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NeuroBactrus, a Novel, Highly Effective, and Environmentally Friendly Recombinant Baculovirus Insecticide

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A novel recombinant baculovirus, NeuroBactrus, was constructed to develop an improved baculovirus insecticide with additional beneficial properties, such as a higher insecticidal activity and improved recovery, compared to wild-type baculovirus. For the construction of NeuroBactrus, the *Bacillus thuringiensis* crystal protein gene (here termed *cry1-5*) was introduced into the *Autographa californica* nucleopolyhedrovirus (AcMNPV) genome by fusion of the polyhedrin–*cry1-5*–polyhedrin genes under the control of the polyhedrin promoter. In the opposite direction, an insect-specific neurotoxin gene, *AaIT*, from *Androctonus australis* was introduced under the control of an early promoter from *Cotesia plutellae* bracovirus by fusion of a partial fragment of *orf603*. The polyhedrin–Cry1-5–polyhedrin fusion protein expressed by the NeuroBactrus was not only occluded into the polyhedra, but it was also activated by treatment with trypsin, resulting in an ~65-kDa active toxin. In addition, quantitative PCR revealed that the neurotoxin was expressed from the early phase of infection. NeuroBactrus showed a high level of insecticidal activity against *Plutella xylostella* larvae and a significant reduction in the median lethal time against *Spodoptera exigua* larvae compared to those of wild-type AcMNPV. Rerecombinant mutants derived from NeuroBactrus in which *AaIT* and/or *cry1-5* were deleted were generated by serial passages *in vitro*. Expression of the foreign proteins (*B. thuringiensis* toxin and AaIT) was continuously reduced during the serial passage of the NeuroBactrus. Moreover, polyhedra collected from *S. exigua* larvae infected with the serially passaged NeuroBactrus showed insecticidal activity similar to that of wild-type AcMNPV. These results suggested that NeuroBactrus could be recovered to wild-type AcMNPV through serial passaging.

Baculovirus is the largest family of insect viruses and is characterized by large double-stranded circular DNA genome with a size ranging from 80 to 200 kb (1). They are naturally occurring pathogens that are highly specific to one or a few related insect species, and the majority of baculovirus hosts are within the order *Lepidoptera* (2). No effects on nontarget species have been demonstrated. Baculoviruses have enveloped rod-shaped virions and two distinct phenotypes, budded virus (BV) and occlusion-derived virus (ODV), in a single cycle of infection (3). Whereas the BV is responsible for transmission of the virus from cell to cell, the ODV is responsible for horizontal transmission from insect to insect.

Baculoviruses have a long history of safe use as specific, environmentally benign insecticides because they have infectious particles that are protected in proteinaceous occlusion bodies called polyhedra, which allows for the formulation of biopesticides with easy application technology (4). However, their use has been limited by several factors, especially their slow pathogenicity (5). Depending on the strain of virus and pest insect species, it can take several days to weeks before the infected insect stops feeding. During this time, significant feeding damage can be caused to the crop. To produce baculoviruses with improved speed of kill or decreased effective feeding times, various foreign genes with a potential to increase insecticidal activity have been inserted into the baculovirus genome using recombinant DNA technology. Attempts to improve the relative effectiveness of baculoviruses include an expression of insect-specific toxins, insect hormones (e.g., juvenile hormone esterase, diuretic hormone, and protho-racicotropic hormone), and enzymes predicted to have deleterious effects on host physiology upon inappropriate expression (6). Among these approaches, the expression of insect-specific neurotoxins, including the mite toxin TxP-1 and the scorpion toxins AaIT, LqhIT1, and LqhIT2 resulted in a significant increase of pathogenicity (6–11).

Bacillus thuringiensis Cry toxins (Bt toxins) have been used as effective means for controlling pest populations. Bt toxin accumulates in large amounts during sporulation of *B. thuringiensis*, forming crystalline occlusions. When ingested by susceptible insects, the crystal is solubilized in the alkaline environment of the gut and activated by proteases that are 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 toxin within the context of baculovirus infection was predicted to gen-

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erate pores in cell membranes, leading to disruption of osmotic balance and then cell death. Although full-length or truncated forms of the Bt toxin genes have been inserted into the baculovirus genome to attempt to enhance the pathogenicity for insect larvae, 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 due to the mechanism of action of the Bt toxin (i.e., activation and activity within the insect midgut) (12–15). This lack of efficacy has been overcome by expression of the Bt toxin as a fusion protein in which the toxin is fused to the major occlusion body protein (polyhedrin) of the baculovirus (2). This recombinant baculovirus (ColorBtrus) expressing the polyhedrin-Bt toxin fusion protein as well as authentic polyhedrin produced recombinant polyhedra that occluded Bt toxin and released toxin in the insect midgut. Bioassays of the ColorBtrus demonstrated that its speed of action and pathogenicity are strikingly enhanced compared to the wild-type virus (2).

In spite of these improvements, however, these recombinant baculoviruses express foreign protein stably, which might cause problems related to the development of genetically modified organisms. Unfortunately, scientific and public aversion to field release of genetically modified organisms has stymied the potential for commercialization of these recombinant baculoviruses (16). Recently, to circumvent this issue, a novel recombinant baculovirus (Bactrus) was constructed by the insertion of the Bt toxin gene between the two polyhedrin genes under the control of the polyhedrin promoter (17). The Bactrus expressing polyhedrin-Bt toxin-polyhedrin fusion protein produced polyhedra containing Bt toxin and became less active during serial passaging as a result of deletion of the Bt toxin gene by homologous recombination between the two polyhedrin genes (18).

In this study, we describe the construction and characterization of an advanced recombinant baculovirus that expresses AaIT under the control of an early promoter from *Cotesia plutellae* bracovirus (CpBV) and produces polyhedra that incorporate the Bt toxin. The infectivity and speed of action of this virus were dramatically improved compared to the wild-type virus. In addition, the recombinant baculovirus became less active during serial passaging as a result of the deletion of AaIT and Bt toxin genes. These experiments pave the way for delivery of a "three-hit" approach: (i) direct uptake of gut-acting Bt toxin, (ii) prevention of neurotransmission by neurotoxin expression during the early phase of viral infection, and (iii) subsequent baculovirus infection of any insects that survive the initial toxin exposure.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* strain JM109 (TaKaRa, Japan) was used in all experiments. All restriction endonucleases and modifying enzymes were from Roche Applied Science (Germany).

Insect cells, insects, and viruses. The *Spodoptera frugiperda* cell line, Sf9, was maintained at 27°C in TC-100 medium (WelGene, Republic of Korea) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (WelGene, Republic of Korea) and subcultured every 3 to 4 days. *Plutella xylostella*, *Spodoptera exigua*, and *Spodoptera litura* larvae were obtained from a laboratory colony and reared at 25°C under a 16-h/8-h light/dark cycle with an artificial diet (19). The wild-type and recombinant AcMNPV used in the present study were propagated in Sf9 cells maintained in TC-100 medium.

Construction of the recombinant baculovirus. An ~2.4-kb fragment of the *cry1-5* gene corresponding to the active domain region was PCR-amplified from *B. thuringiensis* 2385-1 (20) and digested with XhoI and BglII. The resulting fragment was inserted into the pOBII transfer vector (17) and digested with restriction endonucleases with recognition sites located between the two polyhedrin genes to obtain pB(1-5)B. An ~4.8-kb restriction fragment containing a 5′ partial fragment of the *orf603* (corresponding to approximately bp −63 to +165 of the *orf603* open reading frame [ORF]) and the polyhedrin gene–*cry1-5* fusion gene was excised from pB(1-5)B by double digestion with NaeI and BglII and cloned into pAcUW-3006ProAaIT (21) that had been digested with EcoRV and BamHI to obtain pAcUWB5ΔB-3006AaIT. The *cry1-5*-polyhedrin fusion gene fragment was serially excised from the pB(1-5)B by double digestion with XhoI and SnaBI, and it was introduced into pAcUWB5ΔB-3006AaIT that had been digested with the same restriction endonucleases to obtain the final transfer vector, pNBt. The internal structure of the pNBt was verified by restriction endonuclease digestion and nucleotide sequencing analyses. Recombinant AcMNPV expressing Cry1-5 and AaIT under the control of the polyhedrin and CpBV ORF3006 promoters, respectively, was generated by cotransfection of pNBt with bAcGOZA (22) into Sf9 cells. Transfection was carried out using the Cellfectin (Invitrogen) transfection reagent according to the manufacturer's instructions, and the recombinant virus was purified by a plaque assay using Sf9 cells as previously described (23).

Serial passages of recombinant baculovirus. Sf9 cells were infected with the second passage of recombinant baculovirus (P2) at multiplicities of infection (MOI) of 0.1 and 10, respectively. At 4 days postinfection (p.i.), budded viruses (BVs) were collected and titrated with the endpoint dilution method (23). Infection of Sf9 cells with the BVs was performed at two different MOIs: 0.1 and 10. By repeating this step more than 15 times, passages P4, P6, P8, P10, P12, P14, and P15 were collected.

Southern hybridization. One microgram of viral DNA was digested with SalI and separated on a 0.8% agarose gel. The separated DNAs were transferred to Hybond N^+ filters (Amersham Biosciences). The EcoRV- and XbaI-digested polyhedrin gene fragment labeled with digoxigenin (DIG) using a DIG DNA labeling kit (Roche) was used as a probe. The prehybridization, hybridization, washing, and detection procedures were performed using the DIG luminescent detection kit (Roche) as described by the manufacturer.

Total RNA extraction, quantitative PCR (qPCR), and Northern dot blotting. Total RNA was isolated from infected Sf9 cells or infected larvae of *S. exigua* using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The extracted total RNAs were treated with DNase I (TaKaRa) to remove contaminating DNA, and the effectiveness of this procedure was validated by PCR.

Single-strand cDNA was synthesized from total RNAs using a SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. The qPCR was conducted with a 2×DyNAmo HS SYBR Green qPCR kit (Finnzymes, Finland) and a Chromo4 real-time thermal cycler (Bio-Rad). The 28S rRNA gene was used as a housekeeping reference gene as previously reported (24). The cycling profile used for qPCR was as follows: a preheating step for enzyme activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 30 s. The relative transcription level was calculated by using the $2^{-\Delta CT}$ method (25).

For Northern dot blot analysis, 10 μg of total RNA was denatured with glyoxal loading dye (Ambion) and loaded directly onto nylon membranes using a manifold dot blot system. The membranes were hybridized with DIG-labeled probes prepared using the DIG DNA labeling kit). The prehybridization, hybridization, washing, and detection procedures were carried out using the DIG luminescent detection kit as described by the manufacturer.

Confocal laser scanning microscopy. For the detection of expressed polyhedrin and the Cry toxin fusion protein, Sf9 cells were infected with viruses and collected at 96 h p.i. The resulting cell pellets were fixed in 10% formalin for 1 h, dehydrated in grade ethanol, and then embedded in a paraffin block. These paraffin blocks were cut into 4-μm-thick sections and mounted on a glass slide. These sections were deparaffinised in xylene

and hydrated by serial soaking in ethanol, distilled water and phosphate-buffered saline (PBS). The washed slides were bound with anti-polyhedrin primary antibody in mouse serum or anti-Cry1-5 primary antibody in rabbit serum diluted in PBT (PBS containing 2% Triton X-100) at 4°C overnight, washed three times with PBS, and then incubated for 2 h with Alexa Fluor 488-conjugated anti-mouse or Alexa Fluor 568-conjugated anti-rabbit secondary antibodies (Invitrogen), respectively. After incubation, the slides were washed three times with PBS and subjected to confocal scanning microscopic analysis. For the detection of expressed AaIT, the cells were infected with viruses and incubated in chambered cover glasses (Nunc, Denmark) for 18 and 36 h p.i. The cells were washed with PBS, fixed for 5 min in Bouin's fixative and then washed three times with PBT. The washed cells were bound with anti-AaIT primary antibody in sheep serum diluted with PBT at 4°C overnight. Then, the cover glasses were washed three times in PBT, followed by incubation for 2 h with Alexa Fluor 350-conjugated anti-sheep secondary antibody. After incubation, the cover glasses were washed three times with PBST and subjected to confocal laser scanning microscopic analysis.

SDS-PAGE and immunoblot analysis. Sf9 cells (10^6 cells) infected with a recombinant baculovirus were gently washed twice with excess PBS (140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.3]) and analyzed on 12% polyacrylamide gels. The polyhedra were released from the infected cells with cell lysis buffer (50 mM Tris-HCl [pH 8.0], 0.4% SDS, 10 mM EDTA, 5% β-mercaptoethanol), washed with an excess amount of PBS, and analyzed on 12% polyacrylamide gels. In addition to confirm the activation of the Cry1-5 protein, trypsin was added at a concentration of 2 μg/ml, and the samples were analyzed on 12% polyacrylamide gels. Recombinant polyhedra were purified from infected larvae of *S. exigua* as previously reported (23) and analyzed on 12% polyacrylamide gels.

For immunoblotting, SDS-PAGE was performed at 100 V for 45 min, and the proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences) using a Semiphor semidry transfer unit TE77 (Hoefer Sciences) at 50 V for 1.5 h. After transfer, the PVDF membrane was blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 (PBST). The membrane was bound with AcMNPV polyhedrin polyclonal antisera diluted 1:2,000 (in mouse serum) or Cry1-5 polyclonal antisera diluted 1:2,000 (in rabbit serum) for 1 h at room temperature and washed in an excess volume of PBST. The membrane was then incubated with anti-mouse or anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Amersham Biosciences) diluted 1:10,000 for 1 h at room temperature. Detection was carried out using an enhanced chemiluminescence plus Western blotting detection system (Amersham Biosciences) according to the manufacturer's instructions.

Bioassays. Recombinant polyhedra produced in Sf9 cells infected with recombinant virus from the fifth passages were released with cell lysis buffer and washed with PBS. Serial dilutions of polyhedra (17,700, 8,880, 4,440, 2,220, 1,110, 555, and 278 polyhedra/cm^2) were applied on a disc of Chinese cabbage leaf (2 by 2 cm^2) by spreading and were allowed to air dry. One hundred *P. xylostella* second-instar larvae per treatment were exposed by feeding on the treated cabbage leaf plugs. For bioassays against *S. exigua* and *S. litura*, 100 third-instar larvae per treatment were fed on cabbage leaf plugs treated with five concentrations (1,000,000, 500,000, 100,000, 25,000, and 12,500 polyhedra/cm^2). The mortality was calculated by counting the dead larvae at intervals of 24 h up to 2 weeks, and the median lethal concentration (LC_{50}) was calculated using a Probit analysis (26). The median lethal time (LT_{50}) was determined at a dose of 8,880 (for *P. xylostella*) or 5×10^6 (for *S. exigua* and *S. litura*) polyhedra per larvae using a Probit analysis plot as described by Russell et al. (26). For diet refusal assays, 20 larvae were fed on cabbage leaf plugs (3 by 4 cm^2) contaminated with polyhedra at a concentration of 5×10^3 polyhedral inclusion body (PIB)/cm^2 for *P. xylostella* larvae or 5×10^6 PIB/cm^2 for *S. exigua* and *S. litura* larvae.

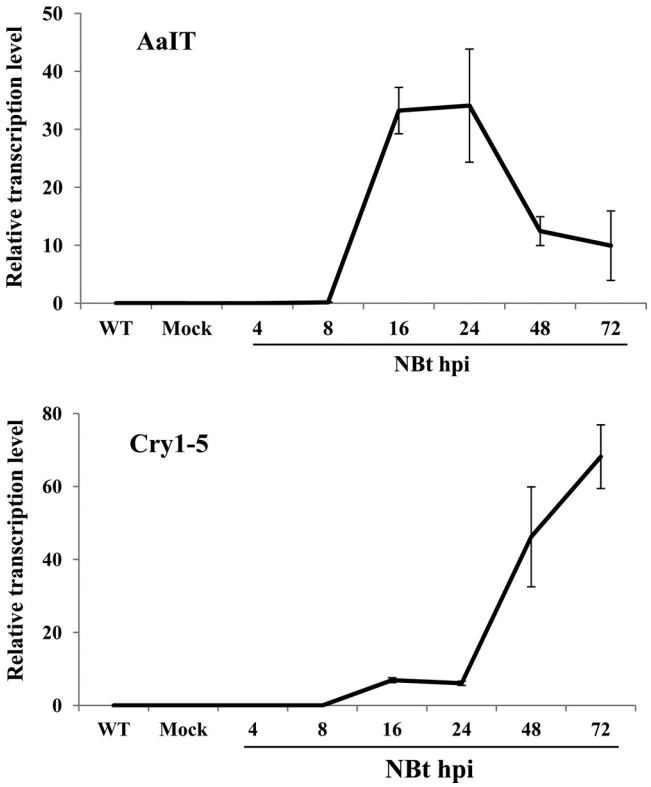

FIG 1 Transcription of the *AaIT* and *cry1-5* in Sf9 cells infected with NeuroBactrus. The data from qPCR run in triplicate were normalized to *28S rRNA* transcription. Sequence-specific amplification was detected by the fluorescent signal of SYBR green I with a Chromo4 real-time PCR detector. For data comparison, the results were adjusted to approximately 50 (*AaIT*) or 80 (*cry1-5*) arbitrary units.

RESULTS

Construction of a recombinant baculovirus expressing insecticidal toxins. To generate a novel recombinant baculovirus, a transfer vector, pNBt, was constructed (see Fig. S1 in the supplemental material). In this transfer vector, the *cry1-5* Bt toxin gene was fused in frame between the two polyhedrin genes under the control of the polyhedrin promoter (see Fig. S2 in the supplemental material). The vector also included scorpion neurotoxin, *AaIT*, under the control of the early promoter originating from CpBV (CpBV ORF3006 promoter). A 5′ partial fragment of the *orf603* gene was located upstream of the ORF3006 promoter in the same orientation for homologous recombination. The recombinant baculovirus, NeuroBactrus, was generated by cotransfection of bAcGOZA DNA with pNBt and purified using a plaque assay. Phase-contrast microscopy and transmission electron microscopy revealed that NeuroBactrus produced polyhedra in which the virions were normally occluded (see Fig. S3 in the supplemental material).

Expression of insecticidal toxins by the recombinant baculovirus. The transcription of *cry1-5* and *AaIT* in Sf9 cells infected with the NeuroBactrus was investigated by qPCR analysis (Fig. 1). Whereas the *cry1-5* gene was transcribed abundantly under the control of the very late polyhedrin promoter after 48 h p.i., transcription of the *AaIT* began at 8 h p.i. and reached a peak at 24 h p.i. under the control of the CpBV early promoter.

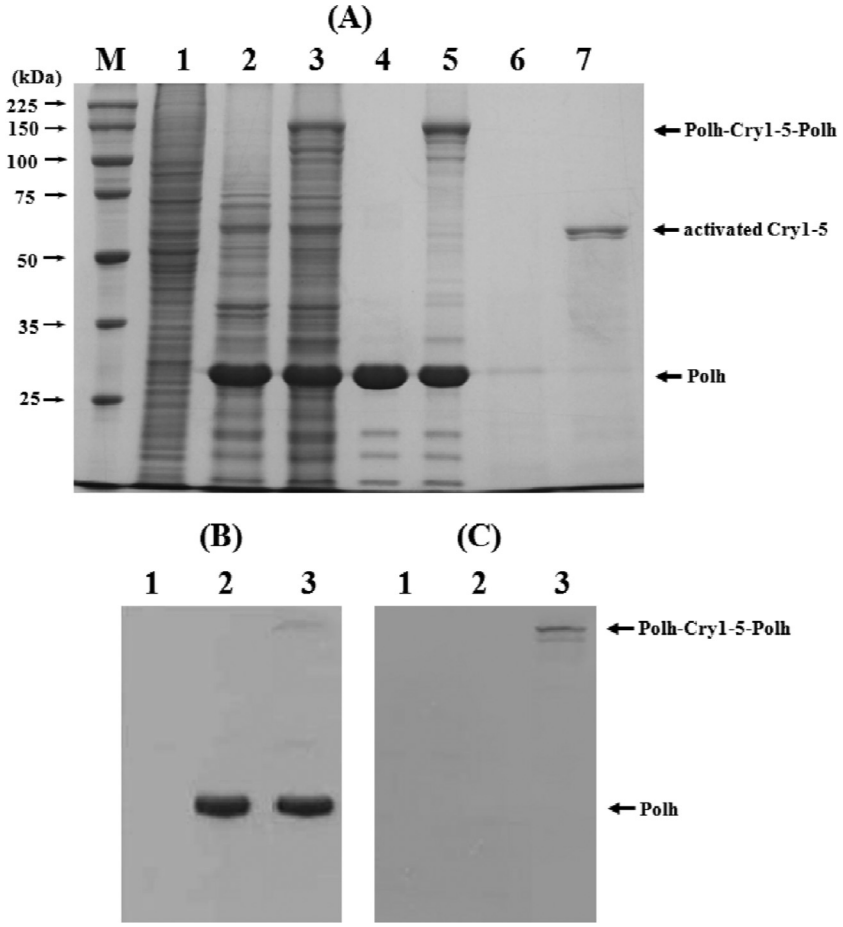

FIG 2 SDS-PAGE and immunoblot analyses of the fusion protein. (A) Infected Sf9 cells, purified polyhedra, and trypsin-treated polyhedra were electrophoresed in 12% polyacrylamide gels. (B and C) Immunoblotting was performed against purified polyhedra using anti-polyhedrin (B) or anti-Cry1-5 (C) polyclonal antisera. Lanes: M, protein molecular weight marker; 1, mock-infected Sf9 cells; 2, 4, and 6, wild-type AcMNPV; 3, 5 and 7, NeuroBactrus; 1, 2, and 3, cell lysates; 4 and 5, purified polyhedra; 6 and 7, trypsin treated.

Immunocytochemistry using fluorescence microscopy with specific antibodies revealed that both AaIT and Cry1-5 were expressed in Sf9 cells infected with the NeuroBactrus (see Fig. S4 in the supplemental material). In contrast, there were no signals corresponding to AaIT or Cry1-5 in wild-type *Autographa californica* nucleopolyhedrovirus (AcMNPV)-infected cells, while a fluorescent signal for AaIT (blue) was detected throughout the cytoplasm of NeuroBactrus-infected cells. Interestingly, the fluorescent signals for polyhedrin (green) and Cry1-5 (red) were nearly overlapping in NeuroBactrus-infected cells, indicating that the fusion protein was successfully incorporated into the occlusion bodies.

Characterization of polyhedra expressing Bt toxin. To confirm the incorporation of the polyhedrin–Cry1-5–polyhedrin fusion protein into the polyhedra produced by the NeuroBactrus, SDS-PAGE and immunoblot analyses were performed (Fig. 2). By SDS-PAGE, a 150-kDa band that is absent from the wild-type AcMNPV-infected cells was detected in the cell lysate and purified polyhedrin samples from the NeuroBactrus (Fig. 2A). Immunoblot analysis with antiserum specific for polyhedrin or Cry1-5 confirmed that this band includes the polyhedrin and Cry1-5 Bt toxins (Fig. 2B and C). In addition, this 150-kDa protein was identified as the polyhedrin–Cry1-5–polyhedrin fusion protein by amino acid sequence analysis using in-gel digestion and Nano-ESI QIT mass (data not shown). In addition to the fusion protein, a 30-kDa band corresponding to the native polyhedrin was detected in the infected cell lysate and purified polyhedra from the NeuroBactrus, confirming the homologous recombination between the two polyhedrin genes (Fig. 2A and B). Bt toxins are normally synthesized as inactive precursors and cleaved by proteases present in the host's gut to release the active toxin fragments. After cleavage with trypsin, the fusion protein band was reduced to ~65 kDa, which is the same size as the activated Cry1-5 Bt toxin (Fig. 2A). These data indicate that the active fragment of the Cry1-5 toxin could be released from the fusion protein by proteases, suggesting that the fusion protein will be active in the gut of host insects.

To investigate the productivity of recombinant polyhedra of the NeuroBactrus in insect cells by means of monolayer culture, Sf9 cells were infected with the virus at various MOIs, and the polyhedral productivity in infected cells was examined at 7 days p.i. Overall, the polyhedral productivity of the NeuroBactrus was comparable to that of the wild-type AcMNPV at all of the MOIs investigated. In addition, the higher MOI the infected into Sf9

TABLE 1 Production of recombinant polyhedra of the NeuroBactrus in Sf9 cells

Virus	Mean no. of polyhedra (10^6 PIB/ml) ± SD[a]		
	MOI = 0.1	MOI = 1.0	MOI = 10.0
AcMNPV	6.3 ± 0.4	4.2 ± 0.2	2.5 ± 0.4
NeuroBactrus[b]	5.8 ± 1.0	4.3 ± 0.3	2.2 ± 0.3

[a] 10^6 Sf9 cells were infected with the virus at a corresponding MOI with a 3-ml working volume.
[b] The NeuroBactrus P5 inoculum was used for infection of the Sf9 cells.

cells, the lower the polyhedral productivity of both viruses observed (Table 1).

Insecticidal activity of the recombinant baculovirus. To evaluate the insecticidal activities of the NeuroBactrus by comparing it with the wild-type AcMNPV, a series of bioassays against the larvae of *P. xylostella*, *S. litura* and *S. exigua* was carried out (Table 2). Both the LC_{50} and LT_{50} for the recombinant baculovirus against *P. xylostella* larvae were significantly lower than those of the wild-type AcMNPV. For *S. exigua* larvae, NeuroBactrus showed an ca. 50% decrease in the LT_{50} compared to that of wild-type AcMNPV. In addition, the time-mortality response for the NeuroBactrus against *S. exigua* larvae was dramatically faster than for the wild-type AcMNPV, presenting a mortality rate of over 40% at 2 days postinoculation (see Fig. S5 in the supplemental material). Whereas no significant differences were observed in the dose and time responses for the NeuroBactrus against *S. litura* larvae compared to the wild-type AcMNPV, the feeding-related damage to cabbage leaves treated with NeuroBactrus was significantly reduced (see Fig. S6 in the supplemental material).

Recovery of the wild-type genotype by homologous recombination. The NeuroBactrus contains two foreign genes, *AaIT* and *cry1-5*, which are fused at both ends with a polyhedrin gene or a partial fragment of the *orf603* gene, respectively. This genomic arrangement might enable recovery to the native genome by double homologous recombination between the two polyhedrin genes and between the two *orf603* partial fragments (see Fig. S7 in the supplemental material). In order to investigate the occurrence of this homologous recombination, plaque purification was carried out with the NeuroBactrus inoculum passaged 10 times in Sf9 cells. PCR and Southern hybridization analyses revealed that three types of recombinants were generated after passages of the NeuroBactrus in cultured cells (Fig. 3). As shown in Fig. S7 in the supplemental material, NBt-DelA was generated by homologous recombination between the polyhedrin genes. In contrast, the NBt-Del5 was generated by homologous recombination between the partial fragments of *orf603*. In particular, the doubly recombined NBt-DelA5 mutant was presumed to have an intact genomic structure of the AcMNPV, thereby representing the recovery of the wild-type genotype.

Expression of insecticidal toxins during serial passaging. To investigate the expression of insecticidal proteins during serial passaging, Sf9 cells or *S. exigua* larvae were infected with the serially passed NeuroBactrus. While the polyhedrin gene was transcribed constitutively, transcription from both of the *AaIT* and *cry1-5* genes was continuously reduced during *in vitro* serial passaging (see Fig. S8 in the supplemental material). qPCR analysis revealed that transcription of these two genes was more dramatically reduced during *in vivo* serial passaging (Table 3). Restriction endonuclease digestion patterns of viral DNA at different *in vitro* passages clearly demonstrated that the reduced transcription of these toxin genes was caused by specific deletion of toxin genes rather than the generation of defective interfering (DI) particles, which could result in the deletion of copies of these genes (Fig. 4). In SDS-PAGE analysis, the expression level of the polyhedrin–Cry1-5–polyhedrin fusion protein was also decreased during serial passaging both *in vitro* and *in vivo*. Reduction of the fusion protein expression occurred more rapidly in Sf9 cells infected with the recombinant virus passaged at a low MOI than in cells passaged at a high MOI (Fig. 5). Moreover, this decrease was more dramatic *in vivo*, showing almost no fusion protein from the fifth passage (Fig. 6). In the same context, the insecticidal activity of the recombinant polyhedra produced by the NeuroBactrus against *S. exigua* larvae was also decreased during serial passaging with a trend that was similar to the results observed by SDS-PAGE (Table 4).

DISCUSSION

For the development of an improved baculovirus insecticide, two major aims were considered here for the construction of recombinant AcMNPV. The first aim of the genetic modification was to increase the pathogenicity and the speed at which the target pests were killed or to reduce their feeding damage. A number of approaches were adopted, including the expression of insect-specific toxins or other proteins that are predicted to have noxious effects on the physiology of the host insect (6). Among these approaches, the expression of insect-specific scorpion neurotoxin, AaIT, derived from the North African scorpion, resulted in a significant increase in the pathogenicity of baculovirus (9, 10). To date, all of the field trials using recombinant AcMNPV constructs expressing AaIT have shown a reduced level of feeding damage in comparison to wild-type AcMNPV-treated control plants (6, 27–29). Thus, in the present study, the *AaIT* gene was selected for the construction of NeuroBactrus.

Alternatively, there have been several reports on the improvement of insecticidal activity by expressing the Bt Cry protein toxin

TABLE 2 Median lethal concentrations and median lethal times for NeuroBactrus against three lepidopteran species

Virus	Median (95% confidence interval)[a]					
	P. xylostella		*S. litura*		*S. exigua*	
	LC_{50} (10^3 PIB/cm^2)	LT_{50}* (days)	LC_{50} (10^5 PIB/cm^2)	LT_{50}† (days)	LC_{50} (10^4 PIB/cm^2)	LT_{50}† (days)
AcMNPV	>17.7	NC[b]	>10.0	NC[c]	3.6 (2.2–5.2)	4.8 (4.6–5.1)
NeuroBactrus	1.9 (1.2–2.9)	1.7 (1.6–1.8)	>10.0	NC[c]	2.5 (1.5–3.6)	2.5 (2.1–2.8)

[a] LC_{50}, median lethal concentration; *, median lethal times (LT_{50}) were calculated at 8,890 PIB/cm^2; †, LT_{50} values were calculated at 5×10^6 PIB/cm^2.
[b] The LT_{50} value was not calculable (NC) because of only 8.0% mortality at a dose of 8,890 PIB/cm^2.
[c] The LT_{50} value was not calculable (NC) because of no mortality at a dose of 5×10^6 PIB/cm^2.

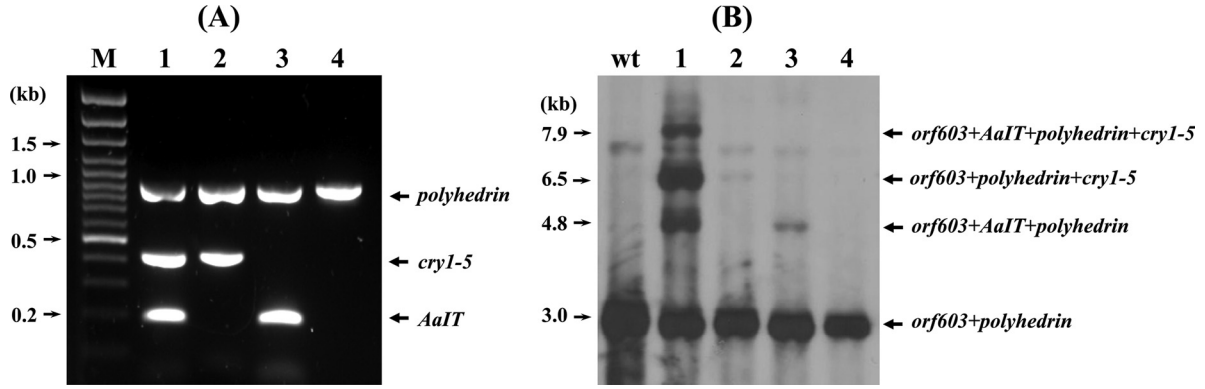

FIG 3 (A and B) Verification of the genomic structure of the NeuroBactrus and its recombinants by PCR and Southern blot analyses. (A) The polyhedrin, *cry1-5*, and *AaIT* genes were PCR amplified using specific primer sets. (B) Viral DNA samples were digested with SalI and electrophoresed on a 0.8% agarose gel. The DNAs were hybridized with a labeled polyhedrin gene probe. Lanes: M, 100-bp DNA ladder; wt, wild-type AcMNPV; 1, NeuroBactrus; 2, NBt-DelA; 3, NBt-Del5; 4, NBt-DelA5.

in baculovirus. Because this Bt toxin has been demonstrated as an effective means of controlling pest populations, full-length or truncated forms of the Bt toxin genes were inserted into the baculovirus genome to enhance the pathogenicity toward insect larvae (13). However, Bt toxin expression in these studies did not improve the virulence of the virus. To solve this problem, Chang et al. (2) took a novel and apparently highly successful approach for the delivery of Bt toxin using a recombinant baculovirus, ColorBtrus, which occludes the toxin within its polyhedra. Their strategy was based upon the coexpression of native polyhedrin and a foreign protein fused with polyhedrin from the same baculovirus. ColorBtrus showed significantly higher insecticidal activity against *P. xylostella* larvae. However, the pathogenicity of ColorBtrus against *S. exigua* larvae was similar to that of wild-type AcMNPV because the occluded Bt toxin, Cry1Ac, has a low toxicity against *S. exigua* larvae. Therefore, in this research, new attempts were made to construct a recombinant baculovirus that incorporates the other Cry1-type Bt toxin, Cry1-5, which has high toxicity against both *P. xylostella* and *S. exigua* larvae (20).

The second aim of the present study was the construction of an environmentally friendly recombinant baculovirus that can be reverted back to wild-type virus. In spite of the improvements in pathogenicity, most of the recombinant viruses that have been constructed to date are characterized by the expression of stable, recombinant protein, which might lead to environmental problems due to the introduction of genetically modified organisms.

TABLE 3 qPCR analysis for the transcription of *AaIT* and *cry1-5* in *S. exigua* larvae infected with serially passaged NeuroBactrus[a]

Virus	Passage	*AaIT* RTL (10^{-5})	*AaIT* SD (10^{-5})	*cry1-5* RTL (10^{-3})	*cry1-5* SD (10^{-3})
AcMNPV		—	—	—	—
NeuroBactrus	P0	3.70	0.43	2.66	1.2
	P2	0.64	0.28	0.13	0.05
	P4	0.10	0.04	—	—

[a] Relative transcription level (RTL) data from qPCR run in triplicate were normalized to the transcription of the polyhedrin gene. For comparison of the data, the results were adjusted to approximately 45 (*AaIT/polh*) or 40 (*cry1-5/polh*) arbitrary units. –, not detected.

To overcome this drawback, another recombinant baculovirus, Bactrus, was constructed by the insertion of the *B. thuringiensis cry* gene between two polyhedrin genes of AcMNPV to be deleted by homologous recombination during serial passaging (17, 18). In the present study, NeuroBactrus was constructed by the insertion of the Bt toxin gene between two polyhedrin genes under the control of the polyhedrin promoter and by the insertion of an insect-specific neurotoxin gene into another homologous recom-

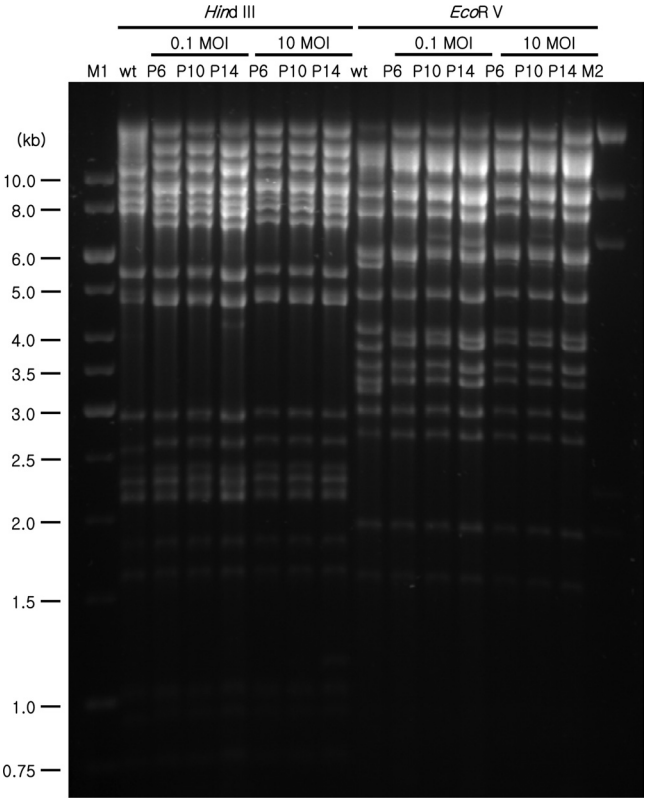

FIG 4 Restriction endonuclease digestion patterns of viral genomic DNA extracted from NeuroBactrus serially passed in Sf9 cells. Lanes: M1, 1-kb DNA ladder; wt, AcMNPV; P6 to P14, NeuroBactrus at the corresponding passage numbers; M2, λ DNA digested with HindIII.

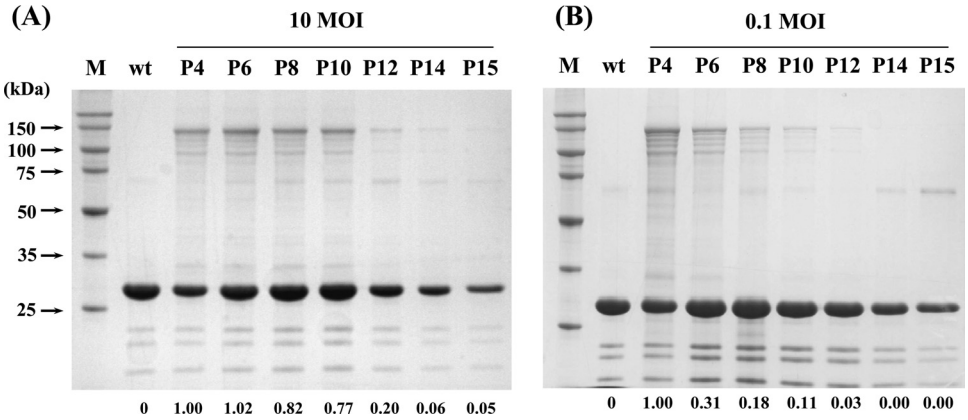

FIG 5 SDS-PAGE analysis of the polyhedra produced from Sf9 cells infected with serially passaged NeuroBactrus. The relative density values at the bottom of the lanes were determined by densitometric scanning of the gels. Lanes: M, molecular weight marker; wt, AcMNPV; P4 to P15, NeuroBactrus at the corresponding passage numbers.

bination region, the *orf603* fragment, under the control of the early promoter from CpBV.

The NeuroBactrus expressed AaIT in infected Sf9 cells, which could be secreted into hemolymph in insects and affect the insect nervous system. Previously, an AcMNPV construct expressing AaIT, Ac3006ProAaIT, in which AaIT is expressed under the control of the CpBV early promoter, showed improved pathogenicity with a 27% reduction of the LT_{50} compared to that of wild-type AcMNPV (21). The CpBV ORF3006 promoter used for expression of AaIT in this recombinant virus showed activity as strong as ca. 35% of that of the polyhedrin promoter in the hemolymphs of *S. exigua* larvae (30). The fused Cry1-5 toxin could be activated in the midgut of host insects through proteolytic cleavage of the toxin by proteases. The insecticidal activities of the NeuroBactrus against *P. xylostella* and *S. exigua* larvae were higher than those of the wild-type virus. This was because the Cry1-5 toxin occluded in the polyhedra and because the AaIT neurotoxin expression at an early stage of viral infection contributed to shortening the time to death of the pest insects. Because both Cry1-5 toxin and AcMNPV were found to be nontoxic to *S. litura* larvae, the LC_{50} and LT_{50} values for the NeuroBactrus against *S. litura* larvae could not be calculated. However, the larvae refused the NeuroBactrus-contaminated diet, and the feeding dose and speed were less than those in the AcMNPV-contaminated diet. These results suggest that the NeuroBactrus is an effective biological pesticide in terms of protection from pest insects and the maintenance of crop yields.

Furthermore, NeuroBactrus reverted to the wild-type genotype during *in vitro* and *in vivo* serial passaging. Deletion mutants with neither or both of the foreign genes were generated during viral replication within the hosts, which was confirmed by PCR and Southern hybridization. The proportion of deletion mutants, especially NBt-DelA5, gradually increased, and for that reason, the expression of both Cry1-5 and AaIT decreased during passaging. For *in vitro* serial passaging, it has been reported that the number of viral replication events in infected cells at a low MOI is larger than at a high MOI (17, 31). The larger the numbers of replication times, the more opportunities for homologous recombination, which resulted in a decreased level of foreign protein expression. The production of Cry1-5 Bt toxin was maintained somewhat constantly until the eighth through tenth passages *in vitro* at a high MOI, at which time the fusion proteins were produced at levels of 82 and 77%, respectively, compared to that at the fourth passage. Although the expression level of AaIT was also reduced during serial passaging, the rate of reduction was smaller than that of Cry1-5. Because the length of the polyhedrin gene involved in homologous recombination is longer than that of the *orf603* fragment, it is assumed that the possibility of homologous recombination between the polyhedrin genes was higher than that between the *orf603* fragments. Although the expression of both

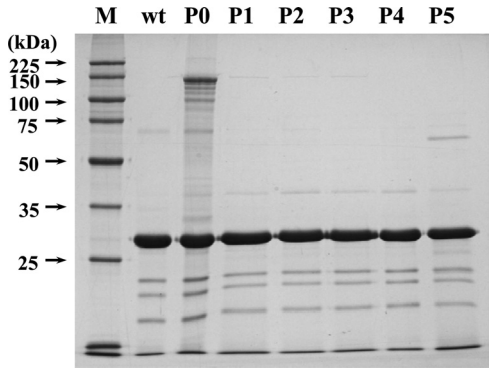

FIG 6 SDS-PAGE analysis of the polyhedra produced from *S. exigua* larvae infected with serially passaged NeuroBactrus. Lanes: M, molecular weight marker; wt, AcMNPV; P0 to P5, NeuroBactrus at the corresponding passage numbers.

TABLE 4 Median lethal times for NeuroBactrus during *in vivo* serial passages against *S. exigua* larvae

Virus	Passage	LT_{50} (days)[a]	95% confidence interval (days)
AcMNPV		4.8[a]	4.6–5.1
NeuroBactrus	P0	2.5[b]	2.1–2.8
	P1	4.8[a]	4.2–5.4
	P2	4.4[a]	4.3–4.5
	P3	4.2[a]	4.1–4.4
	P4	4.0[a]	3.8–4.1

[a] Median lethal time (LT_{50}) values were calculated at 5×10^6 PIB/cm^2. Significant differences are indicated by superscript letters ($P < 0.05$, Tukey's honestly significant difference test groups).

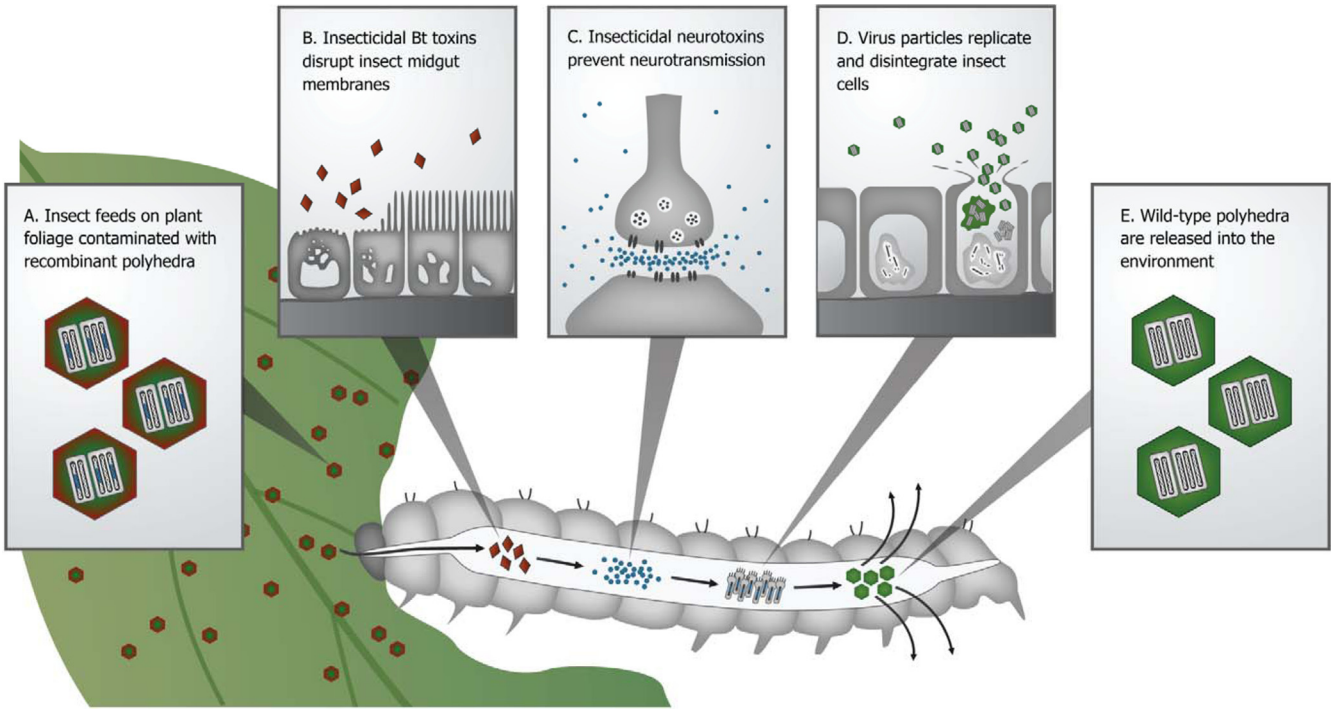

FIG 7 Conceptual diagram for the pathogenesis of NeuroBactrus against its host insects.

Cry1-5 and AaIT also continuously decreased during *in vivo* serial passaging, the decrease in their expression was more drastic compared to that observed during *in vitro* serial passaging. This may be due to the fact that relatively larger numbers of viral replication are involved *in vivo* pathogenicity compared to that in *in vitro* pathogenicity.

In conclusion, a novel recombinant baculovirus, NeuroBactrus, was constructed to develop an improved baculovirus insecticide. This virus has three modes of action to kill its insect hosts, as shown in Fig. 7. At first, a large proportion of the pest insects that ingest this virus would be killed by Bt toxin delivered to the insect midgut. Insect larvae that survived the Bt toxin exposure would be controlled by the subsequent neurotoxin expression or baculovirus replication. Finally, this virus will revert to the wild-type genotype, and it can therefore be safely released into the environment. Consequently, NeuroBactrus could be a good model for effective and environmentally friendly recombinant baculovirus pesticide development.

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