Characterization of *Bombyx mori* nucleopolyhedrovirus with a knockout of *Bm17*

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Abstract Open reading frame 17 (*Bm17*) gene of *Bombyx mori* nucleopolyhedrovirus is a highly conserved gene in lepidopteran nucleopolyhedroviruses, but its function remains unknown. In this report, transient-expression and superinfection assays indicated that BM17 localized in the nucleus and cytoplasm of infected BmN cells. To determine the role of *Bm17* in baculovirus life cycle, we constructed a *Bm17* knockout virus and characterized its properties in cells. Analysis of the production and infection of budded virions, the level of viral DNA replication revealed showed that there was no significant difference among the mutant, the control, and the *Bm17* repaired virus strains. These results suggest that BM17 is not essential for virus replication in cultured cells.

Keywords Baculovirus · *Bm17* · BmNPV · DNA virus · Gene knock-out

Introduction

The family baculoviridae comprises a diverse group of arthropod-specific DNA viruses. Its host species are mostly from insects of the order Lepidoptera, but are also from the orders Diptera and Hymenoptera (Blissard et al. 2000). The infected invertebrate species mainly belong to the order Lepidoptera and contain two genera: nucleopolyhedrovirus (NPV) and granulovirus (GV) (Theilmann et al. 2005). Baculoviruses are characterized by a circular double-stranded DNA genome (ranging from 80 to 180 kb) packaged within a rod-shaped capsid and enclosed by a lipid envelope (Herniou et al. 2003; Jakubowska et al. 2006). Two types of baculovirus virions, BVs and occlusion derived virions (ODVs) are produced during the life cycle.

Bombyx mori nucleopolyhedrovirus (BmNPV) contains a covalently closed circular genome of 128,413 bp, about 136 putative ORFs based on the criterion that the ORF be a single, contiguous, non-overlapping coding region. Based on the comparative analysis of 29 baculoviruses, 62 *orfs* have been identified in common and designated as baculovirus core genes (Jehle et al. 2006), at present, the baculovirus gene function in this study was to focus on 62 core genes, such as *Bm61* (Shen et al. 2009b); *lef11* (Lin and Blissard 2002) and *gp64* (Lung et al. 2002), etc. With in-depth study of the baculovirus, some non-core genes also were characterized, such as ODV-56 (Xiang et al. 2011); *orf74* (Shen et al. 2009a) and *orf60* of BmNPV (Guo et al. 2010), etc.

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Bm17 of BmNPV (nt 17,215–17,602) encodes a putative protein of 129 amino acids with a predicted molecular mass of 14.5 kDa (Gomi et al. 1999). *Bm17* is conserved among baculoviruses and is shared by all group I NPVs or 14 group II NPVs. *Bm17* shares amino acid sequence identities ranging from 93% with AcMNPV ORF 26–32% with CbNPV ORF15 protein. Sequence-based queries performed with Inter ProScan program showed that *Bm17* is a protein of unknown function.

In this study, we used a BmNPV bacmid to generate a *Bm17* knockout mutant by homologous recombination in *Escherichia coli* to determine the role of *Bm17* in BmNPV infection cycle. Our data indicated that *Bm17* is not essential for virus replication.

Materials and methods

Cells, virus, bacterial strains, and antibiotics

Bombyx mori nucleopolyhedrovirus (ZJ strain) virus was propagated in BmN (BmN-4) cells. The BmN cell line was cultured at 27 °C in TC-100 insect medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, USA) using standard techniques (O'Reilly et al. 1992). The *E. coli* strains BW25113 harboring plasmid pKD46 encoding the λ Red recombination system and BW251141 harboring plasmid pKD3 encoding the chloramphenicol resistance gene (*Cm*) were kindly provided by Dr. Mary Berlyn (Yale University). The DH10 strain harboring the BmNPV bacmid and the helper plasmid pMON7124 were kindly provided by Dr. Enoch Y. Park (Shizuoka University).

Knockout and repair bacmid construction

Bacmids containing deletions of *Bm17* were made using the λ Red homologous recombination system in *E. coli* as described previously (Bideshi and Federici 2000). A 1,107 bp linear DNA fragment containing the chloramphenicol resistance gene was PCR amplified from the pKD3 using the primers KF:5'-TTATTGAAA AATATTTCTTTTAGTCATTCCAAATGTGCACCT TTCTGTGTAGGCTGGAGCTGC-3' and KR:5'-ATCTT AAAATTAAACTTTTGCAACTCGCTGATAGA GCCACGTCCTCCATATGAATATCCTCC-3'. Primers KF and KR contained 45 bp identical arm of

Bm17 gene and 18 bp identical fragment of the *Cm* gene (underlined). Colonies were selected and verified by PCR analysis. The resulting *Bm17* knockout bacmid was named vBm^{ko}. The knockout bacmid constructs were transposed with the pFB1-gfp-polh transfer vector (Vanarsdall et al. 2006) according to the methods described previously (Vanarsdall et al. 2004), to introduce the *gfp* reporter gene under control of the BmNPV *i.e-1* promoter and the *polyhedrin* (*polh*) with promoter derived from pFastBac1. The resulting bacmid was named vBm17-ko. To construct repair bacmids, vBm17-re, a 995 bp fragment containing *Bm17* gene with its native promoter and poly (A) tail, was PCR amplified using primers RE-F:5'-C CTGCAGGTTTTTCAAAAATCTGCCTTCG-3' (*Pst* I site was underlined) and RE-R:5'-AGCGGCCG CCGGACCAATTTTTTATTTC-3' (*Not* I site was underlined). The repair fragments were cloned into the pFB1-gfp plasmid to generate pFB1-Bm17-gfp and used to transpose parental knockout bacmids. The control virus was constructed by transposing bacmid with the pFB1-gfp-polh plasmid and the resulting bacmid was named vBm17-wt.

PCR analysis

PCR analysis was used to confirm the absence of *Bm17* gene in BmNPV bacmid and its replacement by the *Cm* gene. Two primer pairs were used to confirm that *Bm17* had been deleted from the *Bm17* locus of the BmNPV bacmid genome. Primers 17-PF: (5'-ATGG ACGGCTCTGTTGTT-3') and 17-PR: (5'-TTAACTC GTTAAAGTTACG-3'), which are just outside the flanking sequence for recombination, were used to confirm the insertion of the *Cm* gene cassette. Primers CmU (5'-GCTCATGGAAAACGGTGTAACAA-3')/ 17-PR were used to examine correct insertion of the *Cm* gene cassette. Tn7-mediated transposition was also confirmed by PCR with M13 primers (F:5'-TGTA AACGACGGCCAGT-3', R:5'-CAGGAAACAGCT ATGACC-3').

Analysis of virus growth curve

To assess whether Bm17 is required for virus production and determine the replication kinetics of virus constructed, a virus growth curve analysis was performed. For this experiment, transfection–infection assay was

performed to examine the tissue culture infectious dose (TCID₅₀) of vBm17-ko, vBm-wt or vBm17-re bacmid in BmN cells. Then, 1×10^6 BmN cells were infected with vBm17-ko, vBm-wt or vBm17-re virus at an multiplicity of infection (MOI) of 5 and at 12, 24, 48, 72 and 96 h post infection (hpi). The titers were determined by a TCID₅₀ end-point dilution assay using BmN cells (O'Reilly et al. 1992).

Quantitative PCR (QPCR) assay

To detect Bm17-knockout viral DNA replication, a QPCR assay was performed as described previously (Vanarsdall et al. 2005). To prepare total DNA for analysis, 1×10^6 BmN cells were infected with vBm-wt, vBm17-ko or vBm17-re at MOI of 5 and at 12, 24, 36, 48, 72 and 96 hpi. The DNA was extracted as previously described (Xi et al. 2007). The primers P-F (5'-CGT AGTGGTAGTAATCGCCGC-3') and P-R (5'-AGTC GAGTCGCGTCGCTTT-3') were used to amplify a 101 bp region within the *gp41* of BmNPV. A standard curve was created with a twofold sample of purified vBm-wt DNA templates were used in quantitative PCR. 5 dilutions (each 1:10) were prepared to cover the workable concentrations of the DNA templates.

Cellular localization of BM17

The *Bm17* gene without the stop codon (TAA) was amplified from BmNPV bacmid with the PCR primers LO-F:5'-GGATCCGTTTTCAAAAATCTGCCT-3' (*Bam*HI site was underlined) and LO-R:5'-CTCG AGACTCGTTAAAGTTACGGT-3' (*Xho*I site was underlined). PCR product was inserted into pFB1-ph⁻ to generated pFB1-Bm17. The polyhedrin promoter was removed from pFastBac1 to create pFB1-ph⁻. The *gfp* was cloned into the pFB1-Bm17 to generate pFB1-Bm17-gfp and used to transpose BmNPV bacmids. Thus, *Bm17* is expressed with a *gfp* tag under the control of the *Bm17* native promoter in the resulting bacmid, which is referred to as vBm^{Bm17-gfp}. The control bacmid vBm^{Bm17p-gfp}, in which only *gfp* is expressed under the control of the *Bm17* native promoter, was generated in a procedure similar to that for vBm^{Bm17-gfp}.

Transfection-infection assay was performed to examine the TCID₅₀ of vBm^{Bm17-gfp} and vBm^{Bm17p-gfp} bacmid in BmN cells. Then, cells were infected with vBm^{Bm17-gfp} and vBm^{Bm17p-gfp} virus at an MOI of 10,

respectively. At 48 hpi, cells were examined with a laser confocal microscopy (Leica). All images were digitally recorded and merged by the use of Leica software.

Results

Construction of knockout, wt and repair bacmids

To determine whether *Bm17* is essential for viral replication, deletion and repair constructs of each gene were prepared. The vBm17-ko constructs were selected by their resistance to chloramphenicol that indicated that site-specific deletion of the target gene had occurred. The vBm17-re with its native promoter region was inserted into the polyhedrin locus with an *gfp* gene under the *i.e-1* promoter and *polh* gene. The structure of all the deletion and repair constructs is shown in Fig. 1a and confirmed by PCR (Fig. 1b). Primers CmU and 17-PR produced no PCR product in vBm-wt, produced a single 792 bp fragment in vBm17-ko and vBm17-re (Fig. 1b). Primers 17-PF and 17-PR produced single fragments of 390 and 1,348 bp from vBm-wt and vBm17-ko, respectively, produced 1,348 and 390 bp fragment from vBm17-re (Fig. 1b). Tn7-mediated transposition was also confirmed by PCR with M13 primers (Fig. 1b). The results indicated that the *gfp* and *Bm17* under the control of its own promoter had been transposed correctly into the polyhedrin locus.

Analysis of virus replication in BmN cell

At 96 h post transfection (hpt), we obtained high titer stocks of vBm17-ko, vBm-wt and vBm17-re, respectively. Then these viruses were used to infect BmN cells at an MOI of 5. Infected cells were monitored by fluorescence due to expression of *gfp*. Fluorescence can be detected in infected cells by 12 hpi and no difference was observed among the three viruses. Fluorescence was observed in almost all vBm17-ko, vBm-wt or vBm17-re infected cells by 24 and 72 hpi. No difference was observed among the three viruses at 24, 72 hpi (Fig. 2a). At 96 hpi, almost every single cell infected with vBm-wt, vBm-17ko or vBm17-re contained occlusion bodies (date no shown).

To further assess the effect of *Bm17* knockout on virus replication and determine the replication kinetics

Fig. 1 Construction of *Bm17* knockout, repair and wt BmNPV bacmids. **a** The *Bm17* was partially deleted by replacement with chloramphenicol resistance gene via homologous recombination in *E. coli*, to generate vBm^{ko}. *gfp* genes inserted in the polyhedrin locus of vBm^{ko} by Tn7-mediated transposition to generate vBm17-ko and vBm17-re. The *Bm17* ORF inserted into vBm17-re is driven by its own promoter. The vBmwt bacmid was generated by insertion of the *gfp* genes into the polyhedrin locus of BmNPV bacmid. **b** PCR analysis of the presence or absence of sequence modifications in vBm17-ko, vBm17-re, and vBm-wt. The virus templates are shown above each lane, and the primer pairs used are shown below

of virus constructed, a virus growth curve analysis was performed. BmN cells infected with vBm17-ko, vBm-wt or vBm17-re revealed steady increase in virus production. Virus production of these three viruses had no significant difference from 12 to 96 hpi (Fig. 2b). These results suggested that knockout of *Bm17* did not affect production of BV.

Viral DNA replication

QPCR analysis showed that the levels of viral (vBm17-ko, vBm-wt and vBm17-re) DNA replication were the same up to 96 hpi. As shown in Fig. 3, BV production was under way by 14–15 hpi and DNA replication

from BV-infected cells started at approximately 7–9 hpi (Milks et al. 2003), which would mean that DNA replication from secondary BV infections would initiate at approximately 24 hpi. Our results showed that, for three viruses, the onset of viral replication occurred at 12 hpi (Fig. 3) and the levels of transcription were also similar. The DNA levels generated by vBm17-ko, vBm17-ko or vBm-wt showed a similarly steady increase from 12 to 96 hpi (Fig. 3).

Cellular localization of *Bm17* in BmN cells

To detect the subcellular localization of *Bm17*, two recombinant baculoviruses, vBm^{Bm17-gfp} and

Fig. 2 Analysis of viral replication in BmN cells. **a** BmN cells infected with vBm17-ko, vBm-wt or vBm17-re at 12, 24 and 72 hpi, respectively. **b** Virus growth curves generated from cells infected with vBm-wt, vBm17-ko or vBm17-re virus at an MOI of 5, and cell culture supernatants were harvested and assayed for the production of infectious virus by TCID₅₀ assay

Fig. 3 QPCR analysis of viral DNA replication in infected BmN cells. Total cellular DNA was isolated from vBm-wt, vBm17-ko or vBm17-re infected BmN cells, digested with the restriction enzyme *Dpn* I to eliminate input bacmid, and analyzed by QPCR. Data show mean $\pm$ SD ($n = 3$)

vBm^{Bm17p-gfp} were constructed (Fig. 4a). *gfp* was fused to the C-terminus of *Bm17* and was expressed under the control of the *Bm17* native promoter in vBm^{Bm17p-gfp}. As a control, *gfp* alone was expressed under the control of the *Bm17* native promoter in vBm^{Bm17p-gfp} (Fig. 4a). BmN cells infected with vBm^{Bm17p-gfp} and vBm^{Bm17p-gfp} at an MOI of 10 were examined for GFP specific fluorescence by fluorescence microscopy (Fig. 4b). The observed localization of BM17-GFP in vBm^{Bm17p-gfp} infected cells, fluorescence accumulated at the nucleus and cytoplasm of infected cells at 48 hpi. In the negative controls, *gfp* showed homogeneous fluorescence in the cytoplasm and nucleus when infected with vBm^{Bm17p-gfp} in the negative controls (Fig. 4b).

Fig. 4 Fluorescence analyses of the subcellular localization of BM17-GFP. **a** Schematic map of construction of GFP-tagged vBm^{Bm17-gfp} and vBm^{Bm17p-gfp}. **b** Fluorescence images of BmN cells. Cells were infected with vBm^{Bm17-gfp} at an MOI of 10. At

48 hpi, cells were examined for fluorescence by fluorescence microscopy. Cells infected with vBm^{Bm17p-gfp} were used as a control

Discussion

Homologues of *Bm17* have been identified in genomes of almost all NPVs. Sequence-based queries performed with Inter ProScan program showed that *Bm17* homologues constitute a protein family (DUF734) of

unknown function. In this paper, the transcription of *Bm17* and the function of the protein were studied.

The role of *Bm17* in the context of BmNPV infection in BmN cells was examined with a *Bm17* deletion virus, which could propagate as a bacmid in *E. coli*. The *Bm17* deletion virus was able to propagate

after infection of BmN cells and could generate infectious progeny viruses. The slope of virus growth curve of the *Bm17* deletion virus was similar to that of the wt virus and the repair virus. DNA replication of the *Bm17* deletion virus was also similar to that of the wt virus and the repair virus. These results, therefore, indicated that the *Bm17* gene was not essential for virus propagation in BmNPV infected BmN cells. So far, several non-core baculovirus genes have been characterized. For example, no obvious differences in AcMNPV *vfgf* mutants were found in Sf-21 cells, which have or have not *vfgf* with respect to BV production, viral proteins synthesis and shut-off of viral or host proteins, or genome replication (Detvisitsakun et al. 2006). The *ac18* deletion virus has no striking phenotype in Sf-9 cells. The knockout mutant does not reduce AcMNPV infectivity to *Trichoplusia ni* in LD₅₀ bioassay; however, it does take 24 h longer for knockout mutant to kill *T. ni* larvae than wild type virus in LT₅₀ bioassay. *Bm21* of BmNPV, one of the unique genes of group I NPVs, its deletion also has no striking phenotype in BmN cells (Huang et al. 2008), most of these genes were nonessential, and many of them did not show obvious phenotypes (Yang et al. 2009; Katsuma et al. 2008).

Fluorescent microscope examination confirmed that BM17 product was localized in the nucleus and cytoplasm in the stage of infection, a similar protein location was found for other baculovirus proteins, such as ORF29 of BmNPV (Chen et al. 2010). The existence of Bm17 in both the cytoplasm and nucleus suggests that *Bm17* is a multifunctional protein. Taken together, all the above results show that BM17 is not essential for virus replication in cultured cells.

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