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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

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ABSTRACT

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.

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).

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(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