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Gabriel Clavijo, Trevor Williams, Oihane Simón, Delia Muñoz, Martine Cerutti, et al.. Mixtures of Complete and pif1- and pif2-Deficient Genotypes Are Required for Increased Potency of an Insect Nucleopolyhedrovirus. *Journal of Virology*, 2009, 83 (10), pp.5127-5136. 10.1128/jvi.02020-08. hal-04171716

HAL Id: hal-04171716

<https://imt-mines-ales.hal.science/hal-04171716>

Submitted on 27 Jul 2023

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Mixtures of Complete and *pif1*- and *pif2*-Deficient Genotypes Are Required for Increased Potency of an Insect Nucleopolyhedrovirus[▽]

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The insecticidal potency of a nucleopolyhedrovirus population (SfNIC) that infects *Spodoptera frugiperda* (Lepidoptera) is greater than the potency of any of the component genotypes alone. Occlusion bodies (OBs) produced in mixed infections comprising the complete genotype and a deletion genotype are as pathogenic as the natural population of genotypes from the field. To test whether this increased potency was due to the deletion or to some other characteristic of the deletion variant genome, we used the SfNIC-B genome to construct a recombinant virus (SfNIC-B Δ 16K) with the same 16.4-kb deletion as that observed in SfNIC-C and another recombinant (SfNIC-B Δ *pifs*) with a deletion encompassing two adjacent genes (*pif1* and *pif2*) that are essential for transmission per os. Mixtures comprising SfNIC-B and SfNIC-B Δ 16K in OB ratios that varied between 10:90 and 90:10 were injected into insects, and the progeny OBs were fed to larvae in an insecticidal potency assay. A densitometric analysis of PCR products indicated that SfNIC-B was generally more abundant than expected in mixtures based on the proportions of OBs used to produce the inocula. Mixtures derived from OB ratios of 10, 25, or 50% of SfNIC-B Δ 16K and the corresponding SfNIC-B proportions showed a significant increase in potency compared to SfNIC-B alone. The results of potency assays with mixtures comprising various proportions of SfNIC-B plus SfNIC-B Δ *pifs* were almost identical to the results observed with SfNIC-B Δ 16K, indicating that deletion of the *pif* gene region was responsible for the increased potency observed in mixtures of SfNIC-B and each deletion recombinant virus. Subsequently, mixtures produced from OB ratios involving 10 or 90% of SfNIC-B Δ 16K with the corresponding proportions of SfNIC-B were subjected to four rounds of per os transmission in larvae. The composition of each experimental mixture rapidly converged to a common equilibrium with a genotypic composition of ~85% SfNIC-B plus ~15% SfNIC-B Δ 16K. Nearly identical results were observed in peroral-passage experiments involving mixtures of SfNIC-B plus SfNIC-B Δ *pifs*. We conclude that (i) the deletion of the *pif1* and *pif2* region is necessary and sufficient to explain the increased potency observed in mixtures of complete and deletion genotypes and (ii) viral populations with decreased ratios of *pif1*- and *pif2*-deficient genotypes in the virus population increase the potency of genotypic mixtures and are likely to positively influence the transmission of this pathogen.

When hosts are infected by multiple genotypes of a pathogen, competition between genotypes of low relatedness may favor rapid exploitation of host resources, resulting in an increase in the virulence of the infection, reflected in the degree of damage inflicted on the host (12). Because individual self-interest prevails under such conditions, a rapidly replicating genotype will quickly use up host resources for the production of progeny particles, thereby penalizing a more prudent coinfecting genotype, an interaction known as the “tragedy of the commons” (33, 44, 49). In contrast, when relatedness between genotypes is high, cooperative exploitation of host resources is favored because the rate of exploitation of host resources is often determined by the production of intracellular products

by the infecting group, an interaction known as “collective action” (2). Each of these models entail different temptations to cheat or defect from the common goal, by excessive greed in the acquisition of public goods (intracellular products) in the case of the tragedy model, and by overly frugal contribution to the pool of public goods in the case of the collective action model (3). In the case of viruses, such game theory approaches to social dilemmas have provided unique insights into the role of cooperation and defection in the evolution of virulence (2, 11, 24, 44), pathogenesis (45), disease management, and the development of potential therapeutic agents (7, 15, 26).

Coinfection by multiple genotypes is a common characteristic of many host-parasite systems, especially insect viruses (5, 6, 14, 19–21). When multiple virus particles infect individual host cells, deletion mutants can arise that have lost genes that are essential for transmission or replication (35). These defective particles survive by sequestering the gene products of complete genotypes in coinfecting cells. Recently, we demonstrated that deletion genotypes were prevalent in a genotypic

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cally diverse population of *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) originally isolated in Nicaragua (SfNIC). The most abundant deletion genotype, named SfNIC-C, comprised a 16.4-kb deletion compared to the complete genotype, named SfNIC-B (40). This deletion included two genes, *pif1* and *pif2*, that encode peroral infection factors that are essential for per os infection (9, 25, 32, 36), which is the usual route of transmission of these viruses. SfNIC-C survives by complementation with PIF-encoding genotypes in the population, a process that requires that complete and deletion genotypes replicate simultaneously in the same cells. The persistence of deletion genotypes at a high frequency in the population signifies that multiple infection of cells is likely to be a common event. However, *pif1*- and *pif2*-defective genotypes can replicate autonomously in cell culture or in larvae, if injected. Remarkably, the progeny viruses from insects that had been simultaneously inoculated with a mixture of complete and deletion genotypes, in a ratio similar to that found in nature (~3:1), were ~2.5 times more pathogenic, as indicated by concentration-mortality metrics, than the complete genotype alone (28, 38, 39). Whether the increased pathogenicity of mixed genotype inocula was specifically due to the deletion has remained unclear and identifying the gene(s) involved represents a key to our understanding of baculovirus infection strategies.

The structure of NPV is complex (46). The multiple NPVs consist of single genomes of double-stranded DNA inside individual nucleocapsids. Groups of approximately one to seven nucleocapsids are then wrapped by an envelope to form occlusion-derived virions (ODVs) that are in turn occluded by a protein matrix to form occlusion bodies (OBs) with approximately 20 to 80 virions within each OB. When consumed by a susceptible insect larva, the OBs dissolve in the alkaline insect midgut releasing numerous virions. The ODV membrane fuses with the membranes of midgut epithelial cells, a process that requires the presence of PIFs in the virion membrane (25, 31). After membrane fusion, the package of nucleocapsids is delivered into the host cell and nucleocapsids migrate to the nucleus, where they uncoat and commence replication. Initially, progeny nucleocapsids migrate from the nucleus and bud from the cell as individual virions, each containing a single genome that spread within the host to initiate secondary infections. It is estimated that each cell of the insect is infected by ~4 budded virions (4, 38). Later, nucleocapsids are retained in the nucleus, wrapped into ODVs and occluded into OBs. After death, the tegument of the insect breaks down and OBs are released onto plant surfaces for transmission to other susceptible insects. It is clear that both the physical structure and the replication cycle of these viruses foster a high prevalence of infection by multiple genotypes.

In the present study we examine the genetic basis for increased pathogenicity and test the hypothesis that coinfection of cells by complete and deleted genotypes results in OBs with increased potency compared to those of the complete genotype alone. Here, we use the term potency to mean the quantity of OBs required to produce a certain prevalence of host mortality, such as the 50% lethal concentration, compared to a standard reference, which in this case are OBs of the complete genotype B. As such, potency is a comparative measure of virus insecticidal activity. We demonstrate that the deletion of the *pif1* and *pif2* region is necessary and sufficient to explain this

increased potency. Finally, we examine the dynamics of mixed infection in serial passage in the natural host and demonstrate that previous findings, in which the ratios of genotypes in experimental mixtures converged to a common equilibrium, were determined by the influence that *pif1*- and *pif2*-deficient genotypes exert on OB potency, thereby indirectly influencing the probability of virus transmission.

MATERIALS AND METHODS

Insects, virus, and cells. *S. frugiperda* larvae were obtained from a laboratory colony maintained under constant conditions ($25 \pm 0.5^\circ\text{C}$, $75\% \pm 5\%$ relative humidity, 16-h/8-h light-dark photoperiod) on a semisynthetic diet (16). The Sf-9 ATCC cell line was cultured at 28°C in TC-100 supplemented with 5% fetal bovine serum (Gibco). OBs of a Nicaraguan isolate (SfNIC) of SfMNPV were amplified in fourth-instar *S. frugiperda* (8). The complete genotype, SfNIC-B, was obtained from plaque-purified material described by Simón et al. (40). SfNIC-B OBs were solubilized with thioglycolate buffer (0.25 M thioglycolic acid, 0.25 M sodium carbonate [pH 10.5]). The released occlusion-derived virions (ODVs) were purified by centrifugation, treated with proteinase K and sarcosyl, and then treated with RNase for 1 h (8). Viral DNA was purified by phenol-chloroform extraction and alcohol precipitation and then subjected to PstI digestion and electrophoresis in 1% Tris-acetate-EDTA agarose gels.

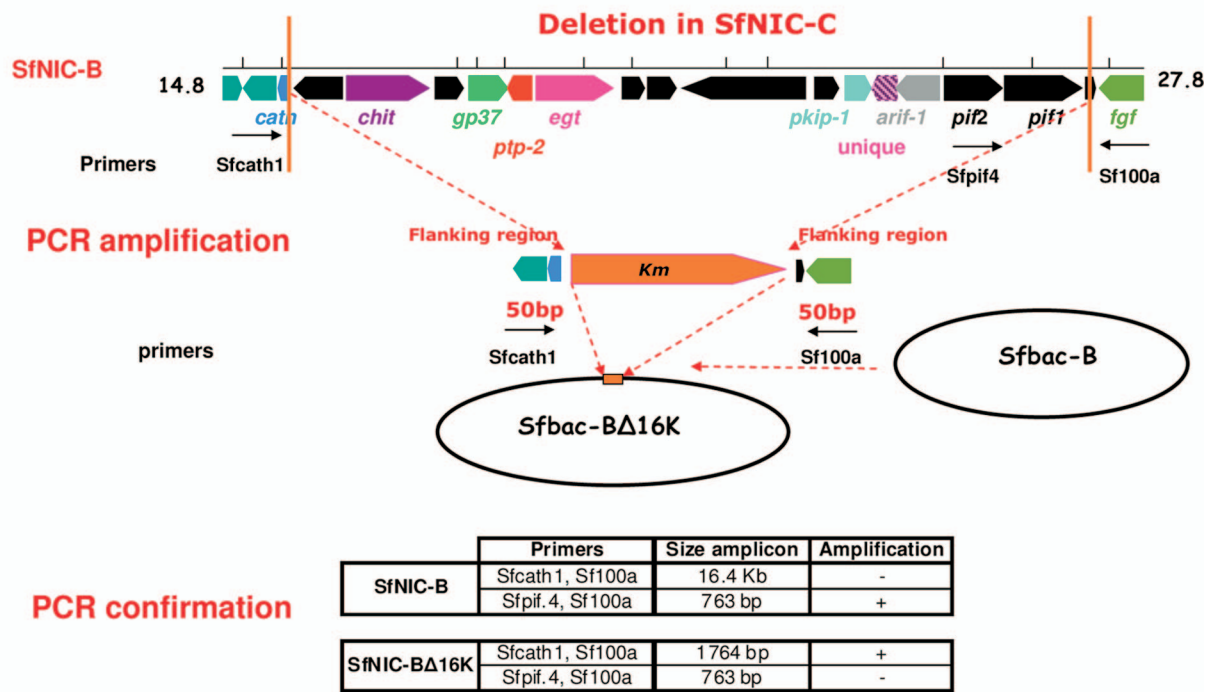
Generation of the 16.4-kb knockout bacmid and virus (SfNIC-BA16K). A bacmid pBACe3.6Sf-B was constructed by using the bacmid pBACe3.6 containing a chloramphenicol resistance gene and the SfNIC-B genome. A 16.4-kb knockout, corresponding to the deleted region of the defective variant SfNIC-C (40), was generated through homologous recombination using the Red/ET recombination system (Gene Bridges GmbH, Dresden, Germany) in *Escherichia coli* (Fig. 1A). First, a 865-bp fragment that contains 25-bp homology arms of the deletion in SfNIC-C and a kanamycin resistance cassette (*Km*) was PCR amplified from SfNIC-B and kanamycin PCR fragments using the primers B-fw1 and B-rv1 (Table 1). Next, a 915-bp fragment with 50-bp homology arms for the deletion, and the *Km* cassette was amplified from the previously amplified 865-bp PCR fragment using primers B-fw2 and B-rv2 (Table 1).

To induce recombination between the 915-bp homology arms/*Km* fragment and the bacmid target sequence, the pBACe36Sf-B was transformed (electroporated) into *E. coli* DH5 α cells, and immediately afterward the same cells were transformed with the 915-bp PCR product and the helper plasmid pSC101-BAD-gbaA (30, 37). Recombination was induced by the addition of L-arabinose to allow expression of the Red/ET system, as described by the supplier (Gene Bridges). Transformed cells were incubated at 37°C for 70 min in LB medium and subsequently spread onto agar plates containing chloramphenicol (15 $\mu\text{g}/\text{ml}$) and kanamycin (15 $\mu\text{g}/\text{ml}$). Plates were incubated overnight at 37°C , and colonies resistant to both antibiotics were selected. The resulting recombinant bacmid was purified with a Midiprep-Nucleobond large construct extraction kit (Qiagen GmbH, Hilden, Germany) and named Sfbac-B16 Δ K. Finally, OBs were obtained by injecting *S. frugiperda* fourth-instar larvae with the Sfbac-BA16K bacmid using Lipofectin and reared individually on semisynthetic diet until death. The resulting OBs were then purified, and the bacmid identity was verified by restriction endonuclease analysis with PstI.

Generation of (*pif1*+*pif2*) deletion plasmid and virus (SfNIC-B Δ *pifs*). In order to construct a SfMNPV virus with a deletion comprising only genes *pif1* and *pif2*, the *pif2*-left and *pif1*-right flanking sequences were separately cloned into plasmids (Fig. 1B). A 1,287-bp fragment containing the left flanking sequence of *pif2* (nucleotides 13701 to 14957) was PCR amplified from SfNIC-B with primers B-pif2fw and B-pif2rv (Table 1). This PCR product and the pSP70 vector were digested with the enzymes *Ava*I and *Hind*III and ligated to generate recombinant plasmid pSP317. A 1,358-bp fragment containing the right flanking region of *pif1* (nucleotides 17762 to 19090) was also PCR amplified from SfNIC-B with primers B-pif1fw and B-pif1rv. Likewise, the PCR product and a pUC19 vector were digested with *Ava*I and *Hind*III and ligated to generate recombinant plasmid pUC318. Then, the 1,358-bp fragment inserted in pUC318 was extracted by digestion with *Hind*III and *Bgl*II and ligated into pSP317, which had been previously digested with the same enzymes, to generate plasmid pSP319. A *lacZ* cassette containing the polyhedrin promoter was extracted from plasmid pCH110 by digestion with *Bam*HI and inserted into *Bam*HI-digested pSP319 to generate the pSP70-B*pifs*-null recombinant plasmid, named pSP70-B Δ *pifs*.

Sf-9 cells were transfected with SfNIC-B DNA and pSP70-B Δ *pifs*. For selection of positive recombinant viruses, X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) was added to the medium, and blue plaques were extracted.

A



B

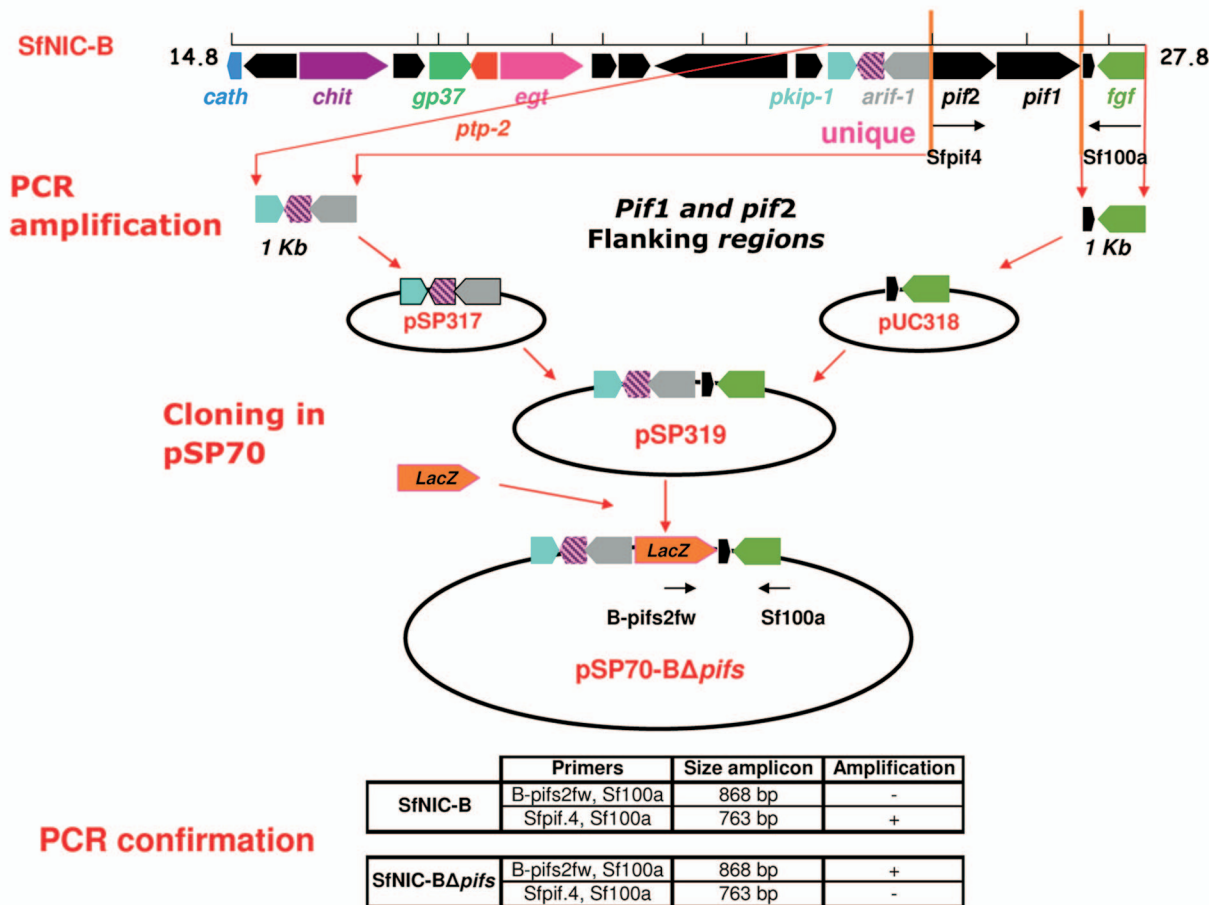


FIG. 1. Strategy for construction of recombinant viruses from complete genotype SfNIC-B. (A and B) Construction of SfNIC-BΔ16K that presented the 16.4-kb deletion present in genotype SfNIC-C (A) and SfNIC-BΔpifs that was deleted in two peroral infection factor genes, *pif1* and *pif2* (B).

TABLE 1. PCR primers used for the generation of recombinant bacmids and plasmids

Primer ^a	Sequence (5'-3') ^b	Restriction site(s)
B-fw1	AGTAACAGCAAAAAGTATGAGTAATTCCCGGGTGGACAGCAAGCGAACCGGATTGC	
B-rv1	TAAAAAATGTATGGCCAAATTGTAACCCGGGTCAGAAGAAGCTCGTCAAGAAGGCC	
B-fw2	ATCTTGGCGCGTCAACGCCCGCTTTAGTAACAGCAAAAAGTATGAGTAATTC	
B-rv2	GCAATCTTTTGGCGTTAAGCAACACTAAAAAATGTATGGCCAAATTGTAA	
B-pif2fw*	AATTGGCTCGAGTGCACACTACTACAGAATCCG	AvaI
B-pif2rv	CGCTCGAAGCTTGGATCCCACCGTCGGTATCGTGTTACATCTC	HindIII, BamHI
B-pif1fw	ACATATAAGCTTGGATCCGAAAACATGGACAATGTCACCGAAG	HindIII, BamHI
B-pif1rv	AAGTTCAGATCTACGGCAAGAGTAATGATCTGTGGTG	BglII
Sfpif.4*	GTGTTCAAGTGGGCACGGCG	
Sfcath1*	GCAGAATCGTTACGACTA	
Sf100a*	GTCTACTTGGATTTCGTCGAAGGTGA	

^a *, Primers used in semiquantitative PCR to estimate the relative proportions of each genotype in genotypic mixtures used as inocula and in OBs sampled at each step of the serial passage experiment. Primers B-pif2fw and Sfcath1 are forward primers of SfNIC-BΔ16K or SfNIC-BΔpifs, respectively. Primer Sfpif.4 is a forward primer of SfNIC-B genotype, and Sf100a is a reverse primer for both recombinant viruses and SfNIC-B genotype.

^b The locations of restriction sites are underlined.

Confirmation of the SfNIC-BΔpifs recombinant was achieved by restriction endonuclease analysis (PstI) and Southern blot hybridization analysis. The recombinant virus was subsequently amplified in fourth-instar SfMNPV larvae by intrahemocoel injection, as PIF-deficient viruses are unable to infect larvae per os. ODVs were obtained by dissolving purified OBs of the recombinant virus. Larvae were injected with 8 μl of ODV suspension obtained from a sample of 10⁹ OBs/ml.

Pathogenicity of single and mixed genotype OBs. Purified OB suspensions containing 5×10^8 OBs/ml of the SfNIC-B isolate and the SfNIC-BΔ16K and SfNIC-BΔpifs recombinant viruses were mixed in the desired proportions, comprising 10, 25, 50, 75, or 90% SfNIC-B and the corresponding percentage of recombinant OBs, as previously described (28). OB mixtures were solubilized and 8-μl volumes of the released ODVs were injected into *S. frugiperda* fourth instars that were incubated individually on semisynthetic diet until death. The proportions of each genotype in the resulting OBs were determined by semiquantitative PCR. Primers were designed to differentiate between genotype SfNIC-B (amplicon of ~750 bp) and the recombinant genotypes SfNIC-BΔ16K (amplicon of 1,076 bp) and SfNIC-BΔpifs (amplicon of 868 bp) (B-pif2fw, Sfpif.4, and Sf100a; Table 1). Reactions were stopped at the mid-logarithmic phase of amplification (19 cycles), since a series of amplifications involving different numbers of cycles indicated that this was the mid-logarithmic phase for the amplification of this product, as described in previous studies (28, 40, 41). The intensities of PCR products were determined relative to the SfNIC-B internal standard by using the ChemiDoc densitometric analysis software (Bio-Rad, Hercules, CA).

To determine the precision of the relationship between densitometric values and the relative quantities of each amplification product present, the densitometric readings were calibrated. For this, amplified products from six reactions, for each virus separately, were purified by using a PCR purification kit (Roche), and the DNA content was estimated by determining the absorbance (A_{260}). Reaction products were sequentially diluted between 2- and 64-fold and electrophoresed, and densitometric readings were obtained. Regressions of the numbers of copies of each product estimated from A_{260} readings and the densitometric values were performed in Excel (Microsoft Corp., Redmond, WA). In all cases the densitometric values were closely correlated with the estimated number of copies: the coefficients of determination (R^2), representing the proportion of variability in each data set that is accounted for by the regression model, were as follows: $R^2 = 0.91$ for genotype SfNIC-B, $R^2 = 0.60$ for SfNIC-BΔ16K, and $R^2 = 0.87$ for SfNIC-BΔpifs. Regression equations were then used to estimate the relative proportions of each product present in PCR amplifications of experimental mixtures of genotypes based on their corresponding densitometric readings (data not shown).

To determine the pathogenicity of single and mixed genotype OBs, bioassays were performed by using the droplet feeding method (23). Batches of 25 newly molted second-instar *S. frugiperda* from the laboratory colony were inoculated with suspensions containing 10% (wt/vol) sucrose, 0.001% (wt/vol) fluorella blue and OBs at concentrations of 9.6×10^2 , 6.0×10^3 , 6.0×10^4 , and 6.0×10^5 OBs/ml. Control insects were treated identically with solutions that did not contain OBs. The bioassay was performed three times. After inoculation, larvae were reared individually on a semisynthetic diet, and the virus-induced mortality was recorded daily. Mortality data were subjected to logit regression (34). Potency values were calculated as the ratio of equally effective concentrations of

OBs originating from genotype B alone compared to OBs originating from experimental genotype mixtures (34).

Dynamics of mixed genotype populations in serial passage. Purified OBs from single genotypes were mixed in the following ratios: (i) 90% SfNIC-B plus 10% SfNIC-BΔ16K, (ii) 10% SfNIC-B plus 90% SfNIC-BΔ16K, (iii) 90% SfNIC-B plus 10% SfNIC-BΔpifs, and (iv) 10% SfNIC-B plus 90% SfNIC-BΔpifs. OBs from these populations were solubilized, and the released ODVs were injected into larvae. The OB progeny resulting from these injected larvae was considered passage zero (P0). OBs were serially passaged four times by feeding them to groups of 25 *S. frugiperda* fourth instars at a fixed concentration (10^8 OBs/ml) that was estimated to result in ~90% mortality, depending on the composition of the OB population. Inoculated larvae were maintained individually on semisynthetic diet until death. OBs were collected, pooled, and used as inocula for the following passage. Samples of OBs from each passage were retained for semiquantitative PCR analysis. PCRs were stopped at 21 cycles (which was the mid-logarithmic phase of amplification for this product as determined by a series of previous amplifications involving different numbers of cycles). Densitometric measurements of electrophoresed products were corrected using the regression equations described in the above to calculate the proportion of each virus present relative to that of SfNIC-B.

RESULTS

Production of SfNIC-BΔ16K and SfNIC-BΔpifs. The correct construction of the 16.4-kb knockout SfNIC-BΔ16K recombinant virus (described schematically in Fig. 1A) was confirmed by PCR and restriction endonuclease analysis. A recombinant virus, SfNIC-BΔpifs, lacking the *pif1* and *pif2* genes of SfNIC-B was constructed using a system of four different plasmids (Fig. 1B). The correct insertion of the *lacZ* cassette and the knockout of the *pif* genes were confirmed by PCR amplification and restriction endonuclease analysis using PstI (data not shown). Primer pairs flanking the predicted recombination sites resulted in PCR amplification of the predicted fragment of ~700 bp. The insertion/knockout was additionally confirmed by sequencing the PCR amplicon (data not shown). Both recombinant viruses were successfully amplified in *S. frugiperda* larvae, and the resulting OBs were used in the following experiments. Calibration-corrected densitometric analyses of the products of semiquantitative PCR indicated that the ratios of genotypes in OBs harvested from infected insects generally contained a greater than expected abundance of genotype B compared to the ratios of OBs used as inoculum, suggesting that OBs of SfNIC-B likely contained a greater number of nucleocapsids than the OBs of either recombinant. This pattern was observed for all mixtures of SfNIC-B plus SfNIC-BΔ16K (Fig. 2A) and

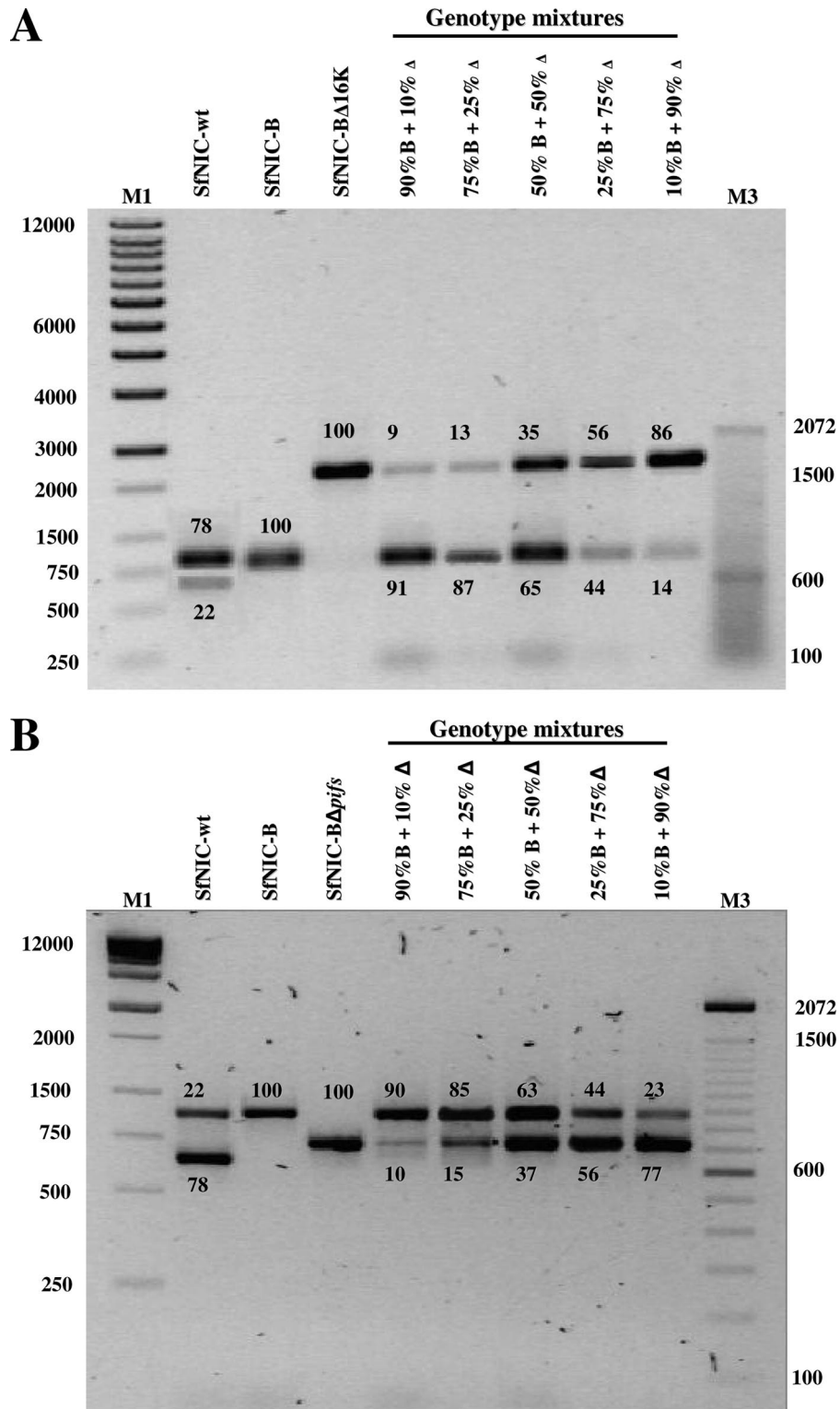


FIG. 2. Semiquantitative PCR of genotypic mixtures produced in insects. Larvae of *S. frugiperda* were coinfectd with OBs comprising different proportions of complete genotype SfNIC-B plus SfNIC-BΔ16K (A) or SfNIC-B plus SfNIC-BΔpifs (B). PCRs were stopped prior to saturation and electrophoresed, and densitometric analysis was performed using the intensity of the amplicon generated from SfNIC-B as the reference. Relative density values are shown above or below the corresponding amplicon. Molecular markers: M1, 1-kb DNA ladder (Stratagene, La Jolla, CA); M3, 100-bp DNA ladder (Invitrogen Corp., Carlsbad, CA) in panel B. In both cases, the minority product present after amplification of the Sf-NIC wild type is a result of deletion genotypes C and D present in the natural population.

TABLE 2. Logit analysis of concentration-mortality bioassays in *S. frugiperda* second instars inoculated with OBs comprising mixtures of SfNIC-B plus SfNIC-BΔ16K or mixtures of SfNIC-B plus SfNIC-BΔpifs in various proportions^a

Inoculum ^b	Mean ± SE		Potency
	Slope	Intercept	
SfNIC-B + SfNIC-BΔ16K			
SfNIC-B		-7.364 ± 0.550	1.00a
SfNIC-WT		-7.092 ± 0.528	2.67b
Genotype mixtures (SfNIC-B/SfNIC- BΔ16K [%])			
90:10		-7.081 ± 0.539	1.48b
75:25		-6.729 ± 0.505	2.40b
50:50		-7.109 ± 0.528	1.42b
25:75		-7.670 ± 0.596	0.66a
10:90		-8.520 ± 0.628	0.22c
SfNIC-B + SfNIC-BΔpifs			
SfNIC-B	1.470 ± 0.152	-7.866 ± 0.772	1.00a
SfNIC-WT	1.690 ± 0.179	-7.171 ± 0.706	2.67b
Genotype mixtures (SfNIC-B/SfNIC- BΔpifs [%])			
90:10	1.458 ± 0.167	-6.349 ± 0.312	1.39b
75:25	1.933 ± 0.218	-5.977 ± 0.303	2.32b
50:50	1.902 ± 0.203	-6.357 ± 0.312	1.36b
25:75	1.463 ± 0.165	-7.151 ± 0.334	0.41a
10:90	1.470 ± 0.152	-8.007 ± 0.359	0.08c

^a OBs of each genotype were mixed in different proportions. ODVs were released by alkaline treatment and injected into insects that subsequently died of infection to produce the initial OB inoculum that was subjected to per os bioassay to determine insecticidal potency. The proportions of genotypes present in each mixture were estimated by PCR and densitometric analyses (Fig. 2A and B).

^b Regressions for SfNIC-B plus SfNIC-BΔ16K (the top half of the table) were fitted with a common slope of 1.670 ± 0.105 (Pearson's $\chi^2 = 29.1$, $df = 19$, $P = 0.064$). A test for nonparallelism was not significant ($\chi^2 = 11.4$, $df = 6$, $P = 0.077$). Potency values followed by identical lowercase letters did not differ significantly for comparisons among treatments for this section of the table ($P > 0.05$). Regressions for SfNIC-B plus SfNIC-BΔpifs (the bottom half of the table) could not be fitted in parallel; potency values therefore reflect the ratio of LC_{50} s (30) (Pearson's $\chi^2 = 21.922$, $df = 19$, $P = 0.288$). Potency values followed by identical lowercase letters did not differ significantly for comparisons among treatments for this section of the table ($P > 0.05$).

mixtures of SfNIC-B plus SfNIC-BΔpifs (Fig. 2B), with the exception of mixtures of 10% of either recombinant virus with 90% SfNIC-B, which accurately reflected the ratio of OBs used in the inoculum. However, for clarity, we refer to all mixtures used in the following experiments in terms of the ratios of OBs used to generate the primary inocula.

Pathogenicity of single- and mixed-genotype OBs. The pathogenicities of OBs obtained after injection of mixtures of SfNIC-B and SfNIC-BΔ16K or SfNIC-BΔpifs in different ratios of OBs (90:10, 75:25, 50:50, 25:75, and 10:90) were compared to those of SfNIC-B alone or the wild-type SfNIC isolate (Table 2). The wild-type isolate was 2.67-fold more potent than SfNIC-B alone, i.e., the concentration of OBs required to produce a specific prevalence of host mortality was 2.67-fold greater for OBs of genotype B alone compared to OBs of the wild-type isolate. The potencies of OBs produced in larvae coinfecting by mixtures of SfNIC-B and between 10 and 50% of SfNIC-BΔ16K were significantly increased compared to the potency of OBs of SfNIC-B alone, with the potency of the mixture involving 75% of SfNIC-B and 25% of SfNIC-BΔ16K

(potency of 2.40) being most similar to that of the wild-type isolate (Table 2). Mixtures comprising higher proportions of the deletion recombinant, particularly the mixture involving 90% SfNIC-BΔ16K, resulted in significantly reduced potency compared to the wild-type or SfNIC-B alone.

The results of potency assays involving different ratios of SfNIC-B plus SfNIC-BΔpifs were almost identical to those involving SfNIC-BΔ16K (Table 2). The wild-type SfNIC was again 2.67-fold more potent than SfNIC-B alone. The potencies of OBs from the treatments involving 10, 25, or 50% of SfNIC-BΔpifs were significantly greater than OBs comprising SfNIC-B alone, with the potency of OBs produced in insects infected by the mixture comprising 25% SfNIC-BΔpifs plus 75% SfNIC-B (potency 2.32) being most similar to that of OBs of the wild-type isolate. Once again, the mixture involving the highest proportion (90%) of the deletion recombinant resulted in significantly reduced potency compared to the wild-type or SfNIC-B alone.

Dynamics of mixed genotype populations in serial passage. OBs obtained from larvae injected with the four experimental populations (SfNIC-B plus SfNIC-BΔ16K or SfNIC-B plus SfNIC-BΔpifs, each at ratios of 90:10 and 10:90) contained a proportion of each genotype similar to that of the injected inocula (Fig. 3A to D), although SfNIC-B was slightly more abundant than expected in the mixture that was generated from an OB ratio comprising 10% SfNIC-B plus 90% SfNIC-BΔpifs (Fig. 3D).

These OBs were considered “passage zero” (P0). After the course of four successive passages of peroral transmission in larvae, the genotypic frequencies in all four experimental populations converged to a common ratio comprising ~85% of SfNIC-B and ~15% of SfNIC-BΔ16K (Fig. 3A and B) or SfNIC-BΔpifs (Fig. 3C and D), independent of their initial proportions in the mixtures. As expected, for both types of virus mixtures, the most dramatic changes in genotype composition were observed in mixtures that initially comprised ~90% (Fig. 3B and D) of the deleted genotypes (SfNIC-BΔ16K or SfNIC-BΔpifs), reflecting the high prevalence of noninfectious OBs present in inoculum produced under such conditions.

Bioassays performed on OBs harvested from infected insects that died at the fourth passage indicated that the potency values of the mixtures with starting ratios of 90% SfNIC-B plus 10% SfNIC-BΔ16K or 90% SfNIC-B plus 10% SfNIC-BΔpifs were both similar to that of the wild-type population, SfNIC-WT (Table 3). Similarly, the potency values of mixtures with starting ratios of 10% SfNIC-B plus 90% SfNIC-BΔ16K or 10% SfNIC-B plus 90% SfNIC-BΔpifs were similar to that of the SfNIC-WT population but were also statistically similar to that of OBs of SfNIC-B alone due to higher levels of overdispersion in the bioassay results of the latter mixtures.

DISCUSSION

The SfMNPV isolate from Nicaragua comprises at least nine genotypes. The complete genotype, SfNIC-B, is the majority genotype in the population; all of the other genotypes present deletions, and three of these—genotypes C, D, and G—are incapable of per os infection when produced in genotypically pure infections. These defective genotypes rely on coinfecting cells with the complete genotype to achieve transmission be-

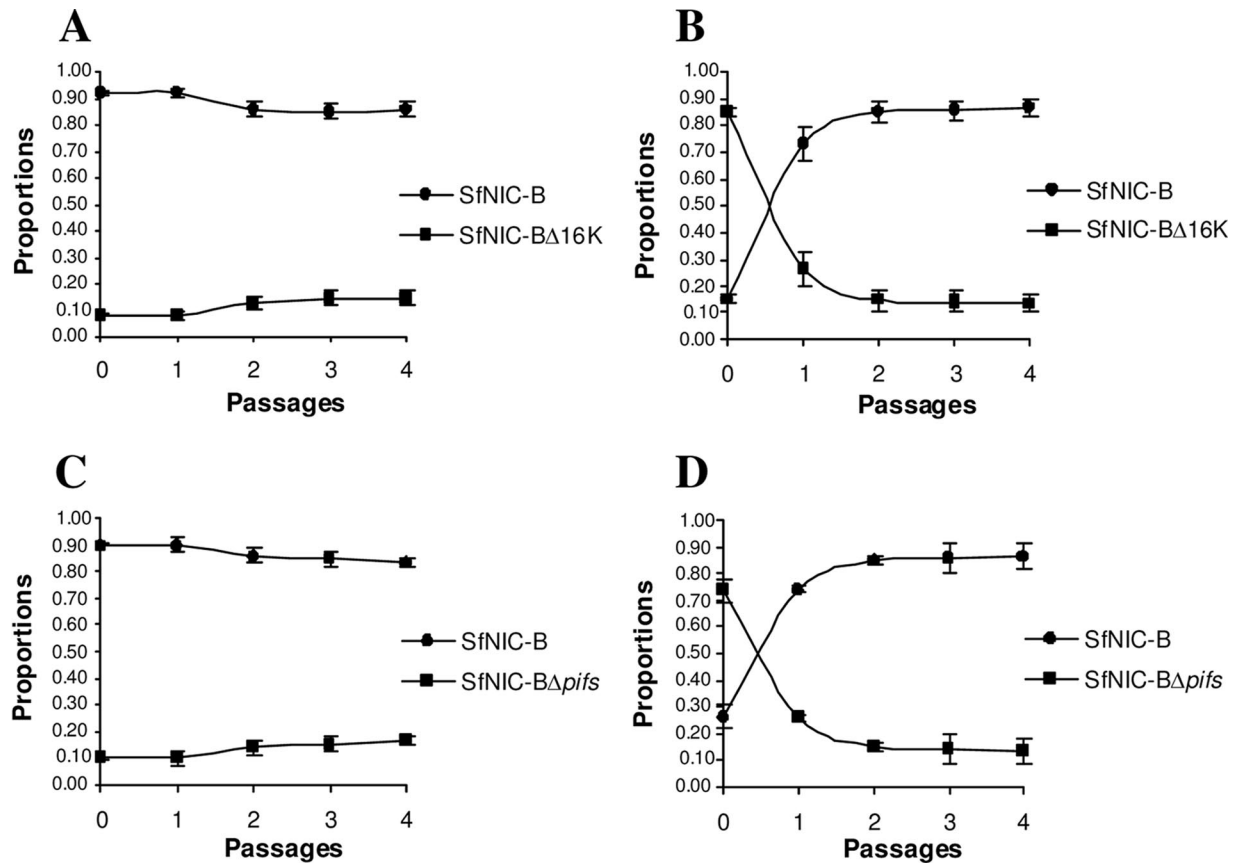


FIG. 3. Dynamics of genotypic composition in mixtures subjected to serial passage in insects. The composition of experimental mixtures comprising SfNIC-B plus SfNIC-B Δ 16K at ratios of 90:10 (A) and 10:90 (B), respectively, or mixtures comprising SfNIC-B plus SfNIC-B Δ *pifs* at ratios of 90:10 (C) and 10:90 (D), respectively. In all cases, viral OBs produced in insects injected with genotypic mixtures were subjected to four passages of peroral insect-to-insect transmission. Samples of OBs recovered after infection with different ratios of genotypic mixtures were subjected to semiquantitative PCR and densitometric analyses at each passage. Vertical bars indicate the standard deviations of densitometric readings for three replicate reactions per sample.

tween insect hosts (39–41). We previously demonstrated that OBs produced by coinfecting insects with mixtures of complete (~75%) and defective (~25%) genotypes were ~2.5-fold more infectious than OBs comprising the complete genotype alone. The absence of PIF1 or PIF2 results in the complete loss of peroral infectivity in other NPVs (17, 31, 32, 36, 43). The lack of per os infectivity of both C and D genotypes was clearly due to the deletion of *pif1* and *pif2*. However, prior to the present study, the cause(s) of the increased transmissibility of OBs comprising mixtures of complete genotypes and deletion variants had been uncertain. Moreover, Frank (13) had proposed that genes present in the genomes of deletion variants may complement defects in full-length partner genomes. Accordingly, SfNIC-C would provide SfNIC-B with factors not present in the SfNIC-B genome. An alternative hypothesis was that the deletion was at the origin of the increased pathogenicity, that is, the absence of some genes in certain genotypes could improve the global pathogenicity of the population.

To clarify the genetic basis for the observed changes in transmissibility, in the present study two deletion recombinants were constructed. First, the SfNIC-B Δ 16K virus was observed to behave in a nearly identical manner to the SfNIC-C genotype, thus confirming that there are no additional phenotypi-

cally influential factors located outside the 16.4-kb deletion of the SfNIC-C genome. In a second phase, a virus was produced that was deleted only in the two *pif* genes region, SfNIC-B Δ *pifs*. Our results indicate that the absence of *pif* genes is indeed responsible for the increased pathogenicity of the mixed population. Since both *pif1* and *pif2* genes were deleted, we cannot determine whether the observed phenotype is due to one or both genes. Although our results do not disprove Frank's (13) hypothesis that genes carried by deletion variants may complement defects in complete genotypes, they demonstrate that for this particular virus, factors other than the expression of *pif* genes do not need to be invoked to explain the observed transmissibility of the SfNIC population.

Differences in the transmissibility of experimental populations of SfMNPV comprising non-natural proportions of complete and defective genotypes were reported in a previous study (38). As the prevalence of defective genotypes increases in the population, the likelihood that a cell will be infected only by defective genotypes increases. The transmissibility of progeny ODVs in such a cell will be zero. Similarly, when the prevalence of complete genotypes is high, a high proportion of cells will be infected solely by complete genotypes and the progeny ODVs from these cells will be less infectious than

TABLE 3. Logit analysis of concentration-mortality bioassays of *S. frugiperda* second instars fed OBs of genotypic mixtures after four passages of peroral transmission involving mixtures of SfNIC-B plus SfNIC-BΔ16K or mixtures of SfNIC-B plus SfNIC-BΔpifs^a

Inoculum ^b	Mean intercept ± SE	Potency
SfNIC-B + SfNIC-BΔ16K		
SfNIC-B	-5.492 ± 0.901	1.00a
SfNIC-WT	-4.798 ± 0.817	2.75b
Genotype mixture starting ratios (SfNIC-B/SfNIC-BΔ16K [%])		
90:10	-4.810 ± 0.818	2.67b
10:90	-4.997 ± 0.830	2.56ab
SfNIC-B + SfNIC-BΔpifs		
SfNIC-B	-5.530 ± 1.070	1.00a
SfNIC-WT	-4.910 ± 0.973	2.81b
Genotype mixture starting ratios (SfNIC-B/SfNIC-BΔpifs [%])		
90:10	-4.934 ± 0.984	2.66b
10:90	-5.117 ± 0.999	2.43ab

^a The starting ratios of OBs of genotypes were mixed in different proportions. ODVs were released by alkaline treatment and injected into insects that subsequently died of infection to produce the initial OB inoculum that was subjected to four passages of infection per os. Genotype ratios converged to a common equilibrium ratio at the fourth passage, where upon OBs were harvested and subjected to bioassay to determine the insecticidal potency.

^b Regressions for SfNIC-B plus SfNIC-BΔ16K (the top half of the table) were fitted with a common slope of 0.525 ± 0.065 (Pearson's $\chi^2 = 12.1$, $df = 14$, $P = 0.596$). A test for nonparallelism was not significant ($\chi^2 = 1.309$, $df = 3$, $P = 0.727$). The genotypic compositions of the mixtures after four passages were 86% SfNIC-B:14% SfNIC-BΔ16K for the mixture starting at a ratio of 90% SfNIC-B:10% SfNIC-BΔ16K (P0) and 87% SfNIC-B:13% SfNIC-BΔ16K for the mixture that started at a ratio of 10% SfNIC-B:90% SfNIC-BΔ16K (P0). Potency values followed by identical lowercase letters did not differ significantly for comparisons among treatments for this section of the table ($P > 0.05$). Regressions for SfNIC-B plus SfNIC-BΔpifs (the bottom half of the table) were fitted with a common slope of 0.548 ± 0.078 (Pearson's $\chi^2 = 11.436$, $df = 14$, $P = 0.651$). A test for nonparallelism was not significant ($\chi^2 = 1.056$, $df = 3$, $P = 0.788$). The genotypic compositions of mixtures after four passages were 83% SfNIC-B:17% SfNIC-BΔpifs for the mixture starting at a ratio of 90% SfNIC-B:10% SfNIC-BΔpifs (P0) and 86% SfNIC-B:14% SfNIC-BΔpifs for the mixture that started at a ratio of 10% SfNIC-B:90% SfNIC-BΔpifs (P0). Potency values followed by identical lowercase letters did not differ significantly for comparisons among treatments for this section of the table ($P > 0.05$).

ODVs from cells that were infected by both complete and defective genotypes. A mathematical model showed that the ratio of complete to defective genotypes observed in the wild population represents an optimal balance between ensuring that every cell is infected by at least one deletion genotype and that no cells are infected solely by deletion genotypes. The percentage of defective genotypes in the natural SfNIC isolate was previously estimated by using uncalibrated densitometric readings at between 19.5 and 22% (28, 39), and the most potent experimental mixtures, produced by injecting insects with ODVs from different ratios of OBs, were similarly estimated at 75 to 80% complete (SfNIC-B) plus 20 to 25% defective (SfNIC-C) genotypes (28, 38). In the present study, however, calibrated densitometric analyses revealed that the true ratio of complete to defective genotypes was approximately 85% SfNIC-B plus 15% SfNIC-BΔ16K or SfNIC-BΔpifs due to an overrepresentation in SfNIC-B that was not detected in previous studies. This was likely due to differences in the number of nucleocapsids present in OBs of SfNIC-B

compared to those of deletion genotypes, an issue that has yet to be addressed in detail. A similar bias in the proportion of genotypes obtained from mixing OBs of different genotypes was recently reported using a qPCR technique (51). Nonetheless, the key issue remains unchanged; that maintaining a stable equilibrium requires the existence of a selective advantage for OBs generated in mixed infections, which in this case is the increased potency of mixed genotype OBs compared to OBs from genotypically pure infections. In consequence, experimental populations with non-natural frequencies of genotypes rapidly converge to an equilibrium that reflects the genotypic composition that results in the highest global transmissibility of ODVs.

Clearly, the NPV system fosters simultaneous coinfection by multiple genotypes, first by coenveloping multiple nucleocapsids into ODVs and then by high multiplicity of infection of budded virions, each containing a single virus genome, within each infected insect. Infection by multiple genotypes has been reported to influence pathogenicity of OBs (29) and the virulence of the infection (20, 21) in NPVs of other species. Multiple enveloping has the additional advantage that it accelerates the onset of infection as some nucleocapsids originating from the ODV are immediately rewrapped upon budding from primary infected cells to ensure establishment of systemic infection in the insect (47, 48). The importance of genotypic diversity and the interactions between genotypes in singly enveloped NPV or granulovirus populations is currently uncertain.

Whenever multiplicities of infection are high and genotypes with differing degrees of relatedness compete for resources, genotypes have the opportunity to cooperate in achieving a common goal or defect and take advantage of the resources that cooperative genotypes contribute. This may be viewed as a special case of collective action because the optimum amount of a key resource that dramatically influences the transmissibility of progeny virus particles (i.e., the concentration of PIFs that are sequestered into ODV membranes) requires that a number of defectors be present in the population. In this case, the relationship between fitness (transmissibility) and genotype frequencies is reduced at the extremes of the frequency distribution for both cooperator and defector genotypes.

The PIF proteins comprising PIF1, PIF2, and the recently identified PIF3 (Ac115) are found at low quantities in the envelopes of ODVs (1, 9, 17, 25, 31, 43), together with an additional infection factor, P74 (10). This low level of expression is due to the weakness of its promoter, at least in the case of *pif1* (17, 32). P74, PIF1, and PIF2 are involved in the entry of the virus into midgut cells by a two-step process that begins with binding to specific receptors on the apical section of the midgut cell microvilli (18, 42), possibly involving one or more integrins (31, 50), followed by fusion of the cell and ODV membranes, although the mechanisms by which this occurs remain unclear (22). It has been suggested that these proteins may interact to form a heterotrimer, whereas PIF3 is involved with some other critical event in early infection (25, 27). The key role of the PIFs (*pif1*, *pif2*, and *pif3*) and P74 in infection is underscored by the fact that these are among 30 core genes that are found in all baculoviruses sequenced to date (1).

The mechanism by which *pif1*- and *pif2*-deficient genotypes achieve the observed increase in virus transmissibility is still

unknown. The structure of the PIF proteins suggests that they may be capable of inducing fusion between adjacent membranes. A high concentration of PIFs in the nucleus of an infected cell may promote fusion between the membranes of ODVs, thereby increasing the number of nucleocapsids per ODV and lowering the average number of ODVs occluded into each OB. This would likely reduce the number of independent foci of infection in the host midgut or decrease the efficiency of entry of ODVs into columnar cell microvilli (31). Alternatively, high concentrations of PIFs in the ODV envelope may saturate cell membrane binding sites or adversely influence the interactions among PIFs and P74, resulting in reduced efficiency of infection.

From our data we cannot formally exclude that deletions of *cis*-acting sequences embedded in the *pif1-pif2* region are, in fact, responsible for the observed increase in the virus potency of genotype mixtures. Elucidation of this mechanism will involve studying the consequences of interactions between complete and *pif* deleted genotypes (or genotypes with *pif* genes inactivated by point mutations) on the composition and infectivity of ODVs, including how different genotypes are distributed among the nucleocapsids packaged into each ODV, the numbers of ODVs occluded in each OB and the per capita infectivity of ODVs produced in mixed genotype infections, and the consequences of increasing the expression of PIFs by placing one or more *pif* genes under the control of strong promoters.

ACKNOWLEDGMENTS

We thank Noelia Gorria for insect rearing and Isabel Maquirriain for technical assistance.

This study was funded by MEC project number AGL2005-07909-CO3-01.

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