

Short Communication

Low multiplicity of infection *in vivo* results in purifying selection against baculovirus deletion mutants

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The *in vivo* fate of *Autographa californica* multiple nucleopolyhedrovirus deletion mutants originating from serial passage in cell culture was investigated by passaging a population enriched in these mutants in insect larvae. The infectivity of polyhedra and occlusion-derived virion content per polyhedron were restored within two passages *in vivo*. The frequency of occurrence of deletion mutants was determined by real-time PCR. The frequency of the non-homologous region origin (non-HR *ori*) of DNA replication was reduced to wild-type levels within two passages. The frequency of the polyhedrin gene did not increase and remained below wild-type levels. A low m.o.i. during the initial infection in insect larvae, causing strong purifying selection for autonomously replicating viruses, could explain these observations. The same virus population used *in vivo* was also passaged once at a different m.o.i. in cell culture. A similar effect (i.e. lower non-HR *ori* frequency) was observed at low m.o.i. only, indicating that m.o.i. was the key selective condition.

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The passage of baculoviruses in cultured insect cells leads to the rapid accumulation of deletion mutants, some of which have been demonstrated to have defective interfering properties (Kool *et al.*, 1991; Pijlman *et al.*, 2001; Wickham *et al.*, 1991). Populations with a high frequency of these deletion mutants can completely lose infectivity for insect larvae (Heldens *et al.*, 1996). The presence of such deletion mutants is therefore likely to be deleterious to the fitness of a virus population *in vivo*, although deletion mutants that can increase fitness are found in some natural baculovirus isolates (López-Ferber *et al.*, 2003). Dai *et al.* (2000) found that alternate passaging of baculoviruses in insect cells and larvae resulted in sustained infectivity for insect larvae, suggesting that purifying selection (Li, 1997) occurs in these animals. In other words, there may be stabilizing selection for a particular trait, in this case the ability to replicate autonomously. Consequently, individuals that do not possess this trait are removed from the population. However, the fate and dynamics of these cell culture-derived deletion mutants after reintroduction into insects have not been systematically addressed. Therefore, a clonal baculovirus was first serially passaged in insect cells to enrich for deletion mutants, and the resulting population was reintroduced into insect larvae. The effects of both *in vitro* and subsequent *in vivo* passaging were investigated by determining the virulence and occlusion-derived virion (ODV) content of polyhedra, and the frequency of

occurrence of deletion mutants by quantitative real-time PCR (qPCR).

In order to generate a clonal population of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), bacmid technology (Luckow *et al.*, 1993) was employed. A bacmid with restored polyhedrin (*polh*) expression was generated using the pFastBac-DUAL/Polh construct (Zwart *et al.*, 2008). This bacmid was used to transfect *Spodoptera frugiperda* Sf-AE-21 (Sf21) cells (Vaughn *et al.*, 1977) according to the method of Pijlman *et al.* (2001). The virus resulting from transfection of Sf21 cells was then passaged serially with minimal dilution (1:4) in Sf21 cells for 20 passages (final population: P_{cell20}). RFLP analysis revealed that viruses with large genomic deletions appeared in the population starting at P_{cell10}, as shown previously (Kool *et al.*, 1991).

Viruses of passages P_{cell2}, P_{cell5}, P_{cell10} and P_{cell20} were subsequently amplified in Sf21 cells. After 96 h, the cells were detached by agitation and sedimented by centrifugation (5 min at 2500 g). The supernatant was removed, stored at 4 °C and subsequently used as budded virus (BV) preparation (BV-P_{cell20}). The pellet was resuspended in 5 ml 1% Triton X-100 and the cells were lysed by sonication. The resulting suspension was layered onto a 5 ml 30% sucrose (in 0.2% Triton X-100) cushion and polyhedra were pelleted by centrifugation (30 min at

15 000 g at 4 °C). The pellet was resuspended in 10 ml Milli-Q water, pelleted (5 min at 2500 g) and finally resuspended in 500 µl Milli-Q water and stored at 4 °C.

The median lethal concentration (LC_{50}) was determined by a droplet feeding bioassay (Hughes & Wood, 1981) for polyhedra of P_{cell2} , P_{cell5} , P_{cell10} and P_{cell20} . *Spodoptera exigua* larvae were reared as described by Smits *et al.* (1986). Twenty-four newly molted *S. exigua* L4 larvae were challenged for each concentration and 24 larvae were taken as healthy controls. Tenfold dilutions of polyhedra (10^6 – 10^2 polyhedra ml^{-1} in sterile distilled water) were used and the bioassay was performed in triplicate. Probit analysis was used to determine LC_{50} . Statistical tests were performed with SPSS version 12.0 unless otherwise specified. Linear regression demonstrated that there was a significant increase in LC_{50} over passages (Fig. 1a; $F_{1,10}=6.937$, $P=0.025$). Unlike *S. exigua* MNPV (Heldens *et al.*, 1996), passaged populations of AcMNPV retain infectivity – albeit reduced – through to passage 20. It was thus possible to use polyhedra of the P_{cell20} population to reinfect insect larvae.

Polyhedra of P_{cell2} , P_{cell10} and P_{cell20} were also analysed by electron microscopy (EM) as described by Zwart *et al.* (2008). The number of ODVs per polyhedron cross-section was counted, for a minimum of 30 polyhedra per sample.

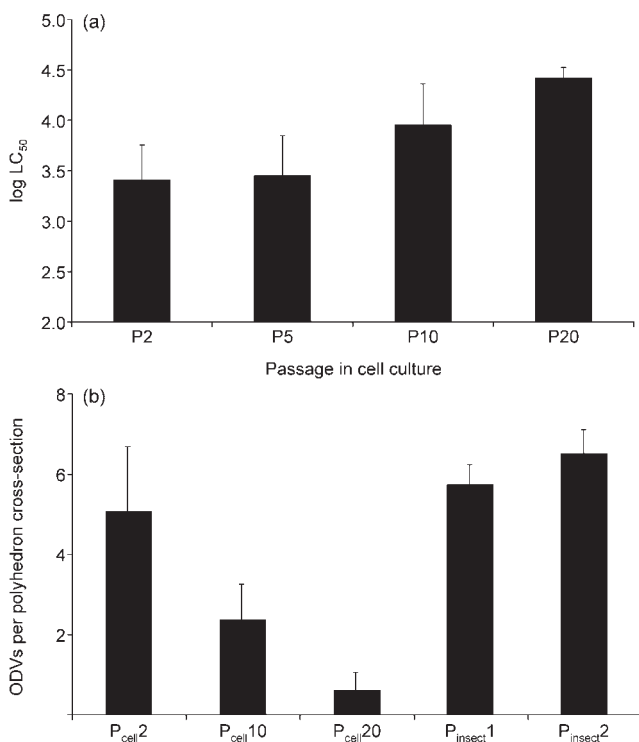


Fig. 1. (a) $\log_{10} LC_{50}$ values of virus with increasing passage number in cell culture (\pm SEM). (b) Mean numbers \pm SEM of ODVs per polyhedron cross-section for passaging in Sf21 cells (P_{cell2} , P_{cell10} , P_{cell20}) and subsequent passaging of P_{cell20} in *S. exigua* larvae at an LC_{99} ($P_{insect1}$, $P_{insect2}$).

There was a significant drop in the number of ODVs per cross-section with increasing passage number [Fig. 1b; Jonckheere–Terpstra (JT) test, $n=151$, standardized JT statistic = -7.237 , $P<0.001$]. By P_{cell20} , most polyhedra no longer contained ODVs.

DNA purification from polyhedra and qPCR were performed as described by Zwart *et al.* (2008) to determine the frequency of occurrence of deletion mutants. Primers used for amplification of the immediate-early gene *ie-1* (Kovacs *et al.*, 1992) (Fig. 2a) have been described previously (Zwart *et al.*, 2008). IE-1, an early transcriptional regulator, is essential for replication and the gene was expected to be relatively stable over passaging and therefore a suitable reference locus. Primers for the non-homologous region origin of DNA replication (non-HR *ori*) (forward, 5'-CCGAGACATACCACAAAGCCG-3'; reverse, 5'-GCACATAAACGACGCAGAATACAT-3') were also used, as this locus has been found to become enriched during passaging (Pijlman *et al.*, 2002). Primers for *polh* (forward, 5'-GGG-TGGCAGCAACAACGAGTA-3'; reverse, 5'-CCGATGTA-AACGATGGGCTTGATG-3'; Smith *et al.*, 1983) were included to test for the presence of the gene. No selection for this gene was expected *in vitro* as BV was passaged, whilst *in vivo* there is weak selection for *polh* (Bull *et al.*, 2001). Primers for the mini-F replicon in the bacmid insert (forward 5'-CTTTACGACGGCGACTCCCATC-3', reverse 5'-GCTTACTGAGGACGCACTGGATG-3'; Luckow *et al.*, 1993) were also used, as this locus is not expected to be under selection in either insect cells or larvae. Ratios of the individual loci to *ie-1* (\pm SEM) were calculated as described

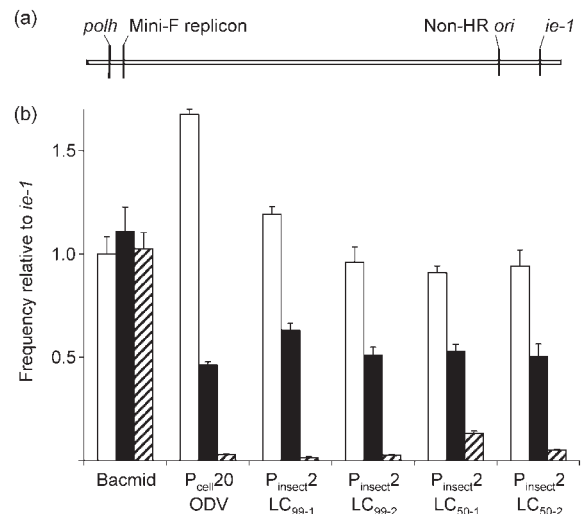


Fig. 2. (a) Location of the loci used for qPCR in the bacmid. (b) Locus frequencies (\pm SEM) of the non-HR *ori* (open bars), *polh* (filled bars) and mini-F replicon (hatched bars) relative to the *ie-1* locus in the original bacmid DNA and in DNA isolated from ODVs of P_{cell20} and the P_{cell20} population after passaging in insects at different doses ($P_{insect2}$).

by Zwart *et al.* (2008), rendering a locus frequency relative to *ie-1* (referred to as the frequency).

qPCR analysis demonstrated that the frequency of the non-HR *ori* had increased to 1.68 by P_{cell20} (Fig. 2b). The frequency of *polh* decreased to 0.47. The mini-F replicon decreased in frequency to 0.03, probably also due in part to the instability of a bacmid insert (Pijlman *et al.*, 2003). The P_{cell20} virus population thus had an unbalanced gene content, as confirmed by qPCR, and had polyhedra with fewer ODVs and lower infectivity.

Amplified polyhedra from the P_{cell20} population were used to perform two passages in *S. exigua* L4 larva (final population: P_{insect2}). Thus, insects were challenged with an LC₅₀ (2×10^4 polyhedra ml⁻¹, final population: P_{insect2-LC50}) and LC₉₉ (6×10^7 polyhedra ml⁻¹, final population: P_{insect2-LC99}), as calculated from the bioassay data for polyhedra from P_{cell20}. Two replicates were performed for each concentration, with polyhedra being pooled from ten larvae per replicate. Polyhedra of P_{insect1-LC99} and P_{insect2-LC99} (replicate 1) were analysed by EM as described above. The LC₅₀ of P_{insect2-LC99} (replicate 1) was also determined by bioassay as described above. For comparative purposes, P_{cell2} was also included in the bioassay. DNA isolation and qPCR as described above were performed for all four P_{insect2} populations.

After two passages in insects at high dose (P_{insect2-LC99}, replicate 1), the infectivity of polyhedra was restored to control values (mean log LC₅₀ ± SEM: P_{cell2} = 3.754 ± 0.064 , P_{insect2-LC99} = 3.963 ± 0.086 ; pairwise *t*-test: $t_2 = -2.392$, $P = 0.139$). The ODV content of polyhedra from this population (P_{insect2-LC99}, replicate 1) was also restored to wild-type levels (Fig. 1b). The observed number of virions per polyhedron cross-section was not significantly different from that in polyhedra from larvae transfected with the original bacmid (mean ± SEM: P_{insect1-LC99} = 5.74 ± 0.58 , bacmid *in vivo* = 7.60 ± 1.17 ; Mann-Whitney *U*-test, $U = 1568.5$, $n = 123$, $P = 0.168$).

To analyse the qPCR data on the P_{insect2} populations (Fig. 2b), a two-way analysis of variance (Genstat 7.0) was performed to test whether there was an effect of dose (LC₅₀ or LC₉₉) on the frequency of the three loci. Neither dose ($F_{1,8} = 0.87$, $P = 0.397$) nor the dose-locus interaction ($F_{2,8} = 0.77$, $P = 0.554$) had a significant effect, whereas locus did ($F_{2,8} = 72.71$, $P < 0.001$). As there was not a significant effect of dose on the frequency of the three loci, locus frequencies for the four populations were compared with that in the P_{cell20} population by means of a one-sample *t*-test. For the non-HR *ori*, there was a significant decrease in frequency ($t_3 = -10.491$, $P = 0.002$). Moreover, the mean P_{insect2} frequency of the non-HR *ori* was not significantly different from the wild-type frequency ($t_3 = 0.006$, $P = 0.996$). The mean increase in *polh* frequency (0.08) was not significant ($t_3 = 2.717$, $P = 0.073$). The mini-F replicon also did not significantly increase in frequency ($t_3 = 0.957$, $P = 0.409$).

The 3000-fold difference in dose between the LC₅₀ and LC₉₉ treatments did not have a significant effect on changes

in locus frequencies. If the number of initially infecting viruses is proportional to the dose, then this could be an indication that the number of initially infecting viruses was very small, even at the high dose. In other words, despite the higher number of viruses initiating infection at high dose, the initial *in vivo* m.o.i. was still so low that it had effectively not changed. This is another indication that the number of founders of infection is small, as has previously been suggested (Smith & Crook, 1988).

Deletion mutants missing *ie-1*, or with multiple copies of the non-HR *ori*, were rapidly purged from the populations during passaging *in vivo* (Fig. 2b). During continuous bioreactor experiments with baculoviruses, cyclic fluctuations in virus titres similar to those described by von Magnus (1954) have been observed (van Lier *et al.*, 1992). The explanation proposed for this phenomenon is that, following a strong drop in virus titres due to defective interference, viruses capable of autonomous replication are enriched at low m.o.i. (de Gooijer *et al.*, 1992). The fact that infection of larvae is initiated by a small number of viruses will similarly lead to low m.o.i. during initial infection. Thus, the purifying selection observed *in vivo* may come about mainly because of these low m.o.i. This is not to suggest that there is no selection for the presence of, for example, *polh* during passaging in insect larvae, but rather that the predominant selective condition resulting in the purifying selection observed is the ability to replicate autonomously.

In order to test whether this explanation has merit for the rescue effect observed by passaging in larvae, the titre of BV-P_{cell20} was determined by an end-point dilution assay and one round of infection of Sf21 cells was performed at m.o.i. of 10, 1, 0.1 and 0.01. BV was purified, DNA isolated and qPCR performed as described above. The experiment was performed in duplicate. Linear-regression analysis was used to determine whether locus frequencies changed as the m.o.i. decreased. For the non-HR *ori*, there was a significant decrease in frequency ($F_{1,6} = 21.937$, $P = 0.0034$), whilst for *polh* ($F_{1,6} = 0.117$, $P = 0.7445$) and the mini-F replicon ($F_{1,6} = 0.1750$, $P = 0.6903$), no change in frequency was observed (Fig. 3a). It was therefore possible to mimic *in vitro* the purifying selection observed *in vivo* simply by altering the m.o.i. This is in agreement with results obtained by van Lier *et al.* (1996) in bioreactor systems: it was empirically shown that low m.o.i. resulted in the slower accumulation of defective viruses. The BV-P_{cell20} sample and a number of time points during passaging were analysed by qPCR to confirm that the ancestral virus (BV-P_{cell0}) had the same genomic AcMNPV profile as the bacmid and to determine when deletion mutants could first be detected (Fig. 3b). Changes in locus frequencies were only visible around passage 12 (P12), in agreement with RFLP data (not shown).

In the experiments described here, *polh* did not increase significantly in frequency by P_{insect2}. Bull *et al.* (2001) found that a *polh*-negative virus persisted, during

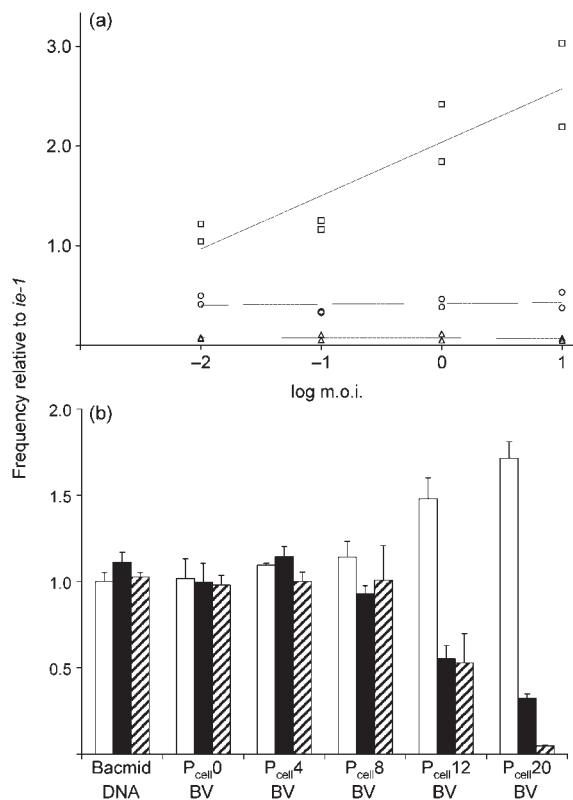


Fig. 3. (a) Locus frequencies relative to the *ie-1* locus after a single passage in Sf21 cells (\log_{10} m.o.i. are indicated). Note that this analysis was performed on DNA obtained from BV collected after a single passage of $P_{\text{cell}20}$ BV at different m.o.i. \square , Non-HR *ori*; \circ , *polh*; \triangle , mini-F replicon. (b) Locus frequencies (\pm SEM) of the non-HR *ori* (open bars), *polh* (filled bars) and mini-F replicon (hatched bars) relative to the *ie-1* locus in the original bacmid DNA, BV of the ancestral virus after transfection ($BV_{\text{cell}0}$) and BV of P4, P8, P12 and P20.

co-infection with a *polh*-positive virus, for six serial transfers in insect larvae. The *polh*-negative virus needs to co-infect a cell with a *polh*-positive virus in order to produce polyhedra and be carried over to the next round of infection. As most cells in the insect will be infected during the final round of infection, Bull *et al.* (2001) reasoned that the rate at which a *polh*-negative virus is lost from a population depends directly on m.o.i. in the final round of cellular infection. Thus, presumably there was not strong selection against the *polh*-negative virus because of a high *in vivo* m.o.i. at the end of infection. ODV of the $P_{\text{cell}20}$ population has an observed *polh* frequency (f_{virus}) of 0.463. If the frequency of *polh* is conserved during infection, then in the final wave of infection, the frequency that cells will be infected by at least one BV containing *polh* in its genome (f_{cell}) is described as: $f_{\text{cell}} = 1 - (1 - f_{\text{virus}})^m$. Here, m is the m.o.i. *in vivo*, estimated at 4.3 BV per cell (Bull *et al.*, 2001, 2003). This results in a value of f_{cell} of 93%. As most cells infected in the final wave of infection have been infected by

a BV containing the *polh* gene, it can be understood that selection is weak.

In contrast to *polh* locus frequency, the ODV content of polyhedra was rescued to wild-type levels within a single passage ($P_{\text{insect}2}$ -LC₉₉, replicate 1). One explanation for this discrepancy is that the presence of deletion mutants, for example those missing *ie-1*, is somehow interfering with the generation of polyhedra. The relative frequency of *polh* to non-HR *ori* – rather than *ie-1* – gives an indication of the frequency of viruses capable of generating polyhedra to all viruses in the population, not only those containing *ie-1*. The frequency of *polh* compared with the non-HR *ori* rises from 0.28 ($P_{\text{cell}2}$) to 0.54 (mean $P_{\text{insect}2}$) during passing *in vivo*. Another compatible explanation for this observation is that the purifying selection observed results in a critical increase in frequency of another gene essential for generation of normal polyhedra (e.g. *25K*).

During baculovirus infection of insect larvae, the m.o.i. *in vivo* changes drastically. As the number of viruses initiating infection appears to be small (e.g. Smith & Crook, 1988), initially the m.o.i. is extremely low. Towards the end of infection, the m.o.i. is rather high (Bull *et al.*, 2001, 2003). These different conditions will result in different selection pressures on the virus population during the course of infection. Genes involved in replication (*ie-1*) or transmission (*polh*) will also be under different selection pressures. There will be a very strong selection on genes involved in replication early in larval infection. Later in infection, selection is somewhat relaxed as m.o.i. become high – if the presence of a single gene copy is sufficient for cellular infection to proceed normally. Conversely, there will not be selection for genes involved in transmission early in infection. Later in infection there will be moderate selection for these genes, but not of the strong purifying type seen for replication early in infection. The fate of baculovirus deletion mutants derived from tissue culture and reintroduced into insect larvae illustrates a virological principle that may apply to many other virological systems.

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