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Characterization of an Improved Recombinant Baculovirus Producing Polyhedra that Contain *Bacillus thuringiensis* Cry1Ac Crystal Protein

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Abstract A novel recombinant baculovirus, Bactrus, was constructed by the insertion of the *Bacillus thuringiensis cry1Ac* gene between two polyhedrin genes of *Autographa californica* nucleopolyhedrovirus (AcNPV) under the control of the polyhedrin gene promoter. Polyhedra produced by Bactrus in insect cells were incorporated with 130 kDa of polyhedrin-Cry1Ac-polyhedrin fusion protein, and 30 kDa of intact polyhedrin, resulting from a homologous recombination between two polyhedrin genes, was also expressed. The insecticidal activity of Bactrus against *Spodoptera exigua* larvae was similar to that of AcNPV, but it showed significantly higher toxicity towards *Plutella xylostella* larvae in comparison with that of AcNPV. The expression level of fusion protein and the insecticidal activity of recombinant polyhedra produced by the Bactrus against *P. xylostella* larvae were decreased after serial passages. In conclusion, the Bactrus had improved insecticidal activity and returned to wild-type AcNPV after several passages.

Key words: Fusion protein, Cry1Ac, polyhedrin, Bactrus, *Plutella xylostella*, *Spodoptera exigua*

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 infect only 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 insecticidal activity [2, 16]. To overcome these defects of baculovirus, several strategies have been planned and tried.

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The application of baculovirus insecticide together with chemicals such as spinosad [22], azadrachtin, and imidacloprid [12], optical brightener [27], and boric acid [5] has been tried. On the other hand, genetically modified recombinant viruses, broadening the spectrum or accelerating the activity, have also been attempted. During the virus infection, expression of some proteins, such as hormones, enzymes, and toxins that interfere specifically with insect metabolism or metamorphosis, might enhance the insecticidal activity of baculovirus [31, 32]. An insect neuropeptide, diuretic hormone [15], and an insect enzyme, juvenile hormone esterase [6] that degrades an essential insect hormone, were the first foreign products shown to increase the natural insecticidal activity of baculovirus. The insect-specific neurotoxins derived from scorpions [17, 21, 29] and mites [30] have also been examined.

Earlier, there were a few studies on the improvement of viral insecticidal activity by expressing the *Bacillus thuringiensis* (Bt) δ-endotoxin protein in baculovirus [13, 19, 20, 24]. Although they produced a large amount of Bt δ-endotoxin protein, there was no enhancement observed in the pathogenicity of the recombinant baculovirus for its production in hemolymph or fat body, and the insecticidal mechanism of Bt δ-endotoxin protein has not yet been considered. Recently, a recombinant baculovirus producing polyhedra with Bt Cry1Ac crystal protein has shown significant increase in insecticidal activity and stable production of recombinant polyhedra [3]. In spite of these improvements, this recombinant has the characteristic to express stable recombinant protein, which might cause problems owing to the development of genetically modified organisms. In this study, therefore, the recombinant baculovirus, which produced polyhedra containing Bt Cry1Ac crystal protein and became less active in serial passage, was constructed, and the recombinant polyhedra produced in insect cells were characterized.

MATERIALS AND METHODS

Insect Cells and Viruses

The *Spodoptera frugiferda* cell line Sf9 was continuously maintained in TC-100 medium (Gibco, U.S.A.) supplemented with 10% FBS (Gibco, U.S.A.) at 27°C. The bAcGOZA [10, 11] was used as a parental virus for the production of recombinant baculovirus, and AcNPV was used as a control virus. The titer of viruses was determined by the end-point dilution method [23].

Polymerase Chain Reaction

For the amplification of a *cry1Ac* toxic fragment, two oligonucleotide primers, 5'-AACTCGAGATGGATTACAAT-CCGAAC-3' and 5'-AACTCGAGTGTTGCAGTAACTGG-AAT-3' were used. PCR reaction was performed with Pyrobest™ DNA polymerase (Takara Co., Japan), using a DNA Thermal Cycler (Perkin Elmer Co., U.S.A.) based on a 30-cycle program, with each cycle consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min.

Transfection

Co-transfection of bAcGOZA and pBactrus into Sf9 cells was carried out by using the Cellfectin™ transfection reagent (Invitrogen, U.S.A.) according to the manufacturer's instruction. Recombinant baculovirus was plaque purified in Sf-9 cells according to the method of O'Reilly *et al.* [23].

SDS-PAGE and Immunoblot Analysis

Insect cells infected with a recombinant baculovirus were gently washed twice with excess phosphate-buffered saline (PBS: 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) and analyzed in 12% polyacrylamide gel. Polyhedra were released from infected cells lysed with cell lysis buffer (50 mM Tris-HCl, pH 8.0, 0.4% SDS, 10 mM EDTA, 5% β-mercaptoethanol) and washed with an excess amount of PBS and analyzed in 12% polyacrylamide gel. To confirm the activation of Cry1Ac protein, gut-juice of *Bombyx mori* was treated and analyzed in 12% polyacrylamide gel.

For immunoblotting, SDS-PAGE was performed at 100 V for 45 min and blotted onto polyvinylidene difluoride (PVDF) membranes (Perkin-Elmer Co., U.S.A.) by using the Semiphor™ semi-dry transfer unit TE77 (Hoefer Sci., U.S.A.) at 50 V for 1.5 h. After transfer, the PVDF membrane was blocked with 5% non-fat dry milk in PBS having 0.1% Tween 20 (PBST). The membrane was bound with 1:2,000 diluted Cry1Ac or polyhedrin polyclonal antisera for 1 h at room temperature and was washed in an excess volume of PBST. Then, the membrane was incubated with 1:10,000 diluted HRP-conjugated secondary antibody (Sigma Co., U.S.A.) for 1 h at room temperature. Detection was carried out using ECL plus the Western Blotting Detection System (Amersham Biosciences, U.K.) by the method of the manufacturer.

Bioassays

Recombinant polyhedra produced in Sf-9 cells were released with cell lysis buffer and washed with PBS. The recombinant polyhedra were treated on a disc of Chinese cabbage leaf (2×2 cm^2). The treatment dosages were diluted in a log scale from 1×10^5 polyhedra per larva. The mortality was calculated by counting the dead larvae from a total of 90 larvae per treatment at intervals of 24 h, and the median-lethal dose (LD_{50}) was calculated using Probit analysis [25]. For the determination of the median-survival time (ST_{50}), 90 larvae of *P. xylostella* and *S. exigua* per treatment, 2nd–3rd instars, were subjected to an LD_{95} dose. The ST_{50} was determined, using a Probit analysis plot as described by Russell *et al.* [25].

Serial Passage of Recombinant Baculovirus

Sf9 cells were infected with the second passage of recombinant baculovirus (P2) at 1 and 10 multiplicity of infection (MOI), respectively. At 4 days post-infection (p.i.), budded viruses (BVs) were collected and titrated by the end-point dilution method [23]. Infection of BVs into Sf9 cells was performed in 1 and 10 MOI. From the replication of this step by more than 10 times, P5, P7, P9, and P11 were collected.

RESULTS AND DISCUSSION

Construction of a Recombinant Baculovirus Containing the *cry1Ac* Gene

The native polyhedrin and the N-terminally located polyhedrin of the fusion protein play an important role in the formation of the recombinant polyhedra [8]. For the production of stable recombinant polyhedra, both native polyhedrin and fusion protein of polyhedrin and foreign protein must be expressed in the same cell. Therefore, we attempted to achieve this double expression by using homologous recombination between two polyhedrin genes in the same orientations for the production of AcNPV polyhedra that incorporated the Bt Cry1Ac toxin. In order to prepare such a recombinant virus, transfer vector, pBactrus, was constructed, as shown in Fig. 1. Thus, the PCR-amplified and *Xho*I-digested toxic domain of the *cry1Ac* gene was inserted into the *Xho*I site located between two polyhedrin genes under the control of the polyhedrin gene promoter in pOB II. In this plasmid, a possibility of homologous recombination between two polyhedrin genes had earlier been demonstrated [4, 9]. By co-transfection of bAcGOZA DNA with pBactrus, recombinant baculovirus, Bactrus, was constructed, and the structure of Bactrus was confirmed by PCR analysis (Fig. 2). In particular, in the PCR amplification with Bac1 and Bac2 primers, 0.8 kb of the polyhedrin gene band was detected along with 3.5 kb of the fusion gene band (Fig. 2, lane 1). This 0.8 kb band was presumed to be the result of

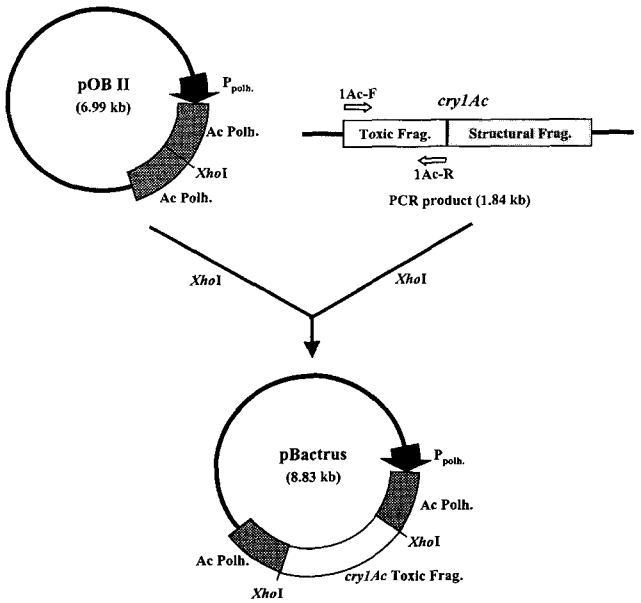

Fig. 1. Construction map of transfer vector, pBactrus.
The 1.84 kb of PCR-amplified *cry1Ac* toxic fragment was cloned into pOB II. The solid arrow indicates the baculovirus polyhedrin gene promoter and the open arrow indicates the primer position used in PCR.

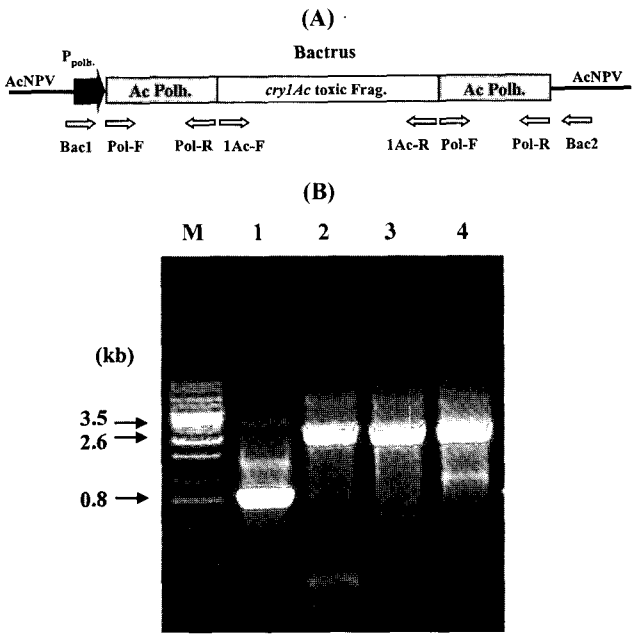

Fig. 2. PCR analysis of the recombinant virus, Bactrus.
Introduction of the fusion gene under the control of the polyhedrin gene promoter in Bactrus (A) was analyzed by PCR (B). Recombinant viral DNA was prepared from polyhedra in 3rd passage and used as a template in PCR analysis. Open arrows indicate primer positions used in PCR. The solid arrow indicates the baculovirus polyhedrin gene promoter. Lane M: 1 kb DNA Ladder; 1, primer Bac1 and Bac2; 2, primer 1Ac-F and Bac2; 3, primer 1Ac-F and Pol-R; 4, primer Pol-F and 1Ac-R.

homologous recombination between two polyhedrin genes in Bactrus, because there was no polyhedrin gene in the parental virus, bAcGOZA, used for the construction of Bactrus.

Expression of Fusion Protein by the Recombinant Baculovirus

To confirm the expression of fusion protein by the Bactrus, SDS-PAGE and immunoblot analyses were performed. In SDS-PAGE, a 130 kDa fusion protein band and 30 kDa polyhedrin band were detected in infected cell lysate and purified polyhedra sample of the Bactrus (Figs. 3A and 3B). From these results, the expression and embedding of fusion protein into polyhedra and the homologous recombination between two polyhedrin genes were confirmed. Activated 60 kDa Cry1Ac protein was detected in the *B. mori* gut-juice treated sample (Fig. 3C). In the immunoblot analysis using Cry1Ac or polyhedrin antibody, Cry1Ac protein and polyhedrin were identified in the position of the 130 kDa fusion protein, which was transferred into blotting paper (Figs. 3D and 3E).

Insecticidal Activity of Recombinant Polyhedra

To evaluate the insecticidal activity of the recombinant polyhedra by comparing with wt AcNPV polyhedra, a bioassay against *P. xylostella* and *S. exigua* larvae was performed. Overall, LD_{50} and ST_{50} of the recombinant polyhedra against *S. exigua* larvae were a little smaller than those of wt AcNPV; however, no significant differences were observed (Table 1). In contrast, LD_{50} of the recombinant polyhedra against *P. xylostella* larvae was significantly lower than that of wt AcNPV (Table 1). In addition, ST_{50} of the recombinant polyhedra was 10-fold shorter than that of AcNPV (Table 1). Although the insecticidal activity of the recombinant polyhedra against *S. exigua* larvae was similar to those of wt AcNPV, there was a significant increase of insecticidal activity against *P. xylostella* larvae. This was most likely due to the fact that the Cry1Ac crystal protein in the recombinant polyhedra has considerably high insecticidal activity against *P. xylostella* larvae, but little activity against *S. exigua* larvae [26]. Consequently, the recombinant baculovirus, Bactrus, acquired improved toxicity against *P. xylostella* and possibly more practicable use in the field [1, 7, 14, 28].

Expression Level of Fusion Protein During Serial Passages

To investigate the expression level of fusion protein during serial passages, SDS-PAGE analysis and bioassay against *P. xylostella* larvae were performed using serially passaged recombinant baculovirus. In SDS-PAGE, the expression level of fusion protein was decreased along with serial passages. However, the decrease of the fusion protein expression was smaller in Sf9 cells infected with Bactrus, which was passaged at 10 MOI, than that passaged at 1 MOI (Fig. 4A). Insecticidal activity of the recombinant polyhedra produced by the Bactrus, which was serially passaged at 1 and 10

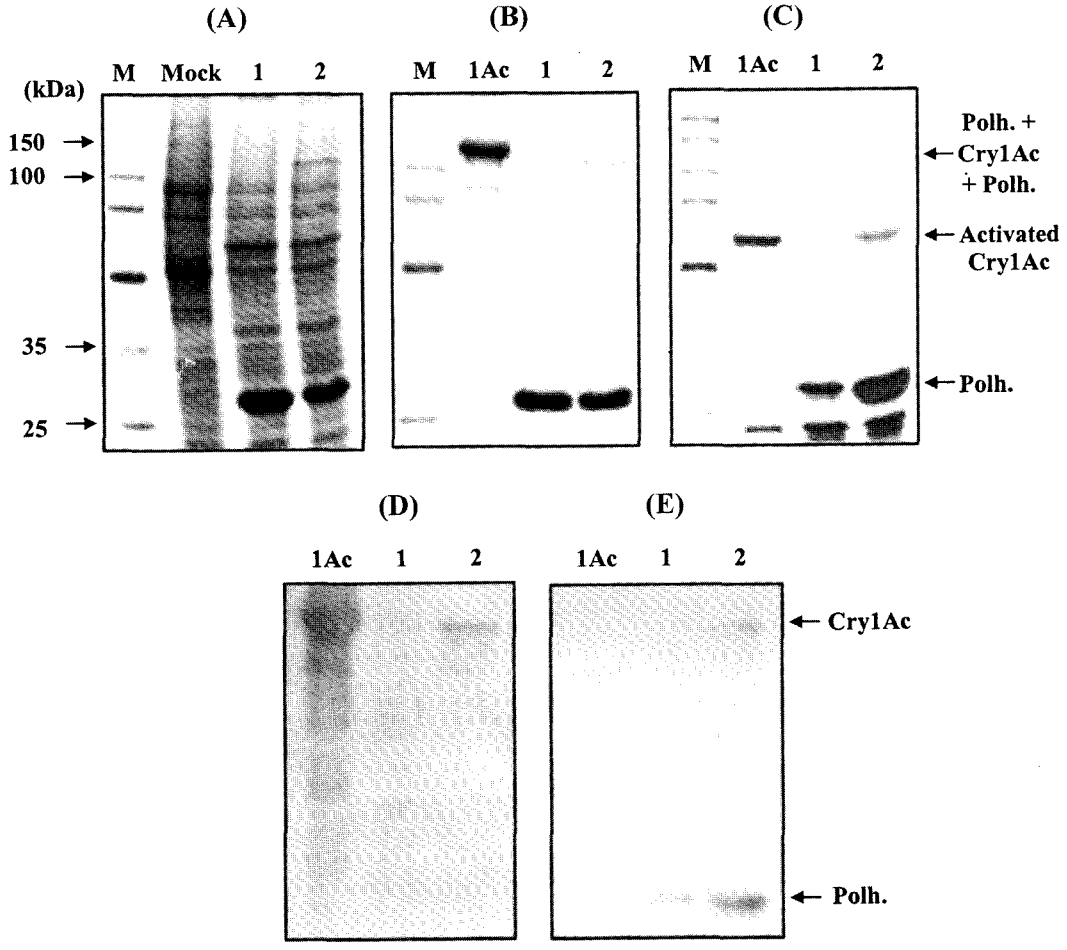

Fig. 3. SDS-PAGE and immunoblot analyses of the fusion protein.
Infected Sf9 cells (A), purified polyhedra (B), and *B. mori* gut-juice-treated polyhedra (C) were electrophoresed in 12% polyacrylamide gel. Immunoblot was performed against purified polyhedra, using polyclonal Cry1Ac (D) or polyhedrin (E) antisera. Lane M: protein molecular weight marker; Mock: mock infected Sf9 cells; 1Ac: *B. thuringiensis* Cry1Ac protein; 1, polyhedra of AcNPV; 2, polyhedra of Bactrus.

MOI, against *P. xylostella* larvae was decreased along with serial passages with a trend similar to the result of SDS-PAGE (Fig. 4B). It has been reported that the number of virus replication time in infected cells is larger at low MOI than at relatively high MOI [18]. In this regard, the larger the number of replication times, the higher the degree of homologous recombination that was observed between two polyhedrin genes. This resulted in lower expression of the fusion protein and decreased insecticidal activity against *P. xylostella* larvae along with serial passages. This character devoids Bactrus of the feasible problems encountered in genetically modified organisms. At the same time, this character might also be a

Table 1. Median lethal dose (LD_{50}) and median survival time (ST_{50}) values for Bactrus against second instar larvae of *S. exigua* and *P. xylostella*.

	S. exigua				*P. xylostella*			
	LD_{50}		ST_{50}[a]		LD_{50}		ST_{50}	
	$\times 10^3$ PIBs[b]/larva	95% CI[c]	Hours	95% CI	$\times 10^3$ PIBs/larva	95% CI	Hours	95% CI
Wt AcNPV	5.5	1.2–33.0	74.4	64.9–83.7	>100	NC[d]	233.5	178.8–435.4
Bactrus	0.8	0.2–2.1	61.5	54.1–68.9	1.1	0.4–2.6	22.1	12.7–29.1

[a]Applied dose was 1×10^5 polyhedra/larva.
[b]Polyhedral inclusion bodies.
[c]Confidence interval.
[d]Non-calculable.

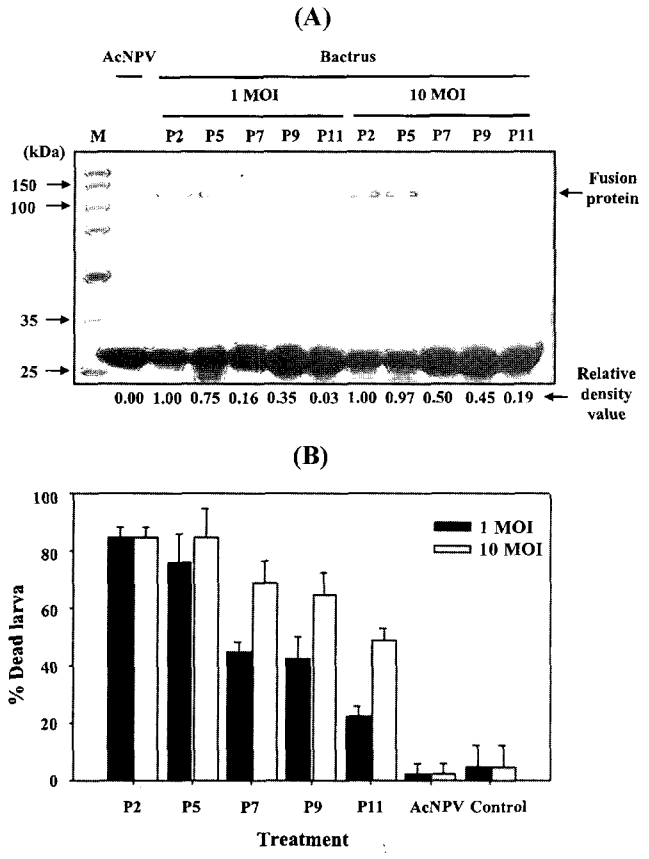

Fig. 4. SDS-PAGE analysis (A) and insecticidal activity against *P. xylostella* second instar larvae (B) of the polyhedra produced by Bactrus at 1 and 10 MOI along the serial passage.
(A) 2×10^6 polyhedra from each passage number were electrophoresed, and the relative density values at the bottom of the lanes were determined by densitometric scanning of the gel. Lane M: protein molecular weight marker; Ac: polyhedra of AcNPV; P2–P11: polyhedra of Bactrus from corresponding passage number. (B) 1×10^5 polyhedra/larva were applied by the surface contamination method.

drawback in the mass production of Bactrus *in vitro* and *in vivo*, and this could be circumvented by obtaining a large volume of virus inoculum at low passage number.

In conclusion, our approach has demonstrated that Bactrus has a higher pathogenicity against *S. exigua* and *P. xylostella* larvae and could return to wt AcNPV in several passages. Thus, Bactrus represents a novel recombinant insecticide that combines positive attributes of baculoviruses and Cry1Ac toxin, and can be used in the field as a safe biopesticide. These results would provide useful information on the development of a recombinant baculovirus insecticide that is friendly tothe environment.

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