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Functional Importance of Deletion Mutant Genotypes in an Insect Nucleopolyhedrovirus Population

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A Nicaraguan isolate of a nucleopolyhedrovirus (SfNIC) that attacks the fall armyworm, *Spodoptera frugiperda*, survives as a mixture of nine genotypes (SfNIC A to I) that all present genomic deletions, except variant B (complete genotype). Sequencing of cloned restriction fragments revealed that genotypic variants lack between 5 and 16 of the open reading frames present in a contiguous sequence of 18 kb of the SfNIC genome. The absence of oral infectivity of SfNIC-C and -D variants is related to the deletion of the *pif* and/or *pif-2* gene, while that of SfNIC-G remains unexplained. The presence of open reading frame 10, homolog of Se030, also appeared to influence pathogenicity in certain variants. Previous studies demonstrated a significant positive interaction between genotypes B and C. We compared the median lethal concentration of single genotypes (A, B, C, D, and F) and co-occluded genotype mixtures (B+A, B+D, B+F, A+C, and F+C in a 3:1 ratio). Mixtures B+A and B+D showed increased pathogenicity, although only B+D restored the activity of the mixture to that of the natural population. Mixtures of two deletion variants (A+C and F+C) did not show interactions in pathogenicity. We conclude that minority genotypes have an important influence on the overall pathogenicity of the population. These results clearly demonstrate the value of retaining genotypic diversity in virus-based bioinsecticides.

Nucleopolyhedroviruses (NPVs) are double-stranded, circular DNA viruses belonging to the family *Baculoviridae* that lethally infect insects, particularly the larval stages of Lepidoptera. To complete the infection cycle in vivo, NPVs require two types of virions that are genetically identical but morphologically distinct (36). The occlusion-derived virions initiate the primary infection in epithelial cells of the host midgut, following ingestion of contaminated foliage. Infected cells give rise to budded virions, which transmit the infection from cell to cell within the host.

As with many viruses, specially large DNA viruses, coinfection of host cells by multiple genotypes results in a high prevalence of mixed-genotype disease (4). Later in the infection cycle, occlusion bodies (OBs) are formed in the nucleus of the infected cells. OBs permit virus survival in the environment. OBs are proteinaceous structures that embed numerous occlusion-derived virions. Each occlusion-derived virion can contain between 1 and 29 genomes (1). Multiple genotypes can therefore be occluded within each OB, a process known as co-occlusion (4). The genotypic composition of OBs therefore reflects the variability of genotypes that infect each host cell. An insect larva becomes contaminated by eating at least one OB, which can contain multiple genomes. After host death, millions of OBs are released into the environment, permitting transmission to susceptible hosts (37).

NPVs have shown considerable promise as biological insecticides for the control of agricultural and forest pests (20). The isolation of individual genotypes by coning in vitro (16, 19) or in vivo (22, 32) has invariably indicated that natural NPV populations are genotypically heterogeneous. These individual genotypes can differ in important phenotypic traits, like virulence or pathogenicity (sensu Thomas and Elkinton [33]), measured by median lethal dose (LD_{50}) or concentration (LC_{50}).

The multinucleocapsid NPV (SfMNPV) of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), is a common pathogen of this pest in the Americas (28). A wild isolate from Nicaragua (SfNIC) was characterized (8) and selected for formulation and field evaluation for the management of *S. frugiperda* by Mesoamerican maize growers (38). The SfNIC isolate consists of at least nine different genotypes (SfNIC A to I) which can be clearly identified by their PstI and EcoRI restriction profiles (31). The various genotypes present marked differences in their pathogenicity to *S. frugiperda* larvae. Three of them, SfNIC-C, -D, and -G, are not infectious per os when inoculated alone. For the others, the 50% lethal concentration (LC_{50}) for second-instar *S. frugiperda* varied by a factor of >3. None of the isolated genotypes attain the pathogenicity of the wild type SfNIC isolate, suggesting that interactions between genotypes results in an increased pathogenicity of the wild-type population (20).

The region between map units 14.8 and 26.8 of the SfNIC genome contains all the variability detected in our studies (31). This genomic region includes PstI-F, PstI-L, PstI-K, and EcoRI-N fragments. The characterization of this variable region is fundamental to understanding the molecular and biological features of these variants, for establishing possible relations between variant phenotype and genotype, and to explain the genetic structure observed in the SfMNPV natural population.

Deletion genotypes SfNIC-C and -D account for ≈20% of the SfNIC genotypes in the natural population. These geno-

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TABLE 1. Summary of locations and directions of the primers used in amplification of the deleted regions of each genotype in the SfMNPV sequenced region

Primer	Sequence	Direction	Nucleotides[a]	Amplification of deleted region of genotype:							
				A	C	D	E	F	G	H	I
Sf8.2	GCGATGACGCCCATGAACTTCACATG	Forward	NL							√	
Sfcath1	GCAGAATCGTTACGACTA	Forward	3–20		√	√	√				√
Sfcath2	GCCGCGTTTAGTAACAGCAA	Forward	210–229	√							
Sf19.1	CGTGGTGTTATCTGCGCGAA	Forward	1487–1506					√	√		
Sf30.3	CCCATGCGCATCGTCGACAA	Reverse	9416–9397					√	√		√
Sf32.1	TGGTGGAGCTGCATGATTGT	Reverse	11895–11876	√						√	
Sf35.1	CGTGTTCACATCTCTCGGAT	Reverse	13704–13686				√				
Sf100a	GTCTACTTGGATTCGTCGAAGGTGA	Reverse	16986–17010		√	√					
PCR fragment size (kb)				1.6	0.9	0.9	1.2	2.4	3.1	6.5	1.1

[a] NL, not located in the sequence, in the ORF homologue to Se8. The left extremity of the SfMNPV PstI-G genome fragment Sf-B variant is not included. It was not used for PCR amplifications because it represented the complete genome.

types alone are not able to infect *S. frugiperda* larvae by ingestion (17), since they do not possess the *pif* (13) and *pif-2* (24) genes that appear to be essential for cell binding and penetration during the process of primary infection of insect midgut cells. However, deletion genotype SfNIC-C has an important positive influence on the pathogenicity of complete genotypes present in the virus population, although the mechanism of this interaction has not been determined (17). The pathogenicity of genotype B alone was less than that of the wild-type SfNIC isolate, but mixtures of genotypes B and C, co-occluded in a proportion of 3:1, approximately the proportion observed in the natural viral population, restored pathogenicity to that of the wild-type SfNIC isolate.

These observations led us to examine the contribution of the other deleted genotypes present in SfNIC to the pathogenicity of the viral population. We determined the nucleotide sequence of the SfNIC variable genome region and the exact deletion in each variant, indicating the genes affected and the possible relation to the pathogenicity of each variant. We then evaluated the interaction between genotypes in order to determine the potential contribution of each genotype to the pathogenicity of the viral population. For this, we inoculated *S. frugiperda* larvae with one of five co-occluded genotype mixtures (B plus A, B plus D, B plus F, A plus C, and F plus C) and measured the inoculum concentration-mortality relationship, a key aspect of virus transmissibility. This study reveals a fundamental relationship between the ability of virus genotypes to cause lethal infections and the structure of NPV populations.

MATERIALS AND METHODS

Insects and viruses. *Spodoptera frugiperda* larvae were obtained from a laboratory colony maintained at constant environmental conditions (25 ± 1°C, 75 ± 5% relative humidity, and a photoperiod of 16 h light and 8 h dark) and reared on a wheatgerm-based semisynthetic diet (9).

Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) was originally isolated from diseased larvae collected on maize in Nicaragua (SfNIC) and characterized by Escribano et al. (8). Viral occlusion bodies were produced by feeding healthy fourth-instar *S. frugiperda* a virus-contaminated diet. OBs were extracted from dead diseased larvae by triturating in water and purified by filtration and differential centrifugation (5). The concentration of purified OBs was determined using an improved Neubauer hemacytometer (Hawksley, Lancing, United Kingdom) under phase-contrast microscopy. OBs were stored at 4°C until used.

The SfNIC genotypic variants (SfNIC-A to -I) were cloned by plaque purification as described previously (17, 31). Briefly, plaque assays were performed by infecting *S. frugiperda* (Sf9) cells with hemolymph containing budded virions, recovered from larvae inoculated with SfNIC 3 days before. Individual plaques were amplified in Sf9 cell cultures and the virion-containing supernatant was injected into fourth-instar *S. frugiperda* for the production of OBs of individual genotypes. Single-genotype OBs were extracted from dead larvae, purified, and quantified as indicated above.

Purification and restriction endonuclease analysis of DNA. Viral DNA was extracted from $\approx 10^9$ OBs in 300 μl of water by digestion of the polyhedrin matrix with 100 μl of 0.5 M Na_2CO_3 and 50 μl of 10% wt/vol sodium dodecyl sulfate at 60°C for 10 min. Undissolved OBs and other particulate material were pelleted by low-speed centrifugation (2,700 × *g*, 5 min). Supernatant containing occlusion-derived virions was recovered and incubated with 500 μg/ml proteinase K at 50°C for 2.5 to 3 h. Viral genomic DNA was extracted with phenol and chloroform, precipitated by addition of 3 M sodium acetate (pH 5.2) and ethanol, washed with 70% ethanol, and dissolved in 0.1× Tris-EDTA buffer. DNA concentration was estimated by absorbance at 260 nm.

For restriction endonuclease analysis, 2 μg of viral genomic DNA was treated with EcoRI or PstI (Amersham) following the manufacturer's recommendations. These enzymes were chosen because they allowed clear discrimination between individual SfNIC genotypes (17, 31). Electrophoresis was performed in horizontal 1% agarose gels in TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) and the DNA fragments were visualized by staining with ethidium bromide.

Cloning and sequencing of DNA fragments. SfNIC-B, the standard genotype that possesses a complete genome, was used for cloning. PstI and EcoRI libraries were constructed in plasmids pUC19 and pSP70 (Promega), respectively, using a DNA ligation kit (Rapid Ligation Kit, Roche Diagnostics, Meylan, France). The plasmids that contained the PstI-F (p279.51), PstI-L (p279.19), PstI-K (p279.56), and EcoRI-N (p264.306) restriction fragments were identified. The complete SfNIC PstI-F (7.5 kb), PstI-L (2.9 kb), and PstI-K (4.9 kb) restriction fragments, the left fragment generated after digestion of the EcoRI-N fragment with PstI of 1.6 kb (p284.1), and the left extremity of the EcoRI-I (p264.100) fragment were sequenced. For primer design, the extremities of the PstI-G fragment were also sequenced.

The sequences of the fragments were determined using an ABI Prism Big Dye terminator cycle sequencing ready reaction kit on a 9600 PE model thermocycler. The reaction products were loaded in an automated DNA sequencer ABI PRISM (Sistemas Genómicos, Valencia, Spain).

Determination of deletion points of each variant. The physical maps of each variant have been constructed (31). Using this information and the sequence data obtained from SfNIC-B, specific primers were designed near the deletion point of each variant (Table 1). These primers included a HindIII restriction cleavage site in the 5′ extremity for cloning purposes. The PCR products obtained were sequenced either directly (SfNIC-G genotype) or after cloning into pUC19 (all the others).

Sequence computer analysis. DNA and protein comparisons with entries in the updated GenBank/EMBL, SWISS-PROT, and PIR databases were performed with BLASTn, FASTA, and BLASTp (2, 23). Multiple sequence alignments were performed with CLUSTALX version 1.7 (34) and Macaw (27).

Production of co-occluded genotype mixtures. The genotypic variants were classified into four different groups depending the length of the deletion present in the genome, relative to complete genotype B, and their pathogenicity, determined previously (31). We selected one genotype from each group (A, B, D/C,

and F) to produce the co-occluded genotype mixtures. Co-occluded genotype mixtures (3, 20) were produced for the following combination of genotypes: B plus A, B plus D, B plus F, A plus C, and F plus C. For this, OBs of each genotype were mixed in the proportion 3:1, since the genotype ratio 3B:1C was previously observed to result in the greatest influence on the OB pathogenicity phenotype (17).

Specifically, OBs of complete genotype B (75% of OBs) were mixed with 25% of deleted genotype OBs (A, D, or F). Similarly, in mixtures in which the prevalence of OBs of genotypes A or F was 75%, deletion variant C was present at a prevalence of 25% of OBs. Occlusion-derived virions were released from the above OB mixtures by alkali disruption with a dissociation buffer (1 volume OBs, 1 volume 0.5 M Na_2CO_3, 5 volumes H_2O). Undissolved OBs and other particulate materials were pelleted by low-speed centrifugation at 2,700 × *g*, 5 min. The occlusion-derived virion-containing supernatant for each genotype mixture was injected into groups of 50 fourth-instar *S. frugiperda* (8 μl/larva). These larvae were individually maintained on semisynthetic diet at constant environmental conditions (see above) until death. Extraction of OBs containing mixtures of co-occluded genotypes from infected cadavers, OB purification, and DNA extraction were then performed as described above.

The relative proportions of each genotype in mixed-genotype OBs were determined by measuring the intensity of a variable fragment amplified by PCR, which is characteristic of each genotype (17). Three different primers were designed to differentiate between *pif*$^+$ genotypes (SfNIC-A, SfNIC-B, and SfNIC-F) (amplicon ≈750 bp) and *pif*$^-$ genotypes (SfNIC-C and D) (amplicon ≈640 bp). DNA extraction of co-occluded genotype mixtures was followed by 25 cycles of PCR. The relative intensities of the two PCR products obtained were determined using the Scion Image PC program (Scion Corp., Frederick, MD).

Insect bioassays. The insecticidal properties of OBs comprising the co-occluded genotype mixtures (B+A, B+D, B+F, A+C, and F+C), the pure genotypes SfNIC-A, SfNIC-B, SfNIC-C, SfNIC-D, and SfNIC-F, and the SfNIC wild-type isolate were determined by insect bioassay. Pathogenicity was expressed as the 50% lethal concentration. Bioassays were performed in second-instar *S. frugiperda* using the droplet feeding method (12). Larvae were starved for 8 to 12 h at 23°C and then allowed to drink from an aqueous suspension containing 10% (wt/vol) sucrose, 0.001% (wt/vol) Fluorella blue, and OBs at one of five different concentrations (1.92×10^3, 9.6×10^3, 4.8×10^4, 2.4×10^5, and 1.2×10^6 OB/ml). This range of concentrations was previously calculated to produce mortalities of between 5 and 95%. Larvae that ingested the suspension within 10 min were individually transferred to a 25-well tissue culture plate with a cube of semisynthetic formaldehyde-free diet.

All bioassays were performed three times using 25 larvae per virus concentration plus 25 mock-infected larvae as control. Inoculated larvae were maintained at 23°C. Mortality data were recorded every 12 h until all insects had either died or pupated. Dead insects were examined by phase contrast microscopy to determine the presence of OBs.

Concentration-mortality results were subjected to logit analysis using the generalized linear interactive modeling program GLIM 3.77 (Royal Statistical Society, London, England) with a binomial error structure specified (6). No evidence of overdispersion was observed in datasets.

RESULTS

Gene content of SfNIC genotypic variants. The 17,850-bp sequence region of the SfNIC-B genome (map units 14.8 and 27.6) was assembled from the information obtained by sequencing cloned restriction fragments. Sixteen complete open reading frames (ORFs), larger than 300 bp, and two partial ORFs (at the left and right extremities of the sequenced region) were identified (Fig. 1; Table 2). The sequenced region included a recently published 5.1-kbp sequence from a Brazilian SfMNPV isolate (35) (nucleotides 4303 to 9420). The two isolates are highly homologous, 20 nucleotide changes, two deletions of 3 nucleotides each, and one insertion of 3 nucleotides. This represents a variability of 0.57% in the nucleotide sequence.

The observed variability between genotypes isolated from the SfNIC virus population was precisely mapped, apart from a small 0.8-kb deletion in SfNIC-D, located in the EcoRI-L fragment (31), and the left extremity of SfNIC-H, which fell outside of the sequenced region. Deletions were characterized using primers (Table 1) designed close to the deletion breaking points of each variant, based on the sequence of the SfNIC-B complete genotype (Fig. 2a).

SfNIC-C and -D lacked 16,362 bp in this region, which represents a total deletion of approximately 17 kb for SfNIC-D due to a deletion of 0.8 kb located in the EcoRI-L fragment, outside this genomic region. Compared with SfNIC-B, both variants completely lacked ORFs 2 to 16, and they both presented partial deletions of ORFs 1 and 17 (Fig. 2a). The deletions started 6 nucleotides after the ATG start codon of *cath*. An alternative ATG in frame can be used (nucleotides 16620 to 16618 in the complementary strand), suggesting that *vcath* could be functional in these deletion genotypes.

The next largest deletion was observed in genotype SfNIC-E, with a 13,650 bp deletion. This variant completely lacked ORFs 2 to 13 and partially lacked the *vcath* and *arif-1* genes (Fig. 2a). No alternative in-frame ATG was found in the sequenced region. Under these conditions, the putative product produced by the virus would be shortened by at least 83 amino acids. Genotype SfNIC-H presented a deletion of approximately 12,700 bp. The region surrounding the deletion was amplified using an oligonucleotide outside of the sequenced region. The deletion of this variant was located in the PstI-G and PstI-K fragments (Fig. 2a). The left point of the deletion was located at nucleotide 64, before the ATG start codon of *lef-1* (homologue to Se014), and the right point at nucleotide 11589, within ORF 11. A composite ORF is formed that has the carboxyl terminus of ORF 11 (215 nucleotides) and 114 nucleotides at the 5′ end coming from the left end of the deletion. We could not determine exactly the genes affected in this variant, as the PstI-G fragment was not sequenced, but we calculated that the region from the *lef-1* gene (homologous to Se014) up to ORF 11 (Se031) was deleted.

SfNIC-A presented a deletion of 10,294 bp. It was located at nucleotide 285, before the TAA stop codon of ORF 2, and at nucleotides 189, after the ATG start codon of ORF 10, with ORFs 2 to 10 all completely or partially deleted (Fig. 2a). SfNIC-I deletion was located between nucleotide 16, before the TAA stop codon of ORF 2, and at nucleotide 29, before the TGA stop codon of ORF 9, with ORFs 2 to 8 completely deleted and ORF 9 partially deleted. The next largest deletion was observed in variant SfNIC-F. This variant presented a 5,637-bp deletion from nucleotides 1985 to 7621 that included ORFs 3 to 8, which were completely or partially deleted. The deletion is located in the left region at nucleotide 531, after the ATG start codon of ORF 3, and in the right region at nucleotide 301, after the ATG start codon of ORF 8. Finally, the SfNIC-G variant presented the shortest deletion, 4,854 nucleotides in length, from nucleotides 3856 to 8709. The deletion point was located at nucleotide 450, after the ATG start codon of ORF 4, and at nucleotide 206, before the TAA stop codon of ORF 10. Consequently, ORF 4 and ORF 10 are partially deleted, whereas ORF 5 to ORF 9 are completely deleted.

The analysis of the nucleotide sequences surrounding the deletion breakpoint did not reveal a consensus feature. In SfNIC-A, -C/D, and -E there is a direct repeat encompassing the deletion points (sequences AAACTTTT, TTGTTaCAaTT, and GTagCGAT, respectively [lowercase letters indicate vari-

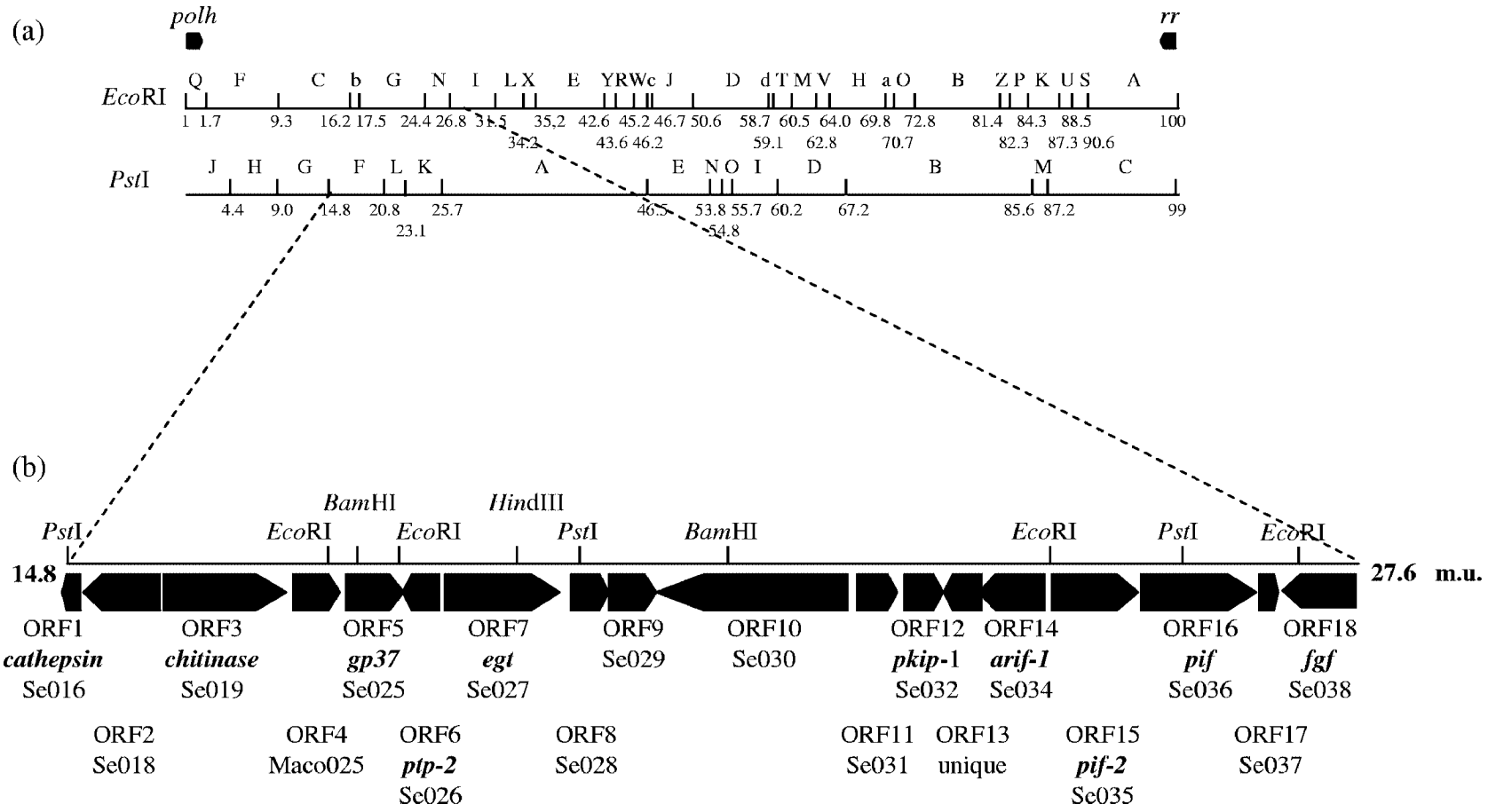


FIG. 1. Location and gene organization present on the PstI-F, PstI-L, PstI-K, and EcoRI-N fragments in the genome of the Nicaraguan SfMNPV isolate, genotype B (SfNIC-B). (a) EcoRI and PstI physical map of the SfMNPV genome. The position and orientation of the polyhedrin (*polh*) and RNase reductase (*rr*) genes are indicated by arrows. (b) Gene order of PstI-F, PstI-L, PstI-K, and EcoRI-N fragments of SfMNPV. The arrows represent the ORFs and point to their directions of transcription. The cathepsin and *fgf* genes were only partially sequenced.

TABLE 2. Summary of locations of ORFs in the sequence and direction of their transcripts, sizes of the ORFs, and putative transcription and termination signals

ORF	Gene family	Positions	Direction	No. of residues encoded	Transcription signals	Promoter	Poly(A) addition signal
1	*cath*	1–250	<	83	TTAAG (−28)	Late	
2	Se018/Se017	370–1425	<	351	TATAA (−27)	Early	+4
					ACGTA (−47)		+17
					GATA (−73)		
3	*chit*	1455–3149	>	564	ATAAG (−17)	Late	+9
4	Maco025	3406–4047	>	213	TATAA (−60)	Early	+18
					GC (−67)		+31
					GATA (−83)		
5	*gp37*	4151–4951	>	266	TTAAG (−557)	Late	+220
6	*ptp-2*	4948–5451	<	167	ATAAG (−18)	Late	+784
					GTAAG (−73)		
7	*egt*	5547–7124	>	525	TATAA (−53)	Early	+32
					ACGTA (−90)		+202
					ACGTT (−159)		
					GATA (−186)		
8	Se028	7311–7817	>	168	TATA (−64)	Early	+243
					ACGTG (−118)		
					TCAGTA (−33)		
					GATA (−183)		
9	Se029	7835–8482	>	215	ATAAG (−183)	Late	+1632
10	Se030	8502–11138	<	878	TATAA (−51)	Early	+3
					TATAA (−64)		
					GC (−69)		
11	Se031	11249–11803	>	184	ATAAG (−46)	Late	+166
12	*pkip-2*	11882–12409	>	175	ATAAG (−56)	Late	−4
13	Unique	12441–12959	<	172	TATA (−130)	Early	−1
					GATA (−132)		
14	*arif-1*	12922–13821	<	299			+158
15	*pif-2*	13718–14914	>	398	CTAAG (−14)	Late	+2033
16	*pif*	14932–16521	>	529	CTAAG (−14)	Late	+426
17	Se037	16529–16786	>	85	CTAAG (−1611)	Late	+161
18	*fgf*	16783–17685	<	300			

able nucleotides]). No conserved motifs were found for the other deletions (Fig. 2b).

Virus pathogenicity. Restriction endonuclease and densitometric analysis confirmed that the proportion of genotypes in each of the genotype mixtures present in co-occluded OBs was approximately the same as the proportion in which they were injected into host larvae (Fig. 3).

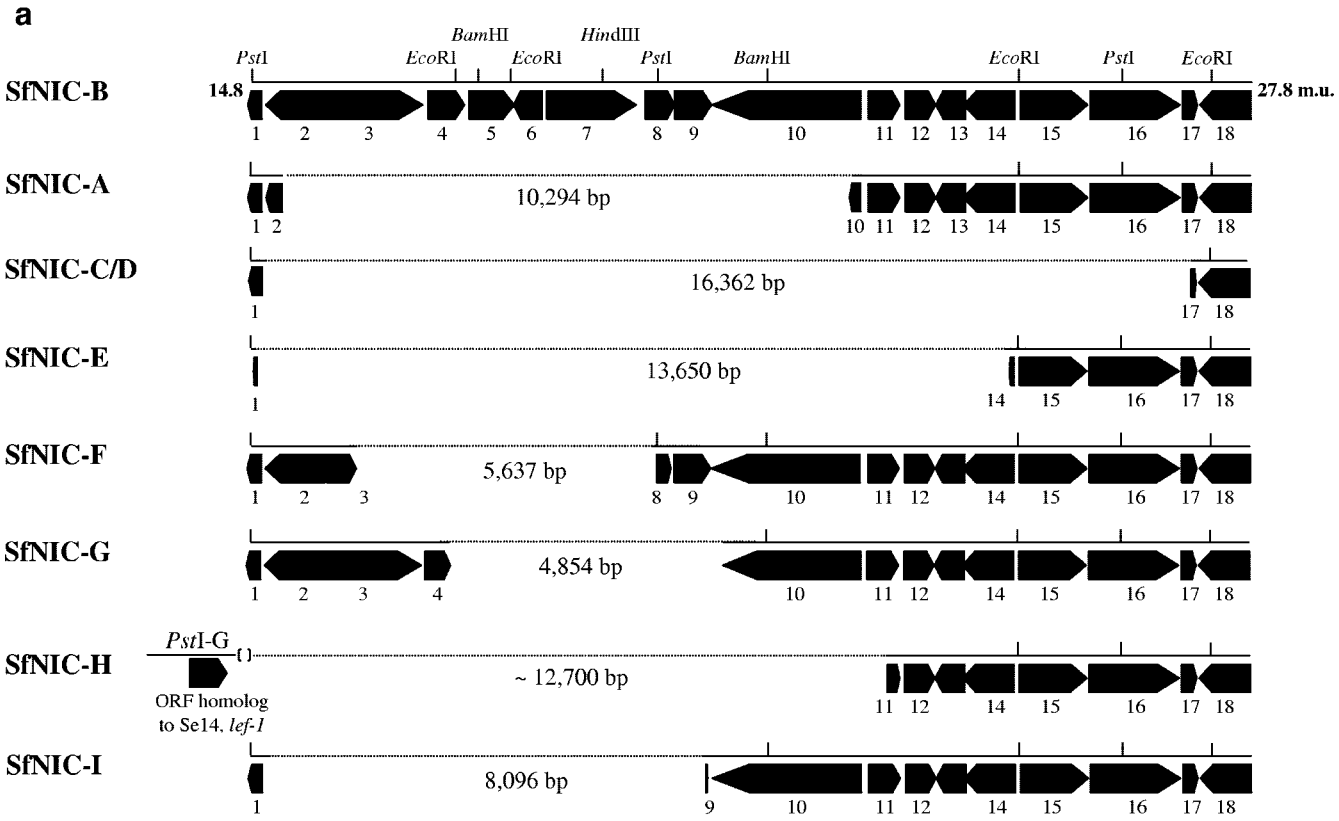
Of the 11 virus treatments (five co-occluded genotype mixtures, five pure genotypes, and the wild-type SfNIC isolate) used to inoculate *S. frugiperda* larvae per os, all resulted in lethal polyhedrosis disease except pure genotypes C and D (Table 3). All single genotypes were significantly less pathogenic than the wild-type SfNIC isolate. Wild-type SfNIC had a potency 4.35, 3.57, and 2.78 times that of single genotypes A, B, and F, respectively (Table 3). We hypothesized that this effect could be due to a significant interaction between genotypes, resulting in an altered pathogenicity when present in mixtures, as observed previously for genotypes B and C (17). The co-occluded mixture of genotypes B and D was as pathogenic as wild-type SfNIC, whereas mixtures B+A, B+F, A+C, and F+C were all significantly less pathogenic than the wild-type SfNIC isolate (Table 3). Co-occluded mixture B+A was 1.79 and 2.20 times more pathogenic than pure genotypes B and A, respectively, indicating a positive interaction between these genotypes, although it was not enough to restore the activity to that of wild-type SfNIC. In contrast, the pathogenicity of the B+F, A+C, and F+C genotype mixtures was not significantly different from that of pure genotypes B, A, and F, respectively (Table 3).

DISCUSSION

From the SfNIC isolate, nine genotypes that could be differentiated by their restriction patterns were isolated by cloning in cell culture (17, 31). A contiguous sequence of 17,850 nucleotides of the SfNIC genome was determined that includes 89% of the variability detected in this isolate, except for the deletion of approximately 800 and 1,500 bp of SfNIC-D and SfNIC-H, respectively, which fell outside the sequenced region. This region included 18 genes. Some of these genes are known to play a role in the per os infection process (*pif* and *pif-2*). The sequenced region overlapped the recently characterized 5.1-kb BamHI-F fragment from a Brazilian isolate that included the *egt* gene (35) and the *pif* gene region (30).

Of the nine genotypes isolated from SfNIC, SfNIC-C, -D, and -G were not infectious per os. The precise determination of each deletion revealed a clean joint, with no reorganizations in the borders of the deletions. In four genotypes, SfNIC-A, -C, -D, and -E, small direct repeats were found overlapping the deletion breakpoint. These may be significant in the generation of the deletions, either by representing marks of transposable element movements or by facilitating homologous recombinations.

Analysis of the deletions suggested that the absence of per os infectivity of the SfNIC-C and -D genotypes could be due to



b

	640	651	10946 10957
SfNIC B	CA**AAAACTTTT**GCtaaattat		ggtaaagT**AAACTTTT**CCG
SfNIC A	CA**AAAACTTTT**GC		T**AAACTTTT**CCG
	232	243	16606 16617
SfNIC B	GTATGAGTAAT**Ttgttcatt**		atggc**ttgTTA**C**AATTT**GGC
SfNIC C	GTATGAGTAATT		**TT**ACAA**TT**TGGC
	61	72	13723 13734
SfNIC B	T**GT**AG**CGAT**ACTttttttca		gtgatg**gt**C**ACGAT**CGAGCG
SfNIC E	T**GT**AG**CGAT**ACT		CA**CGAT**CGAGCG
	1973	1984	7622 7633
SfNIC B	ACCCGTTCCCAAtttgtctc		gatgatttTTCAAACGTAAA
SfNIC F	ACCCGTTCCCAA		TTCAAACGTAAA
	3844	3855	8710 8721
SfNIC B	TATCACGACACGtctctata		ttacgtgtACGTTTGATTTA
SfNIC G	TATCACGACACG		ACGTTTGATTTA
			11590 11601
SfNIC B	TTATTACGCAGC		aataaataCAACAACGACGA
SfNIC H	TTATTACGCAGC		CAACAACGACGA
	343	354	8451 8462
SfNIC B	AAAAATTTATTAtagttatt		agcaatctTAACTTTTAGGA
SfNIC I	AAAAATTTATTA		TAACTTTTAGGA

FIG. 2. (a) Gene order in the SfMNPV variable region, including PstI-F, PstI-L, PstI-K, and EcoRI-N fragments of SfNIC-B. Schematic representation of the gene order of SfNIC genotypic variants, indicating the deletions of each variants and the genes present in each variant. The arrows represent the ORFs and point to their directions of transcription. The number below each arrow indicates the number of the ORF in this variable region; the gene name, or the homologous ORF in *Spodoptera exigua* MNPV that corresponds to each ORF, as indicated in Fig. 1. (b) Sequences around the deletion breakpoint for each genotypic variant. Nucleotide numbers refer to the SfNIC-B sequenced fragment. Repeated sequences are indicated in gray.

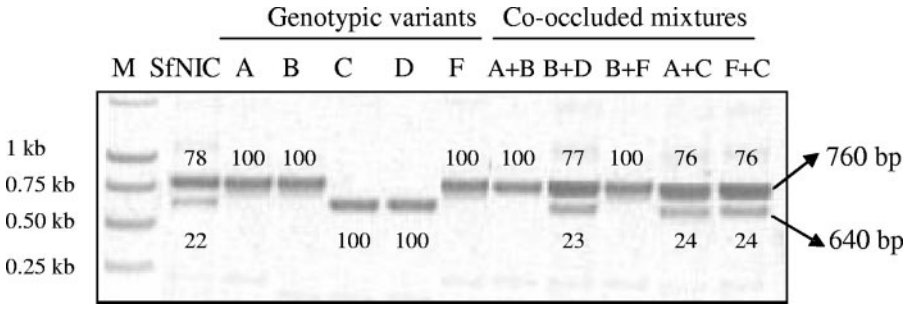


FIG. 3. PCR amplification of the characteristic fragments of SfNIC variants A, B, and F (760 bp), with the *pif* gene in their genome, and of variants C and D (640 bp), which lack the *pif* gene. For the PCR amplifications, total DNA of the wild-type SfNIC isolate, pure genotypes A, B, C, D, and F, and co-occluded mixtures A+B, D+B, F+B, A+C, and F+C was used. The molecular markers (M) used were a smart ladder (Stratagene); molecular sizes are shown to the left and specific PCR fragment sizes are to the right. The intensity of each fragment was measured using the Scion Image program. Both genotypes in each co-occluded mixture were present at close to the proportion at which they were inoculated. Values next to amplicons indicate the relative intensity of each product. It was not possible to measure the proportion of genotypes in the A+B and F+B mixtures because these genotypes both possess the *pif* gene and the amplicon of 640 bp was specifically designed for *pif*-negative variants.

the absence of the *pif* and/or *pif-2* gene. This hypothesis has been confirmed by rescue using a plasmid that encompasses those two genes (p264.306) (31). However, OBs of the SfNIC-G variant are not infectious per os, although they retain both genes. Moreover, the deletion present in SfNIC-G is completely included in that of SfNIC-A, which is infectious per os. Accordingly, the absence of per os infectivity of SfNIC-G observed previously (31) cannot be solely attributed to the deletion. It is possible that point mutations that abolish infectivity are present in this variant. Such mutations are unlikely to be detected by restriction fragment length polymorphism unless they occur within a restriction cleavage site. Rescue experiments are under way to investigate which genes are involved in the SfNIC-G phenotype.

The per os infectivity of other genotypic variants varied by a factor of >3 (31), which is less than observed in some other NPVs, for which up to sevenfold variation has been reported (13). It is interesting that among the pathogenic variants, the two most infectious, which were even more infectious than the nondeleted SfNIC-B genotype, were those presenting the shortest deletions, SfNIC-F and SfNIC-I (31). SfNIC-F lacked ORFs 3 to 8, while SfNIC-I lacked ORFs 2 to 9. Variants with larger deletions, such as SfNIC-A and SfNIC-H, presented lower pathogenicity than variants with shorter deletions (SfNIC-F and SfNIC-I) (Table 3). The principal difference between the SfNIC-A and -I variants is that SfNIC-I presented ORF 10, homolog to Se030, whereas this was absent in SfNIC-A. ORF 10, of unknown function, may therefore play an important role in the pathogenicity of these viruses. Further studies are in progress to determine the importance of this ORF.

TABLE 3. Logit regression analysis of insect mortality following 11 SfMNPV treatments including wild-type SfNIC, five pure genotypes, and five co-occluded genotype mixtures, in second-instar *Spodoptera frugiperda* following per os inoculation[a]

Inoculum	LC_{50} (OB/ml)	95% Confidence limits		Intercept + SE	Relative potency	*P*	Reference
		Low	High				
Wild-type SfNIC	5.47×10^4	3.82×10^4	7.84×10^4	−6.734 + 0.2602	1		This study
Genotype A	2.38×10^5	1.64×10^5	3.48×10^5	−7.643 + 0.2785	0.23	<0.001	This study
Genotype B	1.93×10^5	1.33×10^5	2.82×10^5	−7.515 + 0.2752	0.28	<0.001	This study
Genotype C							This study
Genotype D							This study
Genotype F	1.53×10^5	1.06×10^5	2.22×10^5	−7.369 + 0.2731	0.36	<0.001	This study
Genotype G							34
Genotype H	2.45×10^5	1.89×10^5	3.21×10^5	−7.392 + 0.2000	0.22	<0.001	34
Genotype I	1.66×10^5	1.29×10^5	2.15×10^5	−7.160 + 0.1963	0.33	<0.001	34
75% B + 25% A	1.08×10^5	7.53×10^4	1.56×10^5	−7.156 + 0.2679	0.51	0.014	This study
75% B + 25% D	5.05×10^4	3.09×10^4	8.24×10^4	−4.431 + 0.5141	1.09	N.S.	This study
75% B + 25% F	2.33×10^5	1.59×10^5	3.41×10^5	−7.630 + 0.2787	0.24	<0.001	This study
75% A + 25% C	2.18×10^5	1.50×10^5	3.18×10^5	−7.588 + 0.2766	0.25	<0.001	This study
75% F + 25% C	1.69×10^5	1.17×10^5	2.45×10^5	−7.432 + 0.2735	0.32	<0.001	This study

[a] Logit regression of number of responding insects against $\log_e$ (virus concentration) given in terms of $\log_e$ odds ratio: $\log_e(p/q) = a + bx$. Regressions were fitted in GLIM with a common slope of 0.6173 + 0.0216 (standard error) for all the virus inocula except the B + D mixture, which was fitted separately with a slope of 0.4090 + 0.0467, and the variants described in a previous study (31), H and I, with a slope of 0.5813 + 0.034. A test for nonparallelism was not significant for those virus inocula ($\chi^2 = 4.18$, d.f. = 24, $P > 0.05$). *P* values were calculated by *t* test of the differences between regression intercepts compared to that of the wild-type SfNIC isolate. Relative potencies were calculated as the ratio of effective concentrations relative to SfNIC except in the case of the B + D mixture, for which this represents the ratio of LC_{50} values and is not significant (N.S.) based on overlap of 95% confidence limits (26). As such, relative potencies indicate the relative pathogenicity of each genotype or mixtures of genotypes compared with that of the wild-type isolate. All bioassays were performed in second-instar *S. frugiperda* (~25 insects/concentration, three replicates) using the droplet feeding method. OBs containing co-occluded genotype mixtures were produced by injecting *S. frugiperda* with mixtures of occlusion-devired virions (B+A, B+D, B+F, A+C, and F+C) in a ratio of 3:1. The proportion of each genotype was confirmed by densitometric analysis from semiquantitative PCR amplification (Fig. 3).

None of the co-occluded genotype mixtures tested in this study had the same infectivity as the wild-type isolate except the 3:1 mixture of complete genotype B and deletion variant D. Previously, deletion variant C was demonstrated to restore the pathogenicity of complete genotype B to that of the SfNIC isolate when mixed and co-occluded in the proportion 3B:1C (17). As such, we performed this study with the expectation that deletion variant D would behave like variant C. Defective genotypes C and D, which lacked per os infectivity, were capable of surviving in the SfNIC population by co-occluding with genotypes that express the *pif* and *pif-2* genes. We observed that all mixtures involving SfNIC-C or -D co-occluded with per os infectious genotypes (SfNIC-A, -B, or -F) resulted in transmission of the defective variants, confirming that the transmission of pure genotypes C and D was only possible in the presence of variants that express these essential genes.

We analyzed the relationships between combinations of the other genotypes as a first step to understanding the functional importance of the genetic structure maintained in the virus population. A positive interaction between genotypes was also observed in mixture B+A, where A was present as the minority genotype, as observed in the natural population (31).

In another system involving the NPV of *Panolis flammea*, the pathogenicity of mixed-genotype infections was similarly greater than that of clonal infections (11). Mixed-genotype infections also influenced mortality patterns over time and the yield of progeny OB from infected hosts (11). As the ability of enter the host is a fundamental aspect of transmission, these results demonstrate that interactions between genotypes in mixed infections likely affect the fitness of NPV pathogens. The mechanisms of this interaction have yet to be determined.

Previously, naturally occurring deletion mutants in a commercial bioinsecticide isolate of *Spodoptera exigua* MNPV were classified as parasitic genotypes, as they reduced the pathogenicity of the isolate (22). In this case, pure genotypes were observed to be more pathogenic than mixtures (22). In addition, when parasitic genotypes were mixed with a field isolate of the same virus, the proportion of parasitic genotypes increased upon serial passage, significantly reducing the pathogenicity of the experimental population (21). However, for these studies, individual genotypes were isolated by in vivo cloning, involving per os infection of insect larvae with very low doses of OBs. This process inevitably eliminated noninfectious variants from the experimental population, unlike the in vitro cloning procedure that we employed.

The appearance of defective genotypes containing large deletions within particular regions of the genome is well documented in cell culture (10, 14, 15, 18). Such deletion variants may lose per os infectivity but retain the ability to replicate and transmit in vitro (7), as described for SfNIC variants C and D. Baculoviruses exhibit a high degree of genetic heterogeneity (19, 22, 29) and the fact that minority genotypes are not eliminated suggests that this heterogeneity is important for virus survival. Moreover, heterogeneous populations are likely to prove to be more realistic models for studies of baculovirus host range and pathogenicity than laboratory-cloned variants (25).

Minority variants present within natural NPV populations play a functional role in determining the pathogenicity of the population, a key parameter shaping the transmissibility and survival of viruses. Other phenotypic characteristics, such as OB production, survival time, and the tradeoff between time to kill and virus progeny production (11), are likely to differ between variants and merit study as additional factors affecting virus survival. A great deal of research effort has been made to improve the efficacy of baculovirus-based biopesticides (3); our results demonstrate the importance of retaining genotypic diversity in NPV biopesticide products and in studies of baculovirus ecology.

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