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Life cycle and spore resistance of spore-forming *Bacillus atrophaeus*

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ABSTRACT

Bacillus endospores have a wide variety of important medical and industrial applications. This is an overview of the fundamental aspects of the life cycle, spore structure and factors that influence the spore resistance of spore-forming *Bacillus*. *Bacillus atrophaeus* was used as reference microorganism for this review because their spores are widely used to study spore resistance and morphology. Understanding the mechanisms involved in the cell cycle and spore survival is important for developing strategies for spore killing; producing highly resistant spores for biodefense, food and pharmaceutical applications; and developing new bioactive molecules and methods for spore surface display.

1. Introduction

The physical and chemical resistance of endospores, their ability to germinate after sub-lethal damage and their suitability for genetic manipulation are of practical importance for the pharmaceutical, food, and biodefense industries and for sterilization processes in healthcare facilities. Knowledge of the *Bacillus* life cycle and spore structure is fundamental to understanding spore resistance to physical and chemical agents. It is also important for the identification of the spore DNA repair process during spore germination and outgrowth. Because it is non-pathogenic, easy to culture, has identified resistance characteristics and is used in a variety of applications, *Bacillus atrophaeus* (also referred to as "*Bacillus subtilis* var. *niger*") was used as a reference microorganism for this study. *B. atrophaeus* spores have fundamental roles in the production of biological indicators for sterilization; in studies of biodefense and astrobiology, disinfection methods, DNA extraction during nucleic acid testing, waste treatment evaluation; and as potential adjuvants or vehicles for vaccines and other bioparticles (Gibbons et al. 2011; Sella et al. 2012).

2. Life cycle

The life cycle of spore-forming *Bacillus* consists of three different physiological processes, vegetative growth, sporulation and germination. The transition from one mode of development to another is driven by nutrient availability, which is sensed by the microorganism (Moir 2006; Rosenberg et al. 2012). Multiple signaling pathways transmit nutritional and growth rate information directly to the cell cycle machinery to permit cells to constantly sample their environments and fine-tune the cell cycle process (Wang and Levin 2009).

2.1. Vegetative growth

Vegetative growth is characterized by binary symmetric fission cell growth that occurs when nutrients are available. Chromosomal replication is intimately tied to the vegetative cell division cycle (Wang and Levin 2009). The total separation of sister cells by cleavage of the cell wall in *Bacillus* species may not occur under some circumstances, and the cells may remain linked together through multiple rounds of binary fission, forming long chains (Branda et al. 2001; Chai et al. 2010; Kearns and Losick 2005). Rosenberg et al. (2012) demonstrated transcriptional variability among different bacterial cells during vegetative growth directly related to nutrient availability, and this may represent an additional strategy to enhance population robustness. *Bacillus* vegetative cells are shown in Fig. 1.

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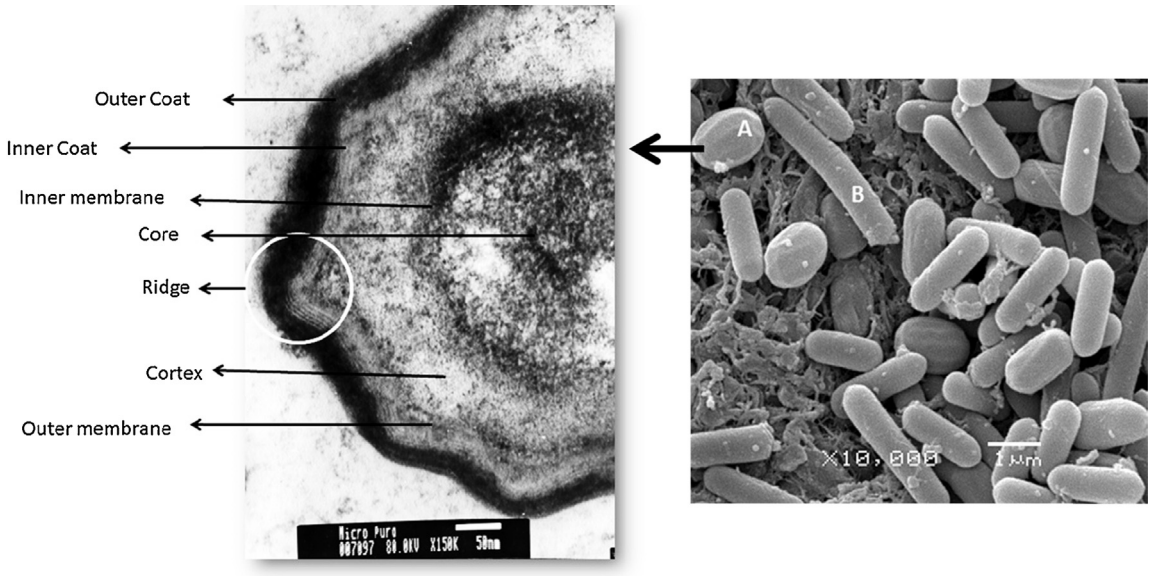

Fig. 1. *Right:* Scanning electron micrograph of *B. atrophaeus* spores (A) and vegetative cells (B) on an agar surface. The scale bar represents 1 μm. *Left:* Cross-section of a *B. atrophaeus* spore. The scale bar is 50 nm. (Fixation with glutaraldehyde and formaldehyde in cacodylate buffer, post fixation with osmium tetroxide, dehydration in an ethanol series and propylene oxide, and embedding in PolyEmbed 812 resin, followed by thin-section transmission electron microscopy.)

2.2. Sporulation

Multiple environmental signals, such as nutrient deprivation, high mineral composition, neutral pH, temperature, and high cell density can trigger the differentiation of vegetative cells into endospores. The cellular mass increases associated with the accumulation of secreted peptides are sensed by cell surface receptors that promote the sequential activation of the master regulator *SpoA*. This activation is called *phosphorelay* and involves the transference of phosphate groups from ATP through histidine kinases and two intermediate proteins, *Spo0F* and *Spo0B*, to a transcription factor, *Spo0A*. *Spo0A-P* controls the expression of a multitude of genes, setting off a chain of events that takes several hours to complete and culminates in the release of the mature spore from its mother cell compartment (Molle et al. 2003; Veening et al. 2009). However, the ultimate decision to sporulate is stochastic in that only a portion of the population sporulates, even under optimal conditions (Chastanet et al. 2010).

The spore formation process (Fig. 2) is described by seven stages.

Stage I: The nuclear material is disposed axially into filaments.
Stage II: Completion of DNA segregation occurs concurrently with the invagination of the plasmatic membrane in an asymmetric position, near one pole of the cell, forming a septum.
Stage III: The septum begins to curve, and the immature spore is surrounded by a double membrane of the mother cell in an engulfment process, similar to phagocytosis, and the smaller forespore becomes entirely contained within the mother cell.
Stage IV: The mother cell mediates the development of the forespore into the spore. The inner and outer proteinaceous layers of the spore are assembled, and the spore cortex, consisting of a thick layer of peptidoglycans contained between the inner and outer spore membranes, is synthesized. Furthermore, calcium dipicolinate accumulates in the nucleus.
Stage V: The spore coat is synthesized, consisting of ~80 proteins deposited by the mother cell, and is arranged in inner and outer layers.
Stage VI: Spore maturation occurs during this stage, and the spores become resistant to heat and organic solvents.
Stage VII: Lytic enzymes disrupt the mother cell, releasing the mature spore (Errington 2003; Higgins and Dworkin 2012; Setlow 2007).

Higgins and Dworkin (2012) reported that the morphological changes during the sporulation process are accompanied by the activation of different sigma specific transcription factors (σ^H, σ^F, σ^E, σ^G and σ^K) in each spore compartment. First, high levels of σ^H and *Spo0A* trigger sporulation and lead to the expression of pro-σ^F. Upon asymmetric division, σ^F is activated only in the forespore, The *SpoIIR* protein under the control of σ^F is produced and secreted by the forespore and activates the proteolytic processing of pro-$\sigma^E \rightarrow \sigma^E$ in the mother cell. After engulfment is complete, a signal produced in the mother cell under the control of σ^E enters the forespore *via* the *SpoIIIAH/SpoIIQ* pore and activates σ^G. Finally, the *SpoIVB* protein produced under the control of σ^G in the forespore activates pro-$\sigma^K \rightarrow \sigma^K$ proteolytic processing in the mother cell *via* an interaction with the *SpoIVFA/SpoIVFB/BofA* complex (Fig. 2).

For a more detailed description of the sporulation process in *Bacillus*, its initiation and regulatory pathways, please refer to Boonstra et al. (2013), Errington (2003), de Hoon et al. (2010), Higgins and Dworkin (2012), Narula et al. (2012), Piggot and Hilbert (2004), Setlow (2007) and Sonenshein (2000).

2.3. Germination

Spores can remain dormant for extended time periods and possess a remarkable resistance to environmental damages, such as heat, radiation, toxic chemicals, and pH extremes. Under favorable environmental conditions, the spore breaks dormancy and restarts growth in a process called spore germination and outgrowth (Fig. 3). The germination process occurs in the following three stages:

Stage I: Activation, defined as the initiation or triggering process in response to nutritional replenishment that occurs when the germinating molecules, including low-molecular-weight amino acids, sugars, and purine nucleosides, are sensed by germination receptors (GRs) located in the inner membrane of the spore. These receptors include, *gerA*, *gerB*, or *gerK*, and the germinating molecules bind these receptors. L-alanine acts through the *gerA*