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Phenotypic grouping of 141 BmNPVs lacking viral gene sequences

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ABSTRACT

We constructed a series of gene knockout BmNPVs (KOVs) for each of 141 genes (Gomi et al., 1999; Katsuma et al., 2011) using the BmNPV T3 bacmid system (Ono et al., 2007) and lambda red recombination system (Datsenko and Wanner, 2000). In a subsequent analysis of the properties needed for infection using a marker gene, *egfp* (enhanced green fluorescent protein gene), inserted into the polyhedrin locus, the knockout viruses (KOVs) were subdivided into four phenotypic types, A to D. Type-A (86 KOVs) showed the ability to expand infections equivalent to the control while type-B (8 KOVs) spread infections more slowly. Type-C (37 KOVs) expressed *egfp* in transfected-BmN cells but the production of infectious viruses was not observed. Type-D (10 KOVs) showed no ability to express *egfp* even in the transfection experiments. KOVs lacking genes (*pkip* (*Bm15*), *gp41* (*Bm66*), *bro-d* (*Bm131*), *Bm20*, *48*, *65*, *91*, *93*, or *101*) previously identified as being essential, were placed in the viable type-A and B categories.

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1. Introduction

Nucleopolyhedrovirus (NPV) is one of the largest DNA viruses. Its genome contains over 100 genes, which are expressed in a stage-dependent manner; immediate-early, delayed-early, late and very-late (Friesen and Miller, 1986). Genes of NPV are involved in transcriptional regulation (Guarino and Summers, 1986; Yoo and Guarino, 1994), viral RNA polymerase components (Guarino et al., 1998), nucleocapsid formation (Thiem and Miller, 1989; Vanarsdall et al., 2006), host gene regulation (Nobiron et al., 2003) and so on. About half of them are predicted to be essential for viral propagation through gene expression, DNA replication and virion components (Rohrmann, 2011). On the other hand, there are some accessory genes or non-essential genes that remain in the viral genome during passage. These observations suggested that each viral gene would work to the advantage at least in certain situations through interaction with other viral/host genes.

The function of baculoviral genes in replication has been studied vigorously by using gene knock-out (KO) technology in baculoviruses such as *Autographa californica* multiple NPV (AcMNPV) and *Bombyx mori* NPV (BmNPV) (Rohrmann, 2011), however, the function of more than one-third of viral genes are still unknown. In addition, KO viruses have been generated based on different genetic backbones using various methods, that is; different species (AcMNPV or BmNPV), strains and methods for mutation (temperature-sensitive mutant, deletion or insertion into a wild-type baculoviral genome or bacmid). In the 1980s, KO viruses were obtained by conventional homologous recombination in insect cells, sometimes making it difficult to distinguish the negative results caused by a failure of the recombination from those caused by the knock-out of an essential gene. This problem was solved when the bacmid system was established in AcMNPV (Luckow et al., 1993) since the viral (bacmid) DNAs could be amplified in *Escherichia coli* even if they lacked essential genes for replication in the host insect cells. Now this technique is available for other baculoviruses such as BmNPV (Motohashi et al., 2005) and *Helicoverpa armigera* SNPV (HearNPV) (Wang et al., 2003).

In general, early genes are transcribed by host RNA polymerase II and mainly involved in regulating replication as trans-regulators, however, late genes are transcribed by viral-derived RNA polymerase and involved in forming viral structure as capsid proteins. In addition, there have been reports that the expression of host genes was not only down-regulated (i.e. shut off), but also up-regulated during baculovirus infections (Nobiron et al., 2003; Sagisaka et al., 2010.). These observations suggested that the baculovirus controlled the host cell mechanisms to produce progeny viruses using host factors and machinery through a complex gene regulatory mechanism among not only viral genes but also host genes. To understand the mechanisms of viral replication, insight into the functions of baculoviral genes and host genes responding to viral infections is essential. Among lepidopteran insects, which include many hosts of baculoviruses, *Bombyx mori* is well-studied physiologically, biochemically and molecular biologically. It is worth noting that the genome has been sequenced through international collaboration between Japan and China (International Silkworm Genome Consortium, 2008), suggesting that the informational

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environment for analyzing the interaction between host and virus is being put into place in the BmNPV-silkworm infection system.

To establish a platform for the comprehensive analysis of the BmNPV gene network and/or interaction between viral and host genes, we constructed a series of gene knock-out BmNPVs (KOVs) for each of 135 genes (Gomi et al., 1999) and another 6 ORFs (Katsuma et al., 2011) using the BmNPV T3 bacmid system (Ono et al., 2007) and lambda red recombination system (Datsenko and Wanner, 2000). Subsequently the growth properties of KOVs in BmN cells, and the gene knockout-specific effects on the production of infectious progeny and polyhedrin gene expression were analyzed using a marker (GFP) controlled by the *polh* promoter.

2. Materials and methods

2.1. Cells, bacmids and transfection

BmN cells were maintained in TC-100 medium (Applichem) containing 10% FBS at 26 °C. Transfection of BmN cells with bacmid DNAs was performed by lipofection using FuGENE HD Transfection Reagent (Promega) according to the manufacturer's instructions.

E. coli strains BW25113 (containing pKD46 encoding the genes for the lambda red recombination system) and BW25141 (pKD3 encoding a chloramphenicol acetyltransferase gene (*cat*)) (Datsenko and Wanner, 2000) were provided by the *E. coli* Genetic Stock Center (Yale University, USA).

2.2. Generation of knockout BmNPV bacmids expressing EGFP

The BmNPV bacmid system (T3 strain (Ono et al., 2007)) was used to generate knockout viruses with the lambda red recombination system (Datsenko and Wanner, 2000).

We first generated a transfer vector for the *polyhedrin* (*polh*) locus of the bacmids. The enhanced green fluorescent protein (EGFP) coding sequence (*egfp*) was excised from pEGFP-1 (Clontech) by digestion with *Bam*H I and *Not* I and ligated into the *Bam*H I-*Not* I site of pFastBac1 (Invitrogen), yielding pFastBac-GFP. The BmNPV T3 bacmid expressing EGFP under the control of the *polh* promoter was produced by transposition in *E. coli* (BmT3DH10Bac; containing the BmNPV genome bacmid and a helper plasmid pMON7124 encoding a transposase) with pFastBac-GFP. The bacmid DNA carrying *egfp* was isolated from a kanamycin-, gentamycin-resistant and lacZ-negative colony, and designated Bmbac^{+egfp} (BmGFP) as described elsewhere (Ono et al., 2007).

For knocking out each gene, we used the lambda red recombination system (Datsenko and Wanner, 2000). KOVs were generated by homologous recombination in *E. coli* containing pKD46 as a helper plasmid encoding a lambda red recombinase to replace each target gene with a chloramphenicol acetyltransferase (*cat*) gene from pKD3 for antibiotic selection. Briefly, DH10B cells (Invitrogen) containing pKD46 were transfected with BmGFP DNA by electroporation using a GenePulser (Biorad) (25 μF, 2.5 kV, 200 Ω) and designated BmT3DH10B^{+egfp}-pKD46. Then, the *cat* gene sequences wedged between the 5′ non-coding regions (50 nucleotides (nts)) and the 3′ non-coding regions (50 nts) of the BmNPV target genes were amplified by PCR with the primer sets (Table 1) using pKD3 as a template. Primers for knocking out a gene were designed not to prevent the expression of adjacent genes, that is, promoter motifs (CAGT, TATA, and TAAG) and at least 30 nts upstream from the ATG translation start codon of the adjacent genes remained. The length of each deleted region was from 34 (Δ*Bm95a*) to 3605 nts (Δ*dnahel* (*Bm78*)). After *Dpn* I treatment to digest pKD3, PCR products (approximately 1 kbp, >500 ng) were purified using Wizard SV Gel and a PCR Clean-Up System (Promega) and transformed into BmT3DH10B^{+egfp}-pKD46 by electroporation as above. Then, cells were incubated with 0.01% Arabinose SOC broth for 4–6 h at 37 °C. Each gene-knockout BmNPV bacmid was obtained from an chloramphenicol- and kanamycin-resistant colony, followed by verification of the absence of the viral gene ORF by PCR with gene-specific primer sets targeting the sequence (about 300 nts) inside of each gene, which were designed for a baculovirus DNA microarray (Yamagishi et al., 2003). On the other hand, the existence of the cat sequence in each KOV was confirmed by PCR with primers to amplify the sequence surrounding the 5′-terminus (cat-up: 5′-gaatcagctccagcctacac-3′ and the gene-specific primers) or the 3′-terminus (cat-down: 5′-ctaaggaggatattcatatg-3′ and the gene-specific primers) of the *cat* sequence. After incubation at 37 °C for 6 h to remove the helper plasmid pKD46 from bacteria, Ampicillin-sensitive colonies were selected. Bacmid DNAs were purified from 50-ml LB cultures using a Qiagen midi-plasmid kit (Qiagen) and each concentration was determined by NanoDrop2000 (Thermo scientific).

2.3. KOV transfection and infection assay

BmN cells were washed with serum-free TC-100 medium and seeded into 96 well plates (5×10^4 cells/well). The cells were transfected with each bacmid (0.25 μg) as described above and subjected to fluorescence microscopic observation. The fluorescence intensity of EGFP was monitored by infinite M200PRO (Tecan). At 4 days post transfection (d.p.t.), the supernatant (10 μl) was collected and added to freshly seeded BmN cells. The culture was continued and the fluorescence intensity of these cells was measured daily as above.

3. Results

3.1. Generation of BmNPV knockout bacmids

BmNPV has about 140 genes, however, most of their functions were remain unknown. To investigate their role in the viral replication cycle, we generated 141 genes-knockout viruses. The absence of the target gene in each KOV was verified by PCR. PCR targeting a sequence of 0.3 kbp inside each deleted ORF was carried out for the knockout bacmids, resulting in negative for each ORF (Fig. 1(b): results for Δ*Bm20*, Δ*Bm48*, Δ*Bm91*, Δ*Bm93* and Δ*Bm101* are shown as examples). On the other hand, PCR with the primer sets targeting the 5′-terminal region (Fig. 1(c)) or the 3′-terminal region (Fig. 1(d)) of the *cat* sequence resulted in the amplification of DNA fragments of expected size for the 5′-terminal region (1500 nucleotides (nts) for Δ*Bm20*, 800 nts for Δ*Bm48*, 1200 nts for Δ*Bm91*, 2000 nts for Δ*Bm93*, 500 nts for Δ*Bm101*) or the 3′-terminal region (650 nts for Δ*Bm20*, 300 nts for Δ*Bm48*, 750 nts for Δ*Bm91*, 450 nts for Δ*Bm93*, 700 nts for Δ*Bm101*). We confirmed the knocked out region of other KOVs in the same way (data not shown). The KOV (Δ*Bm95a*) failed possibly because the sequence to be deleted for *Bm95a* was very small (34 nts). Therefore, we analyzed a KOV lacking both *Bm95a* and *Bm96* (Δ*Bm95a-96*).

3.2. The growth properties of each KOV

To define each KOV phenotype, we introduced the bacmids into BmN cells and analyzed the EGFP expression every 24 h. In addition, at 96 h post transfection (h.p.t.), the culture medium was collected and inoculated into the new cell layer in order to determine whether the infectious virion was produced or not. Because EGFP was driven by the *polh* promoter in each KOV-transfected cell, we observed the infection and expression of *polh* based on the intensity of EGFP in the cells.

The KOV (bacmid)s were roughly subdivided into four phenotypes (A to D); type-A and -B KOVs produced infectious viruses but

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