ΠΡΟΓΡΑΜΜΑ ΔΙΑ ΒΙΟΥ ΜΑΘΗΣΗΣ ΑΕΙ ΓΙΑ ΤΗΝ ΕΠΙΚΑΙΡΟΠΟΙΗΣΗ ΓΝΩΣΕΩΝ ΑΠΟΦΟΙΤΩΝ ΑΕΙ (ΠΕΓΑ)

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An improved baculovirus insecticide producing occlusion bodies that contain *Bacillus thuringiensis* insect toxin

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Abstract

Baculovirus occlusion bodies, large proteinaceous structures which contain virions, have recently been engineered to incorporate foreign proteins. The major constituent protein of occlusion bodies from the baculovirus *Autographa californica* nucleopolyhedrovirus is polyhedrin, and assembly of recombinant occlusion bodies which incorporate a foreign protein depends on an interaction between native polyhedrin and a polyhedrin–foreign protein fusion. This technology has now been applied to the generation of a recombinant baculovirus (ColorBtrus) that produces occlusion bodies incorporating the *Bacillus thuringiensis* (Bt) insecticidal Cry1Ac toxin protein. ColorBtrus coexpresses native polyhedrin and a fusion protein in which polyhedrin is fused to the Bt toxin, which is in turn fused to green fluorescent protein (GFP). Analysis of ColorBtrus occlusion bodies confirmed that they include both Bt toxin and GFP, yet still incorporate virions. Bioassay of ColorBtrus demonstrated that its speed of action and pathogenicity are strikingly enhanced compared to wild-type virus. ColorBtrus represents a novel, powerful biological insecticide that combines positive attributes of both Bt toxin and baculovirus based systems.

Keywords: Baculovirus; *Autographa californica* nucleopolyhedrovirus; AcMNPV; *Bacillus thuringiensis*; Bt; Cry1Ac toxin; Polyhedrin fusion proteins

1. Introduction

Baculoviruses have a long history of safe use as specific, environmentally benign insect control agents. They induce lethal epizootics that can devastate host insect populations. In general, they only infect arthropods, and individual virus strains infect only one or a small number of species. No effects on non-target species have been demonstrated. However, their use has been limited by several factors, especially their slow speed of action (Bonning and Hammock, 1996; Maeda, 1995; Wood and Granados, 1991). Depending on the strain of virus and pest insect species, it can take from several days to weeks before the infected insect stops feeding. During this time, significant feeding damage can be caused to the crop. A number of approaches have been taken in order to produce baculoviruses with improved speed of kill or decreased effective feeding times, including incorporation of a foreign gene(s) into the baculovirus genome so that the product is expressed within the infected cells. Examples include the insertion of insect-specific toxin genes or other genes (e.g., juvenile hormone esterase, diuretic hormone, and prothoracicotropic hormone) predicted to have deleterious effects on host physiology upon inappropriate expression (Black et al., 1997). Amongst these approaches, expression of insect-specific neurotoxins, including mite toxin TxP-I, and scorpion toxins AaIT and LqhIT1/LqhIT2 showed the most significant increase in pathogenicity (Black et al., 1997; Burden et al., 2000;

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Gershburg et al., 1998; Regev et al., 2003; Stewart et al., 1991; Tomalski and Miller, 1991).

Bacillus thuringiensis (Bt) insecticidal crystal proteins (δ-endotoxin) have been used as effective means for controlling pest populations. Bt δ-endotoxin accumulates in large amounts during sporulation of Bt, forming crystalline occlusions. When ingested to susceptible insects, the crystal protein is solubilised in the alkaline environment of the gut and activated by proteases present in the gut juices. The activated toxin causes disruption of the gut, and this leads to cessation of feeding and death. Therefore, expression of Bt protein toxin within the context of baculovirus infection was predicted to generate pores in cell membranes, leading to disruption of osmotic balance and then cell death. Thus, full-length or truncated forms of crystal protein genes were inserted into the baculovirus genome to attempt to enhance the pathogenicity for insect larvae (Ribeiro and Crook, 1993). In addition, recombinant baculoviruses were constructed to express Bt subsp. *kurstaki* HD-73 Cry1Ac toxin (Merryweather et al., 1990) and Bt subsp. *aizawai* 7.21 Cry1Ab toxin (Martens et al., 1990, 1995). However, due to the mechanism of action of the Bt δ-endotoxin (i.e., activation and activity within the insect midgut), insecticidal efficacy was not delivered from expression of intra- or extra-cellular expression of the toxin at late times of baculovirus infection by these constructs.

Diamondback moth, *Plutella xylostella*, is considered among the most serious pest of brassicae crops worldwide (Talekar and Shelton, 1993). Currently, this insect shows resistance to several classes of chemical and Bt subsp. *kurstaki*-based insecticides (Shelton et al., 1993). Increasing difficulty in controlling diamondback moth has promoted interest in using baculoviruses as biocontrol agents (Biever and Andrews, 1984; Padmavathamma and Veeresch, 1991; Talekar and Shelton, 1993). To this end, production of a recombinant *Autographa californica* nucleopolyhedrovirus (AcMNPV) which would deliver a fast-acting and insect-specific toxin directly to the insect midgut would be extremely desirable.

We have recently described the generation of recombinant baculoviruses in which the foreign protein is actually incorporated into the viral polyhedra (Je et al., 2003). One or more complete proteins can be incorporated into the occlusion bodies, which retain the ability to occlude virions. Here we describe the construction and characterisation of a recombinant AcMNPV that produces polyhedra incorporating green fluorescent protein (GFP) as a marker and the Cry1Ac crystal protein toxin. The infectivity and speed of action of this virus are dramatically improved compared to unengineered virus or previously described engineered AcMNPVs. These experiments pave the way for delivery of a '2 hit' approach: direct uptake of gut-acting toxin(s) of choice coupled to subsequent baculovirus infection of any insects which survive the initial toxin exposure.

2. Materials and methods

2.1. Cell lines and viruses

Spodoptera frugiperda IPLB Sf21-AE (Vaughn et al., 1977) clone 9 (Sf9) cells were maintained at 27 °C in TC100 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). All viruses were propagated and titered in Sf9 cells (O'Reilly et al., 1992; Summers and Smith, 1987). Polyhedra and viral DNA were obtained from Sf9 cells by standard methods (O'Reilly et al., 1992; Summers and Smith, 1987).

2.2. Recombinant transfer vectors

An 1836 bp fragment of the Bt subsp. *kurstaki* HD-73 *cry1Ac* crystal protein gene, comprising the N-terminal toxic domain, was amplified from pN6.6 (Adang et al., 1985) using the primers 5′AACTCGAGATGGATAACAATCCGAAC3′ and 5′CTCGAGTGTTGCAGTAACTGGAAT3′. Both primers include an *Xho*I site (underlined) and the fragment was cloned between polyhedrin and GFP in the plasmid pColorPol (Je et al., 2003) to produce pColorBtrus (Fig. 1). In this plasmid, the polyhedrin and fusion genes are under the control of the p10 and polyhedrin promoters, respectively.

2.3. Construction of recombinant viruses

One microgram of BacPAK6 viral DNA (Clontech, USA) and 5 μg of transfer vector DNA were

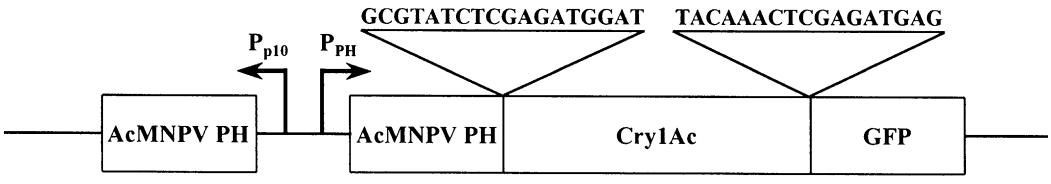

Fig. 1. Structure of the transfer vector for the production of the recombinant baculovirus ColorBtrus. pColorBtrus carries a wild-type AcMNPV polyhedrin gene under the control of the p10 (P_{p10}) promoter, as well as a polyhedrin–Cry1Ac–GFP fusion gene under the control of the polyhedrin (P_{PH}) promoter. The nucleotide sequences at the fusion boundaries are shown. The arrows show the direction of transcription.

cotransfected into Sf9 cells by lipofection as described (O'Reilly et al., 1992). The virus-containing supernatant was harvested 5 days later and recombinant AcMNPV isolated by plaque purification (O'Reilly et al., 1992; Summers and Smith, 1987). The genome structures of the recombinant viruses were confirmed by PCR, restriction endonuclease analysis, and Southern blotting (data not shown).

2.4. Protease digestion and immunoblot analysis

Cry1Ac toxin protein from Bt subsp. *kurstaki* HD-73 was purified by the method of Thomas and Ellar (1983), and quantified using the Bradford assay (Biorad, USA). Purified ColorBtrus polyhedra (1.5×10^6) and purified Cry1Ac (12.5 or 100 ng) were digested with 100 μg/ml trypsin (Amresco, USA) at 37 °C for 2 h. In some experiments, purified wild-type AcMNPV polyhedra were analysed in parallel. Samples were analysed by electrophoresis through 4–12% Bis–Tris gels using a MES buffer system (Novagen, USA). Cry1Ac toxin bands were quantified by densitometry when necessary. For immunoblotting, proteins were transferred to nitrocellulose membranes using a Hoefer Trans-Blot apparatus (Hoefer Scientific Instruments, USA) and incubated with Cry1Ac antiserum, diluted 1:1000. The Cry1Ac antiserum was generated by immunisation of rabbits with purified Cry1Ac. Bound antibody was visualised using an ECL-Plus kit (Amersham–Pharmacia Biotech, USA) as described by the manufacturer.

2.5. Immunogold staining

Samples were fixed for 3 h in 4% formaldehyde, 1% glutaraldehyde in 0.1 M sodium phosphate (pH 7.5) containing 0.15 mM $CaCl_2$, and 0.45 M sucrose (FM) and incubated overnight in pH 10.4 FM without glutaraldehyde. The samples were rinsed in 0.1 M sodium phosphate (pH 7.5), dehydrated in a graded ethanol series (up to 95%), and embedded in Lowicryl K4M (Polysciences, USA). Ultrathin sections mounted on formvar-coated nickel grids were treated for 10 min with Tris-buffered saline (TBS; 0.02 M Tris–HCl, pH 7.5, containing 0.5 M NaCl). The sections were etched with 3% H_2O_2 in double distilled H_2O for 5 min and blocked with 3% BSA in TBS for 30 min. Triple immunogold staining was performed according to Doerrschott and Lichte (1986). Appropriate working dilutions were first defined for each antibody. These were 1:100 for the GFP and Cry1Ac antisera and 1:500 for the polyhedrin antiserum. The Bt toxin antiserum was generated in rabbits by injection of purified Cry1Ac. Secondary antisera were used at a dilution of 1:10 (30 nm gold conjugated goat anti-mouse IgG, BioCell; 20 nm gold-conjugated goat anti-rabbit IgG, Zymed) and 1:20 (10 nm gold-conjugated goat anti-mouse IgG, Zymed). One side of the grid was exposed to a mixture of the rabbit anti-Cry1Ac and mouse anti-GFP antisera followed by a mixture of the 20 nm gold-conjugated goat anti-rabbit and 30 nm gold-conjugated goat anti-mouse secondary antibodies. The reverse of the grid was exposed to the polyhedrin antiserum followed by 10 nm gold-conjugated goat anti-mouse IgG. After immunostaining, sections were post stained with 2% uranyl acetate followed by 0.2% lead citrate and observed with a Hitachi M-600 transmission electron microscope operating at 75 kV.

2.6. Bioassays

The dose– and time–mortality responses of ColorBtrus were compared to wild-type AcMNPV and Cry1Ac toxin in the diamondback moth, *P. xylostella*. *P. xylostella* larvae were obtained from a laboratory colony and reared at 25 °C on chinese cabbage under a 16 h:8 h light dark cycle. To compare the toxicity of ColorBtrus polyhedra with Cry1Ac toxin, twofold serial dilutions of toxin were prepared in 0.05% Triton X-100. Dilutions of ColorBtrus polyhedra containing equivalent amounts of Cry1Ac were also prepared. Wild-type AcMNPV polyhedra were analysed in parallel as a control. Small plugs of chinese cabbage leaves were dipped in each solution and allowed to air-dry. *P. xylostella* larvae, 128 second or third instars, were exposed by feeding on each treated cabbage leave plugs, respectively. When the entire plug was consumed for 24 h, larvae were transferred to fresh chinese cabbage leaves and monitored at 12 h intervals until 5 days after treatment.

To compare the median lethal dose of wild-type AcMNPV and ColorBtrus, (LD_{50}), second or third instar larvae were fed six doses (20,480, 10,240, 5120, 1280, and 640 polyhedra per larva) of AcMNPV or 10 doses (5120, 2560, 1280, 640, 320, 160, 80, 40, 20, and 10 polyhedra per larva) of ColorBtrus on a small plug of chinese cabbage leaves. Larvae that consumed the dose in 24 h were transferred to fresh chinese cabbage leaves and examined twice daily. Cadavers were removed and the cause of death confirmed. The data were analysed by Probit analysis. To determine the median survival time (ST_{50}), 128 second or third instar larvae (evenly distributed amongst treatments) were fed equivalent effective doses of wt AcMNPV or ColorBtrus (10,240 and 80 polyhedra per larva, respectively; approximately equivalent to an LD_{90} dose) on a small plug of chinese cabbage leaves. After feeding, the larvae were transferred to fresh diet and observed as before. ST calculations were made on data averaged from three independent experiments using the Vistat program (Boyce Thompson Institute, Ithaca, NY).

3. Results

3.1. Generation of polyhedra incorporating Bt crystal protein toxin

We have previously demonstrated that a fusion protein comprising one or more foreign proteins fused to polyhedrin can be incorporated into polyhedra if unfused polyhedrin protein is also present in the infected cells (Je et al., 2003). We therefore attempted to produce AcMNPV polyhedra incorporating the Bt Cry1Ac toxin and GFP. The structure of the transfer vector, pColorBtrus, we constructed to make such a virus is shown in Fig. 1. In this plasmid, the Cry1Ac gene is fused in-frame between polyhedrin and GFP. The fusion protein is under the control of the polyhedrin promoter. The plasmid also includes wild-type polyhedrin, under the control of the p10 promoter. The p10 is another abundantly produced baculovirus protein that is expressed with very similar kinetics to the polyhedrin. The virus ColorBtrus was generated by cotransfection of pColorBtrus with the parental virus BacPAK6, an AcMNPV derivative that lacks a polyhedrin gene. ColorBtrus forms polyhedra that are fluorescent under UV (Fig. 2) indicating that the fusion protein was successfully incorporated into the occlusion bodies. This was confirmed by immunoelectron microscopy, which demonstrated the presence of GFP and Cry1Ac toxin as well as polyhedrin (Fig. 3). As expected, the polyhedra occluded virions normally.

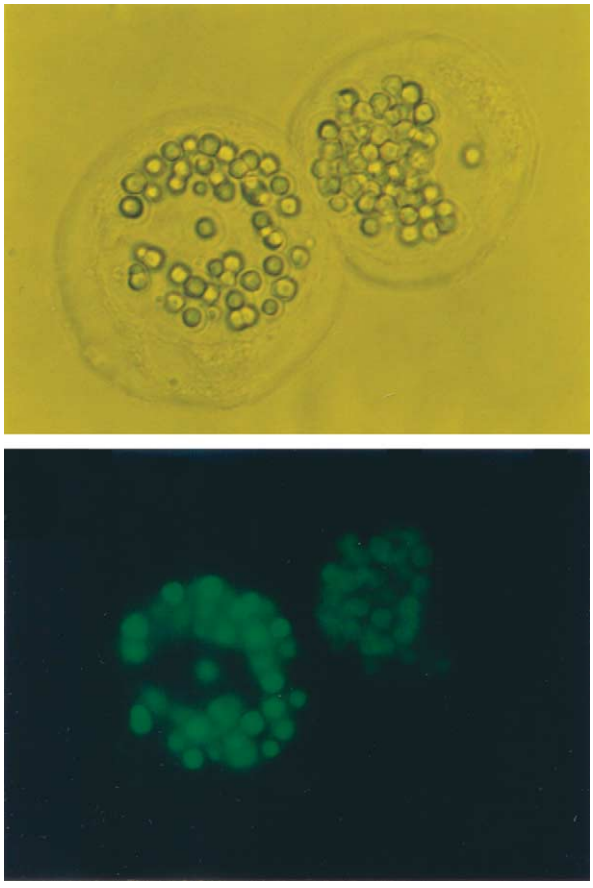

Fig. 2. Formation of fluorescent polyhedra by ColorBtrus. Sf9 cells infected with ColorBtrus were observed 4 days p.i. by light (top) and fluorescent (bottom) microscopy using an Axiophot Universal microscope (Zeiss) (1000×).

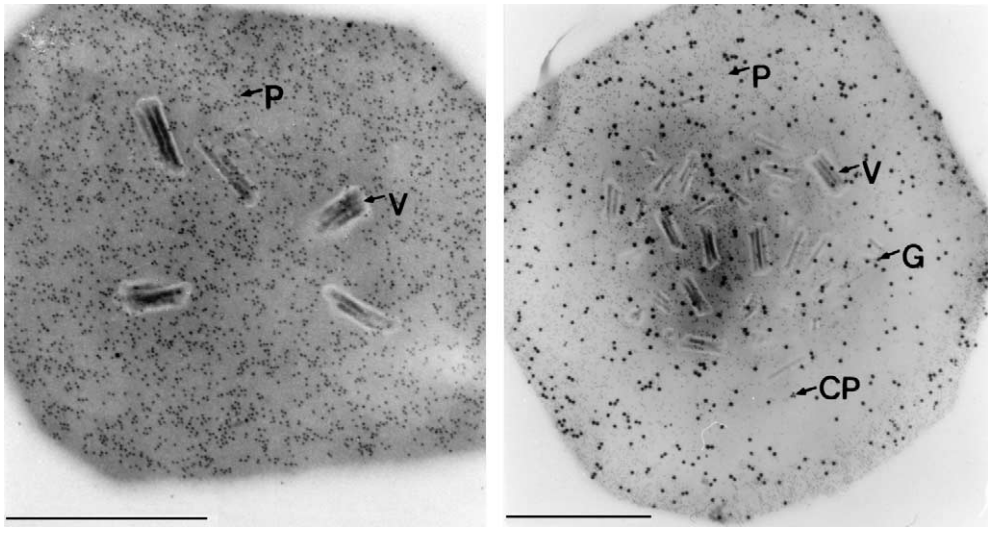

Fig. 3. Immunogold labeling of recombinant polyhedra. Purified polyhedra formed by wild-type AcMNPV or ColorBtrus were analysed by immunogold labeling with antisera against polyhedrin, GFP, and Cry1Ac toxin (left and right panels, respectively). Gold particles attached to the anti-polyhedrin (P, 10 nm), anti-GFP (G, 30 nm), and anti-crystal protein toxin (CP, 20 nm) antibodies were observed by transmission electron microscopy. Virions (V) are indicated. The bars represent 1 μm.

3.2. Characterisation of polyhedra displaying Cry1Ac toxin

Bt toxins are normally synthesised as inactive precursors and cleaved by proteases present in the host's gut to release the active toxin fragments. We investigated whether the polyhedrin–Bt toxin–GFP fusion protein could be cleaved by proteases to release active toxin. ColorBtrus occlusion bodies include a 125 kDa band that is absent from wild-type AcMNPV occlusion bodies (compare lanes 1 and 2, Fig. 4 left panel). This corresponds to the size expected for the polyhedrin–Cry1Ac–GFP fusion protein. Immunoblot analysis with antiserum specific for Cry1Ac toxin confirmed that this band includes Cry1Ac (Fig. 4 right panel, lane 2). Following cleavage with trypsin, the Cry1Ac-reactive band is reduced to approximately 62 kDa (Fig. 4 right panel, lane 5). Cleavage of purified Cry1Ac protoxin with trypsin results in the generation of a band of the same size (Fig. 4 right panel, lane 6). These data indicate that the Cry1Ac toxin active fragment can be released from the fusion protein by proteases, and suggest that the fusion protein will be active in the insects gut.

It was of interest to get an estimate of the amount of Cry1Ac toxin incorporated into the ColorBtrus occlusion bodies. To this end, purified ColorBtrus polyhedra and a Cry1Ac protoxin dilution series were treated with trypsin and analysed by gel electrophoresis. Comparison of the band intensities suggested that 1.5×10^6 ColorBtrus occlusion bodies contain approximately 10 ng of Cry1Ac (data not shown).

3.3. In vivo activity of polyhedra incorporating Cry1Ac toxin

A series of experiments were carried out to evaluate the insecticidal activity of the ColorBtrus virus. Initially, the activity of ColorBtrus polyhedra was compared to equivalent amounts of purified Cry1Ac protoxin. *P. xylostella* larvae were fed dilutions of polyhedra or Cry1Ac and monitored for mortality at 12 h intervals until 5 days after treatment. Both the dose–mortality and time–mortality responses for ColorBtrus occlusion bodies are very similar to those for the equivalent doses of Cry1Ac protoxin (Fig. 5), indicating that the Cry1Ac toxin is fully functional when delivered to insects in the context of a polyhedrin fusion protein. In both cases, the ColorBtrus polyhedra seemed to be markedly more potent than wild-type AcMNPV polyhedra. To investigate this further, a second series of bioassays was carried out comparing AcMNPV and ColorBtrus. The data presented in Table 1 demonstrate that the LD_{50} for ColorBtrus was reduced approximately 100-fold compared to wild-type virus (28.3 polyhedra per larva compared to 2798.3 polyhedra per larvae, respectively). Similarly, at equivalent effective doses, the ST_{50} of ColorBtrus was dramatically faster than wild-type AcMNPV (33.9 h compared to 92.8 h).

In addition, to confirm the production of polyhedra by the ColorBtrus, the fat body from the dead larvae infected with ColorBtrus at 6 days p.i. was observed by fluorescent microscope. The result revealed that dead larvae infected with low doses produced recombinant polyhedra with fluorescence of GFP (data not shown).

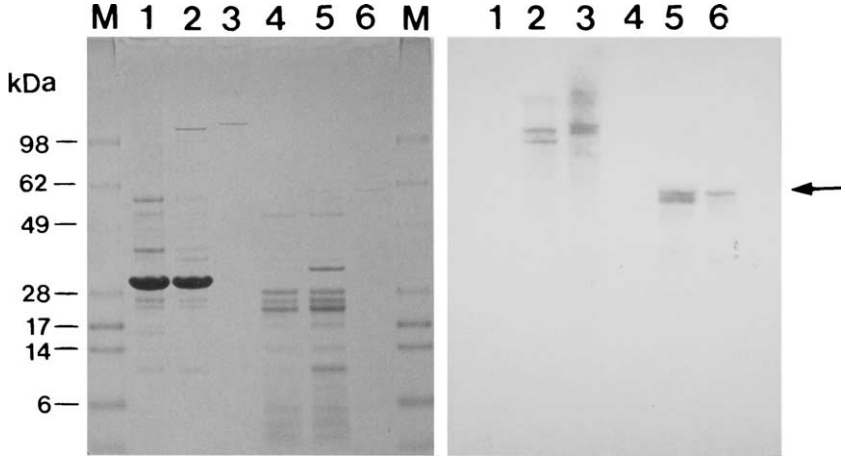

Fig. 4. Trypsin cleavage of recombinant polyhedra. Polyhedra were purified from wild-type AcMNPV or ColorBtrus infected cells and aliquots of each cleaved with trypsin. Cry1Ac toxin was analysed in parallel. The samples were analysed by gel electrophoresis and immunoblotting with Cry1Ac antiserum. The left panel is a Coomassie stained gel and the right panel an immunoblot. Lanes 1 and 4; AcMNPV polyhedra. Lanes 2 and 5; ColorBtrus polyhedra. Lanes 3 and 6; Cry1Ac toxin. Trypsin cleaved samples were in lanes 4–6. The arrow indicates the cleaved 62 kDa form of the toxin. M indicates molecular weight markers. The sizes of the markers are shown on the left.

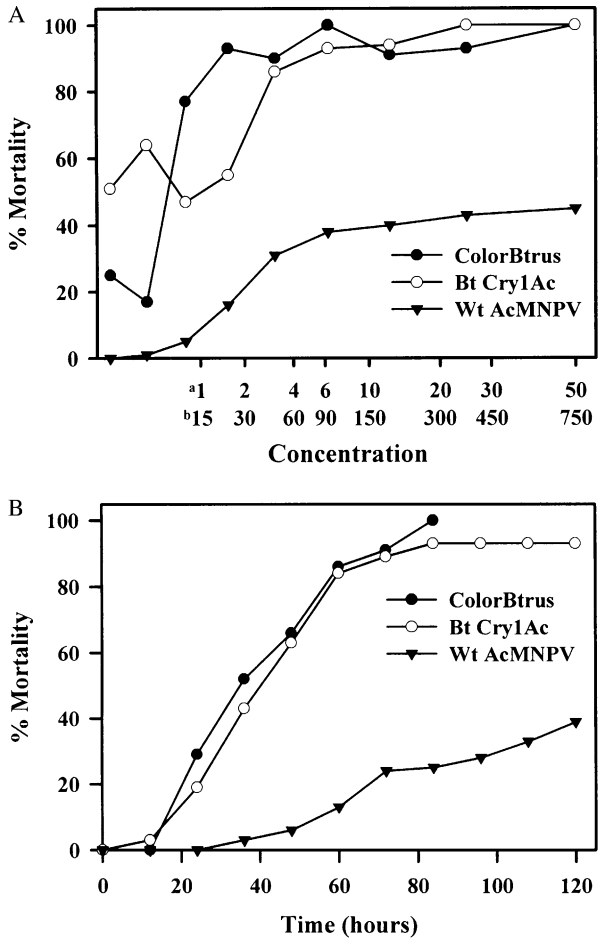

Fig. 5. Insecticidal activity of ColorBtrus polyhedra. (A) Dose response. One hundred twenty-eight *P. xylostella* larvae were each fed on cabbage plugs treated with varying concentrations of ColorBtrus polyhedra or Cry1Ac toxin, and the % mortality recorded 5 days after treatment. The graph is drawn so that equivalent doses of Cry1Ac toxin and ColorBtrus polyhedra are aligned. Treatments with wild-type AcMNPV polyhedra were also carried out for comparison. (a) Concentration of Cry1Ac toxin (ng/ml). (b) Concentration of polyhedra ($\times 10^4$ OB/ml). (B) Time response. One hundred twenty-eight *P. xylostella* larvae were each inoculated with 9.375×10^5 OB/ml wild-type AcMNPV or ColorBtrus polyhedra, or with 6.25 ng/ml Cry1Ac toxin, and the cumulative mortality scored at 12 h intervals for 5 days.

4. Discussion

A major application of recombinant baculovirus technology has been to produce viruses with improved insecticidal properties. The most common approach has been to engineer a baculovirus to overproduce an insect-specific toxin. Because the toxin gene is expressed while the virus is replicating, the toxin is only produced within the body of the infected insect. We have recently developed an alternative approach to engineering baculoviruses whereby the foreign protein is actually incorporated into the viral occlusion bodies. This approach therefore allows an insecticidal protein to be delivered to the gut of the insect, as well as being produced within the body. Here, we applied this technology to the production of occlusion bodies incorporating the insecticidal Bt Cry1Ac toxin protein. Coexpression of native polyhedrin and a polyhedrin–Cry1Ac–GFP fusion protein gave rise to occlusion bodies that had incorporated Cry1Ac and GFP, and contained occluded virus. These occlusion bodies contained approximately 10 ng Cry1Ac toxin per 1.5×10^6 polyhedra. In vitro studies indicated that, despite being fused to other proteins at the N-terminal and C-terminal ends, and being presented on occlusion bodies, the Cry1Ac toxin remained accessible to proteases (Fig. 4). Both the infectivity and speed of action of ColorBtrus occlusion bodies are dramatically enhanced compared to wild-type virus. We have previously shown that the insecticidal properties of an AcMNPV recombinant that produces occlusion bodies containing GFP are very similar to wild-type AcMNPV (Je et al., 2003). Thus, we can exclude the possibility that GFP is contributing to the activity of ColorBtrus. Several previous reports have described recombinant baculoviruses expressing Bt toxins, but the insecticidal properties of these viruses were not significantly improved (Martens et al., 1990, 1995; Merryweather et al., 1990; Ribeiro and Crook, 1993). It seems likely our approach was successful because the toxin is delivered to the insect gut, its normal active site. With previous Bt-toxin viruses, the toxin would have been produced in infected cells within the insect.

Bt subsp. *kurstaki*, from which Cry1Ac is derived, is the most widely used insecticidal strain because it is highly toxic to a range of lepidoptera. However, it is not effective against *Spodoptera* species such as the beet armyworm, *S. exigua* or the tobacco cutworm, *S. litura* (Bai et al., 1993). *S. exigua* is susceptible to AcMNPV, and ColorBtrus gives rise to fluorescent polyhedra in

Table 1
Median lethal dose (LD_{50}) and median survival time (ST_{50}) values of ColorBtrus and wild-type AcMNPV against *P. xylostella* larvae

Virus	LD_{50}			ST_{50}*		
	n[a]	Polyhedra/larva	95% CI	n[a]	h	95% CI
AcMNPV	768	2798.3	1483.8–4884.1	128	92.8	88.0–98.0
ColorBtrus	1280	28.3	21.7–35.7	128	33.9	30.0–40.3

* The ST_{50} was determined at 10,240 polyhedra per larva for AcMNPV and 80 polyhedra per larva for ColorBtrus.
[a] Number of insects.

the fat body of infected larvae (data not shown). Thus, *S. exigua* could be used for the in vivo production of ColorBtrus occlusion bodies.

The ColorBtrus virus has several highly advantageous features as a recombinant insecticide. It has a dual mode of action, killing either by Cry1Ac toxicity or by viral pathogenesis. ColorBtrus occlusion bodies should be toxic to all species susceptible to Cry1Ac toxin, including species not susceptible to AcMNPV. Conversely, insects susceptible to AcMNPV but insensitive to Cry1Ac can be killed by viral replication. The selection of resistance to Cry1Ac should therefore be reduced, as the few individuals within the population which acquire Cry1Ac resistance would also have to simultaneously evolve resistance to baculovirus infection, in order to survive and pass on its Cry1Ac resistance genes to progeny. In addition, the strategy used in this study can be applied to other Bt toxins or other gut active toxins with different modes of action, and the alternate use of these kinds of recombinant viruses may further reduce the emergence of resistance.

In species sensitive to the toxin, the improvement to the pesticidal properties of the virus is remarkable; the median lethal dose of ColorBtrus is approximately two orders of magnitude lower than AcMNPV, and the median survival time of infected insects is reduced to slightly more than 1 day. To our knowledge, no other recombinant baculovirus capable of causing host mortality from 1 day p.i. has been described (Black et al., 1997; Bonning and Hammock, 1996; Hammock et al., 1990; Hughes et al., 1997; Maeda, 1989, 1995; O'Reilly and Miller, 1991; Stewart et al., 1991; Tomalski and Miller, 1991; Wood and Granados, 1991). Furthermore, infected insects cease to feed (data not shown), consistent with pathology due to Cry1Ac (Gill et al., 1992; Hofte and Whiteley, 1989). Thus, the virus should arrest feeding damage to the host plant almost immediately after infection. Finally, ColorBtrus occlusion bodies are fluorescent under UV (Chalfie et al., 1994), enabling the rapid monitoring/detection of infected insects (Chao et al., 1996) and facilitating studies of virus transmission in the field. Thus, ColorBtrus represents a novel recombinant insecticide that combines positive attributes of both baculoviruses and Cry1Ac toxin. The technology used can easily be applied to the expression of other Bt or gut active toxins and/or to the engineering of other baculoviruses.

Acknowledgment

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References

Adang, M.J., Staver, M.J., Rocheleau, T.A., Leighton, J., Barker, R.F., Thompson, D.V., 1985. Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and their toxicity to *Manduca sexta*. Gene 36, 289–300.

Bai, C., Degheele, D., Jansens, S., Lambert, B., 1993. Activity of insecticidal crystal protein and strains of *Bacillus thuringiensis* against *Spodoptera exempta* (walker). J. Invertebr. Pathol. 62, 211–215.

Biever, K.D., Andrews, P.L., 1984. Susceptibility of lepidopterous larvae to *Plutella xylostella* nuclear polyhedrosis virus. J. Invertebr. Pathol. 44, 117–119.

Black, B.C., Brennan, L.A., Dierks, P.M., Gard, I.E., 1997. Commercialization of baculoviral insecticides. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum Press, New York, pp. 341–387.

Bonning, B.C., Hammock, B.D., 1996. Development of recombinant baculoviruses for insect control. Annu. Rev. Entomol. 41, 191–210.

Burden, J.P., Hails, R.S., Windass, J.D., Suner, M.M., Cory, J.S., 2000. Infectivity, speed of kill, and productivity of a baculovirus expressing the itch mite toxin Txp-1 in second and fourth instar larvae of *Trichoplusia ni*. J. Invertebr. Pathol. 75, 226–236.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., Prasher, D.C., 1994. Green fluorescent protein as a marker for gene expression. Science 263, 802–805.

Chao, Y.C., Chen, S.L., Li, C.F., 1996. Pest control by fluorescence. Nature 380, 396–397.

Doerrschott, J., Lichte, C.M., 1986. A triple ultrastructural immunogold staining method—application to the simultaneous demonstration of 3 hypophyseal hormones. J. Histochem. Cytochem. 34, 1101–1104.

Gill, S.S., Cowles, E.A., Pietrantonio, P.V., 1992. The mode of action of *Bacillus thuringiensis* endotoxins. Annu. Rev. Entomol. 37, 615–636.

Gershburg, E., Stockholm, D., Froy, O., Rashi, S., Gurevitz, M., Chejanovsky, N., 1998. Baculovirus-mediated expression of a scorpion depressant toxin improves the insecticidal efficacy achieved with excitatory toxins. FEBS Lett. 422, 132–136.

Hammock, B.D., Bonning, B.C., Possee, R.D., Hanzlik, T.N., Maeda, S., 1990. Expression and effects of the juvenile hormone esterase in a baculovirus vector. Nature 344, 458–461.

Hofte, H., Whiteley, H.R., 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53, 242–255.

Hughes, P.R., Wood, H.A., Breen, J.P., Simpson, S.F., Duggan, A.J., Dybas, J.A., 1997. Enhanced bioactivity of recombinant baculoviruses expressing insect-specific spider toxins in lepidopteran crop pests. J. Invertebr. Pathol. 69, 112–118.

Je, Y.H., Jin, B.R., Park, H.W., Roh, J.Y., Chang, J.H., Seo, S.J., Olszewski, J.A., O'Reilly, D.R., Kang, S.K., 2003. Baculovirus expression vectors that incorporate the foreign protein into viral occlusion bodies. Biotechniques 34, 81–87.

Maeda, S., 1989. Increased insecticidal effect by a recombinant baculovirus carrying a synthetic diuretic hormone gene. Biochem. Biophys. Res. Commun. 165, 1177–1183.

Maeda, S., 1995. Further development of recombinant baculovirus insecticides. Curr. Opin. Biotechnol. 6, 313–319.

Martens, J.W.M., Honee, G., Zuidema, D., Van Lent, J.W.M., Visser, B., Vlak, J.M., 1990. Insecticidal activity of a bacterial crystal protein expressed by a recombinant baculovirus in insect cells. Appl. Environ. Microbiol. 56, 2764–2770.

Martens, J.W.M., Knoester, M., Weijts, F., Groffen, S.J., Hu, Z., Bosch, D., Vlak, J.M., 1995. Characterization of baculovirus insecticides expressing tailored *Bacillus thuringiensis* CryIA(b) crystal proteins. J. Invertebr. Pathol. 66, 249–257.

Merryweather, A.T., Weyer, U., Harris, M.P.G., Hirst, M., Booth, T., Possee, R.D., 1990. Construction of genetically engineered bacu-

lovirus insecticides containing the *Bacillus thuringiensis* subsp. *kurstaki* HD-73 delta endotoxin. J. Gen. Virol. 71, 1535–1544.

O'Reilly, D.R., Miller, L.K., 1991. Improvement of a baculovirus pesticide by deletion of the *egt* gene. Bio/Technology 9, 1086–1089.

O'Reilly, D.R., Miller, L.K., Luckow, V.A., 1992. Baculovirus Expression Vectors: A Laboratory Manual. Oxford University Press, New York.

Padmavathamma, K., Veeresch, G.K., 1991. Effect of larval age and dosage of nuclear polyhedrosis virus on the susceptibility of diamondback moth, *Plutella xylostella*. Entomol. Exp. Appl. 60, 39–42.

Regev, A., Rivkin, H., Inceoglu, B., Gershburg, E., Hammock, B.D., Gurevitz, M., Chejanovsky, N., 2003. Further enhancement of baculovirus insecticidal efficacy with scorpion toxins that interact cooperatively. FEBS Lett. 537, 106–110.

Ribeiro, B.M., Crook, N.E., 1993. Expression of full-length and truncated forms of crystal protein genes from *Bacillus thuringiensis* subsp. *kurstaki* in a baculovirus and pathogenicity of the recombinant viruses. J. Invertebr. Pathol. 62, 121–130.

Shelton, A.M., Wyman, J.A., Cushing, N.L., Apfelbeck, K., Dennehy, T.J., Mahr, S.E.R., Eigenbrode, S.D., 1993. Insecticide resistance of diamondback moth (Lepidoptera: *Plutella*) in North America. J. Econ. Entomol. 86, 11–19.

Stewart, L., Hirst, M., Ferber, M.L., Merryweather, A.T., Cayley, P.J., Possee, R.D., 1991. Construction of an improved baculovirus pesticide containing an insect-specific toxin gene. Nature 352, 85–88.

Summers, M.D., Smith, G.E., 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agric. Exp. Station Bull., 1555.

Talekar, N.S., Shelton, A.M., 1993. Biology, ecology and management of the diamondback moth. Annu. Rev. Entomol. 38, 275–301.

Thomas, W.E., Ellar, D.J., 1983. *Bacillus thuringiensis* var *israelensis* crystal δ-endotoxin: effects on insect and mammalian cells in vitro and in vivo. J. Cell Sci. 60, 181–197.

Tomalski, M.D., Miller, L.K., 1991. Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. Nature 352, 82–85.

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J., McCawley, P., 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro 13, 213–217.

Wood, H.A., Granados, R.R., 1991. Genetically engineered baculoviruses as agents for pest control. Annu. Rev. Microbiol. 45, 69–87.