Soil Bacterium *Bacillus thuringiensis*

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Bacillus thuringiensis (*Bt*) is a rod-shaped, Gram-positive soil bacterium that belongs to the phylum Firmicutes and the genus *Bacillus.* It is a spore-forming bacterium. During sporulation, it produces a wide range of crystalline proteins that are toxic to different orders of insects. Sporulation, structure assembly, and germination are essential stages in the cell cycle of *B. thuringiensis*. The majority of studies on these issues have focused on the model organism *Bacillus subtilis*, followed by *Bacillus cereus* and *Bacillus anthracis.* The machinery for sporulation and germination extrapolated to *B. thuringiensis*. However, in the light of the findings concerning the role of the sporulation proteins (SPoVS), the germination receptors (Gr), and the cortical enzymes in *Bt*, the theory strengthened that conservation in sporulation, structure assembly, and germination programs drive the survival and success of *B. thuringiensis* in the environment and the insect host.

spore-forming bacteria | Gram-positive bacillus | Bacillus thuringiensis | insecticidal crystal proteins

1. Introduction

The phylum Firmicutes (now referred to by a new, name, *Bacillota*) includes known spore-forming bacteria of the genera *Bacillus* and *Clostridium*. The genus *Bacillus* includes to *Bacillus cereus, Bacillus subtilis, Bacillus anthracis, Bacillus megaterium, and Bacillus thuringiensis*. The majority of these are soil bacilli and have relevance at the level of the food industry, pathogenesis, biological weapons, and biotechnology (nanotechnology, therapeutics) [1][2][3][4][5][6][7]. Meanwhile, the members of the genus *Clostridium*, such as *Clostridium perfringe*, *Clostridium botulinum*, and *Clostridium tetanus* mainly have a role in food spoilage, food-borne disease, intoxication, gas gangrene, pseudomembranous colitis, botulism, human pathogenesis (toxin production), and in the biotechnological industry (chemical products) [8][9][10][11][12][13][14][15][16].

A feature shared between the genera *Bacillus* and *Clostridium* is the sporulation, structure assembly, and germination for survival and DNA protection [17][18]. The manner in which they carry out these biological events at the molecular level is the subject of the entry, addressing general knowledge of the soil bacterium Bacillus thuringiensis and insight into the molecular programs that make this bacterium more than a successful insect pathogen in the environment and in the host [3][4][6].

Sporulation in the phylum Firmicutes plays a fundamental role as a cytological and morphological process during the life cycle. Genes and proteins constitute players in spore formation and germination [10][17][19][20][21][22][23][24].

Currently, the high-throughput technologies, integrated as the omic technologies, should allow for deep insight into the unveiling of the complex machinery of the sporulation and germination of spore-forming bacteria [25][26].

The genes and the proteins for each stage are conserved among species of *Bacillus*. However, in *Clostridium* spp., there are some differences due to the environmental conditions vs. the soil rhizosphere [1][6][8][17][27][28][29][30].

However, how do the spores permit the microorganism to survive and persist for long time periods? A cue is the structural architecture of the spore. Recent electron cryotomography (ECT) permits three-dimensional (3D) study reconstruction of the Gram-negative and Gram-positive bacterial cell walls. This analysis, in conjunction with biochemical and genetic evidence, supports the hypothesis that sporulation could be the ancient biological evolution process that gave rise to the second membrane in diderm cells (Gram-negative bacteria). The interconversion of the thin and thick peptidoglycan layer facilitated this process.

The second membrane in diderm bacteria is richer in lipopolysaccharides (LPS) and outer proteins. In other words, the chemical composition of the outer and inner membranes of the spore plays a role in resistance and protection under harsh conditions. The dynamic of sporulating regulatory proteins, the morphogenetic coat, and other proteins are involved in the early, middle, and late stages in sporulation or in spore biogenesis [31]. On referring to Bacillus thuringiensis (Bt) and its remarkable soil life, there are thousands of studies regarding its mechanism of action and its biotechnological application as a bioinsecticide. However, Bt has a spectrum of action due greatly to the battery of proteins produced (ICPs) at the onset of sporulation. Recent works have revealed by combining proteomics and metabolomics that there is a metabolic regulation mechanism of sporulation and ICPs synthesis. Specifically, these metabolic pathways are involved in the synthesis, energy storage, carbon supply, and nutrients (amino acids, sugars), and these are under close regulation (transcriptional and translational) during sporulation and crystal synthesis [32][33].

2. The Soil Spore-Forming Bacterium *Bacillus thuringiensis*

The identity of *Bacillus thuringiensis* relies on a set of pore-forming proteins, known as Cry and Cyt toxins, to kill insect larvae. Therefore, *Bt* is considered an insect pathogen [34][35][36][37][38]. *B. thuringiensis* belongs to the genus *Bacillus*, a rod-shaped Gram-positive soil bacterium that contains genomic DNA and extrachromosomal DNA (plasmids). Interestingly, many plasmids encode the delta-endotoxins or Cry proteins, a strategy of *B. thuringiensis* to survive in the harsh environment of the soil's rhizosphere and for insect and mammalian targeting [34][35][39][40]. Commitment in the life cycle of *B. thuringiensis* consists of a series of morphological and cytological changes that end with spore formation and crystal production. This series includes gene expression and biochemical and genetic programs [41][42].

The Plasmid-Encoded *Bt* Crystalline Proteins

The insecticidal delta-endotoxins of *Bacillus thuringiensis* or Cry (Crystalline) proteins have been the subject of intense research during the last three or four decades [6][35][39]. These crystals comprise an array of immature

protoxins with a molecular weight of 130 kDa, encoded in large plasmids [43][44][45]. To be active in the insect larvae host, protoxins are first solubilized and then processed in the C-terminal region favored by the enzymatic action, yielding a toxin with a molecular weight of 60–70 kDa [46][47]. The 3D structure of several Cry toxins was elucidated by X-ray resolution crystallography [48], including Cry1Ac [49], Cry2Aa [50], Cry3Aa [46], Cry3Ba [51], Cry4Aa [52], and Cry4Ba [53]. Moreover, based on sequence identity, it has been determined that the majority of the Cry toxins share three-domain structures with five highly conserved blocks in domain I [46][47]. Domain I is formed by a bundle of seven alpha-helices, with one central helix surrounded by six other alpha-helices [46]. The secondary structure of the alpha helices of domain I resemble bacterial pore-forming proteins, such as bacterial colicin I.

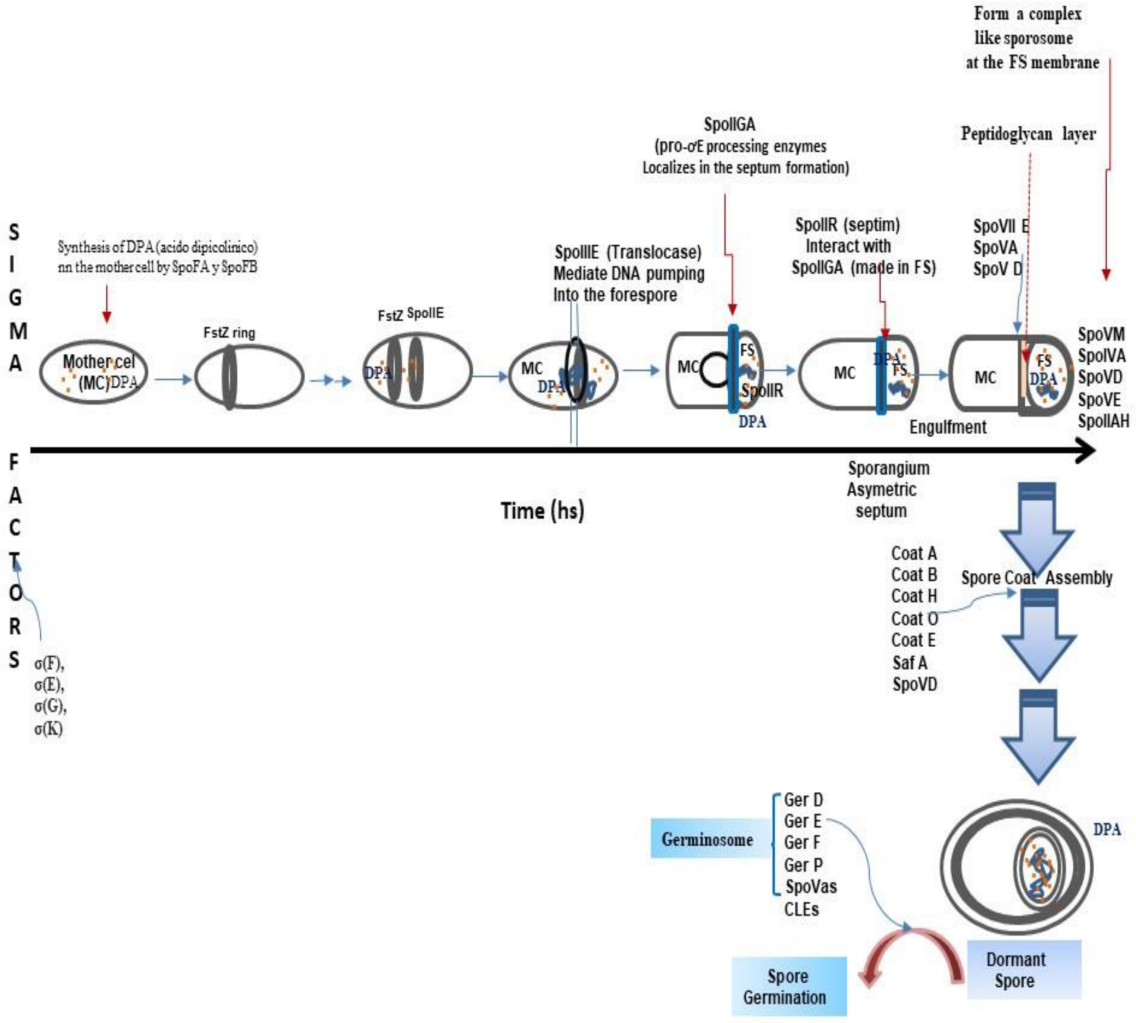
3. The General Sporulation Mechanism in the Genus *Bacillus*

The challenge and the goal objective of sporulation in the genus *Bacillus* and in other spore-forming bacteria such as Clostridium is DNA protection and survival [17][54][55]. The latter process is accomplished by the Firmicutes phylum, despite pressure selection, evolution, and diversity in the set of molecular components comprising the program and that crosstalk [24][54][56][57][58][59][60]

One of these systems is the Rap-Phr quorum sensing system, which regulates different bacterial processes, remarkably the commitment to sporulation in the *Bacillus* species [61][62][63][64][65][66][67][68]. How do Rap proteins act in sporulation? Rap proteins act as quorum sensors, forming a response regulator with a TPR (tetratricopeptide repeat) domain, a hydrophobic pocket able to bind the signaling peptide, thus inducing a conformational change and modulating regulator activities [66][69][70]. Therefore, RAP proteins act on phosphatases, an intermediary component of the sporulation phosphorelay system in Spo0F. Rap63 exhibited moderate activity during sporulation and is inhibited by the Phr63 peptide [42][71]. In *Bacillus subtilis* (frequently used as a model of the genus *Bacillus*), the starting sporulation programs is characterized by the phosphorylation of the master regulator Spo0A [24][31][72][73][74][75][76][77][78]. Across the genus Bacillus, the sporulation process is regulated by a cascade of sigma factors as follows: sigma F (σF); sigma E (σE): sigma G (σG), and sigma K (σK). Sigma factor K (σ(K) is a sigma factor conserved among the Bacillus genera, except in the genus *Clostridium* [24][31][42][78][79].

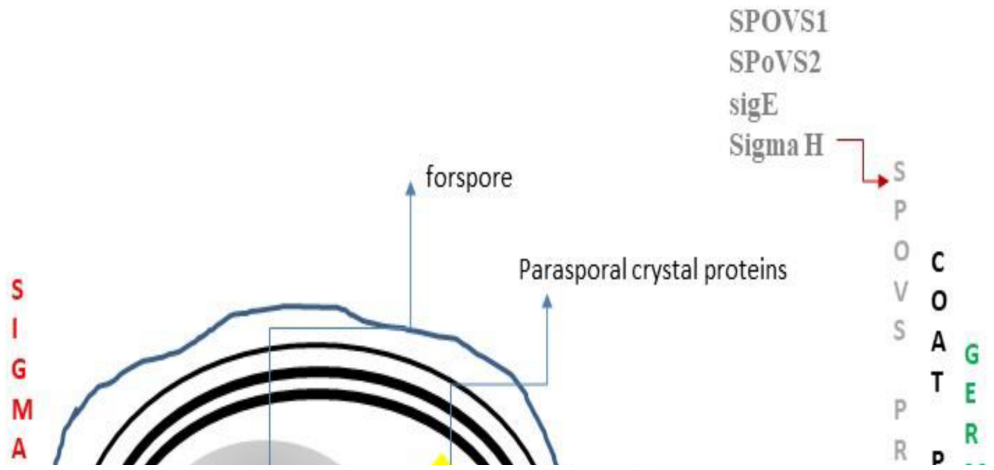
The sporulation program conserved among the members of the genus *Bacillus* comprises the following seven cytological and morphological changes [56][57][80] (**Figure 1**A): Stage 0 to Stage I, Axial filamentation; Stage II, Polar septum formation; Stage III, Forespore engulfment (σ^F, σ^E); Stage IV to Stage V, Cortex and coat assembly, and Stage VI to Stage VII, Spore maturation and mother-cell lysis. The morphological and cytological changes were impaired in spoIID, spoIIM, and spoIIP mutants [60][81][82][83][84][85][86] and in the spoIIB–spoVG double mutant. However, the deletion of the *spoVS* gene, controlled by σ^H, permitted the spoIIB–spoVG double-mutant, to complete engulfment [75][76][87][88]. SpoVA proteins are involved in the uptake and release of nutrients from the core during the uptake of Ca^{2+} dipicolinic acid. The lytic enzymes SleB and CwlJ, found in *bacilli*, hydrolyze the spore cortex [42]. The spore is formed by an assembly process that involves a four-layer coat. The coating proteins described for *Bacillus subtilis* include cot, cot B, saf A, cot H, cot O, cot E, ger E, and cot E ger E [31] (**Figure 1**A). Assembly starts from the external outermost amorphous (crust) layer, followed by the rodlet, the honeycomb, the

fibrous, and the nanodot particle layers, and finally, the undercoat/basement layer. Interestingly, under the exosporium of *B. thuringiensis* [89][90][91][92][93], a hexagonal honeycomb is exposed.



(A)

B. thuringiensis **spore: highly resistant to extreme and harsh environmental conditions (heat, chemical, salinity, ultraviolet radiation, competence)**



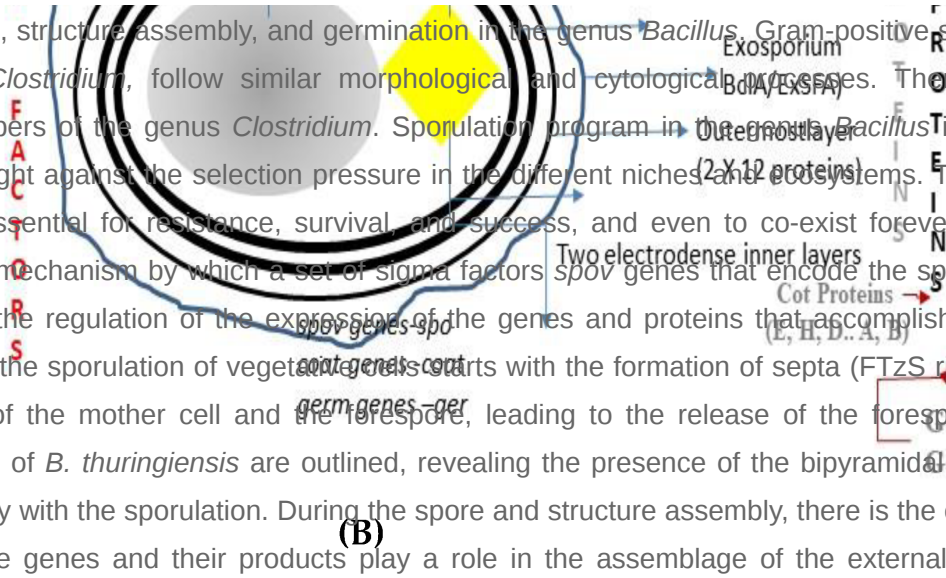


Figure 1. (**A**) Sporulation, structure assembly, and germination in the genus *Bacillus*. Gram-positive spore-forming bacteria, *Bacillus* and *Clostridium*, follow similar morphological and cytological processes. There are some differences among members of the genus *Clostridium*. Sporulation program in the genus *Bacillus* is conserved. The spore allows us to fight against the selection pressure in the different niches and ecosystems. Therefore, the sporulation process is essential for resistance, survival, and success, and even to co-exist forever. Briefly, the sporulation process is a mechanism by which a set of sigma factors *spov* genes that encode the specific SPoVS proteins are involved in the regulation of the expression of the genes and proteins that accomplish each of the steps. A principal step in the sporulation of vegetative cells starts with the formation of septa (FTzS ring), followed by asymmetric division of the mother cell and the forespore, leading to the release of the forespore. (**B**) The components of the spore of *B. thuringiensis* are outlined, revealing the presence of the bipyramidal crystal (ICP) synthesized concomitantly with the sporulation. During the spore and structure assembly, there is the expression of several *cot* genes. These genes and their products play a role in the assemblage of the external and internal layers, similar to that of the bacteriophage T4. Moreover, some members of the genera *Bacillus* possess an exosporium, -an outer layer missing in *B. subtilis*- that confers protection and a direct connection with the environment. The expression and production of the insecticidal crystal proteins (ICP) (in yellow) are under the regulation of the sigma factors, and together with sporulation, both are under metabolic regulated mechanisms at the transcriptional and translated levels. Some of the SpOV proteins also participate in crystal production. Thus, the spore of *Bt* is well-armored as an evolutive advantage for survival and success [89][90][91][92][93][94][95][96][97][98].

The lattice constant of the honeycomb structures was approximately nine nanometers (nm) for both *B. cereus* and *B. thuringiensis* spores, visualized using atomic force microscopy (AFM) by [92]. It was also possible to visualize the species-specific spore assembly and nanometer-scale structure of the spore's surfaces. Ensamblage of the fibrous layer involves the Cot H- and Cot E-dependent proteins and the Cot E-specific protein [85]; this is similar to the assembly of the spore-coating proteins, in that it mimics a non-mineral two-dimensional (2D) crystallization seeding pattern that begins to assemble the coating proteins from the inner to the outer layers in a similar manner as has been described for the bacteriophage lambda [99]. This assembly process is well characterized in *B. subtilis* (**Figure 1**A).

In bacterial-cell division, the structural and cytokinetic functions require the formation of the septum, which involves the assembly of a complex of proteins. Similar to *B. subtilis* sporulation, in *B. thuringiensis* sporulation, the sporulation-specific proteins Spo0A and SpoIIE play a role in gene regulation and in the determination of the structural properties of the specialized sporulation septum. Spore germination, nutrients, and mRNA number abundance participate, possibly providing ribonucleotides [100][101]. In *B. thuringiensis*, the mRNA number is 10–50 times higher than in other species of *Bacillus* and *Clostridium* [96]. How is the distribution found of mRNA in the spore compartments? A low abundance of mRNA is present in the mother cell and a high abundance of mRNA in the forespore (**Figure 1**A).

The transcription of these mRNA is under the control of the sigma factors F or G, and this can be similar among species of Bacillus. A minority of mRNA in the spores of these species is present at more than the molecule-per-spore, averaging only 6% of all individual mRNA identified in these spores. Thus, 94% of mRNA participates in the

generation of proteins that will affect the germination of the whole spore [69][102]. The close relatives of B. subtilis, *B. cereus*, *Bacillus anthracis*, and *Bacillus thuringiensis* Al Hakam, as well as the spores of *Bacillus megaterium* and *Clostridium difficile*, lack several nucleotide biosynthetic enzymes, which are synthesized only at defined times in spore outgrowth [1][103][104][105].

The 60 most abundant mRNA in all five *Bacillus* species transcribed in the developing spore were found only in dormant species. Sigma E/K-dependent transcripts in spores might arise from weak–dependent transcription in the forespore of some of these genes [106]. A possibility could lie in the connection between the mother cell and the forespore, termed a feeding tube in the cytoplasm [101][107][108][109][110], which serves the mother cell and transfers small molecules, such as ATP and amino acids, into the developing spore. mRNA or mRNA fragments also move from the mother cell into the forespore via this feeding tube [111]. The precise time in sporulation at which the feeding tube closes occurs late in forespore development. Developing spores cannot make ribonucleotides, amino acids, or ATP, in that at least several TCA cycle enzymes are absent [1][103][111].

In referring to the structural assembly of the multilayered spore of the genus *Bacillus,* microscopy technology advancements permitted us to approach the spore structure assembly [112]. The structure assembly of the spore coat is accompanied by the synthesis of proteins that contribute to the multilayered structure. These proteins exert a strong influence on the core protection of the endospore, the maintenance of spore-core dehydration and dormancy, and survival in the environment, distribution, and conferring germination [104][113][114][115][116]. The cortex is synthesized within the intermembrane space surrounding the forespore after the engulfment stage during sporulation [100][101][117]. The proteins for cortex synthesis are produced in both the forespore and the mother-cell compartments. Peptidoglycan, lipids, and proteins (GerPA, GerP) (cortex lytic enzymes) form part of the outer coat, the inner coat, and the cortex, playing a structural and biochemical function. For example, in *B. cereus*, it has been shown that six GerP proteins share proximity with cortex–lytic enzymes in the inner coat [112].

4. The General Spore Germination Program in the Genus *Bacillus*

In spore germination, molecular and morphogenetic changes are carried out as crosstalk among signals, germinant nutrients, and spore components in the committed endospore in order to awaken or break dormancy [118]. For an endospore, the fate and the decision to germinate encodes in the dormant spore. The program of spore germination refers to the multistep mechanism through which spores return to life, an awakening process that enables them to reenter into metabolic activity [118][119][120][121][122]. The knowledge of the components and signals in spore germination derives from studies of the model organism *Bacillus subtilis* [11][86][123][124]. However, there are current efforts to update and focus on other members of the genus *Bacillus*, especially those that constitute a problem in pathogenesis, health, agriculture, and in the food industry as well [122][125][126].

Which are the signals that initiate the process of awakening the dormant spore? What is known is that external signals (germinants, small molecules) that sense germination-specific proteins (GR)-like receptors localize in the outer and the inner coat of the spore [104][126][127][128][129][130][131]. The phenotypical characteristic of the spore-

germination stage is considered a weak stage or *spot* in the life cycle of *Bacilli* species. During this stage, the spores become susceptible to physical, chemical, and environmental conditions, starting from the inner membrane coat of the spore, to render these accessible to nutrients of low molecular weight, ions, nutrients, Dodecylamine, and water, in order to flow through the core cortex [123][127][132][133][134].

The general mechanism of spore germination can be outlined as follows:

(1) Germinant sensing; (2) Commitment to germinate; (3) Release of spore depot of dipicolinic acid (DPA); (4) Hydrolysis of peptidoglycan cortex spores; (5) Spore-core swelling and water uptake; (6) Cell-wall peptidoglycan remodeling, and (7) Restoration of core protein and inner-spore membrane–lipid mobility. This mechanism resembles a detailed general program for spore germination that is well characterized *in B. subtilis, B*. *anthracis,* and *B. cereus.* In *B. thuringiensis*, the differences in the molecular components that are involved in these processes are yet to be defined. One of the unsolved questions is related to the import and export of DPA and how the nutrients are sensed in the commitment spore [122].

Detailed program for spore germination in which all of the molecular components are outlined: (1) Activation. Nutrient germinant plus spores (minutes to hours). Lag phase, and (2) Commitment (a major change in IM permeability and structure). GERP proteins allow the access of nutrients into the inner membrane, low-molecular-weight, i.e., Dodecylamine, ions (H^+, Na^+, and K^+). Channel formation by the multiple spore-specific SpoVA) (n = 7) in *Bacillus subtilis* (Setlow and Christie., 2020; [104][127][135][136]; (3) Release of pyridine-2, 6-dicarboxylic acid (dipicolinic acid [DPA]) chelated at 1:1 with divalent cations, predominantly Calcium (Ca^{2+}DPA) through the IM channels; (4) Enzymatic lysozyme-mediated cleavage of the cortex, favoring permeability into small molecules in the inner coat, triggering spore germination; (5) Stage I. All of the Ca^{2+} DPA is released by the CLE cortex degradation, and this event leads to passage into stage II [137], and 6. Stage II cortex degradation is complete. The germ-cell wall and the core take up water and expand. This marks the initiation of germination, giving rise to growing cells and to the activation of metabolic activity [123][138].

5. Implication of the Knowledge of Sporulation Structural Assembly and Germination in the Soil Bacterium *B. thuringiensis*

The stable and resistant nature of spores and the possibility of germinating and growing in a gut environment render them suitable for treatment in the form of probiotics and as vehicles for vaccine and drug delivery. Spore treatments have shown great promise in animal studies. However, human trials require going further. Nonetheless, spores might open the door to safe, effective, and easy-to-administer therapeutics [60][125][139]. It is pivotal to elucidate and understand the life cycle of spore-forming bacteria, especially those bacilli that threaten agriculture, the food industry, and health care [18][140][141]. *t* has become a promising and potential new avenue of alternatives against the biological control of insects and the application of biotechnology biomedicine. Furthermore, Bacillus thuringiensis is viewed as a biofactory for the production of proteins, but also of other products for bioremediation and for improvement as bioinsecticides. Moreover, parasporal delta endotoxins are highly specific against different

orders of insects. However, Cyt proteins can exert a cytopathic effect on mammalian cells, specifically those changed by some types of cancer. This double sword of *Bt* marked the biotechnological success of *B. thuringiensis;* due to the versatility of *Bt*, great interest has emerged during the last two decades [3][4][35][96]. Work in this area ranges from basic research (mechanism of toxicity in insects) to applied science (the genetic engineering of economic crops with *cry* genes), the assembly of proteins for crystal formation (structural biology), and nanotechnology (drug-vehicle delivery or vehicles of subunit vaccines) [142][143][144]. Furthermore, *B. thuringiensis* can produce floating biofilms with a ring and a pellicle [145]. During sporulation, the spores remaining in the biofilm ring are of great utility for the food industry, because they confer spore resistance on washing and cleaning procedures. The spores can restart a new biofilm when food production has resumed [78].

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