‘Omics’, germinosome visualization and antimicrobial resistance of bacterial spores

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(post-doc)
Focus of today:

1) How do we study proteins involved in resistance & germination?

2) What is the mode of action of human derived antimicrobial peptides?
1) How do we study proteins involved in resistance & germination?

In order to enable comprehensive monitoring of spore proteins and protein levels we have developed a ‘one pot’ spore processing method for mass spectrometric analysis of proteins from all spore layers. The method is applicable to *Bacillus subtilis*, *B. cereus* and *Clostridium difficile*.

Remember the talk yesterday by Bhagyashree Swarge!

"One-Pot" Sample Processing Method for Proteome-Wide Analysis of Microbial Cells and Spores

*Bhagyashree Nandakishor Swarge,*† *Winfried Roseboom, Linli Zheng,*
*Wishwas R. Abhyankar, Stanley Brul, Chris G. de Koster,* and *Leo J. de Koning*
The obtained ‘one pot’ proteomics data represent a broad class of functional groups & proteins from all spore layers ($\sigma_F \sigma_G$ fore-spore & $\sigma_E \sigma_K$ mother cells).

(A) Over 1500 proteins have been identified in wild-type B. subtilis spores. These proteins belong to various functional categories with proteins related to the Translation & Metabolism processes and proteins with unknown functions being the largest contributors. These proteins can provide the machinery to initiate germination.

(B) The protein identification using the ‘one pot’ method spans throughout the spore layers as seen by their sigma factor regulation with $\sigma_K$ and $\sigma_E$ regulating proteins from the coat layers while $\sigma_G$ and $\sigma_F$ regulating proteins from the core and the cortex layers.
Latent metabolic pathways in dormant spores (shared proteins)

Glycolysis (16/18)

Amino acid biosynthesis (71/104)
1. It appears that the dormant spores already host a ‘minimal set of’ proteins / essential metabolic pathways. This implies that after hydration the energy metabolism can be activated before protein synthesis begins (Swarge et al. 2018).

2. Simple, robust method of sample processing.

3. Well suited for time resolved quantitative elucidation of structural, morphological and physiological changes during spore germination and outgrowth (use of $^{15}$N labelling). Talk Bhagyashree Swarge yesterday!

4. Key to the initial events in germination is single spore protein activity analysis.

Conclusions

For absolute protein quantification see our recent publication on QconQAT publication.
Spore germination is heterogeneous

Abhyankar et al. (2018) Food Microbiology
Omardien et al. (2018) Sci. Reports (Spore-TrackerX under revision)
**B. subtilis spore germination**

1. Nutrient binding to the germination receptors (GRs) in the inner membrane (IM),
2. Signal transduced from GRs to SpoVA channel,
3. Ca-DPA in the spore core is replaced by water

Germinosome studies at single spore level (with Peter Setlow)

- GerD, the scaffold protein of the germinosome

- Germinant receptors (functional components) in the germinosome

  Operon of germination proteins
  Pandey, 2014 PhD thesis
Presence of ‘germinosomes’: Work flow

- Super-resolution microscope
  Structured Illumination Microscopy (SIM)
  (Subdiffraction resolution <100-110 nm)
- Imaging Z stack

Juan Wen (PhD student)

SIMcheck, ImageJ plugin

Raymond Pasman (MSc student)
Germinosome foci number

GerKB-mCherry  GerD-GFP

Z1  
Z2  
Z3  
Z4  
Z5  

SIM Z-stacks of germinosome foci

Percentage of spores with various number of germination foci

Percentage (%)  
0  20  40  60  80

0  1  2  3  4  5

Foci per Spore

GerKB-mCherry  GerD-GFP
SpoVA channel (puzzling preliminary data)

SpoVAD-sGFP seems to be also present in foci
SpoVAEa-sGFP seems to be also present in foci.....
needs further experimentation using STORM!
Open questions:

1) What is the exact germinosome composition in the *Bacillus* spores and is there a link with germination heterogeneity?

2) Where in the membrane are germinosomes and visualize the germinosome SpoVA channel proteins interaction?

3) Are germinosomes present in *B. cereus* and other spore formers?

FM 4-64 dye
Higher affinity to membrane lipid areas with high fluidity

*B. subtilis* IM, stained by FM 4-64, showed brighter spots in SIM Z-stack image i.e. regions of high fluidity; colocalization with germinosomes?
2) What is the mode of action of human thrombocidin derived antimicrobial peptides? (with Dr. Bas Zaat Academic Medical Center at the Univ. of Amsterdam)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Charge</th>
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<tr>
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<td>BP2</td>
<td>+7</td>
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Circular Dichroism data suggest some α-helical conformation in the presence of membrane mimetics.

Soraya Omardien (PhD viva Friday!)

Positively charged (cationic)
Amphipathic (hydrophilic and hydrophobic)
Prevent spore outgrowth if present during germination
Peptides target germinated spores
- Targets the spore inner membrane

Effect on *B. subtilis* spores studied with labelled TC-84

N-terminal Alexa labelled TC-84

30 min of treatment

**Alexa488-TC84**

PC | Alexa488 | Magnified
---|---|---

**Alexa488-TC84 with TC84**

PC | Alexa488 | Magnified
---|---|---

**Alexa488-TC84 with TC84 - SIM**

Nile red | Alexa488 | Merged
---|---|---

ESC 2018
Membrane fluidity effects of the peptides on outgrowing cells analysed with Laurdan

\[
\text{Laurdan } \text{GP} = \frac{I_{460} - I_{520}}{I_{460} + I_{520}}
\]

Emission intensity

Wavelength (nm)

Subburaj et al (2000) DOI: 10.1002/9780470027318.a9350

ESC 2018
Membrane fluidity changes using Laurdan

5 min of treatment

Peptides create large fluid domains in a rigid bulk membrane.
Alexa Fluor 488 labelled TC84 - Laurdan

Alexa 488-LRAMCIKWWSGKHPK

5 min of treatment

Peptide likely present at the created fluid domains.
Alterations made to the membrane causes membrane bound proteins involved in cell wall synthesis to delocalize.

5 min of treatment
At the peptide created fluid domains membrane invagination and loss of integrity is observed.

**B. subtilis** wild type 168

**B. subtilis** PrpsD-sfGFP
green fluorescent protein under control of ribosomal protein S4 promoter

5 min of treatment
Conclusions & summary

• TC19, TC84 and BP2
  • Bactericidal, preventing spore outgrowth
  • Active against spore inner membrane
  • Create fluid domains in vegetative cell membranes
  • Likely present at the fluid domains
  • Delocalize membrane bound proteins (involved in cell wall synthesis)
  • Cause membrane invaginations and (some) cytosol leakage
  • Internalization (slow) of peptides occurs
  • Induce cell wall and cell membrane stress response
  • Perturb the membrane without visible cell wall damage*
Model

Currently under revision at BBA Biomembranes; Omardien PhD defense April 20, 2018
Acknowledgements

Erik Manders
Norbert Vischer
Ronald Breedijk
Raymond Pasman
Van Leeuwenhoek Centre for Advanced Microscopy

Wishwas Abhyankar (post doc at UvA)
Bhagyashree Swarge
Linli Zheng
Zhiwei Tu
Xiaowei Gao

Leo de Koning
Chris de Koster
Mass Spectrometry of BioMacromolecules

Gertien Smits (pHi work)
Jan Smelt (recently turned 80!-ICPMF ‘17)
Rachna Pandey (now post-doc Ireland)
Juan Wen
Richard de Boer
Yan Wang
Soraya Omardien
Bas Zaat

Stanley Brul
Molecular Biology and Microbial Food Safety
Leendert Hamoen & coworkers
Microbiology (Bacterial Cell Biology)

Peter Setlow & coworkers
Molecular Biology and Biophysics;
UConn Health, USA
spores are cool

bacillus subtilis

Thank you
Intact spores are disrupted by zirconium bead beating in 8M urea under reductive conditions to inactivate proteases and break S-S bonds.

Disrupted spores are subjected to LysC digestion, diluted to 1 M urea and further digested with trypsin (C–side R/K specific).

The peptide mixture is pre-fractionated by Zwitter IoniC Hydrophilic Interaction Liquid Chromatography (ZIC-HILIC) in 10 fractions.

Each fraction is then analyzed with reverse phase LC FTMS/MS and the combined processed data is searched against the bacterial protein database with MASCOT.

Urea at 8 M prevents untimely degradation.
MIC curves of Alexa488-TC84

MIC curves of TC84
Transcriptomics shows that peptides induce cell wall stress response systems

Extracytoplasmatic function Sigma Factors

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bacitracin, LL-37, PLL, vancomycin, enduracidin, moenomycin, fosfomycin, cerufloxime, cephalosporin, lysozyme

Two component systems

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Bacitracin

**Bacitracin Structures**

- **Bacitracin A:** as Shown
  MW 1422

- **Bacitracin B/C:** Substitute Val for Ile
  MW 1408

- **Bacitracin C/D:** Substitute 2 Val for Ile
  MW 1394

**Bacitracin F, G, H:**
From Oxidative degradation of Bac A, B, C respectively contain a thiazole ring
LL37

Gram negative

A  LL-37

18  25  37
19  23  29
LLGDFFRKSFKEKIGKEFKRIVQRKDFLRLNFLPRTE

MIC 5 μM

B  GF-17

Gram positive

F  Gram positive

G  Autolysin release

LPS

LTA

Pep5, RTD2

OM

PGN

UppP

MLT

Lactococcin G

Enterocin 1071

Gravicin ML

Barrel-stave model

Dermcidin, Regllα

Toroidal model

LL-37

Carpet model

Piscidins, LL-37

IM

Replication

DNA

RNA polymerase

Transcription

RNA

Translation

mRNA

Protein synthesis

X

X

Bac7(1-35)

Apidaecins

Oncocins

MccJ25

Binding to 70S