

Cell-dependent production of polyhedra and virion occlusion of *Autographa californica* multiple nucleopolyhedrovirus *fp25k* mutants *in vitro* and *in vivo*

Xin-Hua Cheng,^{1,2} C. M. Senthil Kumar,^{1†} Basil M. Arif,³ Peter J. Krell,⁴ Chuan-Xi Zhang⁵ and Xiao-Wen Cheng^{1,2}

Correspondence
Xiao-Wen Cheng
chengx@muohio.edu

¹Department of Microbiology, Miami University, Oxford, OH 45056, USA

²Graduate Program in Cell, Molecular and Structural Biology, Miami University, Oxford, OH 45056, USA

³Laboratory for Molecular Virology, Great Lakes Forestry Center, Sault Ste Marie, Ontario, P6A 2E5, Canada

⁴Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

⁵Agriculture Key Laboratory of Agricultural Entomology, Institute of Insect Science, Zhejiang University, Hangzhou, Zhejiang 310058, PR China

Members of the family *Baculoviridae* are insect-specific dsDNA viruses that have been used for biological control of insect pests in agriculture and forestry, as well as in research and pharmaceutical protein expression in insect cells and larvae. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the type species of the family *Baculoviridae*. During infection of AcMNPV in permissive cells, *fp25k* mutants are positively selected, leading to the formation of the few polyhedra (FP) phenotype with reduced yield of polyhedra and reduced virion occlusion efficiency, which leads to decreased oral infectivity for insects. Here we report that polyhedra of AcMNPV *fp25k* mutants produced from different insect cell lines and insects have differences in larval *per os* infectivity, and that these variations are due to different virion occlusion efficiencies in these cell lines and insects. Polyhedra of AcMNPV *fp25k* mutants produced from *Sf* cells (*Sf*21 and *Sf*9, derived from *Spodoptera frugiperda*) and *S. frugiperda* larvae had poorer virion occlusion efficiency than those from Hi5 cells (derived from *Trichoplusia ni*) and *T. ni* larvae, based on immunoblots, DNA isolation and larval oral infection analysis. AcMNPV *fp25k* mutants formed clusters of FP and many polyhedra (MP) in the fat body cells of both *T. ni* and *S. frugiperda* larvae. Transmission electron microscopy revealed that the nature of virion occlusion of AcMNPV *fp25k* mutants was dependent on the different cells of the *T. ni* fat body tissue. Taken together, these results indicate that the FP phenotype and virion occlusion efficiency of *fp25k* mutants are influenced by the host insect cells.

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INTRODUCTION

Baculoviruses are large DNA viruses in the family *Baculoviridae* and are promising biocontrol agents that have been developed in several countries to reduce the reliance on chemicals for the control of insect pests that damage agriculture and forestry. They are insect-specific viruses that must be ingested by the insects before infection

can be initiated in the hosts. The most-studied baculovirus, also the type species of the family *Baculoviridae*, is *Autographa californica* nucleopolyhedrovirus (AcMNPV).

Infection of a cell starts when an AcMNPV budded virus (BV) enters a susceptible cell and is transported to the nucleus by a process involving the formation of actin microfilaments (Charlton & Volkman, 1993). After reaching the nucleus, the nucleocapsids uncoat to release their DNA. Host RNA polymerase II initiates early gene transcription from the viral DNA template, followed by DNA replication by the viral DNA polymerase. Viral RNA polymerase transcribes late genes after DNA replication

†Present address: Indian Institute of Spices Research (ICAR), Calicut, Kerala 673012, India.

Five supplementary figures are available with the online version of this paper.

initiates (Passarelli, 2007). One of the very late genes is the polyhedrin gene (*polh*), whose product (polyhedrin protein) is synthesized in large amounts in the late phase of infection to form polyhedra in the permissive insect cells. The process of forming polyhedra occludes the newly assembled virions in the nucleus. The polyhedra protect the occluded virions, known as occlusion-derived viruses (ODVs), from natural inactivation hazards such as desiccation when they are released from the killed larvae on vegetation. Therefore, occlusion of virions is an important evolutionary adaptation for the nucleopolyhedrovirus (NPV) to increase its fitness in the environment.

The virion has an envelope that is derived from blebbing of the inner nuclear membrane (INM) of infected insect cells (Hong *et al.*, 1997); the blebbing process is facilitated by viral protein FP25K (Rosas-Acosta *et al.*, 2001). It has also been suggested that polyhedrin has a strong affinity for the enveloped virions during virion occlusion (Blissard & Rohrmann, 1990; Wood, 1980). Therefore, the virion envelope also plays a significant role in the virion occlusion process.

In cell culture, repeated passage of baculoviruses at high m.o.i. in cells often leads to the development of the few polyhedra (FP) per cell phenotype (de Rezende *et al.*, 2009; Kumar & Miller, 1987; Lua *et al.*, 2002; Cheng *et al.*, 2013). Development of the FP phenotype in cell lines is linked to mutations in the *fp25k* gene. These mutations include host transposon insertions at the TTAA site of the *fp25k* gene, and insertion of a single nucleotide, base substitution, and deletion of nucleotides in the promoter regions or the coding region of *fp25k* (Beames & Summers, 1988; Bischoff & Slavicek, 1997; Carstens, 1987; Fraser *et al.*, 1983; Cheng *et al.*, 2013). Inactivation of *fp25k* also results in elevated BV numbers in the culture medium and these BVs may compete with wild-type (wt) AcMNPV in cell infection (Harrison & Summers, 1995).

Polyhedra of AcMNPV *fp25k* mutants have much-reduced or no infectivity to larvae, due to reduced or absent virion occlusion (Chakraborty & Reid, 1999; Cheng *et al.*, 2001; Katsuma *et al.*, 1999; Potter *et al.*, 1976). The cause of the reduced virion occlusion is unknown. Therefore, we hypothesized that polyhedra of AcMNPV FP mutants produced from various insect cell lines and insects have different *per os* infectivity to insects, due to dissimilar virion occlusion efficiencies in varying cells of different insect species. As there is significant interest to develop baculoviruses as pest-control agents, it is necessary to understand the mechanisms of *fp25k* mutations in insect cells so that NPVs with reduced capacity to form FP mutants can be developed.

In this report, we confirm that virion occlusion in the polyhedra of AcMNPV *fp25k* mutants is dependent on different insect cell lines and cells in insects. As FP25K is associated with the ODV envelope that is derived from the INM of insect cells and FP25K is also located in the nucleus, it is likely that certain viral or cellular proteins on

the INM or in the nuclei of different cells from various insects might be involved in FP formation and virion occlusion of AcMNPV *fp25k* mutants.

RESULTS

AcMNPV *fp25k* mutants had greater virion occlusion efficiency in Hi5 than in Sf21 and Sf9 cell lines

The two AcMNPV *fp25k* mutants – AcP2, which has an adenine insertion in one of the seven adenine mononucleotide repeats (A7 MNR), and AcP13, which has a 287 bp host DNA insertion at the TTAA site – were used to investigate virion occlusion in different insect cell lines and host insects (Cheng *et al.*, 2013). Differences of virion occlusion of the *fp25k* mutants in different insect cell lines and insects were discovered. It was first revealed that the yields of polyhedra of AcMNPV *fp25k* mutant viruses were reduced significantly in Hi5 cells (Cheng *et al.*, 2013). However, in this study more virions were detected in the polyhedra produced in Hi5 cells than in those produced in Sf9 and Sf21 cells when the same number of polyhedra was used to perform the immunoblotting experiments. A mAb against the AcMNPV major capsid protein VP39 failed to detect the presence of VP39 in polyhedra from Sf21 and Sf9 cells infected with the AcMNPV *fp25k* mutant viruses AcP2 or AcP13, which was indicative of undetectable or extremely low levels of virions [Fig. 1a, b, (i)]. However, the antibody reacted strongly with a VP39 band for AcE2 and AcP3 with an intact *fp25k* gene, suggesting the presence of occluded virions [Fig. 1a, b, (i)]. The same anti-VP39 antibody detected VP39 in polyhedra from Hi5 cells infected with either AcP2 or AcP13, but at a lower level than that of AcE2 and AcP3 viruses [Fig. 1c(i)]. Virion occlusion of AcP2 and AcP13 in Hi5 cells was verified by Western blotting analysis of a previously purified *fp25k* mutant AcFP480-2 (Beames & Summers, 1988; Fig. S1, available in JGV Online). The idea that VP39 levels reflect relative levels of occluded virions is further supported by the relative amounts of virion DNA recovered from polyhedra isolated from the different cells infected with AcMNPV with or without an intact *fp25k* gene. Only trace amounts of polyhedral virion DNA were isolated from AcP2 and AcP13 derived from infected Sf9 and Sf21 cells [Fig. 1a, b, (ii)], whereas about 40-fold more polyhedral virion DNA was obtained from AcE2- and AcP3-infected Sf9 and Sf21 cells [Fig. 1a, b, (ii)]. However, in infected Hi5 cells, there was only about a fivefold reduction of polyhedral virion DNA isolated from the *fp25k* mutants AcP2 and AcP13 compared with AcP3 [Fig. 1c(ii)].

The level of polyhedral VP39 was generally lower for AcE2 than for AcP3 in both Sf and Hi5 cells (Fig. 1). Hi5 cell infection with AcE2 resulted in approximately 10% of Hi5 cells showing the FP phenotype with <10 polyhedra per cell compared with the estimated 90% of cells showing the MP phenotype with >30 polyhedra per cell, suggesting that

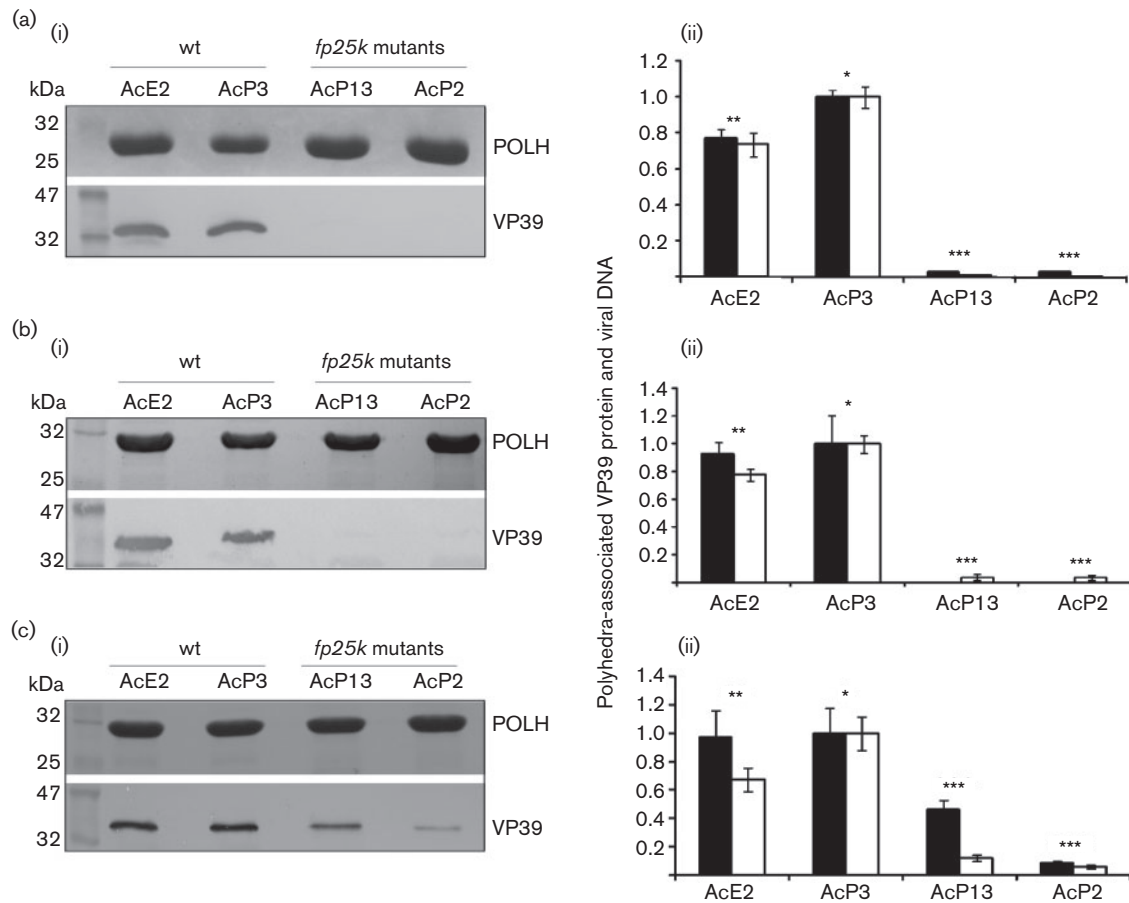


Fig. 1. SDS-PAGE, Western blotting and DNA isolation analysis of virion occlusion of AcMNPV *fp25k* mutant polyhedra. Equal numbers of polyhedra from *S21* (a), *S9* (b) and *Hi5* (c) cells were processed for SDS-PAGE, Western blotting and DNA extraction analysis. (i) SDS-PAGE and Western blotting analyses. Upper panel, polyhedrin loading control in SDS-PAGE. Lower panel, Western blot detection of major capsid protein VP39 with a monoclonal anti-VP39 antibody. (ii) Quantitative virion occlusion assay by densitometry and DNA isolation. Filled bars, comparison of integrated density values (IDVs) of the VP39 signals in the Western blot relative to the IDVs of the polyhedrin signals from SDS-PAGE; the mean of AcP3 IDVs was set to 1. Empty bars, comparison of viral DNA yields from polyhedra; the mean of AcP3 DNA yields was set to 1. Error bars denote SD, $n=3$. Treatments with the same numbers of asterisks had no significant difference at $P=0.05$.

the $10.32 \pm 0.57\%$ (mean \pm SD, $n=3$) of *Hi5* cells showing FP were responsible for the lower virion occlusion efficiency based on Western blotting, dissimilar to that in AcP3 (Figs 1 and S2).

***fp25k* mutants had greater virion occlusion efficiency in *T. ni* than in *S. frugiperda* larvae**

Both Western blotting and DNA isolation assays suggested that virion occlusion of AcP2 did not occur in *S. frugiperda* larvae, but occurred in *T. ni* larvae (Fig. 2). The VP39 antibody detected high levels of VP39 in the polyhedra of AcP3 from *S. frugiperda* larvae, but failed to detect VP39 in polyhedra from AcP2 and AcDef (defective of virion occlusion, negative control) (Wang *et al.*, 2009) from *S. frugiperda* larvae (Fig. 2a). This indicated poor or no virion

occlusion of the *fp25k* mutant viruses propagated in *S. frugiperda* larvae. A rather different situation was observed for polyhedra from *T. ni* larvae, where the antibody detected VP39 in polyhedra from both AcP3- and AcP2-infected larvae, but not in polyhedra for the negative-control virus AcDef (Wang *et al.*, 2009) [Fig. 2b(i)]. Virion occlusion of AcP2 was not as efficient as that of AcP3 in *T. ni* larvae [Fig. 2b(i)]. Densitometry indicated that there was a reduction of about 50% of VP39 in AcP2 compared with that of AcP3 with polyhedra from *T. ni* larvae [Fig. 2a(ii)]. Western blotting virion occlusion assays were supported by polyhedral DNA assays and indicated that AcP2 polyhedra from *S. frugiperda* larvae contained extremely low levels of viral DNA [Fig. 2a(ii)], whereas AcP2 polyhedra from *T. ni* larvae contained viral DNA, but the viral DNA yield was 30% of that of AcP3 [Fig. 2a(ii)].

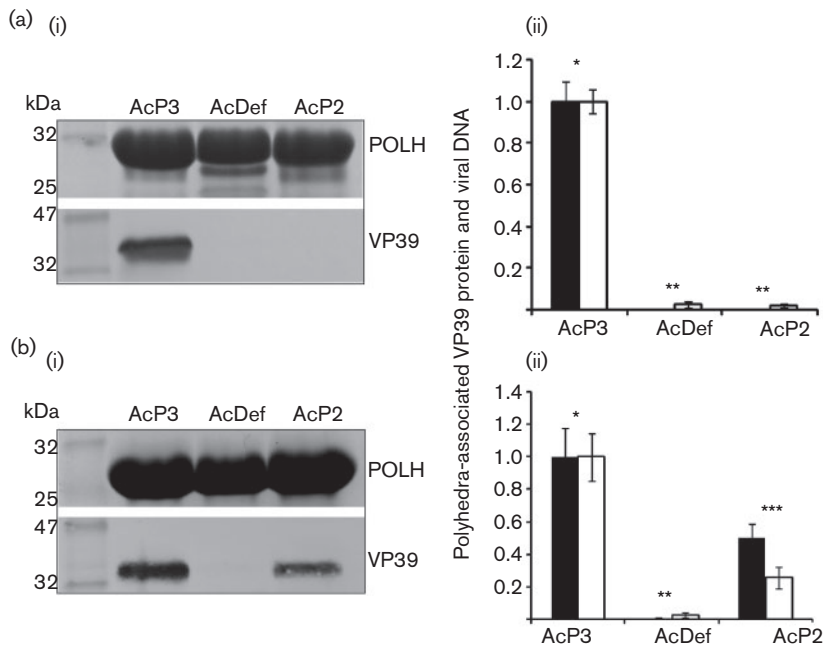


Fig. 2. Western blotting and DNA isolation analysis of virion occlusion of polyhedra of AcMNPV *fp25k* mutant viruses produced in *S. frugiperda* (a) and *T. ni* (b) larvae. (i) SDS-PAGE and Western blotting analyses. Upper panel, polyhedrin loading control by SDS-PAGE; lower panel, Western blot detection of VP39 with a monoclonal anti-VP39 antibody. (ii) Quantitative virion occlusion assay by densitometry and DNA isolation. Filled bars, comparison of IDVs of the VP39 signals in the Western blot relative to the IDVs of the polyhedrin signals from SDS-PAGE; the mean of AcP3 IDVs was set to 1. Empty bars, comparison of viral DNA yields from polyhedra; the mean of AcP3 DNA yields was set to 1. Vertical bars denote SD, $n=3$. Treatments with the same numbers of asterisks had no significant difference at $P=0.05$.

fp25k mutant viruses formed clusters of MP in the fat body tissue of *T. ni* and *S. frugiperda* larvae

The formation of the FP phenotype exhibited diverse characteristics in different cell lines (Cheng *et al.*, 2013), but little information about the formation of polyhedra by *fp25k* mutants *in vivo* is available. Therefore, we tested the *fp25k* mutant viruses in *S. frugiperda* and *T. ni* larvae. We focused on the larval fat body tissue because it is the major insect tissue where NPV replicates (Federici, 1997; Wang *et al.*, 2008). The fat body tissue of AcP3-infected *S. frugiperda* larvae at day 4 post-infection (p.i.) showed typical packaging of >100 polyhedra in the nuclei of fat body cells (Fig. 3a). Examination of 100 fat body cells revealed that >97% of fat body cells showed >100 polyhedra per fat body cell nucleus (MP phenotype) (Fig. 3a). However, AcP2-infected cells showed both FP and MP phenotypes in different *S. frugiperda* fat body cells (Fig. 3b). We defined the MP phenotype as >100 polyhedra per cell, semi-MP as 50–99 polyhedra per cell and FP as <50 polyhedra per cell (Fig. 3c). Of 100 fat body cells, 20% showed the MP phenotype, 30% showed semi-MP and 50% showed FP (Fig. 3c). In *T. ni* larvae infected with AcP3, similar to *S. frugiperda* larval infection, about 97% of *T. ni* larval fat body cells showed the MP phenotype (Fig. 4a). Both MP and FP phenotypes were also observed in the fat body cells of *T. ni* larvae infected with AcP2 (Fig. 4b), but the frequencies of cells showing MP, semi-MP and FP phenotypes (Fig. 4c) were different from those seen in *S. frugiperda* larval infection. More than 40% of *T. ni* fat body cells showed the MP phenotype, 31% showed semi-MP and 27% showed FP. It was further observed that the fat body cells infected with AcP2 showing MP or FP phenotypes were clustered in at least 20 cells in the fat body tissue of *S. frugiperda* (Figs 5a, S3 and S5A) and *T. ni* (Figs 5b, S4 and S5B) larvae.

As tissues other than fat body support the replication of and production of polyhedra by AcMNPV in *T. ni* and *S. frugiperda* larvae, it was necessary to compare the differences in yields of polyhedra in entire larvae between AcP3 and AcP2. We found that, compared with AcP3, AcP2 had about 80% (mean yield of polyhedra of three infected larvae, $P<0.05$) yield reductions of polyhedra in *S. frugiperda* larvae (Fig. 3d) and 50% (mean of polyhedra yields of three infected larvae, $P<0.05$) in *T. ni* larvae (Fig. 4d).

Polyhedra of AcMNPV *fp25k* mutant viruses from Hi5 cells and *T. ni* larvae were infectious, whereas those from Sf21 cells, Sf9 cells and *S. frugiperda* larvae were not

Oral feeding (*per os*) of a high dose of polyhedra (1×10^6 polyhedra per larva) of AcP2 and AcP13 viruses purified from Sf21 cells, Sf9 cells and *S. frugiperda* larvae failed to kill any third-instar *T. ni* larvae. All larvae that survived AcP2 and AcP13 infection pupated and progressed to the next generation without signs of viral infection. The positive-control AcP3 polyhedra from Sf21 cells, Sf9 cells and *S. frugiperda* larvae killed all the tested larvae at the same high dose. Virus-killed larvae from AcP3 infection showed large numbers of polyhedra in the fat body and other tissues (data not shown).

Per os feeding at the same dose of AcP2 and AcP13 polyhedra from Hi5 cells and *T. ni* larvae killed all the treated larvae, as did the AcP3 positive control at the same high dose. Hi5 infection of BVs from the haemolymph of moribund *T. ni* larvae infected with AcP3, AcP2 or AcP13 showed development of the MP and FP phenotypes, similar to that shown in Fig. 2(e) of Cheng *et al.* (2013). This suggested that neither AcP2 nor AcP13 changed to wt

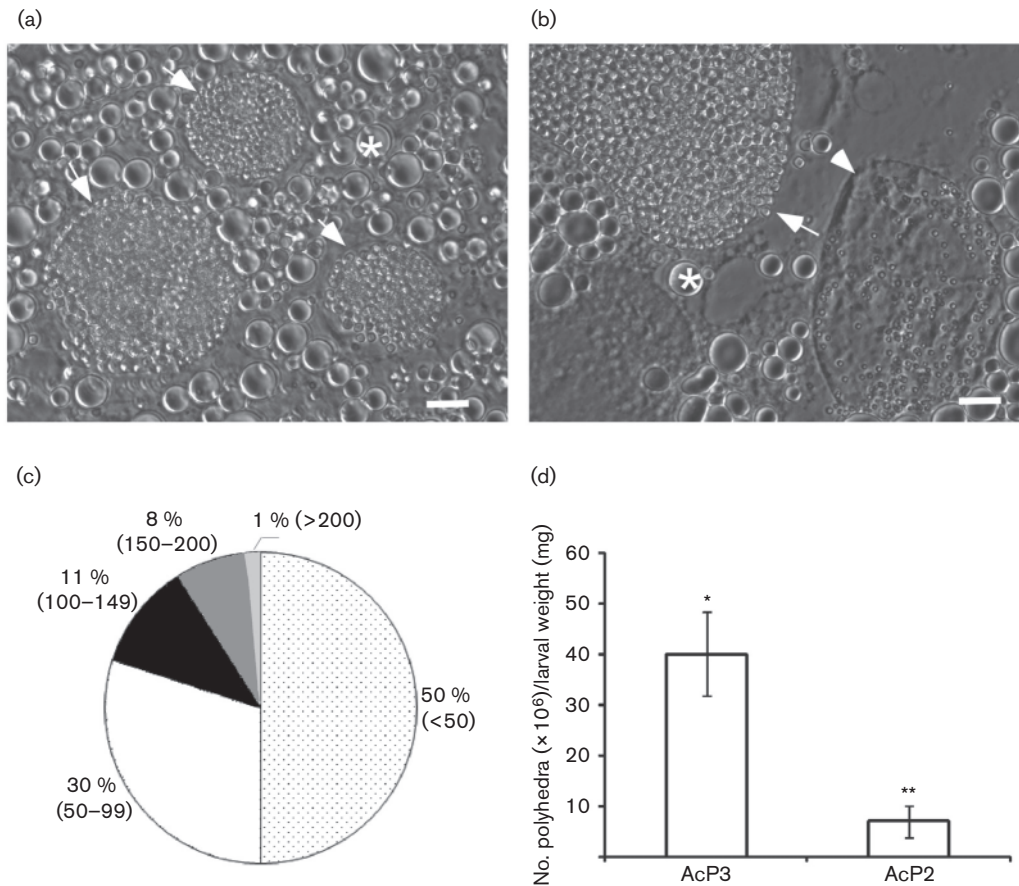


Fig. 3. Comparison of formation of polyhedra by AcMNPV *fp25k* mutant viruses in the fat body cells of *S. frugiperda* larvae. (a) Formation of MP phenotype by AcP3 in *S. frugiperda* larvae. (b) Formation of MP and FP phenotypes by AcP2 in *S. frugiperda* larvae. Arrows point to cells showing MP; arrowheads point to a cell showing FP; the asterisk indicates a fat droplet. Bars, 10 μ m. (c) Frequencies of different polyhedra yields per fat body cell of *S. frugiperda* larvae infected with *fp25k* mutant AcP2. Percentages are frequencies of cells showing different numbers of polyhedra per cell in parentheses for MP (>100), semi-MP (50–99) and FP (<50) phenotypes from 100 cell counts. (d) Comparison of yields of polyhedra between AcP3 and AcP2 in *S. frugiperda* larvae. Yields of polyhedra on the *y*-axis are the mean counts of polyhedra of three individual larvae infected with either AcP3 or AcP2.

during passage in *T. ni* larvae. When progeny polyhedra of AcP2 and AcP3 produced in *T. ni* larvae were used to perform a dose–response bioassay against third-instar *T. ni* larvae, viral infection symptoms, including a whitish appearance and sluggish movement, were observed in larvae. The calculated LD₅₀ values were respectively 8.5 and 630 polyhedra per larva for AcP3 and AcP2, showing a 74-fold difference, and LD₉₀ values were respectively 491 and 103 000 polyhedra per larva, a 200-fold difference (Table 1). This confirmed that the polyhedra of AcP2 from Hi5 cells and *T. ni* larvae contained virions that killed the test insects, but had an LD₅₀ higher than that of AcP3.

AcMNPV *fp25k* mutants had proper virion occlusion in certain *T. ni* fat body cells

Western blots and DNA extraction detected some virions in the polyhedra from Hi5 cells and *T. ni* larvae infected by

AcMNPV *fp25k* mutants, but not in polyhedra from *Sf* cells or *S. frugiperda* larvae. In order to visualize these virions, transmission electron microscopy (TEM) was performed with cells containing polyhedra. The fat body tissue of *T. ni* larvae infected with AcP3 or AcP2 was processed for TEM, which revealed different cells in the fat body tissue with diverse degrees of virion occlusion. AcP3 showed the expected extent of virion occlusion, with very few nucleocapsids and virions in the viroplasm surrounding the polyhedra (Fig. 6a). However, in *T. ni* larvae infected with AcP2, most fat body cells showed no virion occlusion, with naked nucleocapsids in the nucleus adjacent to the empty polyhedra (Fig. 6b). Some AcP2 polyhedra contained many fewer occluded virions than were seen with AcP3 (Fig. 6c), while AcP2 polyhedra in other fat body cells showed efficient virion occlusion (Fig. 6d). Therefore, these data suggest that, in *T. ni* larval fat body tissue, AcP2 caused virion occlusion in a subset of the cells.

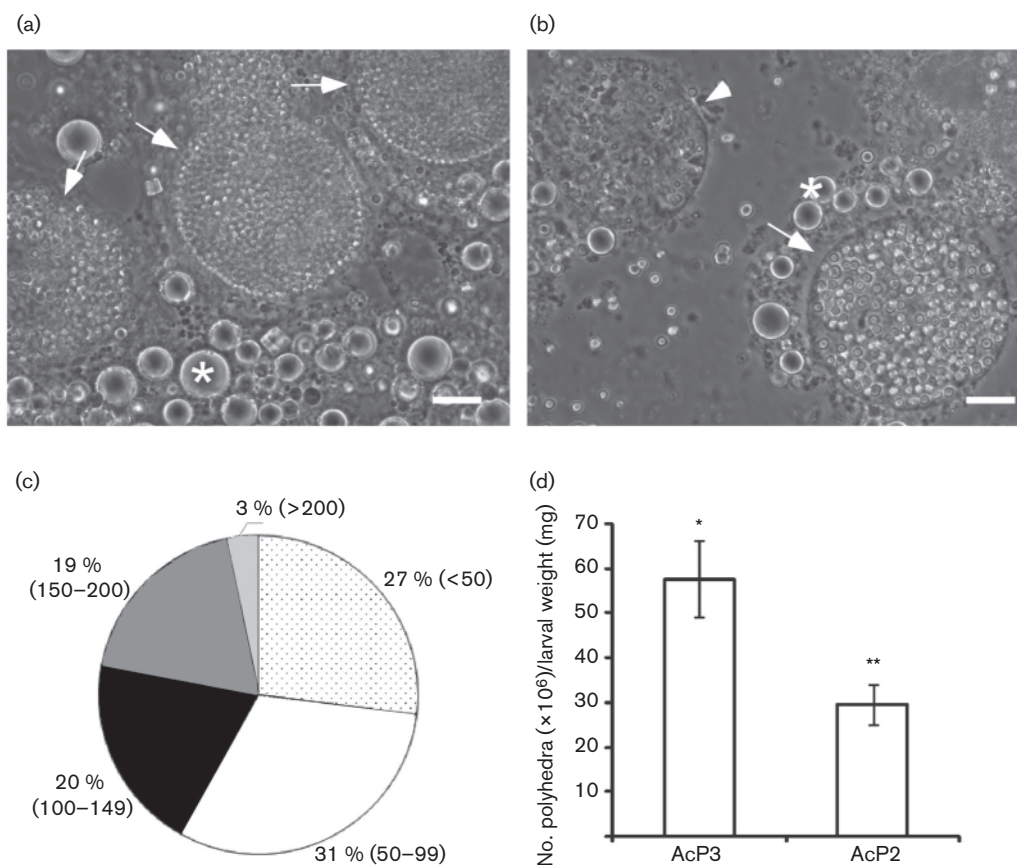


Fig. 4. Comparison of formation of polyhedra by AcMNPV *fp25k* mutant viruses in the fat body cells of *T. ni* larvae. (a) Formation of MP by AcP3 in *T. ni* larvae. (b) Formation of MP and FP phenotypes by AcP2 in *T. ni* larvae. Arrows point to cells showing MP; arrowheads point to a cell showing FP; asterisks indicate fat droplets. Bars, 10 μ m. (c) Frequencies of different polyhedra yields per fat body cell of *T. ni* larvae infected with *fp25k* mutant AcP2. Percentages are frequencies of cells showing different numbers of polyhedra per cell in parentheses for MP (>100), semi-MP (50–99) and FP (<50) phenotypes. (d) Comparison of yields of polyhedra between AcP3 and AcP2 in *T. ni* larvae. Yields of polyhedra on the y-axis are the mean counts of polyhedra of three individual larvae infected with either AcP3 or AcP2.

DISCUSSION

Both the FP and MP phenotypes are produced in *T. ni* larvae infected by a plaque-purified AcMNPV FP mutant, suggesting that the generation of cells of both FP and MP phenotypes by the FP mutant is influenced by the host cells (Ramoska & Hink, 1974). However, no attempt was extended to investigate the cause of their observation. Our observation of FP and MP phenotype production in the fat body tissue of both *T. ni* and *S. frugiperda* larvae is consistent with this early report (Figs 3 and 4). We also observed that clusters (>20 cells) of fat body cells of *S. frugiperda* and *T. ni* larvae infected with AcP2 showing either MP or FP (Figs 5, S3 and S4); this clustering is similar to the Hi5 cell monolayer showing semi-MP and binucleate cells with polyhedra [Fig. 2e(iii) of Cheng *et al.* (2013)]. One possible explanation could be that the A8 MNR of the AcP2 inactivated *fp25k* had reverted to the A7 MNR, leading to the restoration of a functional *fp25k* during viral DNA replication (Gragg *et al.*, 2002).

However, Hi5 cells infected with haemolymph from larvae infected with AcP2 did not show the MP phenotype (data not shown), suggesting that no such reverting occurred and that formation of the MP phenotype is not due to genetic changes of the AcP2 virus, but due to the presence of different cell types in the fat body of both *T. ni* and *S. frugiperda* larvae (Haunerland & Shirik, 1995). Therefore, these clusters of fat body cells supporting MP phenotype production of AcP2 are similar to the cluster of Hi5 cells infected with AcP2 [Fig. 2e(iii) of Cheng *et al.* (2013)].

It has been reported that, in *Sf9* cells, mutations in *fp25k* coincide with impaired nuclear import of the polyhedrin protein (Jarvis *et al.*, 1992). AcMNPV polyhedrin has a nuclear-localization signal (NLS), K₃₂RKK₃₅, that can be recognized by cellular nuclear transport receptors or importins, followed by the nuclear import of the protein complex (Jarvis *et al.*, 1991). In order to accumulate large numbers of polyhedra in the nuclei of some of the fat body cells in both *S. frugiperda* and *T. ni* larvae, one can

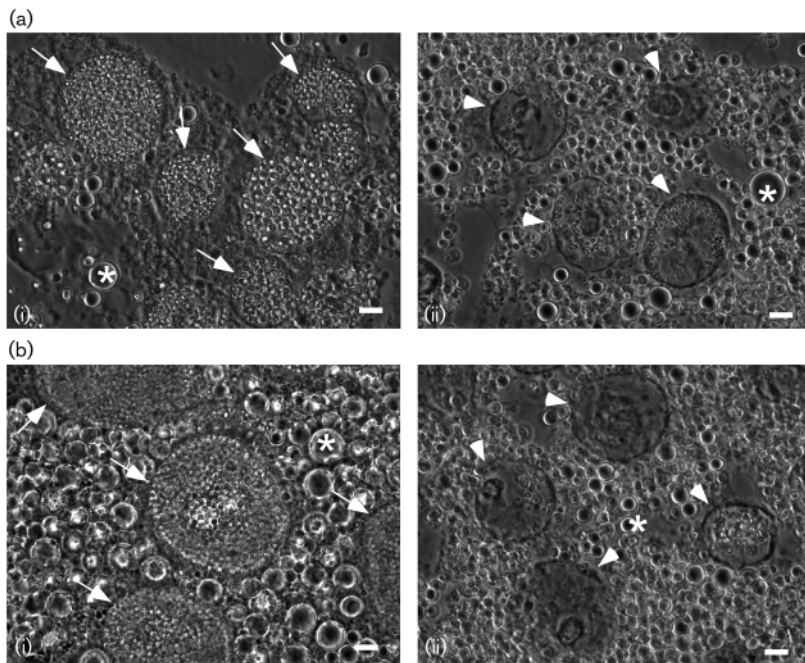


Fig. 5. Clustering of MP and FP phenotype formation in the fat body cells of *S. frugiperda* and *T. ni* larvae infected with the AcMNPV *fp25k* mutant virus AcP2. Third-instar larvae of *S. frugiperda* and *T. ni* were injected intrahaemocoelically with AcP2 BV. Infected larvae were dissected and the fat bodies were examined by microscopy. (a) Clusters of MP (i) or FP (ii) in the fat body cells of *S. frugiperda* larvae. (b) Clusters of MP (i) or FP (ii) in the fat body cells of *T. ni* larvae. Arrows point to cells showing MP; arrowheads point to cells showing FP; asterisks indicate fat droplets. Bars, 10 μ m.

speculate that the polyhedrin protein should first be synthesized in large amounts. In order to synthesize large amounts of polyhedrin, leading to its crystallization and the formation of polyhedra, there should be high *polh* promoter activity, causing the accumulation of high levels of *polh* mRNA for translation. In cells infected with AcP2 that produce low levels of *polh* mRNA both *in vitro* and *in vivo*, there might be viral or cellular repressor proteins that interact either directly with the *polh* promoter or indirectly with late expression factors, leading to lower mRNA synthesis. One plausible explanation of the accumulation of many polyhedra (MP phenotype) in some of the fat body cells in both species of larvae infected by the AcMNPV *fp25k* mutants might be that the putative viral or cellular repressor protein(s) was expressed at low levels, allowing high *polh* promoter activity and resulting in the accumulation of large amounts of polyhedrin protein, which eventually crystallized in the nuclei to form polyhedra (Figs 3 and 4). There might be a cellular

protein(s) with a function similar to that of FP25K in these cells to regulate or facilitate importin binding to the NLS of the polyhedrin protein, in order to transport it to the nucleus for the formation of MP by AcP2 and AcP13.

Infectivity of baculovirus FP mutants to susceptible insect hosts reportedly varied from none to a 30-fold reduction compared with wt baculoviruses (Cheng *et al.*, 2001, 2005; Potter *et al.*, 1976). This vast difference might be due to the nature of the polyhedra of the *fp25k* mutant viruses used in the bioassay; polyhedra with varying virion occlusion efficiencies might be produced in various cells in different cell lines, or in insects with different proteins responsible for higher *polh* promoter activities and for importing polyhedrin to the nucleus, as well as proteins for facilitating virion occlusion for the tests. Polyhedra of AcMNPV *fp25k* mutant viruses that are produced in *Sf9* cells do not have proper virion occlusion and are therefore unlikely to be infectious to insects when the larvae are fed polyhedra without virions (Harrison & Summers, 1995). In contrast,

Table 1. Dose–mortality response of *T. ni* larvae to polyhedra of AcP3 (wt AcMNPV) and AcP2 (*fp25k* mutant) produced in *T. ni* larvae

Numbers in parentheses represent lower and upper 95% confidence limits.

Virus	Dose (polyhedra per larva)			Slope
	LD ₁₀	LD ₅₀	LD ₉₀	
AcP3	0.2 (0–1.9)	8.5 (0.3–44.5)*	491.1 (73–2.2 × 10 ⁵)	0.73
AcP2	4.0 (0.04–21.4)	630.0 (161–2336.7)*	1.0 × 10 ⁵ (3.4 × 10 ⁴ –5.1 × 10 ⁶)	0.58

*A significant difference was found in LD₅₀ values between AcP3 and AcP2.

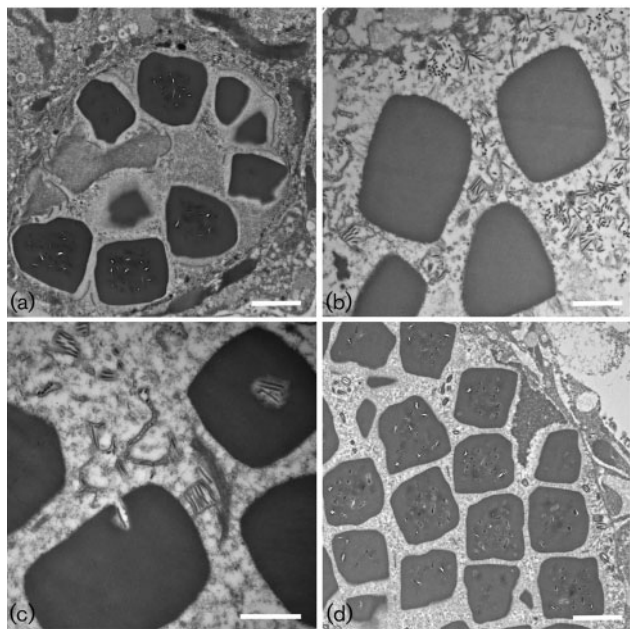


Fig. 6. TEM analysis of virion occlusion of AcP3 and AcP2 in the fat body of *T. ni* larvae. (a) wt AcMNPV AcP3 showing virion occlusion in the polyhedra in the nucleus of a fat body cell. (b) Empty polyhedra of AcP2 in certain cells. (c) Partial virion occlusion of AcP2 in certain cells. (d) Proper virion occlusion of AcP2 in certain cells. Bars, 2 μ m.

the polyhedra of the AcMNPV *fp25k* mutant AcUWL.lacZ produced in *T. ni* larvae killed *T. ni* larvae with a 10-fold reduction in infectivity compared with the wt (Cheng *et al.*, 2001, 2005). Our bioassay results, obtained by using different sources of polyhedra, support earlier reports that polyhedra of AcMNPV *fp25k* mutant viruses produced in *Sf9* cells are devoid of virion occlusion and thereby fail to kill insects *per os* (Harrison & Summers, 1995), whereas AcMNPV FP polyhedra produced in Hi5 and *T. ni* kill the tested insects, suggesting that there is some virion occlusion (Table 1) (Cheng *et al.*, 2001). As polyhedra produced in certain fat body cells of the *T. ni* larvae infected by the *fp25k* mutant viruses contain normal virion occlusions (Fig. 6d), these polyhedra with proper virion occlusion are thought to be those responsible for killing insects in the infectivity tests.

To exclude the possibility that the detection of virion occlusion in polyhedra produced in AcP2- and AcP13-infected Hi5 cells and *T. ni* larvae is due to contamination with AcP3 or any other wt AcMNPV, PCR and sequencing of the *fp25k* locus of AcP2 and AcP13 were performed, and did not detect any wt contamination (Cheng *et al.*, 2013).

Our discovery that some Hi5 cells and *T. ni* fat body cells have proper virion occlusion of *fp25k* mutants may suggest the diversity of INM or nuclear proteins in different cells that influence AcMNPV virion envelope protein trafficking to the nucleus for virion occlusion. Some Hi5 cells and fat

body cells of *T. ni* larvae have virion occlusion, whereas other cells do not (Figs 1 and 2), even though large amounts of polyhedrin are synthesized in some fat body cells of *S. frugiperda* larvae infected with AcP2 (Fig. 2). The genetic background of cells from the two insects is not identical and therefore there might be different proteins expressed in these insect cells (Pasumarthy & Murhammer, 1994), including cellular proteins that could be also involved in ODV membrane protein sorting and could provide functional redundancy for FP25K. Braunagel *et al.* (2009) proposed that importin- α -16 is one of the possible factors and further studies are needed to confirm this. Although large amounts of polyhedrin are synthesized in some of the fat body cells of *S. frugiperda* larvae infected with AcP2, suggesting a possible lack of cellular repressor(s) in these fat body cells showing the MP phenotype (Fig. 6), no virion occlusion has been detected (Fig. 2). This may suggest that these fat body cells of *S. frugiperda* lack the cellular factor(s) involved in virion occlusion found in some of the fat body cells of *T. ni* larvae with virion occlusion (Fig. 6d). Therefore, it is likely that these fat body cells of *T. ni* larvae have cellular factor(s) in addition to those involved in virion occlusion of *fp25k* mutants to enhance *polh* gene expression and nuclear polyhedrin importing of *fp25k* mutants for proper occlusion body production, similar to the wt. Expression of *polh* mRNA of *fp25k* mutants is reduced in some insect cells, but not in others (Cheng *et al.*, 2013), whereas virion occlusion occurred in some *T. ni* fat body cells (Fig. 6d). This may generate indirect negative feedback for FP25K, affecting *polh* expression. If virions are not being occluded properly, this may generate a signal that causes a reduction in *polh* expression.

METHODS

Cells, viruses and insects. IPLB-SF21AE (*Sf21*) cells from *S. frugiperda*, *Sf9* cells cloned from *Sf21* cells and BTI-TN-5B1-4 (Hi5) cells derived from *T. ni* were cultured in TNM-FH medium (Invitrogen) supplemented with 10% FBS at 27 °C. AcMNPV strain AcE2 and FP mutant AcFP480-2 (IFP2.2) (Beames & Summers, 1988) were provided by Dr Max Summers (Texas A&M University, College Station, TX, USA). AcDef, an AcMNPV virion occlusion-defective virus that has a deletion of three genes (*p26*, *p10* and *p74*) in the genome, was described previously (Wang *et al.*, 2009). AcP3 (the wt) and AcP2 (the *fp25k* mutant) were plaque-purified from AcE2 in our laboratory (Cheng *et al.*, 2013). Virus titre was determined according to Cheng *et al.* (2013). *S. frugiperda* and *T. ni* insects were purchased from BioServ and reared on a commercial insect diet purchased from Southland Products Inc., according to Cheng & Carner (2000).

SDS-PAGE, Western blotting and virion DNA content analysis of polyhedra. To investigate the virion occlusion efficiency of wt AcMNPV and *fp25k* mutants in different insect cell lines, six-well plates of *Sf21* or *Sf9* cells at 1×10^6 cells per well and Hi5 cells at 5×10^5 cells per well were infected with the wt and *fp25k* mutants at an m.o.i. of 10 (O'Reilly *et al.*, 1992). At day 3 p.i., the cells were lysed in 0.1% SDS and the polyhedra released were pelleted by centrifugation at 5000 g for 5 min. The polyhedra-containing pellets were suspended in 0.1% SDS and purified further by centrifugation at

16 100 g through a 30% (w/v) sucrose cushion for 3 min to remove potential non-occluded virus contamination for the virion occlusion assay.

The number of virions occluded was determined based on the detection of major viral capsid protein VP39 and the viral DNA content. Equal numbers of polyhedra (1×10^6) of AcP3, AcE2 and *fp25k* mutants produced in all three cell lines were treated with an equal volume of alkaline solution (0.1 M Na_2CO_3 , pH 10.5) to dissolve the polyhedra for virion release. The virion-containing solution was divided equally and processed for protein separation in two identical 12% acrylamide SDS-PAGE gels. One gel was stained with Coomassie blue and the other was used to transfer proteins to a nitrocellulose membrane for Western blotting, in which an anti-VP39 mAb was used to detect the major viral capsid protein (Wang *et al.*, 2009). Analysis of the virion occlusion efficiency of polyhedra purified from insects was performed similarly to that for polyhedra from cell culture, with a minor modification such that polyhedra of AcDef from *T. ni* larvae (previously confirmed to be defective in virion occlusion) were used as a negative control (Wang *et al.*, 2009). This experiment, from cell infection to SDS-PAGE and Western blotting, was performed in triplicate and the signal intensities or integrated density values (IDV) of polyhedrin in SDS-PAGE and VP39 in Western blots were quantified by densitometry. Polyhedrin served as a loading control. The relative virion occlusion efficiency (IDV of VP39/IDV of polyhedrin) between AcMNPV wt and *fp25k* mutants from the three cell lines was tested quantitatively by Student's *t*-test using Excel (Microsoft), with a significance level of $P < 0.05$. Virion DNA assays of polyhedra were performed to correlate with Western blotting assays for virion occlusion of *fp25k* mutant viruses (Wang *et al.*, 2009). Virion occlusion efficiency of *fp25k* mutant viruses by both Western blotting and virion DNA assays was expressed relative to that of AcP3.

Comparison of production of polyhedra between AcMNPV AcP3 and AcP2 in insects. Third-instar *T. ni* and *S. frugiperda* larvae weighing 100 ± 20 mg were used to compare yields of polyhedra between AcP3 and AcP2 viruses. Ten larvae were injected intrahaemocoelically with 5 μl BV (10^6 p.f.u.) (Wang *et al.*, 2009). The injected larvae were fed on an artificial diet and incubated at 27 °C. At day 4 p.i., when larvae became moribund, three larvae of similar weight (about 200 mg) from each virus treatment were weighed and used individually for extraction of polyhedra (Cheng *et al.*, 1990). The yields of polyhedra were enumerated using a haemocytometer. Differences in yield in various insects were analysed with Student's *t*-test in Excel (Microsoft).

To investigate the differences in production of polyhedra by individual cells in the insects, a larva from each virus treatment (AcP3 and AcP2) was dissected and the fat body tissue, which is the major tissue of AcMNPV replication and production of polyhedra, was removed. The fat body tissues were mounted on a glass slide for examination of MP (>100 polyhedra per cell) and FP (<100 polyhedra per cell) phenotype formation. One hundred fat body cells from each virus infection were selected randomly and counted for MP and FP phenotype formation. Comparison of MP and FP phenotype formation by AcP3 and AcP2 viruses was performed using Student's *t*-test in Excel (Microsoft).

Comparison of infectivity between AcMNPV AcP3 and AcP2 viruses in insects. We first compared the oral infectivity of AcMNPV FP polyhedra produced in Sf21, Sf9 and Hi5 cells, as well as in *S. frugiperda* and *T. ni* larvae. Polyhedra from these cells were prepared as described for the virion occlusion efficiency assay. The polyhedra concentrations were adjusted to 10^9 polyhedra ml^{-1} , and 1 μl of this suspension (10^6 polyhedra) was administered to individual third-instar *T. ni* larvae by the diet-plug method (Cheng

et al., 2001). Fifteen larvae were used for each virus treatment and control larvae were treated with water. One larva showing symptoms of viral infection from each treatment was used for haemolymph sampling for Hi5 cell infection. Mortality was recorded at the time when control larvae pupated (approx. 6 days p.i.).

A more detailed assay was conducted when mortality was observed, using particular polyhedra. Bioassays of both AcP3 and AcP2 viruses were performed using third-instar *T. ni* larvae. A 10-fold serial dilution of purified polyhedra of each of the viruses isolated from *T. ni* larvae was prepared (AcP3, 1.8×10^3 – 1.8×10^7 polyhedra ml^{-1} ; AcP2, 2.9×10^4 – 2.9×10^8 polyhedra ml^{-1}). The diet-plug method was used to inoculate 1 μl of each virus dilution to each third-instar larva (Cheng *et al.*, 2001). Control larvae were fed with diet plugs treated with water. Mortality was recorded at day 6 p.i. when uninfected larvae pupated. Medium lethal dose (LD_{50}) was calculated for each virus bioassay using POLO-PC (LeOra Software).

TEM analysis of virion occlusion efficiency of wt and *fp25k* mutants *in vivo*. To visualize virion occlusion efficiency of AcMNPV virus *in vivo*, third-instar larvae were injected with BVs of AcMNPV wt (AcP3) or *fp25k* mutant (AcP2) (10^6 p.f.u.) and, at day 4 p.i., the fat body tissues were removed and processed for TEM (Granados & Lawler, 1981).

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