



Proteomics and microscopy tools for the study of antimicrobial resistance and germination mechanisms of bacterial spores



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ABSTRACT

Bacterial spores are ubiquitous in nature and can withstand both chemical and physical stresses. Spores can survive food preservation processes and upon outgrowth cause food spoilage as well as safety risks. The heterogeneous germination and outgrowth behavior of isogenic spore populations exacerbates this risk. A major unknown factor of spores is likely to be the inherently heterogeneous spore protein composition. The proteomics methods discussed here help in broadening the knowledge about spore structure and identification of putative target proteins from spores of different spore formers. Approaches to synchronize *Bacillus subtilis* spore formation, and to analyze spore proteins as well as the physiology of spore germination and outgrowth are also discussed. Live-imaging and fluorescence microscopy techniques discussed here allow analysis, at single cell level, of the 'germinosome', the process of spore germination itself, spore outgrowth and the spore intracellular pH dynamics. For the latter, a recently published improved pHluorin (IpHluorin) under control of the *ptsG* promoter is applicable. While the data obtained from such tools offers novel insight in the mechanisms of bacterial spore awakening, it may also be used to probe candidate antimicrobial compounds for inhibitory effects on spore germination and strengthen microbial risk assessment.

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1. Introduction

The diseases and spoilage caused by bacterial spore formers are commonly associated with thermally processed foods. The

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endospores, formed by Bacilli, Clostridia and related organisms, are able to survive physical and chemical preservation treatments, such as pasteurization or addition of weak organic acids as preservatives (Hornstra et al., 2009; Setlow, 2006) that are commonly used to prevent unwanted bacterial growth in food products. In fact, after escaping destruction, germinated spores can repair incurred damage followed by outgrowth and thus cause food decay. Therefore, spore removal and/or understanding the spore's germination and outgrowth systems, is a daunting task for the food industries. The spore's stress resistance is in part due to its multi-layered structure (Fig. 1). The different layers of bacterial spores, the exosporium, coat, outer membrane, cortex, inner membrane (IM), and the core, are all comprised of proteins. These help to provide protection against a variety of stresses. The extensive cross-linking between the proteins in the outermost layers of spores is one of the factors that ensure spore longevity. The process of germination, being heterogeneous, builds an additional degree of challenge for the prediction of microbiological food stability. The current industrial focus is on spore-inhibition by 'fail-safe' processing which in practice often results in over-processing of food. In contrast, the consumer's wish is such that food has to be mildly processed leading to better organoleptic qualities and nutritional values. Notwithstanding this wish, food products should remain, microbiologically, of optimal stability and safety. To that end, food industries and health sectors undertake risk assessment procedures. To come to robust models for optimal food processing, accurate qualitative and quantitative data are required, preferentially based on mechanistic insights. We are in the fortunate circumstance that it is now increasingly possible to acquire such data sets and thus facilitate the generation of increasingly robust models to optimize food quality whilst maintaining the necessary microbiological food stability.

Even though spores have been a topic of research for many years, researchers as well as industries could for long not answer (i) whether all the players important for spore resistance and germination have been identified, (ii) if it is possible to make homogeneous spores under varying environmental conditions to uncouple the effects of sporulation medium and of germination condition on spore heterogeneity, (iii) if it is possible to perform time resolved mechanistic analyses of single spores upon germination and outgrowth and, (iv) if one can improve robustness of microbiological risk assessment by analyzing the mode-of-action of preservation strategies. This review outlines how a combination of different proteomics and microscopy based research tools can help answering these questions and provide detailed insights into the spore's integrity and the molecular physiology of spore

germination.

2. Spore proteomics

Proteins are the key players in spore structure making up approximately 75% of spore dry weight in *B. subtilis* (Murrell, 1969). Hence, the identification and characterization of spore proteins is of prime importance. The earliest characterizations of spore proteins date back to the 1960s (Delafield et al., 1968; Somerville et al., 1970; Torriani and Levinthal, 1967). Since then many proteomic studies, predominantly focusing on spore surface layers, have helped to identify over 90 proteins from the spore outer layers of *B. subtilis* (Driks and Eichenberger, 2016). Though there have been numerous efforts in this field, these have either involved the use of complex, multi-stepped and/or gel-based separation methods (Kuwana et al., 2002; Lai et al., 2003) or have focused on specific layers of spores. In that regard, it is noticeable that the inter-protein cross-linking between proteins from spore coats makes it impossible to use gel-based methods that are commonly applicable to soluble protein fractions from cells. Thus developing a method to incorporate both the insoluble as well as the soluble protein fraction from spores has remained a challenge for years. In 2011, a gel-free protocol was proposed, that allows protein characterization of the insoluble fraction of the *B. subtilis* spore coat (Abhyankar et al., 2011). This was rapidly followed by an extension of the method to proteomics of *B. cereus* ATCC 14579 and *C. difficile* 630 spores (Abhyankar et al., 2013). The method, being independent of gels, has identified many new proteins from all three spore forming bacteria, providing novel information and potential new target proteins for spore identification such as SpoIVA, CotJc, Gpr, Tig and exosporium proteins such as BclA, lunH and ExsFB. A powerful example of how such protein analysis can be tailored for clinical purposes is the recently published work about proteomics driven identification of diagnostic peptides for the *B. cereus* group of spores (Pfrunder et al., 2016). In addition the studies paved the way for Quantitative concatenated (QconCAT) peptides based absolute spore coat protein quantification, as was recently reported for *B. cereus* by Stelder et al. (2018). Protein stoichiometry and absolute abundance of spore coat proteins are crucial parameter for diagnostic assays. The spore IM, that harbors the proteins and enzymes essential for germination including the germinant receptors (GRs) (Ross and Abel-Santos, 2010), is another important layer that has remained relatively 'unexplored'. Recently, Zheng and colleagues adapted a pre-existing method (Paidhungat and Setlow, 2001) and combined it with the gel-free approach, to identify > 100 predicted

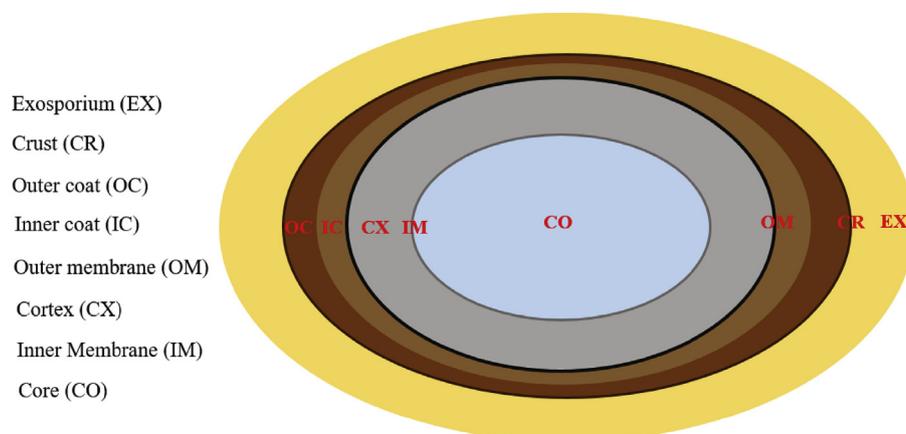


Fig. 1. Multi-layered structure of bacterial endospores.

membrane proteins (Fig. 2). The most surprising findings concerning the composition of this selective permeability barrier have been presented in their study (Zheng et al., 2016). Mass spectrometry based proteomics methods have also allowed us to study the effect of environmental conditions on the spore protein composition and spore structure (Abhyankar et al., 2016; Stelder et al., 2015). Regardless of these efforts, factors like complexity and heterogeneity of the sample, sensitivity of the method and sample loss during processing are still a challenge to the field of spore proteomics. To tackle these issues, a new and generally applicable 'one pot' sample processing method has now been developed that achieves mass spectrometric identification of proteins throughout all the layers from spores thus reaching well over 1000 protein identifications from whole spores and vegetative cells (Swarge et al., manuscript under final revision). These include, among others, the structural spore proteins, enzymes, GRs, as well as ribosomal proteins. For *B. subtilis* the list includes the proteins encoded by genes that are predicted by SubtiWiki (Michna et al., 2016) to be transcribed under control of mother cell specific transcription factors σ^E and σ^K , but also of genes under control of the forespore specific transcription factors σ^F and σ^G (Fig. 3). This one-pot method is extremely efficient since sample loss is minimized by carrying out all processing steps in a single tube. Research and development of such methods is needed to enable detailed time resolved monitoring of the changes in the proteomes both during sporulation as well as germination. Such analyses will facilitate the discovery of current 'missing links' such as the processes involved in coat-crosslinking and mechanisms involved in the turnover of the germinosome constituents during germination.

3. Spore heterogeneity

Sporulation and spore germination are both highly controlled and complex processes. Within a population, where one cell decides to sporulate the other cell may not necessarily follow that path. In fact the phenomenon of cannibalism in *Bacillus* sporulation is well known (Gonzalez-Pastor, 2011). Similarly, on renewed availability of nutrients not all spores germinate at the same time. It is unclear though whether this is a strategy to ensure survival of the whole population in case of rapid environmental changes. The spore germination machinery is thought to be conserved between spore forming bacteria. However, recent studies have exposed significant differences between the germination of spores of *Clostridium perfringens* and that of spores of a number of *Bacillus* species (Paredes-Sabja et al., 2011). Germination studies conducted so far have concluded that GRs, cortex-lytic enzymes (CLEs), and SpoVA

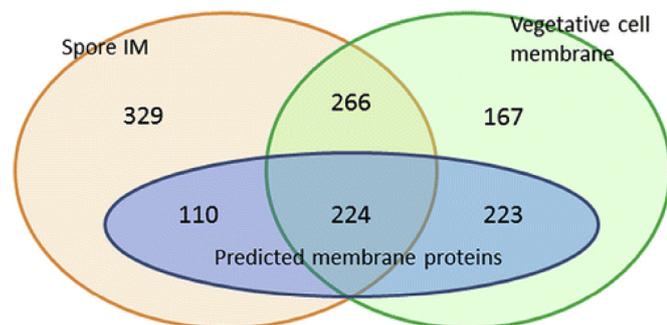


Fig. 2. Venn diagram of proteins identified from the isolated *B. subtilis* spore IM isolation and vegetative cell membrane fractions. Membrane proteins were predicted by several bioinformatics tools. Reprinted with permission from (Zheng et al., 2016) Copyright 2016, American Chemical Society.

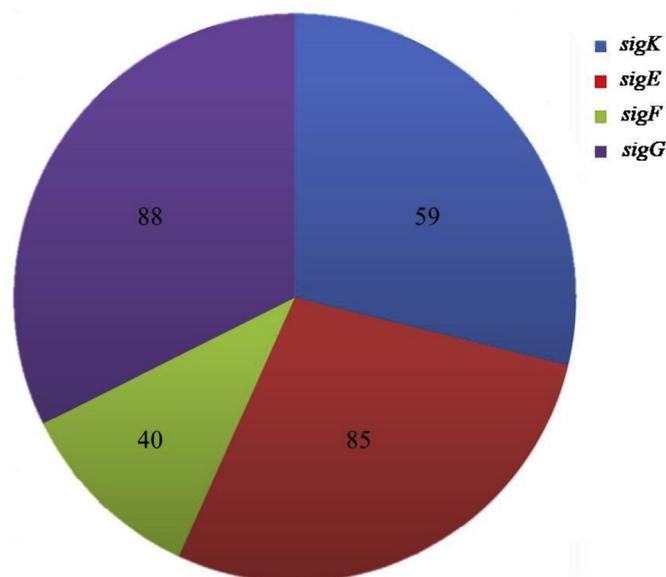


Fig. 3. Classification of numbers of identified proteins based on the different spore-specific sigma factors that regulate their expression. The image shown only considers subsets of proteins for which the regulons are predicted by SubtiWiki.

channels are critical for the process (Wells-Bennik et al., 2016). Other studies have found that despite the strict controls for commitment to sporulation, some cells erroneously initiate sporulation and fail, likely due to lower fitness levels (Krawczyk, 2017). In addition, research shows significant variations in expression levels of different genes belonging to different functional groups and regulons governing bacterial spore germination. In fact, some genes related with the regulation of the sporulation pathway are more weakly transcribed in the natural food-spoilage isolates than in the *B. subtilis* 168 (Krawczyk, 2017).

In order to address sporulation-induced heterogeneity a previous study has addressed the role of the PAS-BC domain of histidine kinase KinA in sporulation (Eswaramoorthy et al., 2009). This study has used a *B. subtilis* strain in which the expression of KinA is induced under the control of an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter. This particular strain is ideal to achieve a homogeneous population of spores as the majority of cells form spores upon IPTG induction. With such a homogeneous population of spores, it is now possible to carry out studies concerning the role of GRs and other components of spores in germination. Importantly, with a homogeneous spore population it is relatively simple to carry out resistance tests in different media and model foods. For instance, in the study performed by Tu and colleagues (unpublished data), *B. subtilis* spores obtained as a homogeneous population via *kinA* induction in both rich medium (Luria Bertani broth i.e. LB) and minimal media (Sterlini and Mandelstam medium i.e. SM & MOPS-buffered medium), differ significantly in their wet heat resistance when subjected to 85 °C for 20 min (Fig. 4).

4. Live-imaging of spore germination and outgrowth

The large variation in sporulation and germination kinetics necessitates the study of individual cells and spores. With the aid of state-of-the-art microscopy techniques, many approaches to study germination of individual spores are now available. Using a combination of Raman tweezers and differential interference contrast microscopy, germination and subsequent release of Ca^{2+} -

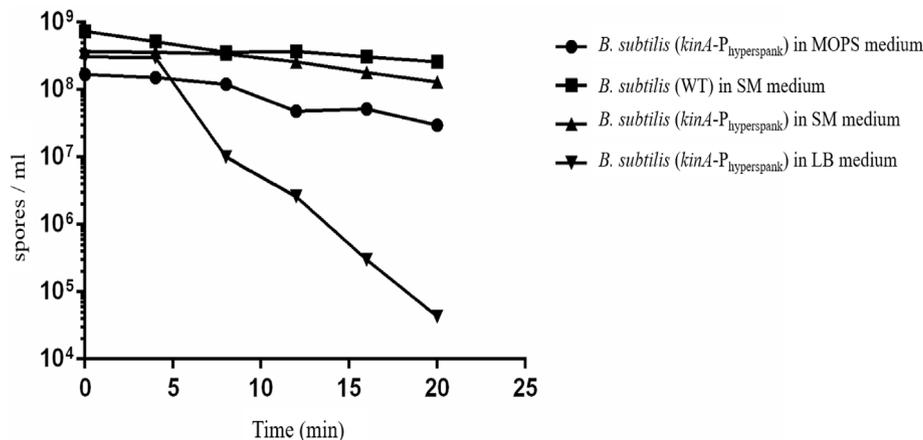


Fig. 4. Wet heat resistance at 85 °C for 20 min of spores obtained from cultures of *B. subtilis* (*kinA-P_{hyperspank}*) sporulated in different media. The wild type (WT) strain PY79 sporulated in SM (Sterlini and Mandelstam) medium is used as a control. The *kinA-P_{hyperspank}* strain was obtained from (Eswaramoorthy et al., 2009).

dipicolinic acid (Ca^{2+} -DPA) of individual spores of different *Bacillus* species has been investigated (Wang et al., 2011; Zhang et al., 2010, 2011). Studies in which spores, sorted individually in wells of a 96 well-microtiter plate using flow cytometry, have been monitored over time for vegetative growth under product-relevant conditions have shown significant heterogeneity (Smelt et al., 2008; Wells-Bennik et al., 2016). Whilst the timing and the duration of germination itself, the time to first vegetative doubling, as well as the initial generation times could not be analyzed separately with this approach, the method gave clear insight in the resulting heterogeneity of the sum of these individual stages. In order to explain the various spore germination and outgrowth stages in detail individual monitoring of germinating spores is required. Live-imaging microscopy using a closed air-containing chamber and phase-contrast microscopy has efficiently allowed for the assessment of

the phase-bright to phase-dark transition of spores, their outgrowth by measuring the time interval between the phase-dark formation and the first cell division, as well as vegetative cell divisions for every individual cell of a population (Fig. 5) (Pandey et al., 2013). Stringer and colleagues also used phase-contrast microscopy to study different stages in germination of anaerobically growing non-proteolytic *C. botulinum* and have also found considerable variability within germinating spores (Stringer et al., 2005, 2011). Special software that allows the user to tag single spores or cells from the images, obtained from live-imaging, and quantify the change in the phase contrast intensities as spores germinate and cells grow out have also been developed. Thus detailed (semi) automated analysis of germination and outgrowth phases is now possible (Kong et al., 2011; Pandey et al., 2013, 2016). Intriguingly, using advanced fluorescence microscopy, co-localization of

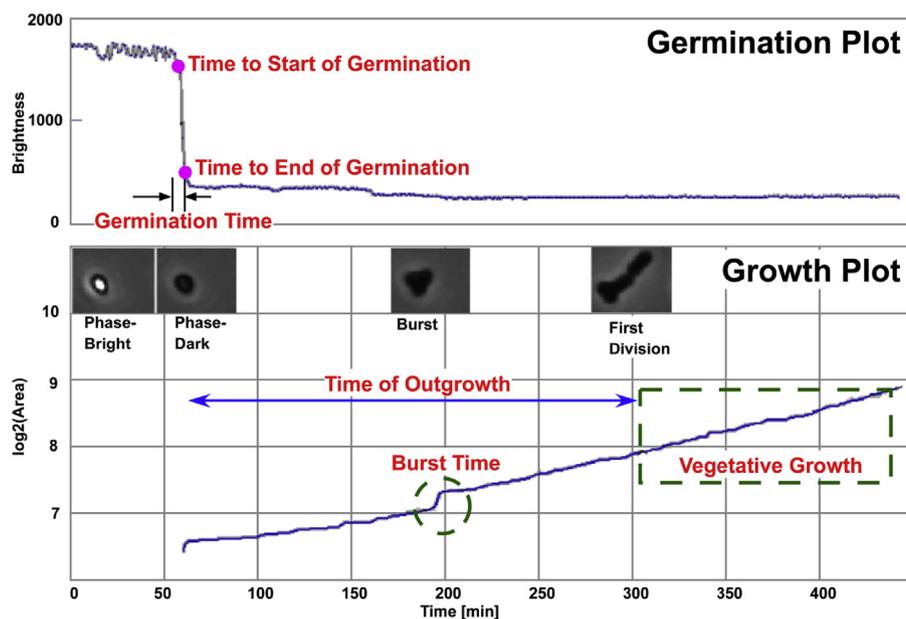


Fig. 5. Dual plot showing spore germination and outgrowth of one heat-activated *B. subtilis* spore as analyzed with SporeTracker. Above: Phase-bright to phase-dark transition, marked with a small filled pink circles at 90% (start of germination) and 10% (end of germination) of the entire (pixel) intensity drop range (brightness). Below: various snapshots at different stages of germination and outgrowth. The exponential growth phase (appearing linear in the \log_2 -transformed plot of the measured area) is used to calculate the generation time. The burst of the cell out of the spore coat is accompanied by a relative short and significant increase in spore area (marked by the green circle).[©] (Pandey et al., 2013). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

germination receptor proteins GerA, GerB and GerK was suggested, forming the spore's 'Germinosome' with GerD holding the cluster together (Griffiths et al., 2011; Moir and Cooper, 2015). Additionally, the Ca^{2+} -DPA transporting channel protein SpoVA is said to interact with the germinosome cluster (Vepachedu and Setlow, 2007). Yet,

questions about the number of GR co-localization foci in the spore IM and about the biophysical properties of the membrane in the region where GRs co-localize remain unanswered. Moreover, there is yet no information about the conservation of germinosome clusters in all spore-forming bacteria. Super resolution microscopy

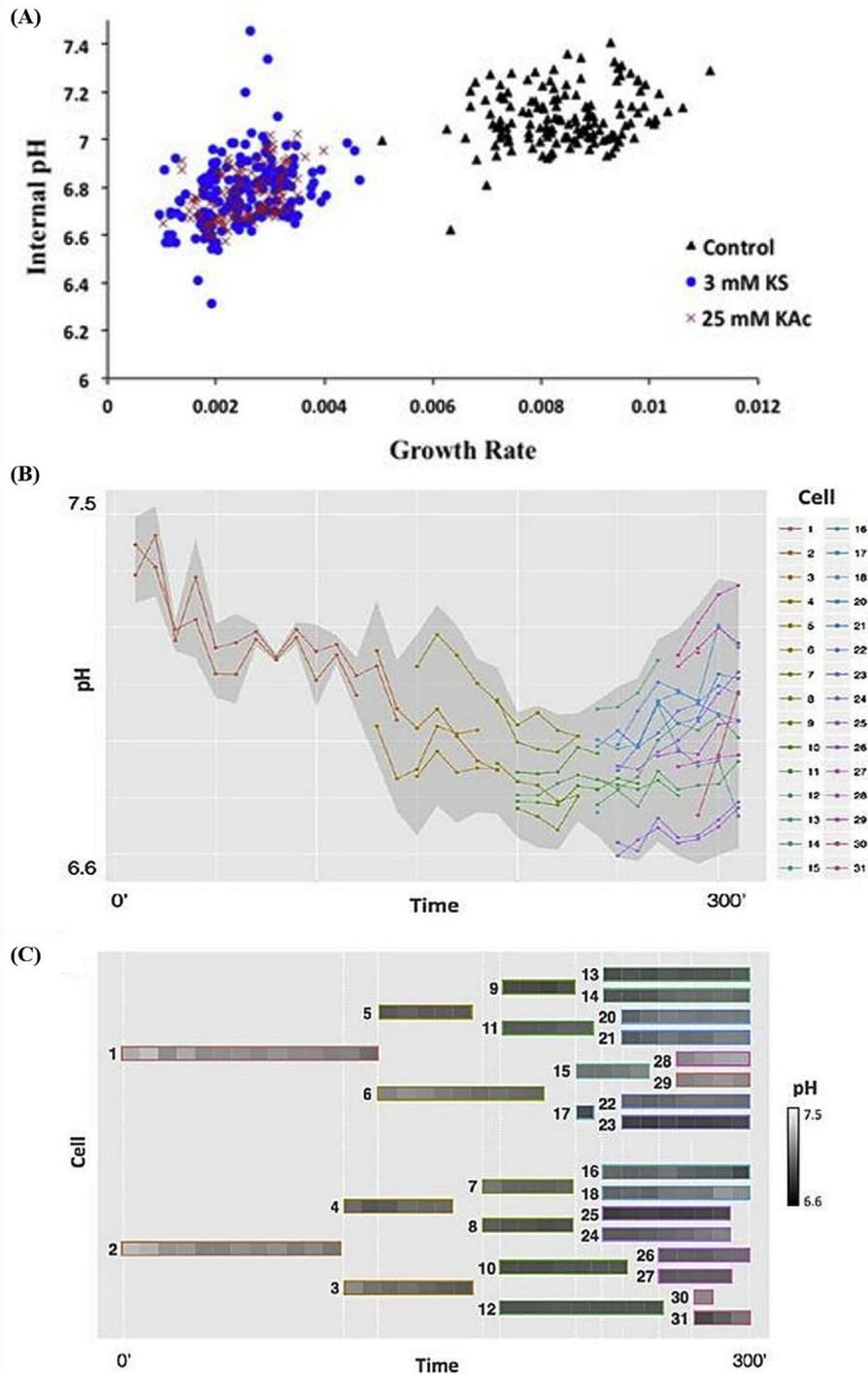


Fig. 6. (A) Growth rate versus pH_i of *B. subtilis* PptsG-IpHluorin expressing cells growing in microcolonies for unstressed cells and cells stressed with 3 mM potassium sorbate or 25 mM potassium acetate. Cells were grown in a closed air containing chamber as described by Pandey et al. (2013) in a minimal defined medium with 80 mM MOPS (3-(N-morpholino) propanesulfonic acid) buffered to pH 6.4 between the acid-stressed cells and the control populations, both the mean and the variance of pH_i and growth rate are significantly different (*t*-test, $P < 0.01$). (B and C) Time-resolved ratiometric image showing growth and division of single *B. subtilis* PptsG-IpHluorin expressing cells in the absence of added weak organic acids at an external pH of 6.4. (B) The pH profiles of individual color-coded cells superimposed on the standard deviation of the mean signal intensity of the entire microcolony (in gray). (C) Lineage tracking and ratio changes in gray scale. Note the transient drop in pH_i presumably due to the increased levels of acetate made by the growing bacteria themselves. For further details see the original manuscript. Copyright © American Society for Microbiology, [Applied and Environmental Microbiology, 82, 2016, 6463–6471, DOI: 10.1128/AEM.02063-16]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

can help answering these questions as it can attain resolutions up to 250 nm. In fact, with Structured Illumination Microscopy (SIM) a resolution of ~100 nm in the lateral direction and ~300 nm in the axial direction is achievable (Gustafsson, 2000; Gustafsson et al., 2008; Schermelleh et al., 2008). Still, at these resolutions it is difficult to rule out the interference of artifacts arising from imperfect imaging or reconstruction algorithms. Therefore Wen et al. (manuscript under preparation), are making efforts in this direction with the use of a combination of SIM and Wide field imaging.

5. Understanding the mode of action of preservation strategies

The live-imaging approach discussed above is extremely advantageous when it comes to quantitatively studying effects of antimicrobial compounds on spore germination and outgrowth. Besides spores, these methods are also applicable to study the effect of antimicrobial compounds or peptides against vegetative cells of food-borne pathogens (Ouardien et al., 2016; Pandey et al., 2015a,b; Rogiers et al., 2017; Schneider et al., 2016). Weak organic acids have been extensively studied for their action as preservatives (Stratford et al., 2013; Ter Beek et al., 2008; Ullah et al., 2012; van Beilen et al., 2014). Using fluorescence microscopy and fluorescent protein probes to study the effect of these acids on intracellular pH has proved to be a revelation (Bizzarri et al., 2009). The cellular pH is an ultimate factor that drives many processes in any living cell. Thus with the advent of a ratiometric pH-sensitive fluorescent protein pHluorin (Miesenböck et al., 1998; Orij et al., 2011) and its improved version IpHluorin (van Beilen and Brul, 2013) it has now become routine to monitor the intracellular pH (pH_i) under the influence of food preservation treatments. The use of such a genetically encoded reporter is advantageous, due to inherent labeling, a high signal-to-noise ratio, and the reporter protein concentration independence of the pH read out. Dependence on molecular oxygen though makes this method inapplicable to anaerobic bacteria. Nevertheless, using the live-imaging technique (see Section 4) the changes in intracellular pH of individual vegetative cells exposed to stress conditions have been monitored recently. Individual *B. subtilis* cells stressed with sorbic acid as well as acetic acid (Fig. 6) have been shown to differ significantly with respect to both pH_i and growth rate from unstressed cells (Pandey et al., 2016). Furthermore, catechins, predominant compounds in Tea, such as gallic acid, epigallocatechin-3-gallate, theaflavin 3,3'-digallate as well as free gallic acid have been shown to have strong effects on spore outgrowth and generation time of vegetative cells after their emergence from the spores (Pandey et al., 2015a). These two studies exemplify how microscopy can be tailored to distinguish the phase(s) where weak organic acids or flavonoid compounds can have maximum effect on the emergence of vegetative cells from dormant spores, i.e. germination, outgrowth or cell division. This information will aid the understanding of the modes of action of different preservation strategies. In other words, this information can be coupled to controlling the unwanted growth of bacteria in food and hence help the food industry to combat food spoilage and food borne disease. For the implementation of such tests under real life conditions it will be challenging to extend its use to a platform that includes a microfluidics-based lab-on-chip system (Dutse and Yusof, 2011). In this way rapid measurement of pH_i values under dynamically changing conditions (change of medium or temperature, external pH) can be directly coupled to monitoring of the pH_i linked to the dynamics of spore germination and outgrowth. Such data will benefit the development of mechanism based quantitative models for microbial food stability.

6. Use of mathematical models to study spore germination kinetics

Mathematical models, developed based on the data obtained from different approaches discussed here, help to standardize the different steps in food preservation treatments (Smelt et al., 2002, 2008). In this regard, the dynamics of spore germination, being relevant for preservation strategies, draws more and more interest for the construction of such models. The stress resistance of dormant bacterial spores versus the stress sensitivity of germinated spores, makes this step a major target to optimize processing strategies aiming at improved food quality and minimization of microbial spoilage and safety risks (Brul et al., 2012). Recently, it has been reported that spores, given a brief exposure to germinants, respond more readily to a subsequent short germinant pulse (Wang et al., 2015). However, the response to the initial germinant pulse decreases when the time interval between two pulses is increased, indicating a memory system in the germination process. The molecular basis of such a memory has been interrogated and it is concluded that germinosome and SpoVA channel proteins are prominent candidates for germination memory generation and storage (Wang et al., 2015). The exact molecular details of the mechanisms of memory remain, however, to be discovered. To address this issue, de Koster et al. (manuscript under review) have established a model in order to identify a minimal molecular network crucial for germination kinetics leading to memory of germinant exposure. This model will be used in contemporary research to reduce the number of experimental variables, to design future experiments, and to compare it to experimental outcomes. This, systems biology, approach will finally allow one to tune the model such that it reaches optimal agreement between theoretical predictions and experimental data. Missing experimental data can be identified and new experiments planned.

7. Conclusions

Summarizing, the advanced proteomics tools allow for rapid one-pot interrogation of all proteins from spore layers and also help monitoring proteome changes over time. This is particularly useful when it comes to performing a time resolved analysis of the potential effects of different antimicrobial strategies on the proteome of germinating and outgrowing spores and cells. Such time resolved analyses combined with microscopy and strategies to obtain homogeneous spore populations, will help to unravel the molecular mechanisms behind bacterial spore germination and outgrowth. It should be possible to extend our insight to the level of the initial binding steps of germinants to their cognate receptors. Additionally in this way it will be possible to assess the onset of initial spore metabolism and protein synthesis in awakening spores. Such insight will provide fundamental understanding of the process and will facilitate the generation of (more) robust predictive models for bacterial spore germination and outgrowth in food products throughout the food chain.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.fm.2018.03.006>.

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