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Review

Sporulation, Structure Assembly, and Germination in the Soil Bacterium *Bacillus thuringiensis*: Survival and Success in the Environment and the Insect Host

Gloria G. Guerrero M.

Unidad Académica de Ciencias Biológicas, Laboratorio de Immunobiología, Universidad Autónoma de Zacatecas, Av. Preparatoria S/N, Col. Agronomicas, Zacatecas 98066, Mexico; gloriaguillermina@uaz.edu.mx

Abstract: *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 recent 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. In the present minireview, the latter pinpointed and reviewed.

Keywords: spore-forming bacteria; Gram-positive bacillus; *Bacillus thuringiensis*; insecticidal crystal proteins (ICPs); spore-germination

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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 in biotechnology (nanotechnology, therapeutics) [1–7]. Meanwhile, the members of the genus *Clostridium*, such as *Clostridium perfringes*, *Clostridium botulinicum*, 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) [8–16].

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 how they carry out these biological events at the molecular level is the subject of the present review, 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 life cycle. Genes and proteins constitute players in spore formation and germination [10,17,19–24]. 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 condi-

tions vs. the soil rhizosphere [1,6,8,17,27–30]. Indeed, a recent study using 108 genomes of different genera of the phylum Firmicutes found that the majority of the bacilli share a core of at least 60 genes deriving from the 500 genes that participate in sporulation [24], while many spore coat proteins (small acid-soluble proteins) and germination proteins are not present in *Clostridia* and in some *Bacilli*. These shed light on the conservation, but also on the evolution and diversity, in both sporulation and germination in the phylum Firmicutes, thus implying a lifestyle shared between the genera Bacillus and Clostridium [2,24].

Spores are the forms most preserved, resistant, and propagated to the utmost degree in any geographical latitude [11,25,31]. Spores are highly resistant to extreme conditions, including physical conditions (such as temperature, pH, radiation), chemical conditions (salinity), or even biological conditions (selection pressure) [32]. Indeed, bacteria have survived under the conditions of an ancient primitive earth thanks to their ability to sporulate [11,24,31,33].

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 play 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 [34]. 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 metabolic regulation mechanism of sporulation and ICPs synthesis. Specifically, these metabolic pathways are involved in synthesis, energy storage, carbon supply, and nutrients (amino acids, sugars), and these are under close regulation (transcriptional and translational) during sporulation and crystal synthesis [35,36]. Nonetheless, there are many questions to answer in terms of the molecular evolution and conservation of the core set of molecular components, the master germinating receptors (GR), the master SpoVA, hydrolytic enzymes, the master dipicolinic acid, import and export, gene–operon organization, biochemical composition, PG, the structural organization of the lipids of the layers between the spores, the germinating spore, and the vegetative cells [37,38]. This knowledge can impact many applications that range from immune stimulants, such as probiotics [39], to nanotechnology as the ideal and model vehicle for drug delivery and vaccines, and in evolutionary biology, developmental biology, and ecology [6,30,40]. No less relevant is that *Bacillus thuringiensis* is successful either outside or inside of its host (insects). Some authors suggest that there is a battle between *Bt* and the insects [6]. Others propose that there is a co-evolution [41] of bacteria–insects, referring in particular to the hundreds of Cry toxins that are produced concomitantly with sporulation [6,42,43], enabling these toxins with binding properties to insect midgut receptors. In nature, in the soil's rhizosphere, there is a fruitful crosstalk among the bacterial community that involves competence for nutrients and for survival, in which the spore plays a primordial role.

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 [41,42,44–46]. *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 [41–43,47]. 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 [40,48]. Remarkably in *B. thuringiensis,* there is an arsenal (around six plasmids) of extrachromosomal DNA (pHT77 plasmids) encoding the delta-endotoxins or Cry proteins), the pAW63 plasmid harboring the RAP-PHR system [49–55], and also encoding another regulon system [56].

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 [6,42,43]. These crystals comprise an array of immature protoxins with a molecular weight of 130 kDa, encoded in large plasmids [49–51]. 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 [57,58]. The 3D structure of several Cry toxins was elucidated by X-ray resolution crystallography [59], including Cry1Ac [60], Cry2Aa [61], Cry3Aa [57], Cry3Ba [62], Cry4Aa [63], and Cry4Ba [64]. 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 [57,58]. Domain I is formed by a bundle of seven alpha-helices, with one central helix surrounded by the six other alpha helices [57]. The secondary structure of the alpha helices of domain I resemble bacterial pore-forming proteins, such as bacterial colicin I. Furthermore, the length of the helices of Cry domain I are sufficient to transverse cellular membranes [57,58]. Domain II is the most hypervariable region of the Cry toxins. The secondary topology is three anti-parallel β-sheets packed together in a β-prism with pseudo-three-fold symmetry [43,65–67]. Insect binding specificity is determined through the interaction of the loops of domain II and the receptors immersed in the insect's midgut. Domain III forms a β-sandwich [57]. The latter is an arrangement of two anti-parallel β-sheets packed in a "jelly roll" topology. Specifically, in the case of Cry1Aa and Cry1Ac, a loop extension in Cry1Ac creates an N-acetygalactosamine (GalNAc) binding pocket implicated in receptor binding and further toxin proteolysis [68–71]. The action of the Cry toxins depends on the presence of a set of insect midgut receptors. This molecular crosstalk between insect receptors and the delta-endotoxins of *B. thuringiensis* strengthened a co-evolution theory for survival and success [3,6,41,72–78].

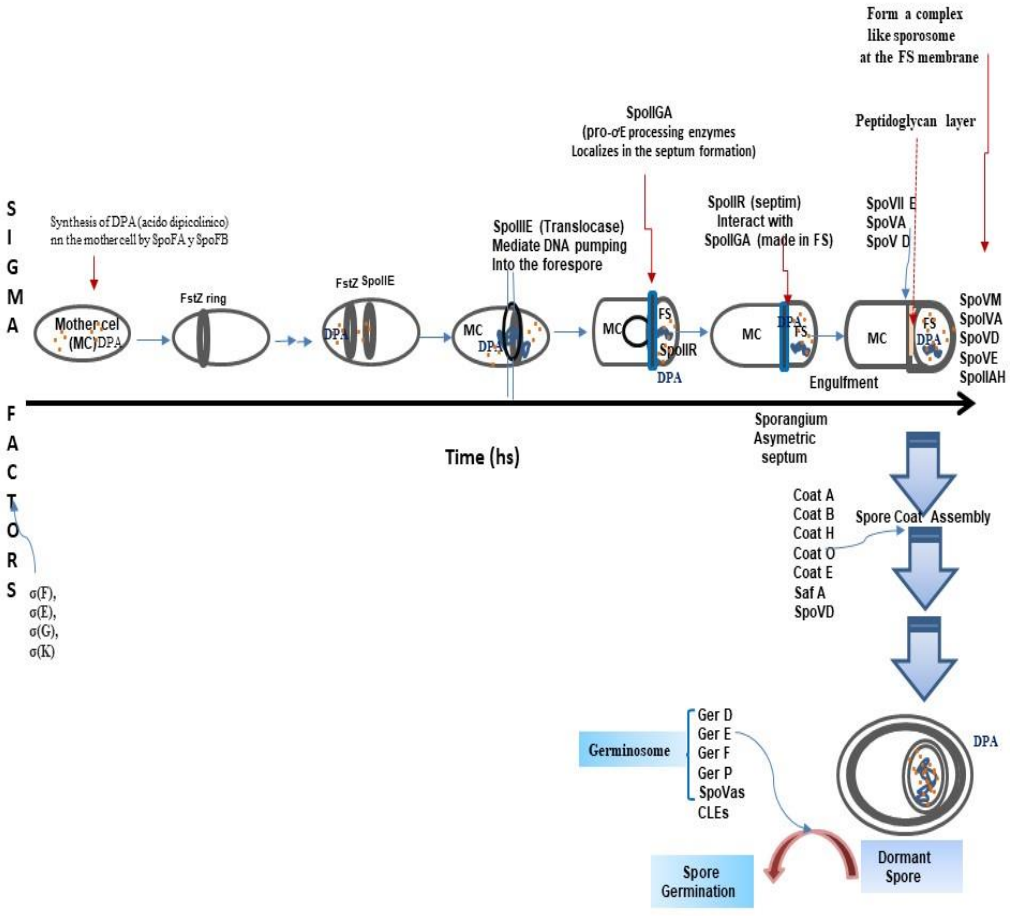
3. The General Sporulation Mechanism in the Genus *Bacillus*

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 [17,79,80]. 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 [24,79,81–85]

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 [86–93]. 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 [91,94,95]. 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 [48,96]. In *Bacillus subtilis* (frequently used as a model of the genus *Bacillus*), the starting sporulation programs is characterized by the phosphorylation of the master regula-

tor Spo0A [24,34,97–103]. 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* [24,34,48,103,104].

The sporulation program conserved among the members of the genus *Bacillus* comprises the following seven cytological and morphological changes [81,82,105] (Figure 1A): Stage 0 to Stage I, Axial filamentation; Stage II, Polar septum formation; Stage III, Forespore engulfment ($\sigma^{F,}$ 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 impaired in spoIID, spoIIM, and spoIIP mutants [85,106–111] and in the spoIIB–spoVG double mutant. However, the deletion of the *spoVS* gene, controlled by σ^{H}, permitted to the spoIIB–spoVG double-mutant, to complete engulfment [100,101,112,113]. 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 [48]. 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 [34] (Figure 1A). 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* [114–118], a hexagonal honeycomb is exposed.



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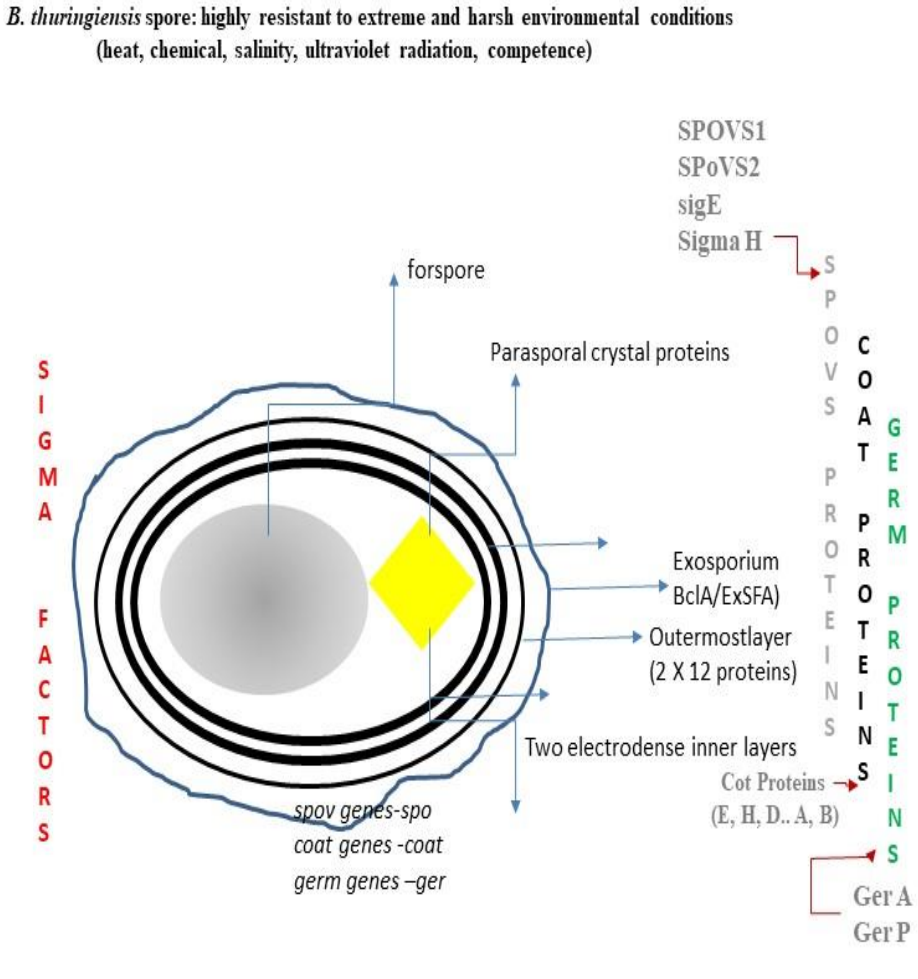

Figure 1. (**A**) Sporulation, structure assembly, and germination in the genus *Bacillus*. Gram-positive spore-forming bacteria, *Bacillus* and *Clostridium,* follow similar morphological and cytological processes. There are some differences among members of the genus *Clostridium*. Sporulation program 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 mechanism at transcriptional and translated level. 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 [54,55,114–121].

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 [117]. 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 [110]; 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 [122]. This assembly process is well characterized in *B. subtilis* (Figure 1A).

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 [123,124]. In *B. thuringiensis*, the mRNA number is 10–50-times higher than in other species of *Bacillus* and *Clostridium* [119]. 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 1A).

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 [94,125]. 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 [1,126–128].

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 [129]. A possibility could lie in the connection between the mother cell and the forespore, termed a feeding tube in the cytoplasm [124,130–133], 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 [134]. 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 [1,126,134].

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 [135]. 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 [127,136–139]. The cortex is synthesized within the intermembrane space surrounding the forespore after the engulfment stage during sporulation [123,124,140]. 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 [135].

3.1. Sporulation Program in B. thuringiensis

Recent work on *B. thuringiensis* sporulation has revealed the participation of several transcriptional factors. These factors determine the fate of vegetative cells in terms of sporulation, crystal formation, and germination [53–55,141–143]. As mentioned previously, sporulation in *B. thuringiensis* is highly conserved among the members of the genus *Bacillus*

(B. subtlis, B. cereus). Indeed, knowledge of the sporulation program has been extrapolated from the model organism of the genus *Bacillus, B. subtilis*, and *B. cereus* [135]. The initiation of *B. thuringiensis* sporulation is controlled by the gradual increase in the activity of the master regulator Spo0A. This regulator is phosphorylated by the multicomponent phosphorelay system [55,121]. This system is usually affected by two negative-regulatory mechanisms: The Rap phosphatases involved in the first mechanism, active, and regulated by peptides derived from Phr proteins and followed by an export–import maturation process [55]. The second negative mechanism involves the Spo0E-P -B, -E, and -H) family of proteins, Spo0A-specific phosphatases, and small proteins (ranging between 50 and 90 amino acids), which share the conserved motif SQELD [55,121].

The transcription factor CpcR positively regulates a spo0E family gene, and variations in this gene expression modulate the production of spores in *B. thuringiensis*. Thus, there is a correlation between *cpcr* gene expression and sporulation. Indeed, one question to be analyzed is whether CpcR interacts with the network of the sporulation program to control cell differentiation [54,55,121]. Moreover, Spo0A activity impairs the presence of the *cpcR* in the LM1212 strain (double spore-forming population and crystal production). Furthermore, a putative phosphatase of the Spo0E family dephosphorylated and phosphorylated Spo0A-Spo0A-P [54,55,121]. This molecular strategy employed by Bt to control sporulation as a cell differentiation process aimed to ensure its survival in the environment and the host [55] (Figure 1A,B). Moreover, the crosstalk between the set of sigma factors (controlling spoVa genes) and the SPoVA proteins during the sporulation of *Bacillus thuringiensis* is concomitant with parasporal crystal formation [119]. Several studies determine which of these talk to which. In particular, mutants in the set of genes involved have demonstrated that the sigma factors (σF, σE) play a role in forespore engulfment (stage 3). Mutants in the gene *spots* controlled by sigma factor (σH) are affected in terms of the efficiency of sporulation and polar septum formation (stage 2) [112,113,144–146].

The Sporulation Mechanism in *B. thuringiensis*, a Multistep Process

(1) Initiation of sporulation by the Rap-Phr system; (2) Commitment to sporulation regulated by the phosphorylation state of the major response regulator Spo0A [53,54,56]; (3) The different signals, such as nutritional deprivation, recognized by sporulation kinases [147]; (4) These kinases phosphorylate Spo0F, which is used as a substrate by the phosphotransferase Spo0B to phosphorylate Spo0A [88]; (5) Response regulator aspartate phosphatases (Rap) inhibit this signal transduction pathway by dephosphorylating the Spo0F-P response regulator [90]; (6) Rap protein activity inhibited by its related Phr peptide, and, (7) The mature Phr peptide of five, six, or seven amino acids [92,120,145,148] translated into a premature form that needs to be secreted, processed, and re-imported by oligopeptide permeases in order to be active [36,92,93,147].

Differences in the molecular mechanism of the cell's fate affect the distribution of the SPoVA proteins in sporulating bacteria (Figure 1B). Recent studies employing double mutants and genomic studies reported that sporulating proteins are distributed according to their function and to the host environment [83,84]. The differences in the numbers of homologous genes lead to differences in the role of the *spoVS* genes in the *B. cereus* group and in *B. subtilis*. The *spoVS* gene found in *B. thuringiensis,* that is, two homologous genes *spoVS1,* and *spoVS2,* are both conserved in *B. cereus* [119]. Their expression is dependent on sigma factor H (σH). Mutants in *B. thuringiensis* HD (Δ*spoVS1*) exhibited effects on morphological changes during sporulation [121,145], which include delay in sporulation efficiency, the formation of polar septa, and spore release. However, the mutants also failed to complete engulfment in some cells and demonstrated disporic septa [119]. Septal thinning and membrane migration play a role in forespore engulfment. The mutants also induced the decreased production of the parasporal crystal Cry1Ac [119]. Several molecular components are involved. For example, a sporulation-specific *cwlC* gene that encodes an N-acetyl muramyl-alanine amidase characterized in the *B. thuringiensis* subsp. *israelensis (Bti)* strain Bt-59. CwlC was the only cell-wall hydrolase in *Bti* found to

contain both MurNAc-LAA and Amidase 02C domains [149]. Moreover, and according to transcriptional analyses, *cwlC* was expressed at the late sporulation stage and was controlled by SigK. In addition, two other cell-wall hydrolase genes were identified with high expression levels, e.g., the *cwlB* and *cwlE* genes controlled by SigK. In contrast, another hydrolase encoded by the *cwlF* gene is not under the control of the SigK factor; however, this gene is plasmid-encoded [149]. Another molecular component is SpoIIID, a small, sequence-specific DNA-binding protein that can direct the transcription of many genes, and it affects spore formation in *B. subtilis* and *B. thuringiensis*. The SpoIIID mutant strain exhibited no mother-cell lysis in the Schaeffer sporulation medium (SSM), but did in Luria-Bertani (LB) medium. The deletion of *spoIIID* decreased crystal protein production in HD73. Furthermore, SpoIIID positively regulated the *sigK* gene, while sigma factor K (σK) negatively regulated the expression of *sigE* [150].

On the other hand, during parasporal crystal formation, the genes *cry1A* (Bravo et al., 1996) [145], *cry4A* [151], cry8E [152], and *cry11A* are controlled by σE or σK [147,153]. The sporulating-specific transcription factors, regulating toxins such as Spo9A, can positively regulate *cry1Ac* [146,154]. Thanks to the progressive advances in genomics, it has been possible to determine the differences in the functions of sporulation-related genes and non-sporulating genes in the genus *Bacillus,* including *B. cereus,* and *B. subtilis* [35,36,154]. *B. thuringiensis* possesses the PlcR regulon linked to endotoxin production [80]. *Bt* Cry toxins are considered nonpathogenic in humans, while Cyt (cytolytic) proteins cause damage to mammalian cells [155]. Cry toxins represent nearly 20–30% of the cell's dry weight. During sporulation, crystal proteins are localized on the spore surface, decreasing spore resistance, but increasing the insecticidal properties of the crystals [156]. The expression of the protoxin gene is controlled by sporulation-dependent promoters. All of the protoxins in *B. thuringiensis* subsp. *israelensis* and other genes or unknown functions encoded in a large plasmid (128 kb) [143,157]. Transcription depends on the mother cell at the middle stages of sporulation, mostly transcribed from the promoter cry4A-P1 and under the control of sigma 35, which is highly homologous and functions similarly in *B. subtilis* [153]. Cry (crystal), and Cyt (cytolytic) toxins are synthesized during sporulation and assembled into one or more crystalline parasporal bodies [158–160]. Several studies on *B. thuringiensis* subsp. *kurstaki* (HD1) has shown that the three Cry1A proteins co-crystallize to form a single bipyramidal crystal [161]. Even more so, during sporulation, the Cry2A inclusion assembles with and is partially embedded in the short axis of the bipyramidal crystal [162]. Remarkably, in mosquitocidal isolates of *B thuringiensis,* parasporal- body structures exhibit greater complexity [163–165]. Recent works have described Cry and Cyt inclusions as being bound together by a peripheral multilaminate fibrous matrix of unknown composition [110,117,160]. Therefore, there is a coordination of the synthesis and assembly of several protoxins and their association with the parasporal-body fibrous matrix during the synthesis of other proteins of the plasmid, suggesting that the latter are in the parasporal body [115,116,118,158–160]. Moreover, the genes *ssrSA* and *ssrSB* in the same operon are co-transcribed as a precursor, processed by specific ribonucleases to form mature 6S-1 and 6S-2 RNA. These non-coding RNA play a role in sporulation and parasporal crystal formation because the deletion of these genes inhibited the growth of *B. thuringiensis* in the stationary phase [86,96–99,102]. In the soil, *Bt* Cry toxins on the spore surface are protected by the exosporium. In the midgut, they are exposed and available for binding to the insect receptors [41,43,47,119]. Furthermore, studies on the Bacillus anthracis group (*B. cereus* and *B. thuringiensis*) [114] have shown that BclA, EsSY, and ExSFA are protein of the basal layer of the exosporium (the outermost external layer), serving as protection, host targeting, and dissemination) (Figure 1B). The underlying molecular mechanisms through which Cry and Cyt proteins are targeted to the parasporal-body matrix or the manner in which the structural integrity of this matrix is maintained is an issue that remains to be defined.

3.2. Routing the Regulatory Metabolism at Transcriptional and Translational Level for Sporulation and Insecticidal Crystal Protein Synthesis (ICPs) Production

To the myriad associated proteins produced for sporulation, high expression level of genes is required for insecticidal crystal proteins synthesis (ICPs). Moreover, sporulation and ICPs are energy-consuming, and require higher expression levels of genes to perform these biological processes. At this point, in the CT-43 strain chromosome, the metabolism of amino acid, carbon, and energy resources are in operons under systematic and regulated coordination at a transcriptional and translational level for routing the metabolism for sporulation and ICPs synthesis [35,36]. Thus, genes organized in operons and genes were either induced or up-regulated in response to amino acid starvation during sporulation. Thus, for the ICPs synthesis, a provision or supply of amino acids are necessary and a prerequisite for crystal protein production. More than 300 genes are involved in the aminoacid metabolism (KEGG base data). Previous isotopic work reported that 80% of amino acids for ICP synthesis came from protein turnover [166]. In *B. thuringiensis*, the amino acids are encoded in genes that conform to different operons, carrying thus complete biosynthetic pathways for the most common amino acids (n = 20). Interestingly, radioisotopic experiments have shown that amino acid supply for sporulation and ICP synthesis comes from protein recycling. A set of proteases, ATP proteases, regulatory proteins are transcriptionally up-regulated (Figure 2) and translational up or downregulated, which allow protein recycling to meet amino acid requirements during sporulation: a set of proteases (proteases, peptidases, and some ATP-dependent proteases) with high expression levels rapidly degrade many abnormal polypeptides. Other proteases exerting proteolysis functions are induced or up-regulated at the transcriptional level YabG (sporulation-specific proteases), CH1854 (intracellular serine protease), and CH3928 (serine protease), NprB (bacillolysin), CalY (camelysin), thermitase (thermostable serine protease), and Vpr (a high-molecular-mass minor extracellular protease) [167,168].

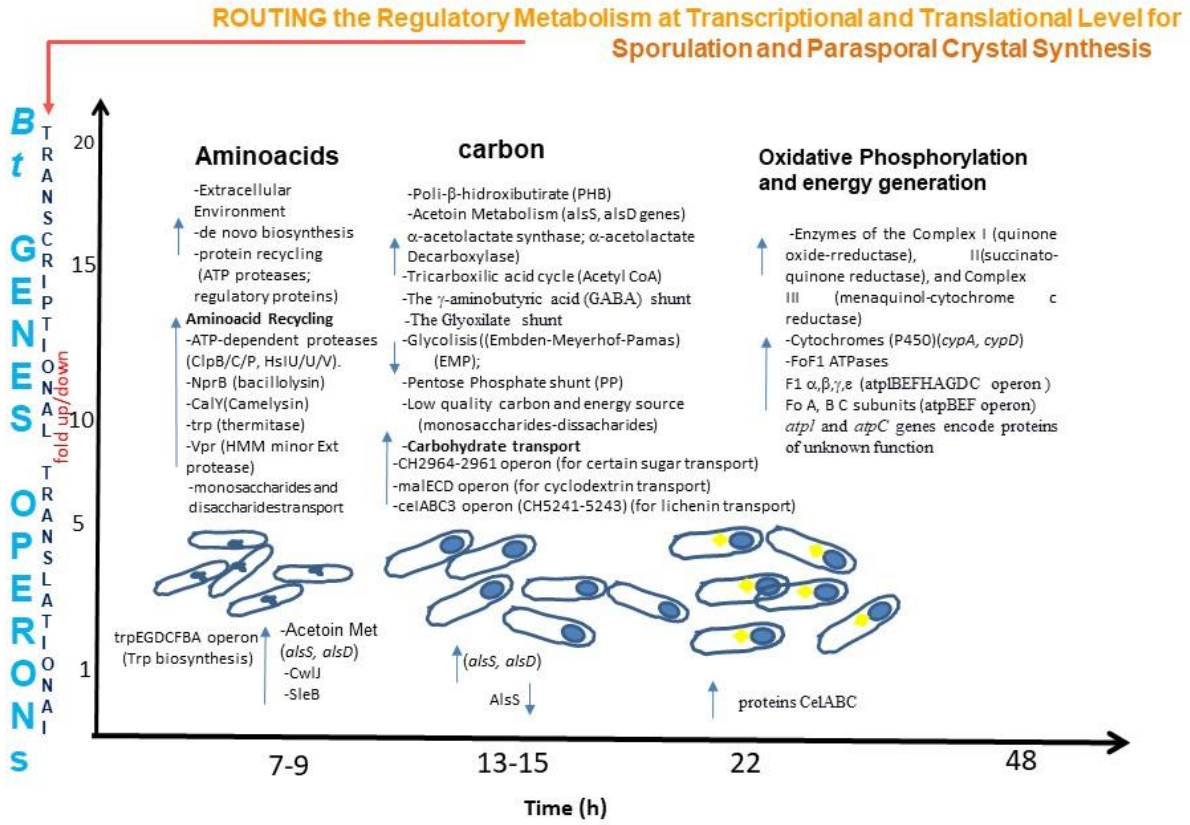

Figure 2. A myriad of genes is associated with the different biological processes in bacteria. Sporulation and crystal synthesis require routing the regulatory metabolism (amino acid biosynthesis, amino

acid transport, carbon supply, carbohydrate transport, and energy generation) at transcriptional (fold-up/down) and translational levels (amount of protein). How the different nutrient sources are routed and regulated for cell growth requires high expression levels of genes. At 7 to 8 h, many genes encoding proteases are induced, and they have functions such as transport and carbohydrate conversion for energy supply. While at 13 and 22 h, some other proteins start to increase and decrease other proteins required for the subsequent step in sporulation and crystal formation. The routing of the regulatory metabolism is quite complex. It is an up and down fold regulation as well as an increase-decrease production of proteins that successfully each cell growth accomplish this task efficiently. It seems that *B. thuringiensis* has evolved strategies to save energy and to profit from external nutrient sources [35,36].

Regulatory proteins control protein quality and regulate many biological processes [169,170]. Moreover, these proteins provide a large number of amino acids. In this way, protein recycling would be the source of amino acids during sporulation (Figure 2). Studies in the CT-43 strains using RNA-seq and bioinformatics have revealed that during nutrient starvation, genes and operons are induced and upregulated [171]. However, when the nutrient is rich, cells store intracellular (PHB) and extracellular (Acetoin) carbon molecules recycled under nutrient-depletion conditions. Indeed, some low-quality carbons as monosaccharides and disaccharides remained unused during exponential growth and fully utilized during sporulation and ICP synthesis.

Carbon and energy requirements *Bacillus* have evolved strategies to store and supply carbon and energy to carry sporulation and ICPs. Among these strategies for carbon supply are production and PHB reuse. PHB is produced as an intracellular carbon and energy storage substance in bacteria [172,173], and found a linear correlation between the final ICP and PHB concentration [172]. In the experiments performed by Wang et al., 2013 [36] with the CT-43 strain, they found that the intracellular PHB level started to increase rapidly, reaching a maximum level at 17 h, followed by a rapid decrease. PHB granules were visible by phase contrast microscope in some sporulating cells even at 15 h. Moreover, studies using RNA-seq [36] showed that most PHB synthesis-associated genes were highly expressed at 7 and 9 h and drastically reduced expression levels at 13 h. Meanwhile, PHB degradation-associated genes such as *pcaD* (*phaZ*), *scoT*, and *phbA1* increased at 13 h. At the translational level, PhbB protein decreased production at 13 h, whereas PhbA, PhbB, and PhaC dramatically down-regulated at 22 h. Conversely, the proteins associated with the PNB degradation pathway, the protein PcaD (PhaZ), increased at 13 h. Of relevance is that PhaP and PhaQ involved in the assembly and disassembly of PHB granules maintained high-level expression at both transcriptional and translational (Figure 2). These studies indicate that the PHB regulatory metabolism (synthesis and degradation) at these levels play a role as an important carbon source in sporulation and parasporal crystal formation. Another potential alternative for carbon sources is the **Pentose Phosphate (PP) shunt**. All the participating enzymes of this pathway were identified by iTRAQ [36], except Zwf, glucose-6-phosphate 1-dehydrogenase) but remained almost unchanged during sporulation, strengthening the suggestion that the PP pathway is not involved at all in providing the reducing power (NADPH) and metabolic intermediates involved in many biosynthetic processes. In the PP pathway, there are three alternatives or routes that could be involved during sporulation and ICP synthesis. The predominant route to arrive at the nodal point is gluconate-6p is: (1) glucose converted into glucose-6p; (2) glucose 6p is converted into Glucono-1, 5-lactone-6p by Zwf; and (3) the intermediate further transformed into gluconate-6p by 6-phosphogluconolactonase (CH3298). An alternative route is for glucose to be converted into gluconate by Gdh (glucose 1-dehydrogenase), and gluconate catalyzed into gluconate-6p by GntK (gluconokinase). The key limiting enzymes, Zwf and Gdh in both routes were not detected during the exponential growth phase (7 h) at both the transcriptional and translational levels [36]. However, the gene *zwf* was slightly induced at 9 h and then up-regulated at 13 h, while the gene *gdh* was initially induced at 13 h at the transcriptional

level. This data would imply a very significant regulatory mechanism of the PP pathway that could have a role as alternatives strategies during biological processes as the ones mentioned above. Interestingly, when CT-43 cells were grown in a GYS medium containing yeast extract, they followed a third route on the *gnt* operon that participates in gluconate metabolism. This *gnt* operon (CH2189–2191) is composed of *gntP* (gluconate permease), *gntK*, and *gntZ/gndA* (6-phosphogluconate dehydrogenase), and it lacks the transcriptional regulator, which expression is comparable to the negative regulator *gntR* described in *B. subtilis* [174]. The data of RNA-seq obtained by Wang et al., 2013 showed that the *gnt* operon expression level reached a maximum at seven hours and then gradually decreased. Furthermore, for bacteria, direct uptake of substances from the extracellular environment might be the most rapid and metabolically economical pathway. Therefore, extracellular gluconate is likely transported into the cells directly by GntP and converted into gluconate-6p by GntK, with gluconate-6p further catalyzed into ribulose-5p by GntZ/GndA, leading, to the repression of the genes *zwf* (glucose-6-phosphate 1-dehydrogenase) and *gdh* (glucose 1-dehydrogenase). Therefore, glucose does not enter the PP pathway when the extracellular environment contains gluconate, which might explain why 100% glucose catabolism was through the EMP pathway. The contribution of the PP pathway was still 5% in a glucose-glutamate-salts medium [175]. The low-quality carbon sources such as monosaccharides, disaccharides -glucosamine from chitin degradation and deacetylation, and glucose form lichenin cleavage [176,177] participate in energy metabolism and amino acid biosynthesis (particularly the branched-chain amino acids)(BCAAs) during sporulation. Furthermore, glucose and other monosaccharides (low-quality carbon sources) provided by the GYS medium could be exhausted during the exponential growth phase. They can enter the EMP pathway during sporulation to produce a large amount of pyruvate, which is also used for the high-level synthesis of dipicolinic acid (approx 25% of sporal core dry weight), a molecular component that plays a role in spore germination and resistance [178] (Figure 2). Fatty acids, b-oxidation, and the C2 and C4 compounds, the PHB (p-hydroxy-b-butyric acid) assembly and disassembly (PHB depolymerase) [179], (7) the intermediate of the TCA cycle (tricarboxylic acid), as acetyl-CoA utilized and remarkably upregulated during sporulation and ICP synthesis.

The TCA Cycle—Of relevance is that any mutant defective in the first three enzymes of the TCA cycle fails to express early sporulation genes suggesting that the activities of these enzymes are critical for sporulation [180,181]. On the contrary, a-ketoglutarate dehydrogenase, which catalyzes the fourth step of the TCA cycle, is not essential [182]. Remarkably, during sporulation, a considerable amount of acetyl-CoA is generated by pyruvate dehydrogenation, fatty acid b-oxidation, and the reuse of acetoin and PHB. It seems that acetyl-CoA would mainly flow into the TCA cycle to yield energy. The data reported by Wang et al., 2013 [36] along with other studies, have speculated that the TCA cycle is significantly modified or supplemented during sporulation via:

The glyoxylate shunt bypasses a portion of the TCA cycle to convert isocitrate to malate [183]. At transcriptional level, two glyoxylate shunt-specific genes; *aceA* (isocitrate lyase) and *aceB* (malate synthase) were up-regulated at 13 h. While at translational level, AceB production increased at 13 h and 22 h, respectively. This result implies that the glyoxylate shunt became more active during sporulation (Figure 2).

The **γ-aminobutyric acid (GABA) shunt** is an additional routing for the TCA cycle and is correlated with spore and parasporal crystal formation in *B-thuringiensis* [182,184]. GABA synthesis is through glutamate decarboxylation catalyzed by glutamate decarboxylase. Remarkably the sole glutamate decarboxylase GadB (CH2716) identified in CT-43 was not expressed at any phase at either the mRNA or protein level. Indeed GABA production was relatively weak in *Bacillus* strains [185]. However, the mRNA of GABA-specific permease *gabP* increased at 13 h, in agreement with an observation that *gabP* activated during nitrogen-limited growth [185]. Moreover, the GABA degradation-associated enzymes GabD (succinate-semialdehyde dehydrogenase) and GabT (4-aminobutyrate aminotransferase) were transcriptionally and translational up-regulated at 13 h, respectively. These

results suggest GABA metabolism became more active during sporulation and that the utilized GABA might mainly come from the extracellular environment. Furthermore, the GABA shunt and the methyl citrate cycle are interconnected through a common node, the succinate, leading, thus, to accurate PHB reuse. Indeed, SucC and Suc D proteins were slightly decreased at 13 h, possibly implying that a significant amount of succinate is converted into succinyl-CoA during sporulation. Data from *B cereus* and with CT-43 strains indicated a significant increase in the levels of enzymes and cytochromes involved in energy production via the electron transport system during the transition from vegetative cells to spores [186,187]. Thus, at the transcriptional level, two cytochrome P450 genes [186], *cypA* and *cypC*, were markedly up-regulated at 13 h, and a dNADPH-cytochrome P450 reductase gene *cypD* induced at 13 h. (Figure 2).

Oxidative phosphorylation and energy generation through the FoF1-ATPase (ATP synthase) complex which catalyzes ATP synthesis from ADP and Pi, driven by the proton gradient generated by the respiratory chain, and organized in the operon atplBEFHAGDC [187–189]. The atpHAGDC encodes the γ, α, β, and ε subunits of the F1 portion, while the atpBEF operon encodes the A, C, and B subunits of the Fo portion. The *atpl* encodes a protein with an unknown function. The genes of these operons are regulated at the transcriptional and translational levels [36]. Thus, *the atpC* gene was down-regulated by more than 20-fold, and the others were decreased by about 2-5 fold during sporulation. Besides, increased expression of the enzymes that form part of Complex I, II, and III (Figure 2). In contrast, at the translational level, the g, a, b, and e subunits of the F1 portion and the C and B subunits of the Fo portion were all maintained at similar levels at 13 h and increased by more than 1.8 fold at 22 h. However, the subunit of the Fo portion failed to be quantified. These data highlight the high energy requirements of spore and parasporal crystal formation. Indeed, as outlined in Figure 2, there is a subtle regulation of the metabolism (amino acids, carbon, and energy) at the transcriptional, translational level that allows *Bt* under nutrient depletion, starvation can perform biological processes such as sporulation, ICP synthesis, and especially those involved in the dipicolinic acid, a molecular component of the spore core [35,36] (Figure 2).

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 [190]. 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 [190–194]. The knowledge of the components and signals in spore germination derives from studies of the model organism *Bacillus subtilis* [11,111,195,196]. 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 [39,194,197].

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 [127,197–202]. 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 [195,198,203–205]. Specifically, in vivo spore germination in *B.thuringiensis* is carried out and favored under the alkaline and midgut-larval environment [140–142]. In vitro germination is activated by germinants (Dodecylamine, amino acids) in the culture medium and temperature growth at 25–27 °C in the medium [143,148,150,198]. The physical factors, such as temperature and pH, can aid the proteins of the coat's inner membrane (IM) in undergoing the confor-

mational changes that leave them in the appropriate conformation for interactions. This set of proteins comprises the so-called germinosome, including the GR proteins (particularly GERD), SpoVA proteins. GerP proteins favored the nutrient's access to the inner membrane [202,206–212]. SpoVA spore components are present during sporulation and germination events [193]. Moreover, to awaken the dormant endospore is to favor inner membrane (IM) structure permeability through the expression of the proteins (SpoVA) that form the channels (seven in *B.subtilis* spores) [203,213] for the movement, passage, and release of monovalent cations, including H^+, K^+, and Na^+ CaDPA (sodium calcium dipicolinic acid) [203,210,214–218]. These are proteins that take part in the release of DPA through channels encoded in one or more operons in all spore-forming bacilli [203,214–216,219,220]. In the particular case of *B. subtilis*, it is a heptacistronic operon. The mutations in the *ger* P locus cause a reduction in the permeability of the spore coat to germinant molecules [221]. In general, at least three proteins are present: SpoVAC; SpVAD, and SpoVAEb [193,222–224]. In *B.thuringiensis*, these proteins are organized as a biscistronic operon [225]. Once CaDPA release is complete in stage I, it triggers entry into stage II. In this latter stage, the expression and function of the molecular enzymatic machinery represented by the lytic transglycosylases (CLE, CwlJ, and SleB) [203,226,227], inactive in the dormant spore and recognize the muramic-δ-lactam modification present in the cortex. Furthermore, for in vitro spore germination, enzymatic treatment with the lysozyme removes the outer membrane and the spore-coat proteins. The concentration or amount of this enzyme is optimized to avoid damage to the incipient germinant spore. The enzymatic treatment renders the ions and nutrients permeable to the external and inner membranes, especially if they are low-molecular-weight germinant inducers. The cortex–lytic enzymes (CLE, CWlj, and SleB) should be present for the necessary cleavages and to permit the exchange of components such as dipicolinic acid (DPA). CLE can degrade the large peptidoglycan cortex layer, favoring the entry of ions and water, leading to spore-core swelling. Thus, the spore becomes a growing cell with restored metabolic activity [198,203,204,228,229] (Figure 3).

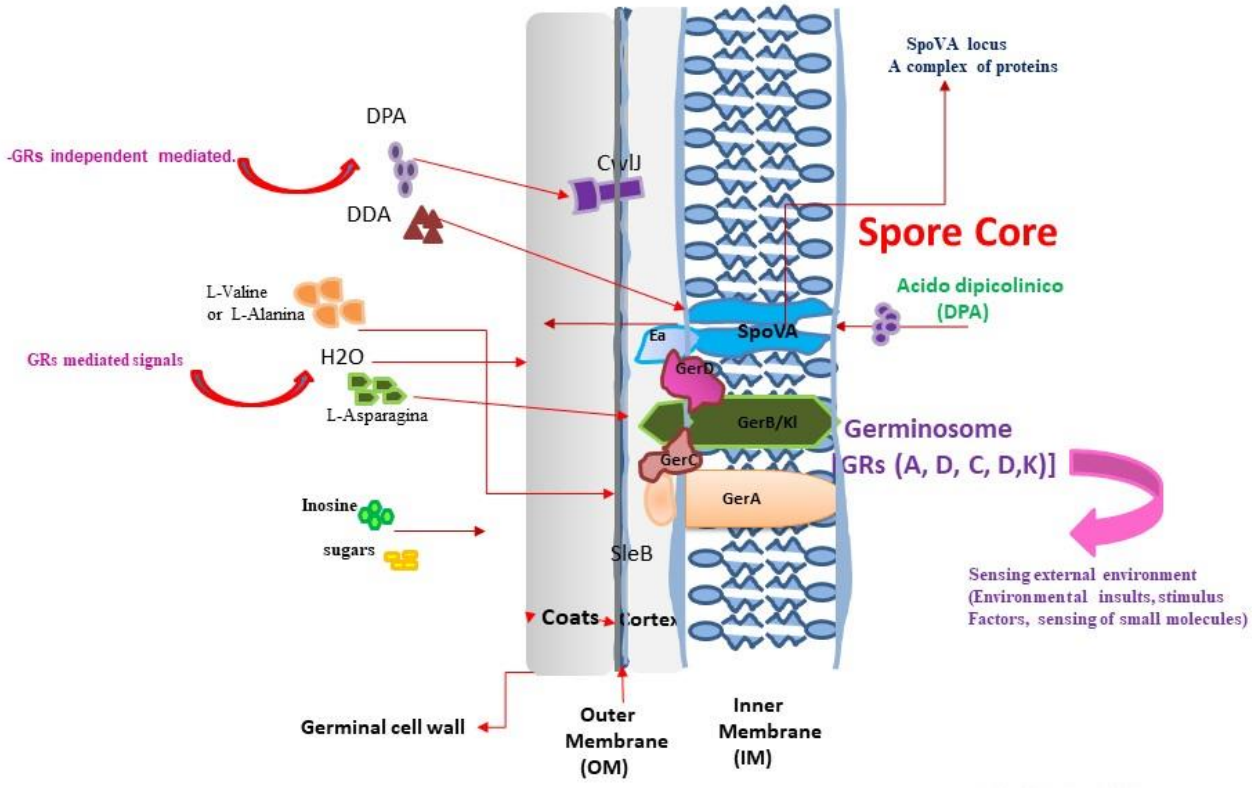

Figure 3. Scheme of the dynamic interaction and cooperation between germinant receptors (GR), and the SpoVA proteins during the spore-germination in the genus *Bacillus*. For the dormant spore to

wake up, start: 1, Sensing of the nutrients (aminoacids, sugars) or external stimuli from the environment or the host by the germinant receptor (GR), clustered in like germinosome (A, D, C, D, K) Schem) in the IM or other molecular components (CWLJ) in the outer membrane; 2. Interaction and cooperation between GRs, and a cooperation with SpoVA proteins; 3,Transmission downstream of the external signals, 4, Activation of the release of dipicolinic acid (DPA), and 5, Initiation of the germination process [37].

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 [194]. Furthermore, the evolution, conservation, and diversity in the machinery of germination programs among the members of the genera *Bacillus* and *Clostridium* have recently approximated by genome sequencing [193,230,231]. It would appear that DPA is the master component whose movement determines the sporulation or germination of a vegetative cell. Indeed, in a recent study using *B. subtilis*, it was suggested that the subunits of the SpoVa proteins that form channels and cytoplasmic plugs play a role in the efficiency of DPA import (sporulation) and export (germination) [37,38,135]. DPA transport into the spores involves cycles of unplugging and then replugging the C-Eb membrane channel. Nutrient detection during germination triggers DPA release by unplugging the C-Eb [37,38,135] (Figure 3).

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; [127,198,230,232]; (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 [208], 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 [195,224].

How is this program conducted? A strategy for success in the awakening of the spores is to produce effective dynamic interaction between the sensor and the nutrients. The termination "ome" refers to a set of clustered elements that performs an integrated function. Therefore, in spore germination, there is recent evidence of the local clustering of receptors in a germinosome in the IM of *B. subtilis* [233,234], and *B. cereus* [37,219]. Moreover, in some points, *B.thuringiensis* form foci as a complex of germinant receptors (GR) known as germinosomes [119,227,235–237]. These include the SpoVA proteins, involved in the uptake of Ca^{2+}-dipicolinic acid into the forespore during sporulation, which are also responsible for its release during germination. Lytic enzymes SleB and CwlJ, found in bacilli and in some clostridia, this enzymes hydrolyze the spore cortex; other clostridia use SleC for this purpose. The diversity in the machinery of germination programs conformed by proteins. SpoVA, enzymatic, and the GR between the genera *Bacillus* and *Clostridium* have been approached by means of genome sequencing [193]. To understand how the germinated receptors (GR) integrate and transmit the downstream signal to awaken the

dormant spores, one alternative is that receptors cluster in the germinosome to allow interaction with each other and with SPoVA proteins at the inner membrane (IM). Thus, germinants reach the GR after passing the outer membrane and interact specifically with GR, which respond to this and transmit to the SPoVA proteins [238] (Figure 3). Despite this, some germinants can interact directly with SPoVA independently of the GR, such as Dodecylamine (DDA) (Figure 3). In the cooperative interactions between the GR and the nutrients, the subunit secondary structure of the GR plays a role. For example, it is known that GR are commonly formed of A, B, and C subunits, encoded by tricistronic *ger* operons, as is the case for *B. subtilis*. In the spores of *B. subtilis*, GerA is the major GR and it has the following three subunits: GerAA; GerAB, and GerAC. The L-alanine activation of GerA requires all three subunits. Prediction studies have revealed that the secondary structure of GerAB is an alpha-helical transmembrane protein that can form water channels. Furthermore, molecular simulation studies have revealed that L-alanine can bind transiently to specific sites on GerAB [234]. In addition, the *B. licheniformis* genome contains the *gerA* family operons *gerA*, *ynd*, and *gerK*, in contrast to the ABC (D) organization, which characterizes the *gerA* operons of many *Bacillus* species. Indeed, *B. licheniformis* genomes contain a pentacistronic *ynd* operon, the *yndD*, *yndE3*, *yndE2*, *yndF1*, and *yndE1* genes, encoding A, B, B, C, and B GR subunits, respectively (subscripts indicate paralogs). In contrast to the B subunits of Ynd, the B subunit of GerA was essential for amino-acid-induced germination [220,235,239,240]. Therefore, in addition to the operon organization of the GR receptors, dynamic structural studies have provided novel insights into the role of individual GR subunits involved in the cooperative interaction among GR, in triggering spore germination [220,235,239,240] (Figure 3). As outlined in Figure 1A,B, dormant spores can awake in several ways: (1) by external stimuli, external environmental signals; (2) in the host by nutrients; (3) by germinants receptors (GR) present in the inner membrane or in the outer membrane; (4) by other components such as CwLJ, or directly signal through SPoVA (independently of GR). In addition, the cooperative interactions of the GR (A, B, C, D), or in some cases, Ynd and GerK components which drive signalization with SPoVAC. This activation can initiate germination by release of DPA through the SPoVA channel, which is a central locus of a set of proteins, key in the import and export of DPA (Figure 3).

The Molecular Components of the Germination of B. thuringiensis

The molecular components of the germination of *B. thuringiensis* that are known are: (1) The enzyme Alanine racemase (AlrA), encoded by the gene homologous to the major component of the exosporium of *B. cereus* spores, *alrA*, which plays a crucial role in moderating the germination rate of *B. thuringiensis* spores [235]. Converted D-alanine into L-alanine, and this in turn stimulated spore germination in *B. thuringiensis*. This gene, cloned from *B.thuringiensis* subsp. *kurstaki* [235], is transcribed only in the sporulating cells. (2) The CLE and spore cortex–lytic enzymes are essential for germination in bacilli. A homolog of the *cwlJ* gene involved in spore germination was isolated from *B.thuringiensis*. The deduced product of this gene exhibits striking sequence similarity to the lytic enzyme, CwlJ of *B. subtilis*. Another open reading frame (ORF), 27 bp downstream of *cwlJ*, which deduced the product, shows high similarity to the YwdL of *B. subtilis*. Reverse transcription-polymerase-chain reaction (RT-PCR analysis indicated that cwlJ and ywdL formed a bicistronic operon in *B. thuringiensis* [225]. In addition, a gene-encoding spore, the cortex–lytic enzyme, designated sleB, was cloned from *B. thuringiensis*. The disruption of sleB did not affect the vegetative growth of *B. thuringiensis*. However, the fall in optical density (OD) (600 nm) in the mutant spores was much slower than in the wild-type strain during the spore germination induced by L-alanine [236] (3). The spore germination of Bacillus can start by sensing germinants such as L-alanine and by binding to specific receptors. The GerA receptor responds to L-alanine in *B. subtilis*. A homologous gerA operon of B. subtilis was isolated from *B. thuringiensis* subsp. *kurstaki*. Disruption of the gerA operon led to blockage of the L-alanine-initiated germination pathway and revealed a delayed inosine-induced germi-

nation response. The germination rate of the gerA complementary- strain spore deriving from the introduction of the gerA operon into the disruption mutant was even faster than that of the wild-type-strain spore [237]. Moreover, one polysaccharide deacetylase gene was cloned from *B. thuringiensis* and was designated *pdaA*. Disruption of *pdaA* did not affect vegetative growth and sporulation, but did affect spore germination [236]. (4) gerM is a very conservative gene of 4.5 kilobase (kb). A DNA fragment cloned from the partial DNA library of *B. thuringiensis* subsp. *kurstaki* 1.175. Sequence analysis showed that the fragment contains one complete ORF that encodes a 349-amino- acid (aa) protein, which has high homology with the GerM protein from *B. subtilis.* (5) The function of the *gerM* gene in *Bt* spores was analyzed utilizing gene disruption. The resulting *germ* mutant grows, sporulates and germinates slower than wild-type *Bt* spores in the presence of L-alanine or inosine. This result suggested that *gerM* expression is required for the normal germination initiated by L-alanine or inosine in *B. thuringiensis* [119,236,237] (Figure 3).

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 [39,85,241]. 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 [18,240,242]. *Bt* 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, arasporal 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 [3,4,42,119]. 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) [243–245].Furthermore, *B. thuringiensis* can produce floating biofilms with a ring and a pellicle [246]. 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 [103]. A recent work [247] reveals improvement in the outdoor use of Cry34Aa toxins (effective against Diptera) as encapsulated proteins in a polyhedral microcrystal toxic to *Bombyx mori* cypovirus. This system provides stability and long-term protein activity under hostile environmental and physical conditions and UV irradiation [247]. *Bt cry* genes have characteristics to enhance resistance to insect pests in genetically modified crops, the application of *cry* genes in plant genetic engineering, and the effect of the *Bt* against different species of invertebrates (nematodes, ticks, mites). New strains have been identified of *Bt* that produce Cyt2BA against *Bradysia difformis* (pests in mushroom production) [4,248]. Another examples of this is the *B. thuringiensis* strains GBA46 and NMTD81, and the strain FZB42, Of toxic against the phytopathogen *Aphelenchoides besseyi* and cause severe damage to various crops of plants and vegetables [37,71,239]. Of relevance is that the progress and the development of novel and improved biological technologies for the bioremediation of heavy metals and other pollutants [249,250], *B. thuringiensis* is a model for hijacking the process of the synthesis of crystal proteins in order to generate diverse proteins with applications in biotechnology and medicine (nanoparticle delivery system) [251]. *B. thuringiensis* is a potential and suitable cell factory for different novel, valuable bioproducts [249–251].

6. Conclusions and Remarks

The majority of studies regarding *B. thuringiensis* centered on elucidating the mechanism of action of the Cry proteins. *Bt* Cry toxins as bioinsecticides. However, in light of the emergence of several worthwhile studies on the *Bacillus* model *B. subtilis*, followed closely by that of *B. cereus*, the molecular biology of *B. thuringiensis (Bt)* sporulation, structural assembly, and germination has approximated it. As reviewed herein, the molecular machinery involved in the morphological and cytological processes is conserved in the genus Bacillus, which belongs to the phylum Firmicutes. Furthermore and remarkably, the metabolic regulation at transcriptional and translational levels of the sporulation and crystal proteins formation. Despite several differential spatio-temporal differences in gene expression and in sigma transcriptional and protein patterns, this morphological complex differentiation process reveals a common cell fate, preservation, and survival under nutrient limitation and harsh environmental conditions. A striking feature of the infectious life cycle of B. *thuringiensis* resides in the concomitant expression and production of the crystalline-delta endotoxins (Cry proteins), a strategy conferred on *Bt* for survival and success in the environment (biological control) and the host. Furthermore, the molecular co-evolution and molecular crosstalk in metabolism are maintained through the evolution between Cry endotoxins and the set of receptors immersed in the insect midgut, thus conferring an evolutive advantage for survival and success.

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