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Stability of a *Spodoptera frugiperda* Nucleopolyhedrovirus Deletion Recombinant during Serial Passage in Insects▽†

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The stabilities of the *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) complete genome bacmid (Sfbac) and a deletion recombinant (Sf29null) in which the *Sf29* gene was replaced by a kanamycin resistance cassette were determined during sequential rounds of *per os* infection in insect larvae. The *Sf29* gene is a viral factor that determines the number of virions in occlusion bodies (OBs). The Sf29null bacmid virus was able to recover the *Sf29* gene during passage. After the third passage (P3) of Sf29null bacmid OBs, the population was observed to reach an equilibrium involving a mixture of those with a kanamycin resistance cassette and those with the *Sf29* gene. The biological activity of Sf29null bacmid OBs at P3 was similar to that of Sfbac OBs. The recovered gene in the Sf29null virus was 98 to 100% homologous to the *Sf29* genes of different SfMNPV genotypes. Reverse transcription-PCR analysis of uninoculated *S. frugiperda* larvae confirmed the expression of the SfMNPV *ie-0* and *Sf29* genes, indicating that the insect colony harbors a covert SfMNPV infection. Additionally, the nonessential bacterial artificial chromosome vector was spontaneously deleted from both viral genomes upon passage in insects.

Nucleopolyhedroviruses (NPVs) (family *Baculoviridae*) infect the larvae of many important lepidopteran pests, and several have been developed as the basis for commercial biopesticides (28). Natural populations of the fall armyworm *Spodoptera frugiperda* (Lepidoptera) suffer *S. frugiperda* multiple nucleopolyhedrovirus (SfMNPV) disease, and some SfMNPV isolates have the potential to control this pest (12, 16, 33, 44). The complete genome sequences of two isolates have been published (16, 44). A core set of 31 genes is common to all baculoviruses, other genes are present in all group II NPVs, and others are unique to this virus. Homologues of the SfMNPV ORF29 (*Sf29*) are found only in group II NPVs. The role of the *Sf29* gene in the structure and replication of this virus has been studied by using bacmid technology to produce a virus mutant (Sf29null) that lacks the gene (36). *Sf29* encodes a viral factor that regulates the number of virions within the viral occlusion bodies (OBs). The DNA content of Sf29null bacmid OBs was reduced compared to that of wild-type OBs, and this difference was correlated with a reduced number of occlusion body-derived virions (ODVs) occluded in each OB of the Sf29null virus. ODVs are released from OBs in the insect gut and are responsible for establishing primary infection in midgut cells. Consequently, deletion of *Sf29* results in a virus with lower infectivity so that greater numbers of OBs are required to initiate lethal infection in insects that consumed Sf29null compared to the number required for lethal infection in wild-type OBs (36).

Genes have been inserted or removed from NPV genomes to produce recombinant viruses with improved insecticidal properties (26, 38), and these viruses have been subjected to field testing (7, 39, 40). In the present study, we aimed to determine the stability of a deletion recombinant NPV in host larvae, an issue of direct relevance to environmental risk assessment studies on the genetic stability of these viruses. For this, we compared the stability of an SfMNPV bacmid (Sfbac) derived from the wild-type virus with that of the Sf29null bacmid in *S. frugiperda* larvae during sequential rounds of *per os* infection. Different genotypic and phenotypic characteristics of the OBs were examined at each passage.

MATERIALS AND METHODS

Larvae, insect cells, and viruses. A laboratory colony of *S. frugiperda* larvae was maintained on a wheat germ-based semisynthetic diet (15) at 25°C. Sf21 cells were maintained in TC100 medium supplemented with 10% fetal calf serum (FCS) at 28°C. SfMNPV wild-type (SfWT) OBs were propagated in fourth-instar *S. frugiperda* larvae as described previously (34, 35, 36). Sfbac and Sf29null bacmid OBs were obtained by injecting larvae with a DNA suspension including bacmid DNAs (100 ng/μl) purified from individual colonies of *Escherichia coli* culture and Lipofectin reagent in a ratio of 2:1 (vol/vol). A 10-μl volume of this suspension was used to inject each larva (667 ng/larva) (36). SfWT, Sfbac, and Sf29null OBs were purified, and DNA extraction was performed (34, 36).

Successive passages *in vivo*. OBs obtained from virus DNA-inoculated larvae were designated passage zero (P0) OBs. These OBs were purified and fed to *S. frugiperda* larvae. Groups of 25 fourth-instar larvae that had recently molted were fed with a suspension of 1×10^8 OBs/ml (equivalent to 1×10^5 OBs/larva) of each virus, which resulted in ~90% mortality. Insects that died of NPV disease were pooled in groups of 22 to 24, and OBs were extracted and purified. These OBs, representing the total production of the infected experimental insect population, were considered passage one (P1) OBs and were used as an inoculum to infect the subsequent group of larvae (25 for each passage and virus treatment). The virus populations were followed for four additional rounds of *per os* infection

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(P2, P3, P4, and P5). A sample of OBs produced at each passage was used to determine the genotypic and phenotypic characteristics of OBs as described below. The entire experiment was performed on three different occasions.

DNA content. The DNA content of OBs originating from infected insects at each passage was estimated by extraction of DNA from samples of 5×10^8 OBs of each virus (36). First, purified OB suspensions were quantified by counting in triplicate using an improved Neubauer hemocytometer. Three different DNA extractions were then performed for each virus at each passage, and DNA quantification procedures were performed three times on each sample. The results were subjected to Kruskal-Wallis and Mann-Whitney nonparametric analyses by using the SPSS program (version 15.0).

ODV content. The mean virion titer per OB at each passage was determined by end point dilution (36). For this, ODVs were released from 5×10^8 OBs in a volume of 500 μl by mixing with an equal volume of 0.1 M Na_2CO_3. A 30-μl volume of 5.4 M HCl was then added to adjust the pH to near neutrality. This suspension was passed through a 0.45-μm filter and serially diluted 1:5 in TC100 medium. Volumes (10 μl) of each dilution were used to infect 10^4 Sf21 cells that had been previously prepared in 96-well plates. Twenty-four separate wells were inoculated with each dilution. Dishes were sealed with masking tape and incubated at 28°C for 10 days. The experiment was performed three times. Fifty percent tissue culture infectious doses ($TCID_{50}$s) were estimated by the Spearman-Kärber method (23) and were subsequently converted to infectious units per 5×10^8 OBs for presentation in the figures. Results were subjected to Kruskal-Wallis and Mann-Whitney nonparametric analyses by using the SPSS program (SPSS version 15.0).

Quantification of the relative proportion of the genomes carrying the *Sf29* gene. The relative proportion of genomes carrying the *Sf29* gene at each passage was estimated by semiquantitative PCR using purified DNA from OBs and primers that differentially amplify the *Sf29* gene (Sf29del3-Sf29del4) and kanamycin resistance cassette (Sf29del1-Sf29del2) (36). Calibration of the technique revealed that the presence of *Sf29* could be consistently detected in a sample of 1 pg of genomic DNA, representing ~6,000 copies of the gene. The lengths of the amplified fragments were calculated to be 2,500 bp for genomes that possess the *Sf29* gene and 1,000 bp for those carrying the kanamycin resistance cassette. Reactions were stopped in the linear phase of amplification (25 cycles) determined in preliminary assays. PCR products were separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and photographed on a UV transilluminator. The relative proportions of each genotype were then estimated by densitometric analysis of the intensities of each product by using the Scion Image PC program (Scion Corp., Frederick, MD). All reactions and measurements of product intensities were performed three times.

Quantification of colonies grown on chloramphenicol and chloramphenicol-kanamycin plates. The numbers of colonies grown on chloramphenicol and chloramphenicol-kanamycin plates were used to estimate the proportions of genomes carrying the bacmid replicon and the kanamycin resistance cassette at each passage. Sfbac is a chloramphenicol-resistant virus, whereas Sf29null is chloramphenicol and kanamycin resistant, as its *Sf29* was replaced by a kanamycin resistance cassette. For this, 150 ng (each) of Sfbac and Sf29null purified DNAs, extracted from 5×10^8 OBs at each passage, was used to transform DH510B GeneHogs electrocompetent cells in triplicate. Transformed cells were incubated at 37°C for 1 h in SOC medium (20 g tryptone, 5 g yeast extract, 0.5 g NaCl, and 18 g glucose per liter), and colonies were selected in the presence of chloramphenicol for Sfbac and chloramphenicol and chloramphenicol-kanamycin for the Sf29null bacmid. Three different DNA extractions were performed per virus at each passage. Results were subjected to analysis of variance and means separation by the Bonferroni procedure by using the SPSS program (version 15.0).

Genomic stability. The genomic stability of each virus was examined by restriction endonuclease (REN) analysis. DNA was extracted from 5×10^8 OBs after each passage. The stabilities of Sfbac and Sf29null bacmids were also determined by REN analysis of DNA from 15 individual colonies grown on chloramphenicol plates after transformation of *E. coli* cells with 150 ng of Sfbac and Sf29null DNAs purified from OBs at each passage. Colonies were amplified, and bacmid DNAs were purified by alkaline lysis. For each virus, 1.5 μg of DNA was mixed with 10 units of PstI (New England Biolabs) and incubated for 4 to 6 h at 37°C. Reactions were stopped by the addition of 4 μl of loading buffer (0.25% [wt/vol] bromophenol blue, 40% [wt/vol] sucrose in water). Electrophoresis was performed using horizontal 1% agarose gels in TAE buffer (0.04 Tris-acetate, 0.001 M EDTA, pH 8.0) at 15 V for 16 to 20 h. DNA fragments were stained with ethidium bromide and photographed on a UV transilluminator.

OB infectivity. The 50% lethal concentration (LC_{50}) of the OBs from each virus at each passage was determined by *per os* bioassay (20). Second-instar *S. frugiperda* larvae were starved for 8 to 12 h at 25°C and then allowed to drink from an aqueous suspension containing 10% (wt/vol) sucrose, 0.001% (wt/vol) Fluorella blue (Hilton-Davis, Cincinnati, OH), and OBs. For SfWT and Sfbac at all passages and Sf29null at P3, P4, and P5, the concentrations assayed were 1.2×10^6, 2.4×10^5, 4.8×10^4, 9.6×10^3, and 1.9×10^3 OBs/ml. For the Sf29null virus at P0, P1, and P2, the concentrations were 6.0×10^6, 1.2×10^6, 2.4×10^5, 4.8×10^4, and 9.6×10^3 OBs/ml. Each range of concentrations was previously determined to kill between 95% and 5% of the experimental insects. Bioassays with 25 larvae per virus concentration and 25 control larvae that had not ingested OBs were performed four times. Larvae were reared at 25°C, and virus mortality was recorded every 12 h until the insects had either died or pupated. Virus-induced mortality was subjected to probit analysis using the PoloPlus program (LeOra software).

RT-PCR detection of covert virus infection in insectary-reared *Spodoptera frugiperda*. Total RNA was individually extracted from 30 putatively uninfected fourth-instar larvae by using Trizol reagent (Invitrogen) by following the manufacturer's instructions. All materials and reagents were previously sterilized and treated with diethyl pyrocarbonate to eliminate RNases. RNA samples were treated with DNase prior to reverse transcription-PCR (RT-PCR) to eliminate contaminant DNA. To verify the absence of DNA, PCR was performed on all samples. The same quantity of treated RNA (1 μg) was used for the detection of the immediate-early *ie-0* and the highly transcribed very late polyhedrin gene transcripts. RT-PCR was performed in two different steps. First, cDNA synthesis was performed using the Improm-II reverse transcriptase (Promega) and the internal reverse oligonucleotides specific to *ie-0* and polyhedrin viral genes as previously described (25). Following this, the mixtures were subjected to PCR amplification with a *Taq* DNA polymerase (Bioline) and the forward and reverse primer mixture for each gene (25). RT-PCR products were electrophoresed in 1% agarose gels, stained with ethidium bromide, photographed on a UV transilluminator, and analyzed using the Molecular Analyst program (Bio-Rad). Finally, to confirm the presence of SfWT infections in colony insects, an RT-PCR assay was performed on 30 putatively healthy insects from the colony by using primers that specifically amplified the *Sf29* gene, as previously described (36). Calibration assays revealed that the presence of *Sf29* transcripts could be consistently detected in a sample of 0.1 pg of cDNA, representing ~600 copies of the transcript. Amplicons were cloned into the pGEM-T Easy vector (Promega) and sequenced (Sistemas Genómicos SL, Valencia, Spain).

RESULTS

DNA content of Sf29null bacmid OBs increased during serial passage. The identities of Sfbac and Sf29null bacmid DNAs from *E. coli* culture were confirmed by REN analysis and PCR (data not shown). The restriction profile of the Sf29null virus at P0 was clearly less intense than those of SfWT and Sfbac (see Fig. S1 in the supplemental material), indicating a reduced DNA content in Sf29null OBs, as previously observed (36). The restriction profile of the Sf29null bacmid increased in intensity during successive passages, and at P3, the intensity was similar to those of samples originating from SfWT and Sfbac OBs (Fig. S1). The SfWT profile did not vary over successive rounds of *per os* infection, whereas in Sfbac and Sf29null viruses, the fragment representing the bacterial artificial chromosome (BAC) replicon decreased in intensity. In addition, the band corresponding in size to the PstI-L fragment, in which *Sf29* is located, increased in intensity (Fig. S1).

No significant differences in the mean concentrations of DNA in OB samples were detected between SfWT and Sfbac in any passage ($P > 0.05$) (Fig. 1). In contrast, Sf29null OBs yielded significantly less DNA at P0 ($P < 0.05$), and the yield increased over successive passages and was similar to those of SfWT and Sfbac after three passages ($P > 0.05$).

ODV content of Sf29null bacmid OBs increased during serial passage. Samples of 5×10^8 OBs of SfWT and Sfbac viruses produced similar titers of infectious units (ODVs) at each passage ($P > 0.05$) (Fig. 2). In contrast, at P0, the same number of Sf29null OBs had around 10-fold fewer ODVs than

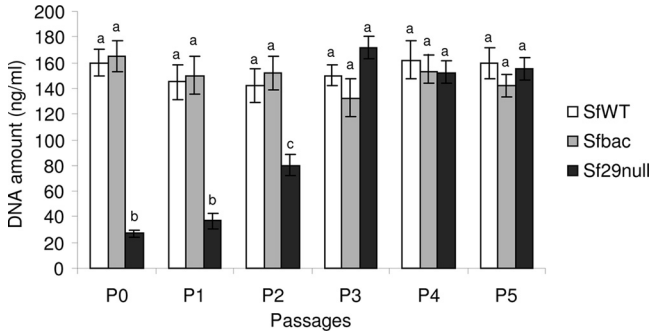

FIG. 1. Mean concentrations of DNA (ng/ml) extracted from samples of 5×10^8 OBs of SfWT, Sfbac, and Sf29null bacmids at each passage. Three different DNA extractions per virus were performed at each passage. DNA concentrations of each sample were measured three times. Different letters above the bars indicate significant differences between treatments and passages (Mann-Whitney test, $P \leq 0.05$).

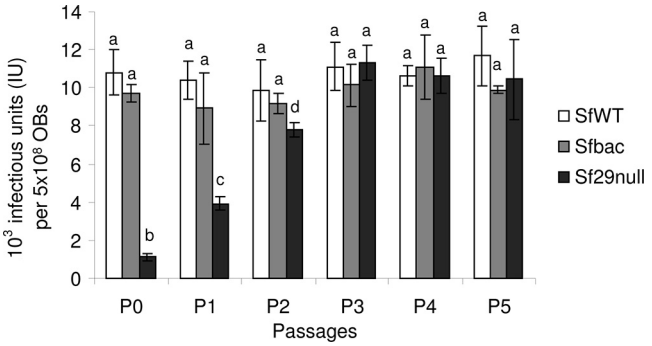

FIG. 2. ODV contents in 5×10^8 OBs of SfMNPV wild-type (SfWT), SfMNPV bacmid (Sfbac), and Sf29null bacmid viruses at each passage (P0, P1, P2, P3, P4, and P5). Sf21 cells were serially infected (1:5, 1:25, 1:125, and 1:625) with ODVs released from OBs. ODV titers (ODV/ml) were calculated by end point dilution in triplicate. Different letters above the bars indicate significant differences between treatments and passages (Mann-Whitney test, $P \leq 0.05$).

SfWT or Sfbac OBs ($P < 0.05$). However, in line with the results for the DNA content of OBs, the ODV titers of Sf29null OBs increased over successive passages and at P3, P4, and P5, Sf29null OBs produced titers of infectious units similar to those of SfWT and Sfbac OBs ($P > 0.05$) (Fig. 2).

Frequency of genomes carrying *Sf29* in Sf29null bacmid OBs increased during serial passage. At P0, the proportion of genomes containing *Sf29* was below the detection level in the Sf29null OBs, but during successive rounds of *per os* infection, this proportion increased, and at P4 and P5, the proportion of the genomes containing *Sf29* was similar to the proportion of genomes containing the kanamycin resistance cassette (~50%) (Fig. 3). Over the course of three passages, the frequencies of genomes containing the kanamycin resistance cassette and those containing the *Sf29* gene converged to a common ratio comprising approximately 50% in all three repetitions (Fig. 3). To amplify the recovered gene, a PCR was performed on the DNA obtained from the Sf29null OBs at P5 by using primers that amplified outside the coding region (36). Sequence analysis of this PCR product showed 100% homologies with the *Sf29* gene of a Nicaraguan isolate (SfNIC) (35) and a fast-killing isolate of SfMNPV from the United States (16) and 97% homology with the *Sf29* gene of the Brazilian isolate SfMNPV-19 (44; data not shown).

The numbers of colonies produced by Sfbac and Sf29null bacmid DNAs decreased during serial passage. The numbers of *E. coli* colonies grown on chloramphenicol plates after transformation of Sfbac (Fig. 4A) and Sf29null (Fig. 4B) DNAs decreased during successive passages, suggesting the loss of the BAC replicon. In addition, the number of colonies produced by Sf29null DNA samples on chloramphenicol-kanamycin plates and the relative proportions of colonies grown in chloramphenicol relative to colonies grown in chloramphenicol-kanamycin also decreased during serial passage, from a ratio of 1:1 at P0 to ratios of 1:0.71, 1:0.31, 1:0.17, 1:0.09, and 1:0.05 at P1, P2, P3, P4, and P5, respectively. These results indicate that the kanamycin resistance cassette tended to be lost upon passage, whereas *Sf29* tended to be acquired during sequential passage, as evidenced by the increasing proportion of genomes containing this gene. However, the results shown in Fig. 4 represent an indirect measure of the increased number of *Sf29* genomes. Loss of the kanamycin resistance cassette does not imply that the cassette was replaced by the *Sf29* gene. The kanamycin resistance cassette could have been lost without replacement by *Sf29*, as occurred with the chloramphenicol resistance cassette, which was lost during serial passage in previous studies (1, 31, 43). Different genotypes appear to have been generated spontaneously during passages; some lost the chloramphenicol resistance cassette or the kanamycin resistance cassette, whereas others appear to have acquired the *Sf29* gene, so that the Sf29null OBs became a mixture of genotypes at each passage. In contrast, the semiquantitative PCR (Fig. 3) represents a direct measure of the genomes containing *Sf29* or the kanamycin resistance cassette.

Genomic stability. At P0, all the colonies analyzed presented the expected Sfbac REN profiles (see Fig. S2A in the supplemental material), whereas at P3, the fragment representing the BAC replicon varied in size among the different colonies (Fig. S2B), suggesting recombination in this genomic region. This variability was also observed at P4 and P5 (data not shown). All the colonies derived from Sf29null OBs sampled at P0 presented the expected Sf29null bacmid REN profile (Fig. S2A), whereas at P3, a high degree of genomic instability was observed, not only in the fragment representing the BAC replicon but also in several other SfMNPV genomic restriction fragments (PstI-F, PstI-K, and PstI-L). This suggests a high frequency of recombination in this region (Fig. S2B), in which *Sf29* is located (PstI-L) and which has been identified as a variable genomic region in SfWT genotypic variants (34, 35). The fragment representing the BAC replicon was present in all the Sfbac and Sf29null colonies analyzed, as all colonies were selected on chloramphenicol plates.

OB infectivity. At all passages, SfWT OBs were more infectious than Sfbac OBs, as observed in previous studies (36). No significant changes were observed in the infectivity of Sfbac OBs at the different passages ($P > 0.05$) (Table 1). However, as expected, differences were observed in the infectivities of Sf29null OBs. At P0, the LC_{50} of Sf29null OBs in *S. frugiperda* larvae was approximately 20-fold greater than the LC_{50} of Sfbac OBs (Table 1). However, Sf29null OB infectivity increased significantly during successive rounds of *per os* infec-

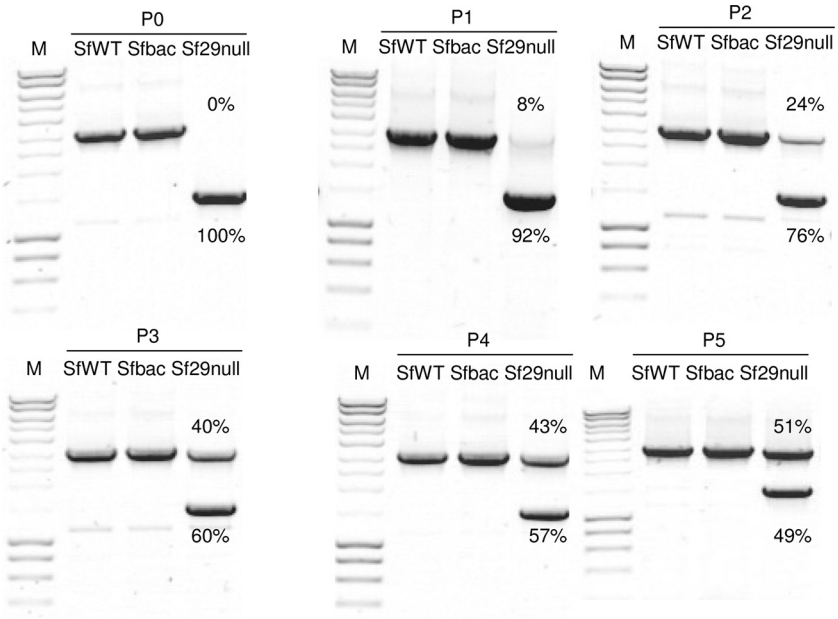

FIG. 3. Semiquantitative PCR analysis of the relative proportions of kanamycin resistance cassette- and *Sf29*-carrying genomes in the OBs from the SfMNPV wild-type (SfWT), SfMNPV bacmid (Sfbac), and Sf29null bacmid viruses at each passage (P0, P1, P2, P3, P4, and P5). Percentages next to amplicons indicate the relative proportions of each product as estimated by densitometric analysis (Scion Image program). M, molecular mass marker.

tion, and at P3, P4, and P5, the LC_{50}s of Sf29null OBs were similar to those of Sfbac OBs (Table 1). These results suggested that Sf29null had recovered *Sf29* function following three rounds of *per os* infection.

RT-PCR detection of SfMNPV *ie-0* and *Sf29* transcripts in insectary-reared *S. frugiperda* larvae. RT-PCR analysis of total RNA extracted from untreated insects taken from the laboratory colony indicated that this *S. frugiperda* colony harbors a covert SfMNPV infection. Primers that specifically amplified the SfMNPV genome were used. *ie-0* transcripts were detected in 8 of the 30 *S. frugiperda* larvae (~25%) analyzed from the colony (see Fig. S3 in the supplemental material), whereas polyhedrin gene transcripts were not detected by using the same RNA samples. Of the 30 putatively healthy colony insects that were subjected to RT-PCR using *Sf29*-specific primers, six larvae (~20%) had detectable amplification products. Sequence analysis of a 900-bp section from the central region of each product revealed that the amplicons were 100% homologous to the *Sf29* gene of SfWT (data not shown).

DISCUSSION

The genetic stability of an SfMNPV recombinant deletion mutant was examined following sequential rounds of *per os* infection in host larvae. After three rounds of infection, the genotypic and phenotypic characteristics of the Sf29null bacmid OBs were similar to those of Sfbac OBs, suggesting the occurrence of recombination events by which the Sf29null bacmid had recovered the *Sf29* gene. We discarded the possibility of viral contamination of the Sf29null viral inoculum, since Sf29null bacmid DNA was purified by alkaline lysis from *E. coli* culture and the identity of the sample was confirmed by PCR. Moreover, the experiment was performed on three separate occasions, thus reducing the possibility of chance contamination.

We questioned whether the recovered gene came from the host or from another virus causing a covert infection in the insect colony. The exchange of genetic material between coinfecting baculoviruses may be a common event among strains of the same or closely related virus species (10, 11, 21). There is also evidence of gene exchanges between baculoviruses and their hosts (13, 19) and other infectious agents of insects (17, 19, 24, 27) over much longer timescales. The observed absolute homology with the *Sf29* gene of the Nicaraguan isolate (35) suggested that the *S. frugiperda* laboratory colony harbors a covert infection produced by a genotypic variant of SfMNPV, which was confirmed by RT-PCR analysis of *ie-0* expression and by sequencing RT-PCR-generated products of *Sf29* transcripts from apparently healthy laboratory insects. Therefore, the Sf29null virus most likely acquired *Sf29* from a covert SfMNPV present in the laboratory *S. frugiperda* population. Viruses may persist when hosts are rare by adopting sublethal or latent infection strategies (3, 4, 5, 41), in a manner similar to that of herpes viruses (6, 32) or Epstein-Barr virus (18). Covert infections have been described as a mechanism for long-term persistence of baculoviruses in insects (3, 5). Insects that survive a virus challenge as larvae could potentially retain a sublethal virus infection, which is then transmitted vertically to the next generation (8, 14, 22, 41, 42). Indeed, covert infection and vertical transmission of NPV have previously been described in *S. frugiperda* laboratory populations (14, 25).

The proportion of Sf29null genomes that carried *Sf29* increased during serial passage but did not become dominant; an equilibrium was reached between genomes carrying *Sf29* and those with the kanamycin resistance cassette. Model predic-

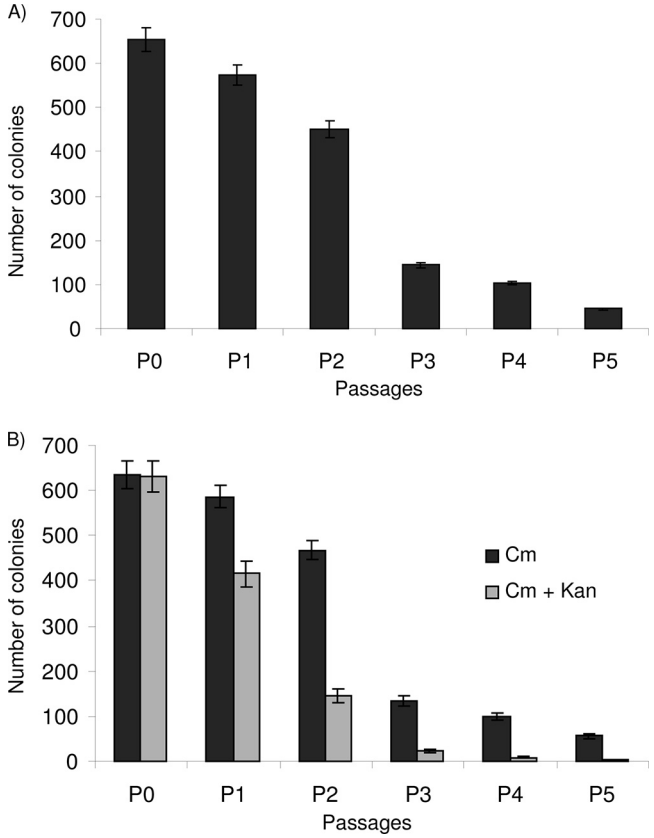

FIG. 4. Numbers of colonies grown in chloramphenicol (Cm) and chloramphenicol-kanamycin (Cm + Kan) plates, after transformation of DH5B10 electrocompetent cells with 150 ng of Sfbac (A) and Sf29null (B) DNAs at each passage (P0, P1, P2, P3, P4, and P5) in triplicate. Three DNA extractions were performed per virus at each passage. The proportions of colonies grown in chloramphenicol relative to colonies grown in chloramphenicol-kanamycin after transforming 150 ng of Sf29null DNA were 1:1, 1:0.71, 1:0.31, 1:0.17, 1:0.09, and 1:0.05 during passages P0 to P5, respectively.

tions indicate that it would take more than 40 rounds of infection for the frequency of a selectively neutral genotype to drop from 50% to 1% (2). As each cell is infected by multiple virions during the systemic phase of baculovirus infection, mutant genomes can persist by complementation and reach equilibrium in baculovirus populations with only a relatively minor replication advantage (2).

The genomic instability observed for the Sf29null virus was located within PstI-F, PstI-L, and PstI-K fragments, which form a variable genomic region of the SfNIC isolate (34, 35). This genomic region has also been observed to be variable in the highly colinear genome of a *Spodoptera exigua* multiple NPV (SeMNPV) isolate from the United States (29). The fact that *Sf29* represents an important viral factor that determines the potency of OBs and that it is located in an instable genomic region would favor the appearance and proliferation of recombinants generated during serial passage.

The nonessential BAC vector including the expression cassette was spontaneously deleted from the Sfbac and Sf29null genomes. Pijlman et al. (31) observed that the BAC vector from *Autographa californica* multiple NPV (AcMNPV) was also spontaneously deleted from the viral genome upon passage in insect cells. In this case, the drop in foreign protein production levels was explained by the predominance of those mutants upon passage. Instability of mini-F plasmids, which are also known as BACs, in eukaryotic cells has been reported to occur in several other cases (1, 37, 43). The BAC vector itself may display a certain intrinsic genetic instability, as it represents a nonessential foreign sequence. Alternatively, the heterologous gene may confer a certain level of toxicity to the infected cells, thereby creating an added selection pressure against intact bacmids (30, 31). In the present study, deletion of nonessential genomic regions during serial passage was observed to be a common feature in whole insect larvae, presumably because there was no adaptive advantage for the viruses to maintain these sequences.

TABLE 1. Probit regression for Sf29null and SfWT at each passage compared with Sfbac in second-instar *S. frugiperda* larvae[a]

Passage	Virus	Intercept ± SE	LC_{50} (OBs/ml)	Relative potency	Fiducial limit (95%)	
					Low	High
P0	Sfbac	−4.778 ± 0.385	1.03×10^5	1.0		
	Sf29null	−4.637 ± 0.492	1.96×10^6	0.053	0.023	0.123
	SfWT	−4.390 ± 0.362	3.81×10^4	2.709	1.704	4.306
P1	Sfbac	−4.720 ± 0.384	1.17×10^5	0.880	0.547	1.417
	Sf29null	−4.982 ± 0.507	1.24×10^6	0.083	0.041	0.169
	SfWT	−5.043 ± 0.396	3.50×10^4	2.944	1.892	4.582
P2	Sfbac	−4.712 ± 0.391	1.49×10^5	0.690	0.425	1.120
	Sf29null	−4.991 ± 0.462	5.56×10^5	0.186	0.104	0.330
	SfWT	−4.826 ± 0.383	3.55×10^4	2.905	1.855	4.549
P3	Sfbac	−4.720 ± 0.384	1.17×10^5	0.880	0.528	1.373
	Sf29null	−4.836 ± 0.396	1.53×10^5	0.677	0.418	1.096
	SfWT	−4.452 ± 0.368	3.30×10^4	3.124	1.969	4.958
P4	Sfbac	−4.729 ± 0.390	1.21×10^5	0.851	0.528	1.373
	Sf29null	−4.920 ± 0.395	1.18×10^5	0.870	0.544	1.391
	SfWT	−4.853 ± 0.387	3.66×10^4	2.813	1.793	4.411
P5	Sfbac	−4.608 ± 0.379	1.27×10^5	0.812	0.501	1.317
	Sf29null	−4.799 ± 0.388	1.22×10^5	0.849	0.529	1.362
	SfWT	−5.058 ± 0.391	3.56×10^4	2.900	1.865	4.510

[a] Probit regressions were fitted using the PoloPlus program (LeOra software, 1987). A test for nonparallelism was not significant ($\chi^2 = 19.89$, df = 17, $P = 0.280$). Lines were fitted with a common slope of 0.951 ± 0.0815 (standard error [SE]). Relative potencies were calculated as the ratios of effective concentrations relative to that of the Sfbac virus at passage P0.

Genes have been inserted into, and removed from, NPV genomes to improve the insecticidal properties of these viruses (9, 26, 38), and field testing of the efficacy of recombinant virus insecticides has been undertaken (7, 39, 40). The findings of the present study represent a clear example of why environmental risk assessment procedures should examine the stability of recombinant baculoviruses during successive passages in insects, with the aim of identifying regions of instability and likely recombination with naturally occurring latent or persistent virus infections in the target pest insect populations. They also suggest that deletion recombinant insecticides are likely to genetically "decay" following release, as they have the ability to acquire deleted genes from covertly infected host insects, though more studies are needed to confirm this hypothesis. Finally, these results underscore the need to ensure virus-free insect colonies during mass production of recombinant baculoviruses or studies on baculovirus biology.

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