Review

Regulation of cry Gene Expression in *Bacillus thuringiensis*

Chao Deng 1,2, Qi Peng 1, Fuping Song 1,* and Didier Lereclus 2,3,*

1 State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China; E-Mails: dengchaowin@sina.com (C.D.); qpeng@ippcaas.cn (Q.P.)
2 INRA, UMR1319 Micalis, La Minière, Guyancourt 78280, France
3 AgroParisTech, UMR Micalis, Jouy-en-Josas 78352 cedex, France

* Authors to whom correspondence should be addressed; E-Mails: fpsong@ippcaas.cn (F.S.); Didier.Lereclus@jouy.inra.fr (D.L.);
Tel.: +86-10-6289-6634 (F.S.); +33-1-34-653779 (D.L.);
Fax: +86-10-6281-2642 (F.S.).

Received: 3 June 2014; in revised form: 11 July 2014 / Accepted: 15 July 2014 / Published: 23 July 2014

**Abstract:** *Bacillus thuringiensis* differs from the closely related *Bacillus cereus* group species by its ability to produce crystalline inclusions. The production of these crystals mainly results from the expression of the *cry* genes, from the stability of their transcripts and from the synthesis, accumulation and crystallization of large amounts of insecticidal Cry proteins. This process normally coincides with sporulation and is regulated by various factors operating at the transcriptional, post-transcriptional, metabolic and post-translational levels.

**Keywords:** *cry* gene; transcription; metabolism; crystallization; protein expression; regulation; RNA stability

1. Introduction

*Bacillus thuringiensis* (Bt) is a spore-forming bacterium, which is genetically very closely related to *B. anthracis*, the agent of anthrax, and to *B. cereus*, an opportunistic human pathogen that causes food-borne gastroenteritis [1]. One of the most evident differences between Bt and its relatives is its ability to produce crystal inclusions during the stationary phase of growth (Figure 1) [1]. By contrast, the plasmids carrying the toxin genes of *B. anthracis* and *B. cereus* are absent from Bt [2]. The crystals
produced by Bt mainly consist of Cry proteins, most of which are toxic for specific insects [3], and consequently Bt has been widely and successfully used as a biopesticide for more than 50 years [3,4]. The crystal inclusion can account for up to 25% of the dry weight of Bt cells, which means that a massive production of proteins should occur during stationary phase (each cell has to synthesize $10^6$ to $2 \times 10^6$ δ-endotoxin molecules to form a crystal) [5]. The mechanism for the massive expression of Cry proteins in Bt has been investigated and involves numerous factors: transcriptional regulation, cry gene copy number, the stability of cry gene mRNA, and the accumulation and crystallization of Cry proteins [5,6]. Many findings relevant to the regulation of cry gene expression have been reported over the last 20 years. They indicate that the regulation of Bt cry gene expression is more complex than formerly thought: some sporulation-dependent cry genes are under the control of the transition-phase sigma factor SigH during the transition phase; some non-sigma factors contribute to the regulation of cry gene expression; metabolic pathways may influence Cry protein production; and the description of a unique Bt strain, LM1212, has changed our view of Bt cry gene transcriptional regulation and expression patterns. The aim of this review is to consider and collate current knowledge about the regulation of cry gene expression in Bt.

### Figure 1. Different patterns of crystal production in Bt. (A) Strain HD73 with a typical parasporal crystal phenotype: the crystal is produced beside the spore, in the mother cell compartment; (B) Strain YBT-020 with the spore-crystal association phenotype: the crystal is produced between the exosporium and the spore coat; (C) Strain LM1212 with the crystal-producer and spore-former differentiation phenotype: crystal and spore are produced in different cell subpopulations. The exosporium is indicated by arrows.

#### 2. Transcriptional Regulation

The primary regulation of gene expression is at the transcriptional level. The cry genes have been classified into two types according to their transcriptional regulation mechanisms: sporulation-dependent cry genes are controlled by sporulation-specific sigma factors SigK and/or SigE; and sporulation-independent cry genes are under the control of the vegetative SigA factor [5]. Some accessory factors also contribute to the transcriptional regulation of cry gene expression (Table 1).
Table 1. Transcription factors involved in cry gene expression.

| Gene     | Sigma factors | Other TFs                  | References |
|----------|---------------|----------------------------|------------|
| cry1A    | σE, σK        | Sp00A (+); PDH E2 (+)      | [7–9]      |
| cry2A    | σE           | NA                         | [10]       |
| cry2B    | σE           | NA                         | [10]       |
| cry3A    | σH           | NA                         | [5]        |
| cry4A    | σE, σK, σH    | PPK(+); Hpr/CcpA(−)        | [11–14]    |
| cry4B    | σE, σK       | NA                         | [11]       |
| cry6Aa2  | NA            | ORF2 (−)                   | [15]       |
| cry8Ea1  | σE, σK, σH    | NA                         | [16]       |
| cry11A   | σE           | Sp00A (−)                  | [11]       |
| cry15A   | σE           | NA                         | [17]       |
| cry18A   | σE, σK       | NA                         | [18]       |

Notes: TFs: Transcription factors; (−): negative regulation; (+): positive regulation. NA: undetermined.

2.1. Sporulation-Dependent cry Genes

The sporulation of Bacillus species initiates with the asymmetric division of cellular compartment into two parts: the mother cell and the forespore. In the model organism B. subtilis (Bs), this process is temporally and spatially regulated by a set of sigma factors of RNA polymerase: the main vegetative sigma factor SigA and SigH in the pre-asymmetric division cell; SigE and SigK in the mother cell; and SigF and SigG in the forespore [19]. Homologous sigma factors (SigA, SigH, SigE, SigK, SigF and SigG) have been found in Bt and play similar roles as those in Bs. Thus, it is generally assumed that the sporulation process in Bt is roughly the same as that in Bs [20–22]. Many cry genes have been defined as sporulation-dependent because their transcription is mainly controlled by the mother cell-specific sigma factors SigE and SigK.

The transcription of many cry genes, including cry1 [7,23], cry4 [11,24,25], cry8 [16], cry11 [11] and cry18 [18], is controlled by both SigE and SigK. The transcription is initiated by SigE at the early stage of sporulation and continued by SigK at the late stage of sporulation [20]. The successive activation of these two mother cell-specific sigma factors ensures the continuous and strong transcription of cry genes in the mother cells. This allows the production of massive amounts of Cry proteins during sporulation. The transcription of a minority of the sporulation-dependent cry genes, notably cry15A (formerly known as cry34) [17] and cry2 [10], is controlled by SigE alone. These genes are expressed for a relatively shorter period than cry genes directed by both SigE and SigK [21].

Some of the sporulation-dependent cry genes, for example, the cry1 [8], cry4 [11], cry8 [16], and cry11 [11], are weakly expressed at the end of the vegetative growth phase. This expression is initiated by the vegetative sigma factor SigH. The SigH-dependent promoter of cry1Ac maps upstream from the SigE- and SigK-dependent promoters [8]; the cry4 and cry11 SigH-dependent promoters overlap with the SigE-dependent promoters [11,24]; and the SigH-dependent promoter of cry8E maps in the intergenic region between the cry8E coding sequence and an upstream gene called orf1 [16]. Thus, there is not a single model that applies to the transcriptional regulation of all cry genes, and their regulation depends on a combination of promoters allowing various expression patterns during sporulation.
2.2. Sporulation-Independent cry Genes

The transcription of cry3 genes is initiated during the vegetative growth, is activated at the end of exponential phase and continues for several hours during the stationary phase [5]. Unlike the sporulation-dependent cry genes, which are controlled by sporulation-specific sigma factors, transcription of cry3 genes is directed by the vegetative SigA promoter and the expression of cry3 genes is stronger in sporulation-defective spo0A and spo0F mutants than the wild-type [5,26–28]. The cry3 genes were the only examples of sporulation-independent cry genes until the discovery of an unusual Bt strain, LM1212 [29].

Strain LM1212 displays a unique and intriguing phenotype: there is differentiation of crystal-producing cells and spore-forming cells in the population. Thus, crystals are produced in a subpopulation of cells that do not sporulate, and not in the mother cell compartment of sporulating cells (Figure 1). Transcriptional analysis of LM1212 cry genes revealed that the promoters of these genes are activated at the end of exponential growth and, in LM1212, are continually expressed for several hours. In sharp contrast, the activity of LM1212 cry gene promoters is very low in the wild-type Bt strain kurstaki HD73 throughout growth. Work with promoter-gfp fusions revealed that the LM1212 cry genes are specifically transcribed in a subpopulation of non-sporulating cells in Bacillus species, and this subpopulation is much smaller in strains other than LM1212 (Figure 2). The analysis of the expression of the promoter-gfp fusion also indicated that the LM1212 cry genes are not controlled by the sporulation-specific sigma factors SigE or SigK. Thus the LM1212 cry genes are sporulation-independent and are controlled by a novel mechanism of transcriptional regulation.

Figure 2. Specific transcription of LM1212 cry gene promoter in non-sporulating cells of Bt strains. (A) The LM1212 cry gene promoter drives transcription specifically and strongly in strain LM1212 in a subpopulation of crystal-producers; (B) The LM1212 cry gene promoter in strain HD73 drives transcription specifically and weakly in a small non-sporulating subpopulation of cells. Left: GFP/FM®-4-64 overlay. Right: bright field image. Sporulating cells are indicated by arrows and GFP-expressing cells are indicated by arrow heads. Cells were grown in SSM medium until T20 with erythromycin. The cell walls were stained with FM®-4-64.

2.3. Regulation of cry Gene Expression by Other Factors

2.3.1. The Transcriptional Regulator Spo0A

The DNA-binding protein Spo0A is the master regulator for entry into sporulation in B. subtilis [30]. The phosphorylated form of Spo0A (Spo0A–P) binds to a DNA sequence known as the “0A-box” and
acts as both a repressor of certain vegetatively expressed genes and an activator of sporulation-specific genes [30]. The temporal and spatial gene regulation during the sporulation process in Bt is very similar to that in Bs, and the Spo0A proteins of Bt and Bs are homologous [28]. An in silico analysis found DNA sequences similar to the “0A-box” upstream from the cry genes (cry4A, cry4B and cry11A) in B. thuringiensis subsp. israelensis (Bti) [11]. The transcription level of the cry11A promoter is higher in the spo0A mutant than that in the sigE mutant [11]. However, deletion of the putative “0A-box” increases expression of the cry11A operon, and resulted in its activity being higher in the spo0A mutant than that in the spo0A mutant [11]. These results suggest that the expression of cry11A is repressed by Spo0A via its binding to the putative “0A-box” [11]. By contrast, the cry1Ac promoter has a higher activity in the sigE mutant than in the spo0A mutant, suggesting that this cry gene is positively regulated by Spo0A during exponential and transition phases [7]. However, expression of the sporulation-specific cry genes is very much lower in a spo0A mutant than in a wild-type strain. Altogether, these results indicate that Spo0A has different regulatory roles for different cry genes, including: (i) a moderate role during the transition phase to modulate cry gene expression before the onset of sporulation; and (ii) a major role involving triggering the activity of the sporulation-specific sigma factors.

2.3.2. The E2 Subunit of the Pyruvate Dehydrgenase (PDH) Complex

The PDH complex is widely distributed in bacteria and catalyzes the oxidative decarboxylation of pyruvate to acetyl-coenzyme A, linking the glycolysis pathway with the tricarboxylic acid (TCA) cycle [31]. The PDH E2 subunit in Bs is involved in regulating gene expression during sporulation [32]. In Bt, the PDH E2 subunit specifically binds to sites 200–300 bp upstream from the cry1A gene promoter [9]. Mutations of these sites decrease both the binding of the E2 subunit and the transcription of cry1A promoter [9]. Thus, the PDH E2 subunit can positively regulate cry1A transcription by specifically binding to the 5’ non-coding region of the cry1A gene. These observations imply that the PDH E2 subunit may function as a transcription factor, independent of its enzymatic activity. They also imply a link between catabolism and Cry protein synthesis.

2.3.3. Regulation by Two Negative Factors

Yu et al. [15] studied a fragment from Bt strain YBT-1518 carrying cry6Aa2 and its downstream orf2, which are co-transcribed, and the inverted repeat sequence found between the two ORFs. The inverted repeat sequence disturbs orf2 expression [15], probably by down-regulating orf2 transcription. Over-expression of orf2 reduced the cry6Aa2 expression whereas deletion of orf2 significantly enhanced cry6Aa2 expression [15]. This work, thus, demonstrates that expression of the orf2 gene down-regulates the expression of the cry6Aa2 gene, although the mechanism remains unclear. This is an example of cry gene expression being regulated by two negative factors (the inverted repeat sequence and the orf2 production).

3. mRNA Stability

The stability of mRNA is a major determinant of gene expression. The half-life of Bt cry genes mRNA is about 10 min, which is significantly longer than the mean half-life of E. coli mRNAs.
(two to three minutes) [33]. The causes of cry gene mRNA stability have been discussed elsewhere [5]. However, it is interesting to revisit these mechanisms in the light of recent results.

3.1. 3’ Terminal Structure

Inverted repeat sequences are widely found, and are conserved, in the 3’ untranslated regions of many cry genes (for example, the cry1A genes). They contribute to stabilizing cry mRNAs [5,34]. These sequences can form stable stem loop structures which might protect the cry mRNA from exoribonucleolytic degradation by 3’-5’ exoribonucleases, which are sensitive to RNA secondary structure [5].

3.2. 5’ mRNA Stabilizer

Two transcripts are generated from the cry3A gene: (i) a minor transcript starting 558 bp upstream from the start codon; and (ii) a major transcript starting 129 bp upstream from the start codon [5,35] (Figure 3). A Shine-Dalgarno (SD)-like sequence close to the 5’ of the −129 position is responsible for stabilizing the cry3A mRNA [36]; this sequence (designated as STAB-SD) does not direct translation initiation but can interact with the 3’ end of the 16S rRNA [36]. Therefore, it was suggested that the binding of a 30S ribosomal subunit to the STAB-SD sequence stabilizes the mRNA by preventing degradation by 5’-3’ exoribonuclease [36]. However, it was previously believed that bacteria only possessed mRNA degradation activities that progressed in the 3’ to 5’ orientation. The discovery of the 5’-3’ exoribonucleolytic activity of the RNase J1 confirmed the original hypothesis: RNase J1 degrades the −558 transcript of cry3A mRNA from 5’ to 3’; the 30S ribosomal subunit binds to the STAB-SD sequence of cry3A mRNA and blocks 5’-3’ exoribonucleolytic progress of RNase J1 (Figure 3) [37]. Similar STAB-SD sequences are also present in the 5’ untranslated regions of other genes (other cry3 genes and genes in Gram-positive bacteria other than Bt) and may therefore be a widespread determinant of mRNA stability.

**Figure 3.** The STAB-SD sequence in cry3A mRNA acts as a 5’ mRNA stabilizer. RNase J1 degrades the −558 transcript of cry3A mRNA from 5’ to 3’; the 30S ribosomal subunit can bind to the STAB-SD sequence of cry3A mRNA and block the further 5’-3’ exoribonucleolytic progress of RNase J1.
4. Metabolic Regulation of Cry Protein Production

The metabolic pathways that provide carbon components, amino acids, and energy for sporulation and massive synthesis of Cry proteins in Bt are expected to be subject to substantial regulation. Indeed, a recent report reveals metabolic regulation mechanisms involved at both the transcriptional and translational levels [38]: they include controls over the metabolism of proteases and amino acids, the supply of carbon and energy sources, the regulation and modification of metabolic pathways, and the regulation of oxidative phosphorylation and energy generation. The regulation of Cry protein expression via the metabolism-associated sigma 54 factor, and according to polyphosphate metabolism and glucose catabolite repression has been described (see below).

4.1. Regulation by the Sigma 54 Factor

The sigma factor sigma 54 (also called SigL) regulates various nitrogen and carbon sources, and energy metabolism pathways in bacteria. We recently found that mutation of sigma 54 in Bt strain HD73 decreased Cry1Ac protein production in LB medium (Peng and coll., unpublished data). The positive effects of sigma 54 on crystal production are at the transcriptional level, and may be indirect involving sigma 54-dependent metabolic pathways. For example, the γ-aminobutyric acid (GABA) shunt, which is controlled by sigma 54 and regulated by GabR [39], is an additional supplement to the TCA cycle and was shown to be correlated with spore and parasporal crystal formation in B. thuringiensis [40]. Accordingly, the metabolism of GABA becomes more active during sporulation [38]. Another example is the acoABCL operon encoding the acetoin dehydrogenase complex [41]: acoABCL is strongly up-regulated at both the transcriptional and translational levels during sporulation [38]. We confirmed that this operon is controlled by sigma 54 and is activated by AcoR (Peng and coll., unpublished data). However, sigma 54 does not affect Cry1Ac transcription and production in sporulation medium (SSM), indicating that the effects of metabolic pathways on Cry protein production depend on growth conditions.

4.2. Polyphosphate Kinase (PPK) and Polyphosphate Metabolism

Some mineral nutrients, especially inorganic phosphate, are important for Cry protein production in Bt [42–44]. Polyphosphate is synthesized by PPK from the terminal phosphate of ATP [45] and can be degraded to inorganic phosphate by endopolyphosphatase and exopolyphosphatase enzymes [46]. Doruk et al. (2013) reported that the overexpression of PPK in Bti increased both the intracellular concentration of polyphosphate and Cry protein production. They also found that the transcription of sigE was stimulated in the strain overexpressing PPK [12]. Thus, changes to polyphosphate metabolism may influence Cry protein production in Bt, probably via SigE.

4.3. Catabolic Repression by Glucose

In Gram-positive bacteria, catabolite repression of many catabolic operons involves the phosphocarrier protein HPr, the catabolite control protein CcpA, and a cis-acting catabolite responsive element (cre) [13,14]. The phosphorylated HPr binds to the CcpA to form a complex with strong DNA binding affinity [47,48]. The phosphorylated HPr-CcpA complex modulates the transcription of
target genes by binding to the cre sequence [49]. Glucose represses cry4A gene expression at the mRNA level in Bti [50], and the phosphorylated HPr-CcpA complex of Bti represses cry4A transcription by specifically binding to a 15 bp cre sequence overlapping the -35 element of the cry4A promoter [14]. Glucose catabolite repression of cry4A is abolished both by site-specific mutation of the cre sequence [14] and by the HPr-S45A mutant, which produces phosphorylation-disabled HPr [13]: both increase the activity of the cry4A promoter. Thus, in Bti, the synthesis of Cry protein is controlled by HPr-CcpA-mediated glucose catabolite repression.

5. Crystallization of the Cry Proteins

The accumulation of large amounts of proteins requires production of stable proteins or, at least, a mechanism to prevent degradation of the proteins produced. Bt mainly produces Cry proteins during the stationary phase, when large amounts of various proteolytic enzymes are synthesized. This means that Bt must have mechanisms to prevent proteolysis of the synthesized Cry proteins. One strategy is to form crystals resistant to proteolytic enzymes.

The mass of many Cry proteins (for example, the Cry1 proteins) is between 130 and 140 kDa. Most of these large Cry proteins can spontaneously form crystals independent of the host bacterium; the genes encoding these proteins are generally monocistronic [5,51]. The C-terminal halves of these proteins are called crystallization domains as they are not involved in toxicity but are necessary for the formation of the crystal. Many other Cry proteins of smaller mass, for example, the Cry2, Cry11 and Cry19 proteins, have no C-terminal crystallization domain like that in the 130–140 kDa Cry proteins. The massive accumulation or crystallization of these Cry proteins generally requires the presence of additional proteins encoded by genes in the same operon [5,51]. These additional proteins are in many cases small, have no insect toxicity and are not the main components of the crystals; rather, they enhance the accumulation or crystallization of their accompanying Cry proteins. Consequently, they are described as accessory proteins or helper proteins. These helper proteins include the 19 kDa P19 and 20 kDa P20 proteins encoded by the p19 and p20 genes in the cry11Aa operon [25]; the 20 kDa Orf1 and 29 kDa Orf2 proteins encoded by the orf1 and orf2 genes in the cry2A operon [10]; and the 60 kDa ORF2 (60 k) protein encoded by the orf2 gene of the cry19A operon [51].

The function of P19 and Orf1 in Cry protein production and crystallization is not clear. Orf2 is necessary for the crystallization of Cry2A [52,53]. It contains 11 tandem repeats of a 15/16 amino acid motif that is acidic in nature [10,52]. Orf2 and Cry2A can be co-precipitated, evidence of interaction between the two proteins [53]. Indeed, Orf2 serves as a crystallization factor by interacting with the Cry2A protein, possibly acting as a template or scaffold [52,53]. ORF2 (60 k) encoded by the orf2 of the cry19A operon is very similar to the C-terminal domain of the 130 kDa Cry proteins and is essential for the crystallization of Cry19A, which is itself similar to the N-terminal domain of the large Cry proteins [51]. There is various evidence that ORF2 (60 k) functions primarily as the C-terminal crystallization domain of the large Cry proteins: (i) there are approximately equimolar amounts of ORF2 (60 k) and Cry19A in the crystals; (ii) in-frame fusion of Cry19A to ORF2 (60 k) forms stable crystals; (iii) in-frame fusion of the N-terminal region of Cry1C to ORF2 (60 k) results in the formation of visible inclusions; and (iv) in-frame fusion of Cry19A to the C-terminal crystallization
Toxins 2014, 6

region of Cry1C results in a protein that forms crystals [51]. Similar gene organizations are also found in other cry operons such as the cry10Aa, cry39Aa and cry40Aa operons: the upstream reading frame encodes the Cry N-terminal domain, and the frame found about 100 bp downstream encodes a protein similar to the Cry C-terminal crystallization domain [51,54].

The P20 protein can improve the yield and/or crystallization of a variety of insecticidal crystal proteins including Cyt1Aa [55], Cry1Ac [56], Cry2Aa [52], Cry4Aa [57], Cry11Aa [58] and truncated Cry1C [59,60]. It can also enhance the expression of the Bt vegetative insecticidal protein Vip3A [61] and B. sphaericus binary toxins BinA-BinB [62]. The effect of P20 on Cyt1A expression was addressed by a series of studies in E. coli by Whiteley’s group decades ago: the P20 protein does not affect transcription or mRNA stability [63], nor regulate translational initiation of cyt1A [64]; there is a protein-protein interaction between P20 and Cyt1A, and this occurs only with the nascent Cyt1A peptide [65]; P20 is not required for production of large amounts of Cyt1A in E. coli strains defective in proteolytic activity [65]. These findings suggest that P20 acts as a molecular chaperone, which helps Cyt1A to form protease-resistant crystals. Another study supports this possibility and provides more details [56]: in Bt, the Cry1Ac protein is substantially degraded throughout the production process, especially during protein synthesis, before crystallization [56]. The introduction of P20 significantly improves Cry1Ac production, and this was the consequence of protection of the nascent peptides [56]. The p20 gene has been detected in various Bt serovar strains, suggesting that P20 may be a widespread factor influencing the Cry protein crystallization.

6. Patterns of Crystal Production

The Cry protein inclusion of Bt is called a parasporal crystal because it is generally produced beside the spore, in the mother cell. This phenotype is mainly determined at the transcriptional level. The sporulation-dependent cry genes, mostly controlled by the mother cell-specific SigE and SigK, are transcribed exclusively in the mother cell of sporulating cells, thus restricting the production of crystals to this compartment (Figure 1A). The cry3 genes, which are under direction of SigA-like promoters, may also be expressed in a subpopulation of non-sporulating cells, although this has never been reported, except for when the cry3 gene is cloned in a spo0A mutant [28].

The discovery and characterization of an unusual strain, LM1212, expanded our understanding of the parasporal crystal phenotype in Bt. In this strain, the crystal is not produced in the mother cell of sporulating cells, but only in a subpopulation of non-sporulating cells (Figure 1C) [29]. The separation of crystal and spore into different LM1212 cell populations is a consequence of cell differentiation associated with a previously undescribed cry gene transcription pattern; indeed, the LM1212 cry genes are transcribed only in a subpopulation of non-sporulating cells, called crystal-producer cells (Figure 2).

In most Bt strains, crystals separate from spores after lysis of the mother cell. However, a few Bt strains display a spore-crystal association (SCA) phenotype in which the crystals are produced between the exosporium and the spore coat (Figure 1B) [66]. The SCA phenotype is typically found among the Bt subsp. finitimus strains. Debro et al. [67] reported that genes on a 98 MDa plasmid are responsible for the SCA phenotype in one of these strains. Zhu et al. [66] found that the genes necessary and sufficient for the SCA phenotype in another Bt subsp. finitimus strain YBT-020 are harbored by two different plasmids: the cry26Aa gene on a 188 kb plasmid and a five-gene cluster on
a 139 kb plasmid. However, the genome sequence of a Bt strain displaying a SCA phenotype does not contain genes homologous to any of these genes. Therefore, genes determining the SCA phenotype appear to differ among subspecies, or have not so far been correctly identified [66]. Although the biology of the SCA phenotype remains unclear, a tight association between crystal and spore may reduce the risk of the insecticidal toxins being exploited by other microorganisms that do not produce the crystal.

7. Concluding Remarks

Bt is the most widely and successfully used bio-insecticide, so agronomic applications are always to the forefront in any considerations of the species. A powerful strategy for improving the use of Bt-based biopesticides is to enhance Cry protein production. This requires a good understanding of the regulation of cry gene expression. Indeed, with the characterization of cry gene expression mechanisms, a variety of strategies have been used to improve the Cry protein yield from Bt strains: using strong promoters and increasing the cry gene copy number [68,69], introducing the STAB-SD sequence [70,71], using the cry3A promoter in a sporulating-defective mutant [28], co-expression of a helper protein [56], and using combinations of strategies [72,73]. Another way to extend agronomic applications of Bt is to broaden its insecticidal spectrum. One approach to this is the co-expression of different Cry toxins under the control of different types of cry promoters (sporulation-dependent and -independent), an approach that may also increase total Cry protein amounts because competition for regulation factors and nutrition between these promoters and genes is reduced [74–76]. We believe that new Bt-based biopesticides could be developed by using novel regulation mechanisms. A better understanding of the mechanism of Cry protein crystallization may facilitate the development and synthesis of stable and biologically active crystal inclusions. This has already been demonstrated in the case of the Vip3 toxin: this soluble protein exported in the extra cellular medium during growth can be specifically produced and accumulated in the mother cell of Bt as crystal inclusions by placing an in-frame fusion of the gene with the sequence encoding the C-terminal of cry1C under the control of a sporulation-dependent cry gene promoter [77].

The expression of cry genes by Bt is regulated in a sophisticated way, through mechanisms from the cry gene copy number to the involvement of helper proteins and at various levels from transcriptional to metabolic. These strategies, and the variety of cry genes, make Bt an efficient biological weapon for killing a wide range of susceptible insects in various ecological niches. This explains why Bt has become the most successful and widely distributed biopesticide in the world. However, it is not clear why during evolution Bt has maintained or developed different cry gene expression systems and crystal localization patterns rather than a single, powerful system. The production of Cry toxins in Bt has been used as a model system to study the cooperation and evolutionary ecology of bacterial virulence [78,79]; the division of labor between crystal-producers and spore-formers in LM1212 has been shown to benefit the population when competing with a typical Bt strain [29]. This illustrates how a better knowledge of the mechanisms of cry gene expression and crystal localization in Bt can provide important new insights into basic issues and questions of fundamental importance.
Acknowledgments

We are grateful to Ming Sun for the picture of B. thuringiensis YBT-020. This work was supported by a grant from the National Natural Science Foundation of China (No. 31270111) and by the French National Institute of Agronomical Research.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Vilas-Boas, G.T.; Peruca, A.P.; Arantes, O.M. Biology and taxonomy of Bacillus cereus, Bacillus anthracis, and Bacillus thuringiensis. Can. J. Microbiol. 2007, 53, 673–687.
2. Kolstø, A.B.; Lereclus, D.; Mock, M. Genome structure and evolution of the Bacillus cereus group. Curr Top. Microbiol. Immunol. 2002, 264, 95–108.
3. Bravo, A.; Likitvivatanavong, S.; Gill, S.S.; Soberon, M. Bacillus thuringiensis: A story of a successful bioinsecticide. Insect. Biochem. Mol. Biol. 2011, 41, 423–431.
4. Sanahuja, G.; Banakar, R.; Twyman, R.M.; Capell, T.; Christou, P. Bacillus thuringiensis: A century of research, development and commercial applications. Plant. Biotechnol. J. 2011, 9, 283–300.
5. Agaisse, H.; Lereclus, D. How does Bacillus thuringiensis produce so much insecticidal crystal protein? J. Bacteriol. 1995, 177, 6027–6032.
6. Baum, J.A.; Malvar, T. Regulation of insecticidal crystal protein production in Bacillus thuringiensis. Mol. Microbiol. 1995, 18, 1–12.
7. Yang, H.; Wang, P.; Peng, Q.; Rong, R.; Liu, C.; Lereclus, D.; Zhang, J.; Song, F.; Huang, D. Weak transcription of the cry1Ac gene in nonsporulating Bacillus thuringiensis cells. Appl. Environ. Microbiol. 2012, 78, 6466–6474.
8. Perez-Garcia, G.; Basurto-Rios, R.; Ibarra, J.E. Potential effect of a putative sigma(H)-driven promoter on the over expression of the Cry1Ac toxin of Bacillus thuringiensis. J. Invertebr. Pathol. 2010, 104, 140–146.
9. Walter, T.; Aronson, A. Specific binding of the E2 subunit of pyruvate dehydrogenase to the upstream region of Bacillus thuringiensis protoxin genes. J. Biol. Chem. 1999, 274, 7901–7906.
10. Widner, W.R.; Whiteley, H.R. Two highly related insecticidal crystal proteins of Bacillus thuringiensis subsp. kurstaki possess different host range specificities. J. Bacteriol. 1989, 171, 965–974.
11. Poncet, S.; Dervyn, E.; Klier, A.; Rapoport, G. Spo0A represses transcription of the cry toxin genes in Bacillus thuringiensis. Microbiology 1997, 143, 2743–2751.
12. Doruk, T.; Avican, U.; Camci, I.Y.; Gedik, S.T. Overexpression of polyphosphate kinase gene (ppk) increases bioinsecticide production by Bacillus thuringiensis. Microbiol. Res. 2013, 168, 199–203.
13. Khan, S.R.; Banerjee-Bhatnagar, N. Loss of catabolite repression function of HPr, the phosphocarrier protein of the bacterial phosphotransferase system, affects expression of the cry4A toxin gene in Bacillus thuringiensis subsp. israelensis. J. Bacteriol. 2002, 184, 5410–5417.
14. Kant, S.; Kapoor, R.; Banerjee, N. Identification of a catabolite-responsive element necessary for regulation of the cry4A gene of *Bacillus thuringiensis* subsp. *israelensis*. *J. Bacteriol.* 2009, 191, 4687–4692.

15. Yu, Z.; Bai, P.; Ye, W.; Zhang, F.; Ruan, L.; Sun, M. A novel negative regulatory factor for nematicidal Cry protein gene expression in *Bacillus thuringiensis*. *J. Microbiol. Biotechnol.* 2008, 18, 1033–1039.

16. Du, L.; Qiu, L.; Peng, Q.; Lereclus, D.; Zhang, J.; Song, F.; Huang, D. Identification of the promoter in the intergenic region between orf1 and cry8Ea1 controlled by sigma H factor. *Appl. Environ. Microbiol.* 2012, 78, 4164–4168.

17. Brown, K.L. Transcriptional regulation of the *Bacillus thuringiensis* subsp. *thompsoni* crystal protein gene operon. *J. Bacteriol.* 1993, 175, 7951–7957.

18. Zhang, J.; Schairer, H.U.; Schnetter, W.; Lereclus, D.; Agaisse, H. *Bacillus popilliae* cry18Aa operon is transcribed by sigmaE and sigmaK forms of RNA polymerase from a single initiation site. *Nucl. Acids Res.* 1998, 26, 1288–1293.

19. Piggot, P.J.; Hilbert, D.W. Sporulation of *Bacillus subtilis*. *Curr. Opin. Microbiol.* 2004, 7, 579–586.

20. Lereclus, D.; Agaisse, H. Toxin and virulence gene expression in *Bacillus thuringiensis*. In *Entomopathogenic Bacteria: From Laboratory to Field Application*; Charles, J.F., Delécluse, A., Nielsen-Leroux, C., Eds.; Kluwer Academic Publishers: Heidelberg, Germany, 2000; pp. 127–142.

21. Aronson, A. Sporulation and delta-endotoxin synthesis by *Bacillus thuringiensis*. *Cell. Mol. Life Sci.* 2002, 59, 417–425.

22. Wang, J.; Mei, H.; Qian, H.; Tang, Q.; Liu, X.; Yu, Z.; He, J. Expression profile and regulation of spore and parasporal crystal formation-associated genes in *Bacillus thuringiensis*. *J. Proteome Res.* 2013, 12, 5487–5501.

23. Bravo, A.; Agaisse, H.; Salamitou, S.; Lereclus, D. Analysis of cryIAa expression in sigE and sigK mutants of *Bacillus thuringiensis*. *Mol. Gen. Genet.* 1996, 250, 734–741.

24. Yoshisue, H.; Ihara, K.; Nishimoto, T.; Sakai, H.; Komano, T. Expression of the genes for insecticidal crystal proteins in *Bacillus thuringiensis*: CryIVA, not cryIVB, is transcribed by RNA polymerase containing sigma H and that containing sigma E. *FEMS Microbiol. Lett.* 1995, 127, 65–72.

25. Dervyn, E.; Poncet, S.; Klier, A.; Rapoport, G. Transcriptional regulation of the cryIVD gene operon from *Bacillus thuringiensis* subsp. *israelensis*. *J. Bacteriol.* 1995, 177, 2283–2291.

26. Agaisse, H.; Lereclus, D. Expression in *Bacillus subtilis* of the *Bacillus thuringiensis* cryIIIA toxin gene is not dependent on a sporulation-specific sigma factor and is increased in a spo0A mutant. *J. Bacteriol.* 1994, 176, 4734–4741.

27. Malvar, T.; Baum, J. Tn5401 disruption of the spo0F gene, identified by direct chromosomal sequencing, results in cryIIIA overproduction in *Bacillus thuringiensis*. *J. Bacteriol.* 1994, 176, 4750–4753.

28. Lereclus, D.; Agaisse, H.; Gominet, M.; Chaufaux, J. Overproduction of encapsulated insecticidal crystal proteins in a *Bacillus thuringiensis* spo0A mutant. *Nat. Biotechnol.* 1995, 13, 67–71.
29. Deng, C.; Slamti, L.; Raymond, B.; Liu, G.; Lemy, C.; Gominet, M.; Yang, J.; Wang, H.; Peng, Q.; Zhang, J.; et al. Division of labour and terminal differentiation in a novel Bacillus thuringiensis strain. *ISME J.* 2014, in press.

30. Molle, V.; Fujita, M.; Jensen, S.T.; Eichenberger, P.; Gonzalez-Pastor, J.E.; Liu, J.S.; Losick, R. The Spo0A regulon of Bacillus subtilis. *Mol. Microbiol.* 2003, 50, 1683–1701.

31. Berg, A.; de Kok, A. 2-Oxo acid dehydrogenase multienzyme complexes. The central role of the lipoyl domain. *Biol. Chem.* 1997, 378, 617–634.

32. Gao, H.; Jiang, X.; Pogliano, K.; Aronson, A.I. The E1beta and E2 subunits of the Bacillus subtilis pyruvate dehydrogenase complex are involved in regulation of sporulation. *J. Bacteriol.* 2002, 184, 2780–2788.

33. Glatron, M.F.; Rapoport, G. Biosynthesis of the parasporal inclusion of Bacillus thuringiensis: Half-life of its corresponding messenger RNA. *Biochimie* 1972, 54, 1291–1301.

34. Mathy, N.; Benard, L.; Pellegrini, O.; Daou, R.; Wen, T.; Condon, C. 5’-to-3’ exoribonuclease activity in bacteria: Role of RNase J1 in rRNA maturation and 5’ stability of mRNA. *Cell* 2007, 129, 681–692.

35. Wang, J.; Mei, H.; Zheng, C.; Qian, H.; Cui, C.; Fu, Y.; Su, J.; Liu, Z.; Yu, Z.; He, J. The metabolic regulation of sporulation and parasporal crystal formation in Bacillus thuringiensis revealed by transcriptomics and proteomics. *Mol. Cell. Proteomics* 2013, 12, 1363–1376.

36. Ali, N.O.; Bignon, J.; Rapoport, G.; Débarbouillé, M. Regulation of the acetoin catabolic pathway is controlled by sigma L in Bacillus subtilis. *J. Bacteriol.* 2001, 183, 2497–2504.

37. Kurt, A.; Ozkan, M.; Ozcengiz, G. Inorganic phosphate has a crucial effect on Cry3Aa delta-endotoxin production. *Lett. Appl. Microbiol.* 2005, 41, 303–308.

38. Ozkan, M.; Dilek, F.B.; Yetis, U.; Ozcengiz, G. Nutritional and cultural parameters influencing antidipteran delta-endotoxin production. *Res. Microbiol.* 2003, 154, 49–53.

39. Banerjee-Bhatnagar, N. Inorganic phosphate regulates Cry1VA protoxin expression in Bacillus thuringiensis israelensis. *Biochem. Biophys. Res. Commun.* 1999, 262, 359–364.

40. Ahn, K.; Kornberg, A. Polyphosphate kinase from *Escherichia coli*. Purification and demonstration of a phosphoenzyme intermediate. *J. Biol. Chem.* 1990, 265, 11734–11739.
46. Akiyama, M.; Crooke, E.; Kornberg, A. An exopolypophosphatase of *Escherichia coli*. The enzyme and its *ppx* gene in a polyphosphate operon. *J. Biol. Chem.* 1993, 268, 633–639.

47. Fujita, Y.; Miwa, Y.; Galinier, A.; Deutscher, J. Specific recognition of the *Bacillus subtilis gnt* cis-acting catabolite-responsive element by a protein complex formed between CcpA and seryl-phosphorylated HPr. *Mol. Microbiol.* 1995, 17, 953–960.

48. Jones, B.E.; Dossonnet, V.; Kuster, E.; Hillen, W.; Deutscher, J.; Klevit, R.E. Binding of the catabolite repressor protein CcpA to its DNA target is regulated by phosphorylation of its corepressor HPr. *J. Biol. Chem.* 1997, 272, 26530–26535.

49. Hueck, C.J.; Hillen, W. Catabolite repression in *Bacillus subtilis*: A global regulatory mechanism for the gram-positive bacteria? *Mol. Microbiol.* 1995, 15, 395–401.

50. Banerjee-Bhatnagar, N. Modulation of Cry IV A toxin protein expression by glucose in *Bacillus thuringiensis israelensis*. *Biochem. Biophys. Res. Commun.* 1998, 252, 402–406.

51. Barboza-Corona, J.E.; Park, H.W.; Bideshi, D.K.; Federici, B.A. The 60-kilodalton protein encoded by *orf2* in the *cry19A* operon of *Bacillus thuringiensis* subsp. *jegathesan* functions like a C-terminal crystallization domain. *Appl. Environ. Microbiol.* 2012, 78, 2005–2012.

52. Ge, B.; Bideshi, D.; Moar, W.J.; Federici, B.A. Differential effects of helper proteins encoded by the *cry2A* and *cry11A* operons on the formation of Cry2A inclusions in *Bacillus thuringiensis*. *FEMS Microbiol. Lett.* 1998, 165, 35–41.

53. Staples, N.; Ellar, D.; Crickmore, N. Cellular localization and characterization of the *Bacillus thuringiensis* Orf2 crystallization factor. *Curr. Microbiol.* 2001, 42, 388–392.

54. De Maagd, R.A.; Bravo, A.; Berry, C.; Crickmore, N.; Schnepf, H.E. Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. *Annu. Rev. Genet.* 2003, 37, 409–433.

55. Wu, D.; Federici, B.A. A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. *J. Bacteriol.* 1993, 175, 5276–5280.

56. Shao, Z.; Liu, Z.; Yu, Z. Effects of the 20-kilodalton helper protein on Cry1Ac production and spor formation in *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* 2001, 67, 5362–5369.

57. Yoshisue, H.; Yoshida, K.; Sen, K.; Sakai, H.; Komano, T. Effects of *Bacillus thuringiensis* var. *israelensis* 20-kDa protein on production of the Bti 130-kDa crystal protein in *Escherichia coli*. *Biosci. Biotechnol. Biochem.* 1992, 56, 1429–1433.

58. Wu, D.; Federici, B.A. Improved production of the insecticidal CryIVD protein in *Bacillus thuringiensis* using *cryIA(c)* promoters to express the gene for an associated 20-kDa protein. *Appl. Microbiol. Biotechnol.* 1995, 42, 697–702.

59. Park, H.W.; Bideshi, D.K.; Federici, B.A. Molecular genetic manipulation of truncated Cry1C protein synthesis in *Bacillus thuringiensis* to improve stability and yield. *Appl. Environ. Microbiol.* 2000, 66, 4449–4455.

60. Rang, C.; Bes, M.; Lullien-Pellerin, V.; Wu, D.; Federici, B.A.; Frutos, R. Influence of the 20-kDa protein from *Bacillus thuringiensis* ssp. *israelensis* on the rate of production of truncated Cry1C proteins. *FEMS Microbiol. Lett.* 1996, 141, 261–264.

61. Shi, Y.X.; Yuan, M.J.; Chen, J.W.; Sun, F.; Pang, Y. Effects of helper protein P20 from *Bacillus thuringiensis* on Vip3A expression. *Acta Microbiol. Sinica* 2006, 46, 85–89.
62. Park, H.W.; Bideshi, D.K.; Federici, B.A. The 20-kDa protein of Bacillus thuringiensis subsp. israelensis enhances Bacillus sphaericus 2362 bin toxin synthesis. *Curr. Microbiol.* **2007**, *55*, 119–124.

63. McLean, K.M.; Whiteley, H.R. Expression in Escherichia coli of a cloned crystal protein gene of Bacillus thuringiensis subsp. israelensis. *J. Bacteriol.* **1987**, *169*, 1017–1023.

64. Adams, L.F.; Visick, J.E.; Whiteley, H.R. A 20-kilodalton protein is required for efficient production of the Bacillus thuringiensis subsp. israelensis 27-kilodalton crystal protein in Escherichia coli. *J. Bacteriol.* **1989**, *171*, 521–530.

65. Visick, J.E.; Whiteley, H.R. Effect of a 20-kilodalton protein from Bacillus thuringiensis subsp. israelensis on production of the CytA protein by Escherichia coli. *J. Bacteriol.* **1991**, *173*, 1748–1756.

66. Zhu, Y.; Ji, F.; Shang, H.; Zhu, Q.; Wang, P.; Xu, C.; Deng, Y.; Peng, D.; Ruan, L.; Sun, M. Gene clusters located on two large plasmids determine spore crystal association (SCA) in Bacillus thuringiensis subsp. finitimus strain YBT-020. *PLoS One* **2011**, *6*, e27164.

67. Debroy, L.; Fitz-James, P.C.; Aronson, A. Two different parasporal inclusions are produced by Bacillus thuringiensis subsp. finitimus. *J. Bacteriol.* **1986**, *165*, 258–268.

68. Arantes, O.; Lereclus, D. Construction of cloning vectors for Bacillus thuringiensis. *Gene* **1991**, *108*, 115–119.

69. Sanchis, V.; Agaisse, H.; Chaufaux, J.; Lereclus, D. A recombinase-mediated system for elimination of antibiotic resistance gene markers from genetically engineered Bacillus thuringiensis strains. *Appl. Environ. Microbiol.* **1997**, *63*, 779–784.

70. Hernandez-Soto, A.; Del Rincon-Castro, M.C.; Espinoza, A.M.; Ibarra, J.E. Parasporal body formation via overexpression of the Cry10Aa toxin of Bacillus thuringiensis subsp. israelensis, and Cry10Aa-Cyt1Aa synergism. *Appl. Environ. Microbiol.* **2009**, *75*, 4661–4667.

71. Park, H.W.; Ge, B.; Bauer, L.S.; Federici, B.A. Optimization of Cry3A yields in Bacillus thuringiensis by use of sporulation-dependent promoters in combination with the STAB-SD mRNA sequence. *Appl. Environ. Microbiol.* **1998**, *64*, 3932–3938.

72. Sanchis, V.; Agaisse, H.; Chaufaux, J.; Lereclus, D. Construction of new insecticidal Bacillus thuringiensis recombinant strains by using the sporulation non-dependent expression system of cryIIIA and a site specific recombination vector. *J. Biotechnol.* **1996**, *48*, 81–96.

73. Sanchis, V.; Gohar, M.; Chaufaux, J.; Arantes, O.; Meier, A.; Agaisse, H.; Cayley, J.; Lereclus, D. Development and field performance of a broad-spectrum nonviable asporogenic recombinant strain of Bacillus thuringiensis with greater potency and UV resistance. *Appl. Environ. Microbiol.* **1999**, *65*, 4032–4039.

74. Lereclus, D.; Vallade, M.; Chaufaux, J.; Arantes, O.; Rambaud, S. Expansion of insecticidal host range of Bacillus thuringiensis by in vivo genetic recombination. *Nat. Biotechnol.* **1992**, *10*, 418–421.

75. Yan, G.; Song, F.; Shu, C.; Liu, J.; Liu, C.; Huang, D.; Feng, S.; Zhang, J. An engineered Bacillus thuringiensis strain with insecticidal activity against Scarabaeidae (*Anomala corpulenta*) and Chrysomelidae (*Leptinotarsa decemlineata* and *Colaphellus bowringii*). *Biotechnol. Lett.* **2009**, *31*, 697–703.
76. Wang, G.; Zhang, J.; Song, F.; Wu, J.; Feng, S.; Huang, D. Engineered *Bacillus thuringiensis* GO33A with broad insecticidal activity against lepidopteran and coleopteran pests. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 924–930.

77. Song, R.; Peng, D.; Yu, Z.; Sun, M. Carboxy-terminal half of Cry1C can help vegetative insecticidal protein to form inclusion bodies in the mother cell of *Bacillus thuringiensis*. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 647–654.

78. Raymond, B.; West, S.A.; Griffin, A.S.; Bonsall, M.B. The dynamics of cooperative bacterial virulence in the field. *Science* **2012**, *337*, 85–88.

79. Raymond, B.; Bonsall, M.B. Cooperation and the evolutionary ecology of bacterial virulence: The *Bacillus cereus* group as a novel study system. *BioEssays* **2013**, *35*, 706–716.

© 2014 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).