**Bombyx mori** nucleopolyhedrovirus ORF9 is a gene involved in the budded virus production and infectivity

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The ORF9 of **Bombyx mori** nucleopolyhedrovirus (BmNPV) (Bm9) is conserved in all completely sequenced lepidopteran nucleopolyhedroviruses. RT-PCR analysis demonstrated that Bm9 is an early and late transcribed gene that is initiated at 3 h post-infection, and immunofluorescence microscopy showed that Bm9 is localized mainly in the cytoplasm of infected cells. To determine the role of Bm9 during virus infection, Bm9 was knocked out by recombination in a BmNPV genome propagated as a bacmid in *Escherichia coli*. The budded virus (BV) production of Bm9-deleted bacmids was reduced more than 10-fold compared with wild-type (wt) bacmid; however, the kinetics of viral DNA replication were unaffected. The defect in BV production was recovered by the Bm9 rescue bacmid. In addition, electron microscope observations revealed that polyhedra formation was not affected by the deletion of Bm9. Bioassays showed that the Bm9-deleted bacmid took approximately 14–22 h longer to kill fifth instar *B. mori* larvae than wt bacmid, and the LD50 was about 15 times higher than that of the wt bacmid. In conclusion, Bm9 is an important but not essential factor in virus production and infectivity *in vivo* and *in vitro*.

**INTRODUCTION**

Baculoviruses are double-stranded DNA viruses that are pathogenic for invertebrates, particularly insects of the order *Lepidoptera*. The family *Baculoviridae* is taxonomically divided into two genera, *Nucleopolyhedrovirus* and *Granulovirus* (Theilmann et al., 2005). Baculovirus gene expression is regulated mainly at the transcriptional level, and involves sequential expression of early, late and very late genes. The function of early genes is the initiation of infection, and they are required to accelerate replication events and to prepare the host cell for virus multiplication (Friesen, 1997). The late stage occurs following the initiation of viral DNA replication. Two virion phenotypes are produced: occlusion derived virus (ODV) and budded virus (BV) (Funk et al., 1997). The ODV transmits infection from insect to insect by infecting midgut columnar epithelial cells, whereas the BV is responsible for causing systemic infection within the host (Keddie et al., 1989).

Baculoviruses are being extensively studied for their potential use as bioinsecticides around the world and as expression vectors for heterologous gene expression in insect-derived cells, as well as in host caterpillars. Recently, *Bombyx mori* nucleopolyhedrovirus (BmNPV) has been successfully developed for surface display of recombinant proteins (Rahman & Gopinathan, 2003), and baculoviruses are also regarded as potentially useful gene therapy vectors (Condrey & Kost, 2007; Huser & Hofmann, 2003; Tani et al., 2003). These applications drive the study of the molecular basis of baculovirus infection.

Since the complete genome of BmNPV was determined (Gomi et al., 1999), extensive studies have focused on the function of individual genes. The open reading frame 9 (ORF9) of BmNPV (Bm9), homologous to ORF17 of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), is considered to be a gene specific for lepidopteran nucleopolyhedroviruses (NPVs), suggesting that the gene plays an important role in the infection cycle of lepidopteran baculovirus. A previous study showed that AcMNPV ORF17 was dispensable for trans-activation in expression of a late gene (Guarino & Summers, 1988) and a recent study indicated that it was an early gene encoding a protein located in the cytoplasm of infected cells (An et al., 2006). However, its function is still unknown.

**METHODS**

Cell line, virus, bacterial strains, bacmids and plasmids. BmNPV (ZJ strain) and BmNPV bacmid virus were propagated in...
BmNPV ORF9 involved in BV production and infectivity

Monolayers of BmN cells (1 × 10⁶) were infected with BmNPV BV at an m.o.i. of 5. Total RNA from mock- or BmNPV-infected cells was extracted at 3, 6, 12, 24, 48 and 72 h post-infection (p.i.). After treating with RNase-free DNase I (TaKaRa), cDNA was synthesized by using the PrimeScript reverse transcriptase (TaKaRa) with oligo-dT(18) according to the manufacturer’s protocol. The coding region of the Bm9 gene was amplified by PCR with Bm9-specific primers Bmorf9F and Bmorf9R (Supplementary Table S1, available in JGV Online). Total RNA from mock-infected cells was used as a negative control. The PCR products that were obtained were analysed on a 1 % agarose gel.

RNA isolation and RT-PCR. Monolayers of BmN cells (1 × 10⁶) were infected with BmNPV BV at an m.o.i. of 5. Total RNA from mock- or BmNPV-infected cells was extracted at 3, 6, 12, 48 and 72 h post-infection (p.i.). After treating with RNase-free DNase I (TaKaRa), cDNA was synthesized by using the PrimeScript reverse transcriptase (TaKaRa) with oligo-dT(18) according to the manufacturer’s protocol. The coding region of the Bm9 gene was amplified by PCR with Bm9-specific primers Bmorf9F and Bmorf9R (Supplementary Table S1, available in JGV Online). Total RNA from mock-infected cells was used as a negative control. The PCR products that were obtained were analysed on a 1 % agarose gel.

Immunofluorescence microscopy. Monolayers of BmN cells were infected with BmNPV and collected at various times p.i. The cells were washed three times with 1 × PBS and fixed in cold methanol/acetone (1:1) for 15 min followed by three washes with 1 × PBS, then incubated with primary antibody in 1 × PBS for 2 h at room temperature. Polyclonal antibody against Ac17 (An et al., 2006) was used as the primary antibody, since Bm9 and Ac17 share 95 % amino acid identity. Primary antibody was removed by washing three times with 1 × PBS; the cells were then incubated with protein G fused with enhanced green fluorescent protein (eGFP) for 2 h and the DNA-specific DAPI stain (Sigma) for 1 h. Subsequently, the cells were directly observed and photographed using a Zeiss LSM 510 confocal laser scanning microscope.

Generation of bacmid with a Bm9 deletion. To construct a Bm9-deleted BmNPV bacmid by homologous recombination in E. coli, we amplified a CmR cassette from prADZ3 with primers DeBmorf9FF and DeBmorf9RR (Supplementary Table S1), which have 51 nt (8934–8984) and 53 nt (9008–9060) nt, respectively, homologous to Bm9 and 18 nt of the prADZ 3 sequence that flanks the CmR cassette. The PCR products were purified for the following electrotransformation. BW25113/pKD46 competent cells were made according to the method described by Datsenko & Wanner (2000). The BmNPV bacmid DNA was electrotransformed into the BW25113/pKD46 competent cells to generate the bacterial strain BW25113, containing pKD46 and BmNPV bacmid, designated BW25113/pKD46/BmBac.

To construct Bm9-rescued BmBac, we generated another donor plasmid, pFB1-De9Re-PH-GFP. Briefly, a 1562 bp fragment containing the Bm9 gene with its native promoter and poly(A) signal was PCR amplified with primers DeBm9Re-F and DeBm9Re-R (Supplementary Table S1). This PCR product was digested with EcoRI and PstI and ligated with pFB1-PH-GFP to generate the donor plasmid pFB1-De9Re-PH-GFP. This was transformed into DH10B/BmBacKO-PG, respectively. The constructed plasmids were verified by PCR with pUC/M13 forward and reverse primers (Supplementary Table S1).

Viral growth curves. To investigate viral growth, monolayers of BmN cells (1 × 10⁶) cultured in 1 ml serum-free TC-100 medium were infected with BmBacKO-PG, BmBacRePG and BmBacWT-PG bacmids in 35 mm dishes with an m.o.i. of 5. After 1 h incubation, the inoculum was removed and the cells were washed with TC-100 medium three times, and exchanged with TC-100 medium supplemented with 10 % serum; this was defined as time zero. Each infection was done in triplicate. The supernatant was collected at indicated times p.i. and the viral titre was determined by the end point dilution method (TCID₅₀) in triplicate (O’Reilly et al., 1992).

Quantitative PCR (qPCR) analysis of viral DNA replication. To assess viral DNA replication, we quantified viral genomic DNA accumulation by using qPCR. The infection was performed as described above. At designated times p.i., cells were collected and bacmid DNA was extracted by using the method described by King & Possee (1992). Primers QR and QP (Supplementary Table S1) were designed for qPCR, and the reaction was carried out using the SYBR Premix Ex Taq kit (TaKaRa) on the iCycler machine (BioRad) using Power SYBR Green Master Mix (Applied Biosystems) in a total volume of 25 μl. The reactions were performed in a 96-well plate format using the following parameters: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 30 s. The melting curve was analyzed from 65 °C to 95 °C. The specificity of the PCR reaction was confirmed by melting curve analysis. The reactions were performed in triplicate for each sample and the expression level of each gene was calculated by the 2⁻ΔΔCT method.
the following conditions: 95 °C for 10 s and 40 cycles of 95 °C for 5 s and 55 °C for 20 s.

We generated a recombinant plasmid (pGEX4t-2-118) with an insertion in BmNPV ORF118 and used a dilution series of this plasmid to generate a standard curve for qPCR. The number of genomic copies of each sample were determined using this standard curve.

Electron microscopy (EM). Monolayers of BmN cells were infected with BmBacWT and BmBacKO at an m.o.i of 5. At 96 h p.i., the cells were harvested by centrifugation (5000 × g, 5 min, 4 °C) and the pellet was fixed in 2.5% glutaraldehyde for 1 h at 4 °C, followed by fixing with 1% osmium tetroxide for 1 h at room temperature. Next, the pellets were dehydrated in graded ethanol (50–100%), and then soaked in acetone for 20 min. Infiltration was performed in graded Spurr (50–100%) (Sigma) and exposed for 16 h at 70 °C. Ultrathin sections were cut with a diamond knife. After staining with uranyl acetate and lead citrate, the ultrathin sections were viewed under a JEM-1230 transmission electron microscope (JEOL) at an accelerating voltage of 80 kV.

B. mori larvae bioassay. The LD50 and median lethal time (LT50) of BV were estimated by haemococically injecting different doses of BVs diluted in PBS (50, 500, 5000 and 50 000 p.f.u.) into B. mori larvae, BaiYu × QiuFeng strain, within 8 h of molting to the fifth instar. Twenty-five larvae per dose were used and each dose was repeated in triplicate. The mortality was counted every 4 h.

The computation processes, including modelling, test for the goodness of fit and estimation of LD50 and LT50, were conducted using Probit analysis (Finney, 1971). To investigate the effects on LD50 and LT50, comparisons between the viruses were performed using one-way ANOVA followed by LSD test to identify significant differences (P<0.01).

RESULTS

Sequence and transcription analysis of Bm9

The ORF9 of BmNPV (Bm9) is located between nt 8725 and 9355 in the genome, encoding a putative protein of 210 aa with a predicted molecular mass of 24.1 kDa; the gene is transcribed in the same orientation as the polh gene (Gomi et al., 1999). Sequence analysis revealed that a TATA box, a baculovirus consensus early promoter motif CAGT and two late transcriptional motifs, GTAAG and AATAAG, were located at 56, 436, 220 and 444 nt upstream of the start codon ATG, respectively. All of the early and late motifs are far from the start codon. The prediction of domains, motifs and post-translational modifications in the amino sequence were carried out by Motifscan online (http://www.expasy.org). An N-myristoylation site, a putative bacterial immunoglobulin (Ig)-like domain 1 (Big-1) domain profile, an asparagine-rich region profile, two putative casein kinase II phosphorylation sites, two putative protein kinase C phosphorylation sites and three putative N-glycosylation sites were predicted in the encoded amino acid sequence.

To determine the transcriptional profile of Bm9, the BmN cells were infected with BmNPV at an m.o.i of 5, and then collected at various times p.i. for RT-PCR analysis. Transcriptional analysis by RT-PCR revealed that a product with predicted size of 592 bp was detectable at 5 h p.i., and was still stable at the very late phase (72 h p.i.) (Supplementary Fig. S1, available in JGV Online). This observation indicated that Bm9 was transcribed throughout all stages of infection. In contrast, no PCR product was detected in the mock-infected sample (Supplementary Fig. S1). Also, no amplicon was obtained when the RT-PCR step was omitted, which ruled out the possibility of contamination of the cDNA sample with BmNPV genomic DNA (data not shown).

Immunofluorescence microscopy

To investigate the subcellular localization of the Bm9 protein, we carried out an indirect immunofluorescence assay to examine its distribution. Cells were collected for immunofluorescence analysis at different times p.i. Fluorescence signals were detected throughout infection within the cytoplasm but not in the nucleus, as shown at 72 h p.i. (Fig. 1), indicating that Bm9 localizes mainly within the cytoplasm of infected cells. In the control samples, no fluorescence was detected with anti-Ac17 serum (Fig. 1). Preimmune sera did not react with uninfected or infected samples (data not shown). The cross-reactivity of anti-Ac17 antibody to Bm9 was shown by Western blot analysis (Supplementary Fig. S2, available in JGV Online).

Construction of Bm9 knockout, repair and wt BmNPV bacmid

To determine the function of Bm9 in viral replication, we generated a Bm9-deleted BmNPV bacmid via the λ Red homologous recombination system in E. coli. A short sequence of 23 bp (nt 8985–9007) within the Bm9 locus was replaced with the CmR gene cassette (Fig. 2a). A portion (260 bp) of the 5′ end and 350 bp of the 3′ end of the Bm9 ORF were retained in order to preserve transcription of the adjacent genes, ORF8 and ORF10. The precise deletion was confirmed by PCR; as expected, a PCR product of 1.5 kb (which includes the inserted CmR cassette) was amplified from the Bm9-deleted bacmid, while a 0.59 kb product was amplified from the original bacmid (Fig. 2d), demonstrating that Bm9 was successfully replaced with the CmR cassette by homologous recombination.

Since the BmNPV bacmid was phenotypically polh-negative, we constructed the donor plasmid pFastBac-Bmph to transpose the polh gene cassette into the original bacmid and the Bm9-deleted bacmid to generate BmBacWT (Fig. 2b) and BmBacKO (Fig. 2c), respectively. The BmNPV polh transposition was verified by PCR with pUC/M13 forward and reverse primers, which resulted in a 3.7 kb product as expected (Fig. 2d). The BmBacWT and BmBacKO bacmids were used to transfect BmN cells for further ODV observation.
To facilitate observation of viral infection, the donor plasmid pFB1-PH-GFP, containing AcMNPV polh and gfp, was transposed into the polh locus in the original bacmid and Bm9-deleted bacmid to produce BmBac<sup>WT-PG</sup> (Fig. 2b) and BmBac<sup>KO-PG</sup> (Fig. 2c), respectively. The PCR with pUC/M13 forward and reverse primers confirmed successful transposition, which yielded the expected 4.2 kb products (Fig. 2d). The BmBac<sup>WT-PG</sup> and BmBac<sup>KO-PG</sup> bacmids were used to transfect BmN cells for viral DNA replication assay and BV titre assay.

To confirm the phenotype resulting from the Bm9 deletion, we constructed a Bm9 rescue bacmid. The donor plasmid pFB1-De9Re-PH-GFP, containing polh, egfp and Bm9 (with its native promoter), was transposed into the polh locus in the Bm9-deleted bacmid to create a rescue bacmid, named BmBac<sup>RePG</sup> (Fig. 2c). The successful transposition was verified by PCR with primer pUC/M13 forward and reverse primers, and a 5.7 kb product was amplified as expected (Fig. 2d).

**Observation of BmBac<sup>KO-PG</sup>, BmBac<sup>WT-PG</sup> and BmBac<sup>RePG</sup> transfection in BmN cells**

To examine the effect of Bm9 deletion upon virus replication, the BmN cells were transfected with Bm9-deleted (BmBac<sup>KO-PG</sup>), Bm9 rescue (BmBac<sup>RePG</sup>) and wt (BmBac<sup>WT-PG</sup>) bacmids. At 72 h post-transfection, these cells all presented cytopathic effects (CPE) and green fluorescent protein (GFP) expression (Supplementary Fig. S3, available in JGV Online). To further confirm the infectious capability of the BmBac<sup>WT-PG</sup>, BmBac<sup>KO-PG</sup> and BmBac<sup>RePG</sup>, a passage assay was performed. The supernatants from these cells were added to fresh BmN cells and incubated for 72 h. A large amount of GFP was observed in the majority of cells infected with the three bacmids, indicating that infectious BV spread among cells. The knockout virus remains infectious after passing through several generations. Thus, the viral passage experiments suggested that the Bm9 deletion does not abort BV production in cultured BmN cells.

**Analysis of viral growth curves**

To quantitatively assess the effect of the Bm9 deletion on BV production, a viral growth curve was generated for supernatants from BmN cells infected with BmBac<sup>WT-PG</sup>, BmBac<sup>KO-PG</sup> and BmBac<sup>RePG</sup>. At designated times p.i., the supernatants were collected in order to determine BV titre by TCID<sub>50</sub> assay. By 16 h p.i., the BV titre of BmBac<sup>KO-PG</sup> was approximately one log (10-fold) lower than that of BmBac<sup>WT-PG</sup>. However, the titre of BmBac<sup>RePG</sup> could reach the BmBac<sup>WT-PG</sup> level at 96 h p.i. (Fig. 3a), though there was a delay in BV production from 16–72 h p.i. Therefore, the data showed that the Bm9 deletion significantly reduced BV production in cultured BmN cells, and such a defect could be rescued by inserting the Bm9 gene cassette back into the knockout bacmid.

**Viral DNA replication**

To examine whether the reduced BV was due to viral DNA replication, we used qPCR to monitor viral genomic DNA accumulation in infected cells. The results revealed that, throughout the infection process, the Bm9-deleted bacmid presented similar kinetic growth curves of viral DNA replication to the wt and rescued bacmids. From 0 to 3 h, a phase prior to DNA replication, low numbers of viral...
**Fig. 2.** Construction of BmBac\(^{KO}\), BmBac\(^{WT}\), BmBac\(^{KO-PG}\), BmBac\(^{WT-PG}\) and BmBac\(^{RePG}\), and PCR identification of the constructs. (a) The scheme for construction of a Bm9 knockout bacmid. A 23 bp portion of the Bm9 locus was replaced with Cm\(^R\) via homologous recombination. (b) Schematic diagram of the wt bacmids BmBac\(^{WT-PG}\) and BmBac\(^{WT}\). BmBac\(^{WT-PG}\) was constructed by inserting AcMNPV polh and gfp into the BmNPV bacmid's polh locus by Tn\(^7\)-mediated transposition in the Bac-to-Bac system. BmBac\(^{WT}\) was generated by inserting only BmNPV polh into the BmNPV bacmid's polh locus. (c) Schematic diagram of the constructed bacmids BmBac\(^{RePG}\), BmBac\(^{KO-PG}\) and BmBac\(^{KO}\). The rescue bacmid, BmBac\(^{RePG}\), was constructed by inserting AcMNPV polh, Bm9 gene cassette and gfp into the Bm9-deleted bacmid's polh locus by Tn\(^7\)-mediated transposition in the Bac-to-Bac system. The AcMNPV polh and gfp were inserted into the Bm9-deleted bacmid's polh locus to generate BmBac\(^{KO-PG}\). The BmNPV polh was inserted into the Bm9-deleted bacmid's polh locus to generate BmBac\(^{KO}\). (d) PCR identification of the Bm9 deletion and reconstructed bacmids of BmBac\(^{KO}\), BmBac\(^{WT}\), BmBac\(^{WT-PG}\), BmBac\(^{KO-PG}\) and BmBac\(^{RePG}\). The sizes (bp) of the PCR products are indicated.

**Fig. 3.** Time-course analysis of BV production (a) and viral DNA replication (b) from Bm9-deleted bacmid (BmBac\(^{KO-PG}\), —) and wt bacmid (BmBac\(^{WT-PG}\), ······) and the rescue bacmid (BmBac\(^{RePG}\), ——). (a) BmN cells were infected with an m.o.i of 5, and supernatant was collected at the indicated times. The BV titres were determined by TCID\(_{50}\). (b) BmN cells were infected with an m.o.i of 5; cells were collected at the indicated times and viral DNA was extracted for qPCR analysis. Results are shown as mean ± SD.
genomes per cell (<10) were detected (Fig. 3b). By 12 h p.i., approximately 100–500 genomes per cell were detected in cells transfected by all three bacmid-generated viruses (Fig. 3b). By 36 h p.i., the accumulation of viral DNA reached a plateau, with approximately 3000 genomes per cell (Fig. 3b). Thus, the assay illustrated that the Bm9 deletion does not affect viral DNA replication in cultured BmN cells.

**Examination of polyhedra formation**

EM observation was performed to examine the distribution of nucleocapsids of BmBacKO and BmBacWT in infected cells. The results showed that there was no discernable morphological effect on nucleocapsid distribution for the two bacmids (Fig. 4). The BmBacKO-infected cells displayed features characteristic of baculovirus infection, including an enlarged nucleus, the presence of an electron-dense virogenic stroma, lots of nascent nucleocapsids accumulated within the stromal matter and polyhedra occluded with nucleocapsids (Fig. 4b, c). Thus, no obvious discrimination of polyhedra formation was observed in the Bm9 deletion bacmid.

**Effect of Bm9 deletion on BV infectivity in B. mori larvae**

To determine whether the Bm9 deletion has an effect on infectivity in B. mori larvae, fifth instar larvae were haemocoelically injected with BmBacKO-PG, BmBacWT-PG and BmBacRePG BVs. Firstly, LD50 was determined by injecting with various doses of BV (50, 500, 5000 and 50 000 p.f.u.). We observed that the LT50 of BmNPV budding virus in B. mori larvae after injection with BmBac WT-PG and BmBacKO-PG (5000 p.f.u. per larva) for 6 days. The BV titre of BmBacWT-PG was approximately 19 times higher than that of BmBacKO-PG, with 5.62 × 10^6 and 3.03 × 10^8 TCID50 (ml haemolymph) ^-1, respectively (Supplementary Fig. S4, available in JGV Online).

**DISCUSSION**

Bm9 and its homologues are found specifically in lepidopteran NPVs (Herniou *et al.*, 2003), suggesting that in many other baculovirus host systems, the function of this gene either is not required or is replaced by other viral and/or cellular factors.

In this study, we examined transcription of the Bm9 gene of BmNPV and the cellular localization of the gene product, as well as its effect on viral replication of BmN cultured cells. Transcriptional analysis via RT-PCR demonstrated that Bm9 is an early and late transcribed gene, with transcripts initially observed at 3 h p.i. (Supplementary Fig. S1); this is identical to observations of its homologue Ac17 (An *et al.*, 2006; Guarino & Summers, 1988). To detect the Bm9 distribution in virus-infected cells, we performed immunofluorescence assays with polyclonal antibody against Ac17. The results showed that Bm9 protein was found exclusively in the cytoplasm at 72 h p.i. (Fig. 1).

To further investigate the function of Bm9, we constructed a Bm9-deleted bacmid virus. The deletion of Bm9 did not affect viral DNA replication (Fig. 3b). However, the Bm9-null virus was deficient in the production of infectious virions, with BV titres reduced to 10 % of wt levels (Fig. 3a). This defect could be restored by insertion of the Bm9 gene cassette into the polh locus, indicating that such a phenomenon is derived from Bm9 deletion.

As far as BV production was concerned, Bm9 had a similar function to pe38 and pp31 genes, which both greatly reduced BV yields (Milks *et al.*, 2003; Yamagishi *et al.*, 2003), suggesting that in many other baculovirus host systems, the function of this gene either is not required or is replaced by other viral and/or cellular factors.

**Fig. 4.** Electron micrographs of ultrathin sections of BmN cells infected with BmBacKO and BmBacWT. BmN cells were infected with BmBacKO and BmBacWT at an m.o.i. of 5. At 96 h p.i., cells were collected and ultrathin sections were prepared for EM observation. (a) BmN cells infected with BmBacWT. (b) and (c) BmN cells infected with BmBacKO.
However, the pe38-null virus reduced the viral DNA synthesis level, whereas the Bm9- and pp31-null viruses presented identical levels of DNA replication compared to wt bacmid virus. 

Subsequently, EM analysis indicated that the rod-shaped nucleocapsids, virogenic stroma, ODVs and polyhedra containing ODVs were formed in the BmBacKO virus-infected cells and showed no difference to those in BmBacWT virus-infected cells. Therefore, the results indicated that Bm9 was not essential for occlusion body morphogenesis in BmNPV-infected cells. Guarino & Summers (1988) found that the Ac17 gene, which was included in the PstI-G region, was not required for late gene expression. Their results and ours indicate that Bm9 is dispensable for DNA replication and some late or very late gene expression at the cellular level.

Bioassay results showed that the deletion of Bm9 elongated the LT50 and increased the LD50 compared with the controls, which is consistent with the reduction of BV production detected in the in vitro assay, suggesting that both infectivity and virulence were reduced in the deletion bacmid. These results support the hypothesis that defects in BV production led to reduced virulence in infected larvae (Milks et al., 2003). To determine whether Bm9 is a component of BV or ODV structure, we carried out a Western blot of BV and ODV using anti-Ac17 serum (Supplementary Fig. S5, available in JGV Online). Our results showed that Bm9 was a component of neither BV nor ODV, suggesting that the reduction in the infectivity was not due to a lack of Bm9 in the budded virion structure, but a reduction in the number of budded virions produced.

It is interesting to note that, based on sequence analysis, Bm9 has a Big-1 domain in the N-terminal region, which is present in bacterial adhesion molecules of the intimin/invasin family, and is involved in pathogenicity. The Big-1 proteins are surface-expressed proteins mediating mammalian host cell invasion or attachment (Hamburger et al., 1999; Kelly et al., 1999; Luo et al., 2000). However, it was not determined whether Bm9 was also expressed on the membrane surface. Whether this domain of Bm9 had an

| Table 1. Dose mortality of BmBacWT-PG, BmBacRePG and BmBacKO-PG for fifth instar B. mori larvae |
|-------------------------------------------------|--------|--------|-------------|--------|
| Virus                                           | Regression equation | $x^2$ value | $P$ value | LD50 (p.f.u.)* | 95% confidence limit (p.f.u.) |
| BmBacWT-PG                                      | $y=0.72 \times -3.66$ | 2.5401   | 0.2808    | 137.5 a       | 92 183 |
| BmBacRePG                                       | $y=0.53 \times -3.79$ | 0.1836   | 0.9123    | 142.2 a       | 105.4 191.9 |
| BmBacKO-PG                                      | $y=0.81 \times -2.35$ | 0.8836   | 0.6429    | 1987.5 b      | 1319.5 3473 |

*LD50 values followed by a different letter are statistically different ($P<0.01$). If $P>0.05$, the model was determined to be statistically significant.

| Table 2. Time mortality of BmBacWT-PG, BmBacRePG and BmBacKO-PG for fifth instar B. mori larvae |
|-------------------------------------------------|--------|--------|-------------|--------|
| Virus concentration                             | Virus      | LT50 (h)* | 95% confidence limit (h) |
| per larva (p.f.u.)                              | BmBacWT-PG | 132.8 a   | 128.6 137.0 |
|                                                 | BmBacRePG  | 131.2 a   | 126.9 135.5 |
|                                                 | BmBacKO-PG | 146.5 b   | 142.6 150.4 |
| 50000                                          | BmBacWT-PG | 137.5 a   | 132.9 142.1 |
|                                                 | BmBacRePG  | 133.9 a   | 130.2 137.6 |
|                                                 | BmBacKO-PG | 160.4 b   | 156.9 163.9 |
| 5000                                           | BmBacWT-PG | 144.3 a   | 138.8 147.8 |
|                                                 | BmBacRePG  | 142.6 a   | 137.5 147.7 |
|                                                 | BmBacKO-PG | NA        | NA NA |
| 500                                            | BmBacWT-PG | NA        | NA NA |
|                                                 | BmBacRePG  | NA        | NA NA |
|                                                 | BmBacKO-PG | NA        | NA NA |

*LT50 values followed by a different letter are statistically different ($P<0.01$).
effect on infectivity and virulence would be of significant interest in future studies.

In conclusion, we examined the transcription of Bm9 in infected cells and found that it was an early transcribed gene. Detection of the localization of Bm9 in BmNPV-infected cells showed that it is mainly localized in the cytoplasm. There was a significant reduction in infectious BV production in cells infected with the Bm9 null virus relative to wt virus; however, the kinetics of viral DNA replication were unaffected. The bioassays following inoculation with BV showed that the Bm9-deleted bacmid took approximately 14–22 h longer to kill the fifth instar larvae than wt bacmid, and that the LD50 was about 15 times higher than that of wt bacmid, demonstrating that Bm9 is not an essential gene for viral invasion and replication but affects the efficiency of virus infection to some degree in larvae. EM analysis of Bm9 knockout virus-infected cells did not highlight obvious differences from wt virus-infected cells. All these results suggested that Bm9 is not necessary for BmNPV propagation but affects infectivity and virulence in vivo and in vitro.

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