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[Review]

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

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Early studies of the molecular biology of *Bacillus thuringeinsis* 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 se- quences, 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 sequnence, Helper proteins

Introduction

Crystal (Cry) protein toxins of *Bacillus thuringiensis* are the active component of most bacterial insecticides (Hofte and Whiteley, 1989; Schnepf *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 (Agaisse 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 *cryI* 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; Schnepf *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 (Agaisse 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 by binding to RNA polymerase determine which gene promoters are recognized (Helmann and Chamberlin, 1988). These factors are σ^A, the primary sigma factor of vegetative cells, and five factors that are activated during sporulation, σ^E, σ^F, σ^G, σ^H and σ^K, in order of their occurrence during sporulation (Errington, 1993). The σ^A and σ^H factors are active in the predivisional cell, σ^E and σ^K are active in the mother cell, and σ^F and σ^G are active in the forespore. In *B. thuringiensis*, two genes encoding sigma factors, σ^{35} and σ^{28}, which show, respectively, 88 and 85% amino acid sequence identity with σ^E and σ^K of *B. subtilis*, have been cloned (Adams *et al.*, 1991). In *B. thuringiensis*, there are two primary sporuation-dependent promoters, BtI and BtII. The BtI promoter is transcribed by σ^{35} complexed with the RNA polymerase (Brown and Whiteley, 1988), whereas the BtII promoter is transcribed by the σ^{28} complexed with the RNA polymerase (Brown and Whiteley, 1990).

The first crystal protein gene to be cloned from *B. thuringiensis* and characterized was *cry1Aa* (Schnepf 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_2 and T_6 of sporulation and BtII is active from about T_5 onwards (where T_n 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 midexponential 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^E-like of σ^K-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^E-or σ^K-dependent (Table 1).

In *B. thuringiensis* subsp. *israelensis*, a subspecies toxic to dipterans, four different crystal proteins are produced: Cry4A, Cry4B, Cry11A and Cyt1A. Yoshisue *et al.* (1993a, b) showd that the *cry4A* and *cry4B* promoters are homologous to σ^E-dependent promoters. However, Yoshisue *et al.* (1995) later reported that *cry4A*, but not *cry4B*, is transcribed at low levels in a σ^E mutant of *B. subtilis*, and proposed that *cry4A* is partially controlled by a σ^H-dependent promoter. Characterization of *cry1A* demonstrated that transcription is initiated by both the σ^E (Ward and Ellar, 1986) ans σ^K (Waalwijik *et al.*, 1986) forms of RNA polymerase from promoters homologous to BtI and BtII.

The *cry3A* gene, originally isolated from the colepteran-active *B. thuringiensis* subsp. *morrisoni* (strain tenebrionis), is a typiclal 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_8 of sporulation.

Unlike BtI and BtII, the *cry3A* promoter is similar to promoters recognized by σ^A, 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^E consensus	KMATATT	CATACA-T	Moran, 1993
cyt1A BtI	GCATCTT	CATAGAAT	Ward and Ellar, 1986
σ^K consensus	GKMACA	CATANNNT	Moran, 1993
cyt1A BtII	GCACCA	AATATTAT	Waalwijk *et al.*, 1986
σ^A consensus	TTGACA	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 AbrB, 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 positon-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 aganist 5'-3' ribonuclease activity. Such SD sequences are also present in similar positions in at least two other members of *cry3* Gene family, *cry3Ba1* and *cry3Ba2* (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).

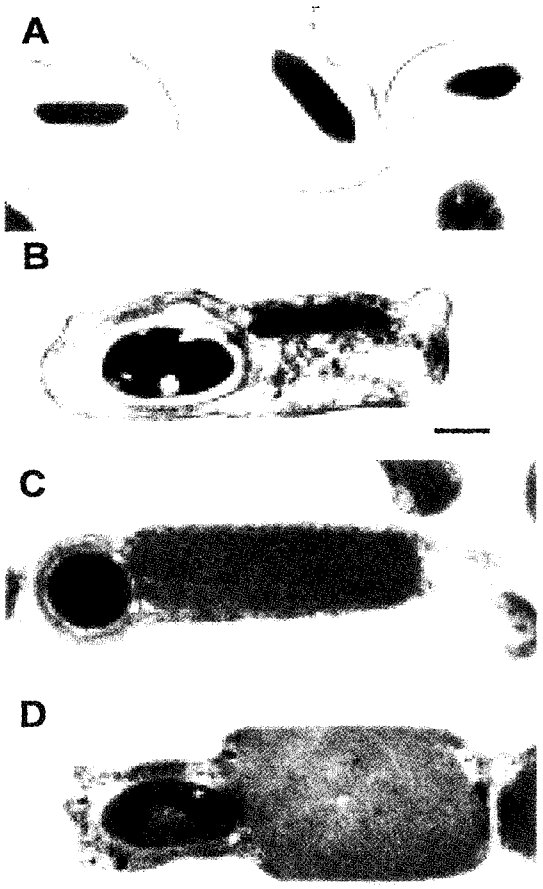

Fig. 1. Electron micrographs of sporulated wild-type and engineered strains of *B. thuringiensis* illustrating crystals typical of these strains. A. Wild-type *B. thrungiensis* subsp. *morrisoni* (strain tenebrionis) DSM2803. *B. Acrystalliferous* strain (4Q7) of *B. thuringiensis* subsp. *israelensis* 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 *cry1Aa* from *B. thuringiensis* subsp. *kurstaki* HD1 acts as a positive retroregulator, 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 hetedologous 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. Recentluy, a stem-loop binding protein that impedes the progress of the *Escherichia coli* exoribonuclease PNPase *in vitro* has been identified (Causton *et al.*, 1994). Thus, the binding of an adeditional 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 *cry1C* 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-

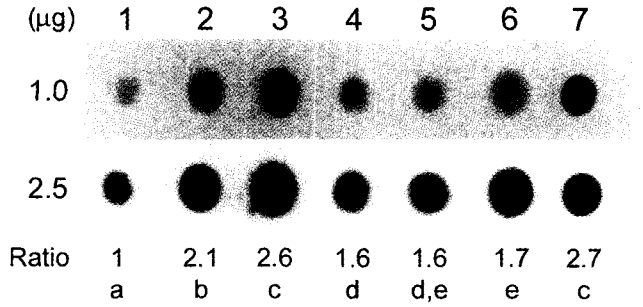

Fig. 2. Transcript levels for different truncated *cry1C* constructs. Lane 1, pPET1C-t[*cyt1A* p + truncated *cry1C*]; 2, pPFT1 Cs-t[*cyt1A* p + STAB-SD + truncated *cry1C*]; 3, pPF T1Cs-3t(+)[*cyt1A* p + STAB-SD + truncated *cry1C* + stem-loop (5'-3')]; 4, pPFT1Cs-3t(-)[*cyt1A* p + STAB-SD + truncated *cry 1C* + stem-loop(3'-5')]; 5, pPFT1Csf-3t(+)[*cyt1A* p + STAB-SD + *orf2* + trun-cated *cry1C* + stem-loop(5'-3')]; 6, pPFT1Cs-20k [*cyt 1A* p+ STAB-SD + truncated *cry1C* + *20-kDa*]; 7, pPF T1Csf-20k[*cyt1A* p + STAB-SD +*orf2* + truncated *cry1C* + *20-kDa*] The ratios shown in Lanes 2-7 are relative to the dot in Lane 1, which was assigned a value of 1. Each value represents the average value (ratio) obtained from three separate experiments. Different letters beneath the ratios indicate the values were significantly different at P=0.05.

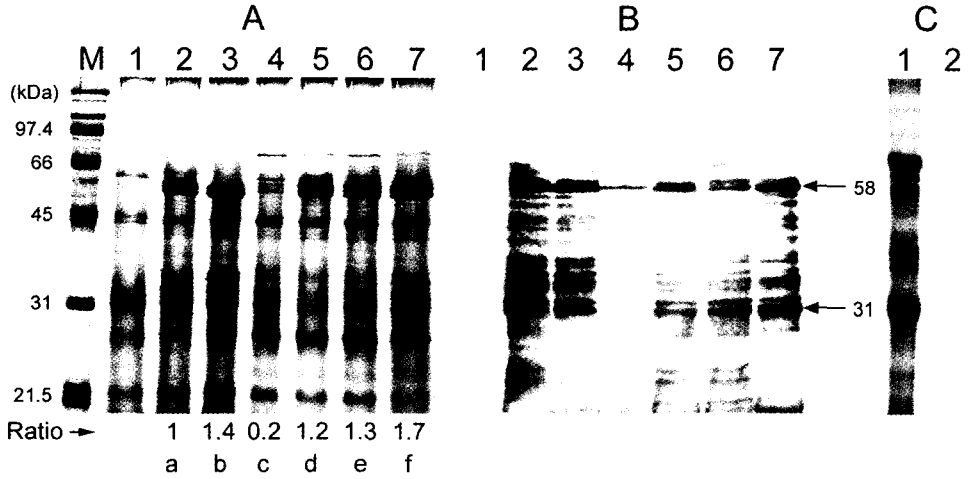

Fig. 3. Synthesis of Cry1C-t by different constructs as determined by SDS-PAGE and Western blot analysis. A, 12% SDS-PAGE gel and, B, Western blot of the same gel. The relative amounts of Cry1C-t produced by each strain are indicated at the bottom of the lanes in A. Lane 1, pPFT1C-t[*cyt1A* p+truncated *cry1C*]; 2, pPFT1Cs-t[*cyt1A* p+STAB-SD+truncated *cry1C*]; 3, pPFT1Cs-3t(+)[*cyt1A* p + STAB-SD + truncated *cry1C* + stem-loop(5'-3')]; 4, pPFT1Cs-3t(-)[*cyt1A* p + STAB-SD + truncated *cry1C* + stem-loop(3'-5')]; 5, pPFT1Csf-3t(+)[*cyt1A* p + STAB-SD + *orf2* + truncated *cry1C* + stem-loop(5'-3')]; 6, pPFT1Cs-20k[*cyt1A* p + STAB-SD + truncated *cry1C* + *20-kDa*]; 7, pPFT1Csf-20k[*cyt1A* p + STAB-SD + *orf2* + truncated *cry1C* + *20-kDa*]; M, Molecular size marker. The control, C, for the Western blot analysis is *Bacillus thuringiensis* subsp. *israelensis*, which produces Cry11A and Cyt1A. Lane 1, SDS-PAGE gel; 2, Western blot of the same gel. The ratios shown in lanes 3-7 are relative to the amount of the 58-kDa protein in Lane 2, which was assigned a value of 1. Each value represents the average value (ratio) obtained from three separate experiments. Different letters beneath the ratios indicate the values were significantly different at P=0.05.

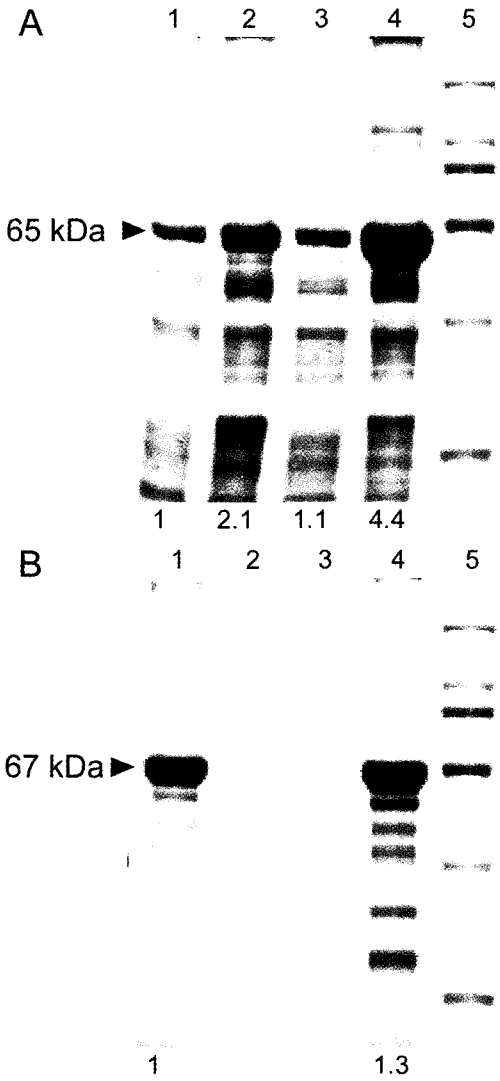

Fig. 4. SDS-PAGE analysis of Cry2A and Cry11A yields produced by *Bacillus thuringiensis* subsp. *israelensis* 4Q7 strains expressing different constructs. The relative amount of crystal protein produced by each strain is indicated at the bottom of the gel. A: Strains producing Cry2A. Lane 1, pDBF69 (wild-type operon); lane 2, pPFT2As (*cyt1A*p + STAB-SD + *cry2A*); lane 3, pPFT2Af (*cyt1A*p + *orf2* + *cry2A*); lane 4, pPFT2Asf (*cyt1Ap* + STAB-SD + *orf2* + *cry2A*). B: Strains producing Cry 11A. Lane 1, pWF53 (*cry1Acp* + *20-kDa* + *cry11A* + *19-kDa*); lane 2, pPFT11As (*cyt1Ap* + STAB-SD + *cry11A*); lane 3, p PFT11At (*cyt1Ap* + *cry11A* + *20-kDa*); lane 4, pPFT11Ast (*cyt 1Ap* + STAB-SD + *cry11A* + *20-kDa*). Lane 5 in both panels shows molecular mass standards.

terminal halves of these protoxins participate in crystal formation, perhaps via the formation of intermolecular disulfide bonds (Aronson, 1993).

The smaller crystal proteins do not possess the conserved C-terminal domain characteristic of the 130-140 kDa proteins, and several studies indicate that some of these crystal proteins may require helper proteins to form crystals. 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 Cru11A synthesis (Fig. 4). This protein was originally shown to be required for efficient Cyt1A 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; Yoshisue *et al.*, 1992; Wu and Federici, 1995; Park *et al.*, 1999), as well as Cyt1A production and crystal formation in *B. thuringiensis* (Wu and Federici, 1993).

Summary

Studies of the mechanisms that cntrol 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 studis 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.

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