

# Defective or effective? Mutualistic interactions between virus genotypes

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Defective viruses lack genes essential for survival but they can co-infect with complete virus genotypes and use gene products from the complete genotype for their replication and transmission. As such, they are detrimental to the fitness of complete genotypes. Here, we describe a mutualistic interaction between genotypes of an insect baculovirus (nucleopolyhedrovirus of *Spodoptera frugiperda* (Lepidoptera)) that increases the pathogenicity of the viral population. Mixtures of a complete genotype able to be transmitted orally and a deletion mutant unable to be transmitted orally resulted in a phenotype of increased pathogenicity. Because the infectiousness of mixed genotype infections was greater than that of single genotype infections, we predict that the transmissibility of mixed genotype occlusion bodies will be greater than that of any of their single genotype components. Such interactions will be subject to frequency-dependent selection and will influence the impact of these viruses on insect population dynamics and their efficacy as biological insecticides.

**Keywords:** nucleopolyhedrovirus; defective genotypes; mutualistic interactions; pathogenicity; Lepidoptera

## 1. INTRODUCTION

Analysis of genotypic variation in populations of asexual microparasites can generate key insights into the dynamics of host–parasite interactions and the development of epizootics of disease (Read & Taylor 2001; Wilson *et al.* 2002). Co-infection by different genotypes resulting in a high prevalence of mixed genotype disease appears to be a common characteristic of many host–parasite systems, particularly viruses (Ennos 1992; Hodgson *et al.* 2002). Defective viruses arise by spontaneously generated deletion of genes that are essential for the survival of the virus particle. However, in the presence of a complete virus genotype, the deletion mutant has a replicative advantage owing to its shorter genome, and can use gene products from the complete genotype to achieve transmission (Roux *et al.* 1991; Frank 2000).

We may intuitively suspect that diversity at the population level reflects selection processes acting on parasite reproduction within a host that favours certain genotypes, whereas selection between hosts (transmission) may favour other variants (Ebert & Hamilton 1996). In reality, the forces that promote and maintain genotypic diversity are complex and may include trade-offs in parasite fitness components (Wilson *et al.* 2000), host immune response (Washburn *et al.* 1998; Zhang *et al.* 2002), differential selection for genotypes (Hodgson *et al.* 2002), recombination (Crozier & Ribeiro 1992) and within-host interactions between competing parasite genomes (Muñoz *et al.* 1997).

Usually models of competition between microparasite genotypes implicitly assume that each genotype is com-

petent and capable of being independently transmitted (Dieckmann *et al.* 2002). However, some viruses may lose part of the genome but continue to replicate by sequestering the gene products of the complete genotypes. These defective viruses arise by spontaneously generated deletion of genes that encode products that are essential for the survival of the virus, such as coat proteins and replication enzymes. The defective virus cannot replicate or transmit by itself. However, in the presence of a complete genotype, the deletion mutant has a replicative advantage due to its shorter length, and can use the products from complete genotypes to replicate and transmit itself (Barrett & Dimmock 1986).

Nucleopolyhedroviruses (NPVs) are unusual in that there are two virion forms produced for transmission within and between hosts. Occlusion-derived virions (ODVs) infect gut cells following consumption of contaminated foliage. An initial round of replication gives rise to nucleocapsids that individually bud through the basement membrane and disperse to infect the cells of other tissues (Volkman 1997). Crucially, in the budded form, multiple genotypes may infect each host cell, thereby allowing defective genotypes to take advantage of the gene products of complete genotypes. Later in the infection cycle, nucleocapsids are retained in the nucleus where they are wrapped by a membrane, either singly or in groups of several nucleocapsids (MNPVs). These enveloped virions are occluded into large proteinaceous occlusion bodies (OBs) that protect the virus in the environment (Volkman 1997). Following the death of the host, large numbers of progeny OBs are released onto the host plant to achieve transmission between hosts (Goulson 1997).

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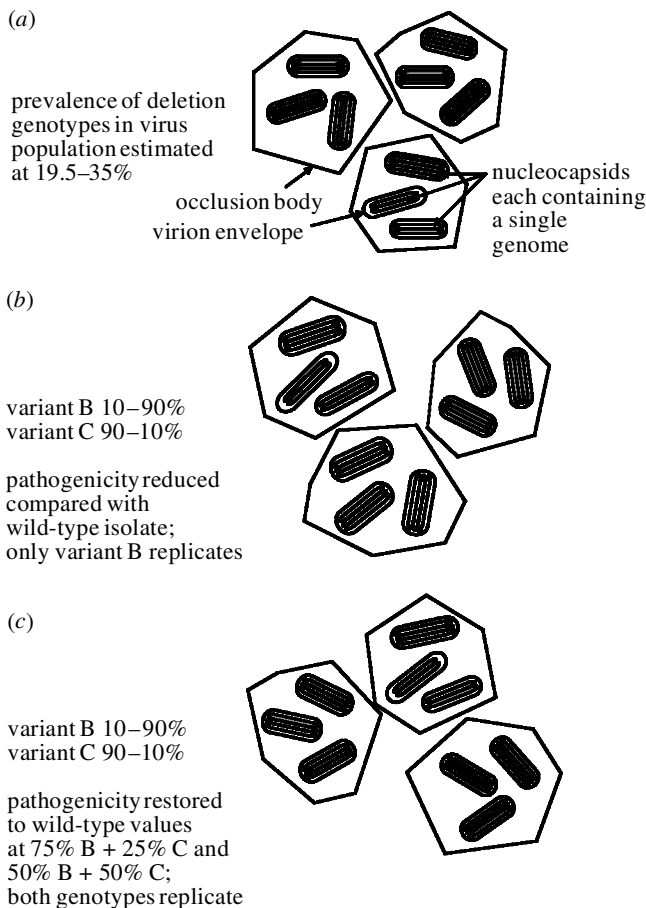


Figure 1. Composition of the virus OBs employed in the study. (a) Wild-type isolate consisting of a co-occluded mixture of complete genotypes and deletion mutants. (b) OB mixtures of genotype B (complete) and genotype C (deletion mutant) showing reduced pathogenicity compared to wild-type. (c) Co-occluded mixtures of genotypes B and C in which pathogenicity was restored to wild-type values. White, wild-type genotype (variant B); shaded, deletion genotype (variant C).

In a multinucleocapsid NPV isolated from the fall armyworm, *Spodoptera frugiperda* (SfMNPV), we have isolated incomplete genotypes that alone are not able to achieve transmission between insect hosts. The deletion variants are present at an intermediate prevalence in the virus population. Contrary to current theory on genetic diversity in microparasite populations (Read & Taylor 2001), we show that the presence of a defective genotype in mixtures with a complete genotype results in a phenotype with increased pathogenicity compared with that of the complete genotype alone.

## 2. MATERIAL AND METHODS

### (a) Isolation of genotypes from wild-type virus

SfMNPV was collected from diseased *S. frugiperda* larvae infesting maize plants in Nicaragua (Escribano *et al.* 1999). The virus was amplified by feeding OBs to third and fourth instar *S. frugiperda* from a healthy laboratory colony, reared on a semi-synthetic diet until death. For the isolation of individual genotypes (figure 1a), infected larvae were surface decontaminated with 70% ethanol and haemolymph was taken by bleeding. The

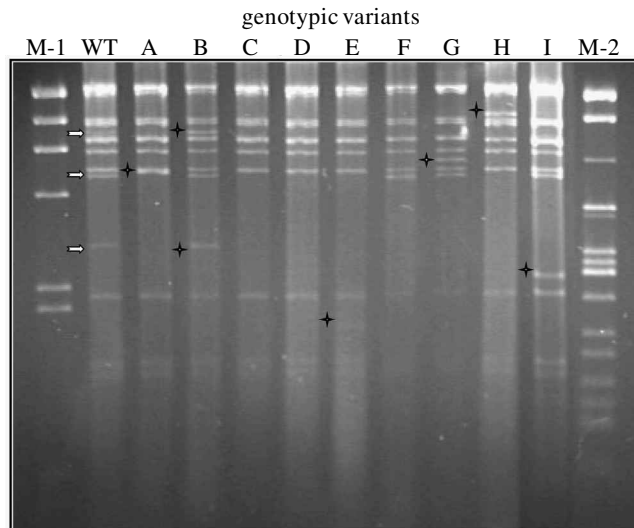


Figure 2. Agarose gel (1%) electrophoresis of *Pst*I restricted genomic DNA of the different genotypic variants (labelled A–I) isolated from the wild-type (WT) SfMNPV population. Arrows signify the presence of submolar restriction fragments in the wild-type isolate characteristic of genetic heterogeneity, stars indicate the restriction fragments used as markers for the genotypic variants. M-1, marker lane ( $\lambda$ -*Hind*III); M-2, marker lane ( $\lambda$ -*Eco*RI/*Bam*HI/*Hind*III).

haemolymph was diluted in sterile phosphate-buffered saline and used to infect *S. frugiperda* (Sf9) cells. Plaque assays were then performed. Plaque assay dishes were screened for the presence of plaques 10 days after inoculation. Individual plaques were then amplified in Sf9 cells. At 8 days after inoculation, cell culture supernatant was diluted 1 : 5 and then inoculated by injection of 8  $\mu$ l volumes into fourth instar *S. frugiperda*. When larvae died 5–8 days later, viral OBs were extracted by trituration of dead larvae in sterile distilled water and used in insect bioassay and restriction endonuclease analyses. DNA was extracted from ODVs by incubation with sodium dodecyl sulphate + proteinase K followed by phenol–chloroform treatment. The virus DNA was restricted using *Eco*RI or *Pst*I and separated in agarose gels to verify the proportion of each genotype in the experimental population. Deletion mutants were identified by the absence of a 7.8 kb *Pst*I fragment located between map units 81 and 86 of the genome. This fragment was specific to the Nicaraguan isolate and was not observed in a United States isolate (Sf-2) described previously (Maruniak *et al.* 1984).

The relative proportions of each genotype in the wild-type population were determined by measuring the intensity of a variable restriction fragment present in digested genomic DNA. A cloned 3.1 kb *Eco*RI fragment (previously referred to as fragment K (Maruniak *et al.* 1984), insert of plasmid p264.306) was labelled with digoxigenin-dUTP by random priming and used to probe a Southern blot of *Eco*RI-treated SfMNPV genomic DNA. In the deleted genotypes, this fragment is linked to the neighbour and appears as a 9 kb composite fragment. The hybridization proceeded at 60 °C overnight followed by critical washes at 25 °C in 2  $\times$  standard saline citrate (SSC) (for 15 min) and 60 °C in 0.1  $\times$  SSC (for 30 min). The relative intensity of probe hybridization was measured using the SCION IMAGE PC program (Scion Corporation, Frederick, USA).

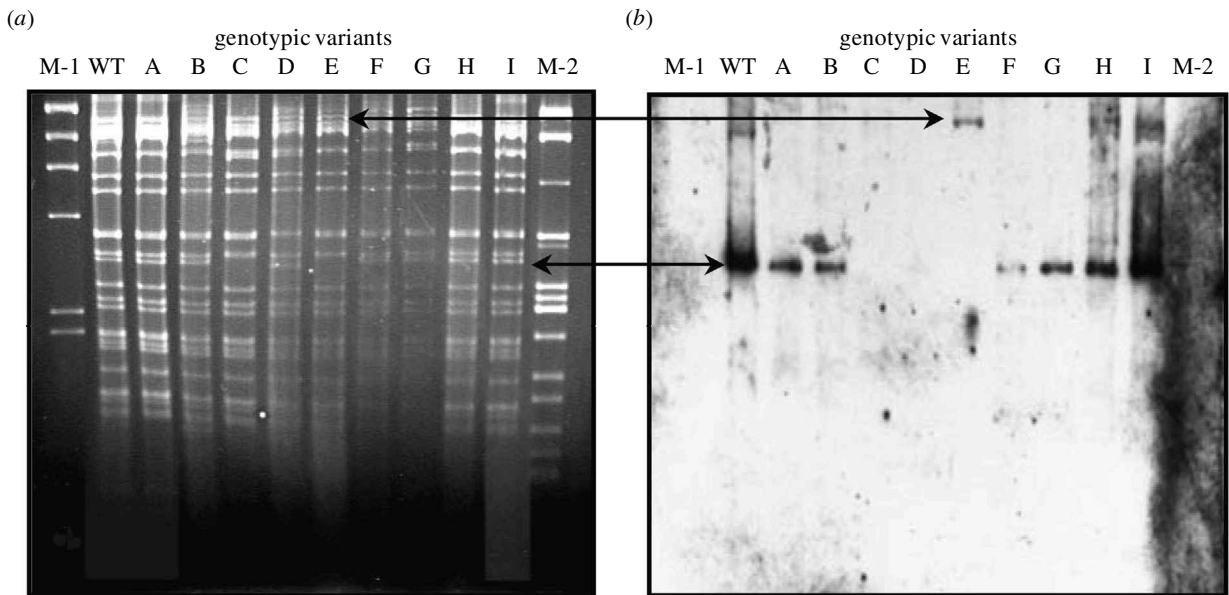


Figure 3. (a) Restriction endonuclease profiles of *EcoRI*-digested genomic DNA from wild-type (WT) and each genotypic variant (A–I). M-1, marker lane ( $\lambda$ -*HindIII*); M-2, marker lane ( $\lambda$ -*EcoRI/BamHI/HindIII*). (b) Southern blot analysis of gel shown in (a) probed with a PCR amplification fragment of the *pif* gene. Arrows indicate the *pif* probe hybridization to a Southern blot and the corresponding position of restriction endonuclease fragments visualized by ethidium bromide staining of an agarose gel. No hybridization of the *pif* probe was observed in variants C and D.

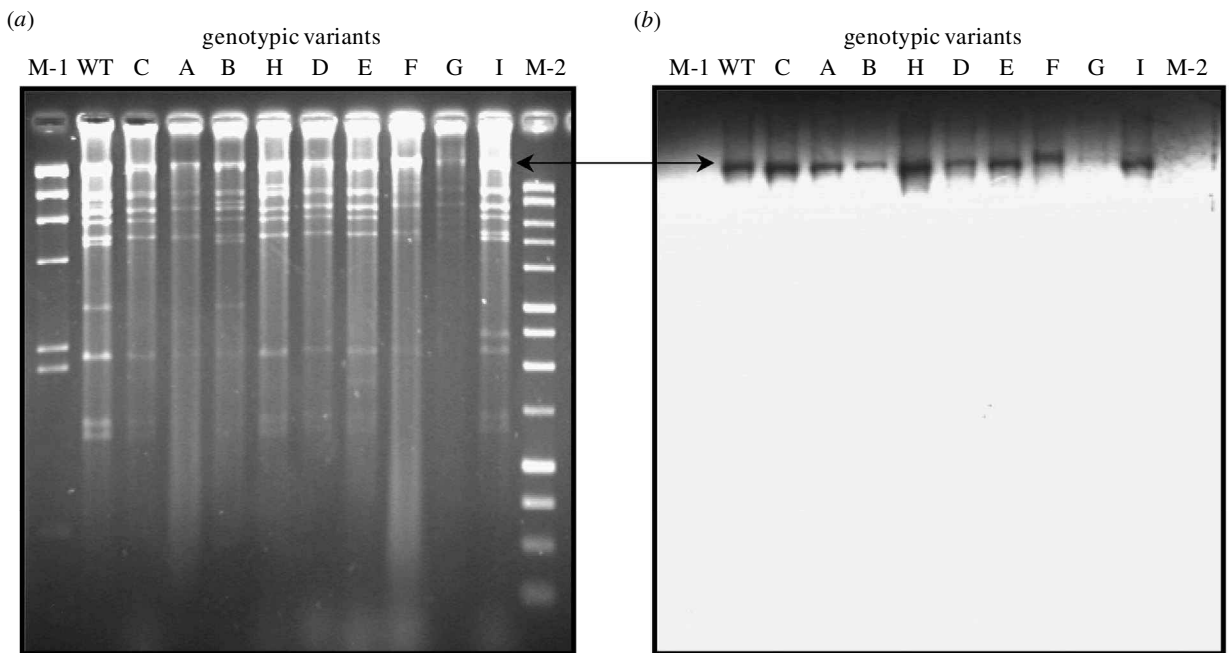


Figure 4. (a) Restriction endonuclease profiles of *PstI*-digested genomic DNA from wild-type (WT) and each genotypic variant (A–I). M-1, marker ( $\lambda$ -*HindIII*); M-2, marker (1 kb DNA ladder). (b) Southern blot analysis of gel shown in (a) probed with a PCR amplification fragment of the *p74* gene from *Spodoptera exigua* MNPV. Arrow indicates the position of the corresponding restriction fragment to which the probe hybridized on the Southern blot. The *p74* probe hybridized to all genotypic variants and the wild-type isolate.

#### (b) Insect bioassays

Bioassays were performed using a droplet feeding method (Hughes *et al.* 1986) in second instar *S. frugiperda* from the laboratory colony. Five concentrations were employed over the range  $9.6 \times 10^2$  to  $6 \times 10^5$  OBs  $\text{ml}^{-1}$  with 24 insects treated with each concentration. The bioassay was performed three times. After inoculation, larvae were reared individually on a semi-syn-

thetic diet and virus-induced mortality was scored each day. Dead larvae were smeared onto glass slides and examined microscopically for the presence of OBs. Mortality data were subjected to logit regression (Robertson & Preisler 1992). The slopes of the concentration–mortality regressions for OB mixtures did not differ significantly, indicating that the presence of OBs or co-occluded virions of variant C did not significantly

Table 1. Logit regression analysis of virus induced mortality of *Spodoptera frugiperda* larvae inoculated with: (a) wild-type SfMNPV and purified variant B OBs; (b) mixtures of variant B and variant C OBs in the proportions indicated; and (c) OBs containing mixtures of B and C variant co-occluded virions in the proportions indicated.

((a) Logit regression of number of responding insects against  $\log_e(\text{virus concentration})$  given in terms of  $\log_e$  odds ratio:  $\log_e(p/q) = a + bx$ . Regressions were fitted in GLIM (Numerical Algorithms Group, Oxford, UK) with a common slope of  $0.5908 \pm 0.0219$  (s.e.). A test for non-parallelism was not significant ( $\chi^2 = 6.36$ , d.f. = 11,  $p = 0.85$ ).  $p$ -values were calculated by  $t$ -test of the differences between regression intercepts compared with that of variant B. Treatments were subsequently assigned to four groups comprising treatments similar to variant B in pathogenicity (a, c, d, h, k, l), similar to wild-type (b, i, j), of reduced pathogenicity (e, f) and very reduced pathogenicity (g) and subjected to a *post hoc* examination with 'group' specified as a factor and  $\log_e(\text{virus concentration})$  as a covariable. Group was highly significant ( $\chi^2 = 143$ , d.f. = 3,  $p < 0.001$ ) and all group means were clearly different from one another, indicating significant differences in biological activity between groups. Relative potency was calculated as the ratio of the number of OBs of each genotype (or mixture of genotypes) required to produce the same mortality response. As such, relative potencies indicate the relative pathogenicity of each genotype (or mixture of genotypes) compared with that of variant B. All bioassays were performed in second instar *S. frugiperda* (between 68 and 73 insects/concentration) using the droplet feeding technique. (b) Mixtures of OBs were obtained by mixing different proportions of OBs from variants B and C. (c) OBs from the B and C variants were mixed in the proportions indicated, the occlusion-derived virions were extracted and injected into the haemolymph of fourth instar *S. frugiperda*. Progeny OBs containing different proportions of co-occluded B and C variants were purified from the dead larvae, and used for the bioassays. The proportions of genomic DNA were estimated by densitometric estimates from semi-quantitative PCR amplification given in figure 5.)

inoculum		intercept $\pm$ s.e.	relative potency	$p$	
<b>(a)</b>					
a	variant B	$-7.324 \pm 0.2870$	1.00	—	
b	wild-type isolate	$-6.771 \pm 0.2062$	2.55	0.0168	
<b>(b) mixtures of OBs (%)</b>					
	variant B	variant C			
c	90	10	$-7.412 \pm 0.2850$	0.86	0.656
d	75	25	$-7.482 \pm 0.2909$	0.76	0.438
e	50	50	$-7.712 \pm 0.2971$	0.52	0.091
f	25	75	$-8.045 \pm 0.3043$	0.29	0.009
g	10	90	$-8.625 \pm 0.3214$	0.11	< 0.001
<b>(c) co-occluded mixed genotypes (%)</b>					
	variant B	variant C			
h	90	10	$-7.180 \pm 0.2805$	1.28	0.463
i	75	25	$-6.694 \pm 0.2707$	2.91	0.009
j	50	50	$-6.887 \pm 0.2748$	2.10	0.046
k	25	75	$-7.079 \pm 0.2786$	1.51	0.224
l	10	90	$-7.044 \pm 0.2775$	1.61	0.170

affect the probability of infection by variant B at any of the concentrations tested. Relative potency was calculated as the ratio of equally effective concentrations (Robertson & Preisler 1992).

### (c) Production of occlusion body and co-occluded genotype mixtures

Two types of OB mixtures were prepared (figure 1*b,c*). First, purified OB suspensions were quantified by counting in an improved Neubauer haemocytometer. OBs of each genotype were then mixed in the desired proportions and used in the insect bioassay described below (figure 1*b*). Second, to obtain co-occluded genotype mixtures, in which each genotype was co-enveloped into virions and subsequently co-occluded into OBs,  $4.5 \times 10^8$  OBs  $\text{ml}^{-1}$  of each genotype were mixed and dissolved in 0.5 M of  $\text{NaCO}_3$  to release the ODVs. Five volumes of water were then added to each suspension, and 8  $\mu\text{l}$  volumes were injected into fourth instar *S. frugiperda* that were reared on a semi-synthetic diet until death. OBs were extracted from the dead larvae and purified by differential centrifugation. These OBs were used for the bioassays without any further amplification (figure 1*c*).

The relative proportions of each genotype in OB mixtures and co-occluded OBs were estimated by semi-quantitative PCR. Pri-

mers were designed to differentiate between variant B (amplicon *ca.* 750 bp) and variant C (amplicon 640 bp). Amplifications were performed using genomic DNA extracted from ODVs. Reactions were stopped at the mid-logarithmic phase of amplification (*ca.* 20 cycles), before the rate of amplification began to decrease (plateau). The relative intensities of the two products obtained were compared using the SCION IMAGE PC program.

### 3. RESULTS

A total of nine distinct genotypes were identified from the wild-type SfMNPV by their *Pst*I restriction profiles (figure 2). The complete genotype B was selected as the standard because its restriction profile corresponds to the predominant profile in the SfMNPV population. Genotypes C and D show deletions 15 kb in length with respect to genotype B.

Out of 200 plaques analysed from the wild-type isolate, deleted genotypes account for a total of 35% (70 out of 200) of the variants isolated in cell culture (figure 1*a*). The most prevalent of the deletion variants was represented by genotype C (figure 2). Virus amplification in cell culture may favour the selection of certain defective genotypes.

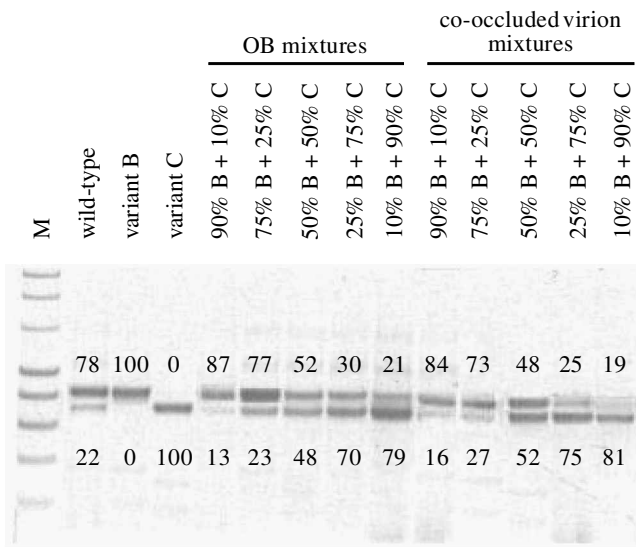


Figure 5. Semi-quantitative PCR analysis of the proportion of B and C genotypes in mixtures of OBs and OBs containing co-occluded virions of both genotypes in different proportions. Figures next to amplicons indicate the relative proportions of each product estimated by densitometric analysis.

To avoid this bias, the relative prevalence of deleted genotypes was estimated by densitometric analysis of genomic SfMNPV DNA in the wild-type isolate. The prevalence of deleted variants estimated by this method was 19.5% of the population.

Genotypes C and D were not able to infect *S. frugiperda* larvae by ingestion, whereas both variants retained the ability to replicate in cell culture or by injection into larvae. Two genes have been described that affect the peroral infectivity of NPVs: *p74* (Faulkner *et al.* 1997) and *pif* (Kikhno *et al.* 2002). Both P74 and PIF are located in the ODV envelope and are involved in cell binding and penetration during the early steps in the infection of midgut cells. Genome analysis by Southern blot clearly revealed that *p74* is present in all the variants (figure 3*a,b*), while the non-infectious variants do not contain *pif* (figure 4*a,b*). The absence of *pif* was confirmed by PCR analysis using oligonucleotides that amplify the complete open reading frame.

The pathogenicities of the wild-type isolate and the genotypic variant B were determined by bioassay in second instar *S. frugiperda*. Logit analysis revealed that the wild-type isolate had a relative potency (being the ratio of the number of OBs of each genotype, or mixture of genotypes, required to produce the same mortality response) 2.55 times that of variant B ( $\chi^2 = 9.0$ ,  $p = 0.003$ ; table 1*a*). We hypothesized that this effect could be due to a significant contribution to the pathogenicity of the population by uncommon genotypes present in the wild-type population, including deletion mutants.

Mixing of OBs from infective and deletion variants did not permit peroral infection by the deletion genotype (figure 1*b*); insects died exclusively from variant B, and no C variant profile was detectable. This observation was in agreement with a previous study on a *pif*-deficient NPV genotype from *Spodoptera littoralis* (Kikhno *et al.* 2002). The pathogenicity of OB mixtures corresponded to the

proportion of B variant OBs present in the inoculum (table 1*b*).

Mixed genotype virions were obtained by injecting *S. frugiperda* larvae with mixtures of B and C variant ODVs in various proportions. The genotypes replicate independently and are co-wrapped into virions that are subsequently occluded into OBs (figure 1*c*). Densitometric analysis of discriminant DNA restriction fragments obtained from progeny OBs confirmed that both genotypes were present at close to the proportions in which they were inoculated (figure 5).

Bioassay of the mixed genotype OBs revealed that the presence of the deletion variant (C) at a proportion of 25 or 50% restored the pathogenicity of the B + C variant mixture to that of the wild-type population (table 1*c*). The reduced activity of OBs produced in insects inoculated with high proportions of variant C genotype is presumably a consequence of the prevalence of OBs containing only defective (C) genotypes and lacking the PIF protein in the virion envelope. Non-infectious OBs are produced in cells infected only with variant C budded virus, the probability of which is directly proportional to the prevalence of C genotypes in the inoculum.

#### 4. DISCUSSION

The complete (B) genotype appears to act as a helper that facilitates transmission of the deletion (C) genotype. By increasing the pathogenicity of genotypic mixtures, deletion mutants are likely to have an important positive influence on the probability of transmission of complete genotypes present in the virus population. When mixtures of ODVs were injected into hosts, the progeny OBs contained approximately the same proportion of genotypes as the original mixture (figure 5), indicating that each genotype had a similar rate of replication on a single passage. This therefore represents a mutualistic phenomenon, although the mechanism of this interaction cannot be inferred from the available data. However, pathogenicity gains may be partly offset by reductions in the total number of progeny of each genotype, or related components of baculovirus fitness, which have yet to be determined.

The wrapping of multiple genomes as a single virion is unique to NPVs and has been suggested as a mechanism to overcome the sloughing of midgut cells, which can occur at a rate faster than that of virus replication (Washburn *et al.* 1999). The multiple encapsidation strategy, however, allows defective genomes to be transmitted between hosts. Previous work on *Spodoptera exigua* MNPV revealed that some genotypes act as parasites, resulting in a reduction in the pathogenicity of the wild-type population (Muñoz *et al.* 1998), but our results show that, in certain situations, cooperation between deletion mutant and complete genotypes may be of mutual benefit.

The persistence of deletion mutants in the baculovirus population is possible due to co-infection of host cells by multiple genotypes (Bull *et al.* 2001) during within-host cell-to-cell transmission of the budded virus form, a phenomenon that permits selection for cheats (defector genotypes) that sequester the products of complete genotypes (Turner & Chao 1999). The prevalence of defectors in the virus population will clearly be subject to frequency-dependent selection (Godfray *et al.* 1997) because of

reduced viral fitness when defective genotypes are rare or extremely common. The results of the experiments with co-occluded virion mixtures support this; mixtures containing an intermediate prevalence of deletion genotypes (25 or 50%) were more pathogenic, and therefore more likely to achieve transmission, than mixtures containing greater or lesser proportions. This is because there is a positive relationship between the degree of pathogenicity of the virus variant and the probability that it will achieve infection when ingested by a susceptible host; clearly a key component of pathogen fitness (Anderson & May 1983; Van Baalen 2002). This represents a variation on the Prisoner's Dilemma (Turner & Chao 1999), a classic of game theory, in which the decision whether to cooperate by expressing the full range of gene products, or defect, by sequestering essential products of others depends on the frequency of encounters with defectors.

However, this approach has been criticized because, in reality, viral genotypes do not cooperate or defect in a discrete manner, but are subject to a variable spectrum of opportunities to cheat or cooperate with co-infecting genotypes of varying degrees of relatedness (Brown 2001). Brown *et al.* (2002) have argued that such interactions should be considered as collective action, in which the fitness of each genotype is modulated by the availability of shared resources within each infected cell. In this case, the defective variants may exploit PIF production by complete genotypes but may also contribute positively to the group by producing shared resources such as polymerases and structural proteins. This intriguing possibility merits further empirical and theoretical attention.

Our results also represent one of the few examples of the infectiousness of mixed genotype infections being greater than that of single genotype infections, as observed in *Plasmodium chabaudi* and its mosquito host (Taylor *et al.* 1997a). Our system has additional parallels with the malarial parasite model in that we expect the transmissibility of mixed genotype OBs to be greater than that of any of their single genotype components, a phenomenon also seen in *P. chabaudi* mixed genotype infections (Taylor *et al.* 1997b). However, this has yet to be tested explicitly in our SfMNPV system.

In practical terms, our findings indicate that pure genotypes are unlikely to be valid models for studies of baculovirus pathogenicity and host range (Possee & Rohrmann 1997). Pure genotypes employed in the testing of genetically modified baculoviruses (Cory *et al.* 1994), or virus preparations with low genetic diversity resulting from *in vitro* production (Lua *et al.* 2002), will also be less effective biopesticides (Moscardi 1999) than genotypically heterogeneous mixtures.

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