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THE MOLECULAR BIOLOGY OF THE *BACILLUS THURINGIENSIS* 8-ENDOTOXIN GENES AND OF THEIR EXPRESSION: AN OVERVIEW

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ABSTRACT

Bacillus thuringiensis produces a large variety of insecticidal crystal proteins, which accumulates in large amounts during sporulation within the mother cell. The regulation of the *cry* gene is mostly determined at the transcriptional level. However, transcription alone cannot account for the ability of this bacterium to accumulate the 8-endotoxins and a series of post-transcrip- tional and post-translational mechanisms contribute to the efficient synthesis of these proteins.

KEY WORDS: *Bacillus thuringiensis,* crystal, sporulation, sigma factor, mRNA stability, chaperonins.

INTRODUCTION

Biocontrol of vectors pests by *Bacillus thuringiensis (Bt)* 8-endotoxins represents the most successful use of a biological control agent to date. The combination of high molar activity on target pests combined with minimal non-target and negligible human toxicity have rendered them very attractive, both as applied microbial pesticides and as candidates for transgenic plant delivery.

Bt is a gram-positive soil bacterium characterized by its ability to produce crystalline inclusions during sporulation. These inclusions consist of polypeptides exhibiting a highly specific larvicidal activity. Many *Bt* strains with different host spectra have been isolated and identified. There are over 30 recognized subspecies or varieties of *Bt* based, in part, on flagellar antigens and other properties (deBaijac andFranchon, 1990). Different *Bt* subspecies, and even strains with a single subspecies, exhibit substantial differences in insecticidal activity. The nature of the insecticidal crystal protein produced dictates both the strain's insecticidal activity spectrum as well as its distinctive crystal morphology. Most strains are active against larvae of certain members of the Lepidoptera, but some show toxicity against dipteran or coleopteran species. More recently, isolates have been found to be active against mites or nematodes. However, for several crystal producing strains, no toxic activity has yet been demonstrated.

Mechanism of action of the crystal proteins

General details on the mechanism of action of the crystal proteins seem apparent (Baum and Mai var, 1995). *Bt* crystals dissolve in the midgut of the larvae, releasing one or several polypeptides (also refered to as 5-endotoxins) of 27 to 140 kDa. In many instances, the crystal

proteins are protoxins which are processed by midgut proteases to a fully active state. The activated toxins bind to the brush border membranes of the insect midgut epithelium, a step that frequently requires the presence of fortuitous receptor proteins. This binding is followed by an apparent intercalation event in which the active toxin moiety, or a portion of it, contributes to the formation of ion channels as well as aggregates to form larger pores within the brush border membrane, leading to osmotic imbalance, cellular swelling and lysis. Insect feeding stops within minutes followed by paralysis and eventual death from starvation within a few hours or days.

The δ-endotoxins were originally classified as CryI, CryII, CryIII, CryIV proteins and Cyt (cytolysins), based on their insecticidal activities (Höfte and Whiteley, 1989). This classification system is being revised to rely solely on amino acid sequence homologies rather than on insecticidal properties as the criterion for assigning Cry designations (Dean, personal communication, 1996). A survey of the databases reveals that near fifty δ-endotoxin genes have now been cloned and sequenced, allowing sequence comparison and establishment of a dendogram showing the relatedness of the *Bt* crystal proteins. Several reviews dealing with the molecular aspects, genetic diversity and mode of action of the *Bt* toxins have been already published (English and Slatin, 1992; Agaisse and Lereclus, 1995).

The production of the parasporal crystals is concomitant with sporulation and the proteins' accumulation in the mother cell, accounting for up to 25% of the dry cell mass of naturally occurring isolates. It was estimated that under laboratory conditions, each cell synthesizes 10^6 to 2×10^6 δ-endotoxin molecules to form a crystal (Priest et al., 1988). This large production explains that specific attention was given to the regulation of the *Bt* δ-endotoxin gene expression. Some interesting findings with respect to the transcriptional, post-transcriptional and even post-translational regulation were revealed. The aim of this paper is to analyze how such mechanisms explain the large amounts of toxins synthesized during the sporulation phase.

δ-endotoxin gene organization

The CryI proteins can be aligned on the basis of their amino acid sequences. The amino-terminal halves of the CryI (and CryIVA and CryIVB) proteins, released from the 130–140 kDa protoxins by proteolytic cleavage, comprise the active toxin moiety and show limited but significant homology (blocks a to e) to the full length CryII, CryIII and CryIVD proteins. The crystal structures for CryIIIA, CryIA and CryIIIB2 reveal a conserved three domain structure comprising the active toxin moiety (Li et al., 1991; Borisova et al., 1994; Grochulski et al., 1994; Galitsky et al., 1994). Domain I consists of an amino-terminal eight helix bundle, essential for the formation of ion channels. Domain II is composed of the central three sheet domain, whereas Domain III is represented by the carboxyl proximal β sandwich domain and both of them strongly influence larvicidal specificity. The unique carboxy halves of the 130–140 kDa protoxins are highly conserved, cys residue rich and appear to be essential for crystal formation.

B. thuringiensis is well known for its numerous plasmids and complex plasmid patterns; at least about 10 to 20% of the potential genetic coding capacity is found on plasmids ranging in size from <1.5 MDa to >130 MDa (Carlton and Gonzalez, 1985). The δ-endotoxin genes are very often located on large plasmids (>30 MDa), but some δ-endotoxin genes are located on the chromosome (Carlson and Kolstø, 1993). Native strains of *B. thuringiensis* frequently contain multiple δ-endotoxin encoding plasmids and some of them can harbor more than one

δ-endotoxin gene. These multiple δ-endotoxin genes may direct the synthesis of related proteins that form a heterologous crystalline inclusion or direct the synthesis of unique proteins that form separate crystals of distinct morphology. The well known strain HD1 subspecies *kurstaki*, harboring 12 resident plasmids, contains *cryIA(a)*, *cryIA(c)*, *cryIIA* and silent *cryIIB* and *cryV* genes on a 110 MDa plasmid and a *cryIA(b)* on a self transmissible 44 MDa plasmid. The CryIA(a), CryIA(b) and CryIA(c) proteins contribute to the formation of the bypyramidal shaped crystal of HD1 while the CryIIA protein forms the distinct cuboidal shaped crystal. In the case of *Bt* subsp. *israelensis* (*Bti*), the *cryIVA*, *cryIVB*, *cryIVD* and *cytA* genes are all located on a 75 MDa plasmid and contribute to the formation of a complex parasporal body composed of multiple inclusions (Gonzalez and Carlton, 1984). A number of the plasmids are transmissible, *via* a conjugation-like process, which is not well understood. However, this feature has facilitated the construction of transconjugant strains and the development of new strains with improved insecticidal properties.

Many of the *cryI* genes are arranged as monocistronic units, with transcriptional start sites immediately upstream from the gene. The *cryIA* terminator sequence, characterized by a large inverted repeat, was shown to protect the RNA from nuclease digestion, leading to a significantly long half-life. There are several examples of toxin genes that are expressed as part of an operon. Both the *cryIIA* and *cryIIC* genes are present as the third gene in operons containing two upstream open reading frames (*orf*s). The *cryIVD* gene is located as the middle gene in an operon that includes two *orf*s encoding 19 and 20 kDa proteins (Dervyn et al., 1995). Interestingly, the first *orf* (p19) of the *cryIVD* operon shows 32% sequence similarity with the *orf1* of the *cryIIA* and *cryIIC* operons. This result agrees with the observation that the CryIVD sequence is most closely related to that of CryIIA. A related *orf1* has been identified as part of an operon for a CryIG-like protein (designated Cry9c), that belongs to the 130–140 kDa protoxin group, and this *orf* is identical to the *orf1* of the *cryIIC* operon. The *cry40* and *cry34* genes of *Bt* subsp. *thompsoni* also comprise an operon (Brown and Whiteley, 1992). The *cryIIIA*, *cryIIIB* and *cryIIIB2* are each associated with downstream *orf*s that encode proteins of 28–32 kDa, but some of them appear to be independently transcribed. The putative role of these additional *orf*s in facilitating production, protein stability and crystal formation will be discussed later.

Sporulation

The accumulation of δ-endotoxin coincides with sporulation in *Bt*. Recent studies have shown that many of the proteins that regulate sporulation during stage 0 and later in *B. subtilis* are present and appear to function similarly in *Bt* (Hoch, 1993). In *B. subtilis*, the Spo0A protein is the major control point of sporulation and, primarily, in its phosphorylated form serves to both positively and negatively control genes for sporulation. The level of Spo0A~P is regulated by a phosphorylation pathway that is comprised of a number of histidine protein kinases, that sense conditions suitable for sporulation, and a series of intermediate phospho-acceptor molecules. The histidine protein kinases KinA, KinB and KinC, respond to unidentified signals by autophosphorylation of a conserved histidyl residue in the carboxy-terminus of the proteins. The phosphate group is then sequentially transferred to Spo0F, Spo0B and finally, to Spo0A. Mutations in a gene encoding for a component of the phosphorelay block the sporulation, while mutations in any of the sensor proteins result in a delayed sporulation phenotype. It is well known that the kinases compensate for each other and that high level expression of *kinA*

bypasses *spo0* mutations in the phosphorelay, by a direct phosphorylation of Spo0A. Very recently, it was demonstrated that phosphatase encoded genes interfere with the transfer of the phosphate group in the phosphorelay and that a protease inhibitor, encoded by a small gene in the same operon as the phosphatase gene is able to inhibit the phosphatase. Interestingly, the inhibitor is secreted and can be used as an external signal by an another cell to induce the sporulation process (Perego et al., 1996). It was shown that the *spo0K* operon, which encodes a membrane associated oligopeptide transport system, is responsible for this internalisation. Other factors may also influence the Spo0A~P levels, such as the GDP/GTP ratio.

Recent studies have demonstrated that the CryIIIA protein is overproduced in *Bt* strains, blocked at stage 0 (Lereclus et al., 1995). These observations led to the identification of *Bt* homologues for the *B. subtilis* Spo0F and Spo0A proteins, as well as a novel histidine protein kinase, HknA, that is similar to the *B. subtilis* KinA protein (Malvar and Baum, 1994). The *Bt* Spo0F protein shares 76% homology with its counterpart from *B. subtilis*, while *Bt* Spo0A shares 82% protein sequence identity with *B. subtilis* Spo0A. Upstream of the *Bt spo0A* gene, a potential *orf* homologue to SpoIVB was reported. In *B. subtilis*, the same genetic arrangement between *spoIVB* and *spo0A* was described. It is assumed that in *Bt* a phosphorylation cascade, similar to that of *B. subtilis*, regulates early sporulation in *Bt*.

The *Bt* HknA protein shares some homology with the *B. subtilis* KinA, B and C proteins, with the majority of sequence similarities localized in the carboxy halves of the proteins. The HknA, KinA and KinC proteins are likely cytolysic proteins, while the KinB protein appears to be associated with the cell membrane, *via* six putative membrane-spanning segments located at the N-terminus of the protein. Hybridization experiments suggest that *kinA*-like DNA sequences may be shared between *Bt* and *B. subtilis*, and *hknA* is apparently not, suggesting that *Bt* employs at least one novel histidine kinase (HknA) in its sensing system for initiating sporulation. Disruption of *hknA* in *Bt* resulted in delayed sporulation and elevated levels of either HknA or KinA were shown to bypass a *spo0F* mutation in *Bt*, as well as an uncharacterized early *spo* mutation in *Bt*. These results argue that HknA functions as a histidine-kinase involved in *Bt* sporulation.

Since similar proteins appear to regulate stage 0 in *Bt* and *B. subtilis*, it may be assumed that the remaining stages of sporulation are likewise similar in the two organisms. This assumption is confirmed by the identification of *Bt* homologues for the *B. subtilis* sporulation specific sigma factors σ^E (part of the *spoIIG* operon) and σ^K (*spoIIIC*) (Adams et al., 1991). The *Bt* σ^E (σ^{35}) protein shares 87.9% identity with σ^E from *B. subtilis* and is also part of an operon that is immediately proceeded by a partially identified *orf* that shows 45% identity to the corresponding region of the *B. subtilis* SpoIIGA protein. It is assumed that σ^E protein also undergoes a processing step in *Bt*, as in *B. subtilis*. Downstream of the *spoIIG* operon, a partial *orf* was identified and encodes a protein that is 92% homologous to the corresponding region of the *B. subtilis* σ^G protein (SpoIIIG). Thus, the same genetic arrangement of the *spoIIG* and *spoIIIG* alleles has been described for *B. subtilis*. The *Bt* σ^K (σ^{28}) protein shares 85% homology with the *B. subtilis* σ^K protein. Unlike the σ^K allele in *B. subtilis*, the σ^K allele in *Bt* is transcribed as a monocistronic unit without prior DNA rearrangement. It is assumed that the *Bt* σ^K protein is processed from a precursor form (pro-σ^K) to its mature form, as is the case in *B. subtilis*. As expected, the *Bt* σ^E and σ^K forms of RNA polymerase show functional homology to the corresponding *B. subtilis* polymerases by directing transcription from σ^E and σ^K dependent

B. subtilis promoters, respectively. In general, though, the available data suggest that the processes involved in *Bt* sporulation parallel those of *B. subtilis*, with some minor differences.

Sporulation-specific δ-endotoxin gene transcription

The sporulation-specific nature of δ-endotoxin synthesis suggests that transcription of the corresponding genes is held in check until factors are being expressed during sporulation. The primary means of temporally and spatially regulating gene expression in *B. subtilis* is by the transitory expression of specific σ factors, changing the RNA polymerase specificity (Hoch, 1993). The σ^A form of RNA polymerase is responsible for the vegetative gene transcription. The σ^H form of RNA polymerase, present at low levels during the vegetative phase, functions primarily during the stationary phase, prior to septation (stage II). The σ^E, σ^F, σ^G and σ^K proteins are sporulation-specific sigma factors of *B. subtilis*, active in either the forespore or mother cell compartments of the sporulating bacterium. Transcription of genes within the forespore compartment required for early and late prespore development depends upon σ^F and σ^G, respectively, while temporal control of early and late transcription in the mother cell are controlled by σ^E and σ^K, respectively. Timing and compartmentalization of σ factor activities in *B. subtilis* ensures precise control over gene expression during spore development. Interestingly, a coordinate expression in both compartments occurs through a criss-cross mechanism. As already mentioned, the identification of homology for σ^E, σ^K and σ^G suggests that *Bt* employs a similar strategy in regulating sporulation and δ-endotoxin gene expression.

The first δ-endotoxin gene to be cloned and sequenced was *cryIA(a)* and two overlapping promoters *BtI* and *BtII* were identified in front of this gene (Wong et al., 1983). The proximal promoter (*BtI*) is transcribed by the *Bt* σ^E form of RNA polymerase, while the more distal *BtII* promoter is transcribed by the σ^K form of RNA polymerase. The expression of *cryIA(a)* transcripts from *BtI* at the beginning of stage II of sporulation, would coincide with the processing of pro-σ^E to the mature σ^E that apparently occurs in both *Bt* and *B. subtilis*. The σ^K directed expression of CryIA(a) would place transcription from *BTII* at stage IV, prior to forming the spore cortex. The dependence of δ-endotoxin transcription on the σ^E and σ^K forms of RNA polymerase: 1) links δ-endotoxin expression to sporulation; 2) limits δ-endotoxin expression to the mother cell compartment, and 3) ensures δ-endotoxin production throughout much of sporulation which probably contributes to the large amounts of δ-endotoxin produced by *Bt*.

It seems likely that most of the *cryI* genes that have been identified in *Bt* are under similar transcriptional regulation (Baum and Malvar, 1994). An alignment of the 5′ regulatory regions for sporulation specific δ-endotoxin genes allows to deduce some consensus sequences for σ^E and σ^K-dependent δ-endotoxin promoters in *Bt*. Analysis of the promoter region for the *Bt* subsp. *thompsoni* operon that encodes the *cry40* and *cry34* proteins indicates σ^E dependent transcription of this operon. Transcriptional initiation of the *Bt* subsp. *kurstaki* HD1 *cryIIA* operon also appears to be under σ^E control.

The dipteran-active *Bti* produces four different δ-endotoxin proteins. The *cryIVA* and *cryIVB* promoters are homologous to σ^E dependent promoters and detection of specific RNA during mid-sporulation supports the σ^E dependent nature of their expression. *CryIVD* is the second gene of an operon, the transcription of which is initiated from two promoters, PI and PII (Dervyn et al., 1995). Trancription from PI is concurrent with early and mid sporulation, while a lower level of transcription from PII coincides with the later stages of sporulation. The –35

and –10 DNA sequences associated with the PI and PII promoters of the *cryIVD* operon are homologous to the σ^E and σ^K dependent BtI and BtII promoters identified respectively, for *cryIA(a)*. Characterization of the *cytA* demonstrated that transcription is initiated by both the σ^E and σ^K forms of RNA polymerase. More recently, it was demonstrated that a weak but significant σ^H dependent transcription of *cryIVA*, *B* and *D* genes occurs one hour before (the onset of sporulation). This residual expression is inhibited by the *spo0A* gene product. However, the biological role of this expression and regulation is not fully understood.

Sporulation-independent δ-endotoxin gene expression

The *cryIIIA* gene, found in the coleopteran-active *Bt* subsp. *tenebrionis*, is a typical example of a non-sporulation dependent *cry* gene (Agaisse and Lereclus, 1994a). Analysis with transcriptional fusions to the *lacZ* reporter gene indicates that the kinetics of *cryIIIA* and *cryIA(a)* expression are different. The *cryIIIA* promoter is weakly but significantly expressed during the vegetative phase of growth, is activated at the end of exponential growth, and remains active only about t_8 in the sporulation medium.

The transcriptional start site of *cryIIIA* has been located at -558 from the ATG codon (Agaisse and Lereclus, 1994b). The –35 and –10 regions identified resemble σ^A dependent promoter sequences. Various *cryIIIA-lacZ* fusions containing deletions within the 650 bp upstream of *cryIIIA* have shown that the –558 promoter region is required for full expression of *cryIIIA* in *Bt*. The expression of *cryIIIA* is not dependent on sporulation specific σ factors either in *B. subtilis* or in *Bt*. Several studies have shown that *Bt* strains that harbor mutations affecting the *spo0A* phosphorylation cascade overproduce CryIIIA protein, as well as other members of the CryIII class of δ-endotoxins. For example, disruption of the *Bt spo0F* gene leads to a 3-fold increase in CryIIIA protein production (Malvar et al., 1994). This phenotype is abolished by the introduction of *kinA* or *hknA* on a multicopy plasmid. Subsequently, a *spo0A* mutant of *Bt* was shown to overproduce CryIIIA. Consequently, it appears that *cryIIIA* is not regulated by the genes regulating sporulation initiation, but rather by some regulator(s) affecting gene expression during the transition from exponential growth to the stationary phase. However, the gene involved in CryIIIA regulation remains to be determined.

The features of *spo0* mutants could be used to overproduce other δ-endotoxins in *Bt*. For example, the *cryIIIB2* promoter fragment was cloned upstream of the *cryIIA* operon on a multicopy plasmid and introduced into a *spo0-Bt* mutant. The resulting recombinant strain produced about 5 times more CryIIA protein than did the parental strain. The amount is comparable, in terms of g of protein, to that of CryIIIA.

The δ-endotoxin gene copy numbers

There are several reports demonstrating that the expression level of a gene is influenced by its copy number and gene amplification has been used to overproduce proteins. As mentioned before, the δ-endotoxin genes are located on plasmids, and some strains carry several copies of these genes. Thus it was assumed that amplification of δ-endotoxin genes might contribute to the high production of toxins. However, it is not the case, since HD73, which harbors only one copy of the δ-endotoxin gene, produces crystals which are not significantly smaller than those produced by strains harboring several copies of different δ-endotoxin genes. It appears that the production of toxins in *Bt* is not strictly proportional to the copy number of the genes. This suggests that the capacity of the *Bt* strains to produce crystal proteins is somewhat limited.

Several arguments support this conclusion. For example, when a *cryIA(c)* gene is cloned in a strain harboring other *cryI* genes, significantly less CryIA(c) protein is produced than when it is cloned alone into an acrystalliferous strain of *Bt.* However, the reduced expression of *cryIA* genes is not observed when they are introduced in a strain harboring only a *cryIII* type gene, since they did not share the same rate limiting elements of the expression systems.

These effects arise from titration of specific σ factors recruited for *cry* gene transcription and are consistent with saturation of *cry* gene expression being primarily at a transcription level.

mRNA stabilization

The rate of mRNA degradation, measured by the half-life, has substantial consequences on gene expression. It is likely that the high level of δ-endotoxin production is due in part to the mRNAs being stable. It was demonstrated several years ago that the mRNAs encoding the δ-endotoxin are among the relatively long lived mRNAs present during the stationary phase and have an average half-life of 10 min.

A number of elements can act as mRNA stabilizers. These elements are generally part of untranslated regions and are classified into two groups, according to their location in the mRNA: i) 3′ terminal structures and ii) 5′ mRNA stabilizers. Both of these elements have been found in *cry* gene mRNAs.

i) It was demonstrated several years ago, that the 3′ terminal end of the *cryIA(a)* gene acts as a positive retroregulator (Wong and Chang, 1985). Deletion analysis showed that the region confering the enhancing activity coincided with the potential transcriptional terminator sequence. The region harbors inverted repeat sequences with the potential to form stable stem-loop structures. It is likely that the *cry* terminator is involved in mRNA stability by protecting the *cry* mRNA from exonucleolytic degradation from the 3′ end. Interestingly, the putative terminator sequences downstream from various *cry* genes are widely conserved, suggesting the important role of this structure. However, the mechanism by which the *cry* mRNA is protected remains to be established.

ii) The *cryIIIA* promoter is complex: full expression requires the presence of a 600 bp region upstream from the translational start site. Two main regions can be distinguished. The upstream region is involved in transcription and harbors the *cryIII* promoter recognized by σ^A-RNA polymerase. The downstream region is involved in *cryIIIA* expression at a postranscriptional level. More precisely, this region is responsible for the accumulation of *cryIIIA* mRNA as a stable transcript with a 5′ end corresponding to –129. It was shown that this region is able to stabilize heterologous mRNAs and it was postulated that it has all the characteristics of a 5′ mRNA stabilizer (Agaisse and Lereclus, 1995).

The main determinant of stability appears to be a consensus Shine-Dalgarno (SD) sequence present close to the 5′ end of the *cryIIIA* mRNA. It is postulated that the SD sequence is involved in stability through interaction with the 3′ end of 16S rRNA. Thus, binding of a 30S ribosomal subunit to the SD sequence located in the 5′ untranslated region of CryIIIA may stabilize the corresponding transcript. It is noteworthy that such a stabilizer is present at a similar position in two other members of the *cryIII* gene family, suggesting a common mechanism. Recently, a similar determinant of stability was identified in the early RNAs from *B. subtilis* phage SP82. Thus, SD sequence may be a general determinant of mRNA stability in *B. subtilis*, but its mechanisms of RNA stabilization remain unknown.

Protein stability and crystal formation

The ability of *Bt* to produce large quantities of insecticidal proteins is largely due to the ability of these proteins to form crystalline inclusions. The transition to an insoluble state presumably renders the δ-endotoxin less susceptible to proteolytic degradation and allows it to accumulate within the mother cell. Moreover, proteolytic stability of the nascent protein is a likely prerequisite for efficient crystal formation as it is well known, that protoxins in an improperly folded state are likely to be degraded by intracellular proteases.

Very frequently, *Bt* isolates contain multiple CryI proteins, while isolates producing multiple CryII or CryIII are rare. It was assumed that the ability to form heterologous crystals comprised of highly divergent protoxins is a property peculiar to the 130–140 kDa δ-endotoxins class. The C-terminal moiety of these protoxins participates in crystal formation, probably *via* the formation of intermolecular disulfide bonds, enabling proteins with diverse amino-terminal moiety to form a single crystal.

The smaller δ-endotoxins do not possess this conserved C-terminal domain and several studies indicate that some of them require assistance in crystal formation. Disruption of *orf2* in the *cryIIA* operon leads to a dramatic reduction in CryIIA production in *Bt*, and an absence of CryIIA inclusions (Crickmore and Ellar, 1992). The role of *orf2* in CryIIA crystal formation is further supported by the observation that the cryptic *cryIIB* gene can be activated to direct the production of CryIIB inclusions in *Bt* when cloned downstream of the *cryIIA-orf1-orf2* genes in an operon. The role of *orf2* on crystal formation may not be generalized, since its disruption did not show effects in the expression of *cryIIC* operon. Finally, disruption of *orf1* within the *cryIIA* operon had no obvious effect on CryIIA production; this result is intriguing since the *orf* has been identified as part of the *cryIIA, IIC, IVD* operons.

A similar phenomenon has been reported with CryIVD and CytA production in *Bti*. The *cytA* gene and *cryIVD* operon, located on a 75 MDa plasmid, are coordinately regulated, but are transcribed from divergent promoters. Expression of the *cytA* gene in *Escherichia coli* indicated that the 20 kDa protein encoded by the third *orf* in the *cryIVD* operon is required for efficient CytA production (Visick and Whiteley, 1991). Similarly, the 20 kDa protein was shown to enhance the production of CryIVA and CryIVD in *E. coli*. More recent studies demonstrated that the protein enhances also the production of CytA in an acrystalliferous *Bt* strain. Moreover, CytA expression in the absence of the 20 kDa protein is lethal for the cell. The related CytB is produced at significant levels in *E. coli* and forms visible inclusions without the aid of the 20 kDa protein, indicating that the putative role of the 20 kDa protein in facilitating crystallisation can not be generalized (Koni and Ellar, 1993).

The mechanism by which the *cryIIA orf2* and the *cryIVD* 20 kDa protein exert their effect on crystal formation are not well understood, although evidence suggests that the 20 kDa protein protects the nascent CytA peptide from proteolysis. Whether protecting the protoxin molecule from proteolysis, assisting in proper folding of the nascent protoxin molecule to allow its deposition in the crystalline inclusion, or serving as a scaffolding protein for crystal formation, an absence of function would result in increased proteolytic degradation of the protoxin and reduced protoxin yield. What is the most striking about these accessory proteins in their apparent degree of specialization? The presence of these chaperonin-like functions in *Bt* may reflect the importance of ensuring that potentially cytotoxic δ-endotoxins are sequestered in crystalline inclusions and are not allowed to interact with the plasma membrane of the bacterial cell.

Finally, the CryIII protein class represents a special case. Cloning a *cryIII* gene type on a multicopy plasmid is sufficient to direct a high level production of crystal protein and the formation of crystalline inclusions (De Souza et al., 1993). Then, the 28–32 kDa protein genes located downstream of these genes are not required for crystal formation and it was suggested that intermolecular interaction such as salt bridges are sufficient for CryIII crystal formation in *Bt.*

CONCLUSION

Bt produces a variety of δ-endotoxins which probably reflects its adaptability to colonize the ecological niches occupied by the insect larvae. Several mechanisms are used by the bacterium to produce the large amount of toxin needed to kill the larvae. Some of these mechanisms act at the transcriptional level. Additional events also influence the δ-endotoxin accumulation, such as the copy number, flanking elements that stabilize the mRNAs, the presence of other δ-endotoxins within the mother cell and accessory proteins that somehow facilitate the deposition of nascent protoxin molecules into crystalline inclusions. A better understanding of all these mechanisms will probably allow to optimize the construction of new strains producing specific toxins against key insect targets.

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