INTRODUCTION

The family Baculoviridae comprises viruses with circular, double-stranded DNA genomes and rod-shaped, enveloped virions (Blissard et al., 2000). Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the type species and is the most widely studied of the family Baculoviridae (Ayres et al., 1994). The AcMNPV open reading frame 150 (Ac150) encodes the 11K protein with a hydrophobic N terminus and a C-terminal peritrophin-A domain (Ayres et al., 1994; Dall et al., 2001). Ac150 also encodes a cluster of charged amino acids between the hydrophobic N terminus and the C6 motif (Zhang et al., 2005). The 11K proteins are conserved among many baculovirus genomes and, in addition, homologues are found in entomopoxviruses (Dall et al., 2001). The peritrophin-A domain can be represented as $\text{CX}_{7,18}\text{CX}_{3}\text{CX}_{6,11}\text{C}$, where X is any amino acid residue other than cysteine, also referred to as the six-cysteine (C6) motif (Tellam et al., 1999). The C6 motif is also found in various chitinases, mucins and other proteins incorporated within the peritrophic membrane (PM), as well as in receptors and other proteins involved in cellular adhesion (Kramer & Muthukrishnan, 1997; Shen & Jacobs-Lorena, 1999; Tellam, 1996; Tellam et al., 1992). The functions of peritrophin-A are diverse but frequently involve interaction with chitin (Elvin et al., 1996; Tellam, 1996). However, it has been shown that AC150 lacks a chitin-binding activity (Lapointe et al., 2004). M2R is a stilbene-derived optical brightener known to bind to chitin and damage the PM (Wang & Granados, 2000). It was shown that M2R does not enhance the larval mortality of an Ac150 deletion mutant, indicating that AC150 may not target the PM (Zhang et al., 2005).

So far, reports on the function of Ac150 have been inconsistent. Ac150 was found as a structural component of both budded virus (BV) and occlusion-derived virus (ODV) of AcMNPV by Western blotting and immunogold electron microscopy (Lapointe et al., 2004), but others could not detect it in ODV using several approaches (Braunagel et al., 2003). Lapointe et al. (2004) reported that deletion of Ac150 alone had no effect on the oral infectivity of the virus, but in combination with Ac145, it had a host-dependent impact on oral infectivity. Zhang et al. (2005) found that when Ac150 was deleted, the virus encoded a cluster of charged amino acids between the hydrophobic N terminus and the C6 motif (Zhang et al., 2005). The function of Ac150 was amplified from BmNPVs isolated from five different regions of China. Sequence analysis showed that the isolates had two different subtypes of Bm126, Bm126-SX and Bm126-GD, and both were different from that of the BmNPV T3 isolate. All of the BM126 ORFs contained a hydrophobic N terminus and a C6 motif at their C terminus, but the sequence between the N terminus and C6 motif varied in each isolate. The function of Bm126 was studied using bacmid BmBacJS13 derived from a BmNPV containing Bm126-SX. A 3′ rapid amplification of cDNA ends showed that the transcript of Bm126 was first detected at 6 h post-infection. A Bm126-knockout bacmid was constructed in which the majority of the coding region of Bm126 was deleted. Subsequently, the gene was repaired with Bm126-SX or Bm126-GD and tested for infectivity. The deletion of Bm126 had no obvious effect on the budded virus growth curve and the mean lethal dose of the occlusion bodies (OBs); however, the mean survival time of the larvae infected with Bm126-null virus was significantly delayed compared with that of the control virus. The delay was rescued by repairing the deletion with Bm126-SX but not with Bm126-GD. In addition, the virus repaired with Bm126-GD showed a significant increase in OB yield, both in vitro and in vivo.

### Variants of open reading frame Bm126 in wild-type Bombyx mori nucleopolyhedrovirus isolates exhibit functional differences

Bifang Hao,1,2 Jinshan Huang,1 Xiulian Sun,1 Fei Deng,2 Yanfang Zhang,2 Hualin Wang,2 Hong Chen1 and Zhihong Hu2

1Northwest A&F University, Yangling, Shaanxi 712100, PR China
2State Key Laboratory of Virology and Joint Laboratory of Invertebrate Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, Hubei 430071, PR China

The open reading frame (ORF) 126 (Bm126) of Bombyx mori nucleopolyhedrovirus (BmNPV) is a homologue of Ac150 and belongs to the baculovirus 11K protein family. Bm126 was amplified from BmNPVs isolated from five different regions of China. Sequence analysis showed that the isolates had two different subtypes of Bm126, Bm126-SX and Bm126-GD, and both were different from that of the BmNPV T3 isolate. All of the BM126 ORFs contained a hydrophobic N terminus and a C6 motif at their C terminus, but the sequence between the N terminus and C6 motif varied in each isolate. The function of Bm126 was studied using bacmid BmBacJS13 derived from a BmNPV containing Bm126-SX. A 3′ rapid amplification of cDNA ends showed that the transcript of Bm126 was first detected at 6 h post-infection. A Bm126-knockout bacmid was constructed in which the majority of the coding region of Bm126 was deleted. Subsequently, the gene was repaired with Bm126-SX or Bm126-GD and tested for infectivity. The deletion of Bm126 had no obvious effect on the budded virus growth curve and the mean lethal dose of the occlusion bodies (OBs); however, the mean survival time of the larvae infected with Bm126-null virus was significantly delayed compared with that of the control virus. The delay was rescued by repairing the deletion with Bm126-SX but not with Bm126-GD. In addition, the virus repaired with Bm126-GD showed a significant increase in OB yield, both in vitro and in vivo.
occlusion bodies (OBs) were significantly less virulent to larvae by oral infection, but this was not the case when ODVs were administered orally. Ohkawa (1997) found that deletion of Bm126, the Bombyx mori NPV (BmNPV) homologue of Ac150, reduced virulence of BmNPV in orally infected B. mori larvae.

In this study, the Bm126 ORF was amplified from BmNPVs isolated from five different regions of China. Sequence analyses showed that there were two subtypes of Bm126 in these isolates, Bm126-SX and Bm126-GD, and that both were different from that of the fully sequenced BmNPV T3 genotype (Gomi et al., 1999). The variation of different Bm126 subtypes occurred in the charged amino acid region between the N-terminal hydrophobic domain and the C-terminal C6 motif. Bacmid BmBacJS13 (Huang et al., 2007), originally derived from a BmNPV harbouring Bm126-SX, was used as a backbone to study the function of Bm126. The function of the different subtypes was examined by constructing a Bm126-knockout bacmid that was subsequently repaired with Bm126-SX or Bm126-GD. The PCR products were gel purified and cloned into the pGEM-T Easy vector (Promega). Two to three clones from each isolate were sequenced. The deduced amino acid sequences of BM126 were aligned using CLUSTAL_X version 1.83 and the alignment was adjusted using GeneDoc software.

Transcriptional analysis of Bm126. BmN cells were infected with vBmBacJS13-polh at an m.o.i. of 5 TCID<sub>30</sub> units per cell. Total RNAs were extracted with Trizol (Invitrogen), following the manufacturer’s instructions, at 0, 4, 6, 8, 12, 24, 36, 48 and 72 h post-infection (p.i.). A 3' rapid amplification of cDNA ends (3'RACE) was performed using avian myeloblastosis virus reverse transcriptase (Promega) and an oligo(dT)15 three-site adaptor primer (5'-CTGATCTAGGGTACCCGAGCTCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
BmBacΔ126-GD-polh) using 6 µl lipofectin (Invitrogen Life Technologies). The cells were incubated for 4 h after transfection and replenished with 2 ml fresh medium supplemented with 10% FBS, penicillin (100 µg ml⁻¹) and streptomycin (30 µg ml⁻¹). Virus-containing supernatant was collected from the transfected BmN cells at 96 h post-transfection (p.t.) and used to infect BmN cells. The cells were observed by light and fluorescent microscopy at 48 h p.i.

**Comparison of BV one-step growth curves of recombinant viruses.** BmN cells were infected with BmBacJS13-polh, BmBacΔ126-polh, BmBacΔ126-SX-polh or BmBacΔ126-GD-polh at an m.o.i. of 5 TCID₅₀ per cell. After 1 h of incubation, the virus-containing medium was removed. The cells were washed three times with serum-free medium, and fresh medium containing FBS was added. In all experiments, time 0 was defined as the point at which fresh medium was added. At the appropriate time points (0, 12, 24, 48, 72 and 96 h p.i.), supernatants were collected and the titres of the BV were determined using an end-point dilution assay (EPDA). OBs were used as a marker of infection during the assay. Each viral infection was carried out in triplicate and the experiment was repeated once. BV titres at different times p.i. were pooled and log-transformed for two-way analysis of variance (ANOVA) in GLM (SPSS) with virus and time as factors.

**In vivo bioassay.** OBs of the recombinants were purified from fifth-instar B. mori larvae infected with vBmBacJS13-polh, vBmBacΔ126-polh, vBmBacΔ126-SX-polh or vBmBacΔ126-GD-polh. The median lethal concentrations (LC₅₀) of the viruses were determined by feeding third-instar larvae an artificial diet containing five different concentrations of OBs. Fifty larvae were used per concentration of each virus and experiments were repeated twice. The mortality of the infected larvae was observed daily. LC₅₀ values and their 95% confidence limits were determined by probit analysis (SPSS). The LC₅₀ values of the viruses were compared further using a two-sided z-test (Snedecor, 1989).

The median survival time (ST₅₀) of the viruses was determined for third-instar larvae using a food-contamination method. OBs (4 µl 5 x 10⁶ OBs ml⁻¹) of the viruses were applied to a small plug of artificial diet in individual containers, and only larvae that completely ingested the contaminated diet within 5 h were used in the experiments (n>40). Time 0 was defined as the point at which the larvae were placed on the contaminated diet and mortality was recorded at intervals of 4 h. ST₅₀ values of the viruses were calculated using the Kaplan–Meier estimator and further compared using a log-rank test (Kalbfleisch & Ross, 1980). The experiments were repeated twice.

**Assay for OB production in vitro or in vivo.** BmN cells (1 x 10⁶) were infected with vBmBacJS13-polh, vBmBacΔ126-polh, vBmBacΔ126-SX-polh or vBmBacΔ126-GD-polh at an m.o.i. of 5. At 48 h p.i., infected cells and medium were harvested completely and centrifuged at 4000 g for 10 min to pellet the cells and OBs. Part of the cell pellets were lysed using a supersonic disrupter to count the OBs using a haemocytometer. The number of OBs in cells was subjected to SDS-PAGE (12%). Gels were stained with Coomassie Brilliant Blue G-250 or transferred onto a Hybond-N nitrocellulose membrane (Amersham) by semi-dry electrophoresis transfer for Western blot analyses (Ausubel et al., 1994). A polyclonal anti-POLH polyclonal antibody and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (SABC) as the secondary antibody. The POLH expression signal was detected with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl β-

**RESULTS**

**Sequence analysis of Bm126**

The different Bm126 ORFs were amplified by PCR from BmNPV-GD, BmNPV-JS, BmNPV-SX, BmNPV-SC and BmNPV-ZJ. The PCR products were cloned and sequenced. The sequences from any one isolate were the same, but the sequences from different isolates showed there were two subtypes of Bm126. BmNPV-JS, BmNPV-SX and BmNPV-ZJ shared one subtype (Bm126-SX), whilst BmNPV-GD and BmNPV-SC shared another subtype (Bm126-GD), and both were different from that of BmNPV T3 (Bm126-T3). The deduced amino acid sequences of Bm126s and AC150 are presented in Fig. 1. All sequences contained a variable region flanked by a hydrophobic N terminus and a C6 motif. The Arg-Gly-Asp (RGD) motif in AC150 was conserved in

**Transcriptional mapping of the 3’ ends of Bm126**

The timing of Bm126 transcription and the 3’ end of the Bm126 transcripts were determined using 3’RACE. As shown in Fig. 2(a), an RT-PCR product of 360 bp was first detected at 6 h p.i. and this band continued to be detected until 72 h p.i. The sequence of the Bm126 region of BmBacJS13 is shown in Fig. 2(b). Two early promoter motifs, CAGT and a TATA box-like sequence, were observed at 166 and 96 nt upstream of the translation start codon ATG (Fig. 2b), whilst a baculovirus late promoter TAAG motif was identified 134 nt upstream of the ATG. Whether these early and late motifs are used for Bm126 transcription needs to be analysed further.

Three clones containing the fragment of the 48 h p.i. RT-PCR product were sequenced. All of the sequences were

http://vir.sgmjournals.org
identical and the poly(A) tails were found immediately after the stop codon of Bm126 (Fig. 2b). There were two poly(A) signals (AATAAA) located upstream of the stop codon of Bm126 (Fig. 2b). The 3′RACE results suggested that they may be used as the poly(A) signal for the Bm126 transcript.

Construction and identification of Bm126-null and Bm126-SX- or Bm126-GD-repaired bacmids

To examine the role of the Bm126 gene in the viral infection cycle, Bm126 was knocked out from bacmid BmBacJS13 (derived from BmNPV-SX) (Huang et al., 2007) by homologous recombination in E. coli (Hou et al., 2002) (Fig. 3a). The resulting bacmid (BmbacΔ126) was selected by kanamycin and chloramphenicol resistance and confirmed by PCR (data not shown) and restriction endonuclease analysis. As shown in Fig. 3(b, lanes 1 and 2), XbaI digestion analysis revealed that the 19 kb C fragment (which contained Bm126) present in BmbacJS13 was replaced by the 21 kb C′ fragment in BmbacΔ126. This change was due to the substitution of Bm126 by CmR and egfp. The restriction profiles indicated that the deletion mutant was constructed correctly.

To test whether Bm126 had any effect on oral infection, polh and Bm126-SX, or polh and Bm126-GD, were reintroduced into BmbacΔ126 by transposition. The resulting bacmids, BmbacΔ126-polh, BmbacΔ126-SX-polh and BmbacΔ126-GD-polh (Fig. 3a), were selected by PCR (data not shown) and digested with various restriction endonucleases. Fig. 3(b) shows the XbaI digestion profiles of the recombinant and control viruses. In BmbacΔ126-polh and BmbacΔ123-polh, where the polh gene was introduced, the XbaI H fragment of BmbacJS13 (4.9 kb) was substituted by a 7.0 kb H′ fragment (Fig. 3b, lanes 3 and 4). In BmbacΔ126-SX-polh and BmbacΔ126-GD-polh, where the polh and Bm126-SX or Bm126-GD gene were introduced, the H fragment was substituted by a 7.5 kb H′ fragment (Fig. 3b, lanes 5 and 6). All of the changes were as expected and the results indicated that all of the recombinant bacmids were constructed correctly.

Recombinant virus replication in BmN cells and one-step growth curves of BV production

BmN cells were transfected with BmBacJS13-polh, BmBacJS13-egfp, BmBacΔ126-polh, BmBacΔ126-SX-polh or BmBacΔ126-GD-polh. At 36 h p.t., fluorescence was observed in all of the transfected cells except for those with BmBacJS13-polh, which did not harbour the egfp gene (data not shown). The supernatants of transfected cells were harvested at 96 h p.t. and used to infect healthy BmN cells. At 48 h p.i., fluorescence was observed in all transfected cells except for those with BmBacJS13-polh, whilst OBs and other cytopathic effects could be observed in all of the samples (Fig. 4a), indicating that Bm126 is not essential for virus replication.

To analyse BV yields of the recombinant viruses, one-step BV growth curves were determined by EPDA. The results (Fig. 4b) showed that all of the recombinant viruses had similar dynamics of BV production. Statistical analysis indicated that there was no significant difference among the titres of the four viruses at different times (F=0.329, d.f.=3, 112, P=0.804).

Bioassay analyses

To determine the LC50 of the recombinant viruses, third-instar larvae were infected with five different concentrations of OBs of vBmBacJS13-polh, vBmBacΔ126-polh, vBmBacΔ126-SX-polh or vBmBacΔ126-GD-polh. The results are presented in Table 1. Statistical analysis indicated that there was no significant difference in the LC50 value of vBmBacΔ126-polh from that of vBmBacJS13-polh (Z=0.007, P>0.05), vBmBacΔ126-SX-polh (Z=0.47, P>0.05) or vBmBacΔ126-GD-polh (Z=0.037, P>0.05). Therefore, deletion of Bm126 did not affect the LC50 of the virus.

Fig. 1. Alignment of the predicted amino acid sequences of the BM126 subtypes and AC150. Sequence alignment was carried out using CLUSTAL_X version 1.83 and adjusted using GeneDoc software. Gaps introduced to optimize the alignment are indicated with dashes. Shading is used to indicate 100% identity (black), 75% identity (dark grey) and 50% identity (light grey). The Arg-Gly-Asp (RGD) motif is underlined. The C6 motif is shown by numbering C1 to C6. The sequences used were: AcMNPVorf150 (GenBank accession no. L22858) and BmNPV T3 orf126 (GenBank accession no. L33180).
ST50 values of the recombinant viruses were determined by orally feeding third-instar B. mori using a food-contamination method (Table 1). The ST50 of vBmBacJS13-polh was 116 ± 0.62 h, whilst that of vBmBacΔ126-polh was 128 ± 1.30 h. Analysis revealed a significant difference in the ST50 of vBmBacΔ126-polh and vBmBacJS13-polh (χ² = 19.95, P = 7.94 × 10⁻⁶), indicating that deletion of Bm126 resulted in a significant delay of ST50. When Bm126-SX was reintroduced into the genome, the resulting vBmBacΔ126-SX-polh had a similar ST50 to that of vBmBacJS13-polh (χ² = 0.89, P = 0.35), suggesting that delay of ST50 was due solely to the deletion of Bm126 and that a repair rescue was possible. However, when Bm126-GD was reintroduced into the Bm126 deletion mutant, the resulting vBmBacΔ126-GD-polh had an ST50 (128 ± 1.33 h) similar to that of the Bm126 deletion mutant (Table 1). Statistical analysis (using a log-rank test) indicated that there were significant differences in the ST50 values of vBmBacΔ126-

**Fig. 2.** Transcriptional analyses of Bm126. (a) RT-PCR analyses of Bm126 transcription in vBmBacJS13-polh-infected BmN cells. The number above each lane indicates the time (h p.i.) when total RNA was isolated. M, DNA maker. H, Healthy (uninfected) cells. (b) The sequence of the 3’ RACE results of the Bm126 transcript and the nucleotide sequence of Bm125 are shown. The predicted amino acid sequences of Bm126 and Bm125 (partial) are shown below and above the nucleotide sequence, respectively. The start codons of Bm126 and Bm125 are shown by arrows. Stop codons and the additional poly(A) site are indicated by ‘#’. The primer sequence of 126RF, which was used for construction of the repaired viruses, is shown in italic. The deleted portion of BM126 in the Bm126-null virus is underlined. The poly(A) signals (A1 and A2) are boxed. Bold indicates the consensus early CAGT or late TAAG transcription start motifs and a TATA box-like sequence.

**Fig. 3.** Construction and identification of recombinant bacmids. (a) Schematic representative genomes of BmBacJS13 and the recombinant bacmids. The location and orientation genes are indicated by arrows. (b) Identification of recombinant bacmid DNAs by XbaI digestion. Lanes: M1, λ DNA digested by BamHI, EcoRI and HindIII; 1, BmBacJS13; 2, BmBacΔ126; 3, BmBacJS13-polh; 4, BmBacΔ126-polh; 5, BmBacJS13-polh; 6, BmBacΔ126-GD-polh; M2, λ DNA digested by HindIII.
GD-polh and vBmBacΔ126-SX-polh ($\chi^2=43.12$, $P=5.14 \times 10^{-11}$) and of vBmBacJS13-polh ($\chi^2=35.92$, $P=2.05 \times 10^{-9}$). Therefore, Bm126-GD was unable to repair the ST50 delay caused by Bm126 deletion.

**OB yields of infected BmN cells and larvae**

BmN cells were infected with vBmBacJS13-polh, vBmBacΔ126-polh, vBmBacΔ126-SX-polh or BmBacΔ126-GD-polh at an m.o.i. of 5. At different times p.i., it was

**Table 1.** Bioassays of the recombinant viruses in third-instar *B. mori* larvae

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mortality–concentration regression</th>
<th>Survival analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LC$_{50}$ ± SEM (OBs ml$^{-1}$)</td>
<td>Slope ± SEM</td>
</tr>
<tr>
<td>vBmBacJS13-polh</td>
<td>$3.31 \pm 1.4 \times 10^5$</td>
<td>$1.55 \pm 0.17$</td>
</tr>
<tr>
<td>vBmBacΔ126-polh</td>
<td>$3.29 \pm 0.9 \times 10^5$</td>
<td>$1.83 \pm 0.21$</td>
</tr>
<tr>
<td>vBmBacΔ126-SX-polh</td>
<td>$5.08 \pm 1.6 \times 10^5$</td>
<td>$1.80 \pm 0.19$</td>
</tr>
<tr>
<td>vBmBacΔ126-GD-polh</td>
<td>$3.15 \pm 1.3 \times 10^5$</td>
<td>$2.14 \pm 0.25$</td>
</tr>
</tbody>
</table>

*ST$_{50}$ values were determined in third-instar larvae at a concentration of $5 \times 10^8$ OBs ml$^{-1}$. The mortality of the dose was 100%.

---

**Fig. 4.** Analysis of virus replication in infected BmN cells. (a) Fluorescence (upper panels) and light (lower panels) microscope images of BmN cells infected with the recombinant viruses indicated. Cells were infected at an m.o.i. of 5 and observed at 48 h p.i. (b) Comparison of one-step BV growth curves. BmN cells were infected with vBmBacJS13-polh, BmBacΔ126-polh, BmBacΔ126-SX-polh or BmBacΔ126-GD-polh at an m.o.i. of 5. At the appropriate time points, supernatants were collected and BV titres were determined by EPDA. Each virus infection was carried out in triplicate. Titres are shown as means ± SD.
observed that the cells infected by BmA126-GD-polh appeared to produce more OBs than the others (data not shown). The number of OBs was then counted as described in Methods. The results showed that BmA126-GD-polh yielded the highest number of OBs (Table 2). Statistical analysis indicated there were significant differences between BmA126-GD-polh and the other viruses (F=27.418, d.f.=3, 476, P=1.46 \times 10^{-4}).

The levels of POLH expression were examined using standard SDS-PAGE and Western blotting. When total proteins from same number of infected cells were loaded on the gel, the expression level of POLH from BmA126-GD-polh-infected cells appeared to be much higher than that of the cells infected by the other viruses (Fig. 5a). Western blot results also showed that levels of POLH expression were indeed higher in BmA126-GD-polh-infected BmN cells (Fig. 5b), whereas VP39 expression was similar among the cells infected with BmA126-SX-polh, BmA126-polh, and BmA126-GD-polh (Fig. 5b).

The production of OBs in the larvae was also investigated. As shown in Table 2, the number of OBs produced by BmA126-GD-polh-infected larvae was significantly higher than that of the other viruses (vBmA126S13-polh, BmA126-polh, BmA126-SX-polh, and BmA126-GD-polh). In summary, BmA126-GD-polh produced significantly higher levels of OBs both in vitro and in vivo in comparison with the other recombinant viruses.

**DISCUSSION**

In this report, Bm126 was amplified from five different Chinese isolates of BmNPV. Sequence analyses showed that the isolates were composed of two subtypes of Bm126: Bm126-SX and Bm126-GD. Including the previously reported Bm126-T3 (Gomi et al., 1999), it is evident that there exist at least three subtypes of Bm126 in natural BmNPV isolates. It has been reported that there is one or more copies of the 11K protein in baculovirus; for example, there are five copies of the 11K gene in Xestia c-nigrum granulovirus (Dall et al., 2001). This is the first evidence that different subtypes of Bm126 exist in natural baculovirus isolates. As our study showed that different subtypes of Bm126 may have different properties under certain conditions, the multiple copies or different subtypes of the 11K genes may have evolved to fine-tune the genes' function under different host or tissue environments.

The 3’RACE results showed that Bm126 was transcribed from early to late stages of infection (Fig. 2a). This result is consistent with the observation that both early and late promoters exist in the upstream region of Bm126 (Fig. 2b). Microarray analysis also showed that Bm126 had a transcription pattern similar to that of gpe64 (Iwanaga et al. 2004), which is known to be a gene under the control of both early and late promoters.

Deletion of Bm126 did not affect BV infectivity in BmN cells (Fig. 4a, b), indicating that Bm126 is not an essential gene for virus replication. This is consistent with the results of Ohkawa (1997) who showed that the growth curve of a Bm126 deletion mutant was indistinguishable from that of the wild-type virus. Deletion of Bm126 resulted in successful oral infection but with a delayed ST50, and this delay was rescued in a repaired virus. Ohkawa (1997) also reported that Bm126-deleted virus took about 1 day longer to kill orally inoculated first-instar larvae compared with BmNPV T3-infected larvae. These results are in agreement with the observation that the cells infected by BmA126-GD-polh appeared to produce more OBs than the others (data not shown).

**Table 2. OB yields in BmN cells and in B. mori larvae**

<table>
<thead>
<tr>
<th>Virus</th>
<th>OB yield (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BmN cells (10^7)††</td>
</tr>
<tr>
<td>vBmA126S13-polh</td>
<td>1.77 ± 0.11 \times 10^7 a</td>
</tr>
<tr>
<td>vBmA126-polh</td>
<td>1.49 ± 0.11 \times 10^7 a</td>
</tr>
<tr>
<td>vBmA126-SX-polh</td>
<td>1.48 ± 0.06 \times 10^7 a</td>
</tr>
<tr>
<td>vBmA126-GD-polh</td>
<td>2.73 ± 0.08 \times 10^7 b</td>
</tr>
</tbody>
</table>

* BmN cells infected with virus at an m.o.i. of 5 TCID50 per cell.
† Viruses with different letters (a vs b) in the same column have significantly different means (P<0.05, one-way ANOVA) in pairwise comparisons between viruses.
‡OBs produced in larvae products were determined in third-instar larvae using the LC99 concentration.

**Fig. 5. SDS-PAGE and Western blot analysis of the expression of POLH.** (a) SDS-PAGE analysis of total proteins of BmN cells infected with recombinant viruses. (b) Results of Western blot analysis with polyclonal antibodies against POLH (α-POLH) and VP39 (α-VP39). M, Protein marker (kDa). Lanes 1–4, BmN cells infected with vBmA126S13-polh (lane 1), vBmA126-polh (lane 2), vBmA126-SX-polh (lane 3) or vBmA126-GD-polh (lane 4) at an m.o.i. of 5; lane 5, uninfected cells. The cells were collected at 48 h p.i.
with previous findings that AC150 is a per os infectivity factor but is not essential for oral infection (Lapointe et al., 2004; Zhang et al., 2005). It has been shown that the deletion of Bm126 and Ac150 results in reduced foci of primary infection (Ohkawa, 1997; Zhang et al., 2005). Therefore, it is possible that the reduced foci of primary infection resulted in delayed ST50 in the larvae infected with a Bm126-null BmNPV.

Different phenotypes were observed when different subtypes of Bm126 were used to repair a Bm126-null virus. The delay of ST50 caused by deletion of Bm126-SX was rescued when repaired with Bm126-SX but not with Bm126-GD. However, the Bm126-GD-repaired virus showed a significant increase in OB production, both in vitro and in vivo. The BV growth curve of the Bm126-GD-repaired virus was not significantly different from that of the others (Fig. 4b). Therefore, the increase in OB production is unlikely to be due to an increase in BV production. The major difference between Bm126-GD and Bm126-SX is that the former contains an RGDDKD sequence (including an RGD motif) and lacks a short stretch of six ND repeats. The RGD motif of many proteins has been shown to interact with subsets of host cell-surface integrins that are critical for a variety of cell functions (Hynes, 1992) such as regulation of gene expression, activation of focal adhesion kinases, activation of cytoskeleton elements, endocytosis, attachment, motility, cell survival, cell-cycle progression, cell growth, apoptosis and differentiation (Giancotti & Ruoslahti, 1999; Ruoslahti & Pierschbacher, 1987). In our experiments, cells infected with Bm126-GD-repaired virus showed a different phenotype; for example, the infected cells remained attached to the surface of the flask and survived for longer compared with the cells infected by other viruses, which became detached from the surface due to infection (data not shown). We suspect that the RGD motif in Bm126-GD might activate an integrin-mediated signalling pathway, which causes the upregulation of gene transcription and cell survival, and results in increased OB yield. Whether this assumption is true needs further investigation. In the Western blot results (Fig. 3b), expression of the major nucleocapsid protein, VP39, of Bm126-GD-repaired virus was not obviously different from that of the other viruses. However, we cannot exclude the possibility that there was a slight increase in VP39 expression that was not detected by Western blot analysis. Our unpublished data showed that there is no significant difference in OB production between wild-type BmNPV-GD and BmNPV-SX. However, it should be noted that there are significant differences in the restriction enzyme profiles of the genomes of wild-type BmNPV-GD and BmNPV-SX, indicating that there are other differences in their genome in addition to Bm126. It was suggested that the RGD motif in Ac150 might play a role in per os infectivity (Lapointe et al., 2004; Zhang et al., 2005). However, Bm126-SX, which has been shown to be a per os infectivity factor, does not contain an RGD motif, whilst Bm126-GD, which contains an RGD motif, did not appear to influence oral infection. Therefore, it is likely that the function as a per os infectivity factor of AC150 homologues is not related to the RGD motif, as least in the case of Bm126. The exact function of Bm126 and its homologues remains to be investigated further.

ACKNOWLEDGEMENTS

This work was supported financially by the following grants: 973 (2003CB114202), the National Natural Science Foundation of China project (30630002) and 863 project (2006AA10A210), and the Programme Strategic Scientific Alliances between China and The Netherlands (2004CB720404). We thank Dr Basil Arif for scientific editing of the manuscript and Professor Cheng Lu of the South-west University for providing B. mori eggs.

REFERENCES


Different *Bm126* ORFs demonstrate variable functions.