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Review

Investigating germination and outgrowth of bacterial spores at several scales

Clément Trunet ^{a, b}, Frédéric Carlin ^c, Louis Coroller ^{a, *}

^a Université de Brest, EA3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, UMT14.01 SPORE-RISK, ISBAM, 6 Rue de l'Université, F-29334 Quimper, France

^b ADRIA Développement, UMT14.01 SPORE-RISK, Z.A. de Creac'h Gwen, Quimper, France

^c UMR408 SQPOV "Sécurité et Qualité des Produits d'Origine Végétale", INRA, Avignon Université, 84000 Avignon, France

A B S T R A C T

Background: Spore-forming bacteria are a major cause of food spoilage and food poisoning. Spores that resist physical and chemical treatments used in the food industry may germinate and multiply. Spore germination, outgrowth and growth constitute a complex and highly heterogeneous process.

Scope and approach: Various techniques and methods can be used to observe the germination, outgrowth and early multiplication process of spore-forming bacteria and/or to quantify the impact of environmental conditions on its progress over time within a spore population. These techniques can be classified by different criteria: (i) the scale of analysis, from populations or cells to molecules, and (ii) the number of analyzed objects (cells) and (iii) the potential of the method to describe and/or quantify the impact of lethal or sub-lethal treatments or environmental conditions. Such treatments are applied to a spore population or a single spore and take into account parameters at the cellular level (growth capacity, morphological properties) to molecular level (proteomics, transcriptomics, spore molecular composition).

Key findings and conclusion: A better understanding and quantification of the germination, outgrowth and growth process require the implementation of several complementary methods. Methods providing information at single and population levels, as well as at molecular and cellular levels, are essential to assess and control the fate of spore-forming bacteria development in food systems.

Keywords:

Spore-forming bacteria
Bacillus
 Cellular analysis
 Molecular analysis
 Single cell
 Flow cytometry
 'Omics'
 Microscopy

1. Introduction

Spore-forming bacteria are largely studied in fundamental research on Gram-positive bacteria and for the numerous industrial applications of the spores themselves, of their enzymes and of their metabolites. Spores of *Bacilli* or *Clostridia* can remain dormant for years and have a much greater resistance to very diverse physical and chemical agents than their vegetative cells. After germination and multiplication, they can be responsible for food poisoning and food spoilage (Mallozzi, Viswanathan, & Vedantam, 2010; Setlow, 2014; Wells-Bennik et al., 2016). Recovery after chemical or physical stress is a complex phenomenon comprising the germination of

spores, the restoration of metabolic activity in suboptimal or favorable conditions and the emergence of the first vegetative cell able to multiply (Setlow, 2013). Germination, outgrowth and growth are characterized by a high variability of the behavior of individual cells. This variability is, moreover, highly influenced by environmental conditions. Monitoring the germination and growth of bacterial spores meets two major objectives: (i) to obtain a better understanding of a major physiological process of cell differentiation in the microbial world and (ii) to implement a quantitative evaluation of the impacts of physical and chemical treatments and/or of environmental conditions on the ability of spores to germinate and grow, and hence become a troublemaker for public health, food production and distribution, and other industrial activities.

This review provides a description of the different techniques and methods for monitoring spore germination and growth after exposure to sublethal treatments and/or suboptimal environments. The discussion focuses on the information provided by different investigation methods with regards to the scales of examination

* Corresponding author. Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, Unité Mixte Technologique SPORE-RISK, Ecole Supérieure d'Ingénieurs en Agroalimentaire de Bretagne atlantique, 2, rue de l'Université, 29334 Quimper cedex, France.

E-mail address: louis.coroller@univ-brest.fr (L. Coroller).

(from cell to molecules, from populations to single cells, from millions to dozens of single events) and their potential for describing and/or quantifying the impact of treatment or environmental conditions.

2. Overview of spore germination and characterization of spore properties

Germination encompasses the deep morphological and physiological modifications of dormant spores to become vegetative cells. The physiological stages observed during germination and growth can be defined by criteria linked to the changes in spore properties. At first, refractivity loss is due to the rehydration of the spore core. Then, the size of the cell increases as the spore converts to a rod-shaped vegetative cell during the germination, outgrowth and growth process. Simultaneously the respiratory activity is restored, the membranes become fluid and the resistance to wet heat is lost. At a molecular level the CaDPA which was accumulated during sporulation, maintaining low water content in the spore core, is released and RNA expression and protein and other (macro) molecule synthesis starts again (Setlow, 2014). These physiological and molecular events may be used to differentiate dormant and germinated spores, or outgrowing and vegetative cells during the germination, outgrowth and growth process (Fig. 1).

The term “germinant” designates nutrient and non-nutrient factors triggering spore germination (Setlow, 2003). Nutrient germinants are low molecular weight compounds including, but not restricted to, amino acids, sugars and purine nucleosides possibly acting in combination and whose effects can be reinforced by co-germinants such as cations. Non-nutrient germinants include the dipicolinic acid-calcium complex (CaDPA, specific to spore-forming bacteria) or high hydrostatic pressure (Setlow, 2013). Germination leads to the rehydration of the spore and the release of the CaDPA concentrated in the spore core. Then, the cortex is hydrolyzed by cortex lytic enzymes (CLEs) such as SleB and CwlJ, the two major CLEs in *Bacillus* sp. Cortex hydrolysis allows the core to expand and complete rehydration to approximately 80% of spore weight (Setlow, 2014). Spores lose their high resistance to moist heat and chemicals early after core hydration. The spore becomes permeable as the inner-membrane surrounding the core expands. In the dormant stage, the low inner membrane permeability prevents small molecules from penetrating the core (Sunde, Setlow, Hederstedt, & Halle, 2009). Finally, the hydration of the spore and the release of CaDPA lead to the loss of spore refractivity (Fig. 1). Metabolic activity and macromolecule synthesis are restored during outgrowth. The spores swell up and escape from the outermost layers, the proteinaceous coat and exosporium (Abee et al., 2011). This step leads to the formation of a new vegetative cell able to multiply. The transformation of a germinated spore into a vegetative cell is characterized by morphological changes: the cell size increases, the new cell comes out of the coat and exosporium, and the restoration of a metabolic activity marks a deep physiological modification (Pandey et al., 2013). The shifts from dormant spores to germinated spores, then from germinated spores to vegetative cells are highly heterogeneous (Setlow, 2012). This variability can be the result of differences in the expression of genes governing sporulation and/or environmental conditions among the population of cells during sporulation as the germination and outgrowth behavior depends on the molecular reserves produced during spore formation. This variability results in different spore dehydration rates, different amounts of enzymes involved in germination and a different number of germinant receptors which are put in place during sporulation (Chastanet et al., 2010; Setlow, 2014). This variability in turn affects heat resistance, germination and growth ability (Hornstra, de Vries, Wells-Bennik, de Vos, & Abee, 2006;

Setlow, 2013).

The study of physical and/or physiological spore transformations requires different levels of analysis, and thus different observation and quantitative methods. Monitoring germination and outgrowth consists in following characteristics which are representative of the spore transformation, over time and within a given spore population. The previously described spore transformations are quantifiable criteria for distinguishing the successive physiological stages of spores during germination and growth. These changes can be observed in populations as well as in individual spores using different techniques and methods, providing information on cellular behavior as well as on molecular characteristics (Fig. 2A–B). Flow cytometry and microscopy methods examine sub-populations and single cells at cellular or molecular levels (Fig. 2C–D). Molecular methods apply to populations or single cells giving information at genomic, transcriptomic, proteomic or other molecular levels (Fig. 2E–F–G).

3. Culture dependent methods and indirect measurements (Fig. 2A–B)

Cultural methods require media (broth or solid) containing the necessary elements to provide spore germination, outgrowth and cell multiplication (Fig. 2A). Cells forming a colony on agar plates have definitely experienced germination, outgrowth and multiplication. The detection threshold is 10 to 10^2 spores CFU mL⁻¹ and can be continued with the dilution procedure. The sensitivity is high as the presence of a few spores can be detected among populations of a million or even billion cells that can be eliminated by a moderate heat treatment or by exposure to an ethanol solution. Culture dependent methods have been applied to estimate the impact of sporulation conditions, the intensity of heat treatment and the recovery environment on the ability of spores to form a colony on agar plates, particularly after a heat process. More generally they can be used to quantitatively assess growth, survival or recovery in laboratory media or in real foods after physical or chemical treatments of the spore-forming bacteria of concern. These data are used to develop mathematical models predicting bacterial behavior to optimize, control and improve food quality and safety (Baril et al., 2012; Leguerinel, Couvert, & Mafart, 2000; Leguérinel, Couvert, & Mafart, 2006; Mafart & Leguérinel, 1998; Mtimet et al., 2015; Trunet et al., 2015).

Germination is characterized by the rehydration of spores, revealed by the transformation of spores from phase-bright to dark-phase (Fig. 2B). This corresponds to a decrease in the A_{600} of spore suspensions, necessarily at relatively high concentrations (i.e. 10^7 - 10^8 spore/ml) to exceed the detection limit of spectrophotometric measurements. For example the impact of suboptimal sporulation and incubation conditions on the germination of *B. weihenstephanensis* or of *B. cereus* has been evaluated by monitoring changes in A_{600} of spore suspensions (Garcia, van der Voort, & Abee, 2010; van Melis, Almeida, Kort, Groot, & Abee, 2012). A 62% decrease in initial A_{600} for *Bacillus weihenstephanensis* or a 45% decrease for *B. cereus* corresponded to a 99.9% germination rate. Unsurprisingly, spectrometric methods do not involve direct observation of spores and therefore do not allow any assessment of the variability in individual cell behaviors. Moreover, these techniques can only be applied to transparent liquids.

Furthermore, by studying changes in absorbance of a spore suspension, the shift from a dormant spore to a vegetative cell able to multiply can also be examined. Vegetative cell multiplication leads to an increase in A_{600} with the increase in turbidity. Monitoring the evolution of A_{600} allows to estimate the lag time corresponding to the time to first cell division, encompassing the germination time and the time needed for growth. With this

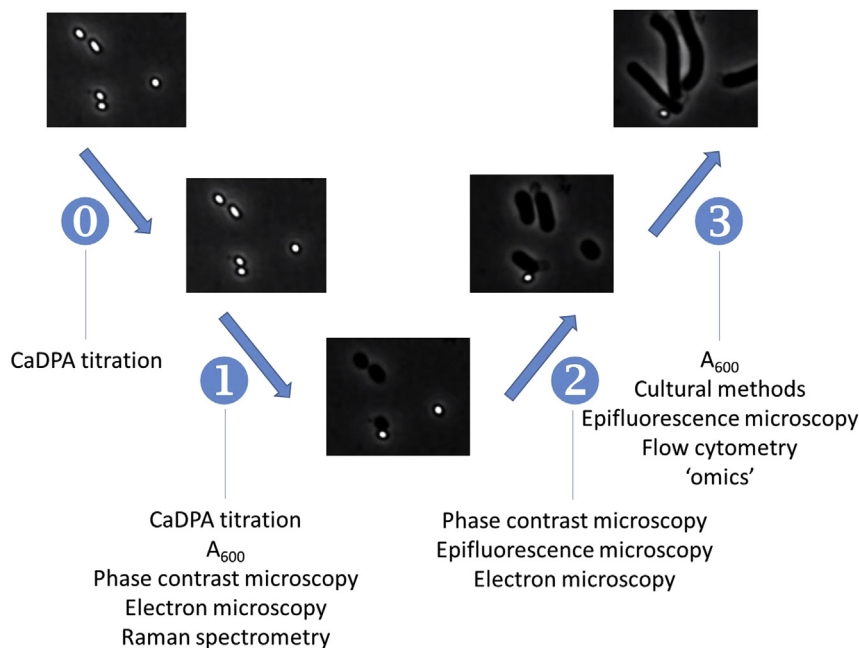


Fig. 1. Successive stages of the germination and outgrowth of bacterial spores, observed under phase-contrast microscopy and methods applied for monitoring. 0 Commitment to germination can be followed by the release of CaDPA (Zhang, Liang, Yi, Setlow, & Li, 2014). Spores remain phase-bright. 1 Germination can be monitored by phase contrast microscopy on individual spores or on limited spore populations (Pandey et al., 2013; Tychinsky et al., 2007); by Raman spectroscopy (Chen et al., 2006) to follow CaDPA release into culture medium (Zhang et al., 2012). Spores turn from phase-bright to phase dark. 2 Outgrowth can be monitored using phase contrast microscopy associated with a time lapse image system (Pandey et al., 2013); by electron microscopy (Saif Zaman et al., 2005) or by epifluorescence microscopy (van Melis, den Besten, Nierop Groot, & Abee, 2014). The spore core hydrates and the cell emerging from the spores expands. 3 Growth can be monitored by changes in turbidity (A_{600}) or by cultural methods (Roubos-van den Hil et al., 2010); by epifluorescence microscopy (van Melis et al., 2014); by transcriptomic methods (Segev, Rosenberg, Mamou, Sinai, & Ben-Yehuda, 2013) or by flow cytometry (van Melis et al., 2011). Rod-shaped cells divide and multiply.

method, a prolonged germination and lag phase and a lower germination rate were observed with spores produced in suboptimal sporulation conditions, then heat-treated or incubated in suboptimal conditions (Collado, Fernández, Rodrigo, Camats, & Lopez, 2003; Daelman et al., 2013; Gaillard, Leguérinel, Savy, & Mafart, 2005; Roubos-van den Hil, Dalmas, Nout, & Abee, 2010). Using modern microplate readers that can be set to a wide range of incubation temperatures, culture conditions are miniaturized in microwells and A_{600} readings are automated and finally computed. A large number of data are potentially generated and the influence of a large number of variable factors on inactivation, germination and spore incubation can be tested. This method is not very indicative for the quantification of the number of germinating spores that give rise to multiplying vegetative cells. An increase in A_{600} can be due to anything from one to millions of spores, the only difference being in the time to a measurable change in A_{600} . Moreover the heterogeneity within the cell population is not observable. As A_{600} reading is also poorly sensitive and does not discriminate cells from other materials, monitoring germination by spectrometry uses highly concentrated spore suspensions (about 10^7 spore/ml) and therefore requires the preparation of high amounts of clean debris-free spore suspensions. However single spores can be isolated in microplate wells by limit dilution techniques or sorted by flow cytometry (Smelt, Bos, Kort, & Brul, 2008; Stringer, Webb, & Peck, 2011) (Fig. 2B). By monitoring A_{600} in cultures formed from these isolated cells over time, the times to spore germination and first cell division from single spores can be estimated. A shorter lag time and a lower variability of the lag time of heat-activated *Bacillus subtilis* and *Clostridium botulinum* spores were revealed (Smelt et al., 2008; Stringer, Webb, George, Pin, & Peck, 2005). The variability in lag times depends on sporulation conditions, on the intensity of the inactivation treatment by heat

and on the incubation conditions (Garcia et al., 2010; Smelt et al., 2008; Stringer et al., 2011).

Germination can also be monitored by studying the changes in the concentration of CaDPA in the incubation medium (Fig. 2B) that corresponds to its release concomitant with the hydration to the spore core. Several methods are available for DPA assay. One for instance uses the photoluminescence of DPA in the presence of terbium. The complex Tb-DPA is excited at 270 nm and the emitted fluorescence wavelength is 545 nm. The concentration in the released DPA is maximal when the germination of the spore population is completed. The relative DPA release at time t is calculated as the ratio between RFU at t and maximum RFU at full germination (Yi & Setlow, 2010; Zhang, Thomas, Li, & Setlow, 2012). By monitoring the DPA release, early events such as “commitment” and germination can be studied or the impact of heat treatment on germination time quantified (Kort et al., 2005; Paidhungat et al., 2002).

The bioluminescence of spores of engineered strains containing the *lux* gene (Fig. 2B) has also been exploited. Particularly, the *lux* gene was under the control of specific promoters, such as the *sspB* promoter. The *sspB* gene codes for a small acid-soluble protein (SASP) found in the spore core. As the spore germinates, the core is hydrated and the spores emit bioluminescence almost instantaneously as the intact luciferase, packaged during sporulation, leads to light emission. Relative Light Units are measured over time to evaluate the percentage of germinated spores within the population (Ciarciaglini et al., 2000; Hill et al., 1994). This method was notably used to quantify the impact of mild acidity, lactic acid and pasteurization on the germination of *B. subtilis* spores or to study the first steps in the germination process of *Bacillus subtilis* with different genetic backgrounds (Ciarciaglini et al., 2000; Setlow, Melly, & Setlow, 2001). Difficulty in strain transformation,

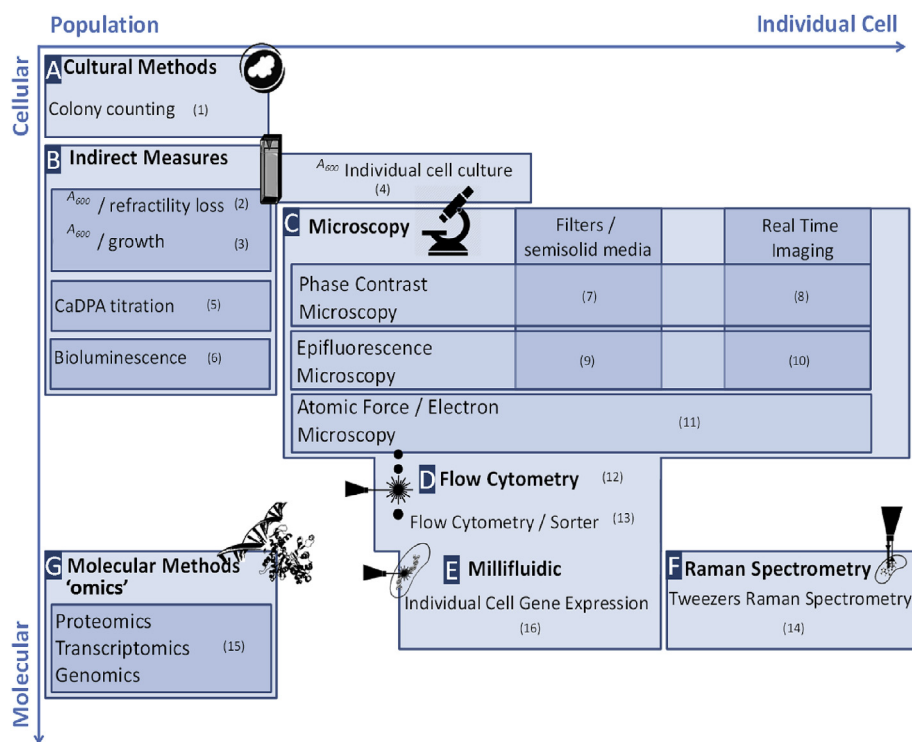


Fig. 2. A synthetic view of the potential of different techniques and methods used to monitor germination, outgrowth and growth of bacterial spores at different scales of investigation, with some selected references. Along the X-axis, methods are discriminated by the number of examined cells. Along the Y-axis they are discriminated by the scale of investigation: from cell (cellular level) to specific cell components (molecular level). **A)** Cultural methods, on solid media, allow the enumeration of spores able to germinate, outgrow and give a vegetative cell able to form a colony. The detection threshold for these methods depends on the applied dilution procedure and can be between 10 and 10^2 CFU mL⁻¹ (1. Leguérinel et al., 2006) **B)** A_{600} measurements monitor the spore refractivity loss (A_{600} decreases when spores lose their refractivity). The initial spore concentration must be high, $\pm 10^7$ spore mL⁻¹, to observe a significant A_{600} decrease (2. Garcia et al., 2010). The increase in A_{600} also allows the growth restoration of a spore population to be monitored. The spore concentration must be $>10^6$ spore mL⁻¹ to exceed the sensitivity of the spectrophotometer (3. Daelman et al., 2013). The full recovery process of single spores can be obtained by combining microplate A_{600} readings with wells inoculated with highly diluted spore suspensions (4. Smelt et al., 2008). Spore germination can be monitored by titration in the culture medium of CaDPA released by spores (5. Zhang et al., 2014). Bioluminescence methods consist in observing bioluminescence of *lux*-containing engineered spores (6. Ciarciaglini et al., 2000). **C)** Phase contrast microscopy can be used to discriminate dormant spores (phase-bright) from germinated spores and vegetative cells (phase-dark) by phase contrast microscopy. Micro-colonies formed after germination, outgrowth and growth restoration of spores fixed on semi-solid media or on strips (Anopore®) can be observed by phase contrast microscopy of spores on (7. Webb et al. 2012). This technique can be associated with live imaging automatic data treatment (SporeTracker for instance) (8. Pandey et al., 2013). Epifluorescence microscopy gives supplementary information about membrane permeability or metabolic activity thanks to fluorescent probes (9. Bruno, 2014). This technique associated with live imaging reveals the metabolic activity or the membrane permeabilization during germination (10). Generally, a concentration $>10^6$ spore mL⁻¹ is necessary to observe spores by microscopic methods. Atomic force microscopy (AFM) highlights the morphological evolution of spores during germination (11. Pinzón-Arango et al., 2009). **D)** Flow cytometry takes into account morphological criteria (size, refringency) associated with physiological criteria (membrane permeability, metabolic activity ...). A high number of spores ($>100\,000$) can be individually analyzed by flow cytometry (12. van Melis et al., 2011; Abee et al., 2011). A cell sorter would allow further characterization of single spores or of their progeny (13). **E)** Omics associated with millifluidic techniques will allow the observation of gene expression during the germination of single spores (16). **F)** Raman spectroscopy can be implemented to monitor single spore germination by following CaDPA release or to detect protein denaturation (14. Zhang et al., 2009). **G)** Omics techniques analyze gene expression or protein composition in spore populations (15. Abhyankar et al., 2013).

differences of phenotypes compared to the wild strain and the potential instability of the transformation could limit the application of this method.

The methods described in this section 3 “Culture dependent methods and indirect measurements” do not allow direct observation of spores or biochemical analyses related to secreted or released cellular compounds. Moreover, the different cellular states cannot be distinguished and the differential quantification of dormant spores, germinated spores and vegetative cells is not possible. The next section will deal with methods of direct spore observation, such as microscopy and flow cytometry.

4. Microscopy and flow cytometry methods (Fig. 2C–D)

Microscopy and flow cytometry discriminate between different physiological stages of germination and outgrowth by spore optical properties (refractivity), morphology (spheroid vs rod), and/or physiological activity (enzymatic or respiratory activity, membrane permeability). Both methods are based on the direct observation of

cells, which may be marked with detectable probes. Phase contrast microscopy differentiates dormant spores (phase-bright spores) from germinated spores (phase-dark) by their higher refractive index and from vegetative cells by their smaller size (Chen, Huang, & Li, 2006; Hornstra, Ter Beek, Smelt, Kallemeijn, & Brul, 2009; Tychinsky et al., 2007). Various physical media may be used for microscopic observation of spore germination and outgrowth. The spores can be deposited on a microscope slide, and then dried. The medium of germination (such as an L-alanine or inosine solution) and/or growth is either the suspension medium of the spores or is added after drying the slide (Kong, Doona, Setlow, & Li, 2014). Spores may also be placed on a semi-solid medium (such as a 1% agarose gel) previously prepared on a microscope slide (Pandey & Pieper et al., 2015; Pandey & Ter Beek et al., 2015; Stringer et al. 2005; Webb, Stringer, Le Marc, Baranyi, & Peck, 2012). Pandey and Pieper et al. (2015), Pandey and Ter Beek et al. (2015) also added a closed air-containing chamber made of an adhesive GeneFrame® (primarily designed for *in situ* hybridization and amplification) to the slide. Spores have also been deposited on strips of

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Anopore[®] permeable membranes (den Besten et al., 2010). Time lapse image systems promote the acquisition of images at relatively high frequency, possibly of spores followed individually when placed on a medium (Fig. 2C). Image analysis is required to monitor the diverse cellular states. Phase-bright and phase-dark spores can be discriminated by their pixel intensity on spore images (Pandey et al., 2013; Pandey & Pieper et al., 2015; Pandey & Ter Beek et al., 2015; Stringer, Webb, & Peck, 2009; Ter Beek et al., 2011; Webb et al., 2012). The different spores or cells can be enumerated and the size can be measured using dedicated software. For instance the SporeTracker software quantifies the decreasing pixel intensity during spore germination and the increasing area as the outgrowing cell size increases. SporeTracker can even detect the new cell popping out of the external spore layers, coat and exosporium; this event is called the “burst” (Pandey et al., 2013).

The time required to complete each step of the transformation of each cell within the population (germination, then outgrowth and first division) can be measured with these microscopical or flow cytometry techniques. Interestingly, the first spores to germinate are not the quickest to form a dividing cell, suggesting a rather time-independent pattern in the successive completion of germination and outgrowth events (Smelt, Stringer, & Brul, 2013). The impact of sublethal heat treatment, environmental conditions (incubation temperature or pH), or preservatives (tea antimicrobial compounds, sorbic acid for instance) on the times to completion of the spore transformations can be estimated and statistically analyzed (Pandey & Pieper et al., 2015; Pandey & Ter Beek et al., 2015). The influence of spore density and relative proximity on the germination of *Clostridium botulinum* spores has been demonstrated using these observation techniques. Practical experiments are performed on populations of several dozen to several thousands of cells, which gives some statistical and biological breadth to the observed phenomena (Pandey et al., 2013; Stringer et al., 2009).

Dynamic phase microscopy is performed with a laser phase microscope equipped with a He-Ne laser emitting at 633 nm. Dynamic phase microscopy estimates phase thickness, i.e. the difference in the optical path (nm) according to the nature of the different structures. Bacterial spore phase thickness can be defined as the spatial distribution of optical path difference between interfering beams, which reflects the state of an object. This technique has shown some potential to study the activation of spores by heat, a sublethal treatment promoting the germination of many species of spore-forming bacteria. Dormant spores (refractive) and germinated spores of *B. licheniformis* had significantly different phase thickness values (Tichinsky, Kretushev, & Luskovich, 2006; Tychinsky et al., 2007). Moreover, heat inactivated spores had a lower phase thickness value than dormant spores. The phase thickness values of spores which had been heat-activated but placed in an environment unsuitable for germination revert to values of untreated spores, confirming the reversibility of the activation treatment shown by other techniques (Setlow, 2013). Epifluorescence microscopy can also provide opportunities to study spore germination, outgrowth and growth by detecting and interpreting the fluorescence signal emitted by fluorescent probes marking spore structural elements (Fig. 2C). Dormant spores are impermeable to many molecules. The outer layers (coat, outer membrane and cortex) are permeable to small molecules (from 2 kDa to 8 kDa) (Driks, 1999; Henriques & Moran., 2007). The first hurdle in staining a spore is its impermeability to larger molecules such as fluorescent dyes. Syto9 passes through intact membranes, intercalates DNA and emits a 540 nm-wavelength light when excited at 488 nm. Dormant spores hardly stain with Syto9, with only a green halo at the spore surface possibly due to mother cell DNA on the spore surface (Cronin & Wilkinson, 2007). As the spore germinates and the core hydrates, Syto9 reaches spore DNA and

germinated spores show a green fluorescence (Cronin & Wilkinson, 2007; Reineke, Mathys, Heinz, & Knorr, 2013). In addition to DNA probes, such as Syto9 or propidium iodide (PI), fluorescent markers such as CFDA may reveal perturbations of the membrane integrity and be applied to the study of spore metabolic activity recovery during outgrowth (Cronin & Wilkinson, 2007). Nucleoid morphology can be analyzed by epifluorescence microscopy to monitor spore germination. The staining of nucleoids is not possible in dormant spores due to the impermeability of the structure, but germinated spores, as forespores, contain ring-shaped nucleoids. The nucleoids of germinated spores are associated with α/β small acid-soluble proteins (SASPs), which seem to have a role in germination and outgrowth: *B. subtilis* α - β SASP spores require a significantly longer time to recover a vegetative cell state (Ragkousi, Cowan, Ross, & Setlow, 2000).

Transmission Electronic Microscopy (TEM) is based on the variation of electron transmission through biological material and is particularly well suited to the observation of multilayered structures, which bacterial spores are, and more specifically to the evolution of spore structures during germination and outgrowth (Dey, Hoffman, & Glomski, 2012; Saif Zaman et al., 2005). The phenomenon called “bottle cap” that consists in the opening of a precise region of the spore exosporium leading to the escape of the new vegetative cell has thus been observed in *Clostridium sporogenes* spores (Brunt, Cross, & Peck, 2015). The “bottle cap” phenomenon may not be so different of the “burst” observed with *B. subtilis* spores by phase contrast microscopy (Pandey et al., 2013). The aperture allowing the cell to escape from external spore layers has also been observed by scanning electron microscopy (SEM) (Bassi, Cappa, & Cocconcelli, 2009; Brunt et al., 2015; Steichen, Kearney, & Turnbough, 2007). This method, in contrast to TEM, allows the observation of entire spores (versus a cross-section with TEM). These two methods are complementary for the analysis of spore structure evolution during germination and outgrowth. Atomic Force Microscopy generates images from the signal variations emitted by a mechanical probe displaced at a nanoscale on the surface of the “observed” object. Only changes in external layers of the spores can therefore be detected. Nevertheless, this method has successfully revealed alterations in coat architecture and topology during germination or after heat treatment (Pinzón-Arango, Scholl, Nagarajan, Mello, & Camesano, 2009; Plomp, Leighton, Wheeler, Hill, & Malkin, 2007; Tabit & Buys, 2010). TEM, SEM and AFM are destructive, with some possible artifacts due to harsh sample preparation conditions. Electron cryotomography (ECT) is also destructive, but objects are preserved in a near-native state thanks to ultra-quick freezing. Multi-directional EM images are computerized to give reconstructed 3-D images (Tocheva, Li, & Jensen, 2010). Peptidoglycan rearrangements and cortex thickening and thinning have thus been monitored throughout sporulation, spore maturation, and outgrowth of *B. subtilis* (Tocheva et al., 2013). However, as with TEM, SEM and AFM, the number of cells that can reasonably be observed and analyzed is inversely correlated to the structural details that can be revealed. These images are priceless for basic studies, but of rather poor utility for population studies with statistical significance or for the acquisition of large quantities of data.

With flow cytometry, spores or cells are aligned along a liquid flow in a capillary and detected with a laser beam. The light signals are analyzed by computing. The forward scattered (FSC) light signal provides information on cell size and the side-scattered light signal on cell inner structure or complexity. In addition appropriate fluorescent probes, commonly the same as those used in fluorescent microscopy, may provide information on cell permeability, viability or metabolic activity. Flow cytometry can be used to monitor germination, outgrowth and growth. Germinated spores

differ from dormant spores due to their higher probe fluorescence (of Syto9 for instance) generated by the permeation of spore layers and core hydration during germination (van Melis, Nierop Groot, Tempelaars, Moezelaar, & Abee, 2011). Spheroidal germinated spores and rod-shaped vegetative cells differ in size. Several characteristics can be obtained for each single cell in one pass through the detector, with size and fluorescence simultaneously providing information on the morphological or physiological states of the evolving spore populations. Other characteristics can be obtained with additional specific detectors (Mathys, Chapman, Bull, Heinz, & Knorr, 2007; van Melis et al. 2011). In contrast to phase contrast microscopy, the monitoring of a single cell over time is rather limited, but the number of cells that can be observed and characterized can be substantially higher (several tens of thousands of events is not uncommon). Flow cytometry has been used for instance to estimate the impact of high pressure (HP), heat treatments, or sorbic acid on *Bacillus cereus* or *Bacillus licheniformis* spore survival, germination and outgrowth (Cronin & Wilkinson, 2007, 2008; Mathys et al., 2007; Reineke et al., 2013; van Melis et al., 2011).

5. Molecular methods (Fig. 2E-F-G)

Molecular methods used for monitoring spore germination, outgrowth and growth can be presented as destructive or non-destructive methods performed on spore populations, spore population extracts or on specific components. Some methods can be implemented at single cell level.

As in any other biological process, the spore germination, outgrowth and growth process is increasingly analyzed with 'omic' technologies. Genomics, transcriptomics and proteomics establish a link between the spore behavior under different stresses and the fundamental basis of this behavior (Abhyankar et al., 2016). The so-called 'omics' data are increasingly used as new tools for hazard identification and characterization, as well as for exposure assessment. These 'omics' data may provide a better understanding of the diversity of physiological states and thus lead to the identification of biomarkers, such as specific genes or specific proteins for example, that may be used as quantitative data to build predictive models applied to Microbial Risk Assessment (Brul et al., 2012; McMeekin, Hill, Wagner, Dahl, & Ross, 2010; Membré & Guillou, 2016). Transcriptomic analysis requires gene expression and mRNA synthesis, which are not essential in the earliest germination events (Korza, Setlow, Rao, Li, & Setlow, 2017). However, transcriptomic analyses are well suited to studying what happens in lag or early growth from bacterial spores. RNA extraction from spores requires some attention because of the particular structure of spores (Moeller et al., 2006) (Fig. 2G). RNA is extracted from germinated spores or outgrowing cells over time and analyzed using different methods such as microarrays (Keijser et al., 2007), RNA-seq (Nagler et al., 2016) or RT qPCR (Bassi et al., 2016). Gene expression can be analyzed by specific pipelines such as T-Rex or JBrowse (Skinner, Uzilov, Stein, Mungall, & Holmes, 2009; de Jong, van der Meulen, Kuipers, & Kok, 2015). Thus, the evolution of cellular activity can be followed during germination and outgrowth and the impact of the incubation environment on this activity can be observed. For example, spore transcripts decrease during germination and early outgrowth until they are undetectable as the RNA stored in the spores is used as a source for *de novo* RNA and an initial source of nucleotides (Keijser et al., 2007). The impact of salinity on *Bacillus subtilis* has been investigated by a transcriptomic approach revealing a significant impact on stress gene expression during outgrowth (Nagler et al., 2016). Proteins have a major role in structure and protection, such as coat proteins, and in differentiation from germinant receptors or cortex lytic enzymes (Setlow,

2014). Proteomic methods can be applied in order to study germination and outgrowth (Fig. 2G). The extracted proteins are digested and analyzed by liquid chromatography associated with mass spectrometry (Abhyankar et al., 2011, 2014; Brul et al., 2011). Several proteins are essential for early germination steps: the germinant receptors, DPA channel proteins, cortex lytic enzymes (CLEs) and lipoproteins that are potentially implicated in the transduction of the germinant binding (Setlow, 2003, 2014). Analyzing dormant spores, germinated spores and outgrowing cells reveal an evolution of their proteome during germination and outgrowth process. For example, YpeB is 14-fold less detected in germinated spores than in dormant spores. YpeB is required for the incorporation of the CLE SleB in the inner spore membrane during sporulation, and then for its degradation during germination (Chen, Ray, Helm, Melville, & Popham, 2014). The changes in spore proteome over time can be monitored during the germination and outgrowth process. A set of proteins appeared to be significantly reduced during the germination step (Jagtap et al., 2006). The 'omics' methods are destructive and still applicable only to large spore populations ($>10^7$ spores mL⁻¹). According to recent works, single cell 'omics' analyses are crucial to be able to take into account the heterogeneity of a spore population (Abhyankar, Stelder, de Koning, de Koster, & Brul, 2017; Blainey, 2013).

Remarkable details of spore germination at individual cell levels have been obtained with Raman spectroscopy (Fig. 2F). Raman spectroscopy is based on the excitation of molecules by a laser. Each spore or cell in this case is considered as a molecular mix. The emitted radiation is collected by a detector and the variation of emitted energy provides information on the energetic levels of the molecules composing the spore or the cell. This method, in combination with confocal microscopy, Raman spectrometry and laser tweezers (exploiting the dielectric properties of cells for separation) have been used to monitor the germination of single spores. CaDPA Raman spectra bands can be identified in several species of spore-forming bacteria such as *Geobacillus stearothermophilus*, *B. subtilis* or *B. thuringiensis* (Chen et al., 2006; Gelder et al., 2007; Zhou, Dong, Setlow, & Li, 2013). The disappearance of the CaDPA spectral bands indicates the CaDPA release from the spore core and has been used to follow the germination of *B. subtilis* or *G. stearothermophilus* spores, for instance (Chen et al., 2006; Vepachedu & Setlow, 2004; Wang, Zhang, Setlow, & Li, 2011, 2015; Zhang, Setlow, & Li, 2009; Zhou et al., 2013). Moreover, the comparison of Raman spectra of dormant spores, germinated spores and heat-treated spores has shown that wet heat treatment causes spore protein denaturation, and hence inhibits germination (Zhang et al., 2009). Spore germination can also be followed through the release of DPA with X-Ray analysis. Electrons from an external source strike the spore atoms, leading to energy emission as X-Ray photons with X-Ray characteristics of the element (Bassi et al., 2009). CaDPA can also be detected by mass spectrometry or Fourier Transform Infrared Spectroscopy (FTIR) and can even be a biomarker of the presence of bacterial spores (Goodacre et al., 2000).

Millifluidic or microfluidic technologies could be employed in order to monitor spore germination, outgrowth and growth. These methods are emerging in microbiology and a device has been specifically developed to study *Bacillus subtilis* spore germination and outgrowth. This device is based on the changes in electrical conductivity of a polyaniline nanofiber network. Electrical changes are induced by the spores passing through a measuring interface and the signal depends on the state of the cell (dormant spores, germinated spores or vegetative cells) (Zabrocka, Langer, Michalski, Kocik, & Langer, 2015).

6. Conclusion

Many techniques and methods can be implemented to monitor the germination, outgrowth and growth process. They operate at different scales, from cellular to molecular, and on different number of studied cells, from a whole population to single spores. It is worth noting that all these methods are complementary: some provide better knowledge of the biological process while others quantitatively determine the impact of different factors, such as heat treatment or incubation pH, on the spores' ability to complete germination, outgrowth and growth.

The ideal method, which does not yet exist, would give quantitative molecular information from the single cell level to the cell population level. By combining different methods, such as 'omics' allied to microfluidics, results can be obtained at both cellular and molecular levels. Such methods are emerging, like mass cytometry, derived from mass spectrometry and applied to analyses of individual cells. At the time of writing, this method is available mainly for mammalian cells, as other techniques like flow cytometry for example were. It is expected to be extended and adapted to microorganisms as mass cytometry has been used to discriminate different strains of *E. coli* (Leipold, Ornatsky, Baranov, Whitfield, & Nitz, 2011). We can expect an extension of this method to single bacterial spores, targeting specific spore molecules such as CaDPA. Moreover, advances in microfluidic methods, like the Droplet PCR technique used to study genomes at single cell level (Gutiérrez-Aguirre, Rački, Dreo, & Ravnikar, 2015; Ottesen, Hong, Quake, & Leadbetter, 2006), offer novel approaches and new opportunities to develop methods allowing gene expression analysis or the proteome study of single spores. The behavior of spores in a food matrix can be different from the behavior observed in laboratory media. Thus, the impact of the food matrix has to be taken into account to improve the control of spore-forming bacteria in foods (Warda, den Besten, Sha, Abee, & Nierop Groot, 2015). Clearly, not all the methods we have described in this review can be applied to study spore behavior in a food matrix. Generally, food-based media are developed to mimic the food matrices and culture-dependent methods or, more recently, microscopy and transcriptomic methods have been used to study spore germination and outgrowth (Bassi et al., 2016; Warda et al., 2015). The study of germination, outgrowth and growth, as well as the impact of environmental factors on this process, by methods at different scales provides better comprehension of these phenomena leading to better control of the behavior of spore-forming bacteria.

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