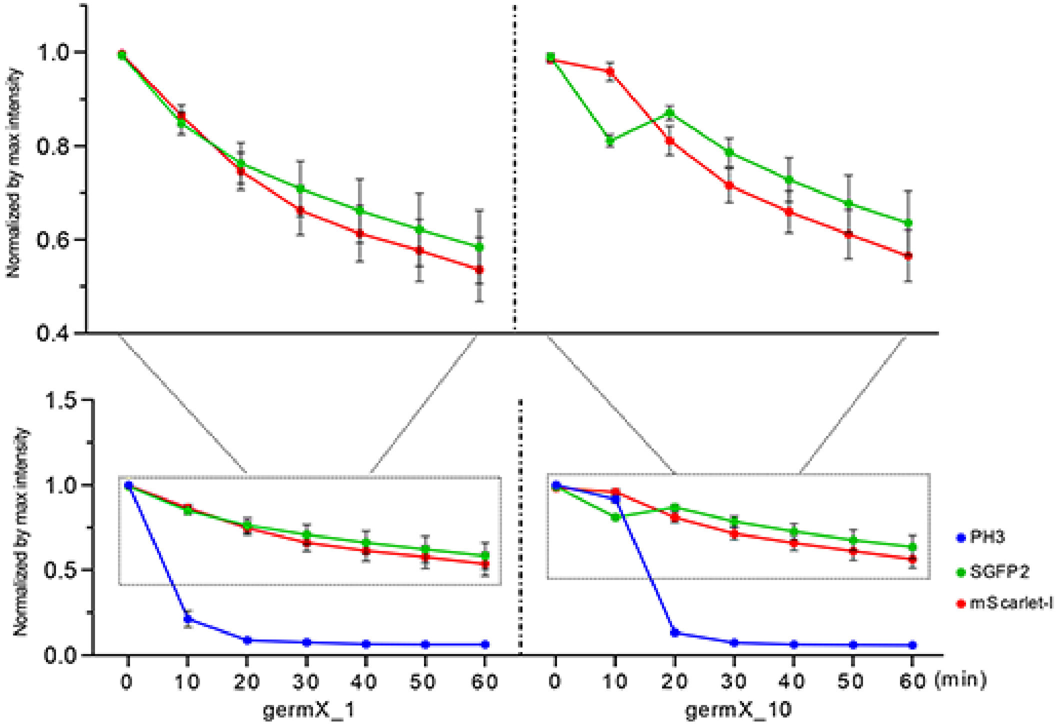
SpoVAEa-SGFP2 foci were lost upon phase transition.

GerD-mScarlet-I foci spread out and continued to exist beyond phase transition.

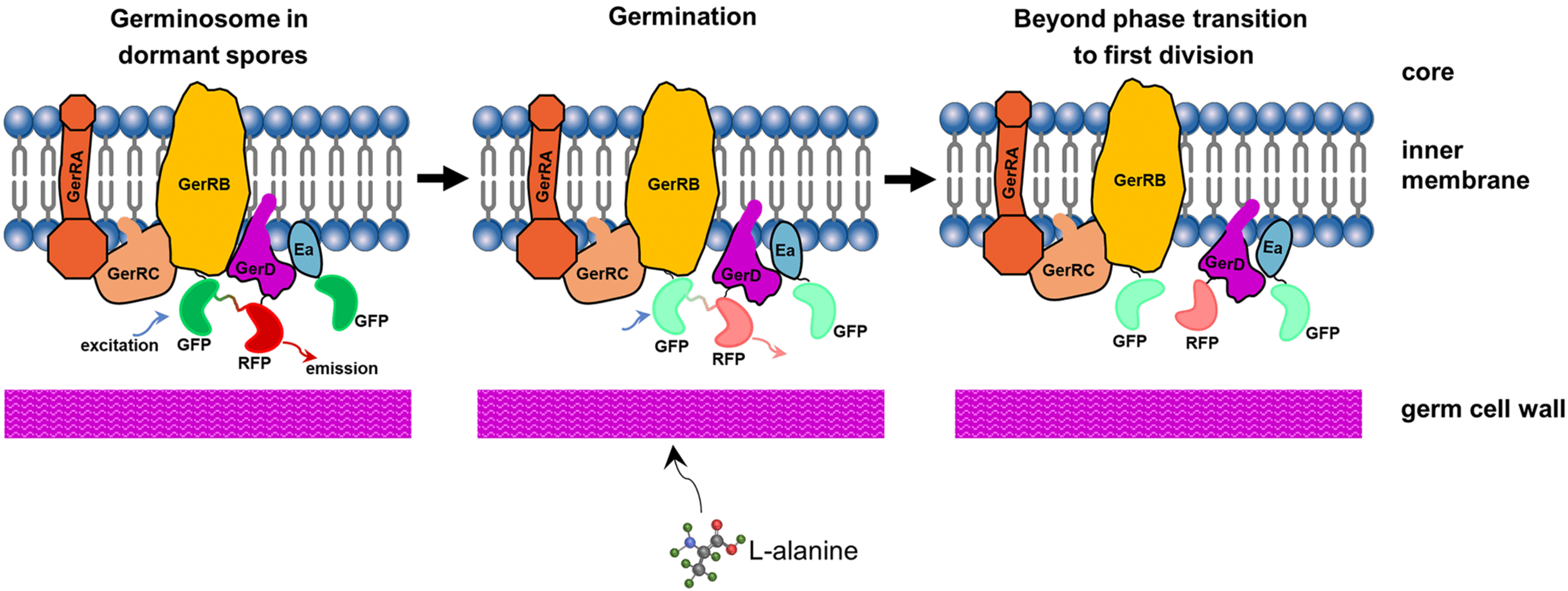
(A)



(B)



A model for the sequence of events during L-alanine induced spore germination in *Bacillus cereus*.



Published: Wang Y, Vischer NOE, Wekking D, Bogian A, Setlow P and Brul S. Visualization of SpoVAEa protein dynamics in doemant spores of *Bacillus cereus* and dynamic changes in their germinosomes and SpoVAEa during germination. (2022) Microbiology Spectrum 10 (3) doi.org/10.1128/spectrum.00666-22

Back to our model *Bacillus subtilis:*

International Journal of
Molecular Sciences

MDPI

Article

Predicting the Structure and Dynamics of Membrane Protein GerAB from *Bacillus subtilis*

Sophie Blinker [1], Jocelyne Vreede [2,*], Peter Setlow [3] and Stanley Brul [1,*]

Molecular modelling of the B-subunits' structure & experimental mutant analysis

ARTICLE

Check for updates

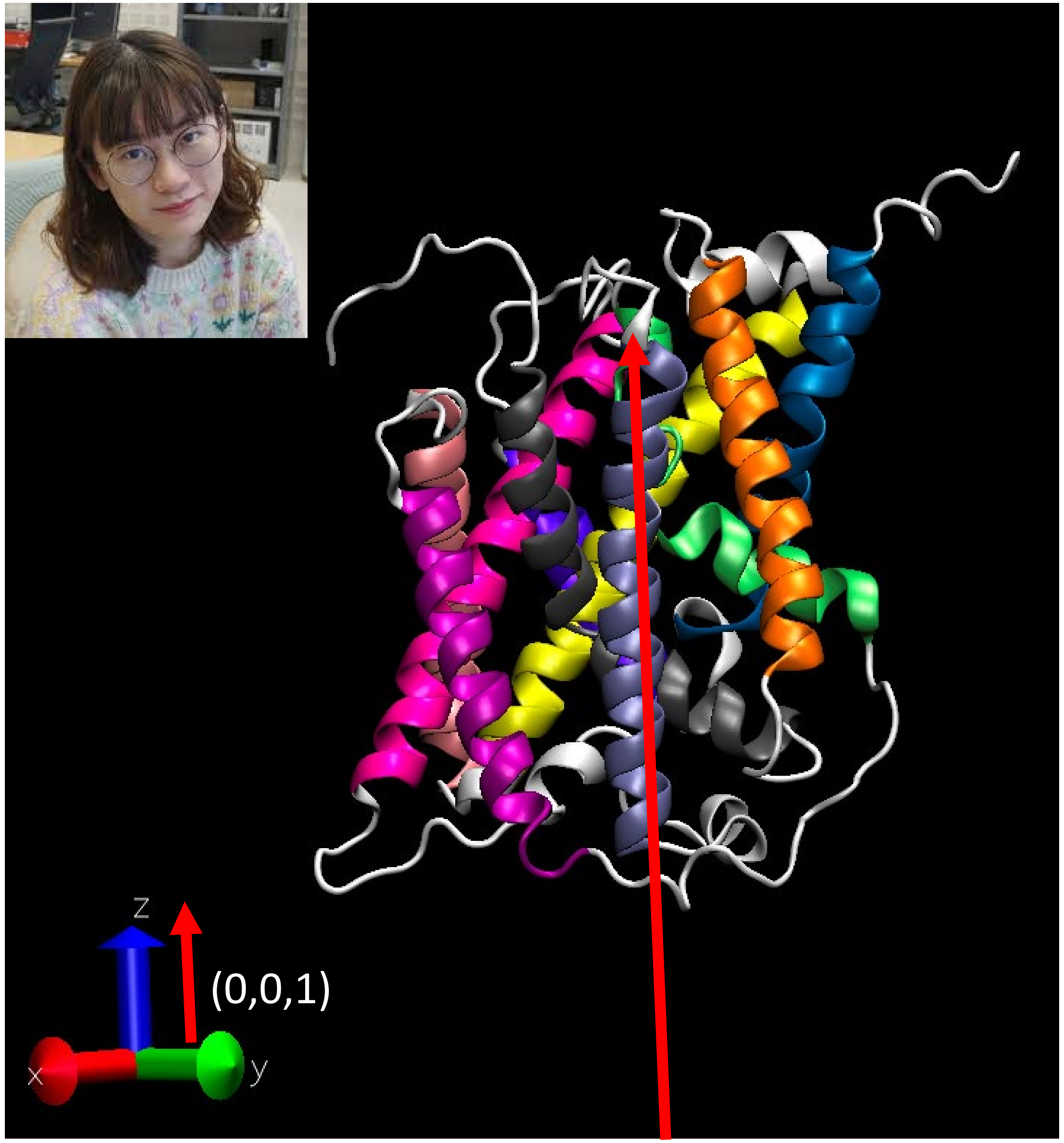
https://doi.org/10.1038/s41467-021-27235-2

OPEN

Dormant spores sense amino acids through the B subunits of their germination receptors

Lior Artzi[1], Assaf Alon[2], Kelly P. Brock[3], Anna G. Green[3], Amy Tam[3], Fernando H. Ramírez-Guadiana[1], Debora Marks[3], Andrew Kruse[2] & David Z. Rudner[1]

Current PhD student Longjiao Chen



Steered Molecular Dynamics shows a putative water channel

Take home messages and challenges ahead

I. First evidence of the existence of germinosomes in spores of the pathogen *B. cereus.*

II. GerD mainly interacts with GerR B subunit and not with A or C- subunits.

III. Dynamics data showed that SpoVAEa moves on the surface of the inner membrane and it might play a role in signal transduction between the germinosome, possibly through GerD, and the SpoVA channel.

IV. There are challenges ahead for bacterial spore germination analysis;
- exact composition of each germinosome,
- do GerAB subunits in *B. subtilis contain a water channel*-> Longjiao Chen
- is such a putative channel conserved in other Bacilli and Clostridia,
- how is the structure of gerAB, gerRB influenced by L-alanine binding, etc.

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Prof. Peter Setlow

Molecular Dynamics @ FNWI UvA

Dr. Jocelyne Vreede

LCAM-FNWI

Dr. ir. Mark Hink

Ing. Ronald Breedijk

Collaborate with us!

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Thank you for your attention.

Towards the understanding of the structure and function of germination receptor protein GerAB by combining computational and *in vivo* analyses.

Longjiao Chen[1], Jocelyne Vreede[2], Peter Setlow[3], Stanley Brul*[1]

[1] Molecular Biology and Microbial Food Safety Group, Swammerdam Institute for Life Sciences, University of Amsterdam, the Netherlands.

[2] Computational Chemistry Van 't Hoff Institute for Molecular Sciences, University of Amsterdam, the Netherlands.
[3] Molecular Biology and Biophysics, UCONN Health, USA.

* s.brul@uva.nl

Keywords: germination, germinant receptor, water channel, *Bacillus subtilis*, molecular dynamics.

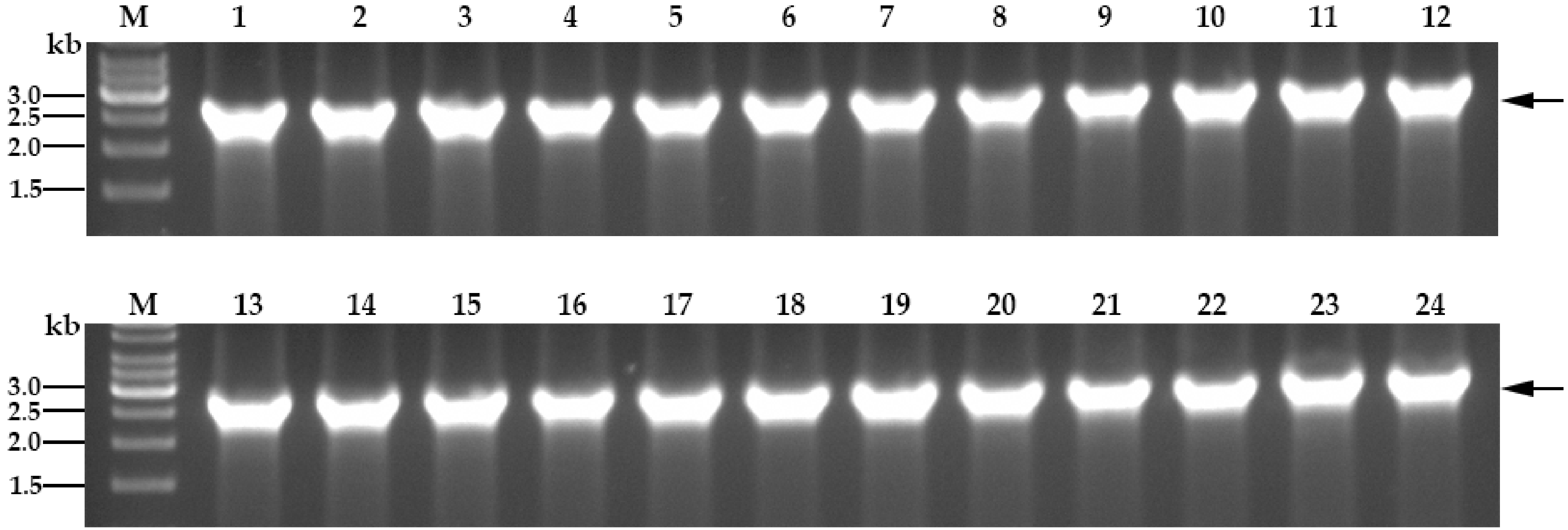
Abstract (<300 words):
Some species in the *Bacillales* and *Clostridiales* bacterial orders form spores in unfavorable environments. These spores are metabolically dormant, resistant to harsh conditions, and their core, analogous to growing cells' protoplast, has a water content between 25-45% of wet wt compared to the ~80% in vegetative cells. However, upon germination by a variety of germinants, spores take up water relatively rapidly, restoring their water content to that of growing cells.[1] With no known functional water channels in the model spore-forming organism *Bacillus subtilis*, the molecular mechanism of spore water uptake during germination is unknown. Recent work showed that a subunit of the prototypical *Bacillus subtilis* spore germinant receptor GerA, the integral inner membrane protein GerAB, is the sensor initiating spore germination in response to L-alanine.[2] Notably previous work found that GerAB contains what appears to be a water channel.[3] Using Molecular Dynamic (MD) simulation methodology, we found water passing through the GerAB protein *in silico*, indicating the proteins putative water channel may well be functional.[3] At the same time, utilizing Steered MD simulation, we can now also pull single water molecules through the GerAB channel and therefore calculate the free energy of water permeation. These computational methods, as well as the predicted GerAB structure, have provided us with indications of GerAB residues that may be crucial in the water channel's function, and thus suggest mutagenesis experiments to experimentally test *in vivo*the computational modelling predictions. Here we present both the *in silico* and *in vivo* data that reinforce each other and thus jointly help to elucidate the function of GerAB as a water channel.

References

1. Christie, G. & Setlow, P. Bacillus spore germination: Knowns, unknowns and what we need to learn. Cell Signal 74, 109729 (2020).
2. Artzi, L. et al. Dormant spores sense amino acids through the B subunits of their germination receptors. Nat Commun 12, 6842 (2021).
3. Blinker, S., Vreede, J., Setlow, P. & Brul, S. Predicting the structure and dynamics of membrane protein GerAB from *Bacillus subtilis*. Int J Mol Sci 22, 3793 (2021).

Validation that spores of all transformants carry the plasmid

1. Spores from 24 independent colonies isolated and lysed
2. PCR run on lysate to identify plasmid DNA, and to give an ~ 2.6 kb fragment
3. PCR products run on gel electrophoresis and stained



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