Simultaneous production of the 34-kDa and 40-kDa proteins from *Bacillus thuringiensis* subsp. *thompsoni* is required for the formation of inclusion bodies

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Abstract Cooperation of two crystal proteins from *Bacillus thuringiensis* subsp. *thompsoni*. strain HnC was shown to be essential for the formation of inclusion bodies. Expression of the operon containing the 34-kDa and 40-kDa protein genes from HnC in a *B. thuringiensis* crystal minus strain resulted in the formation of inclusion bodies identical to those from strain HnC. Interruption of one of the genes in the operon led to the lack of inclusion body and to low production of the remaining protein. Absence of inclusion body and low rate of protein production were also observed when both genes were simultaneously expressed but on different vectors. To show a cooperative effect in the formation of the inclusion body, both proteins must be produced from the same transcript.

Key words: *Bacillus thuringiensis* subsp. *thompsoni*; Crystal proteins; Protein expression; Operon

1. Introduction

Bacillus thuringiensis is a bacterium characterised by the production of a crystal-shape inclusion body or crystal containing specific insecticidal proteins or δ-endotoxins [1]. The final toxicity of *B. thuringiensis* δ-endotoxins is the result of a series of events involving solubilisation of the crystal, activation of the protoxins by gut proteases, recognition of a binding site on the brush border membrane and post-binding events such as channel formation and intracellular signalling [2,3]. Interactions between protoxins within the crystal are important for its solubility and therefore its stability [4].

Several kinds of crystal proteins have been described. Cryl proteins are representative of a group of protoxins in which the whole cysteine-rich C-terminal half plays no role in toxicity to insects and is suspected to be involved in the formation of the crystal through inter-chain disulphide bonds [1,5]. Other δ-endotoxins, the Cry2 proteins, are characterised by the lack of this cysteine-rich domain but can nevertheless make crystals. The *cry2Aa* gene is present in an operon containing also two open reading frames known as *orf-1* and *orf-2* [6], the latter coding for a molecular chaperone which is essential for the formation of the crystal [7,8].

We report here the existence of another mechanism of formation of inclusion body in *B. thuringiensis* in which the presence of two proteins acting cooperatively is necessary. The proteins of 34 kDa and 40 kDa from *B. thuringiensis* subsp. *thompsoni* [9–11] must be translated simultaneously from the transcript of a single operon to produce a pyramidal parasporal inclusion body. When the proteins are produced separately, no parasporal inclusion body formation is observed and the amount of crystal protein produced by the bacterium decreases considerably.

2. Materials and methods

2.1. Bacterial strains and plasmids

B. thuringiensis subsp. *thompsoni* strain HnC was isolated from the European sunflower moth [11]. The acrystalliferous strain Cry-B from *B. thuringiensis* subsp. *kurstaki* HD-1 was used as recipient strain for transformation. pAlter-1 (Promega) was used for site-directed mutagenesis. pHT3101 and pBU4 were used as *E. coli-B. thuringiensis* shuttle vectors [12,13]. pMAU-1 contains the 4-kb *cry2Aa* operon from NRD-12 [14].

2.2. Site-directed mutagenesis and PCR amplification

The 4-kb *Hin*dIII-*Bam*HI fragment containing the *cry2Aa* operon from NRD-12 [14] was subcloned into pAlter-1 and an *Eco*RV site was created at the initiation codon of the *cry2Aa* gene. The 34-kDa protein gene, the 40-kDa protein gene and the whole operon were amplified from total DNA from HnC while creating an *Eco*RV site immediately upstream from the start codon and a *Sma*I site immediately downstream from the stop codon. PCR was conducted using Vent DNA polymerase under standard conditions: denaturation; 94°C for 1 min, annealing; 42°C for 1 min; extension 72°C for 90 s. PCR products were cloned and verified by DNA sequencing.

2.3. Constructs and recombinant strains

The 34-kDa and 40-kDa protein genes were cloned either individually or as an operon in place of the *cry2Aa* gene in the *cry2Aa* operon containing both *orf-1* and *orf-2* to yield the clones p34-5, p40-5 and pOP-5, respectively. Transformation of a crystal-minus *B. thuringiensis* strain yielded the recombinant strains BT-34, BT-40 and BT-OP, respectively. A final construct, p-40Δ34 was prepared from pOP-5 in which the 34-kDa protein gene present in the native operon was interrupted as previously described [7] and was used to obtain the recombinant strain BT-40Δ34. BT-pHT bearing only the shuttle vector pHT-3101 was used as a control [13]. Constructs and recombinant strains are presented in Table 1.

2.4. Electroporation of Bacillus thuringiensis cells

Transformation was performed as described by Lereclus et al. [13]. Electroporated cells were plated onto solid BHI medium with erythromycin (25 μg/ml). The presence of the expected construct in the recombinant *B. thuringiensis* strains selected was confirmed by extraction of plasmid DNA from *B. thuringiensis* and transformation of *E. coli* with the extracted plasmid DNA. The recombinant *E. coli* strains were selected using the antibiotics used for screening recombinant *B. thuringiensis* strains (i.e. erythromycin or tetracycline depending on the shuttle vector). Plasmid DNA was extracted from *E. coli* and mapped with restriction enzymes. Maps were compared to those of similar constructs extracted from the original recombinant DH5-α and JM-101 *E. coli* strains obtained prior to electroporation of *B. thuringiensis*.

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2.5. Preparation of crude extracts and purified parasporal inclusions

To prepare crude extracts, a 5 ml liquid preculture was prepared on usual medium complemented with glucose [15] and 2.5 ml of this preculture, taken at the OD_{600} of 0.5, was inoculated in 125 ml culture of the same medium until complete lysis which was monitored by light microscopy. Both precultures and cultures were complemented with the relevant antibiotic and incubated at 28°C under vigorous shaking. Cultures were harvested at the same time and centrifuged to remove the liquid medium. The pellet was resuspended in 12.5 ml of sterile distilled water and stored at −80°C. Aliquots of equal volume are taken from this stock for SDS-PAGE analysis. Parasporal inclusion bodies were purified as previously described [11] using a discontinuous NaBr gradient [16]. Protein concentration was determined using the bicinconinic acid method (PIERCE, MicroBCA protein kit).

2.6. Analysis of proteins by SDS-PAGE and immunoblotting

Sporulated cultures or purified inclusion bodies samples were separated on 12% polyacrylamide gels under standard conditions and stained with Coomassie Blue R-250. Proteins were transferred onto PVDF membranes (Millipore Immobilon-P membrane) and probed with rabbit polyclonal antibodies raised against solubilised crystals from HnC using the protoblot kit from Promega. Densitometric analysis was performed on samples separated on polyacrylamide gels. Gels were scanned using the software Adobe Photoshop and bands intensity as well as protein profiles were determined with Optilab 2.6 (Graphtec). A protein band of ca. 25 kDa was used as an internal marker of protein concentration. Statistics and plotting were performed with Microsoft Excel 4.0. Data were tested by Student's *t*-test.

3. Results

3.1. Operon expression and individual expression in Bacillus thuringiensis of the 34-kDa and 40-kDa protein genes

SDS-PAGE analysis of crude extracts from BT-pHT, BT-34 and BT-40, which are respectively the control strain, the strain producing only the 34-kDa protein and that producing only the 40-kDa protein, did not reveal any major band or clearly visible bands of the expected size (Fig. 1A, lanes 3 and 4) whereas analysis of crude extracts at similar concentration from HnC, the natural isolate of *B. thuringiensis* subsp. *thompsoni*, showed two major bands of 34 kDa and 40 kDa (Fig. 1B, lane 1). The proteins of 34 kDa and 40 kDa were detected in crude extracts from BT-34 and BT-40, respectively, by polyclonal antibodies raised against HnC solubilised crystal proteins, indicating that although not produced as major proteins, the expected individual gene products were effectively present (Fig. 1C, lanes 2 and 3). To determine the influence of gene deletions on the rate of production of proteins a SDS-PAGE analysis was conducted on crude extracts from HnC and from three independent colonies from BT-34 and from BT-40, respectively (Fig. 1B). A band of ca. 25 kDa, unrelated to either the 34-kDa or 40-kDa protein as shown by

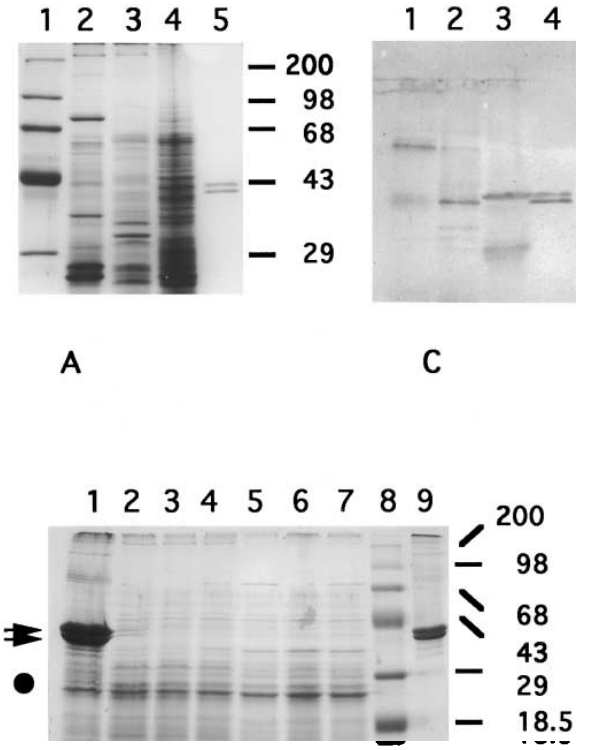

Fig. 1. Electrophoretic analysis of crude extracts from *B. thuringiensis* transformants. (A) Coomassie blue-stained SDS-polyacrylamide gel. Lane 1, molecular mass standards. Lanes 2–4: crude extracts of BT-pHT (lane 2), BT-34 (lane 3), BT-40 (lane 4); lane 5, purified inclusion bodies from HnC. (B) Comparative protein production by recombinant and native strains. Coomassie blue-stained SDS-polyacrylamide gel. Lane 1, crude extract from HnC; lanes 2, 3 and 4, crude extract from three distinct colonies of BT-40; lanes 5, 6 and 7, crude extract from three distinct colonies of BT-34; lane 8, molecular mass standard; lane 9, crude extract from HnC diluted 15 times. Identical amounts of samples were loaded in lanes 1, 2, 3, 4, 5, 6 and 7. The 34-kDa and 40-kDa proteins are marked by arrows whereas the ca. 25-kDa protein used as concentration marker is marked by a dot. (C) Immunodetection of proteins showed in Fig. 1A with antibodies raised against solubilised inclusions from HnC. Lanes 1–3: crude extracts from BT-pHT (lane 1), BT-34 (lane 2), BT-40 (lane 3), lane 4, purified crystals from HnC. To allow clear photographic reproduction and to avoid discrepancy due to difference in the rate of protein production (i.e. overloading or underloading) samples were not loaded at the same concentration. This Western blot analysis therefore reflects the identity of the proteins produced by the recombinant *B. thuringiensis* strains (qualitative analysis) and not the rate of protein production (quantitative analysis).

Table 1
Bacterial strains and constructs

Strains	Constructs	Genes present in the strain[a]	Proteins produced[b]
HnC	None (parental strain)	34-kDa and 40-kDa protein genes	34-kDa and 40-kDa proteins
BT-OP	pOP-5	34-kDa and 40-kDa protein genes	34-kDa and 40-kDa proteins
BT-34	p34-5	34-kDa protein gene only	34-kDa protein
BT-40	p40-5	44-kDa protein gene only	40-kDa protein
BT-40Δ34	p40Δ34-5	40-kDa protein gene 34-kDa protein gene interrupted	40-kDa protein
BT-3440	p40-5 pBU-34	40-kDa protein gene 34-kDa protein gene (genes borne by separate vectors)	34-kDa and 40-kDa proteins
BT-pHT	pHT3101 (vector)	None	None

[a]Only the crystal protein genes are considered.
[b]Only the crystal proteins are considered.

Table 2
Densitometric analysis of the protein contents of crude cell extracts

	Lane[a]							
	1	2	3	4	5	6	7	9
Window A[b]	164	100	86	80	73	75	76	106
Window B[c,d]	124	137	134	128	115	129	122	58
Window A/Window B[e]	1.32	0.73	0.64	0.62	0.63	0.58	0.62	1.82

[a]Numbers correspond to the lanes of the polyacrylamide gel presented in Fig. 1B.
[b]Window A was designed to cover the surface occupied by the doublet comprising the 34-kDa and the 40-kDa proteins in lane 1. The same window (i.e. identical in size) was considered in other lanes at similar position. Data represent the bands intensity and are given in arbitrary units.
[c]Window B has the same size as window A and was centred on the 25-kDa band marked by a dot in Fig. 2B. Data represent the bands intensity and are given in arbitrary units.
[d]Except for lane 9 in which the sample was diluted 15 times, individual data reflecting the intensity of window B (25-kDa protein) were tested by Student's *t*-test and no runaway data was noted. Data on lanes 1–7: mean 127, S.E. 2.81, S.D. 7.44.
[e]Data on lanes 2–7: mean 0.64, S.E. 0.02, S.D. 0.05.

western blot analysis, was used as an internal marker of protein concentration and had a similar intensity in lanes 1 to 7. This indicated that equivalent amounts of samples have been loaded (Fig. 1B, Table 2). The absence of this band in lane 9 is a consequence of the 15 fold dilution of the sample (Fig. 1B). Although, equivalent amounts of cultures were loaded, the two major proteins of 34 kDa and 40 kDa were clearly visible only in lanes 1 and 9, the latter being a 15 fold dilution of the sample loaded in lane 1 (Fig. 1B). No major protein of the expected size could be detected in lanes 2 to 4 and lanes 5 to 7 which correspond to three independent colonies of BT-34 and BT-40, respectively (Fig. 1B). In lane 1, loading a similar amount of sample as in lanes 2 to 7 led to an overloading and a well to well contamination visible in lane 2 (Fig. 1B). Densitometric analysis of the gel presented in Fig. 1B, confirmed that samples 1 to 7 were loaded at similar concentrations since the intensity of the 25-kDa band is very similar from one lane to another and no significant variation was noted (Fig. 2, Table 2). Individual points were plotted on a chart for lanes 1, 6 and 9 and the intensity of the 25-kDa band as well as the shape of the peak on the chart are similar for lanes 1 and 6 whereas the intensity of the peaks representing the 34-kDa and 40-kDa varies greatly between lane 1 and lane 6 (Fig. 2A and B). In the chart representing lane 1, the peaks corresponding to the two crystal proteins are clearly visible and flattened at the top indicating that the proteins are at saturation in the gel (Fig. 2A). In contrast, there is no detection of these two bands in the plot chart of lane 6 (Fig. 2B) although the 25-kDa marker band is as intense as in the representation of lane 1. Chart representation of lane 9 shows the absence of a peak for the 25-kDa band when the sample is diluted 15 times whereas Peaks corresponding the 34-kDa and 40-kDa proteins are however clearly visible (Fig. 2C). Although diluted 15 times, the proteins are probably still at saturation since the peaks are at the maximum intensity (Fig. 2C). These data clearly indicate that the low concentration of the 34-kDa and 40-kDa proteins detected in lanes 2 to 7 is not related to a difference in sample concentration but to a reduced production of crystal proteins when either one of the crystal protein genes is interrupted. Quantitative analysis of these samples shows that the ratio between the intensity the group comprising the two crystal proteins (window A) and that of the 25-kDa protein (window B) is equivalent in lanes 2 to 7 with a mean value of 0.64 and, as expected, is different in lanes 1 and 9 (Table 2). This ratio is also different between lanes 1 and 9, with respective values of 1.32 and 1.82, which indicates that the proteins are indeed at saturation (Table 2). The difference of protein production between HnC and the single-gene recombinants (BT-34 and BT-40) is at least 45 times, based on the densitometric analysis (Table 2). However, because of the saturation observed in lanes 1 and 9, this difference in production is probably underestimated.

3.2. *Cooperative effect of the proteins for the formation of parasporal inclusion bodies*

To test whether the two crystal proteins from HnC were required for the formation of an inclusion body, the 34-kDa protein gene which is present at the second position in the operon was truncated by removing its 3′ moiety. The recombinant strain BT-40Δ34 containing a truncated operon in which a large part of the 34-kDa protein gene has been interrupted did not produce any pyramidal parasporal inclusion body. Crude extracts from strain BT-40Δ34 were analysed by SDS-PAGE and although purposely overloaded, no band of 40 kDa as thick as that from the parental strain could be detected (Fig. 3A, lane 4). BT-OP, the recombinant strain bearing the whole native operon, produced, in similar amounts, inclusion bodies equivalent in size and shape to those from the parental strain HnC [11] which can be easily purified on NaBr gradients (Fig. 3A, lanes 1 and 2). Immunodetection after protein transfer showed clearly the presence of only the 40-kDa protein in strain BT-40Δ34 (Fig. 3B, lane 4) whereas both crystal proteins were present in strains Hnc and BT-OP (Fig. 3B, lanes 1 and 2). The construct containing only the 34-kDa protein gene originally present in strain BT-34, which produced only the 34-kDa protein, was transferred to the tetracycline-resistant shuttle vector pBU4 [12] to yield the plasmid pBU-34. Transformation of strain BT-40, the erythromycin-resistant recombinant strain producing only the 40-kDa protein, with pBU-34 (tetracycline resistant) yielded the recombinant strain BT-3440 resistant to both tetracycline and erythromycin which was monitored during sporulation by phase contrast light microscopy. The presence of both constructs in BT-3440 was determined by transforming *E. coli* cells with plasmid DNA extracted from BT-3440. Separate batches of *E. coli* strains were selected on erythromycin or on tetracycline. Plasmid DNA extracted from *E. coli* strains growing on antibiotics was analysed by physical mapping. Plasmids from *E. coli* strains growing on erythromycin yielded patterns identical to that from p40Δ34 whereas plasmids from strains growing on tetracycline yielded patterns identical to those from pBU-34 indicating that both plasmids were present in strain BT-3440 (data not shown). As previously described for strains BT-40Δ34, BT-34 and BT-40, no

pyramidal inclusion bodies could be detected in strain BT-3440. Crude extracts from strain BT-3440 were analysed by SDS-PAGE and although loaded purposely at a higher concentration than those from strain BT-OP, no protein of 34 kDa or 40 kDa were visible in BT-3440 (Fig. 3A, lane 3), whereas they were clearly detectable as major proteins in BT-OP, indicating a difference in the rate of production of these 34-kDa and 40-kDa proteins (Fig. 3A, lane 2). Immunodetection using polyclonal antibodies raised against HnC crystal proteins clearly revealed however that the two expected proteins were actually present in BT-3440 (Fig. 3B, lane 3) confirming that the crystal protein genes present in the two shuttle vectors were both functional and that the proteins were actually produced but at a low rate.

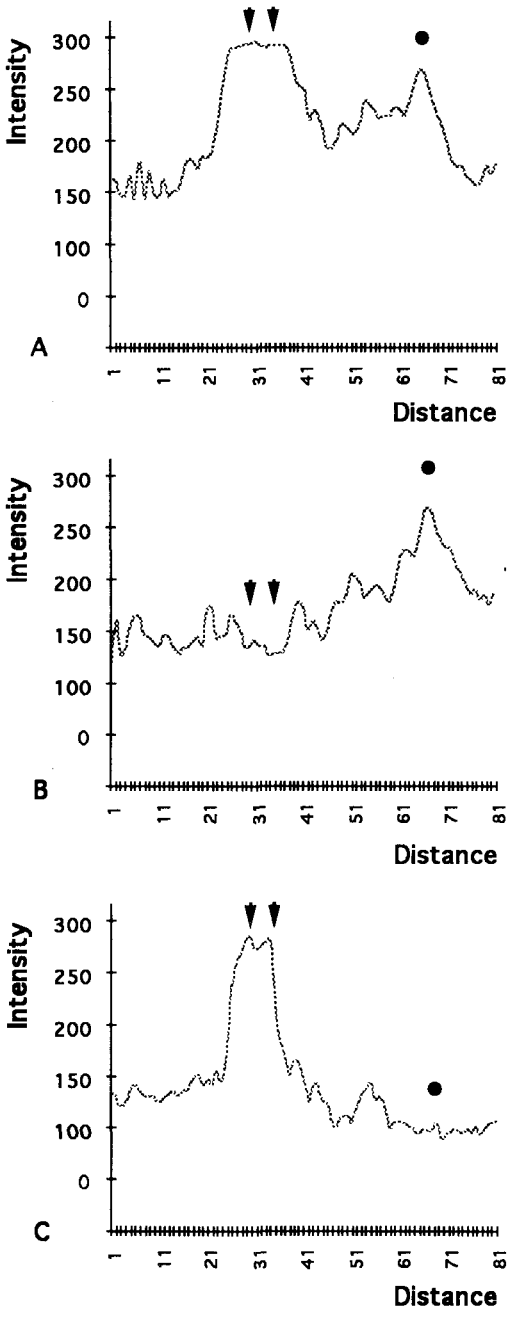

Fig. 2. Densitometric analysis of the protein contents of crude cell extracts: (A) HnC; (B) BT-34; (C) HnC diluted 15 times. The plots correspond to lanes 1, 6 and 9, respectively, in Fig. 1B. The *x*-axis represent the distance of migration in the gel in millimetres whereas the *y*-axis represents the intensity of the bands shown in arbitrary units. Peaks representing the 34-kDa and 40-kDa proteins are marked with arrows and the peak representing the ca. 25-kDa protein used is marked with a dot.

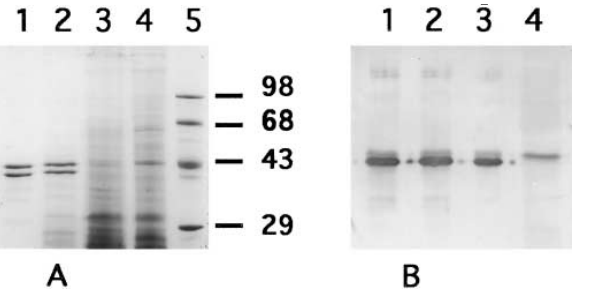

Fig. 3. Analysis of the cooperative effect of the 34-kDa and 40-kDa proteins. (A) SDS-PAGE analysis of crude extracts from BT-OP (lane 2), BT-3440 (lane 3), BT-40Δ34 (lane 4), purified inclusion bodies from HnC (lane 1), molecular mass standards (lane 5). Samples in lanes 3 and 4 were purposely loaded at a higher concentration than sample in lane 2 to facilitate the detection of any potential band of 34 kDa or 40 kDa. (B) Immunodetection with antibodies raised against solubilised crystals from HnC. Lane 1, purified inclusion bodies from HnC. Lanes 2–4: crude extracts from BT-OP (lane 2), BT-3440 (lane 3) and BT-40Δ34 (lane 4). The lower intensity of the 40-kDa protein following immunodectection is related to the frequently-noticed low transfer efficiency of this protein. As described in Fig. 1C, samples were not loaded at the same concentration to compensate the difference in the rate of production and to allow a good photographic reproduction.

4. Discussion

These results show that the pyramidal inclusion body found in HnC, containing the two proteins of 34 kDa and 40 kDa, is the result of an obligate interaction between these two proteins. These proteins were found to be identical, according to the nucleotide sequences of the genes, to those previously described [9]. Although the 34-kDa protein was shown to be active against *Manduca sexta*, the 40-kDa protein showed no toxicity and it was suggested that it could act as a molecular chaperone and facilitate the crystallisation of the 34-kDa protein [9]. The lack of formation of inclusion body when the 34-kDa or the 40-kDa protein genes are expressed individually in different strains or in the same bacterium as well as when one of the gene is interrupted in the operon, suggests that the presence of both crystal proteins is essential for the formation of the inclusion body. Densitometric analysis of the protein contents of crude extracts confirms that when either one of the proteins is missing, the production rate drops drastically even though the remaining protein is still produced and detectable by immunodetection. The translation from a single messenger appears to be essential to ensure a high rate of production and to obtain the proper structure for crystal formation, suggesting an obligate interaction between the two proteins from the early phase of translation. Inclusion bodies from HnC were previously shown to be homogeneous in structure as seen using transmission electron microscopy [11], indicating that the crystal proteins are probably equally involved in the formation of a protein lattice and proportionally distributed throughout the inclusion body. This ultrastructural homogeneity would confirm an early interaction of the proteins for the formation of the inclusion body. The

mechanisms involved in this process are still unknown and further research is also needed to determine whether the low rate of production of proteins when the genes are expressed individually or in trans-complementation is due to degradation by proteases, lower stability of the proteins or to a feedback control of the gene expression.

These results suggest the existence in *B. thuringiensis* of a mechanism of formation of parasporal inclusion bodies different from those involving inter-chain disulphide bonds between cysteine-rich domains (i.e. Cryl proteins) and action of a non-crystal protein (i.e. Cry2 proteins). The 34-kDa and 40-kDa proteins from HnC may represent a mechanism of formation of inclusion body in *B. thuringiensis* where the formation of this parasporal body is the result of an obligate and early interaction of two crystal proteins synthesised from the transcription product of a single operon. One of these crystal proteins could be a helper protein only involved in the formation of the inclusion body at a 1:1 ratio with the other crystal protein and without trans-complementation. A similar phenomenon involving a cooperative relationship between two crystal proteins for both crystal formation and toxicity has been reported in *Bacillus sphaericus* [17] which shares traits with what is reported here on the crystal proteins from HnC. The two crystal proteins of 41 kDa and 52 kDa produced by the mosquitocidal bacterium *B. sphaericus* were shown to work synergistically to make a typical crystal inclusion [17]. When produced separately, the proteins made small-size amorphous inclusion bodies. Another similarity with the crystal proteins from *B. thuringiensis* subsp. *thompsoni* is that the 42-kDa protein is insecticidal, although the LC50 is about 160 fold higher than that of the native purified crystal proteins, whereas the 51-kDa protein has no insecticidal activity [17]. Similarly, Brown and Whiteley [9], reported that the protein of 34 kDa showed activity against *Manduca sexta* whereas the 40-kDa protein did not.

The crystal structure seems to be an important feature in the overall process of toxicity [4,18] and *B. thuringiensis* may have developed different strategies for producing crystals. An evolutionary tree created on the basis of amino acids sequence homologies between different *B. thuringiensis* crystal proteins showed that the group made of the Cry2 proteins and Cry11A, both characterised by the presence of the corresponding genes in association with a molecular chaperone in the same operon, was separated very early from the group comprising the Cryl proteins, Cry3, Cry4A and Cry4B proteins [19,20]. The 34-kDa and 40-kDa protein genes are present in an operon but no other open reading frame was found in this same operon, the crystal proteins cooperate for the formation of the inclusion body and they share no homology with other crystal proteins. Finally, *cry15A* (34-kDa protein gene) makes a separate group [19], which associates this cooperative mechanism of formation of inclusion body to a different group of genes. A question which could therefore be raised is whether the different *B. thuringiensis* toxin genes are coming from a common ancestral gene or different types of organisations and gene structures were developed and evolved independently.

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