Virulence and competitiveness of *Cydia pomonella granulovirus* mutants: parameters that do not match

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The LD<sub>50</sub>, median survival time (ST<sub>50</sub>) and virus production are virulence parameters that are commonly used to describe the biological characteristics of viruses. In this study, these parameters were determined for *Cydia pomonella granulovirus* (CpGV-M) and two naturally occurring mutants (CpGV-MCp4 and -MCp5) that carry Tc1-like insect transposable elements. The three virus genotypes were similar in their LD<sub>50</sub>, ST<sub>50</sub> and virus production. However, the mutant genotypes MCp4 and MCp5 were very effectively out-competed by CpGV-M in direct competition experiments, where *Cydia pomonella* larvae were co-infected with known ratios of occlusion bodies or budded virus of CpGV-M and one of the two mutants. It was demonstrated that MCp5 and MCp4 could not be sustained in the virus population when the progeny viruses of different co-infections were used as inocula to infect next passage larvae. These results show that the virulence parameters LD<sub>50</sub>, ST<sub>50</sub> and virus production alone do not adequately reflect the competitiveness of the virus and are thus not suitable to describe virus population dynamics.

INTRODUCTION

*Cydia pomonella granulovirus* (CpGV) belongs to the family *Baculoviridae*. CpGV is highly pathogenic for the codling moth (*Cydia pomonella*), but larvae of the false codling moth (*Cryptophlebia leucotreta*) are also susceptible. The genome of CpGV consists of circular dsDNA, has a size of 123 500 bp and encodes 143 genes (Luque et al., 2001). Previous infection studies of CpGV in larvae of *Cydia pomonella* and *Cryptophlebia leucotreta* resulted in the isolation of two virus mutants harbouring insect host transposable elements (TEs) (Jehle et al., 1995, 1998). Molecular characterization of the mutants demonstrated that these transposons originated from the insect host genomes and were horizontally transferred into the genome of CpGV. Mutant CpGV-MCp5 (MCp5) contained transposon TCl4.7, which escaped from the genome of *Cryptophlebia leucotreta*. TCl4.7 is 4726 bp, has inverted terminal repeats (ITRs) of 29 bp and carries a defective transposase gene (Jehle et al., 1995). The other mutant, CpGV-MCp4 (MCp4), harbours transposon TCp3.2, which originated from the genome of *Cydia pomonella*. TCp3.2 is 3239 bp, has ITRs of 756 bp and also has a defective transposase gene (Jehle et al., 1998). Both TEs belong to the superfamily of Tc1/mariner-like transposons, which are characterized by sequence homology [D,D(35)E motif] within the transposase gene and duplication of a TA dinucleotide target site (Radice et al., 1994; Doak et al., 1994; Robertson, 1995).

The integration site of TCI4.7 is located between CpGV repeated sequences 3 and 4, which are found in the non-translated region between CpGV open reading frames (ORFs) Cp15 and Cp16 (Luque et al., 2001). The functions of Cp15 and Cp16 are unknown. Repeated sequences 3 and 4 belong to a group of 13 identified imperfect palindromes of about 75 bp that are distributed throughout the CpGV genome (Luque et al., 2001). In CpGV, these repeated sequences are not arranged as multiple tandem repeats as is typical for homologous regions (hrs) that function as origins of replication or as transcription enhancers in many nucleopolyhedroviruses (Cochran & Faulkner, 1983; Theilmann & Stewart, 1992; Kool et al., 1995; Xie et al., 1995; Hayakawa et al., 2000). It remains to be investigated whether these repeats in CpGV have a similar function to hrs. Transposon TCp3.2 is inserted in a non-translated region downstream of late expression factor 2 (*lef-2*, Cp41) and upstream of Cp42 (Jehle et al., 1997). *lef-2* is a conserved baculovirus gene that is essential for DNA replication (Kool et al., 1994; Lu & Miller, 1995). ORF42 is possibly an early transcribed gene with an unknown function. Integration of TCp3.2 occurs at a TA dinucleotide that is part of a putative TATA-box in the promoter region of Cp42. Previous
investigations have revealed that the median lethal concentration (LC50) for neonate *Cydia pomonella* larvae infected with CpGV-M, MCp4 or MCp5 is not significantly different (Jehle et al., 1995). Since LC50 is only one of several commonly used parameters to quantify virus virulence, parameters describing the virulence and biological fitness of these viruses were further compared.

Lethal dose (LD), lethal time and virus offspring production are some commonly used quantitative parameters to describe virus–host interactions (Shapiro-Ilan et al., 2005). However, these parameters take only single virus infections into account. They do not consider virus fitness in competition situations, which might be more typical in natural epizootics. There are many examples that have demonstrated that mixed infection of two baculoviruses or two baculovirus genotypes can have a synergistic benefit for both viruses or at least for one virus (Lara-Reyna et al., 2003; Lopez-Ferber et al., 2003). In certain cases, mixed infection with different genotypes can result in an equilibrated long-term co-existence of these genotypes (Munoz & Caballero, 2000; Hodgson et al., 2004). In the present study, it is shown for the first time that mixed infections of CpGV genotypes can be extremely disadvantageous for mutant genotypes, even though their virulence parameters, determined in single genotype infections, do not differ.

**METHODS**

**Virus stocks, cell lines and insect larvae.** The viruses used in this study were derived from an *in vivo* cloned genotype of the Mexican strain of CpGV (CpGV-M) (Tanada, 1964). The transposon insertion mutants CpGV-MCp5 (MCp5) and CpGV-MCp4 (MCp4) were isolated from an infection experiment of *Cryptophlebia leucotreta* larvae with CpGV-M and *Cryptophlebia leucotreta* granulovirus (CrleGV-CV3) and a subsequent *in vivo* cloning procedure in *Cydia pomonella* larvae (Jehle et al., 1995). Virus occlusion bodies (OB) were purified as described by Payne (1981). The OB were enumerated using a Petroff–Hauser counting chamber (depth 0-01 mm) using the dark field optics of a light microscope (Leica DMRBE) at ×400 magnification. Virus DNA was purified from OB according to Crook et al. (1985). Infectious CpGV haemolymph and passaged budded viruses (BV) were prepared and titrated as described by Winstanley & Crook (1993).

The *Cydia pomonella* larvae from insect rearing at the Agricultural Service Center Palatinate, Neustadt/Weinstr., Germany, were reared at 26 °C on a semi-synthetic diet (Ivaldi-Sender, 1974). For experiments performed at Horticulture Research International, Wellesbourne, UK, the insects were reared at 25 °C on a semi-synthetic diet (Guennelon et al., 1981). Handling and infection of the *Cydia pomonella* cell line DW14R was described by Winstanley & Crook (1993).

**Bioassays.** Fifth instar *Cydia pomonella* were used for dose mortality response analysis expressed as LD and for median survival time analysis (expressed as ST50). Bioassays were performed in autoclavable 50-well plates. Larvae were inoculated by feeding a small piece of medium containing the virus dose. Only larvae that had completely ingested the dose within 24 h were placed on fresh virus-free medium and reared individually at 26 °C. To determine the LD50 groups of 40 larvae were infected with six different doses of virus (0, 1, 10, 25, 50 and 250 OB). Mortality was recorded every 2 days until death or pupation of the larvae. Bioassays were replicated two to three times for each virus dose. The ST50 was determined by inoculating 35 larvae with a calculated LD50 dose (80% lethal dose). Mortality of the larvae was monitored at intervals of 8 h starting at day 5 post-infection (p.i.) until larval death or pupation. Assays were replicated three times for each virus. The number of virus offspring was quantified by infecting early L5 instars with LD50 of each of CpGV, MCp4 and MCp5. Virus OB were purified and enumerated for each larva as described above.

**Statistical analyses.** The dose mortality response was calculated by probit analysis (normal distribution) according to Finney (1971) using the SAS software package (SAS Institute, 2001). Three doses, LD10 (10% lethal dose), LD50 (50% lethal dose) and LD90 (90% lethal dose), were compared. For statistical analysis of the differences between these doses, ratios of lethal dose (RLD) were determined and the 95% confidence interval (CI) was calculated for each RLD as described by Robertson & Preisler (1992). Lethal doses were considered to be statistically different when the 95% confidence interval of their RLDs did not include 1:0 (Robertson & Preisler, 1992). The median survival time was determined using the Kaplan–Meier procedure (SAS Institute, 2001).

**Competition and passage experiments.** In the competition experiments, fifth instar *Cydia pomonella* were co-infected per os (10 000 OB per insect) with different ratios of CpGV-M:MCp5 and CpGV-M:MCp4 (100:0, 90:10, 67:33, 50:50, 33:67, 10:90 and 0:100). OB were isolated from individual cadavers and the CpGV-M:mutant ratio was determined (see below).

In passage experiments, two types of inoculum virus were used: purified OB were administered orally and BV were injected into the haemocoel. When OB were applied, 20 L5 larvae were inoculated with 250 OB (see Bioassays) from the previous passage. For virus isolation and quantification of the OB offspring, 20 larvae were pooled.

In the passage experiment with BV, two types of BV inocula were used: (i) BV-containing supernatant from infected CpDW14R cells (passage 1) and (ii) BV-containing haemolymph. Passage 1 virus for CpGV-M, MCp5 and MCp4 was prepared and subsequently titrated on CpDW14R cells as described by Winstanley & Crook (1993). Based on the mean TCID50 values determined, larvae were inoculated with CpGV-M and a mutant genotype at a 10:90 ratio using a total dose of 350 TCID50 in a total volume of 9 μl BV-containing haemolymph was obtained from 20 infected larvae on day 4 p.i. (Winstanley & Crook, 1993). This infective haemolymph was used as an inoculum to infect next passage larvae (9 μl per larva). The BV inoculum was injected into the proleg of ether-paralysed fifth instar *Cydia pomonella* using a micro-injector.

In the passage experiments, 40 larvae were inoculated. At 4 days p.i., BV-containing haemolymph was collected from 20 larvae and used to inoculate next passage larvae. The remaining 20 infected larvae were further incubated. After larval death, OB were isolated from the pooled cadavers and were subsequently used to determine the CpGV-M:mutant ratio. The CpGV-M:mutant ratio for OB from each batch of pooled cadavers for different infection ratios was determined three times.

**Determination of the CpGV-M:MCp5 and CpGV-M:MCp4 ratios.** The ratios of CpGV-M and mutant genotypes in the offspring of the CpGV-M:MCp5 and CpGV-M:MCp4 co-infection experiments were determined by densitometric quantification of genotype-specific DNA restriction fragments. For this, virus OB were isolated from moribund *Cydia pomonella* cadavers and viral DNA was isolated as described by Jehle et al. (1992). Then, the samples of viral DNAs were digested using different sets of restriction endonucleases. DNA restriction fragments were separated on 0.8 % TAE agarose gels and stained with ethidium bromide (0.1 μg ml⁻¹).
Table 1. Dose mortality response of fifth instar Cydia pomonella infected with CpGV-M, MCp4 and MCp5

<table>
<thead>
<tr>
<th>Virus</th>
<th>LD10 (CI)</th>
<th>RLD (CI)</th>
<th>LD50 (CI)</th>
<th>RLD (CI)</th>
<th>LD90 (CI)</th>
<th>RLD (CI)</th>
<th>Slope (± SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpGV-M</td>
<td>7.8±4 (4.6–11.4)</td>
<td>–</td>
<td>44.7±5 (35.9–54.7)</td>
<td>–</td>
<td>255±7 (185–402)</td>
<td>–</td>
<td>0.74 (±0.076)</td>
</tr>
<tr>
<td>MCp4</td>
<td>3.6±6 (0.8–8.5)</td>
<td>2.2 (0.37–9.6)</td>
<td>35.7±7 (19.1–52.9)</td>
<td>1.3 (0.5–5.6)</td>
<td>353±8 (236–681)</td>
<td>0.72 (0.11–2.0)</td>
<td>0.55 (±0.088)</td>
</tr>
<tr>
<td>MCp5</td>
<td>1.7±4 (0.5–3.7)</td>
<td>4.5 (3.2–370)</td>
<td>23.2±2 (13.3–35.8)</td>
<td>1.9 (1.31–15.5)</td>
<td>318±8 (208–542)</td>
<td>0.8 (0.15–2.4)</td>
<td>0.49 (±0.052)</td>
</tr>
</tbody>
</table>

*Slope and standard error (SE) of probit line.

Gels were photographed (Intas Gel Documentation System) and the optical density of the single DNA fragments was quantified using the Imagemaster 1D software (Amersham Pharmacia Biotech). In optimization experiments, a strong correlation between size and the optical density of the DNA restriction fragments was determined (data not shown). By correlating the sizes and the densities of the genotype-specific and genotype-shared restriction fragments, the molar proportions of the different DNA fragments were calculated. Using these molar proportions, the ratios of the CpGV-M and mutant genotypes in the virus offspring were determined.

For the survival time determination, larvae were infected with an LD80 dose, which was equal to 140 OB (CpGV-M), 130 OB (MCp5) and 160 OB (MCp4). The calculated ST50 values were 184 h for CpGV-M, 208 h for MCp5 and 222 h for MCp4 (Table 2). During the bioassays, no differences were observed in the disease symptoms of the Cydia pomonella larvae infected with CpGV-M, MCp5 or MCp4.

Virus offspring production

Since a potential selection advantage of a virus may depend on the amount of virus produced, the virus yields of fifth instar Cydia pomonella infected with LD80 of CpGV-M, MCp5 and MCp4 were determined (Table 2). Larval cadavers contained a mean of 3.61×10^10 OB per larva (CpGV-M), 1.98×10^10 OB per larva (MCp5) and 3.60×10^10 OB per larva (MCp4) (Table 2). These differences, however, were not significant (Tukey’s test, d.f. = 29, P = 0.05). This demonstrated that there was no difference in virus progeny production between the different genotypes in fifth instar Cydia pomonella.

Table 2. ST50 and virus production of fifth instar Cydia pomonella infected with CpGV-M, MCp4 and MCp5

<table>
<thead>
<tr>
<th>Virus</th>
<th>Survival time analysis</th>
<th>Virus progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ST50 CI (lower–upper)</td>
<td>OB per larva ± SE (×10^10)*</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>CpGV-M</td>
<td>184</td>
<td>175–199</td>
</tr>
<tr>
<td>MCp4</td>
<td>222</td>
<td>216–232</td>
</tr>
<tr>
<td>MCp5</td>
<td>208</td>
<td>192–216</td>
</tr>
</tbody>
</table>

*Superscript letters indicate statistical differences in a Tukey’s Studentized range test (P = 0.05).
Competition experiments between CpGV-M and MCp5 and between CpGV-M and MCp4

In order to compare the propagation efficacy of CpGV-M and the mutants, co-infection experiments of fifth instar *Cydia pomonella* were performed by oral inoculation with OB. A selection advantage of one of the genotypes was investigated by comparing the virus ratios in the inoculum with those of the virus offspring. In order to exclude possible dose effects in the infection progress, different ratios of CpGV-M : MCp5 and CpGV-M : MCp4 were applied as inocula. After the virus OB were isolated from single larval cadavers, molar proportions of the genotypes were determined by densitometric quantification of genotype-specific restriction fragments in the virus offspring. A *Bam*HI digest of viral DNA originating from CpGV-M/MCp5 co-infection experiments resulted in a CpGV-M-specific (7.0 kb) and a MCp5-specific (11.7 kb) fragment (Fig. 1a). A *Bam*HI digest of viral DNA obtained from CpGV-M/MCp4 co-infection experiments resulted in a CpGV-M-specific (5.8 kb) fragment. A *Hind*III/*Bgl*II digest on this DNA resulted in MCp4-specific fragments of 1.2 and 6.8 kb (Fig. 1b).

For each infection ratio used in the co-infection experiments, the CpGV-M : mutant ratio in the virus offspring was determined. In all experiments, a lower MCp5 proportion was found in the offspring than was applied in the inoculum (Fig. 2a). For example, when using an infection ratio of 90 % MCp5 and 10 % CpGV-M, only 39 % of the virus offspring was MCp5, but 61 % was CpGV-M. This shift towards CpGV-M continued with increasing proportions of CpGV-M in the inoculum. Only infections using 100 % of either CpGV-M or MCp5 genotype resulted in a pure corresponding virus offspring. A similar, but even more dramatic selection disadvantage, was noticed for MCp4 (Fig. 2b). Here, an infection ratio of 90 % MCp4 and 10 % CpGV-M resulted in only 10 % MCp4 offspring, but 90 % CpGV-M. In all other co-infections where the proportions of MCp4 were 67 % or less, no MCp4 was found in the virus offspring.

**Passage effect**

In order to investigate whether MCp4 and MCp5 can be sustained in the virus population despite the observed selection disadvantage, the virus offspring of the 10 % CpGV-M, 90 % MCp5/MCp4 co-infections were serially passaged through the larvae (Fig. 3a; P1-OB). Two types of progeny virus were used to infect the following passage larvae: (i) OB isolated from larval cadavers were used to orally infect next passage larvae (Fig. 3a; larvae P2-OB and P3-OB); and (ii) BV isolated from the haemolymph of infected larvae was injected into the haemolymph of the next passage larvae (Fig. 3a; larvae P2-BV and P3-BV).
DNA was isolated from OB obtained from the larval cadavers and the ratio of CpGV-M : mutant was densitometrically determined. As shown in Fig. 3(b), the ratio of MCp5 in the virus offspring decreased from 90% in the inoculum to 46, 10 and 0% after 1, 2 and 3 passages, respectively (larvae P1-OB, P2-OB and P3-OB). When BV was used in the passage experiment, MCp5 was no longer detectable in the virus offspring after the first passage (larvae P2-BV).

The observation that MCp4- and MCp5-specific DNA restriction fragments could be detected in P2-OB, but not in P2-BV (Fig. 3b) suggested that the mutants might be maintained slightly longer in the offspring when they were passaged as OB rather than as BV. However, in these experiments, the CpGV-M : mutant ratios in the BV collected from P1 larvae and used to infect P2 larvae (Fig. 3a) were unknown. In order to circumvent this limitation, an additional experiment was performed in which the ratios of the BV were known. For this, titrated BV samples of CpGV-M and the two mutant MCp4 and MCp5 were produced in infected CpDW14R cells. A CpGV-M : mutant ratio of 10 : 90 was used to inject P1 larvae (Fig. 4a). BV-containing haemolymph was collected from infected larvae 4 days p.i. and passaged two times by injection in larvae as described above (Fig. 4a; larvae P2 and P3). Quantification of MCp5,

![Fig. 3. Competition experiment between CpGV-M and MCp5 and between CpGV-M and MCp4 in Cydia pomonella larvae after several larval passages.](image)

(a) inoculum P1 P2 P3
OB
CpGV:mutant 10:90
OB
OB
OB
BV
BV
BV

(b) Ratio MCp5 (%) OB inoculum OB P1 OB BV OB BV
90 46 10 0 0 0

(c) Ratio MCp4 (%) OB inoculum OB P1 OB BV OB BV
90 29 2 0 0 0

![Fig. 4. Competition experiment between CpGV-M and MCp5 and between CpGV-M and MCp4 in Cydia pomonella larvae transferred through several larval passages. Larvae P1 were inoculated with BV (CpGV-M : mutant ratio of 10 : 90) by injection in the proleg. Progeny BV in the haemolymph of infected larvae was transferred to the next passage larvae (P2 and P3) by injection in the proleg (a). After larval death, the ratio of the MCp5 (b) and MCp4 (c) genotypes in the extracted OB was determined. The ratio of the mutant genotypes in each ‘OB offspring’ was determined three times.](image)

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Virulence and competitiveness of CpGV mutants
MCP4 and CpGV-M in the isolated OB fractions corroborated again that the mutants were out-competed rapidly by CpGV-M. After only one passage, the mutants were undetectable in the virus progeny (Fig. 4b, c).

In order to improve the detection sensitivity of genotype-specific restriction fragments, PCR studies were performed to investigate whether traces of MCP5 and MCP4 were detectable in the DNA samples used for DNA restriction analyses. PCR analyses using a transposon-specific and a CpGV-specific primer (prMCp5 and prTCl4.7 for MCP5 and prMCP4 and prTCP3.2 for MCP4) resulted in 850 bp (MCP5) and 900 bp (MCP4) genotype-specific amplification products, respectively (Fig. 5a, b). Decreasing traces of MCP4 and MCP5 were detected by PCR in all passages except P3-BV (CpGV-M: MCP5). PCR analyses showed a more rapid decrease in the intensity of MCP5- and MCP4-specific products in samples of BV, compared with that in OB passaging experiments. This observation is in agreement with the results of DNA restriction analysis. It has to be noted that the mutant-specific PCR fragments in the CpGV-M: MCP4 samples were more intense than in the CpGV-M: MCP5 samples (Fig. 5a, b). From this observation, however, it cannot be concluded that MCP4 was maintained in higher amounts than MCP5 in the different passages. Indeed, the opposite was observed in quantitative restriction analyses (Fig. 5b). The differences between the intensities of MCP- and MCP-specific PCR signals could be caused by different dynamics of the PCR, different efficacies of PCR primer annealing or differing PCR primer quality.

DISCUSSION

Infection parameter LD, ST50 and virus offspring production revealed only small differences between the analysed viruses CpGV-M, MCP4 and MCP5. The LD50 and LD90 values of the three genotypes were similar. Only the LD10 and LD50 of mutant MCP4 were lower statistically than those of CpGV, suggesting that MCP4 was even a little more virulent than CpGV-M at low infection doses. No evidence for decreased infectivity of the mutants MCP4 and MCP5 was found. These findings are in good agreement with earlier bioassays in neonate larvae of Cydia pomonella, which also resulted in similar LC50 values for CpGV-M and the two mutants (Jehle et al., 1995). Considering the ST50, the mutants MCP4 and MCP5 appeared to kill fifth instar Cydia pomonella larvae slightly slower than CpGV-M. In addition, the progeny virus yields of the parental CpGV-M and the transposon-carrying mutants did not reveal statistically significant differences between the virus genotypes. Taken together, the bioassays demonstrated that the transposon-carrying mutants retained their virulence and their ability to complete the replication cycle and produce infective OB in Cydia pomonella larvae.

When the competitiveness of virus genotypes was determined in co-infection and passaging experiments, a rapid out-competition of the mutants MCP4 and MCP5 by CpGV-M was observed. This result indicated a tremendous selection disadvantage of MCP4 and MCP5 compared to CpGV-M. The observed disappearance of MCP4 and MCP5 in the co-infection and passaging experiments is probably not attributable to the loss of the inserted transposable elements from the mutant genomes. Theoretically, the loss of MCP4 and MCP5 genotypes from the virus populations in the co-infection experiments could have been caused by excision of the transposons in these mutants. However, the genetic stability of these mutants has been confirmed in previous propagation experiments (Jehle et al., 1998; Arends & Jehle, 2002). It has also been demonstrated that MCP4 and MCP5 replicate in Cydia pomonella larvae yielding pure genotypes (Fig. 2). It can be thus concluded that the loss of MCP4 and MCP5 in co-infection experiments is indeed caused by phenotypic out-competition due to the presence of CpGV-M.

The integration of a transposon in a viral genome does not necessarily lead to reduced fitness of the recipient baculovirus. Such insertions can be silent. Under specific conditions, they can even result in a propagation advantage, as observed for a transposon harbouring few-polyhedra (FP) mutants of Autographa california multiple nucleopolyhedrovirus (AcMNPV) and Galleria mellonella MNPV mutants, which were isolated following serial passage of BV in insect cell lines (for reviews see Fraser, 1986; Friesen, 1993). Although these mutants produced OB with a reduced per oral infectivity, they had an obvious replication advantage compared to the parental viruses when propagated in...
insect cell lines. Most of these mutants contained transposon insertions within the fp25k gene, which encodes a protein that is involved in regulation of the production of BV and occlusion-derived virus during the biphasic virus replication cycle (Beames & Summers, 1989; Harrison & Summers, 1995). Disruption of this gene leads to increased BV production resulting in a propagation advantage in insect cell lines (Volkman & Keddie, 1990; Jarvis et al., 1992). Recently, a novel FP-mutant (AcMNPV.fp-1) with a transposon in the fp gene was isolated that could be maintained in an AcMNPV population during natural infection (Bull et al., 2003). Although this mutant had a greatly reduced ability to form OB, it was able to persist as a stable polymorphism with a wild-type AcMNPV during successive rounds of infection in Trichoplusia ni larvae. Most probably, this mutant can be sustained in the virus population since it has a higher replication rate in T. ni larvae and because it can be co-occluded with wild-type AcMNPV after co-infection.

It is not yet clear which molecular mechanisms cause the lack of competitiveness of MCP4 and MCP5. However, the serial passage experiments with MCP4 and MCP5 in which OB were inoculated per os and BV were inoculated by injection into the haemolymph, further suggested that the selection disadvantage of the mutants was not due to a difference in per os infectivity. If only a reduced per os infectivity existed, then the out-competition of MCP4 and MCP5 would not have been observed when BV was passaged instead of the OB (Figs 3–5). Apparently, the competition disadvantage occurred during the later stages of the infection cycle. It might be linked to the speed of BV replication or to virus spread. The small increase in the ST50 of MCP4 and MCP5 is a hint that infection speed of MCP4 and MCP5 might be slightly decreased in these mutants. Since the transposons TCP3.2 and TCI4.7 both integrated into non-coding genomic regions of MCP4 and MCP5, a direct influence on the amino acid sequence of virus-encoded proteins can be excluded. Further experiments are necessary to analyse whether the integration of TCP3.2 and TCI4.7 are the reason for the tremendous differences in replication observed between CpGV-M, MCP4 and MCP5 and how transposon insertion may impede transcription of neighbouring genes. If this proves to be the case, the integration regions of the transposons might be interesting targets for the genetic engineering of CpGV and other GV's because this could reduce the competitiveness of the virus without altering other parameters such as infectivity or virus production.

It is striking that despite no or very small differences in the virulence parameters LD50, ST50 and virus production, there is an extreme difference in direct competition. These findings are in clear contrast to previous analyses where fitness and competitiveness of different baculovirus genotypes from naturally occurring wild-type populations were compared. Munoz & Caballero (2000) demonstrated that even defective genotypes from Spodoptera exigua MNPV (SeMNPV) that are not able to replicate on their own can utilize genome functions of intact SeMNPV genotypes and can persist to a high degree in mixtures containing the helper virus. An essentially neutral co-infection between two naturally occurring genotypes of Panolis flammea NPV (PafINPV) was described by Hodgson et al. (2004). In this study, no evidence for competition for limited host factors by co-infecting PafINPV genotypes was observed. In these two examples, a more or less stable ecological equilibrium between different virus genotypes can be anticipated resulting in co-existence of these genotypes. However, Munoz et al. (1997) showed that an SeMNPV crossover mutant had an LD50 value that did not significantly differ from those of the two parental viruses. In co-infection experiments, however, the parental viruses were rapidly replaced by the mutant virus upon successive passage in larvae of S. exigua. These different and contradictory findings clearly demonstrate that predictions on the ecological behaviour of a given virus genotype cannot be made on the basis of parameter estimates resulting from single genotype infections, but need to be evaluated in the light of possible biological interactions with other genotypes. In conclusion, virulence parameters alone (e.g. LC50, LD50, ST50 and virus production) are not suitable to describe the competitiveness of a genotype in the environment.

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