REVIEW ARTICLE

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Biomarkers of bacterial spore germination

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Abstract
Exposure to specific germinant can induce germination in dormant bacterial spores converting them into vegetative cells which are metabolically active and fragile. This phenomenon of conversion of spores from one phase to another could be a keynote potential strategy for development of different type of techniques ranging from spore detection to their eradication and spore-based biosensing. To extend this biphasic spore germination-based approach in order to facilitate development of detection systems, mechanistic details of markers that signals the process of germination initiation in bacterial spores are required. These markers possess a high level of specificity for differentiation in germinating and dormant spores. In this line, present review underscores the structural properties, sporulation and germination in bacterial spores, and covers a detailed description on various biomarkers namely absorbance (600 nm), dipicolinic acid (DPA), refractility of spores, nucleic acid, ATP, spore's heat resistance, and enzymes which could be valuable in perceiving germination in bacterial spores. Assaying germination using these markers can further explore the efficient use of spores in development of detection devices for food and environment safety.

Keywords Germinant · Dormant · Spore · Germination · Marker · Eradication · Biosensing

Introduction

Bacterial spores are robust and metabolically dormant structures that are produced by a process of sporulation to prevail over harsh and unfavorable climatic conditions of starvation and stress (Nicholson 2002; Thakur et al. 2013; Tehri et al. 2017). Being a survival strategy, spores are well resistant to varying range of temperature and pressure, U.V. radiation, many noxious chemical substances such as hypochlorite, aldehydes, ethylene oxide, and several other extreme environmental conditions (Setlow 1994; Nicholson et al. 2000; Tennen et al. 2000). Spores can remain in dormant form for long time periods; however, they persistently scrutinize their environment for the presence of germinants that trigger

germination (Setlow 2003). During initiation of germination, metabolic activities commence resulting in ATP formation, synthesis of RNA, and proteins. Eventually, replication of DNA occurs which in turn results in vegetative cell growth (Setlow 1983; Paidhungat and Setlow 2002).

Various food spoilages and food-borne diseases are caused by some *Bacillus* and *Clostridium* spp. spores that have been found very resistant to several killing agents (Setlow 2006; Setlow and Johnson 2007; Coleman et al. 2010). These are liable to cause threatening impacts on human life and have ability to cause great economic loss to food industries. Therefore, detection and eradication of spores are crucial for public health, food, and environment safety. But in recent years, their detection has become a challenging task for regulatory authorities in various developed and developing countries. Various techniques available for elimination of spores which mainly include U.V., heat, high pressure, and other stress treatments, etc. are failed due to dormant and robust nature of spores. This indicates an immense need for new tools in order to combat drawbacks of existing techniques.

Physiological behavior of spores that occurs as a part of their life cycle holds great promise to resolve the constraints of available methods. It is well known that the life of spore-forming bacteria turns around two phases, the dormant and the metabolically active vegetative state, and perhaps, this exclusive biphasic phenomenon of bacterial spores could have

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great potential to form the basis of development of various techniques ranging from spores eradication, sterilization, and efficacy testing to spore-based biosensing. Development of such techniques based on the use of spores takes the advantage of the process of germination by examining the physiological changes occurring upon addition of endospore-specific dormancy breaking signals (Yung 2008).

Induction of germination in spores by exposure to specific germinant converts them into vegetative cells which are metabolically very active and fragile and can easily be eliminated from food products and environmental samples. Thus germination-based approach could be a potential strategy to facilitate eradication of bacterial spores more efficiently. It is well known that conversion from dormant to vegetative forms takes place when spores sense favorable environmental conditions. Although, the presence or absence of contaminants directly or indirectly affects this conversion indicating the potential of spores for their use as biosensing element.

Thus, the efficient use of spore germination-based approach in order to develop aforementioned sensitive and reliable techniques requires detection of germination in spores that commences within minutes after return of favorable conditions and is a major challenge. The advent of biomarkers has been a revival of interest in practical utility of germination process in bacterial spores. Presented article is the first review that covers description of all type of biomarkers of spore germination such as absorbance, DPA, refractility of spores, nucleic acid, ATP, spore's heat resistance, and enzymes used to date. Detailed knowledge of these markers can aid to better understanding of machinery of germination process and improvement of existing and emergence of novel spore eradication and spore-based biosensing techniques in order to guarantee food and consumer safety.

Bacterial spores, their structural properties and sporulation

Spores are produced by certain species of Gram-positive and Gram-negative bacteria such as *Bacillus* spp., *Clostridium* spp., *Sporomusa malonica*, *Sporomusa ovata* to prevail over unfavorable environmental conditions (Nicholson 2002). Being a survival strategy, endospores have very hardy and robust structure comprises of many layers such as spore coat, outer membrane, cortex, germ cell wall, inner membrane, core, and sometimes outer exosporium as depicted in Fig. 1. Core is the innermost layer of intact spores consisting of nucleic acids, i.e., DNA, RNA, small acid soluble proteins associated with DNA, enzymes, Ca-DPA (contributing to 25% dry weight of core), and moisture content around 25–50% of wet weight. Inner membrane surrounding core region is composed of lipids, act as barrier to many undesirable substances such as toxic chemicals. Inner membrane is further

surrounded by germ cell wall composed of peptidoglycan (Setlow 2006, 2007; Zhang et al. 2010). Outside the germ cell wall is the cortex region containing peptidoglycan with modified composition as compared to vegetative cells. An outer membrane surrounds this cortex region and plays its unique role in process of spore formation. A spore coat that is of proteinaceous nature further surrounds this outer membrane and protects the spore from the attack of lytic enzymes and other chemical compounds. Most of the species have spore coat as its outermost layer; while in other, coat is further surrounded by an outer layer known as exosporium (Warth and Strominger 1972; Laaberki and Dworkin 2008; Henriques et al. 2007). These unique structural properties of spores well preserve their genetic material and allow them to resist radiations, very low and high range of temperature and pressure, toxic chemical compounds, and several other extreme unfavorable environmental conditions.

The process by which spores are formed under unfavorable environmental condition is known as sporulation. In sporulation, a vegetative cell is divided into two unequal compartments: one small forespore and another large mother cell by the formation of a polar septum. Successively, the mother cell engulfs the forespore, and its cytoplasm constitutes the core of spore (Piggot and Hilbert 2004). Synthesis of core is followed by formation of cortex, a thick wall material around it. Subsequently, a proteinaceous multilayered coat is formed by mother cell on forespore surface. In recent times, an additional outermost layer on spore, known as crust, has been recognized (McKenney et al. 2010). Spore's outer layers, as also mentioned above under structural properties, provide them mechanical strength and exclude undesirable substances. Eventually, upon completion of sporulation process, mother cell lysis takes place which in turn is followed by liberation of mature spore (Piggot and Hilbert 2004).
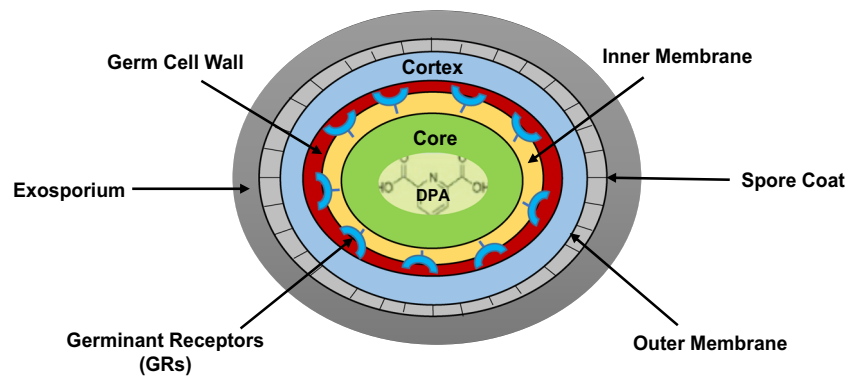
Spores germination

Spores are dormant; with their resistant forms still they scrutinize their surroundings for molecules and/or conditions that trigger germination within minutes followed by outgrowth to generate growing vegetative cells (Setlow 2003; Paredes-Sabja et al. 2010; Ross and Abel-Santos 2010; Setlow and Johnson 2012).

Germinants

Germinants are those molecules which trigger germination in spores. They can be classified into two types, i.e., nutrient and non-nutrient. Further, both types of germinants initiate the process of germination by different approaches.

Fig. 1 Schematic diagram of bacterial spore structure

Nutrient germinants

These are the most common type of germinants known to trigger germination in bacterial spores. They are mostly species and strain specific and mainly include sugars, amino acids, purines, and other low molecular weight compounds (Gould 1969; Paredes-Sabja et al. 2010; Tehri et al. 2018). Onset of germination in spores has also been found to be dependent on the stereospecific nature of germinants. For example, L-alanine is the most common form of alanine used as germinant, while D-alanine is often an inhibitor of germination triggered by L-alanine. This unique specificity associated with germinants has been found to be associated with proteins, called as germinant receptors that bind to germinants. In addition to nutrient germinants, some monovalent cations like K+ that alone can trigger germination can also act as obligatory co-germinants for nutrient germinants (Setlow 2013; Tehri et al. 2017).

Non-nutrient germinants

Apart from nutrient germinants, several other physical and chemical agents such as calcium dipicolinate (CaDPA) (at increased concentrations), alkylamines, and high pressure are also known to initiate the process of germination in bacterial spores. However, the mechanism they use to stimulate germination is extremely different from nutrient type of germinants (Setlow 2013). Mechanism for onset of germination in spores varies depending on the type and level of non-nutrient means of germination. Involvement of germinant receptors in triggering germination has been observed at 100 MPa, however not at 600 MPa. On the other hand, pressure of 600 MPa leads to the release of CaDPA from spore's core which itself possess germinant properties and therefore not involving germinant receptors (Wuytack et al. 2000; Black et al. 2005; Moir 2006). The spores have also been found germinated in response to heat activation (Luu et al. 2015). Interestingly, germination can also be initiated upon exposure to glycine and certain bile acids, e.g., taurocholate. Spores of *Clostridium difficile* have been reported to interact with bile

acids along the gastrointestinal tract in such a way that spores use a host-derived signal to initiate germination (Wilson 1983; Weese et al. 2000; Sorg and Sonenshein 2010).

Germinant receptors

Germinant receptors, termed as GRs, are the proteins that bind germinants (Setlow 2013). Each GR can bind a specific germinant, i.e., sugars, amino acids, and cations, and thus provides nutrient germinant specificity to bacterial spores. However, structurally distinct biomolecules are responded by spores by expression of multiple GRs. Moreover, a unique set of GRs is encoded by each spore-forming bacterial species for recognizing different types of germinants. Remarkably, a single or multiple germinants can also be responded by cooperation of diverse range of GRs. Under forespore-specific transcription factor, i.e., σG regulation, polycistronic operons are mainly responsible for expression of GRs only within late sporulation phase (Ross and Abel-Santos 2010; Madslien et al. 2014). Each type of GRs is found with only about ten molecules in the inner membrane of per spore and is therefore less abundance protein (Paredes-Sabja et al. 2010). Some of the major GRs involved in germination of *Bacillus* and *Clostridium* species are listed in Table 1.0. This reveals importance of GRs as key player in the process of spore germination. On the other hand, an impaired response to recognize germinants has been observed in spores lacking GRs (Hornstra et al. 2006; Barlass et al. 2002).

Steps involved in spore germination

Interactions between germinant and GRs play as key role in triggering of germination. Upon binding to specific germinant-like amino acid, inorganic salts, and sugars, GRs are activated and trigger spore germination irreversibly and therefore known as commitment step. Subsequent to this step, monovalent cations along with pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) and divalent cations, especially Ca^{2+}, in chelated form, i.e., CaDPA are released from spore core. This is followed by entry of water inside core region to initiate

Table 1 Major germinant receptors and their functions in *Bacillus* and *Clostridium* species

Spore former	Germinant receptor (GR)	Function	Reference
Clostridium difficile	CspC and the CspB protease CspA and that may be localized to the outer membrane by GerS	Bile acid mediated germination	Bhattacharjee et al. 2016
Bacillus subtilis	GerA GerK, GerB	Recognize L-alanine or L-valine Respond together to combination of L-asparagine, D-glucose, D-fructose and potassium ions	Li et al. 2014
Bacillus subtilis	Ger D	GRs in *B. subtilis* spores are in a single cluster in the IM, termed the germinosome, and GerD is essential for germinosome assembly	Griffiths et al. 2011
Bacillus megaterium QM B1551	Ger U	D-glucose and L-leucine appeared to activate the receptor Ger U	Christie and Lowe 2007
Bacillus cereus ATCC 14579	Ger G Ger I	L-glutamine-induced germination Purine riboside and aromatic ring amino acid-initiated germination	Hornstra et al. 2006
Clostridium botulinum	Ger A	Nutrient-induced germination	Broussolle et al. 2002

the process of rehydration. Further, release of CaDPA in *Bacillus* spores has also been found to contribute to peptidoglycan hydrolysis in spore cortex due to activating any of the two types, i.e., CwlJ and SleB of cortex lytic enzymes (CLEs). Subsequently, hydrolysis of spore cortex permits the core region for expansion and rehydration, which in turn activates enzymes and hence initiate the metabolic activities in spore core (Moir et al. 2002; Yi and Setlow 2010). During the phase of outgrowth, synthesis of RNA, proteins, and DNA recommences and thus results in conversion of spore to vegetative cell (Gupta et al. 2013). Under optimal conditions, this process occurs very rapidly, most probably within minutes (Carr et al. 2010). Several factors such as medium composition, temperature, pH, germinant type and concentration, and heat treatment are known to influence the rate and thus time of spore germination (Caipo et al. 2002). Further enhancement of germination, i.e., activation has been found to be associated with additional treatments like type of time-temperature combination, certain chemicals, etc. (Foster and Johnstone 1990). However, the mechanisms underlying in activation have not been clearly understood.

Biomarkers for detection of spore germination

Germination is a process that can be measured and detected in various ways. A number of physical, biological, and chemical changes occur in germinating spores at the time of transition from dormant to vegetative cells. As germination is an irreversible event, therefore, germination can be detected by the use of different types of markers also known as biomarkers or

signature markers of germination process which can directly correlate the changes in the aforementioned properties, occurring during germination, to the process of germination (Stewart et al. 1981; Yi and Setlow 2010; Katherine 2010). To date, various such markers have successfully been used as unique tools to assess germination in bacterial spores and are mainly of seven different types as depicted in Fig. 2. Detailed description of each of the seven potential markers of spore germination is explained below.

Dipicolinic acid

The core region of spores contains low water content, i.e., 25–50% of wet weight and large quantity of DPA, i.e., dipicolinic acid (~ 10% dry weight of spore) which is unique to spores, as well as huge amount of divalent cations. DPA in spores is normally found in chelated form with divalent cations chiefly with Ca^{2+}, i.e., CaDPA (Paidhungat et al. 2000; Gerhardt and Marquis 1989). Germinant binding to its respective receptor site triggers germination with concomitant change in inner membrane permeability allowing the release of monovalent cations, i.e., K+, Na+, and H+ with subsequent release of CaDPA (Setlow 2013; Swerdlow et al. 1981; Peng et al. 2009; Cabrera-Martinez et al. 2003). Multiple spore-specific proteins, i.e., SpoVA, are probable candidates known to form channels in membrane of spore to allow DPA release from the core region (Setlow 2014; Cabrera-Martinez et al. 2003). Thus, release of DPA is early event in the process of germination and therefore possesses great potential for its use as one of the best biomarker to detect germination in dormant spores. To date, several methods have been also developed to assay DPA released during the process of germination. The most
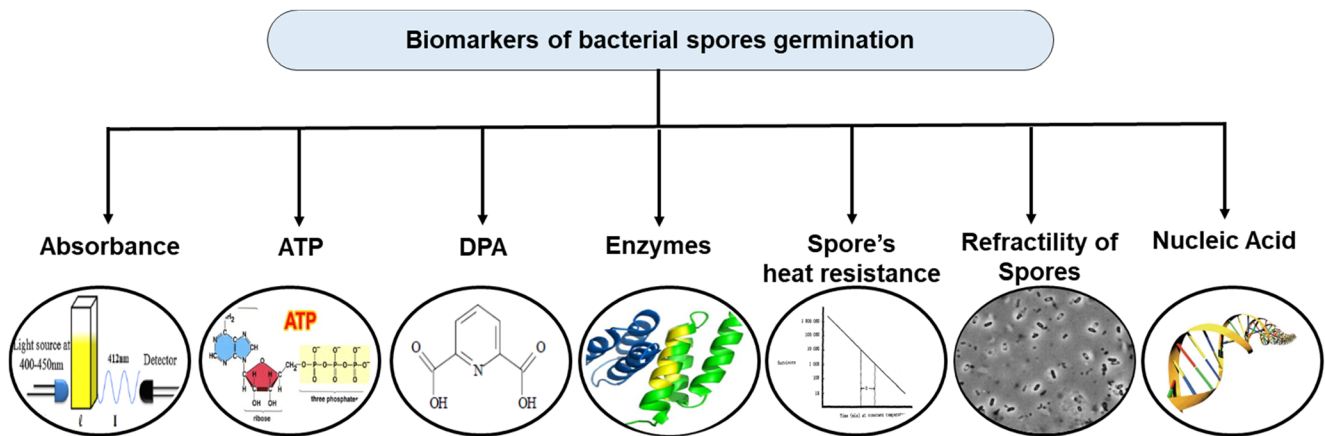
Fig. 2 Biomarkers of bacterial spore germination

commonly used and highly sensitive method for detection of DPA is Tb3+ luminescence assay. This assay works on the principle of transfer of excitation energy from DPA to bound Tb3+ (Clear et al. 2013). Another DPA assay namely dual luminescent and colorimetric assay based on the concept of dye displacement has been also developed by Clear and co-workers. Recently, a highly sensitive and selective DPA assay employing ratiometric colorimetric and fluorescent dual probe based on Eriochrome Black T (EBT)–Eu3+ complex has been designed. The assay works on the principle of formation of magenta colored complex between Eu3+ ions and EBT that upon the addition of DPA changed to blue immediately. The performance of developed assay was evaluated using spores of *Geobacillus stearothermophilus* that resulted in detection of as low as 2.5×10^5 spores in the test sample (Yilmaz and Oktem 2018).

DPA-based methods are highly reliable and known traditionally for their use to assay germination in spores. However, studies exist that have reported the presence of DPA-less spores of *Bacilli* and *Clostridia* spp. An asporogenous phenotype of *Bacillus subtilis* resulting from the loss of any SpoVA protein (except SpoVAEa or SpoVAF) has been observed where DPA was synthesized normally in sporulation, but it was not taken up and the unstable DPA-less spores germinated in the sporangium and lyse (Errington 1993; Setlow 2013). Another study carried out with *Clostridium perfringens* DspoVA showed no DPA accumulation in spores; however, DPA-less spores were stable (Paredes-Sabja et al. 2008; Setlow 2013). This raises a question on the applicability of DPA as a marker for germination of spores from different spore formers.

Absorbance at 600 nm

OD600 is the optical density, or light absorbance, at 600 nm, and describes transmission of light at this wavelength through a solution or object (Kristin 2012). In germinating spores, OD600 measurement, i.e., maximum rate of fall in OD600,

is a simple and reliable way that is used to study kinetic analysis of germination and commitment, and to monitor different spore populations in terms of comparing and quantitating germination rates, at particular concentration of germinant (Powell 1950; Paidhungat and Setlow 2002; Løvdal et al. 2012; Cabrera-Martinez et al. 2003). The absorbance loss reflects the change (decrease) in refractive index of core in multiple spores present in a suspension, following germination steps occurring well after commitment step such as Ca-DPA release, core swelling, and rehydration which accompanies hydrolysis of cortex causing loss in OD600 (Ghosh 2013), and therefore is not the earliest event in the spore germination process (Yi and Setlow 2010; Løvdal et al. 2012). However, during spore germination, significant OD600 loss can occur even when there is no hydrolysis of cortex; therefore, when this assay is used, it is essential always to use either flow cytometry or phase contrast microscopy to study degree of spore germination completion (Ghosh 2013). Thus, at a particular germinant concentration, rate of spore germination is calculated from maximum rate in OD600 fall versus time which is represented graphically and measured from the maximum slope of OD600 vs. time plot (Cabrera-Martinez et al. 2003). OD600 is usually determined after every 10 min. to capture the typical refractility loss during initial phase, i.e., in first few minutes of spore germination (McCormick 1965; Katherine 2010). Studying germination in populations by use of OD600 is also associated with some disadvantages. In a population, all the spores are not identical and will respond differently to germinants and germinant environments. In a population, only a germination tendency can be studied in the population as a whole, with a probability of each spore germinating within a given time. In populations with low germination, OD600 measurements may not even show the response. Spores can also clot together or adhere to the surfaces in the testing container where OD600 is measured, thereby complicating measurements and interpretation of these. When germination exceeds 90%, it is also difficult to show differences between populations in the assay. In other words,

OD600 is a good biomarker for observing germination in populations which show a relatively good germination response. When comparing different strains, the response should differ at least 10–20% for this method of observation to be effective. Additionally, other methods such as phase contrast microscopy and DPA release can also be used as a means of control when studying germination by measuring OD600 in a population. During germination conversion of the spores from bright to dark phase translates an OD600 loss of up to 60% of the original OD600 for a spore population (Kristin 2012). Nearly, 70% loss in OD600 has also been found when DPA release occurs during spore germination (Cabrera-Martinez et al. 2003).

Refractility of spores

Loss of refractility in spores occurs due to the influx of water, swelling of cell, and excretion of dry matter during their germination. This change in refractility of germinating spores can be easily studied by phase contrast microscopy using an oil-immersion objective with × 60 or × 100 magnifications. Water uptake in spores occurring during germination in terms of refractility loss is observed as transition from phase bright dormant spores to phase dark germinating spores under phase contrast microscopy as shown in Fig. 3 (Tehri 2015). The degree of spore refractility can be correlated to the course of germination, which follows biphasic kinetics. In the first phase, spores change into partial phase dark and lose part of the heat resistance. The proteinaceous coat becomes more porous, leading to water hydrolysis and release of calcium dipicolinate from the spore core. In second phase, a complete hydration of the core and degradation of the cortex in spore take place, which render the spore phase dark. Phase contrast

microscopy has successfully been applied to observe germination in both *Bacillus* and *Clostridium* spores. This can be carried out using a normal microscope slide-cover slip setup or on solidified agar covered with a cover slip. Acid popping of spores is another method of choice to study germination in bacterial spores using this technique at laboratory level. In this method, strong acid is passed through the spore suspension in a fixed microscopic mount allowing the spores to rehydrate and lose their high refractility and turning them immediately from bright to dark. An inverse relationship has been reported between the spore inoculum size and germination time. Several factors such as species, inoculum size, germinants, temperature, and the optics used for observation are known to affect the duration of germination. The duration of phase transition for individual bacterial spores ranges from 75 s to approximately an hour. Use of phase contrast microscopy is also advantageous in studying the process of germination at single spore level. Recently, this technique has been used to study the live imaging of germination in individual *Bacillus subtilis* spores. For this, a novel phase contrast microscopy-based closed air containing chamber was used to analyze *B. subtilis* spore germination, outgrowth, and subsequent vegetative growth. A program named Spore Tracker was used for image analysis which allowed automated data processing from germination to outgrowth and vegetative cell doubling (Pandey et al. 2013). Limitations inherent with the use of phase contrast technique is that lipid inclusions may be mistakenly assigned as spores because they are phase bright and are about micron size. Still, phase transition observed under the microscope provides a strong evidence for spore germination. When used in conjunction with other markers of spore germination, phase contrast microscopy proves to be
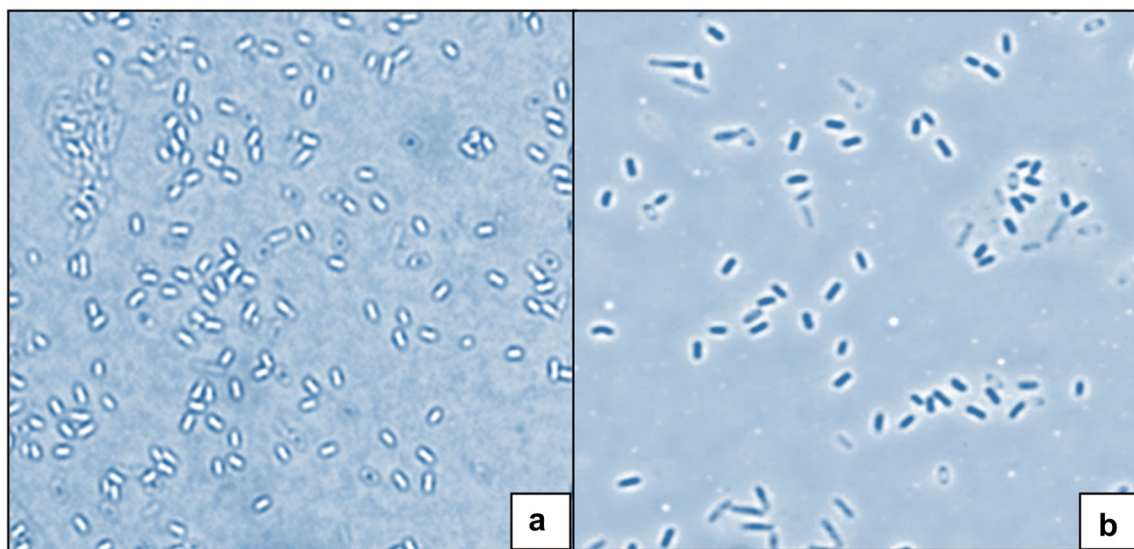

Fig. 3 Phase contrast microscopic observations: **a** Dormant spores of *B. megaterium* MTCC 2949; **b** Germinated spores of *B. megaterium* MTCC 2949

a very useful validation test and provides total and germinable counts of spore suspensions (Yung 2008).

Enzymes

In the course of germination, a series of complex biophysical processes occur upon activation of germinant receptors. As a result of which, several intracellular proteases and extracellular hydrolases that facilitate cellular differentiation to the vegetative form are activated (Fisher and Hanna 2005; Ferencko et al. 2004). Perhaps, these enzyme released during germination process possesses great potential for their use as marker in order to detect onset of germination in spore formers. Recently, activation of esterases has been used as a successful parameter to quantitatively measure the process of germination in *Bacillus* spores. Expression of esterases during germination comes under the umbrella of biochemical events presumably related to molecular mechanisms associated with germinant-mediated activation of silent hydrolytic enzyme(s). This enzyme has been used as a marker to detect germination in several species of *Bacilli*. To measure the activity of this enzyme, a fluorogenic substrate named diacetyl fluorescein (DAF), an acetylated derivative of the green fluorescent dye fluorescein, has been used. In DAF, dye remains in its nonfluorescent state as acetyl groups are attached on the xanthene group (Boyd et al. 2008). Once spore undergoes germination, DAF is hydrolyzed non-specifically by esterases, lipases, and proteases and converted to deacetylated form. The main product of the reaction is fluorescein, i.e., deacetylated form of molecule, a highly fluorescent compound and by-products include acetic acid/acetaldehyde (Thakur et al. 2013). Thus, fluorescent compound DAF is a cell viability stain which is cleaved effectively by germinating spores as compared to dormant spores. DAF has been used to measure activity of esterase which has been proved as a successful marker to study the onset of germination in spores of *Bacillus anthracis*. The expression of esterase in this work was also reported as an early event occurring consistently under conditions known to date to induce germination in spores. The findings of this work based on the use of esterase as biochemical marker of spore germination were also validated by measurement of refractility using phase contrast microscopy (Ferencko et al. 2004). The similar principle based on the use of enzymes and DAF has been used to develop a simple and rapid technique for detection of germination in *Bacillus megaterium* spores. The spores were allowed to germinate in the presence of nutrient germinant-like D-dextrose, and fluorescent signal was captured using EMCCD that allowed automated data processing in terms of detection of distinct fluorescent pattern for dormant and germinating spores (Thakur et al. 2013). Furthermore, 2,3,5-triphenyltetrazolium chloride (Tzm) has also been used to assay the activity of enzymes possessing the potential to use as markers upon onset of germination in

spores. Colorless form of Tzm is reduced to a red formazon derivative by an array of enzymes released during germination of spores. Thus, use of enzymes as marker is a convenient way to measure germination signal in spores and also advantageous over other existing markers. As measurement of germination, using enzymes neither require vegetative growth nor it suffer from spore clumping which can interfere to some extent in O.D. measurements (Gupta et al. 2013).

Spore's heat resistance

The core of bacterial spore contains less amount of water. Large peptidoglycan cortex that surrounds the spore inner membrane and core restricts the uptake of water in the spore core. Secondly, the presence of huge amount of CaDPA displaces significant amounts of water from the maturing spore core. This low water content of spore's core is the major factor known to contribute heat resistance to bacterial spores. The process of germination in bacterial spores leads to loss of heat resistance. During germination of *B. megaterium* and *B. subtilis* spores, the loss of heat resistance has been reported to precede CaDPA release and occurs essentially in parallel with commitment (Luu and Setlow 2014). Thus, loss of heat resistance is a potential marker to assay the extent of germination in bacterial spores exposed to either nutrient or nonnutrient germinant. Germination of *B. anthracis* in the presence of nutrient germinants was studied by determination of loss of heat resistance. For this, spore samples that had been exposed for various periods of time to germinant were incubated at 65 °C for 30 min. The samples were then rapidly chilled on ice, diluted, and spread on trypticase soy agar plates to determine viable counts (Levinson and Hyatt 1966; Welkos et al. 2001). The percentage of survival was determined by comparing the colony count of each germinant-exposed sample to that of the sample collected after exposure to germinant (Welkos et al. 2004).

Stainability of nucleic acid

Both intact dormant and decoated dormant spores that have lost their outer membrane and much of their coat show poor stainability because of their complex structural properties and therefore do not easily take up and bind many types of reagents particularly nucleic acid dyes, as nucleic acid of dormant spores remains in the central core region and is not readily assessed and penetrated by binding dyes (Gould 1969; Setlow et al. 2002; Magge et al. 2009; Ragkousi et al. 2000; Black et al. 2005). On the other hand, spores upon germination convert into vegetative cells and are easily get stained using similar dyes. Thus, binding of nucleic acid with its specific dyes shows great potential for its use as significant marker for detection of germination induced in dormant spores under favorable conditions. However, staining of

nucleic acids by dyes has been found to occur after the loss of most spore heat resistance and thus, the germination step that is measured by this marker would not be expected to be an early event (Setlow et al. 2002; Welkos et al. 2004). Work has been carried out with various dyes such as Syto 9, Syto 11, Syto 12, Syto 13, Syto 14, Syto 15, and Syto 16 to study staining pattern of dormant and germinating spores of *B. subtilis*. Among all dyes, Syto 16 was reported to give more distinct separation in fluorescence between dormant and germinated spores using flow cytometer (Black et al. 2005). Kong et al. (2010) studied the kinetics of uptake of this dye during germination of *Bacillus cereus* and *Bacillus subtilis* spores using a methodology that combines fluorescence microscopy, phase contrast microscopy, and laser tweezers Raman spectroscopy. The findings of this work showed that the uptake of this dye will occur following release of CaDPA and degradation of DNA binding proteins. Degradation of spore cortex by CLEs also reported to play a crucial role in uptake of Syto 16. Significant findings obtained when this dye, i.e., Syto 16, has been used to study germination induced under different conditions in dormant spores of various species of *Bacillus*. Spores of *B. subtilis* when germinated by a pressure of 150 MPa have shown strong stainability with Syto 16 using flow cytometry (Black et al. 2005). A dye named Syto 9 has also been used to study the process of germination in *B. anthracis* spores induced by two different combinations of nutrient germinants. Germination was detected in terms of change in fluorescence (RFU) upon addition of Syto 9 with both combinations of germinants comprising of L-alanine, inosine and L-alanine, adenosine, and casamino acids. The findings of this Syto 9-based assay were further validated with other established procedures. As a result, a significant level of correlation was obtained between germination as measured by different methods, i.e., increase in RFU, increase in percent of phase dark spores, and decline in heat resistance of spores (Welkos et al. 2004).
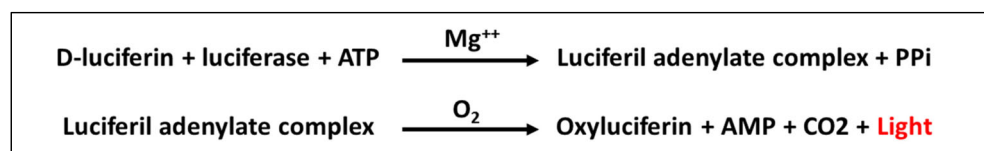
Impact of different treatments used for killing of spores could have drastic impacts in some spore permeability barrier and in turn, changes should be reflected in staining of core region by nucleic acid binding agents. One such study was carried out by using two different dyes namely DAPI and acridine orange, when killing of spores was observed by acid or ethanol but not by alkali. A peripheral staining of untreated spores, i.e., of many external layers but neither of coat nor core, observed with these dyes. However, after onset of germination, the peripheral staining in dormant spores disappeared in 20 min. and was replaced by an intense staining of

the germinated spore core, (Gould 1969; Ragkousi et al. 2000). Thus, suggesting that the peripheral staining in dormant spores is due to the staining of cortex region, which is hydrolyzed in the first minutes of germination. Staining of spores treated with NaOH was found similar to staining untreated dormant spores. On the other hand, spores treated with both ethanol and acid showed staining of the core region (Setlow et al. 2002). Thus, as a marker, stainability of spores offers significant advantages such as easy and rapid mode to detect the process of germination in bacterial spore.

ATP

ATP is found in all living microorganisms, and therefore, it has been used as an outstanding marker for detection of cell viability. ATP bioluminescence assays based on luciferine/luciferase reaction have been developed to detect various microorganisms. The working principle of bioluminescence assay is based on the oxidation of luciferine in the presence of ATP by luciferase with simultaneous production of enzyme-bound luciferil adenylate complex. This complex is further oxidized to oxyluciferin that is converted from excited to stationary state resulting in the emission of light due to rapid loss of energy of this molecule (Fig. 4). In this reaction, photons are emitted with quantum yield of about 90% (Seliger 1989; Wilson and Hastings 1998; Chollet and Ribault 2012). Similar approach of bioluminescence employing ATP as marker has also been extended for detection of the process of germination in bacterial spores. The amount of ATP is very low in dormant spores, and germination is a required process to increase the content of this molecule (Kodaka et al. 1996; Chollet and Ribault 2012). Most of the nucleotides in dormant form of spores remain stored in the form of 3-phosphoglyceric acid. Thus, less than 1% of the adenine nucleotide pool in dormant spores is ATP. On the other hand, spores undergoing germination and converting into vegetative cells account for 80% of ATP. This is because upon onset of germination, the stored 3-phosphoglyceric acid is catabolized into ATP, and increase in porosity of spore coat permits an easy extraction of intracellular pool of ATP (Santo and Doi 1974; Singh et al. 1977). Increase in light intensity by germinating spores of *Bacillus* in ATP bioluminescence assays has been reported in various works. Germination in spores of *B. subtilis* incubated in nutrient broth containing L-alanine for 30 min. have been observed in terms of increased RLU of spores (Fujinami et al. 2004; Chollet and Ribault 2012). Increase of ATP content was also reported during nutrient-induced germination in anaerobic

Fig. 4 Principle of ATP bioluminescence

$$\text{D-luciferin + luciferase + ATP} \xrightarrow{\text{Mg}^{++}} \text{Luciferil adenylate complex + PPi}$$

$$\text{Luciferil adenylate complex} \xrightarrow{\text{O}_2} \text{Oxyluciferin + AMP + CO2 + Light}$$

Clostridium spores (Hausenbauer et al. 1977). A rapid production of ATP in spores of *B. subtilis* upon germination by a pressure of 100 MPa was observed, but no ATP produced during germination induced at 600 MPa (Wuytack et al. 1998; Yung 2008). Until today, ATP assays have mainly been used for detection and enumeration of vegetative cells of pathogenic bacteria for food quality control and hygiene testing. But because of aforementioned distinctive properties of dormant and germinating spores in relation to ATP and earlier studies, ATP-based assay could also find immense application in detection of the process of germination. Bioluminescence assay based on measurement of ATP for detection purpose offers several advantages in terms of high sensitivity and rapidity. However, in some cases this assay can suffer from the problem associated with the presence of substances in some food samples which can pose inhibitory effect or interfere with luciferase activity (Leach and Webster 1986).

Conclusions

Spore-based sensing technologies are ideal tools for the detection of microbial and non-microbial contaminants in order to assure quality of various types of food and environmental samples. They offer several advantages like high sensitivity, selectivity, easy measurements, and low cost, high throughput analysis and are able to resolve problems encountered by conventional methods. Use of spores for development of such sensors requires a detailed understanding of appropriate markers that can be used to detect the process of germination. Keeping in view, the major objective of presented article is to highlight the scope of various biomarkers (absorbance, DPA, refractility of spores, nucleic acid, ATP, spore's heat resistance, and enzymes) that have the potential to assay germination in dormant spores. The better understanding of germination markers will be helpful for development of new as well as improvement of developed spore sensors which in turn can lead to their faster commercialization by transferring them from lab to industry. Moreover, knowledge of markers can immensely contribute also towards understanding of spore germination process and in development of novel spore eradication techniques in order to guarantee food and consumer safety.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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