Characterization of a Bombyx mori nucleopolyhedrovirus variant isolated in Laos

Shota Fujimoto¹, ², Ryuhei Kokusho³, Haruka Kakemizu¹, Tadashi Izaku¹, Susumu Katsuma³, Yoshimitsu Iwashita¹, Hideki Kawasaki³ and Masashi Iwanaga¹, ²*¹

¹ Department of Agrobiology and Bioresources, School of Agriculture, Utsunomiya University, Mine-machi 350, Utsunomiya-shi, Tochigi 321-8505, Japan
² Department of Biological Production Science, United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Saiwai 3-5-8, Fuchu-shi, Tokyo 183-8509, Japan
³ Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

*To whom correspondence should be addressed.
Fax: +81-28-649-5401. Tel: +81-28-649-5454. Email: iwanaga@cc.utsunomiya-u.ac.jp (Masashi Iwanaga)

The Bombyx mori nucleopolyhedrovirus (BmNPV) La strain is a variant isolated in Laos (Iwashita, 1993). Although DNA fingerprinting analysis revealed nucleotide sequence variations in the genomes of BmNPV La and T3 (the type strain of BmNPV), other features of La, including virulence and growth properties, are unknown. Here we compared the pathogenicity and replication of La and T3 in B. mori larvae and cultured cells. Larval bioassays revealed that the median lethal dose of La BV was approximately 2-fold lower compared with that of T3. We found that La induced earlier host liquefaction compared with T3, and the median lethality time of La was approximately 6 h shorter compared with that of T3. Further, La-infected B. mori larvae released more occlusion bodies (OBs) into the hemolymph compared with those infected with T3. These results show that the virulence of La is higher compared with that of T3. Moreover, La may be suitable for use in a BmNPV-based expression vector system, because La produced approximately 4-fold and 1.75-fold higher levels of polyhedrin mRNA and protein, respectively, compared with T3. Moreover, we found that multicapsid occlusion-derived viruses (ODVs) were more numerous in La-infected cells compared with those in T3-infected cells. However, there was no significant difference in the median lethal oral dose. Together, these results show that La exhibits unique features that mediate pathogenesis, production of the Polyhedrin protein, and the formation of multicapsid ODVs.

Key words: Baculovirus, Nucleopolyhedrovirus, BmNPV, Polyhedrin, Baculovirus expression vector system

INTRODUCTION

Baculoviruses represent a diverse group of viruses with large, double-stranded, circular DNA genomes ranging from 80 kb to 180 kb, which are packaged in enveloped, rod-shaped virions. Baculoviruses are classified into four genera according to host specificities and phylogenetic analysis as follows: Alphabaculovirus (lepidopteran-specific nucleopolyhedroviruses [NPVs]), Betabaculovirus (lepidopteran-specific granuloviruses [GVs]), Gammabaculovirus (hemipteran-specific NPVs), and Deltabaculovirus (dipteran-specific NPVs) (Jehle et al., 2006). Alphabaculoviruses can be subdivided into group I or II NPVs according to phylogenetic studies (Herniou et al., 2001).

During the life cycle of NPVs, occlusion-derived viruses (ODVs) and budded viruses (BVs) are produced. ODVs and BVs play unique roles in the viral life cycle. ODVs, which are virions enclosed in occlusion bodies (OBs), establish the primary infection in the midgut of the host through an oral route, whereas BVs are responsible for systemic infection by transmitting the infection to neighboring cells (Granados and Lawler, 1981; Keddie et al., 1989). In NPVs, the ODV comprises virions containing a single nucleocapsid (SNPV) or multiple nucleocapsids (MNPVs).

Among group I NPVs, the Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) and Bombyx mori nucleopolyhedrovirus (BmNPV) are frequently used as baculovirus expression vector system (BEVS) in cultured cells and larvae (reviewed in Rohrmann, 2013). BmNPV is the first baculovirus used to express foreign genes in larval insects, and the complete genome sequence of its type strain T3 is available (Maeda et al., 1985; Gomi et al., 1999). The complete genome sequences of eight BmNPV strains and two Bombyx mandarina nucleopolyhedroviruses (BomaNPVs) are available as well (Xu et al., 2010, 2012; Fan et al., 2012; Ardisson-Araujo et al., 2014). These BmNPV variants have unique features. For example, the BmNPV Cubic strain forms cubic OBs, the Brazilian strain produces OBs containing both single and multicapsid ODVs (Cheng et al., 2012; Ardisson-Araujo et al., 2014), BomaNPV S2 produces singlecapsid ODVs in B. mori-derived cells and multicapsid ODVs in Trichoplusia ni-derived cells (Xu et al., 2012).

In the present study, we characterized the BmNPV variant La, which was isolated in Laos (Iwashita, 1993). We found that La was highly pathogenic for B. mori larvae and cultured cells compared with T3. Further, reverse-transcription quantitative PCR (RT-qPCR) analysis demonstrat-
ed that the polyhedrin gene is transcribed in La-infected cells at levels approximately 4-fold higher compared with those in T3-infected cells. Moreover, we show that La formed larger numbers of multicapsid ODVs in cultured cells compared with T3. Taken together, these results indicate that La has unique features compared with those of other BmNPV strains and may be suitable for developing a BEVS that achieves higher levels of expression.

MATERIALS AND METHODS

Insects, cells, and viruses

B. mori larvae (F1 hybrid N124 × C124) were reared as previously described (Iwanaga et al., 2002). BmN-4 (BmN) cells were maintained as previously described on TC-100 medium supplemented with 10% fetal bovine serum (Iwanaga et al., 2002). The BmNPV La isolate (Iwashita, 1993) was purified three times using a plaque assay. The BmNPV T3 (Maeda et al., 1985) and La isolates were propagated in BmN cells. The cells were infected with BmNPV at a multiplicity of infection (MOI) = 10. Virus titers are expressed as plaque-forming units (PFUs) by plaque assay as previously described (Iwanaga et al., 2004).

Larval bioassays

The median lethal dose (LD$_{50}$) of BV was determined using day 1, fifth instar larvae that received intrahemocoelic injections of BV diluted with TC-100 medium to 1 × 10$^1$, 1 × 10$^2$, 1 × 10$^3$, and 1 × 10$^4$ PFU. The median lethal time (LT$_{50}$) was determined by administering an intrahemocoelic injection (1 × 10$^5$ PFU) into day 1, fifth instar larvae. The median lethal concentration (LC$_{50}$) of OB was determined using per os infection. The newly hatched first instar larvae were fed with small blocks of an artificial diet containing different concentration of OBs (1 × 10$^2$, 5 × 10$^2$, 1 × 10$^3$, 5 × 10$^3$, and 1 × 10$^4$ OB/cm$^3$). Twenty insects per dose were used in each experiment, mortalities were counted every 6 h, and LT$_{50}$, LD$_{50}$, and LC$_{50}$ values were determined using probit analysis (Finney, 1971).

OB production in B. mori larvae and BmN cells

Day 1, fifth instar larvae were starved for several hours, injected with 50 μl of the BV suspension (1 × 10$^5$ PFU), returned to the artificial diet, and maintained at 25°C. To assess the amount of OBs released into the hemolymph, hemolymph samples of infected larvae were collected at 72 h and 96-h postinfection (hpi). To evaluate the amount of OBs in the intact body of a larval cadaver, virus-infected larvae were isolated individually in 50 μl tubes at 72 hpi, homogenized with PBS, and filtered using cotton gauze. To determine the amount of OBs contained in cultured cells, BmN cells (4 × 10$^5$) infected with La or T3 were harvested at the designated times and gently scraped using a rubber policeman. After centrifugation, OBs were extracted by lysing the cells with 0.1% SDS. The OBs were counted using a Burker-Turk hemocytometer (Hirschmann, Eberstadt, Germany). To quantitate extracellular OBs, square areas (150 μm$^2$) were observed using a light microscope (IMT-2; Olympus, Tokyo, Japan).

BV production in B. mori larvae and BmN cells

To determine virus titers in the hemolymph, day 1, fifth instar larvae were starved for several hours and then injected with La or T3 BV (1 × 10$^5$ PFU). The hemolymph of infected larvae was collected at 72 and 96 hpi and analyzed using plaque assays. Virus growth was determined using BmN cells infected with La or T3, MOI = 10. A small amount of culture medium was harvested at the designated times, and BV production was determined as previously described (Iwanaga et al., 2002).

Polyhedrin expression in BmN cells

To estimate the expression of the Polyhedrin protein, BmN cells infected with La or T3 were collected at 48, 72, and 96 hpi and subjected to SDS-PAGE. The gels were stained using Coomassie Brilliant Blue R-250 (Eastman Kodak, Rochester, NY) to visualize the proteins, and the Polyhedrin bands were quantified using NIH Image J 1.51 h. For RT-qPCR analysis, total RNAs were isolated from BmN cells infected with La or T3 at the designated times. First-strand cDNA was synthesized to serve as qPCR templates using a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). The mRNA levels of polyhedrin were determined using a SYBR Premix Ex Taq II (Takara Bio) with the LightCycler 96 (Roche, Basel, Switzerland) as previously described (Innami et al., 2016). The forward and reverse primers used to amplify polyhedrin sequences were rpolhF1: 5′-GAAACAAGGGGAAGCAATG-3′ and rpolhR1: 5′-TCCAGTGGCGATTACCTC-3′, respectively (Kokusho et al., 2016). Thermal cycling was performed as follows: (1) 30 s at 95°C, one cycle and (2) 5 s at 95°C and 20 s at 60°C, 50 cycles. Melting-curve analysis of amplicons was performed to demonstrate the specificity of the PCR products.

To compare amounts of replicated viral genomic DNAs, BmN cells were inoculated with La or T3, harvested at the designated times pi, and then lysed with 200 μl of cell lysis buffer (10 mM Tris pH 7.8, 10 mM EDTA, 0.5% SDS, and 1 mg of proteinase K). DNA was extracted using the standard phenol–chloroform method and subjected to qPCR using the primer sets po1h-qF and po1h-qR for polyhedrin and ago3_gF: 5′-CTCTTCCAGACATCAGGAGGAGGGAAGCAATG-3′ and ago3_gR: 5′-TTTTGCTAATGACTCAACGAGGAGGGAAGCAATG-3′ for B. mori argonaute 3 (Kokusho et al., 2016). B. mori argonaute 3 was used for internal standardization. Thermal cy-
Characterization of BmNPV La strain

Table 1. Dose-mortality of BV of La and T3 in B. mori larvae

<table>
<thead>
<tr>
<th>Virus</th>
<th>LD₅₀ (PFU/larva)</th>
<th>95% Confidence limit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td>T3</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>La</td>
<td>0.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>

clinging to amplify B. mori ago3 was performed as follows: (1) 30 s at 95°C, 1 cycle; (2) 5 s at 95°C, 20 s at 42°C, and 20 s at 72°C, 50 cycles. Melting-curve analysis was performed as described above. To determine the DNA sequence of the polyhedrin promoter of La, genomic DNA was extracted from La ODVs using proteinase K digestion and phenol–chloroform extraction (Gomi et al., 1999) and subjected to PCR using the primers polh-F: 5’-CGG CTCCGGCCACTATTAATGCAA-3’ and polh-R: 5’-GCC TAAAGCGCCGGATGTTAATATG-3’. Sequence analysis was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA) with a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Transmission electron microscopy (TEM)

BmN cells were inoculated with La or T3. OBs were extracted at 120 hpi, purified, and fixed using 2% glutaraldehyde in PBS. The OBs were then fixed using 1% osmium tetroxide in PBS overnight, dehydrated using a standard acetone series, and embedded in epoxy resin. The 70-nm sections were stained with uranyl acetate and lead citrate and viewed using a JEM-2000EX transmission electron microscope (JEOL, Tokyo, Japan) operated at 200 kV.

Statistical analysis

Statistical analysis was performed using KaleidaGraph ver. 4.1 (Synergy Software, Reading, PA) or Prism 5 (GraphPad Software, La Jolla, CA), and p < 0.05 indicates a significant difference.

RESULTS

Viral infectivity and pathogenicity

To evaluate viral virulence, B. mori, fifth instar larvae received intrahemocoelic injections of varying doses of BVs and were monitored for mortality. We found that the LD₅₀ of La was approximately 2-fold lower compared with that of T3 (Table 1). Next, we determined the LT₅₀ values of La and T3 by administering intrahemocoelic injections of BVs to fifth instar larvae. The survival curves indicate that the LT₅₀ of La was approximately 6 h shorter compared with that of T3 (Fig. 1). Also, typical liquefaction of the integument was observed in larvae infected with La or T3 at 84 hpi and 96 hpi, respectively (Fig. 2). These results indicate that La BV was highly pathogenic, which was associated with earlier liquefaction of B. mori larvae.

Next, in order to compare the per os infectivity of La and T3 OBs in B. mori larvae, we used the most sensitive

Fig. 1. Survival curves of La- and T3-infected B. mori larvae. Fifth instar B. mori larvae were injected with La (closed circles) or T3 (open circles) BVs (n = 20). The LT₅₀ values of La and T3 were 108.9 and 114.0 h, respectively.

Fig. 2. Representative photographs showing liquefaction of the integument in B. mori larvae infected with La or T3. Fifth instar B. mori larvae were injected with La or T3 (n = 20 each). Images were acquired at 24, 48, 72, 84, and 96 hpi.
method to determine LC_{50} values of OBs from both strains. Newly hatched larvae were orally administered various concentrations of OBs and monitored for mortality. Since, there was no significant difference between the LC_{50} values of La and T3 OBs (Table 2), we concluded that the oral infectivity of La was equivalent to T3.

<table>
<thead>
<tr>
<th>Virus</th>
<th>LC_{50} (OB/ml)</th>
<th>95% Confidence limit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td>T3</td>
<td>3448.3</td>
<td>2667.6</td>
</tr>
<tr>
<td>La</td>
<td>3299.8</td>
<td>2559.8</td>
</tr>
</tbody>
</table>

Fig. 3. OB production in B. mori larvae infected with La or T3. Fifth instar B. mori larvae were injected with La or T3 (1 x 10^5 PFU). (A) OBs released into the hemolymph of B. mori larvae at 72 hpi and 96 hpi. OBs were counted using a hemocytometer. The results represent the average value of three larvae, and the error bars indicate the standard deviation. The asterisk indicates a statistically significant difference (p < 0.05, Student's t-test). (B) OBs produced in the intact bodies of B. mori larvae. The OBs in B. mori cadavers were counted using a hemocytometer. Boxes represent the median values and the 25-75th percentiles of nine cadavers. Whiskers indicate the highest and lowest values.

Fig. 4. BVs produced by B. mori larvae infected with La and T3. Fifth instar B. mori larvae were injected with La or T3 BVs (1 x 10^5 PFU). BV titers were determined using a plaque assay. The results represent the average values of BVs in three larval hemolymph samples, and the error bars indicate the standard deviation. The asterisk indicates a statistically significant difference (p < 0.05, Student's t-test).

OB production in B. mori larvae and BmN cells

We counted OBs in the hemolymph of a fifth instar larva infected with La and T3. OBs in the hemolymph of La-infected larvae at 72 hpi and 96 hpi were 2.9-fold and 1.6-fold more abundant, respectively, compared with those of T3-infected larvae (Fig. 3A). To determine whether La produced a large number of OBs in intact bodies of infected larvae, we homogenized individual cadavers and counted OBs. There was no significant difference between the numbers of OBs extracted from cadavers infected with La or T3 (Fig. 3B). Next, we determined viral titers of fifth instar larvae infected with La or T3 and found that there was no significant difference in BV production at 96 hpi, although La produced fewer BVs compared with T3 at 72 hpi (Fig. 4). These results indicate that there was no significant difference in OB and BV production, although more OBs were released in the hemolymph of B. mori larvae infected with La compared with T3.

We further investigated OB production in cultured cells infected with La or T3. BmN cells were inoculated with La or T3 and observed using a light microscope at 72, 120, and 168 hpi. OB formation began at 72 hpi in La- and T3-infected cells (Fig. 5A). Notably, most of the La-infected cells were disrupted, and many OBs were released into the extracellular space at 168 hpi (Fig. 5A). We counted extracellular OBs and found much larger OBs associated with BmN cells infected with La compared those infected with T3 (Fig. 5B). We next compared OB production in cells infected with La or T3 and found that OB production in the former was comparable with that in cells infected with T3 (Fig. 5C). These results are consistent with the results obtained using larvae (Fig. 3A and B), indicating that La severely disrupts host cells, leading to an ex-
tensive release of OBs.

**Polyhedrin expression in BmN cells**

We found that a large number of OBs were released to the outside of the La-infected cells (Fig. 3), whereas the total number of OBs in La-infected cells was equivalent to that in T3 (Fig. 5). We then investigated the expression of Polyhedrin, which is the major constituent protein of OB, in BmN cells infected with La or T3. We compared the amount of Polyhedrin protein produced in BmN cells infected with La or T3. SDS-PAGE analysis showed that the levels of Polyhedrin in BmN cells infected with La were higher compared with those infected with T3 (Fig. 6A). Densitometric analysis indicated that La produced approx-
approximately 1.75-fold more Polyhedrin at 96 hpi compared with T3 (Fig. 6A). We next examined the mRNA levels of polyhedrin. RT-qPCR analysis revealed that the levels of polyhedrin mRNA in BmN cells infected with La were significantly higher compared with those infected with T3 (Fig. 6B). At the peak of transcription (84 hpi) of polyhedrin, the level of polyhedrin mRNA in cells infected with La was as much as 4-fold higher compared with cells infected with T3 (Fig. 6B).

When we compared the growth properties of La and T3 in BmN cells, we found that viral growth curves and the levels of virus genomic DNAs were not significantly different (Fig. 6C and D). Further, the polyhedrin promoter sequences of La and T3 were identical (Fig. 6E). Taken together, these results indicate that La produces more polyhedrin transcripts in cultured cells, which is due to the genomic region(s) other than the polyhedrin promoter.

**TEM of La OBs**

TEM did not detect differences between the shapes and sizes of La and T3 OBs, although numerous ODVs containing multiple nucleocapsids were embedded in the OBs of La. Most ODVs of both strains included one or two nucleocapsids, whereas 15% and 0.9% of the ODVs of La and T3, respectively, included >3 nucleocapsids.

**DISCUSSION**

In the present study, we compared viral pathogenicity, OB production, Polyhedrin expression, and the number of nucleocapsids in the ODVs of La and T3. These experiments show that compared with T3, La is more pathogenic, produces higher levels of the polyhedrin transcript, and forms more multicapsid ODVs.

When a baculovirus-infected insect larva dies, its integument melanizes and readily disintegrates, releasing large amounts of OBs into the environment to facilitate efficient horizontal transmission (Volkman and Keddie, 1990). Baculovirus genomes encode auxiliary genes that are not required for virus replication; however, these genes confer a selective advantage. For example, the auxiliary genes v-cath (a cysteine protease) and v-chiA (a viral chitinase)
Characterization of BmNPV La strain

encode V-CATH and V-CHIA, respectively, which promote liquefaction of the host body (Ohkawa et al., 1994; Hawtin et al., 1997). Further, v-cath is required for body liquefaction and contributes to host viability (Hom et al., 2002).

The present study reveals that La is more pathogenic and induces host liquefaction earlier compared with T3 (Table 1 and Fig. 2). Further, we found that B. mori larvae infected with La died faster and released considerably more OBs into the host hemolymph compared with larvae...
infected with T3 (Figs. 1 and 3A). Moreover, the extracellular release of OBs was not caused by higher production of OBs but to the enhanced disintegration of La-infected cells (Fig. 5). Therefore, the protease activity, expression level, or both of La V-CATH may have been higher compared with those of T3. Also, it is possible that increased V-CATH activity reduced the lethal time of La-infected larvae. It has been reported that BmNPV FP25K is essential for maintaining a steady-state level of v-cath expression throughout the infection (Katsuma et al., 2009). Therefore, the fp25k encoded by La should also be analyzed in the future.

The polyhedrin gene is abundantly transcribed under the control of the strong polyhedrin promoter. The virus-induced RNA polymerase initiates at a TAAG sequence motif. Mutagenesis studies demonstrate that sequences upstream of the TAAG motif have a minor effect on activity, whereas any mutation in the TAAG sequence abolishes transcription initiation (Rankin et al., 1988; Katsuma et al., 1999). Further, mutations in the adenine- and thymine-rich sequences downstream of the TAAG motif dramatically reduce transcription (Ooi et al., 1989). The present study shows that the level of polyhedrin mRNA in cells infected with La was higher compared with those infected with T3 (Fig. 6B), although we determined that the polyhedrin promoter sequences of La and T3 are identical (Fig. 6E). Therefore, the mechanism of polyhedrin transcription employed by La should be identified in the future. Interestingly, the virus-encoded vlf-1 gene (encoding very late factor-1) specifically enhances activation of the very late promoters p10 and polyhedrin (Yang and Miller, 1999) and that the host gene encoding PPBP (polyhedrin promoter binding protein) is involved in the transcription of polyhedrin (Ghosh et al., 1998). Thus, these factors encoded by the virus or host may enhance polyhedrin transcription in BmN cells infected with La.

It is noteworthy that because La synthesized an abundant amount of polyhedrin mRNA, the La strain may be more advantageous for recombinant protein expression using BEVS. However, as shown in Figs. 2 and 5, it is possible that the La V-CATH causes not only cell disintegration but also severe degradation of the recombinant protein. It has been reported that recombinant protein production is significantly improved using a BmNPV lacking v-cath (Suzuki et al., 1997). Therefore, the deletion of v-cath from the BmNPV La genome may greatly enhance the production of recombinant protein.

In the present study, we show that the numbers of enveloped nucleocapsids formed in OBs were higher in cells infected with La compared with those infected with T3 (Fig. 7). The products of certain AcMNPV genes contribute to the formation of a multicapsid ODV. For example, the disruption of ac23 or ac78, which encode a product associated with ODVs, reduces the rate of production of multicapsid ODVs (Yu et al., 2009; Li et al., 2014). Further, knockout of ac132, which encodes a product associated with the nucleocapsid, decreases the production of multicapsid ODVs (Yang et al., 2014). BmNPV encodes ORFs 14, 64, and 109, which are ac23, ac78, and ac132 homologs, respectively (Gomi et al., 1999). Therefore, their La homologs may be involved in the formation of multicapsid ODVs, and the La strain may be useful for studies aimed to determine why BmNPV mainly envelops a single nucleocapsid ODVs. Moreover, whole-genome sequencing of La strain and subsequent functional analyses of above-mentioned genes encoded by La may contribute to identification of the mechanism of formation of multicapsid ODVs.
ACKNOWLEDGEMENTS

We thank the Technology Advancement Center, Graduate School of Agricultural and Life Sciences, The University of Tokyo for preparing the TEM samples and data acquisition. This work was supported in part by the A-step Feasibility Study Program (AS262Z01406N) of the Japan Science and Technology Agency (to MI), the Project F Program of the Creative Department for Innovation, Tsukuba University (to MI), and by JSPS KAKENHI Grant Number 16H05051 (to SK).

REFERENCES


