Comparative analysis of the individual protoxin components in P1 crystals of *Bacillus thuringiensis* subsp. *kurstaki* isolates NRD-12 and HD-1

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Two commercially important strains (NRD-12 and HD-1) of the entomopathogenic bacterium *Bacillus thuringiensis* subsp. *kurstaki* each contain three genes of partially identical sequence coding for three classes of 130–135 kDa protoxins (termed the 4.5, 5.3 and 6.6 protoxins) that display toxicity towards various lepidopteran larvae. These gene products combine to form the intracellular bipyramidal P1 crystal. Each of the genes from both strains was cloned and expressed in *Escherichia coli*. Analysis of the cloned genes at the restriction-endonuclease level revealed no detectable differences among genes within a particular gene class. The composition of the P1 crystal from both strains was quantitatively analysed by CNBr cleavage of the purified P1 crystal, with the purified recombinant gene products as reference proteins. Independent verification of the presence of high 6.6-protoxin gene product in the P1 crystal was provided by a rapid *in vitro* lawn cell toxicity assay directed against a *Choristoneura fumiferana* (CF-1) insect cell line. The results indicate that, although all three gene products are represented within the P1 crystal of either NRD-12 or HD-1, only the contents of the 4.5 and 5.3 protoxins vary between the two crystals, whereas the 6.6 protoxin contents are similar and represent approximately one-third of the P1 crystal in either strain.

INTRODUCTION

One of the most interesting features of the bacterium *Bacillus thuringiensis* is its ability to elaborate a large proteinaceous intracellular crystal during sporulation. These parasporal crystalline inclusions, when isolated from different strains among the more than 22 known serotypes, display toxicity towards larvae from the insect Orders Lepidoptera, Diptera and Coleoptera [for reviews see Aronson *et al.* (1986), Whiteley & Schnepf (1986) and Andrews *et al.* (1987)]. Furthermore, within an insect Order, isolates from a single serotype may exhibit drastically different spectra of entomopathological activity (Jaquet *et al.*, 1987). The cloning and sequencing of a number of different toxin genes has served to highlight the complexity of the crystals with respect to their compositions and diverse insecticidal activities (Adang *et al.*, 1985; Schnepf *et al.*, 1985; Adang *et al.*, 1987; Hefford *et al.*, 1987; Brousseau & Masson, 1989). The potential for having more than one protein within a crystal presents difficulties in establishing whether the biological activity of newly discovered *B. thuringiensis* isolates are a result of novel toxin genes, different combinations or levels of expression of known toxin genes, or potential synergistic/antagonistic effects among the different toxin combinations. This problem is further illustrated by reports appearing in the literature on two commercial *B. thuringiensis* subsp. *kurstaki* strains, NRD-12 and HD-1. At the genetic level these two strains appear to be similar, each possessing the three classes of previously characterized lepidopteran-specific genes termed 4.5, 5.3 and 6.6, or as recently suggested cryIA(a), cryIA(b) and cryIA(c) (Höfte & Whiteley, 1989), whose 130–135 kDa gene products combine to create the P1 crystal (Kronstad & Whiteley, 1986; Préfontaine *et al.*, 1987). However, the NRD-12 strain has been shown to possess a different toxicity spectrum from that of strain HD-1, being 2–3.5-fold more toxic to the spruce budworm and the gypsy moth as well as dem-

onstrating a faster kill for the gypsy moth (Dubois, 1983, 1984, 1985). It remains unclear as to whether these differences can be attributed to subtle differences in the crystal protein genes at the nucleotide level or alternatively to differences in expression among the three genes. One approach to resolve these differences would be to clone and express the individual genes in order to carry out a comparative analysis of their toxicity spectra within a particular gene class. Another approach would be to determine the contents of each of these gene products within the crystal itself. The present paper deals with the latter approach, using both chemical and biological techniques to determine and quantify the contents of purified HD-1 and NRD-12 P1 crystals by comparison with the individually cloned lepidopteran-specific gene products from both strains as reference proteins.

EXPERIMENTAL

Bacterial strains, plasmids and media

B. thuringiensis subsp. *kurstaki* strains HD-1 and NRD-12 were obtained from the Forest Pest Management Institute (Sault Ste. Marie, Ont., Canada). *Escherichia coli* strains HB101 and MC1061 were the host bacteria for the pUC- or pING-based vectors respectively. The plasmid pING2, in which an *Nde*I linker was inserted into the unique *Sma*I site of the pING1 plasmid polylinker (Johnson *et al.*, 1985), was kindly given by INGENE (Santa Monica, CA, U.S.A.). The two pUC-based vectors pK52 and pK36, which express the 4.5-protoxin and the 5.3-protoxin gene products of strain HD-1, were kindly provided by Dr. M. Geiser (CIBA-GEIGY, Basle, Switzerland). All bacteria were grown at 37 °C in double-strength YT broth or on plates containing ampicillin at 100 µg/ml.

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Gene isolation

Total genomic DNA was prepared from HD-1 and NRD-12 by the method of Kronstad *et al.* (1983). The genomic DNA was restricted with *NdeI* followed by size fractionation on a discontinuous 5–25% (w/v) sucrose gradient in 10 mM-Tris/HCl buffer, pH 7.4, containing 0.8 M-NaCl and 8 mM-EDTA. The gradients were centrifuged at 200 000 *g* in a Ti 50.1 rotor for 5 h, and the appropriate fractions were dialysed against 10 mM-Tris/HCl buffer, pH 8, containing 1 mM-EDTA and ligated into *NdeI* linearized pING2. Transformed *E. coli* HB101 colonies were hybridized (Grunstein & Hogness, 1975) with oligonucleotide probes designed to identify the 4.5-prototoxin, 5.3-prototoxin and 6.6-prototoxin genes from both strains as described previously (Préfontaine *et al.*, 1987). In order to express the identified gene products, the plasmids carrying the prototoxin genes were sub-cloned into pMPCV by *NdeI* digestion and re-ligation (Fig. 1). The pMPCV plasmid is a cassette vector created by *NdeI* digestion and re-ligation of the *HpaI*–*PstI*-digested cloned NRD-12 5.3-prototoxin gene in pUC18 (the unique pUC18 *NdeI* site was destroyed by end-filling of the 3'-recessed ends), thus creating a smaller 3 kbp cloning vehicle. This vector contains part (76 bp) of the NRD-12 5.3-prototoxin gene promoter sequence and 308 bp of the 5.3-prototoxin gene 3' non-coding sequences. All DNA manipulations were performed according to established techniques (Maniatis *et al.*, 1982).

Protein purification

E. coli cells containing the various crystal-protein genes were grown in 1.5 litres of L-broth containing 100 µg of ampicillin/ml for 2 days at 37 °C. This long incubation period resulted in a 2–3-fold increase in the prototoxin concentration observed after only 24 h of growth. An additional 100 µg of ampicillin/ml was added to the culture after the first 24 h of incubation. The cells were harvested, washed once in 0.1 M-sodium phosphate buffer, pH 6.0, and resuspended in 15 ml of the same buffer. The suspension was passed twice through a French pressure cell at 11 000 lbf/in² (76 MPa) internal pressure and the insoluble material was collected by centrifugation at 7500 *g* for 15 min. This material was washed once in 0.1 M-sodium phosphate buffer, pH 6.0, containing 0.1 M-NaCl and resuspended in distilled water to produce a milky colloidal solution. Renografin-76, a mixture of meglumine diatrizoate (66%) and sodium diatrizoate (10%) normally used for urography (Squibb, Montreal, Que., Canada), can be utilized to produce low-capacity density gradients capable of separating whole cells and cellular debris from insoluble inclusion bodies. The colloidal suspension was mixed 1:1.8 with Renografin and centrifuged at 30 000 *g* in an SW28 rotor for 1 h. The top gelatinous layer was discarded and the supernatant was carefully decanted from the bottom pellet. After dilution of the supernatant with 2 vol. of distilled water, the insoluble prototoxin inclusion bodies were harvested by centrifugation at 10 000 *g* for 15 min. The white inclusion-body pellet was washed extensively with water to remove all traces of renografin. This method routinely yielded between 30 and 100 mg of 90–95%-pure prototoxin. HD-1 and NRD-12 crystal preparations, a gift from Tim Lessard (National Research Council, Ottawa, Ont., Canada), were highly purified as described elsewhere (Carey *et al.*, 1986). These crystals were purified from batch-fermentation cultures of both strains grown under identical conditions. All protein concentrations were determined by the protein-dye method of Bradford (1976), with BSA as a standard.

Protein and immunological analysis

Prototoxins and their CNBr-cleaved derivatives were analysed by SDS/PAGE in 10% (w/v) polyacrylamide gels, which were

subsequently stained with Coomassie Brilliant Blue. The destained gels containing the cleaved peptides were dried between single sheets of clear wetted cellophane and scanned with an LKB Ultrosan XL laser densitometer possessing an internal digital integrator to calculate peak areas. Unstained gels containing the *E. coli*-produced recombinant proteins were electroblotted on to nitrocellulose. The protoxins were located by using rabbit antibodies directed against purified HD-1 crystals followed by staining with horseradish peroxidase linked to Protein A (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and detected with 2-chloro-1-naphthol.

CNBr cleavage

Prototoxins from both inclusion bodies or crystals were solubilized in 50 mM-Na₂CO₃/HCl buffer, pH 9.5, containing 10 mM-2-mercaptoethanol (CM buffer) at 37 °C for 1 h. This buffer completely and selectively solubilizes the 130 kDa protoxins in the HD-1 or NRD-12 crystals, thus separating them from the insoluble 65 kDa mosquitocidal factor P2 crystal, which is subsequently removed by centrifugation for 10 min (Huber *et al.*, 1981). The proteins were either washed and concentrated in a P-30 Centricon concentrator (Amicon) followed by freeze-drying to remove the 2-mercaptoethanol or precipitated in ice-cold 90% (v/v) acetone. The proteins were harvested by centrifugation after incubation for 1 h at –20 °C and subsequently dried under vacuum. The freeze-dried proteins were then solubilized in 70% (v/v) formic acid, and CNBr was added to a final concentration equivalent to a 500-fold excess over the total methionine concentration of the protein or protein mixture to be cleaved. After incubation for 24–30 h at 30 °C, the cleaved peptides were diluted 10-fold in distilled water and freeze-dried. The peptides were then resuspended in water and freeze-dried two additional times to ensure removal of trace levels of formic acid. The peptides were finally resuspended in protein SDS-sample buffer, boiled for 5 min and analysed by SDS/PAGE in a discontinuous 10% (w/v) polyacrylamide gel (Laemmli, 1970).

Insect cells and toxicity assays

Choristoneura fumiferana (CF-1) cells (Lepidoptera, spruce budworm, trypsin-treated larval tissue), obtained from Dr. S. Sohi (Forest Pest Management Institute), were grown in Grace's medium (GIBCO) containing 0.25% tryptose and 10% (v/v) fetal bovine serum. Cells were grown in spinner flasks at 28 °C to a final concentration of 1×10^6 – 2×10^6 cells/ml. Activated toxins were prepared by solubilizing *B. thuringiensis* crystals or *E. coli*-produced recombinant protoxins in CM buffer followed by an overnight incubation with 1% (w/v) *Bombyx mori* gut juice [diluted in 0.2 M-Caps (3-cyclohexylamino-1-propanesulphonic acid)/NaOH buffer, pH 10.5, containing 0.2% dithiothreitol]. To assay for toxicity of the crystals and recombinant gene products *in vitro*, we essentially followed the method developed by Dr. L. Gringorten (Forest Pest Management Institute). Briefly, this method consists of washing the cultured CF-1 insect cells (2×10^8 cells) twice in 0.01 M-Mops/NaOH buffer, pH 7.0, containing 0.15 M-NaCl and resuspended in 4 ml of the same buffer. The cells were then mixed with 4 ml of 2% (w/v) SeaPlaque agarose (FMC Corp., Rockland, ME, U.S.A.) at 37 °C and poured into the cover of a 96-well micro-titre plate containing a base of 1% (w/v) SeaKem agarose, and the mixture was left to solidify for 30 min at room temperature to prepare a lawn of insect cells. Twofold dilutions of the activated toxins in dithiothreitol/Caps buffer were spotted as 1 µl portions on to the surface of the insect-cell lawn and allowed to incubate for 1 h at room temperature. The plate was then stained for 10 min with 20 ml of 0.2% Trypan Blue in NaCl/Mops buffer, and destained

for 60 min in NaCl/Mops buffer followed by several hours in 1.34% (w/v) KCl. The toxicity thresholds, defined as the lowest amount of activated toxin in the dilution series creating a visible 'lytic spot', were then determined.

RESULTS

Cloning and expression of the crystal protein genes

In order to analyse the levels of gene expression of the individual crystal components for the strains NRD-12 and HD-1, it was necessary to clone each of the three classes of lepidopteran-specific genes (i.e. the 4.5-prototoxin, 5.3-prototoxin and 6.6-prototoxin genes) from both strains. These recombinant protoxins could then be used to determine any comparative differences in their respective CNBr-cleavage patterns on a gene-class basis as well as for eventual use as reference proteins in studying the cleavage pattern obtained with the crystals. The

choice of the use of CNBr was based on computer predictions using sequenced 4.5-prototoxin, 5.3-prototoxin and 6.6-prototoxin genes, which indicated that cleavage of the individual protoxins should result in the production of unique cleavage patterns.

For cloning, *Nde*I digests of total genomic DNA from both strains were fractionated on a sucrose gradient, and the fractions containing the 3–5.5 kbp range of fragments were ligated into *Nde*I-linearized pING2 (Fig. 1) and the individual protoxin genes were identified by specific oligonucleotide probes (Préfontaine *et al.*, 1987). Since the genes expressed poorly in the pING2 plasmid, an expression cassette vector (called pMPCV) was created as described in the Experimental section. All other genes were subcloned as *Nde*I fragments into this vector in the proper orientation (i.e. so that the promoters are reconstructed), thus producing the protoxin-expressing plasmids pMP39 (NRD-12 4.5-prototoxin gene), pMP37 (NRD-12 6.6-prototoxin gene) and pMP46 (HD-1 6.6-prototoxin gene). An extensive restriction-endonuclease analysis was performed on each of the three genes from both strains, but no differences were detected when comparing both genes with each gene class (results not shown). These plasmids were examined for their ability to express the protoxin in *E. coli*. Fig. 2(a) shows the levels of expression of the three NRD-12 genes as well as the HD-1 6.6-prototoxin in *E. coli*. Proteins ranging in size from 130 to 135 kDa appeared in total protein extracts of *E. coli* strains possessing the recombinant plasmids, but not in non-transformed *E. coli*. The authenticity of these proteins was verified by Western-blot analysis, as shown in Fig. 2(b), with polyclonal antisera raised against purified crystals from strain HD-1. The HD-1 4.5-prototoxin and 5.3-prototoxin gene products were not included here, since they have been characterized previously (Schnepf *et al.*, 1985; Geiser *et al.*, 1986). Inclusion bodies were purified from the six recombinant strains by a rapid-lysis density-gradient procedure, which resulted in high yields of the protoxin at purity levels exceeding 90%, as verified by laser densitometry scanning of dried SDS/PAGE gels. All the recombinant gene products demonstrated toxicity to various lepidopteran larvae *in vivo* (*Lymantria dispar* L. and *Bombyx mori*; results not shown).

Crystal analysis

In order to cleave the protoxins chemically with CNBr, pure preparations of the recombinant protoxins and the protoxins present in the P1 crystals are prerequisites. Selective solubilization in CM buffer resulted in pure preparations of the protoxins from the three gene classes of strains NRD-12 and HD-1, since any contaminating proteins in the inclusion-body preparations remained insoluble and were removed by centrifugation. Furthermore, all the 130–135 kDa protoxins in the P1 crystals were completely solubilized and separated from the P2 mosquitocidal factor, as judged by SDS/PAGE. A typical CNBr-cleavage peptide pattern of solubilized crystals from strains NRD-12 and HD-1, with the three NRD-12 gene products as reference proteins, is shown in Fig. 3. It may be noted that each of the CNBr-cleavage products from each gene class of strain NRD-12 was identical with its HD-1-strain counterpart (results not shown). For each reference protoxin we have assigned a signature peptide (indicated by arrowheads in Fig. 3), which is defined as the largest peptide produced by CNBr cleavage. Selection of the largest CNBr-generated peptide served a dual purpose. The molecular masses of these peptides have been calculated as 53.7, 39.6 and 42.6 kDa for the 6.6-prototoxin, 5.3-prototoxin and 4.5-prototoxin gene products as summarized in Table 1. Thus not only are the three signature peptides readily identified on the basis of their different molecular masses, but by selecting the largest peptide the completeness of the cleavage reaction can be monitored simply by determining the presence of peptide bands

Fig. 1. Cloning of lepidopteran-specific parasporal crystal toxin genes from *B. thuringiensis* strains NRD-12 and HD-1

All *B. thuringiensis* toxin genes were isolated from total genomic DNA as *Nde*I fragments cloned in the *Nde*I-linearized arabinose-inducible expression plasmid pING2 to create the pBT series of plasmids. To enhance expression of the protoxin, the genes were subcloned into pMPCV containing both the 5' and 3' non-coding sequences of the NRD-12 5.3-prototoxin gene as described in the text to produce the pMP series of expression plasmids.

Fig. 2. Expression of the cloned protoxin *B. thuringiensis* genes in *E. coli*

Total protein extracts from cells harbouring the expression plasmids were separated by SDS/PAGE and stained with Coomassie Brilliant Blue (a) or immunoblotted with polyclonal antisera directed towards purified HD-1 crystals. The control lane represents purified inclusion-body 4.5 protoxin from strain HD-1.

Fig. 3. Cleavage of the purified recombinant protoxins and crystals from *B. thuringiensis* strains NRD-12 and HD-1

The CNBr-cleaved peptides from each NRD-12 gene class and from P1 crystals of both strains NRD-12 and HD-1 were analysed by SDS/PAGE. The arrowheads indicate the signature peptides from the cloned gene products used to quantify the extents of gene expression in the *B. thuringiensis* NRD-12 or HD-1 P1 crystal lanes.

Table 1. Summary of predicted sizes of *B. thuringiensis* protein signature peptides after CNBr cleavage

The signature-peptide region indicated in each case represents the terminal amino acid positions of the largest peptide generated after CNBr cleavage (Met-Xaa) of the protoxin.

Gene class	Signature-peptide region (Xaa-Yaa)	Expected molecular mass (kDa)
4.5	555-927	42.6
5.3	556-902	39.6
6.6	453-927	53.7

of a larger molecular mass than the signature peptide. Any cleavage reactions displaying such large peptides were discarded. Moreover, the presence of any incompletely cleaved low-molecular-mass bands could also be ignored, as they would have no bearing on the final determinations of signature-peptide

Fig. 4. Densitometry scan of the peptide cleavage products of *B. thuringiensis* protoxins

This Figure represents a typical gel used to quantify the amounts of the signature peptides from each gene class of strain NRD-12 (a) and in the cleaved P1-crystal protein lanes of strains NRD-12 and HD-1 (b) as indicated by the arrowheads.

concentrations. Interestingly, the observed molecular masses of the signature peptides were approx. 12% higher than the calculated values. However, no significant differences were detected when calculating the percentage protoxin composition of the P1 crystals on the basis of either the observed or the calculated signature-peptide molecular masses. When these gels are scanned by laser densitometry, the signature peptides defining each gene product are clearly observed when electrophoresed alone (recombinant protoxin) or as a mixture (solubilized P1 crystals). A typical scan of CNBr-cleaved peptides separated by SDS/PAGE is presented in Fig. 4. The arrowheads indicate the signature peptide peaks in the three classes of reference proteins

Table 2. Analysis of P1 crystals of strains NRD-12 and HD-1

Molar proportions were calculated from the mean weight proportions of integrated signature-peptide peaks. All values are normalized to the 6.6-prototoxin cleavage peptide. Percentage contents are given as means $\pm$ S.D.

Gene class	Molar proportions		Content of P1 crystal (%)	
	NRD-12	HD-1	NRD-12	HD-1
6.6	16.4	16.4	37.6 $\pm$ 5.4	32.2 $\pm$ 1.9
5.3	18.4	27.6	39.4 $\pm$ 4.7	54.2 $\pm$ 3.7
4.5	10.3	7.0	23.0 $\pm$ 5.1	13.6 $\pm$ 3.2

(Fig. 4a) and in the solubilized P1 crystals (Fig. 4b). It is important to stress that the signature peptides within a particular gene class have very similar sequences. Furthermore, the signature peptides are produced from the same regions of the individual protoxins as listed in Table 1 and thus share high sequence similarity (approx. 65–85%) with each other. Therefore these features essentially eliminate the variations attributed to differential peptide staining by Coomassie Brilliant Blue and allow highly reproducible quantification of the different gene products in the P1 crystals by laser densitometry. As shown in Table 2, the compositions of the crystals differ between the two *kurstaki* strains. The most striking differences lie with the 5.3-prototoxin and 4.5-prototoxin gene products in that the NRD-12 P1 crystal contained less 5.3 prototoxin as compared with the HD-1 crystal (39.4% compared with 54.2%) whereas the NRD-12 P1 crystal contained more 4.5 prototoxin as compared with the HD-1 crystal (23.0% compared with 13.6%). Of most interest, however, was the presence of high contents of the 6.6-prototoxin gene product in crystals of both strains, as this prototoxin was previously believed to be either absent or produced in amounts that were difficult to detect (Yamamoto *et al.*, 1988). In contrast, our results clearly show that the 6.6 prototoxin comprises approximately one-third of the P1 crystal for both strains.

Insect-cell toxicity assays

The toxicity of the solubilized recombinant gene products were examined by using a rapid lawn assay of cultured insect cells *in vitro*. It was found that the 4.5-prototoxin activated gene product was essentially non-toxic to CF-1 cells (non-toxic at concentrations greater than 1 μ g), as shown in Table 3. The 5.3-prototoxin activated gene product showed weak toxicity, having a threshold toxicity concentration of 1 μ g. However, the 6.6 prototoxins from either strain HD-1 or strain NRD-12 displayed very high toxicity towards the same cells at similar threshold concentrations. By using a variety of mixtures of the recombinant protoxins, we determined that the 4.5-prototoxin and 5.3-prototoxin gene products do not exert any significant antagonistic or synergistic effects on the threshold toxicity concentrations of the 6.6-prototoxin gene products (results not shown). Therefore a second approach was possible to verify the higher-than-expected levels of 6.6-prototoxin gene expression, since any lethal effects of *Bombyx mori*-gut-juice-activated purified P1-crystal protoxins towards cultured CF-1 cells would be due solely to the 6.6-prototoxin gene product. Indeed, it has been shown elsewhere that crystals from strain HD-1 are highly toxic to cultured CF-1 cells (Johnson, 1987), but the actual gene product responsible for the toxicity remained undetermined. The average threshold toxicity values of the crystals for CF-1 cells was found to be approx. 2.7–2.9-fold lower than that seen for the control recombinant 6.6-prototoxin gene products. It should be noted that this method is semi-quantitative in nature and is only accurate to within a single

Table 3. Toxicity of *B. thuringiensis* protoxins to cultured CF-1 insect cells

The percentage relative-toxicity values are calculated as the threshold toxicity (6.6 prototoxin) $\times$ 100 divided by the threshold toxicity (crystal). All prototoxin values represent at least four separate experiments.

Sample	Average threshold toxicity to CF-1 cells (ng)	Relative toxicity (%)
HD-1 crystals	0.47	34.0
NRD-12 crystals	0.38	36.8
HD-1 6.6 prototoxin	0.16	100
NRD-12 6.6 prototoxin	0.14	100
NRD-12 5.3 prototoxin	1000	0.02
NRD-12 4.5 prototoxin	Non-toxic	—

2-fold dilution step. However, by averaging the results from a number of experiments it was determined that the 6.6-prototoxin gene product does exist in the P1 crystal and at a content estimated to be roughly one-third of both the HD-1 and the NRD-12 P1 crystals.

DISCUSSION

The comparison of multi-gene strains possessing different biological activities but a similar genetic makeup, such as the case with *B. thuringiensis* strains NRD-12 and HD-1, poses two major problems. The differential activity may be explained by subtle differences at the nucleotide level, or alternatively by a difference in gene expression levels among identical genes. Such problems can be dissected by cloning and analysing the individual genes and their expressed products.

In the present study two independent approaches were utilized to quantify the levels of gene expression of the three gene classes in the lepidopteran-specific P1 crystals of strains NRD-12 and HD-1. In order to achieve the accurate quantification of the P1-crystal components it was necessary to possess the individual cloned genes and purified gene products from each strain to use as reference proteins. After performance of a comparative analysis of each cloned gene at the restriction-endonuclease level, and each purified prototoxin at the CNBr-cleavage level, no differences between the two strains, on a gene-to-gene basis, can be detected. At the nucleotide level only the 5.3-prototoxin genes can be compared since the 4.5-prototoxin and 6.6-prototoxin genes from strain NRD-12 and the 6.6-prototoxin gene from strain HD-1 have yet to be sequenced. The 5.3 prototoxin from strain NRD-12 differs by eight amino acid residues when compared with the translated HD-1 5.3-prototoxin gene sequence reported by Adang *et al.* (1987); however, the significance of these changes is as yet unknown. Thus three major conclusions can be derived from the CNBr-cleavage results: (1) there are no detectable differences when comparing the individual gene products in a particular gene class, (2) HD-1 and NRD-12 strains express the 6.6-prototoxin gene product to similar extents, comprising about one-third of the P1 crystal, and (3) the P1 crystals differ primarily in the contents of the 4.5-prototoxin and 5.3-prototoxin gene products between the two strains. The results from the lawn cell assay *in vitro* independently verify the CNBr-cleavage results in that NRD-12 and HD-1 strains both produce the 6.6-prototoxin gene product and in large and relatively similar amounts. These results are in direct contrast with those published by Yamamoto *et al.* (1988). Using tryptic peptide mapping by h.p.l.c., these

authors were unable to detect the presence of the 6.6-prototoxin gene product but had determined that the 4.5-prototoxin and 5.3-prototoxin gene products each comprised approx. 50% of the HD-1 P1 crystal. The inability to detect the 6.6-prototoxin gene product may have been due to the fact that their analysis was based on a more indirect mapping method using crystals purified from variants of different *B. thuringiensis* strains partially cured of their plasmids. It is possible that their h.p.l.c. analysis was unable to resolve the 4.5-prototoxin and 6.6-prototoxin gene products, which by our analysis sum up to 45.8% of the P1 crystal. By not having the purified individual prototoxins for use as direct reference peptides, the presence of the 6.6-prototoxin gene product in their reconstitution experiments could have remained undetected, or alternatively have mimicked the 4.5-prototoxin gene-product profile. In agreement with their results, however, we have also determined that the 5.3-prototoxin gene product of strain HD-1 constitutes approx. 50% of the P1 crystal. By possessing each of the cloned gene products, our results clearly show a significant content of the 6.6-prototoxin gene product present in the P1 crystal from both a physical and a biological viewpoint.

The different amounts of the NRD-12 4.5-prototoxin and 5.3-prototoxin gene products as compared with those of strain HD-1 raise the possibility that the fermentation process itself may cause a differential level of gene expression from batch to batch. Both strain NRD-12 (L. Masson, unpublished work) and strain HD-1 (Geiser *et al.*, 1986; Kronstad & Whiteley, 1986; Moar *et al.*, 1989) have been known to lose the 5.3-prototoxin gene. If this occurs early in growth, then it follows that the final product would vary in its crystal composition and presumably, although not necessarily, in its toxicity towards a particular insect. This could account for the conflicting toxicity reports observed in the literature (Dubois, 1984; Stelzer & Beckwith, 1988; Van Frankenhuyzen & Fast, 1989). Indeed, the two-gene HD-1 strains are known to be less toxic towards *Spodoptera exigua* larvae than either the three-gene HD-1 (Wilcox *et al.*, 1986) or NRD-12 strains (Moar *et al.*, 1989).

The usefulness of the CNBr-cleavage method for the analysis of crystal components has been demonstrated here with the products of known genes. Apart from the advantage of being a highly reproducible and rapid means of detecting known gene products in multi-protein mixtures or crystals, this technique may have broader applications. In unpublished work we have found the presence of more than one 130 kDa protein in purified crystals of a *B. thuringiensis* subsp. *entomocidus* strain previously thought to be a single-gene strain (Préfontaine *et al.*, 1987; Masson *et al.*, 1989). Moreover, we have confirmed that another putative single-gene strain, *B. thuringiensis* subsp. *kurstaki* strain HD-73, only expresses the 133.3 kDa 6.6-prototoxin gene product in the crystal. Therefore this method should prove useful in detecting the presence of additional unknown gene products present within a particular crystal.

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