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Biochemical and Molecular Characterization of the Insecticidal Fragment of CryV

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Two C-terminal deletion constructs were made to study the effect of such deletions on the biological activity of the CryV protein of *Bacillus thuringiensis* subsp. *kurstaki*. The results of feeding on neonatal larvae of *Ostrinia nubilalis* (European corn borer [ECB]) indicated that the 50% lethal dose of the full-length CryV protein was 3.34 μg/g of diet (95% fiducial limits, 2.53 to 4.32 μg/g of diet). Removal of 71 amino acids (aa) from the C terminus had little effect on toxicity, whereas deletion of 184 aa abolished the insecticidal activity of the CryV protein completely. Truncations of the full-length CryV protein were also generated with trypsin and the midgut protease of ECB. The proteolytically treated products were characterized by determining their N-terminal amino acid sequences. The CryV protein was found to be cleaved by both proteases through a two-step process. Initially an intermediary form was generated which contained aa 45 of full-length CryV as its N-terminal end. The C-terminal end of this peptide was not experimentally determined. However, analysis of the deduced amino acid sequence of CryV indicated that the C-terminal end of the intermediary form is likely either aa 655 or 659. Further N-terminal processing of the intermediary form resulted in a protease-resistant core form. The core included aa 156 to aa 655 or 659. While the intermediary form retained 100% of the ECB larval toxicity, the core form exhibited only ~22% of the toxicity of the full-length protein.

The gram-positive soil bacterium *Bacillus thuringiensis* has gained considerable academic and commercial importance over the last few decades, owing to its ability to control the larval forms of several insects of agronomic and medical relevance. The toxicity of these bacilli resides in the crystalline, proteinaceous inclusions which are generally made by these organisms under conditions which induce sporulation (8). Based on their target specificity and molecular relatedness, the crystal (Cry) proteins are classified into six major classes (5). Among these, class V displays several unique characteristics. (i) Although the *cryV* gene or *cryV*-like sequences are found in many *B. thuringiensis* subspecies, these genes are normally not expressed in any of the strains (3, 7, 15). (ii) Molecular cloning and expression of this gene in *Escherichia coli* under the control of a heterologous promoter have revealed that CryV is made as an 81.2-kDa protein (15). (iii) CryV was found to exhibit dual toxicity, being specific to larval forms of both lepidoptera and coleoptera (15). The *cryV* gene has been given the designation *cry1Ia1* under the revised nomenclature (4).

Most CryI, CryIII, and CryIV proteins are made as precursors which are activated by the midgut protease(s) of the susceptible insect larvae into active toxins. The CryI and CryIV proteins are proteolytically processed at both the N-terminal and C-terminal ends; the processing of the CryIII proteins, however, typically occurs only at the N terminus (8). Although various Cry proteins are known to maintain some homology, five blocks of amino acids sharing a high degree of homology are found to be located within the active toxin segments of most Cry classes. In addition, it has been observed that the fifth conserved block demarks the C terminus of the active toxins in the ~130-kDa Cry proteins (8). In the smaller proteins, which appear to be naturally truncated, such as CryIII, the fifth block is located at the natural C-terminal end (2). Similar to most Cry proteins, the CryV protein has also been found to contain five conserved amino acid blocks (15). Beyond the fifth conserved block, the full-length CryV protein extends 76 amino acids (aa) further.

In the present study, the toxicity of CryV for the larvae of a major lepidopteran pest, *Ostrinia nubilalis* (European corn borer [ECB]), was established and compared to that of CryIAc. By generating 3′ truncations of the *cryV* gene and by performing biochemical analyses of full-length and protease-treated (trypsin- and ECB protease-treated) CryV proteins, efforts were made to characterize the minimum toxic fragment of the CryV protein. Our results are summarized in this report.

MATERIALS AND METHODS

3′ truncations. The complete *cryV* sequence is available from the EMBL data bank under accession no. X62821. Truncations at the 3′ end of *cryV* were achieved via cloning. Plasmid pIC224 (15) containing full-length *cryV* was digested with *Nde*I and *Sac*I. A double-stranded linker, composed of oligonucleotides 5′ CCTAGATATCTCACTCA 3′ and 5′ TATGAGTGAGATATCTAG GAGCT 3′, which provided an in-frame stop codon, an *Eco*RV site, and a second stop codon was cloned into *Nde*I and *Sac*I sites. The resulting plasmid, named pIC224N1, encoded 648 aa of the CryV protein (referred to as the N1-truncated protein) and ended 5 aa downstream of the fifth homologous block (Fig. 1). Plasmid pIC224N1 was digested with *Eco*RV and religated to form pIC224E5, which encoded 535 aa of the CryV protein (referred to as the E5-truncated protein) and ended at the 3′ end of the third homologous block (Fig. 1). The authenticity of these constructs was verified by restriction analysis. They were used to transform *E. coli* BL21DE3 (14).

Preparation of larval midgut juice and its immobilization on CNBr-activated Sepharose. About 100 fifth-instar ECB larvae were induced to regurgitate their gut contents by electric shock treatment (12). The gut juice obtained was clarified by centrifugation at 15,000 × *g* for 15 min at 4°C and used as the source of larval protease(s). Immobilization of the midgut protease on CNBr-activated Sepharose 4B (Pharmacia) was performed as outlined by Ogiwara et al. (12). The immobilized midgut protease was stored as a slurry at 4°C.

Digestion of CryV with trypsin or immobilized midgut protease. A highly purified CryV protein (15), prepared from the extract of recombinant *E. coli* (pIC224) through ion-exchange and gel filtration chromatography, was a generous gift from R. Tailor, Zeneca Seeds, Bracknell, Berkshire, United Kingdom.

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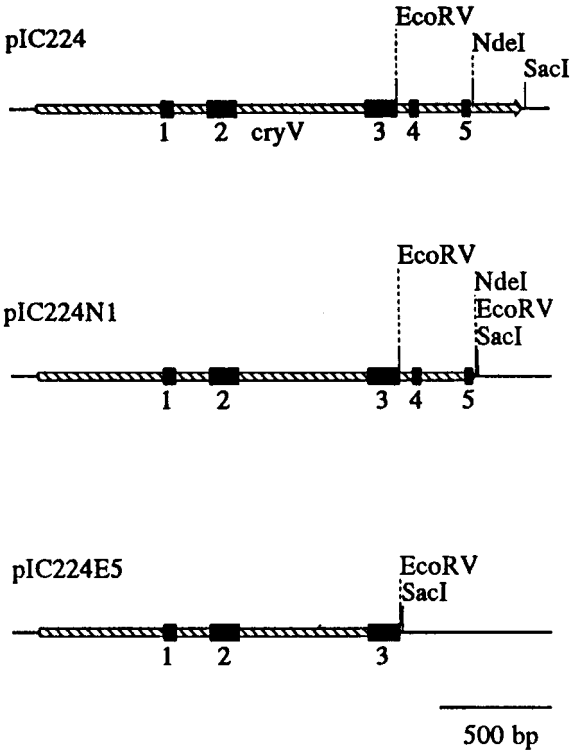

FIG. 1. Schematic representations of the full-length *cryV* gene in pIC224 and the truncated versions in pIC224N1 and pIC224E5. The restriction sites used for cloning were *Eco*RV, *Nde*I, and *Sac*I. The solid bars denote the five blocks of amino acids that share homology among the *cryI*, *-III*, *-IV*, and *-V* genes.

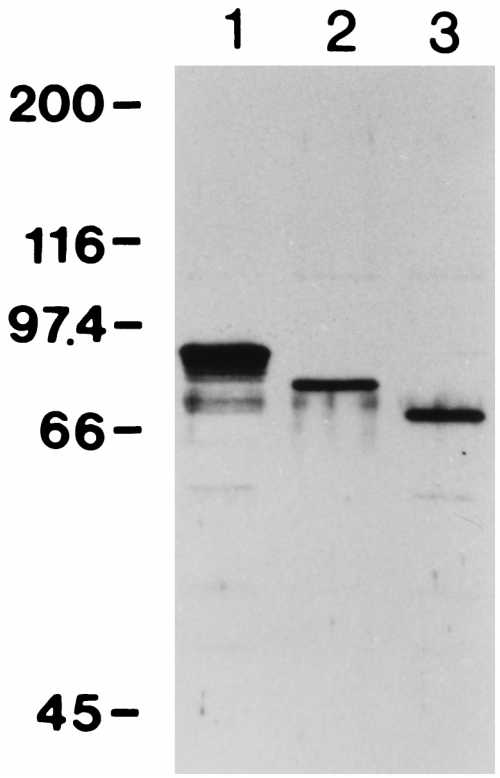

FIG. 2. Immunoblot analysis of full-length and truncated CryV proteins. Total protein (5 μg/lane) from recombinant *E. coli* lysates pIC224 (lane 1), pIC224NI (lane 2), and pIC224E5 (lane 3) were analyzed by immunoblotting. The molecular masses of protein standards (in kilodaltons) are on the left.

The lyophilized protein powder was dissolved in 20 mM Tris (pH 8.0) containing 1 mM EDTA, and the protein content of the solubilized sample was determined by the dye-binding assay (1). Concentrations of the N1- and E5-truncated proteins present in the extracts of recombinant *E. coli*(pIC224N1) and *E. coli*(pIC224E5), respectively, were estimated by enzyme-linked immunosorbent assay using CryV-specific antiserum. Required amounts of the full-length and truncated CryV proteins were digested with tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Sigma) at a 1:1 (wt/wt) ratio of CryV to trypsin. Treatment with the immobilized ECB protease was performed by using equal amounts of the regurgitated gut juice proteins and CryV. The digestions were carried out at 37°C for 15 min with the proteolytic enzymes to generate the intermediary and core forms. Prolonged digestion (i.e., 2 h) with trypsin was performed to generate only the core form. After digestions, phenylmethylsulfonyl fluoride and *o*-phenanthroline were added (to concentrations of 0.5 and 5 mM, respectively) to the samples prior to analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). It had been shown previously (9) that in the presence of SDS, protease-activated Cry toxins can be further degraded to very small peptide forms. The protease-resistant forms were separated from the much smaller proteolytic degradation products by concentration using a high-M_r (i.e., 50,000)-cutoff membrane filter (Spectrum).

Immunoblot analysis. The N1- and E5-truncated proteins, along with full-length CryV and their proteolytic degradation products, were analyzed by SDS-PAGE (10) using a Mini Protean V dual-slab gel unit (Bio-Rad). The separated proteins were transferred (16) to HybondC-super membranes (Amersham) with the help of a Trans-Blot transfer cell (Bio-Rad). Immunoblotting of the membrane-bound proteins was performed using CryV-specific antiserum raised in a rabbit (at a 1:5,000 dilution). Prior to its use in immunoblots, any *E. coli*-specific antibodies present were removed from the CryV-specific antiserum by affinity chromatography (13) using CNBr-Sepharose-bound *E. coli* BL21DE3 proteins. Donkey anti-rabbit antibody conjugated to horseradish peroxidase was used as the secondary antibody (at a 1:20,000 dilution). Visualization of the bound antigen-primary antibody-secondary antibody complex on X-ray film (Kodak XAR) was achieved by a horseradish peroxidase-based enhanced-chemiluminescence detection system (Amersham).

N-terminal amino acid sequencing. The partially purified trypsin- and midgut protease-activated intermediary and core forms were separated by SDS-PAGE and subsequently transferred to polyvinylidene difluoride membrane filters. The membrane-bound proteins, visualized by Coomassie blue staining, were cut out, and their N-terminal amino acid sequences were determined. Protein microsequencing was performed by M-Scan Inc., West Chester, Pa., on an Applied Biosystems 477A sequencer.

Bioassays. Larval bioassays were performed as described by Tailor et al. (15). The toxicity of full-length CryV, the N1- and E5-truncated proteins, and the intermediary and core forms of CryV was tested on laboratory-reared neonatal larvae of ECB (17) by multiple-dose diet incorporation bioassays. All assays were conducted by using three larvae per dish and five replicates for each treatment. Treated larvae were maintained at 23°C for 6 days prior to mortality scoring. The toxicity of the full-length CryIAc protein for ECB larvae was also established in a similar manner. The 50% lethal doses (LD_{50}s) were calculated by using probit analysis (6) after repeating the assays at least five times.

RESULTS

3′ truncations. Immunoblot analysis of proteins found in recombinant *E. coli* lysates revealed that clone pIC224, containing the full-length *cryV* gene, expressed an 81.2-kDa protein (Fig. 2). On the other hand, none of the proteins of the host strain *E. coli* BL21DE3 exhibited any cross-reactivity to the CryV-specific antiserum (data not shown). Clones pIC224N1 and pIC224E5 (encoding the N1- and E5-truncated proteins, respectively) produced proteins of 72.9 and 59.9 kDa, respectively, as predicted by the deduced amino acid sequences (Fig. 2). The other, smaller, weakly cross-reactive bands observed are likely proteolytic degradation products.

Protease digestions. Immunoblot analysis revealed that a short (i.e., 15-min) digestion of CryV with trypsin generates two sets of products (Fig. 3, lane 2). Each set consisted of two closely resolved proteins. The lower band of the larger doublet, namely, the intermediary form, had a mass of 69.1 kDa, whereas the lower band of the smaller doublet, the core form, had a mass of 56.9 kDa. Prolonged (i.e., 2-h) trypsin digestion produced only the core form (lane 1). Similarly, limited diges-

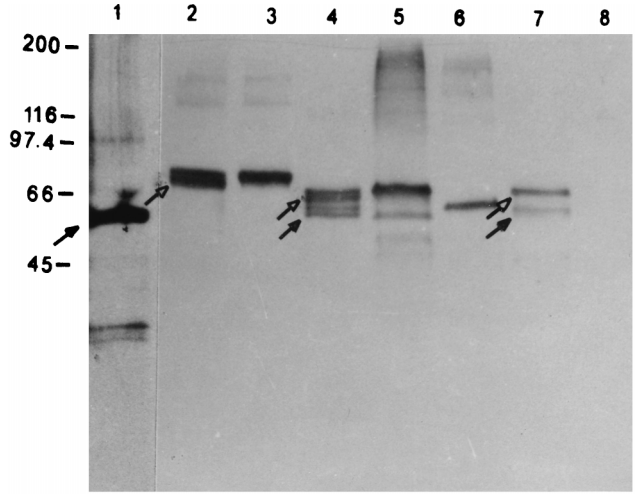

FIG. 3. Immunoblot analysis of protease-treated CryV proteins. Protease-treated full-length and truncated proteins (250 ng/lane) were analyzed. Lanes: 1, CryV digested with trypsin (1:1 [wt/wt], 2 h); 2, CryV digested with trypsin (1:1 [wt/wt], 15 min); 3, undigested full-length CryV; 4, CryV digested with ECB protease (1:1 [wt/wt], 15 min); 5, N1-truncated protein; 6, E5-truncated protein; 7, N1-truncated protein digested with ECB protease (1:1 [wt/wt], 2 min); 8, E5-truncated protein digested with ECB protease (1:1 [wt/wt], 15 min). The intermediary and core forms are indicated by open and closed arrows, respectively. The molecular masses of protein standards (in kilodaltons) are on the left.

tion with ECB protease also produced two groups of peptides (lane 4). Protein microsequencing revealed aa 45 to be the N-terminal amino acid of the intermediary from (i.e., the first 44 aa from the N terminus of CryV were cleaved off). In the core form, the N terminus was aa 156. Digestion of the N1-truncated protein with ECB protease (lane 7) resulted in the formation of products with molecular masses similar to that of the full-length protein. Protease treatment of the E5-truncated protein, on the other hand, resulted in complete degradation of the protein (lane 8), indicating the extreme sensitivity of this protein to proteolytic degradation. Similar results were observed when the N1- and E5-truncated proteins were digested with trypsin (data not shown).

Bioassays. Through laboratory bioassays, the LD_{50} of the full-length CryV protein for neonatal ECB larvae was determined as 3.34 μg/g of diet (95% fiducial limits, 2.53 to 4.32 μg/g of diet). The LD_{50} of full-length CryIAc, on the other hand, was 181.54 ng/g of diet (95% fiducial limits, 127.69 to 240.02 ng/g of diet). The N1 truncation did not have any effect on toxicity. The E5-truncated protein did not exhibit any toxicity at all (data not shown).

In the case of trypsin digestion, the intermediary form had a toxicity comparable to that of the full-length protein (i.e., the LD_{50} was 3.52 μg/g of diet [95% fiducial limits, 2.64 to 4.59 μg/g of diet]). The efficacy of the core form was lower than that of full-length CryV (i.e., the LD_{50} was 14.77 μg/g of diet [95% fiducial limits, 10.25 to 36.42 μg/g of diet]). The toxicity of the ECB protease-digested intermediary and core forms was also similar to that of the trypsin-digested products (data not shown).

DISCUSSION

We have demonstrated that the N1-truncated protein (encompassing aa 1 to 648 and retaining 5 aa beyond the fifth conserved block) is fully toxic. On the other hand, the E5-truncated protein (containing aa 1 to 535 and having a deletion of 108 aa upstream of the fifth block) is nontoxic to the larvae of ECB. Hence, it appears that as in the other Cry proteins (8), in CryV also, sequences extending up to the fifth block are essential for biological activity.

In the ~130-kDa CryI proteins, the carboxyl half of the protein is thought to be involved in stabilizing the crystal structure (8). However, reported *cryV* genes are a distal part of an operon located approximately 500 bp 3′ of a *cryI*-type gene. Due to the presence of an intergenic transcriptional terminator and a lack of an upstream promoter-like sequence, *cryV* genes are not normally expressed in *B. thuringiensis* strains (3). Hence, the exact function of sequences beyond the fifth conserved block in CryV remains unclear.

Treatment of CryV with trypsin and ECB protease resulted in the generation of a protease-resistant core through an intermediary form. While the intermediary form retained aa 45 of CryV as its N terminus, the core had aa 156 as its N end. The exact C-terminal ends of these peptides were not determined experimentally. However, analysis of the deduced amino acid sequence of CryV revealed the presence of trypsin cleavage sites at positions 655 and 659. SDS-PAGE analysis of the tryptic intermediary and core products of the N1-truncated protein (which retains only aa 1 to 648 and does not include aa 655 or 659) indicated that the sizes of these peptides were similar to the intermediary and core forms of full-length CryV (data not shown). The N-terminal amino acid sequences of the pIC224N1 protein's digestion products (i.e., the intermediary and core forms) were identical to those generated from full-length CryV. Hence, the C-terminal end of the intermediary and core forms of CryV is very likely aa 655 or 659.

There was a noticeable difference between the proteolytic sensitivity of the E5-truncated protein and that of the full-length and N1-truncated proteins. This may be attributed to the fact that the E5-truncated protein does not contain 108 aa upstream of the fifth conserved block while the other truncated proteins do retain several amino acids beyond the fifth block. Since it is known that deletion of amino acids within or upstream of the fifth block leads to loss of stability of the Cry proteins (2, 8), the extreme sensitivity of E5-truncated protein to proteolysis may be due to the absence of the fifth (and also the fourth) conserved block in this protein.

Comparison of the toxicities of the full-length CryV protein and its protease-treated forms for neonatal larvae of ECB revealed that the LD_{50} of the intermediary form (i.e., 3.52 μg/g of diet) was comparable to that of the full-length protein (i.e., 3.34 μg/g of diet). However, the toxicity of the core form (LD_{50}, 14.77 μg/g of diet) was ~4.5-fold lower than that of the full-length protein. Lambert et al. (11) have recently shown that the Cry9Cal protein is made as a 129.8-kDa protein. Trypsin activation of this protein removes 43 aa at the N terminus and deletes the entire C-terminal half beyond conserved block 5 to generate a product of 68.7 kDa. Further digestion results in the formation of a 53-kDa trypsin-resistant core form. Similar to that of CryV, the initial tryptic product of Cry9Cal (i.e., the 68.7-kDa fragment) retains 100% of the toxicity of the full-length protein for members of the Noctuidae family (including larvae of ECB). The core form (i.e., the 53-kDa fragment), on the other hand, exhibits no toxicity at all. By elimination of a trypsin cleavage site in the protoxin, those investigators were able to prevent proteolytic degradation of the 68.7-kDa fragment to the 53-kDa form. However, it was reported that such a modification did not lead to increased toxicity of the protoxin for ECB larvae. We have not altered the proteolytic sites in the CryV toxin so as to eliminate the generation of the less-active 56.9-kDa core form. Hence,

whether such an alteration would improve the bioefficacy of CryV for ECB larvae remains to be determined.

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REFERENCES

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
2. **Carroll, J., and D. J. Ellar.** 1989. Proteolytic processing of a coleopteran-specific delta-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis*. Biochem. J. **261:**99–105.
3. **Chambers, J. A., A. Jelen, M. P. Gilbert, C. S. Jany, T. B. Johnson, and C. Gawron-Burke.** 1991. Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* subsp. *aizawai*. J. Bacteriol. **173:**3966–3976.
4. **Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, B. Lambert, D. Lereculus, C. Gawron-Burke, and D. H. Dean.** 1995. Revision of the nomenclature for *Bacillus thuringiensis cry* genes, p. 14. *In* Program and abstracts of the 28th Annual Meeting of the Society for Invertebrate Pathology. Society for Invertebrate Pathology, Bethesda, Md.
5. **Feitelson, J. S., J. Payne, and L. Kim.** 1992. *Bacillus thuringiensis*: insects and beyond. Bio/Technology **10:**271–275.
6. **Finney, D. J.** 1971. Probit analysis, 3rd ed. Cambridge University Press, Cambridge, England.
7. **Gleave, A., R. Williams, and R. Hedges.** 1993. Screening by polymerase chain reaction of *Bacillus thuringiensis* serotypes for the presence of *cryV*-like insecticidal protein genes and characterization of a *cryV* gene cloned from *B. thuringiensis* subsp. *kurstaki*. Appl. Environ. Microbiol. **59:**1683–1687.
8. **Hofte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
9. **Inagaki, S., M. Miyasono, T. Ishiguro, R. Takeda, and Y. Hayashi.** 1992. Proteolytic processing of δ-endotoxin of *Bacillus thuringiensis* var. *kurstaki* HD-1 in insensitive insect, *Spodoptera litura*: unusual proteolysis in the presence of sodium dodecyl sulfate. J. Invertebr. Pathol. **60:**64–68.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
11. **Lambert, B., L. Buysse, C. Decock, S. Jansens, C. Piens, B. Saey, J. Seurinck, K. Van Audenhove, J. Van Rie, A. Van Vliet, and M. Peferoen.** 1996. A *Bacillus thuringiensis* insecticidal crystal protein with a high activity against the members of the family Noctuidae. Appl. Environ. Microbiol. **62:**80–86.
12. **Ogiwara, K., L. S. Indrasith, S. Asano, and H. Hori.** 1992. Processing of δ-endotoxin from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and HD-73 by gut-juices of various insect larvae. J. Invertebr. Pathol. **60:**121–126.
13. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 12.27–12.28. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
14. **Studier, F., and B. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes. J. Mol. Biol. **159:** 113–130.
15. **Tailor, R., J. Tippett, G. Gibb, S. Pells, D. Pike, L. Jordan, and S. Ely.** 1992. Identification and characterization of a novel *Bacillus thuringiensis* delta-endotoxin entomocidal to coleopteran and lepidopteran larvae. Mol. Microbiol. **6:**1211–1217.
16. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
17. **Vanderzant, E., C. Richardson, and S. Fort.** 1962. Rearing of the bollworm on artificial diet. J. Econ. Entomol. **55:**140.