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Construction of the Bac-to-Bac System of *Bombyx mori* Nucleopolyhedroviru*

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Abstract: To construct the Bac-to-Bac expression system of *Bombyx mori* nucleopolyhedrovirus (BmNPV), a transfer vector was constructed which contained an *Escherichia coli* (*E. coli*) mini-F replicon and a lacZ: attTN7: lacZ cassette within the upstream and downstream regions of the BmNPV polyhedrin gene. *B. mori* larvae were cotransfected with wild-type BmNPV genomic DNA and the transfer vector through subcutaneous injection to generate recombinant viruses by homologous recombination *in vivo*. The genomic DNA of budded viruses extracted from the hemolymph of the transfected larvae was used to transform *E. coli* DH10B. Recombinant bacmids were screened by kanamycin resistance, PCR and restriction enzyme (REN) digestion. One of the bacmid colonies, BmBacJS13, which had similar REN profiles to that of wild-type BmNPV, was selected for further research. To investigate the infectivity of BmBacJS13, the *polyhedrin* gene was introduced into the bacmid and the resultant recombinant (BmBacJS13-*ph*) was transfected to BmN cells. The budded viruses were collected from the supernatant of the transfected cells and used for infecting BmN cells. Growth curve analysis indicated that BmBacJS13-*ph* had a similar growth curve to that of wild-type BmNPV. Bio-assays indicated that BmBacJS13-*ph* was also infectious to *B. mori* larvae.

Key words: *Bombyx mori* nucleopolyhedrovirus (BmNPV); Bac-to-Bac; *in vivo*; Expression

Since 1983, when the baculovirus expression vector was first introduced (20), the baculovirus expression vector system (BEVS) has been widely used for the expression of recombinant proteins in insect cells. BEVS has many advantages, such as the capacity for insertion of large DNA fragments, high yields of recombinant protein, and integrated post-translational modifications (17). The BEVS of two viruses, that of *Autographa californica* nucleopolyhedrovirus (Ac-MNPV) and *Bombyx mori* nucleopolyhedrovirus

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(BmNPV), are most widely used to express foreign proteins. More than a thousand genes have been cloned and expressed through recombinant AcMNPV and BmNPV, ranging from the components of transcription machinery to pharmaceutical products (1). However, the traditional preparation of recombinant baculovirus to express foreign genes is very time consuming, because multiple rounds of purification and amplification of recombinant viruses are needed. Recently, the newly developed Bac-to-Bac™ system of AcMNPV has overcome this drawback. The AcMNPV bacmid can autonomously replicate in *E. coli* as a large plasmid at a low copy number, and the recombinant virus can be generated by the site specific transposition in *Escherichia coli* (*E. coli*) and used to infect insect cells. Since this system eliminates multiple rounds of purification and amplification of virus, recombinant viruses can be selected and purified within 7-10 days.

BmNPV, a member of the family Baculoviridae, is a natural pathogen of the mulberry silkworm *Bombyx mori*. Though the BmNPV genome is over 90% identical to the genome of AcMNPV (7), its host specificities is very narrow. Unlike AcMNPV which can infect more than 30 lepidopteran insects (2, 8), the BmNPV can only infect silkworm and its cell lines. Since it was first used to express α-interferon in 1985 (15), the BEVS of BmNPV has been used for the expression of many foreign proteins either in a cell culture system or in a insect larvae system (1).

B. mori BEVS are particularly suitable for the large-scale manufacture of foreign proteins, as the protein expression using silkworm or pupae is 10- to 100-fold higher than from *B. mori* cells. Silkworms are safe to the environment, and easy to breed with low cost. There is a long history of raising silkworms in China. All these make *B. mori* BEVS one of the most optimal systems for mass production of recombinant proteins. Several *B. mori* BEVS have been developed in past years (5, 10,11, 26, 27), however, the applications were limited due to the time consuming process or low infectivity of the virus to silkworm.

In this report, we described the construction of a bacmid of BmNPV, BmBacJS13, using *in vivo* recombination. To study the infectivity of the bacmid, the *polyhedrin* gene was inserted into the bacmid generating recombinant virus BmBacJS13-*ph*. The infectivity of BmBacJS13-*ph* was demonstrated by growth curve analysis of budded viruses and bioassay in *B. mori* larvae. The results indicated that BmBacJS13-*ph* is a functional virus with similar infectivity to that of wt BmNPV. The results also showed that infective recombinant viruses could be generated with BmBacJS13 by site specific transposition in *E. coli*, indicating a functional Bac-to-Bac system of BmNPV was constructed.

MATERIAL AND METHODS

Insect, virus and cell line

Larvae of *Bombyx mori,* were reared on an artificial diet at 27℃ (4) and third or fifth instars larvae were used in the experiments. BmN cells were cultured in TC-100 (JRH) insect medium supplemented with 10% fetal bovine serum (GIBCO/BRL) at 28℃ using standard techniques (17). The wt BmNPV Shaanxi strain, collected in Shaanxi province, China, were propagated on silkworm larvae (3).

Construction of transfer vector

According to the sequence of the BmNPV T3 strain (GenBank accession number L33180), a 1.5 kb segment upstream of *polyhedrin* gene was amplified

by PCR from the wt BmNPV genome DNA using primers(5'-GGGCCGCGGGCGTAGAGATTCGAC GAAAGC-3' and 5'-GCGCTGCAGATAATTACAA ATAGGATTGAGGCC-3') and cloned into the *Sac* II and *Pst* I sites of pBluescriptKS II+ (Stratagene). A 1.7 kb segment downstream of the *polyhedrin* gene was PCR amplified using forward primer (5'-GGGG AATTCCCTGAGGTAAGCGTTAGATTCTGTGCG TTTG-3') with *Eco*R I and *Bsu* 36I sites, and reverse primer (5'-GCGGGTACCTGACGAATCGTAAATA TGAATTCTGT -3') with *Kpn* I site. The product was cloned into the plasmid containing the upstream segment to generate pKS-USR-DSR. The mini-F replicon, kanamycin resistance gene and the Tn7 target sites were cut out as an 8.6 kb *Bsu*36I fragment from plasmid pBAC-*Bsu*36I (18) and inserted into the *Bsu*36I site of pKS-USR-DSR to generate transfer vector pBmTV (Fig. 1).

Recombination and identification of the bacmid *in vivo*

Newly molted fifth instar larvae were co-transfected with 20 μL DNA mixture per larva (including 0.45μg linearized plasmid pBmTV DNA, 0.15 μg wt BmNPV viral DNA and 3μL lipofectin (Invitrogen)) through subcutaneous injection (25). The haemolymph of the larvae were collected at 4 days post transfection and budded viruses (BVs) were retrieved from the supernatant of haemolymph after centrifuging. The DNA of BVs was extracted and transformed into *E. coli* DH10B cells (GIBCO/BRL), and colonies were selected in the presence of kanamycin and X-Gal. The colonies were further analyzed by PCR and REN digestion. One of the positive clones, BmBacJS13 which had similar REN profiles to that wt BmNPV, was chosen for further analysis.

Construction of BmBacJS13-*ph*

The *Polyhedrin* gene of BmNPV was amplified by forward primer (5'-GCGGGATCCTGTCGACAAGC TCTGTCCGTTT-3') with *Bam*HI (underlined), and reverse primer (5'-GGGGAATTCTTAATACGCCG GACCAGTG-3') with *Eco*R I (underlined). The *Bam*H I site of the fragment was blunted and the fragment was inserted into *Bst*1107 I and *Eco*R I sites of pFast bac Dual (Invitrogen) to generate pFast-DUAL-*ph*. The pFast bac Dual-*ph* was then used to produce a recombinant BmBacJS13-*ph* via transposition in *E. coli* according to the Bac-to-Bac™ system manual. The bacmid DNAs of the recombinant were extracted and were used to transfect the BmN cells.

Transfection of BmN cells

Bacmid DNA of BmBacJS13-*ph* was isolated by methods developed for large plasmids (instruction manual of Bac-to-Bac™ system/Life Technologies). The DNA was used to transfect BmN cells with lipofectin, and genomic DNA of wt BmNPV was used as a control. The supernatant was collected from the

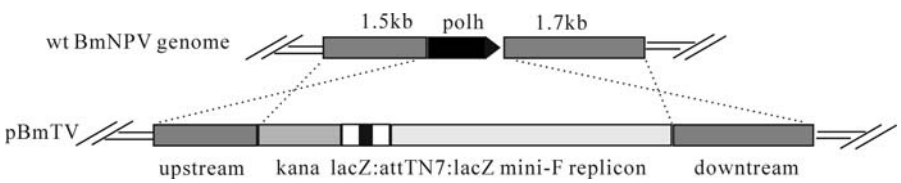

Fig. 1. Schematic of transfer vector pBmTV. The 1.5kb upstream sequence and 1.7kb downstream sequence are homologous arms, through recombination the *polh* was replaced with kanamycin cassette, lacZ: attTN7: lacZ and mini-F replicon.

transfected BmN cells at 96 hrs post transfection (h.p.i.) and was used to infect BmN cells. The supernatants of the infected cells were collected at 96 h.p.i and $TCID_{50}$ was determined using end-point dilution method.

Electron microscope

BmN cells (1×10^6) were infected with wt BmNPV or BmBacJS13-*ph* with a multiplicity of infection (MOI) of 5. The infected cells were harvested at 72 h.p.i. The samples were processed for electron microscopy examination as described by Van Lent *et al.* (22).

Comparison of the growth curves between wt BmNPV and BmBacJS 13-*ph*

BmN cells were infected with BV of Bm-BacJS13-*ph* or wt BmNPV at an MOI of 5. At the appropriate time points post infection, the supernatants were collected and the titers were detected using the end-point dilution method, Each virus infection was repeated five times and the data were statistically analyzed with one-way ANOVA (SPSS); polyhedral inclusion body (PIB) was used as the marker during the assay.

Bioassay

The third instars of *B. mori* larvae were used in oral infection assays to determine the infectivity of Bm-BacJS13-*ph* and wt BmNPV. The larvae (n=50 per viral dose) were fed with artificial diet which contain-ned different concentrations of viruses (3×10^7 PIB/mL, 10^7 PIB/mL, 3×10^6 PIB/mL, 10^6 PIB/mL, 3×10^5 PIB/mL and 10^5 PIB/mL, respectively) for twenty-four hrs. Then the larvae were transferred to fresh diet in plates and reared at 27℃ to investigate the mortality. The infectivity of BmBacJS13-*ph* and wt BmNPV was determined by probit analysis with SPSS10.0 and LC_{50} values of the viruses were further compared with a two side's z-test (21).

RESULTS

Identification of the bacmid BmBacJS13

After the construction of transfer vector pBmTV (Fig. 1), the pBmTV DNA was co-transfected with BmNPV DNA into newly molted fifth instar larvae. The hemolymphs of the larvae were collected 4 days post transfection, and BV DNAs were retrieved to transform *E. coli* DH10B cells. Colonies were chosen randomly and bacmid DNAs were digested with *Hin*d III. By comparison with the wt BmNPV profiles, several variations were found in different colonies (data not shown). One of the colonies, BmBacJS13, which had similar REN profiles to that of wt BmNPV, was analyzed with additional restriction enzymes. As shown in Fig.2, comparison with the wt BmNPVindicates that the *Xba* I profile of BmBacJS13 lacked B (19.3 kb)，D (15.8 kb),G (7.6 kb) and H (6.3 kb) bands, and contained five additional bands, D+H (22.2 kb), B' (19.0kb), G' (9.1 kb), 4.9 kb and 1.6 kb. Due to the deletion of the *polyhedron* gene, the B band in wt BmNPV became a shorter band B' in BmBacJS13. The 4.9 kb and the 1.6 kb bands were from the inserted 8.6 kb *Bsu*36I fragment which contains three *Xba* I sites. The remaining *Bsu*36I fragment (2.1kb) was linked with the remainder of G the fragment (7.0 kb) to generate G'(9.1 kb). The D+H band in BmBacJS13, however, was a sub-molecular band of the D and H bands in the wt BmNPV. These were the expected changes for the bacmid. Therefore BmBacJS13 was constructed correctly and was used for further investigation

Biological activity of BmBacJS13-*ph*

To test the oral infectivity of BmBacJS13, the *polyhedrin* gene was repaired into the bacmid, generating BmBacJS13-*ph*. The BmBacJS13-*ph* was identified to be correct by PCR and REN analysis (data not shown), and transfected into BmN cells to generate BVs.

BmN cells were infected with BmBacJS13-*ph* at an MOI of 5, occlusion bodies were observed in the nucleus of cells by optical microscopy from 48 hrs p.i, and the detachment of the cells were observed at the same time. These cytopathic effect (CPE) were similar to that of wt BmNPV infected cells. Electron microscopy analysis showed that the occlusion bodies were formed in the nuclei. The polyhedra and ODVs of BmBacJS13-*ph* had a similar shape and size as

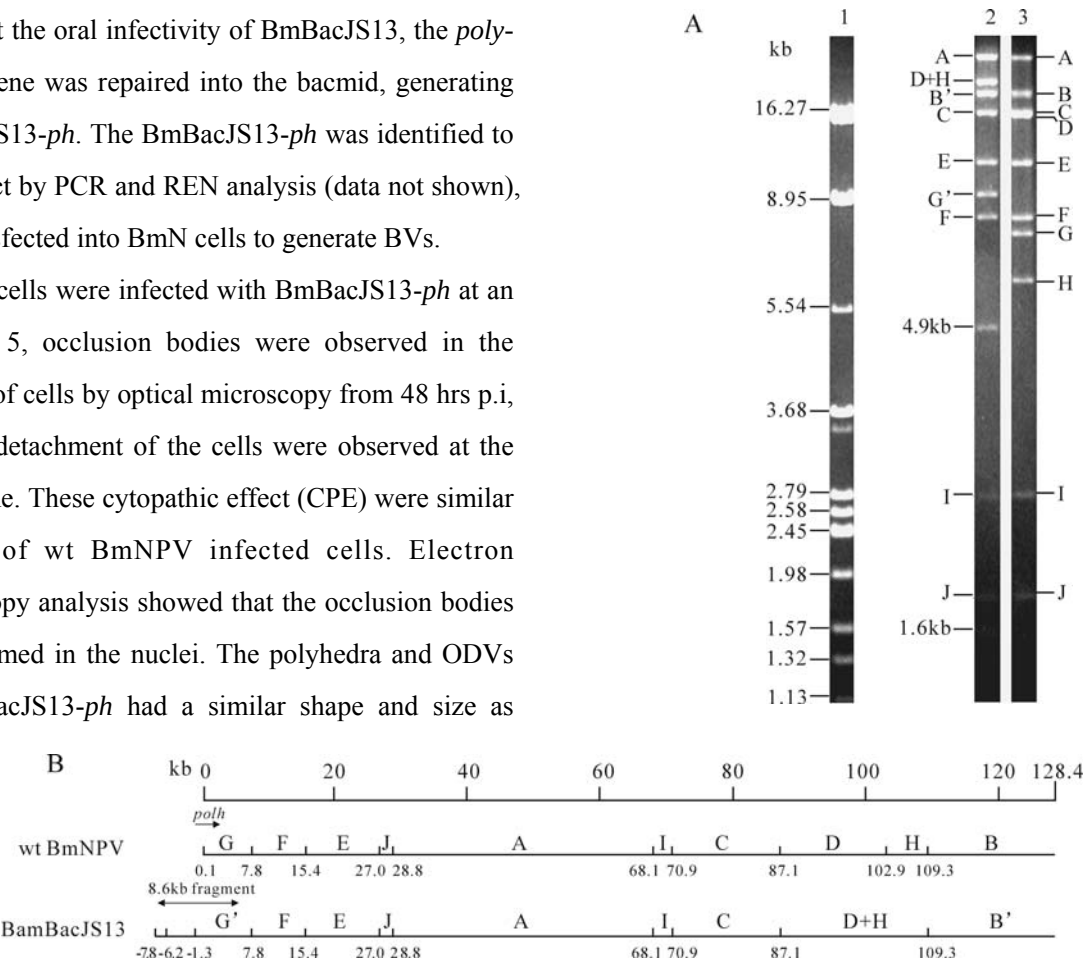

Fig. 2. *Xba* I digestion profiles and linearized physical maps of BmBacJS13 and wt BmNPV. A. *Xba* I digestion profile of BmBacJS13 and wt BmNPV. 1, λ DNA digested by *Bam*H I, *Eco*R I and *Hin*d III; 2, BmBacJS13; 3, wt BmNPV. B. Linearized *Xba* I physical maps of BmBacJS13 and wt BmNPV. The restriction sites are indicated in kb from the zero point. The genome size in kb is shown on a scale at the top. The location of *polh* and 8.6kb *Bsu*36I fragment are shown.

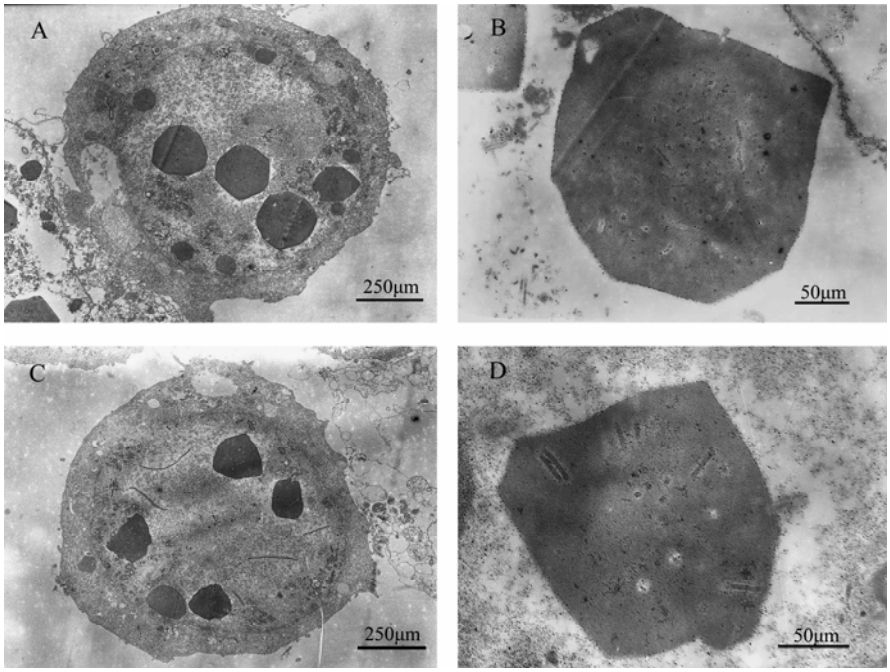

Fig. 3. EM pictures of BmN cells infected with wt BmNPV (A, B) and BmBacJS13-*ph* (C, D).

those of wild type BmNPV (Fig. 3).

One-step growth curve analysis indicated that BmBacJS13-*ph* BV had similar replication dynamics to that of wt BmNPV (Fig. 4). The titers of the two viruses were very similar during the early infection, but the titers of BmBacJS13-*ph* were slightly lower than that of wt BmNPV during 24 to 96 h.p.i. Statistical analysis indicated that the differences were not significant except those for 72 h.p.i..

The bioassay result showed that LC_{50} of BmBacJS13-*ph* against 3th larvae was 3.9x10^6 PIB/ml, which was not significantly different from of that of wt BmNPV (3.7x10^6 PIB/ml) (z=0.3276, P>0.05) (Table 1).

DISCUSSION

A functional Bac-to-Bac system should possess the following characteristics: first, the bacmid should be able to autonomously replicate in the bacteria; second, a foreign gene can be inserted into the bacmid *via* transposition, and lastly, the backbone virus must have similar biological properties as the wt virus, in both cells and insects. Our study showed that BmBacJS13 could autonomously replicate in the bacteria, and a foreign gene (*ph*) was introduced into the Tn7 site of BmBacJS13 with the aid of the helper plasmid. The recombinant virus BmBacJS13-*ph* had similar replication dynamics to that of wt BmNPV and bioassay results showed that its *in vivo* infectivity was similar to that of wt BmNPV. Therefore a functional Bac-to-Bac system of BmNPV has been constructed, and BmBacJS13 can be used to construct BmNPV recombinants for expressing foreign proteins. During our experiments, a similar Bac-to-Bac system of BmNPV was reported by Motohashia *et al* (16). In their system, a bacmid of the BmNPV T3 strain was constructed, and it could express enhanced green fluorescence gene (*egfp*) in larvae and pupae. As we have used a local strain from China (Shaanxi strain), it would be interesting to compare the properties between BmBacJS13 and the T3 bacmid.

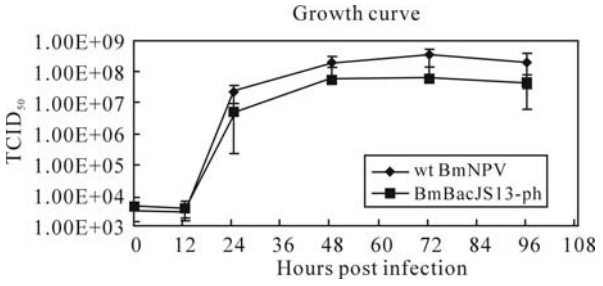

Fig. 4. Growth curves for BmBacJS13-*ph* and wt BmNPV. BmN cells were infected with BmBacJS13-*ph* or wt BmNPV at an MOI of 5. The supernatants were harvested at different times post infection and the BV titers were analyzed by EPDA. The average titer from five independent $TCID_{50}$ assays were shown with the bars indicating standard errors.

Table 1. LC_{50} of wt BmNPV and BmBacJS13-*ph* in early third instar *B. mori* larvae

Virus	LC_{50} (PIB/mL)	95% confidence limits	
		Lower	Upper
wt BmNPV	3669441[a]	654072	28459424
BmBacJS13-*ph*	3876462[a]	1400386	25047426

[a] No significant difference.

The Bac-to-Bac system can also be used for functional genomics studies of BmNPV. Bacmids have been widely used to delete genes by site-specific recombination in *E. coli* in AcMNPV and *Helicoverpa armigera* NPV (6, 9, 13, 12, 14, 19, 24, 23). The target genes were deleted and subsequently repaired by transposition to determine the function of the genes in viral life cycle. Currently we are studying the functions of several BmNPV genes using BmBacJS13 and the results will be reported in the future.

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