Genetic Factors Affecting Insecticidal Crystal Protein Synthesis in *Bacillus thuringiensis*

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Early studies of the molecular biology of *Bacillus thuringiensis* suggested that genetic manipulation of this species could create combinations of genes more useful than those known to occur in natural isolates. Breakthroughs that made these manipulations possible include the cloning of many genes encoding endotoxins, the development of transformation vectors, and various PCR techniques. This paper reviews several genetic factors such as promoters, a 5′ mRNA stabilizing sequence, 3′ transcription termination sequence, and helper proteins that have been used to enhance crystal protein synthesis, and shows how these genetic elements can be manipulated with new molecular tools to develop more efficacious strains of *B. thuringiensis*.

Key words: *Bacillus thuringiensis*, Crystal protein synthesis, Promoters, mRNA stabilizing sequence, Transcription termination sequence, Helper proteins

Introduction

Crystal (Cry) protein toxins of *Bacillus thuringiensis* are the active component of most bacterial insecticides (Hofte and Whiteley, 1989; Schneef et al., 1998). Cry proteins are synthesized during the stationary phase of reproduction and accumulate in the mother cell as one or more crystalline inclusions that can account for as much as 25% of the sporulated cell's dry weight (Agassie and Lereclus, 1995).

The first systematic attempt to organize the genetic nomenclature of endotoxin genes relied on (1) insect toxicity spectrum and (2) sequence similarity (Hofte and Whiteley, 1989). The *cry* genes encoded proteins toxic to lepidopterans; *cryII* genes encoded proteins toxic to both lepidopterans and dipterans; *cryIII* genes encoded proteins toxic to coleopterans; and *cryIV* genes encoded proteins toxic to dipterans. This system had been used until a new nomenclature system, based solely on amino acid identity, was developed so that closely related toxins could be grouped together. According to this new system, the known 127 Cry oxins have been classified into 22 classes (Crickmore et al., 1998). In addition to Cry toxins, a second type of toxin-Cyt toxins (for cytolytic)-exists. These toxins are in the 25-28 kDa mass range and, based on amino acid sequence, are not related to Cry proteins. Two classes (Cyt1 and Cyt2) containing 9 Cyt toxins are known (Crickmore et al., 1998).

In this system, Cry proteins fall into two types or groups-one with molecular masses of 130-140 kDa, and the other with masses in the range of 65-70 kDa. The former is represented by Cry1 proteins. Only the N-terminal half of these is toxic (Hofte and Whiteley, 1989; Schneef et al., 1998). With respect to the latter class, typical examples are Cry2A, Cry3A and Cry11A, which lack the C-terminal half characteristic of Cry1 proteins. Therefore, the 65-70 kDa proteins are in essence naturally truncated versions of the 130-140 kDa toxins, and composed primarily of the toxic portion of the molecule.

The primary genetic factors affecting the size of naturally truncated crystals of Cry proteins were determined to be those involved in protein synthesis. Among these were strong promoters (Brown and Whiteley, 1998; 1990), the cry3A 5′ STAB-SD (Agassie and Lereclus, 1996; Park et al., 1998) and 3’ stem-loop mRNA stabilizing sequences (Wong and Chang 1986; Park et al., 2000), and the 20-kDa (Wu and Federici, 1995; Park et al., 1999) and 29-kDa (Crickmore and Ellar, 1992; Ge et al., 1998; Park et al., 1999) helper proteins encoded by, respectively, the cry11A and cry2A operons.

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The aim of the present paper is to review the molecular genetic factors affecting Cry protein synthesis, and eventually to provide this knowledge as a basis for developing more efficacious insecticidal bacteria.

Promoters
In Bacillus species, the endospore develops in a sporangium consisting of two cellular compartments, the mother cell and the forespore. In B. subtilis, the developmental process is temporally regulated at the transcriptional level by the successive activation of six sigma (σ) factors that bind to RNA polymerase determine which gene promoters are recognized (Helmann and Chamberlin, 1988). These factors are σ^5, the primary sigma factor of vegetative cells, and five factors that are activated during sporulation, σ^7, σ^8, σ^9, σ^11 and σ^6, in order of their occurrence during sporulation (Errington, 1993). The σ^5 and σ^11 factors are active in the predivisional cell, σ^6 and σ^6 are active in the mother cell, and σ^7 and σ^9 are active in the forespore. In B. thuringiensis, two genes encoding sigma factors, σ^55 and σ^58, which show, respectively, 88 and 85% amino acid sequence identity with σ^5 and σ^6 of B. subtilis, have been cloned (Adams et al., 1991). In B. thuringiensis, there are two primary sporulation-dependent promoters, BtI and BtII. The BtI promoter is transcribed by σ^55 complexed with the RNA polymerase (Brown and Whiteley, 1988), whereas the BtII promoter is transcribed by the σ^58 complexed with the RNA polymerase (Brown and Whiteley, 1990).

The first crystal protein gene to be cloned from B. thuringiensis and characterized was cry1Aa (Schneppf and Whiteley, 1981). Wong et al. (1983) identified two overlapping promoters, BtI and BtII, from which cry1Aa is transcribed. BtI is active between about T_5 and T_6 of sporulation and BtII is active from about T_6 onwards (where T_6 is the number of hours after the end of the exponential phase). The start of crystal formation has been observed microscopically during the latter part of sporulation stage II (7 to 8 hr after mid-exponential phase) using a B. thuringiensis strain containing these promoters (Bechtel and Bulla, 1976). Crystal protein continues to be synthesized until the end of state IV (11 hr after mid-exponential phase), and crystals may continue to enlarge until stage VI (12 hr after mid-exponential phase).

All together, several cry promoters have been identified, and their sequences determined (Blizzard et al., 1991; Brown, 1993; Yoshisue et al., 1993a,b; Dervyn et al., 1995). Consensus sequences for promoters recognized by B. thuringiensis RNA polymerase containing σ^5-like of σ^7-like factors have been deduced from alignment of the promoter regions of these genes (Baum and Malvar, 1995). The results indicate that the transcription of many other cry genes is likely to be σ^5 or σ^7-dependent (Table 1).

In B. thuringiensis subsp. israelensis, a subspecies toxic to dipterans, four different crystal proteins are produced: Cry4A, Cry4B, Cry11A and Cry1A. Yoshisue et al. (1993a, b) showed that the cry4A and cry4B promoters are homologous to σ^5-dependent promoters. However, Yoshisue et al. (1995) later reported that cry4A, but not cry4B, is transcribed at low levels in a σ^7 mutant of B. subtilis, and proposed that cry4A is partially controlled by a σ^7-dependent promoter. Characterization of cry4A demonstrated that transcription is initiated by both the σ^7 (Ward and Ellar, 1986) and σ^7 (Waalwijk et al., 1986) forms of RNA polymerase from promoters homologous to BtI and BtII.

The cry3A gene, originally isolated from the coleopteran-active B. thuringiensis subsp. morrisoni (strain tenebroidis), is a typical example of a sporulation-independent Cry gene. The activity of the cry3A promoter is low during the vegetative phase, increases toward the end of exponential growth, and then ceases at about T_6 of sporulation.

Unlike BtI and BtII, the cry3A promoter is similar to promoters recognized by σ^6, the primary sigma factor of vegetative cells. The expression of cry3A is not dependent on sporulation-specific σ factors in either B. subtilis or B. thuringiensis (Agaisse and Lereclus, 1994a; Salamitou et al., 1996). Moreover, cry3A expression is increased and prolonged in mutant strains that rare unable to initiate sporulation (Malvar and Baum 1994; Lereclus et al., 1995). Thus, cry3A promoter activation is not regulated by the genes regulating sporulation initiation, but rather by some regulatory proteins preventing or activating gene expression during the transition from exponential growth to stationary phase. Two types of regulatory proteins act-

| Table 1. Nucleotide sequence of the cry gene promoters |
|---------------------------------|---------------------------------|---------------------------------|------------------|
| Promoter | -35 region | -10 region | Reference |
| σ^6 consensus | KMATAATT | CATACA-T | Moran, 1993 |
| cry1A BtI | GCATCTT | CATAGAAT | Ward and Ellar, 1986 |
| σ^6 consensus | GKMCA | CATANNNT | Moran, 1993 |
| cry1A BtII | GCACCA | AATATTAT | Waalwijk et al., 1986 |
| σ^4 consensus | TTAGCA | TATAAT | Moran, 1993 |
| cry3A | TTGCAA | TAAGCT | Agaisse and Lereclus, 1994 |
ing on gene expression during stationary phase have been identified in *B. subtilis* (Smith, 1993). One type, such as AhrB, Hpr or Sin, prevents gene expression during vegetative growth. The other type of regulatory protein, such as the Deg proteins or ComP/ComA, activates expression of genes silent previously. To date, however, the gene involved in cry3A regulation has not been identified.

### 5′ mRNA stabilizing sequence

The stability of mRNA contributes importantly to the high levels of toxin synthesis in *B. thuringiensis*. The half-life of cry gene mRNA is at least five-fold greater than the half-life of average bacterial mRNAs (Glatron and Rapoport, 1972). Within the past several years it has been shown that mRNA regions at the 5′ end of the transcript can also enhance Cry synthesis. For example, the 5′ region of the cry3A transcript beginning at nucleotide position-129 contains a region that stabilizes this mRNA (Agaisse and Lereclus, 1994). Fusion of this region to the 5′ region of the lacZ gene transcribed from a promoter inducible in *B. subtilis* increased the stability of the lacZ fusion mRNA and resulted in a 10-fold increase of both steady-state mRNA and β-galactosidase synthesis (Agaisse and Lereclus, 1996). In conferring stability on any heterologous sequence to which it is fused, the downstream region benefits from the mRNA stabilizing characteristic of this 5′ region.

The determinant of stability appears to be a consensus Shine-Dalgarno (SD) sequence, designated STAB-SD, close to the 5′ end of the cry3A mRNA (Agaisse and Lereclus, 1996). Mutations introduced into this region suggest that this sequence provides stability through interaction with the 3′ end of the 16S rRNA. Therefore, the binding of a 30S ribosomal subunit to the SD sequence located in the 5′ untranslated region of cry3A apparently stabilizes the corresponding transcript by protecting it against 5′-3′ ribonuclease activity. Such SD sequences are also present in similar positions in at least two other members of cry3 Gene family, cry3Bal and cry3Bα2 (Agaisse and Lereclus, 1996; Crickmore et al., 1998), suggesting that the corresponding transcripts are probably stabilized by a similar mechanism. Recently, it has been shown that a segment of early RNA from *B. subtilis* bacteriophage SP 82 could act as a 5′ mRNA stabilizer (Hue et al., 1995). Similarly, the determinant of stability appears to be a polypurine sequence that resembles a ribosome binding site. Therefore, the STAB-SD sequence may be a general determinant of mRNA stability, where required, in *Bacillus* species. Park et al. (1998) showed that significantly higher yields of Cry3A can be obtained by using dual sporulation-dependent cyt1A promoters to drive expression of cry3A when the STAB-SD sequence is included in the construct (Fig. 1).

![Electron micrographs of sporulated wild-type and engineered strains of *B. thuringiensis* illustrating crystals typical of these strains. A. Wild-type *B. thuringiensis* subsp. *morrisoni* (strain tenebrionis) DSM2803. B. Acrylalliferous strain (4Q7) of *B. thuringiensis* subsp. *israelensis* transformed with pPFT3A, cry3A without the STAB-SD sequence under the control of cyt1A promoters. C. and D. Respectively, cross and sagital sections through 4Q7 cells transformed with pPFT3As, cry3A with the STAB-SD sequence under the control of cyt1A promoters. All micrographs are at the same magnification; bar in B=300 nm.

### 3′ transcription termination sequence

Wong and Chang (1986) showed that a non-coding region near the 3′ terminus of cyt1Aa from *B. thuringiensis* subsp. *kurstaki* HD1 acts as a positive retoregulator, i.e., serves as a cis-acting element that regulates a target gene from a distance. The fusion of this fragment with the 3′ end of heterologous genes increased transcript half-life and consequently the amount of Cry protein synthesized. Recently it has been shown that the 3′ region contains inverted repeat sequences with the potential to form stable stem-loop structures (Ge, 1999).

The activity of 3′-5′ exonucleases is affected by RNA secondary structure. In particular, their rate of mRNA degradation is impeded by 3′ stem-loop structures. Therefore,
it is likely that cry and cyt gene terminators are involved in mRNA stability by protecting the mRNA from exonucleolytic degradation from the 3' end. It is becoming increasingly apparent that the 3' stem-loop structure blocks exonuclease digestion long enough for degradation to be initiated by upstream endonucleolytic cleavage. The putative terminator sequences downstream from various cry genes are widely conserved. Recently, a stem-loop binding protein that impedes the progress of the Escherichia coli exoribonuclease PNPase in vitro has been identified (Custon et al., 1994). Thus, the binding of an additional factor to the 3' stem-loop structure of cry gene mRNA may be involved in the stabilizing effect of this region. However, it remains to be established whether such a mechanism exists in B. thuringiensis. Recently, it has been shown that the orientation of the cry3A transcription terminators was important to enhancing truncated cryIC transcript stability and protein synthesis (Park et al., 2000; Fig 2 and 3).

The 20 and 29 kDa helper proteins

The ability of B. thuringiensis to form large crystals of insecticidal proteins after the onset of stationary phase and during sporulation is due to a combination of its capacity to synthesize large quantities of these proteins and facilitate their rapid crystallization. Although naturally occurring isolates of B. thuringiensis frequently produce crystals containing multiple Cry1 proteins, isolates that produce multiple Cry2 or Cry3 proteins are rare. Thus, the ability to form heterogeneous inclusions composed of different protoxins may be a property unique to the 130-140 kDa crystal protein class. It appears that the conserved C-
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The gene encoding Cry11A occurs as the second gene in an operon that is co-transcribed with genes not involved in toxicity. The same is true for Cry2A. Disruption of orf2 in the cry2A operon leads to a marked reduction in the production of Cry2A inclusions (Fig. 4). Evidence suggests that this gene encodes a 29 kDa scaffolding protein that assists the formation of typical Cry2A crystals (Crickmore and Ellar, 1992; Ge et al., 1998; Park et al., 1999). A 20-kDa protein encoded as the third ORF of the Cry11A operon apparently acts like a chaperone, assisting Cry11A synthesis (Fig. 4). This protein was originally shown to be required for efficient Cry1A production in E. coli (McLean and Whiteley, 1987; Adams et al., 1989; Visick and Whiteley, 1991). It has also been reported to enhance net synthesis of Cry4A and Cry11A in E. coli and in B. thuringiensis (Visick and Whiteley, 1991; Yoshise et al., 1992; Wu and Federici, 1995; Park et al., 1999), as well as Cry1A production and crystal formation in B. thuringiensis (Wu and Federici, 1993).

**Summary**

Studies of the mechanisms that control Cry protein synthesis have been used to develop knowledge that has led, using molecular genetic techniques, to new strategies for obtaining substantial increases in Cry protein yields per cell. These studies have shown that: (1) strong and multiple promoters contribute substantially to toxin synthesis; (2) transcript stability is important to Cry protein synthesis; (3) cry transcription terminators play an important role in cry mRNA stability; and (4) helper proteins can enhance Cry and Cyt protein translation and/or crystallization. The most substantial increases in synthesis of the 65-kDa protein type have been obtained using the Shine-Dalgarno mRNA stabilizing sequence (STAB-D) located in the 5’ region of the cry3A gene. The 20-kDa protein encoded as orf3 of the cry11A operon, which apparently acts as a molecular chaperone, and the 29-kDa protein encoded as orf2 of the cry2A operon, for which there is evidence that it serves both as a molecular chaperone and a scaffolding protein, can also be used to increase crystal production, but are not as important as the 5’ STAB-SD sequence.

**References**


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