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The *Bombyx mori* nucleopolyhedrovirus (BmNPV) ODV-E56 envelope protein is also a *per os* infectivity factor

Xingwei Xiang, Lin Chen, Aiqin Guo, Shaofang Yu, Rui Yang, Xiaofeng Wu*

College of Animal Sciences, Zhejiang University, Huajiachi Campus, Hangzhou 310029, PR China

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ABSTRACT

The *Bombyx mori* nucleopolyhedrovirus (BmNPV) *odv-e56* gene is a late gene and encodes an occlusion-derived virus (ODV)-specific envelope protein, ODV-E56. To determine its role in the BmNPV life cycle, an *odv-e56* null virus, BmE56D, was constructed through homologous recombination. A repaired virus was also constructed, named BmE56DR. The production of budded virion (BV) and polyhedra, the replication of viral DNA, and the morphological of infected BmN cells were analyzed, revealing no significant difference among the BmE56D, the wild-type (WT), and the BmE56DR virus. Larval bioassays demonstrated that injection of BmE56D BV into the hemocoel could kill *B. mori* larvae as efficiently as repaired and WT viruses, however BmE56D was unable to infect the *B. mori* larvae when inoculated *per os*. Thus, these results indicated that ODV-E56 envelope protein of BmNPV is also a *per os* infectivity factor (PIF), but is not essential for virus replication.

1. Introduction

The family Baculoviridae encompasses a group of arthropod-specific viruses found ubiquitously in the environment and have been isolated from more than 600 host insect species predominantly from the Lepidoptera, Hymenoptera and Diptera (Slack and Arif, 2007). During the typical biphasic infection cycle, two structurally and functionally distinct enveloped virion phenotypes are produced: occlusion-derived virus (ODV) and budded virus (BV). Both ODV and BV contain rod-shaped nucleocapsids that are assembled within the nucleus. ODV are contained in polyhedra, transmit virus from insect to insect via oral infection, whereas BV spread infection to neighboring cells (Granados and Lawler, 1981; Keddie and Volkman, 1985; Keddie et al., 1989; Engelhard et al., 1994).

ODV virions infect the host insect's midgut epithelial cells when polyhedra are ingested by the host and solubilized in the midgut lumen, releasing the ODV, attaching to host midgut epithelial cells and fusing their envelopes directly with the cell membrane (Bonning, 2005). The ODV envelope proteins that are essential to oral infection are called *per os* infectivity factors (PIFs). To date, five highly conserved core genes, *p74 (pif-0)*, *pif-1*, *pif-2*, *pif-3* and *pif-4* have been identified. The deletion of the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) *p74* gene results in the complete elimination of the *per os* infectivity of polyhedra, while virions purified from mutant polyhedra were infectious when injected into the hemocoels of *Trichoplusia ni* or *Heliothis virescens* larvae (Faulkner et al., 1997; Haas-Stapleton et al., 2004). PIF-1 was originally identified in *Spodoptera littoralis* NPV, deletion of *pif-1* (*spli7*) resulted in viruses that were unable to infect *S. littoralis* larvae *per os* (Kikhno et al., 2002). PIF-2 was first identified in *Spodoptera exigua* MNPV, and disruption of *pif-2* resulted in the complete loss of *per os* infectivity for the host (Pijlman et al., 2003). PIF-3 (*ac115*) is also an essential factor for oral infection of AcMNPV. Although PIF-3 is not required for ODV attachment and fusion, it may mediate the translocation of ODV along microvilli during primary infection (Ohkawa et al., 2005). PIF-4 (*ac96*) was a new *per os* infectivity factor identified in *A. californica* multiple nucleopolyhedrovirus, and the ac96-null virus was unable to infect midgut tissue when *T. ni* larvae were inoculated *per os* (Fang et al., 2009).

The *Bombyx mori* nucleopolyhedrovirus (BmNPV) genome has been completely sequenced, which is a covalently closed circular DNA (Gomi et al., 1999). Some BmNPV ORFs have been characterized and related either to DNA replication, gene expression, packaging and assembly, or *per os* infection. The *odv-e56* gene is expressed late during infection and encodes a protein that is exclusively associated with the ODV envelope (Braunagel et al., 1996). Comparative analysis of the 49 completely sequenced baculovirus genomes reveals 31 core genes that are conserved in all baculovirus genomes and are therefore likely to serve important roles in baculovirus life cycles (Garcia-Maruniak et al., 2004; McCarthy and Theilmann, 2008; van Oers and Vlak, 2007).

In the present study, a recombinant virus that cannot express *odv-e56* was constructed through homologous recombination. The

* Corresponding author. Tel.: +86 571 86971658; fax: +86 571 86971647.
E-mail address: wuxiaofeng@zju.edu.cn (X. Wu).

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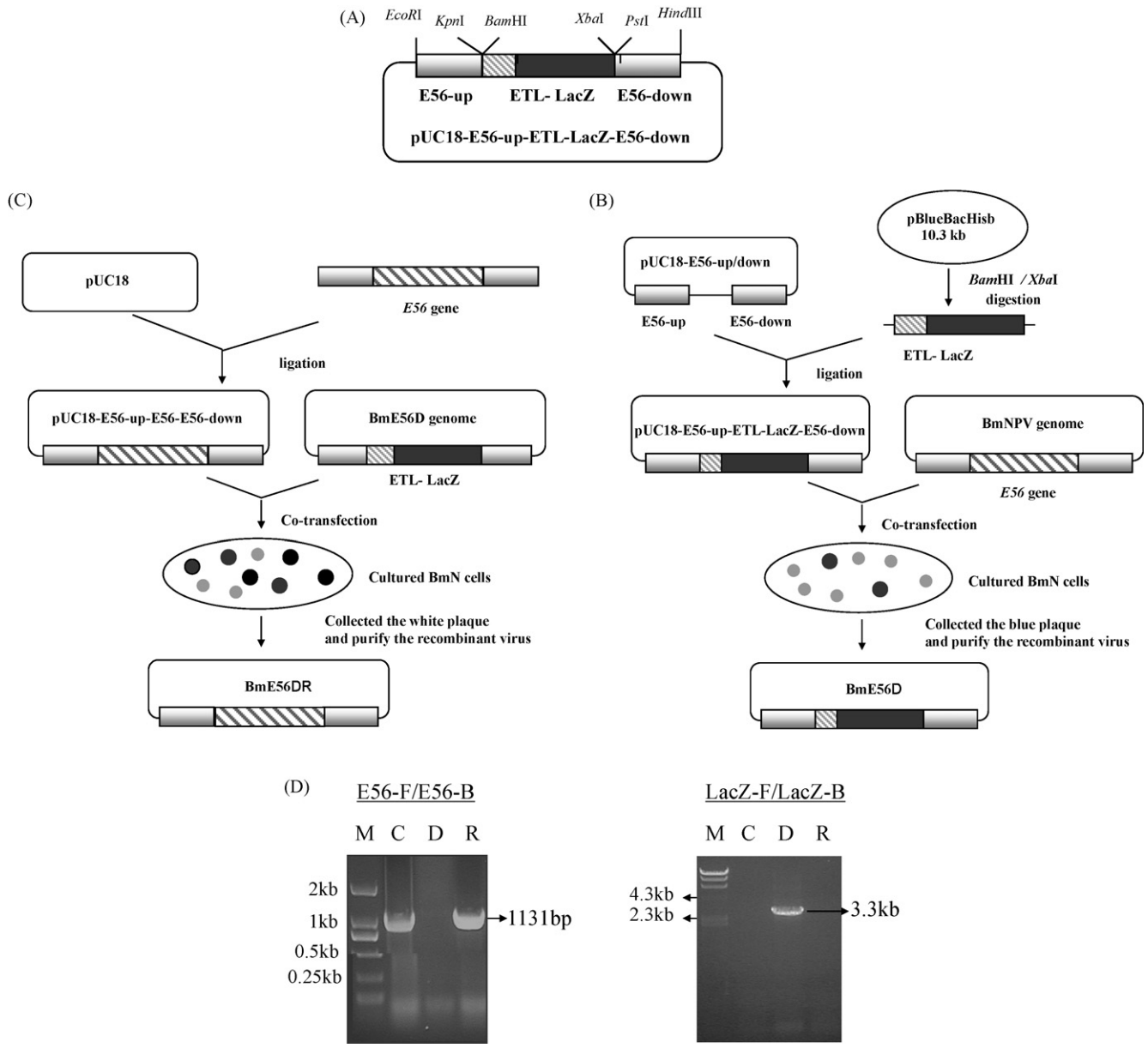

Fig. 1. Construction of recombinant viruses used in this study. (A) Construction of recombinant transfer vector pUC18-E56-up-ETL-LacZ-E56-down for homologous recombination. The upstream and downstream fragments of E56, ETL-LacZ, and corresponding restrictions enzyme sites are indicated. (B) Flow chart of construction of BmE56D. (C) Flow chart of construction of BmE56DR. Scheme for construction of mutant BmNPVs is described in detail in Materials and methods. (D) PCR analysis of the genome of wild-type, BmE56D, and BmE56DR. Each genotype was confirmed by PCR using the primers E56-F/E56-B and LacZ-F/LacZ-B.

results showed that in tissue culture, *odv-e56* was not required for viral DNA replication, BV production and polyhedra formation. Larvae bioassays demonstrated that injection of BmE56D BV into the hemocoel killed *B. mori* larvae as efficiently as repaired and WT viruses; however, it was unable to infect the *B. mori* larvae when inoculated *per os*.

2. Methods

2.1. Cells, viruses, and insect rearing

The BmN cells were maintained at 27 °C in TC-100 insect medium (Gibco, USA) supplemented with 10% (V/V) fetal calf serum (Gibco, USA). BmNPV was stored in our laboratory. A hybrid strain of silkworm (commercial name: Qiufeng × Baiyu) were reared on an artificial diet in a conditioned incubator at 27 °C for the bioassays.

2.2. Construction of recombinant virus

In order to delete *odv-e56* of the BmNPV genome through homologous recombination, a recombinant transfer plasmid was constructed as shown in Fig. 1. The upstream and downstream fragments of *odv-e56* (named E56-up and E56-down, approximately 2.0 kb) were cloned from the BmNPV genome. A 3.3 kb *Bam*HI/*Xba*I fragment containing the ETL promoter-*lacZ* expression cassette was obtained from plasmid pBlueBacHisb. The primers were designed as listed in Table 1. The PCR was run using the following conditions: denaturing at 94 °C for 50 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min; the reaction was run for 30 cycles. Subsequently, the amplified fragments E56-up and E56-down were digested with *EcoR*I/*Kpn*I and *Pst*I/*Hind*III respectively. The digested fragments together with the pETL-*lacZ* expression cassette were cloned into the corresponding sites of pUC18 to generate the final transfer plasmid pUC18-E56-up-ETL-LacZ-E56-down.

Table 1
Primers used in this experiment.

Primers	Sequences (5′–3′)	Length of amplified fragment/bp
E56-upF	AGAATTCTTGTTATCGTGTTCGC (*EcoR*I)	1935
E56-upB	CTCGATAGGTACCAGACAAAAAT (*Kpn*I)	
E56-downF	ACTGCAGTAATAAATATATCACAG (*Pst*I)	2001
E56-downB	GAAGTTCTACTCGTAAGCTTAG (*Hind*III)	
E56-F	GATATCATGAGTTTTTTTTCAAATCTTC(*EcoR*V)	1131
E56-B	AGGATCC TCGAGGGGCCGTTG (*Bam*HI)	
850-F	TTTGGCAAGGGAACTTTGTC	100
851-F	ACAAACCTGGCAGGAGAGAG	
1483-F	CGTAGTGGTAGTAATCGCCGC	100
1484-F	AGTCGAGTCGCGTCGCTTT	
M13-F	TGTAAAACGACGGCCAGT	
M13-B	GAAACAGCTATGACCATGAT	
LacZ-F	AGATCTATGATAGATCCCGTC(*Bgl*II)	3060
LacZ-B	TCAAGCTTATTTTTGACACCAG(*Hind*III)	

Recombinant viruses were generated by co-infection of BmN cells. The transfer plasmids pUC18-E56-up-ETL-LacZ-E56-down (1 μg/μL) and BmNPV genome DNA (1 μg/μL) were co-transfected into BmN monolayer cells. For 15 min at room temperature, 10 μL of plasmid, 1 μL of BmNPV genome DNA, 14 μL of lipofectin (Invitrogen), and 15 μL of MilliQ H_2O (final volume 40 μL) were gently mixed, incubated and added to the cell medium. Plaque assay had been carried on until the positive recombinant virus was screened, named BmE56D.

In addition, a repaired virus BmE56DR, was also constructed through homologous recombination. Firstly, a 5.1 kb fragment containing the E56-up, E56 and E56-down was cloned from BmNPV genome using the primers of E56-upF and E56-downB. The PCR was run using the following conditions: denaturing at 94 °C for 50 s, annealing at 56 °C for 30 s, and extension at 72 °C for 4 min; the reaction was run for 30 cycles. Subsequently, the amplified fragment was digested with *EcoR*I/*Hind*III, cloned into the corresponding sites of pUC18 to generate the final recombinant transfer plasmid pUC18-E56-up-E56-E56-down.

The repaired virus, BmE56DR, was also generated by co-transfection of BmN cells with transfer plasmids pUC18-E56-up-E56-E56-down (1 μg/μL) and BmE56D DNA (1 μg/μL) by liposome-mediated transfection using lipofectin, followed by plaque assay of the cell medium 5 days post-infection.

2.3. BV production in BmN cells

The use of qPCR to titrate baculovirus stocks has been previously described (Lo and Chao, 2004). This method is based on the amplification of approximately 100-bp fragments located in the coding regions of selected genes. Primers 850 and 851 were designed to amplify a 100-bp genomic fragment of *chitinase*. BmN (1.0×10^6 cells/35-mm-diameter plate) cells were infected with the recombinant virus, the WT virus and the repaired virus at a multiplicity of infection (MOI) of 5. At various times post-infection, the supernatant containing BV was harvested, and cell debris was removed by centrifugation (at $8000 \times g$ for 5 min). An aliquot of each of these supernatants (100 μL) was processed using the OMEGA Viral DNA Kit. An aliquot of each purified DNA sample (2 μL) was combined with the TOYOBO SYBR Master Mix (SYBR Green qPCR Master Mix-Plus-QPK-212) and the qPCR primers in a 20 μL reaction. The samples were analyzed in a ABI 7300 qPCR cycler under the following conditions: 1 cycle of 95 °C for 60 s; 40 cycles of 95 °C for 15 s, 59 °C for 15 s, 72 °C for 45 s. The results were analyzed by the 7300 system software.

BV titers at 6 and 72 h post-infection were also titrated in duplicate by endpoint dilution in BmN cells with 96-well microtiter plates.

2.4. Virus DNA replication analysis by qPCR

Viral DNA replication was assayed by qPCR as described previously (Vanarsdall et al., 2006). Briefly, this method is based upon the amplification of a 100-bp region of the *gp41* using the primers 1483 and 1484. To prepare total DNA for analysis, BmN cells (1.0×10^6 cells/35-mm-diameter plate) were infected with recombinant virus, the WT virus and the repaired virus at a multiplicity of infection (MOI) of 5, and at designated time, the total virus DNA was extracted by Classic Genomic DNA Isolation Kit (Bio Basic Inc.) following the manufacturer's instructions. An aliquot of the virus DNA (2 μL) was combined with the TOYOBO SYBR Master Mix (SYBR Green Qpcr Master Mix-Plus-QPK-212) and the qPCR primers 1483 and 1484 in a 20 μL reaction. The samples were analyzed in a ABI 7300 qPCR cycler under the following conditions: 1 cycle of 95 °C for 60 s; 40 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 45 s. The results were analyzed by the 7300 system software.

2.5. Purification and production of polyhedra in BmN cells

BmN cells (1.0×10^6 cells/35-mm-diameter plate) were inoculated with the recombinant virus, the WT virus and the repaired virus at a multiplicity of infection (MOI) of 5, collected the infected cells at 120 h post-infection, washed with PBS (20 mM NaH_2PO_4, 20 mM Na_2HPO_4, 150 mM NaCl, pH 7.2) and fragmented by ultrasonic wave, and collected the precipitate by centrifugation at $15{,}000 \times g$ for 10 min. The pellets containing polyhedra were further purified by Percoll density gradient centrifugation at $15{,}000 \times g$ for 20 min. A nine-to-one ratio of Percoll to PBS was empolyed for this purpose. The purified polyhedra were washed several times with PBS, suspended in PBS buffer and counted with a Neubauer hemocytometer.

2.6. Electron microscopy

BmN cells (1.0×10^6 cells/35-mm-diameter plate) were infected with viruses at a MOI of 5. At 60 h post-infection, the supernatant was removed, cells were washed once with 1 × cacodylate buffer (0.1 M NaCl; 0.05 M cacodylate pH 7.5) and then fixed in 2.5% glutaraldehyde in 0.1 M Na cacodylate for 30 min. The cells were then washed twice with 1 × cacodylate buffer and fixed with 1:1 osmium tetroxide:cacodylate buffer for 30 min, washed once with 1 × cacodylate buffer and stained with 2% uranyl acetate for 30 min. After dehydration through a series of 30–100% ethanol washes, cells were embedded in Spur resin. Ultra thin sections were obtained and subsequently stained with 1% uranyl acetate and lead citrate. Images were obtained using a JEM-1230 transmission electron microscope.

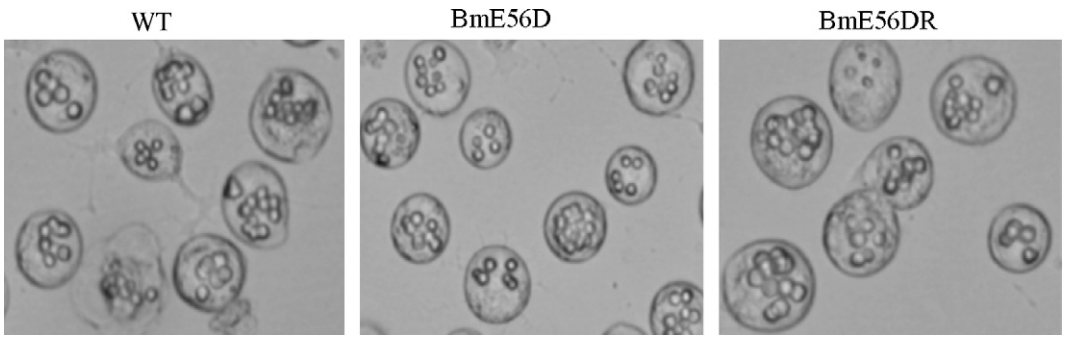

Fig. 2. Analysis of viral replication in BmN cells. Light microscopic observations of polyhedra in BmN cells at 96 hpi. BmN cells were infected with WT, BmE56D and BmE56DR at an MOI of 5.

2.7. Larvae bioassays

The infectivity of BV was examined by injecting 20,000, 2000, or 200 $TCID_{50}$ (50% tissue culture infective dosage) units of BV of BmE56D, the WT and BmE56DR virus into 5th-instar *B. mori* larvae. Grace's medium was used as a negative control. Twenty-five larvae per dose were used and each dose was repeated in triplicate. For the oral-infectivity assay, polyhedra of BmE56D, the WT and BmE56DR virus were purified from BmN cells and suspended in PBS buffer. The newly molted silkworm larvae of the 3th-instar were used for investigation of per oral infection efficiency. The 20,000, 2000 or 200 polyhedra were added on the artificial diet and administrated for feeding. Fifteen larvae per dose were used and each dose was repeated in triplicate. Infected larvae were reared individually in 24-well plates and monitored daily until all larvae had either pupated or died.

To investigate the effects on infectivity, comparisons between the viruses were performed using one-way ANOVA followed by LSD test to identify significant differences ($P < 0.01$).

3. Results

3.1. Construction of odv-e56-deleted and repaired viruses

To determine the role of *odv-e56* during viral infection in BmN cells and *B. mori* larvae, an *odv-e56*-deleted virus was constructed based on homologous recombination in BmN cells. Firstly, the upstream and downstream fragments of *odv-e56* (named E56-up and E56-down, approximately 2.0 kb) and a *LacZ* gene under the control of baculovirus ETL promoter (approximately 3.6 kb) which was obtained from pBlueBacHisb plasmid were cloned into the pUC18 plasmid, to generate a recombinant transfer plasmid (Fig. 1A). And then an *odv-e56*-deleted BmNPV was generated by homologous recombination in BmN cells (Fig. 1B). For this, the recombinant transfer plasmid DNA was co-transfected with wild-type BmNPV DNA into BmN cells and the recombinant virus (BmE56D) was isolated by identification of plaques expressing β-galactosidase. Deletion of E56 was confirmed by PCR (Fig. 1D). Successful isolation of BmE56D showed that E56 is not essential for virus replication in BmN cells.

In addition, a repaired virus BmE56DR was also constructed through homologous recombination (Fig. 1C). Firstly, a transfer vector was constructed that contained 5.1 kb insert DNA, including *odv-e56* and the flanking regions. And then the recombinant transfer plasmid was co-transfected with BmE56D genomic DNA into BmN cells and the repaired virus (BmE56DR) was also isolated by identification of plaques expressing β-galactosidase and confirmed by PCR (Fig. 1D).

3.2. Analysis of viruses in infected BmN cells

At 96 hpi, light microscopy analysis showed that BmE56D, WT and BmE56DR produced polyhedra of normal appearance, and observed that there was no difference in the proportion of the cells containing polyhedra at 96 hpi (Fig. 2A). The polyhedra were purified from different viruses infected cells at 96 hpi, and counted, the results showed that equivalent polyhedra were existed in those cells. These results, therefore, suggested that *odv-e56* does not affect polyhedra formation.

3.3. Virus growth curves

To further analyze if deletion of *odv-e56* quantitatively affects infectious BV production or replication kinetics, virus growth curve analysis was performed by qPCR and $TCID_{50}$. Fig. 3A showed the replication kinetics of BV production from BmN cells infected with BmE56D, the WT and BmE56DR as determined by qPCR, which detects viral genomes regardless of infectivity. No significant difference in the slope of the growth curves of these viruses at 12,

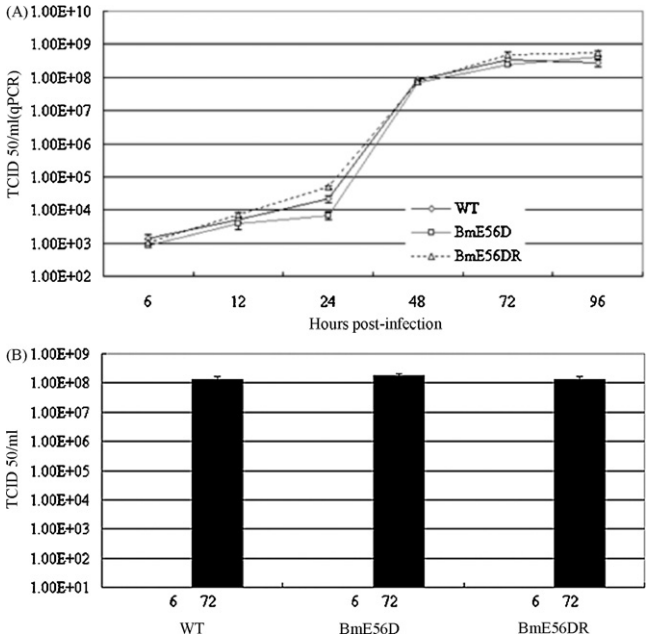

Fig. 3. BV production from BmN cells infected with WT, BmE56D and BmE56DR. (A) BV growth curves assayed by quantifying the number of viral genomes using qPCR analysis of supernatants of BmN cells infected with WT, BmE56D and BmE56DR, at the designated time points. (B) Infectious virus titers at 6 and 72 hpt, determined by a $TCID_{50}$ endpoint dilution assay. Error bars represent the standard errors.

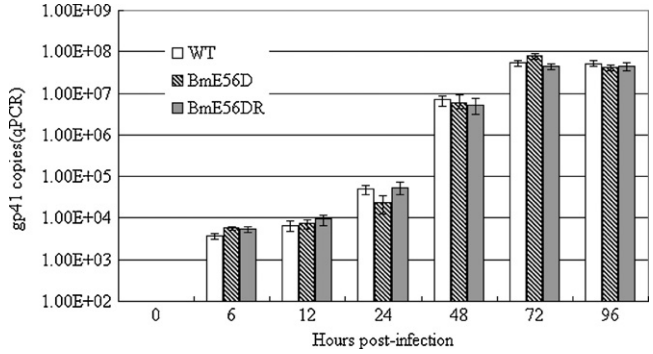

Fig. 4. Quantitative real-time PCR analysis of viral DNA replication. BmN cells were infected with WT, BmE56D and BmE56DR at an MOI of 5. At the designated time points, total cellular DNA was isolated from each virus infected BmN cells, and analyzed by qPCR. Each datum point represents the average from two independent infections. Error bars represent the standard errors.

24, 48, 72 and 96 hpi, indicating that the replication process is not affected by deleting *odv-e56*.

To confirm that equivalent amounts of infectious BV were produced, the virus titers at 6 and 72 hpi were analyzed by $TCID_{50}$ endpoint dilution (Fig. 3B). No infectious BV was detected from any construct at 6 hpt, however, the titers of different viruses at 72 hpi were equivalent, results similar to those obtained using qPCR. The $TCID_{50}$ assays demonstrated that *odv-e56* is not required for the production of infectious BV.

3.4. Effect of odv-e56 deletion on viral DNA replication

To quantitatively analyze the effect of *odv-e56* on viral DNA replication, BmN cells infected with BmE56D, WT and BmE56DR were harvested at various times post-infection, and total DNA was extracted and analyzed by qPCR. The onset of all virus replication occurred at 12 hpi, and the levels of replication were also similar. The DNA quantity generated by BmE56D, WT and BmE56DR increased similarly from 12 to 96 hpi (Fig. 4). This result indicated that *odv-e56* deletion does not affect the virus DNA replication.

3.5. Electron microscopy analysis

To further analyze whether the deletion of *odv-e56* has any effect on virus morphogenesis, electron microscopic analysis was performed with thin sections generated from virus infected BmN cells. BmN cells infected with the virus displayed typical cytological changes and viral morphogenesis of nucleopolyhedrovirus infection. Polyhedra could be found in the ring zone of the nucleus of a cell infected with BmE56D (Fig. 5A), but no such structure in mock-infected cells. Enveloped virions containing nucleocapsids prior to occlusion in the protein crystalline matrix of the developing occlusion bodies were observed within the ring zone, as shown in Fig. 5B, and numerous enveloped virions were embedded in the polyhedra (Fig. 5C). These observations indicated that the deletion of *odv-e56* did not affect the polyhedra morphogenesis and ODV assembly into polyhedra.

3.6. Effect of odv-e56 deletion on viral infection in vivo

To examine the effect of *odv-e56* deletion on viral infection *in vivo*, BV and OBs of WT, BmE56D and BmE56DR from infected BmN cells were assayed in *B. mori* larvae. When the BV supernatant of BmE56D (20,000 $TCID_{50}$ units/larvae) was injected into the hemocoels of 5th-instar *B. mori* larvae, the mortality rate reached to 100%, which was similar to those of WT and BmE56DR (Table 2). Similar results were obtained by injection of 2,000 or 200 $TCID_{50}$ units/larvae. These results suggest that there was no difference in BV virulence when the *B. mori* larvae was infected by direct hemocoel injection. However, significant difference among the mortality rate was observed when 3th-instar *B. mori* larvae were orally inoculated with polyhedra. Ingestion of 20,000 polyhedra/larvae of BmE56D produced only 15.6% of *B. mori* larvae, in contrast, WT and BmE56DR killed 95.6% and 93.3% of the *B. mori* larvae, respectively (Table 2). Similar results were obtained by ingestion of lower doses of polyhedra. These assays indicate that ODV-E56 is required for *per os* infection of BmNPV.

4. Discussion

In this study, the BmNPV envelope protein ODV-E56 is identified as a new *per os* infectivity factor. The deletion of *odv-e56* had no impact on virus replication or BV production. Electron micrographs of *odv-e56* mutants indicated that ODV-E56 is not required for virion morphogenesis or occlusion assembly. However deletion of *odv-e56* in recombinant virus abolished the infectivity of polyhedra (Table 2), and unable to initiate the primary infection, this is the same phenotype observed with other *pif* genes (Kuzio et al., 1989; Kikhno et al., 2002; Pijlman et al., 2003; Ohkawa et al., 2005; Fang et al., 2009). In a previous study, recombinant AcMNPV virus in which the *odv-e56* was replaced with a fusion between the N-terminus of ODV-E56 and β-galactosidase was generated (Braunagel et al., 1996). Electron micrographs of cells infected with this virus also did not show any obvious defects in ODV or occlusion body morphogenesis. Recently, a recombinant AcMNPV clones in which ODV-E56 protein synthesis was eliminated by inserting a β-galactosidase (*lacZ*) expression cassette into the *odv-e56* open reading frame was generated, and can be functionally substituted by *Rachiplusia ou* multiple nucleopolyhedrovirus ODV-E56 (Harrison et al., 2010). However, in this study the BmNPV ODV-E56 is completely deleted

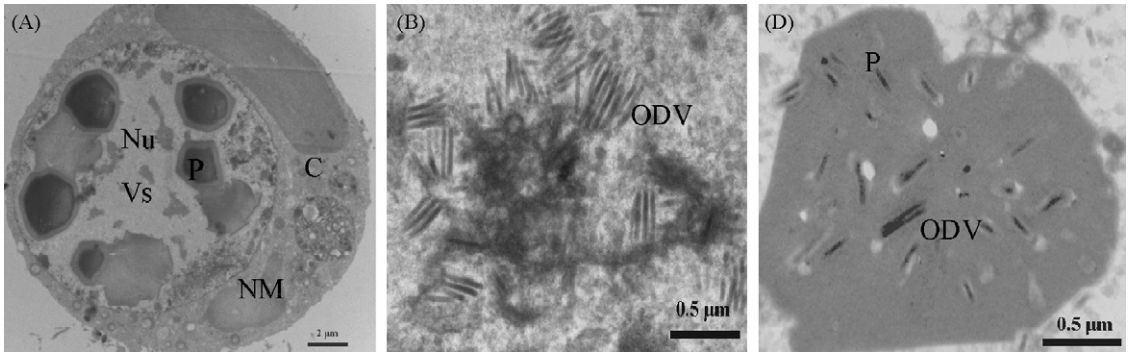

Fig. 5. Electron microscopic analysis of BmE56D infected BmN cells. (A) Polyhedra of the nucleus of a cell infected with BmE56D. (B) ODVs were forming in the ring zone. (C) Normal virions embedded in the polyhedra. Cultures were harvested at 60 hpi. Nu, nucleus; P, polyhedron; NM, nuclear membrane; C, cytoplasm; VS, virogenic stroma.

Table 2
Infectivities of viruses in *B. mori* larvae.

Dosage	Virus	Inoculation methods	Mortality rate (%)*	95% confidence limit (%)	
				Lower	Upper
0	No virus		0	0	0
20,000 TCID50	WT	Injection	100.0a	100.0	100.0
	BmE56D	Injection	100.0a	100.0	100.0
	BmE56DR	Injection	100.0a	100.0	100.0
2000 TCID50	WT	Injection	98.7a	92.9	104.4
	BmE56D	Injection	97.3a	91.6	103.1
	BmE56DR	Injection	97.3a	85.9	108.8
200 TCID50	WT	Injection	84.0a	74.1	93.9
	BmE56D	Injection	85.3a	79.6	91.1
	BmE56DR	Injection	85.3a	79.6	91.1
20,000 polyhedra	WT	*Per os*	95.6a	86.0	105.1
	BmE56D	*Per os*	15.6b	6.0	25.1
	BmE56DR	*Per os*	93.3a	76.8	109.9
2000 polyhedra	WT	*Per os*	53.3a	36.8	69.9
	BmE56D	*Per os*	11.1b	1.5	20.7
	BmE56DR	*Per os*	62.2a	36.9	87.5
200 polyhedra	WT	*Per os*	24.4a	14.9	34.0
	BmE56D	*Per os*	2.2b	−7.3	11.8
	BmE56DR	*Per os*	22.2a	12.7	31.8

* Mortality rate values followed by a different letter are statistically different ($P < 0.01$).

in BmE56D, while in Braunagel et al. and Harrison et al.'s paper the N-terminal ODV-E56 still exist.

The PIFs have previously been determined to play a role in the specific binding of ODV to midgut cells. P74 has been shown to function as an attachment protein and to bind to a 30-kDa host receptor protein on primary target cells within the midgut (Haas-Stapleton et al., 2004). PIF-1 and PIF-2 also mediate the specific binding of ODV to midgut target cells (Ohkawa et al., 2005). Fang et al. (2009) speculated that AC96, P74, PIF-1, and PIF-2 may form complexes to bind host receptors or may act sequentially to mediate ODV entry to initiate primary infection). A recent study showed that the PIFs had to be present in the same virion in order to permit virus infection of the midgut (Song et al., 2008). We believe ODV-E56 is also essential for this activity.

Previously research considered ODV-E56 is a protein that is exclusively associated with the ODV envelope. However, the proteomics of the *A. californica* nucleopolyhedrovirus budded virions identified ODV-E56 was also associated with BV. Subcellular localization of ODV-E56 during infection showed that it distributed in cytoplasm as well as in nucleus. ODV-E56 in the cytoplasm is most likely associated with the endoplasmic reticulum and then transported into the inner nuclear membrane, ultimately to become part of the ODV envelope. It is also possible that ODV-E56 could be delivered to the plasma membrane, and form part of the BV membrane. Analysis of BmE56D BV in cell culture and intrahemocoelic injections did not reveal any phenotypic differences from the WT virus with regard to replication or infectivity. This suggests that ODV-E56 does not have a function in BV.

All the PIF genes are core baculovirus genes, indicating that the midgut infection process is an ancient and highly conserved process. Determining the function of the PIF genes and how they interact with each other and the insect midgut will be critical for the understanding of baculovirus virulence. In addition, the PIF genes are also associated with ODV, therefore studying the PIF genes can provide clues to understand the mechanism of the interaction between ODV protein and polyhedra matrix. This has the potential applications in nanobiotechnology, occlude vaccines and biopharmaceutical products.

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