Stability of a *Spodoptera frugiperda* Nucleopolyhedrovirus

Deletion Recombinant During Serial Passage in Insects

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Abstract

The stability 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 cassette, was 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 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 Sf29null virus was 98-100% homologous to the *Sf29* gene of different SfMNPV genotypes. RT-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 non-essential BAC vector was spontaneously deleted from both viral genomes upon passage in insects.
INTRODUCTION

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 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 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 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 the 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 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 virus 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 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* was maintained on a wheatgerm 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* 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 (v/v). A 10 µl volume of this suspension were 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). These OBs were purified and fed to *S. frugiperda* larvae. Groups of 25 newly molted fourth instars were fed with a suspension of 1x10^8 OBs/ml (equivalent to 1x10^5 OBs/larva) of each virus that resulted in ~90% of mortality. Insects that died of NPV disease were pooled in groups of 22 to 24, OBs were extracted and purified. These OBs, representing the total production of the infected experimental insect population, were considered as passage one (P1) and were used as 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 (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 5x10^8 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 Kruskall Wallis and Mann Whitney non-parametric analyses using the SPSS program (SPSS ver. 15.0).

**ODV content.** Mean virion titer per OB at each passage was determined by end point dilution (36). For this, ODVs were released from $5 \times 10^8$ OBs in a volume of $500 \mu l$ by mixing with an equal volume of $0.1 \text{ M Na}_2\text{CO}_3$. A $30 \mu l$ volume of $5.4 \text{ M HCl}$ was then added to adjust pH to near neutrality. This suspension was passed through a $0.45 \mu m$ filter and serially diluted $1:5$ in TC100 medium. Volumes of $10 \mu l$ 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 $28^\circ$C for 10 days. The experiment was performed three times. Tissue culture infectious dose 50 (TCID$_{50}$) values were estimated by the Spearman-Kärber method (23) and were subsequently converted to infectious units per $5 \times 10^8$ OBs for presentation in the figures. Results were subjected to Kruskall Wallis and Mann Whitney non-parametric analyses using the SPSS program (SPSS ver. 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 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 ~6000 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 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 using the Scion Image PC program (Scion Corp., Frederik, MD). All reactions and measurements of product intensities were performed three times.

Quantification of colonies grown in chloramphenicol and chloramphenicol+kanamycin plates. The number of colonies grown in chloramphenicol and chloramphenicol+kanamycin plates was used to estimate the proportion of genomes carrying the bacmid replicon and the kanamycin cassette at each passage. Sfbac is a chloramphenicol resistant virus, whereas Sf29null is chloramphenicol and kanamycin resistant, as Sf29 was replaced by a kanamycin cassette. For this, 150 ng of Sfbac and Sf29null purified DNAs, extracted from 5x10^8 OBs at each passage, were used to transform DH510B GeneHogs electrocompeent cells in triplicate. Transformed cells were incubated at 37 °C for 1 h in SOC medium 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 using the SPSS program (SPSS ver. 15.0).

Genomic stability. The genomic stability of each virus was examined by restriction endonuclease (REN) analysis. DNA was extracted from 5x10^8 OBs after each passage. The stability of Sfbac and Sf29null bacmids was 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 purified by alkaline lysis. For each virus, 1.5 µg of DNA were mixed with 10 units of PstI (New England Biolabs) and incubated for 4-6 h at 37°C. Reactions were stopped by adding 4 µl of loading buffer (0.25% w/v bromophenol blue, 40% w/v 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 during 16-20 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* were starved for 8 to 12 h at 25 °C and then allowed to drink from an aqueous suspension containing 10% (w/v) sucrose, 0.001% (w/v) Fluorella blue and OBs. For SfWT and Sfbac at all passages and Sf29null at P3, P4 and P5, the following OB concentrations were assayed: \(1.2 \times 10^6\), \(2.4 \times 10^5\), \(4.8 \times 10^4\), \(9.6 \times 10^3\) and \(1.9 \times 10^3\) OBs/ml, whereas for the Sf29null virus at P0, P1 and P2 the concentrations were \(6.0 \times 10^6\), \(1.2 \times 10^6\), \(2.4 \times 10^5\), \(4.8 \times 10^4\) and \(9.6 \times 10^3\) 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 instars using Trizol Reagent (Invitrogen) following manufacturer’s instructions. All materials and reagents were previously sterilized and treated with di-ethyl pyrocarbonate to eliminate RNases. RNA samples were treated with DNase prior to 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* genes transcripts. RT-PCR was performed in two different steps. First, cDNA synthesis was performed using the Improm-II\textsuperscript{TM} 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 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 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 (Fig. S1), 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 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 amounts 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), which increased over successive passages and was similar to that of SfWT and Sfbac after three passages (P > 0.05).
ODV content of Sf29null bacmid OBs increased during serial passage.

Samples of 5x10^8 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 ten-fold fewer ODVs than SfWT or Sfbac OBs (P < 0.05). However, in line with the results on the DNA content of OBs, the ODV titer of Sf29null OBs increased over successive passages and at P3, P4 and P5, Sf29null OBs produced similar titers of infectious units 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 cassette (~50%) (Fig. 3). Over the course of three passages the frequencies of genomes containing the kanamycin cassette and 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 using primers that amplified outside the coding region (36). Sequence analysis of this PCR product showed 100% homologies with the Sf29 gene of the Nicaraguan isolate (SfNIC) (35) and the fast killing isolate of the SfMN PV from USA (16) and 97% homology with the Sf29 gene of the Brazilian isolate SfMNPV-19 (44) (data not shown).

The number of colonies produced by Sfbac and Sf29null bacmid DNAs decreased during serial passage. The number 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 and chloramphenicol+kanamycin also decreased during serial
passage from a ratio of 1:1 at P0 to 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 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 in Fig. 4 represent an indirect measure of the increased number of Sf29 genomes. Loss of the kanamycin cassette does not imply that the cassette was replaced by the Sf29 gene. The kanamycin cassette could have been lost without replacement by Sf29, as occurred with the chloramphenicol cassette, that 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 cassette or the kanamycin 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 the Sf29 or kanamycin cassette.

**Genomic stability.** At P0, all the colonies analyzed presented the expected Sfbac REN profiles (Fig. S2 A), whereas at P3 the fragment representing the BAC replicon differed in size among the different colonies (Fig. S2 B), 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. S2 A), 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, PstI-L). This suggests a high frequency of recombination in this region (Fig. S2 B), 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 infectivity of Sf29null OBs. At P0 the LC$_{50}$ value of Sf29null OBs in S. frugiperda larvae was approximately twenty-fold greater than the LC$_{50}$ value of Sfbac OBs (Table 1). However, Sf29null OB infectivity increased significantly during successive rounds of per os infection and at P3, P4 and P5 the LC$_{50}$ of Sf29null OBs was similar to that 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 SeMNPV infection. Primers were used that specifically amplified the SfMNPV genome. ie-0 transcripts were detected in 8 of the 30 S. frugiperda larvae (~25%) analyzed from the colony (Fig. S3), whereas polyhedrin transcripts were not detected using the same RNA samples. Of the 30 putatively healthy colony insects that were subjected to RT-PCR using Sf29-specific primers, amplification products were detected in six larvae (~20%). 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 a 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 that recombination events had occurred 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 coinfected 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 absolute homology found 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 the *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* infection 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 laboratory populations of *S. frugiperda* (14, 25).

*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 cassette. Model predictions 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 in the Sf29null virus was located within PstI-F, PstI-L and PstI-K fragments that form a variable genomic region of the SfNIC isolate (34, 35). This genomic region has also been observed to be variable in the highly

The non-essential 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 non-essential 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 non-essential genomic regions during serial passage was observed to be a common feature in whole insect larvae, presumably as there was no adaptive advantage for the viruses to maintain these sequences.

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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REFERENCES


TABLE 1. Probit regression for the Sf29null bacmid (Sf29null) and SfMNPV wild-type (SfWT) at each passage (P0, P1, P2, P3, P4 and P5) compared with the SfMNPV bacmid (Sfbac) in second instar Spodoptera frugiperda larvae.

<table>
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<tr>
<th>Passages</th>
<th>Viruses</th>
<th>Intercept ± S.E.</th>
<th>LC₅₀ (OBs/ml)</th>
<th>Relative potency</th>
<th>Fiducial limits (95%)</th>
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<tbody>
<tr>
<td></td>
<td>Sfbac</td>
<td>-4.778±0.385</td>
<td>1.03x10⁵</td>
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<td>1.373</td>
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<tr>
<td>P3</td>
<td>Sf29null</td>
<td>-4.836±0.396</td>
<td>1.53x10⁵</td>
<td>0.677</td>
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<td>SfWT</td>
<td>-4.452±0.368</td>
<td>3.30x10⁴</td>
<td>3.124</td>
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<td>Sfbac</td>
<td>-4.729±0.390</td>
<td>1.21x10⁵</td>
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<td>P4</td>
<td>Sf29null</td>
<td>-4.920±0.395</td>
<td>1.18x10⁵</td>
<td>0.870</td>
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<td>SfWT</td>
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<td>3.66x10⁴</td>
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<td>Sfbac</td>
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<td>1.27x10⁵</td>
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<td>P5</td>
<td>Sf29null</td>
<td>-4.799±0.388</td>
<td>1.22x10⁵</td>
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<td>SfWT</td>
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Probit regressions were fitted using the PoloPlus program (LeOra Software, 1987). A test for non-parallelism was not significant (χ²=19.89, d.f.=17, P=0.280). Lines were fitted with a common slope of 0.951±0.0815 (S.E). Relative potencies were calculated as the ratio of effective concentrations relative to the Sfbac virus at passage P0.
FIG. 1. Mean amounts of DNA (µg) extracted from samples of 5x10^8 OBs of SfWT, Sfbac and Sf29null bacmids at each passage. Three different DNA extractions were performed per virus at each passage. DNA concentration of each sample was measured three times. Different letters above columns indicated significant differences between treatments and passages (Mann Whitney test, P ≤0.05).

FIG. 2. ODV content in 5x10^8 OBs of SfMNPV-WT (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, 1:625) with ODVs released from OBs. ODV titers (ODV/ml) were calculated by end point dilution in triplicate. Different letters above columns indicate significant differences between treatments and passages (Mann Whitney test, P ≤0.05).

FIG. 3. Semi-quantitative PCR analysis of the relative proportions of kanamycin cassette and Sf29 carrying genomes in the OBs from the SfMNPV-WT (SfWT), SfMNPV bacmid (Sfbac) and Sf29null bacmid viruses at each passage (P0, P1, P2, P3, P4 and P5). Figures next to amplicons indicate the relative proportions of each product estimated by densitometric analysis (Scion Image Program).

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 (4A) and Sf29null (4B) DNAs at each passage (P0, P1, P2, P3, P4 and P5) in triplicate. Three DNA extractions were performed per virus at each passage. The proportion of colonies grown in Cm and Cm+Kan after transforming 150 ng of Sf29null DNA was 1:1, 1:0.71, 1:0.31, 1:0.09, 1:0.17, 1:0.05 during passages P0 - P5, respectively.
Fig. 1

DNA amount (ng/ml)

SfWT
Sfbac
Sf29null

Passages

P0 P1 P2 P3 P4 P5
Fig. 2

The figure illustrates the abundance of infectious units (IU) per 5x10^8 OBs across different passages (P0 to P5). The x-axis represents the passages, and the y-axis shows the number of infectious units (x10^3 IU per 5x10^8 OBs). Three different conditions are compared:

- SfWT
- Sfbac
- Sf29null

Legend:
- SfWT
- Sfbac
- Sf29null

The bars marked with lowercase letters (a-d) indicate statistically significant differences between groups. The figure shows a consistent trend across passages, with Sf29null generally having the highest IU values, followed by Sfbac and SfWT.
Fig. 3
Fig. 4

A) 

![Bar graph showing the number of colonies for different passages.](image)

- **Passages**: P0, P1, P2, P3, P4, P5
- **Y-axis**: Number of colonies
- **Legend**: Cm, Cm + Kan

B) 

![Bar graph showing the number of colonies for different passages.](image)

- **Passages**: P0, P1, P2, P3, P4, P5
- **Y-axis**: Number of colonies
- **Legend**: Cm, Cm + Kan