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The "11K" gene family members *sf68*, *sf95* and *sf138* modulate transmissibility and insecticidal properties of Spodoptera frugiperda multiple nucleopolyhedrovirus

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ABSTRACT

The "11K" gene family is notable for having homologs in both baculoviruses and entomopoxviruses and is classified as either type 145 or type 150, according to their similarity with the *ac145* or *ac150* genes of Autographa californica multiple nucleopolyhedrovirus (AcMNPV). One homolog of *ac145* (*sf138*) and two homologs of *ac150* (*sf68* and *sf95*) are present in Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV). Recombinant bacmids lacking *sf68*, *sf95* or *sf138* (Sf68null, Sf95null and Sf138null, respectively) and the respective repair bacmids were generated from a bacmid comprising the complete virus genome. Occlusion bodies (OBs) of the Sf138null virus were ~15-fold less orally infective to insects, which was attributed to a 100-fold reduction in ODV infectious titer. Inoculation of insects with Sf138null OBs in mixtures with an optical brightener failed to restore the pathogenicity of Sf138null OBs to that of the parental virus, indicating that the effects of *sf138* deletion on OB pathogenicity were unlikely to involve an interaction with the gut peritrophic matrix. In contrast, deletion of *sf68* and *sf95* resulted in a slower speed-of-kill by 9 h, and a concurrent increase in the yield of OBs. Phylogenetic analysis indicated that *sf68* and *sf95* were not generated after a duplication event of an ancestral gene homologous to the *ac150* gene. We conclude that type 145 genes modulate the primary infection process of the virus, whereas type 150 genes appear to have a role in spreading systemic infection within the insect.

1. Introduction

There are three families comprising insect pathogenic viruses that produce occlusion bodies: *Baculoviridae*, *Poxviridae* and *Reoviridae* (Hajek, 2004). Reoviruses are double-stranded segmented RNA viruses, whereas the first two families are formed by double-stranded DNA viruses, being the family *Baculoviridae* the only one whose members are only infective to insects. Genomic comparisons of members of the family *Baculoviridae* and insect-specific *Poxviridae* (subfamily *Entomopoxvirinae*) have revealed four groups of genes that are present in some viruses belonging to both taxa, and absent in the members of these virus families that do not infect insects (Dall et al., 2001). These gene groups are known as the "11K", "57K", "tryptophan repeat" and "fusolin/gp37" groups and are considered as key components of insect-virus relationships that have evolved to exploit the host machinery in a highly effective manner (Dall et al., 2001).

The "11K" gene group encodes for a family of small proteins of between 90 and 110 amino acids, with a relative molecular mass of approximately 11,000 and a core C6 motif comprising six cysteine residues in a well defined spacing pattern (Dall et al., 2001). Members of this group are present in all lepidopteran nucleopolyhedroviruses (genus *Alphabaculovirus*) (Dall et al., 2001;

Abbreviations: AcMNPV, Autographa californica multiple nucleopolyhedrovirus; BmNPV, Bombyx mori nucleopolyhedrovirus; *H. virescens*, *Heliothis virescens*; LC50, lethal concentration 50%; MTD, mean time to death; nt, nucleotide; OB, occlusion body; ODV, occlusion derived virion; PIF, *per os* infectivity factor; qPCR, quantitative polymerase chain reaction; RH, relative humidity; REN, restriction endonuclease; SeMNPV, Spodoptera exigua multiple nucleopolyhedrovirus; SfMNPV, Spodoptera frugiperda multiple nucleopolyhedrovirus; SDS, sodium dodecyl sulfate; *S. exigua*, *Spodoptera exigua*; *S. frugiperda*, *Spodoptera frugiperda*; TCID50, 50% tissue culture infection dose; *T. ni*, *Trichoplusia ni*.

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Lapointe et al., 2004). Phylogenetic analysis of the C6 motif revealed two different gene types within the 11K group, type 145 and type 150, which received their names from the corresponding genes (*ac145* and *ac150*) of Autographa californica multiple nucleopolyhedrovirus (AcMNPV), the type species of the *Baculoviridae* (Dall et al., 2001). Both genes of AcMNPV, together with the type 150 gene, *bm126*, of Bombyx mori nucleopolyhedrovirus (BmNPV), contribute to key aspects of the phenotypic characteristics of their respective viruses, including oral pathogenicity, virulence or the infectivity of budded virions (BV) (Hao et al., 2009; Lapointe et al., 2004; Zhang et al., 2005). However, the influence of these genes on virus phenotype differs depending on the host species (Lapointe et al., 2004; Yamagishi et al., 2003; Zhang et al., 2005). Both AcMNPV and BmNPV belong to group I nucleopolyhedroviruses (Jehle et al., 2006), which is a group of viruses characterized by the presence of GP64 protein in the BV envelope (Hayakawa et al., 2000). In contrast, group II nucleopolyhedroviruses are characterized by the presence of F-protein instead of GP64 in the BV envelope. The effect of the "11K" protein family on the biological activity of group II nucleopolyhedroviruses has not been determined.

Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) is a group II nucleopolyhedrovirus (Zanotto et al., 1993). Three geographically distinct isolates of the virus have been completely sequenced (Harrison et al., 2008; Simón et al., 2011; Wolff et al., 2008). This sequence information revealed the presence of *sf138* that is homologous to *ac145*, as well as the presence of *sf68* and *sf95* that are homologs of *ac150*. As SfMNPV is being assessed as a potential biological insecticide in Latin America (Armenta et al., 2003; Barrera et al., 2011; Vieira et al., 2012) we decided to determine the role of the *sf68*, *sf95* and *sf138* genes in the infectivity of the SfMNPV on its natural host, *Spodoptera frugiperda*. With this aim, deletion mutants were constructed from the complete SfMNPV bacmid genome. The effect of each deletion on the phenotypic characteristics of SfMNPV was determined *in vitro* and *in vivo* in *S. frugiperda* cells and larvae.

2. Material and methods

2.1. Insects, cells and viruses

Larvae of *S. frugiperda* were obtained from a laboratory colony that originated from Honduras and was refreshed periodically with pupae from southern Mexico. Insects were maintained at 25 °C, 75% relative humidity (RH) and 16 h light: 8 h dark photoperiod and were reared on a wheatgerm-based semisynthetic diet (Greene et al., 1976). Sf9 cells were maintained in TC100 medium supplemented with 10% fetal calf serum at 28 °C (King and Possee, 1992). The SfMNPV-B genotype is the largest genotype present in a Nicaraguan isolate of SfMNPV (Simón et al., 2011, 2004). This genotype was selected for construction of the SfMNPV bacmid (Sfbac).

2.2. Construction of the deletion bacmids

Sf68null, Sf95null and Sf138null bacmids were generated after deletion of the respective *sf68*, *sf95* and *sf138* ORFs from Sfbac using the Red/ET Recombination system (Gene Bridges GmbH, Heidelberg, Germany). For this, two short homology arms were added to a kanamycin cassette by PCR in a two step procedure using the PrimeSTAR HS high fidelity DNA polymerase (Takara). First, a PCR fragment was amplified using the Tn5-neo PCR template and the respective primer pair (Sf68del.1 and Sf68del.2, Sf95del.1 and Sf95del.2, Sf138del.1 and Sf138del.2 in Table S1) that added 25 nucleotides (nt) homologous to either 3′ or 5′ untranslated regions of the corresponding genes. Then, in a second PCR, additional 25 nt terminal sequences were added using the PCR product of the first amplification and the corresponding primer pair: Sf68del.3 and Sf68del.4, Sf95del.3 and Sf95del.4 or Sf138del.3 and Sf138del.4 (Table S1). Bacteria containing Sfbac were made electrocompetent and transformed with the Red/ET plasmid pSC101-BAD-gbaA (Gene Bridges GmbH). The PCR products containing the kanamycin cassette and the flanking regions of the *sf68*, *sf95* and *sf138* genes, respectively, were used to transform the electrocompetent cells containing Sfbac and pSC101-BAD-gbaA. These cells were induced with arabinose (0.1–0.2% w/v) to express the recombination protein (gbaA). Sf68null, Sf95null and Sf138null recombinant bacmids were selected as resistant colonies on media containing chloramphenicol (resistance gene located in the pBACe3.6 vector) and kanamycin (which replaced the different genes). To confirm the deletion of the genes, *Pst*I restriction endonuclease (REN) analysis of the bacmid DNAs was performed and PCR amplifications using Sf68del.3/Sf68del.4, Sf95del.3/Sf95del.4 and Sf138del.3/Sf138del.4 primer pairs were sequenced. The deletion of *sf68* was located between the nucleotides 62,858 and 63,290 in the SfMNPV-B genome sequence (limits of the ORF: 62,859–63,314 nt) (Simón et al., 2011). The deletion of *sf95* was located between nucleotides 91,885 and 92,200 (limits of the ORF: 91,887–92,258 nt) whereas the deletion of *sf138* between the nucleotides 126,630 and 126,910 (limits of the ORF: 126,631–126,909 nt) (Simón et al., 2011).

2.3. Generation of the repair bacmids

The coding regions corresponding to *sf68*, *sf95* and *sf138* were amplified by PCR using primers spanning the regions of interest (Sf68rep.1 and Sf68rep.2, Sf95rep.1 and Sf95rep.2 and Sf138rep.1 and Sf138rep.2 in Table S1), PrimeSTAR HS high fidelity DNA polymerase (Takara) and SfMNPV-B DNA (Simón et al., 2011) as template. A 5 μl volume of a mixture containing 50 μl of the respective deletion bacmid DNA (100 ng/μl), 50 μl of the PCR product that included the coding region (500 ng/μl) and 50 μl of Lipofectin reagent (Invitrogen) was injected into fourth instar *S. frugiperda* larvae. Injected larvae were reared at 25 °C on semisynthetic diet until death or pupation. OBs were recovered from dead larvae and viral DNA extracted as described below. PCR was performed with Sf68del.3/Sf68del.4, Sf95del.3/Sf95del.4 and Sf138del.3/Sf138del.4 primer pairs to test whether the kanamycin cassette had been replaced by the respective genes and, thus, to check whether the OBs were composed by a mixture of the deleted and the repair viruses. Viral DNAs were then transfected into DH5α electrocompetent cells. Colonies were growth in medium containing chloramphenicol, to allow the growth of deleted and repair mutants, both containing the chloramphenicol resistance cassette in the bacmid region (Simón et al., 2008). Bacmid DNAs were purified by alkaline lysis and analyzed by REN and PCR to select those colonies containing the genes under study. PCR amplification products with primers outside the coding regions were sequenced to confirm the correct reinsertion of the genes in their original locus in the Sf68rep, Sf95rep and Sf138rep bacmids.

2.4. Computational analysis of protein amino acid sequences

To determine the nature of the SF68, SF95 and SF138 proteins, a search for conserved domains was performed using InterProScan (Zdobnov and Apweiler, 2001). Signal sequences were screened using SIGNALP 4.0 (Petersen et al., 2011) and the presence of transmembrane domains was determined using TMHMM (Jones, 2007). Homologs of SF68, SF95 and SF138 were searched for in the updated GENEBANK/EMBL database using BLAST (Altschul et al., 1990). In order to assess whether the two homologs of *ac150* in the SfMNPV genome were a result of a duplication event, the

sequences of all protein homologs of SF68, SF95 and AC150 were aligned using CLUSTAL-X (Thompson et al., 1994). A neighbor-joining phylogenetic tree was generated using njplot (Perrière and Gouy, 1996) using the option of excluding positions with gaps.

2.5. DNA infectivity and production of OBs

Bacmid DNAs were purified from *E. coli* colonies by alkaline lysis and cesium chloride gradient centrifugation (King and Possee, 1992). To study DNA infectivity and produce OBs, fourth instar *S. frugiperda* larvae were injected with 10 µl of a mixture containing 100 µl of bacmid DNA (10 µg of bacmid DNA) and 50 µl of Lipofectin reagent (Invitrogen) (Simón et al., 2012, 2008). Groups of 24 larvae were injected with each bacmid DNA. Inoculated larvae were individually transferred to diet and reared at 25 °C. Virus-induced mortality was recorded every day. The experiment was performed in triplicate. Results were subjected to ANOVA and Tukey's post hoc test using the SPSS v.15 software.

OBs obtained from corpses were extracted and filtered through cheesecloth. These were washed twice with 0.1% sodium dodecyl sulfate (SDS) and twice with double-distilled water, and finally resuspended in double-distilled water. OB suspensions were quantified by counting in triplicate using a Neubauer chamber and stored at 4 °C until used. To confirm the authenticity of the recombinant OBs, DNA was extracted from OBs as described in the following section and *Pst*I restriction endonuclease analysis and PCR were performed.

2.6. Viral DNA content within OBs

Virions were released from samples of 10^6 OBs of Sfbac, Sf68null, Sf95null, Sf138null, Sf68rep, Sf95rep and Sf138rep by mixing with 100 µl of 0.5 M Na_2CO_3 and 50 µl of 10% (w/v) SDS in a final volume of 500 µl and incubating at 60 °C during 10 min. Undissolved OBs and other debris were removed by low speed centrifugation (3800*g*, 5 min). The supernatant fraction containing virions was treated with 25 µl of proteinase K (20 mg/ml) and incubated at 50 °C for one hour. Viral DNA was extracted twice with 500 µl of phenol and once with chloroform and isolated by alcohol precipitation. The resulting pellet was resuspended in 50 µl of 0.1× TE buffer by incubation at 60 °C during 10 min. DNA samples were diluted 1:100 and quantified using qPCR based on SYBR green fluorescence in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Specific primers amplifying in the unique gene *sf43* of SfMNPV were used (Table S1). The PCR product resulting from specific amplification was cloned into the pGEM-T easy vector (Promega). Known dilutions of plasmid DNA were used as internal standards for qPCR. The reaction mixture (10 µl) contained 5 µl SYBR Premix Ex Taq (2×), 0.2 µl of each SfMNPV primer (10 pmol/µl) and 1 µl of DNA template. qPCR was performed under the following conditions: 95 °C for 2 min and 30 s, followed by 45 amplification cycles of 95 °C for 15 s and 60 °C for 30 s and finally a dissociation stage of 60 °C for 15 s and 95 °C for 5 s. Data acquisition and analysis were handled by Bio-Rad CFX Manager software (Bio-Rad). Melting-curve analysis was performed to confirm specific replicon formation in qPCR. Ten different DNA extractions were performed and both standards and samples were measured in triplicate. Data were subjected to ANOVA using the SPSS v.15 software after inverse transformation.

2.7. Infectivity of OBs

The lethal concentration 50% (LC_{50}), mean time to death (MTD) and OB production were determined in second instar *S. frugiperda* larvae by *per os* inoculation following the droplet feeding technique (Hughes and Wood, 1987). For each virus the range of OB concentrations estimated to kill between 5% and 95% of the insects, was determined in preliminary assays. The concentrations chosen for tests for Sfbac, Sf68null, Sf95null, Sf68rep, Sf95rep and Sf138rep were 1.9×10^3, 9.6×10^3, 4.8×10^4, 2.4×10^5 and 1.2×10^6 OB/ml, whereas higher concentrations were used for Sf138null: 4.8×10^4, 2.4×10^5, 1.2×10^6, 6.0×10^6 and 3.0×10^7 OB/ml. Bioassays were performed in triplicate using groups of 24 larvae per virus concentration and 24 control larvae. Larvae were reared individually on diet at 25 °C and virus mortality was recorded every day until larvae had died or pupated. Virus induced mortality data were subjected to probit analysis using the POLO statistical program (LeOra-Software, 1987).

To determine speed of kill, groups of 24 *S. frugiperda* second instars were inoculated with an OB concentration estimated to result in 90% mortality, namely, 1.94×10^6 OB/ml for Sfbac, 2.78×10^6 OB/ml for Sf68null, 2.05×10^6 OB/ml for Sf95null, 3.37×10^7 OB/ml for Sf138null, 1.96×10^6 OB/ml for Sf68rep, 2.15×10^6 OB/ml for Sf95rep and 2.06×10^6 OB/ml for Sf138rep. Inoculated larvae were reared individually on diet at 25 °C and mortality was recorded at 8 h intervals until larvae had either died or pupated. The experiment was performed in triplicate. Time-mortality data were subjected to Weibull analysis using the generalized linear interactive modeling (GLIM) program (Crawley, 1993).

Finally, OB yield was determined in larvae used in the speed of kill assays. Each corpse was homogenized in 100 µl distilled water and OBs were quantified in a Neubauer hemocytometer. For each larva the OB count was performed in duplicate in each of the three repetitions performed. Results were normalized by square root transformation and subjected to ANOVA and Tukey's post hoc test using SPSS 15.0.

2.8. Infectivity of ODVs

ODV infectivity was determined by end point dilution as previously described (Simón et al., 2012, 2008), as determining differences in ODV infectious titers is easier to manage in cell cultures than *in vivo*. Samples of 5×10^8 OBs were used for Sfbac, Sf68null, Sf95null, Sf68rep, Sf95rep and Sf138rep, whereas 2.75×10^9 OBs were used for Sf138null. ODVs were released by incubation with 0.1 M Na_2CO_3 at 28 °C for 30 min. This suspension was filtered through a 0.45 µm filter and serially diluted 1:5 in TC100 medium, resulting in dilutions of 1:10, 1:50, 1:250, 1:1250 and 1:6250. A 10 µl volume of each ODV dilution was used to inoculate wells containing 10^4 Sf9 cells in a 96-well plate. In total, 24 wells were inoculated with each dilution and the experiment was performed six times. Masking tape was used to seal the plates, which were incubated at 28 °C for 7 days and then examined for signs of virus infection. The resulting data were analyzed by the Spearman-Kärber method (Lynn, 1992) in order to determine the 50% tissue culture infectious doses ($TCID_{50}$). $TCID_{50}$ values were subsequently converted to infectious units per 5×10^8 OBs for representation in figures and were subjected to ANOVA and Tukey's post hoc test using SPSS 15.0.

2.9. Effect of an optical brightener on Sf138null OB infectivity

To determine whether SF138 had a role in the degradation of the host peritrophic matrix, a bioassay was performed, using OBs in mixtures with Leucophor AP ($C_{40}H_{42}N_{12}O_{10}S_{22}Na$, Clariant, Barcelona, Spain). This stilbene-derived optical brightener is known to bind chitin and disrupt the peritrophic matrix, facilitating ODV access to the midgut cells (Wang and Granados, 2000). If SF138 was involved in the degradation of the peritrophic matrix, then addition of Leucophor AP would be expected to restore the pathogenicity of Sf138null OBs to that of the parental viruses (Sfbac and Sf138rep). Groups of 24 s instar *S. frugiperda* larvae were

inoculated with 5×10^4 OB/ml of Sfbac, Sf138null or Sf138rep, in mixtures with or without 1% (V/V) Leucophor AP, following the droplet feeding technique (Hughes and Wood, 1987). In the absence of optical brightener, this OB concentration was expected to result in mortalities of ~50% for Sfbac and Sf138rep and ~30% for Sf138null. Mock infected larvae were used as controls. The experiment was performed in triplicate and mortality data were subjected to ANOVA and Tukey's post hoc test using SPSS 15.0.

3. Results

3.1. Generation of the recombinant bacmids

Replacement of the *sf68*, *sf95* and *sf138* ORFs in the Sf68null, Sf95null and Sf138null bacmids was confirmed by restriction endonuclease analysis and PCR with specific primers previously used for deletion of the predicted recombinant junction regions.

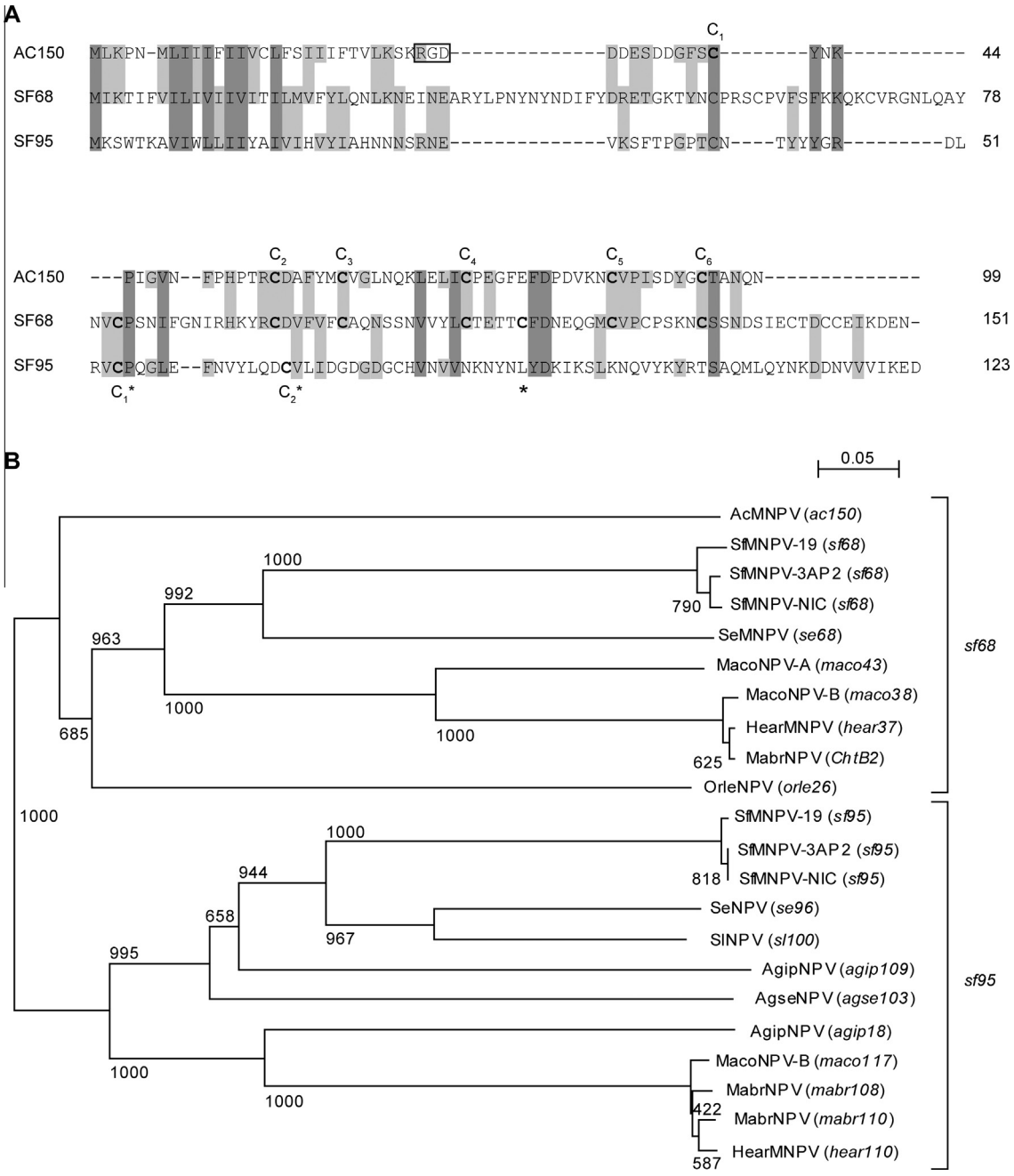

Fig. 1. Computational analysis of proteins. (A) Alignment of the predicted amino acid sequences of the SF68, SF95 and AC150 proteins. Sequence alignment was carried out using Clustal X (Thompson et al., 1994). Gaps introduced to optimize the alignment are indicated with dashes. The different shades of gray indicate the degree of consensus between the different sequences. The C_6 motif is shown by numbering the cysteines from C_1 to C_6. C_1^* and C_2^* indicate possible C_1 and C_2 in the C_6 motif of SF68 and/or SF95. Asterisk marks the cysteine present between C_4 and C_5 in the SF68. The Arg-Gly-Asp (RGD) motif in AC150 is indicated by a square. The sequences used were: *ac150* of AcMNPV (GenBank accession No. NC_001623) and *sf68* and *sf95* of SfMNPV (GenBank accession No. HM595733). (B) Phylogenetic tree of the genes with high similarity to *sf68* and *sf95*. The consensus phylogenetic tree was constructed by the neighbor-joining method. Bootstrap values for 1,000 replicates are shown. The sequences used were: *ac150* of AcMNPV (GenBank accession No. NC_001623); *agip18* and *agip109* of AgipNPV (GenBank accession No. NC_011345.1); *agse103* of AgseNPV (GenBank accession No. NC_005839.2); *hear 37* and *hear 110* of HearMNPV (GenBank accession No. NC_011615.1); *ChtB2*, *mabr108* and *mabr110* of MabrNPV (NC_023681.1); *maco43* of MacoNPV-A (GenBank accession No. NC_003529.1); *maco38* and *maco117* of MacoNPV-B (GenBank accession No. NC_004117.1); *orle26* of OrleNPV (GenBank accession No. NC_010276.1); *se68* and *se96* of SeMNPV (GenBank accession No. NC_002169.1); *sf68* and *sf95* of SfMNPV-19, SfMNPV-3AP2 and SfMNPV-NIC (GenBank accession No. EU258200.1, NC_009011.2 and HM595733); and *sl100* of SpltNPV-II (GenBank accession number NC_011616.1).

The genomic arrangement of the recombinants was also verified by sequencing. The deletion of the *sf68* ORF did not affect the adjacent ORFs *sf67* (61,945–62,847 nt) and *sf69* (63,311–64,315 nt). Both the *sf67* and *sf68* are located in the forward strand, whereas the *sf69* is located in the complementary strand. Hence, the deletion of *sf68* ORF did not affect *sf67* or *sf69* promoters. The deletion present in the Sf95null mutant did not affect the adjacent ORFs *sf94* (91,537–91,794 nt) and *sf96* (92,233–92,733 nt). The *sf94* is a late gene, whose promoter motif ATAAG located in the complementary strand between nucleotides 91,816 and 91,820 was not affected by the *sf95* deletion. However, although *sf95* deletion did not affect the cap site of the *sf96* promoter (CAGT, 92,214–92,217 nt), the TATA box (TATAAAT, 92,184–92,190 nt) of this promoter was deleted in the recombination process. Finally, the deletion of *sf138* did not affect the adjacent *sf137* (125,953–126,594 nt) and *sf139* ORFs (126,913–127,764 nt). The *sf137* is located in the forward strand, whereas both *sf138 and sf139* are present in the reverse strand. Hence, the *sf137* and *sf139* promoters are not affected in the Sf138null mutant. The same method confirmed the correct insertion of the respective genes in the Sf68rep, Sf95rep and Sf138rep bacmids.

3.2. Protein analysis and comparison with AC145 and AC150 proteins

SF68, SF95 and SF138 are small proteins of 92–151 amino acids (Aa). The SF68 protein showed 33% identity and 44% similarity to AC150, whereas SF95 showed 59% identity and 68% similarity to SE96 of Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV), which in turn has 32% identity and 55% similarity with AC150. Furthermore, the SF138 amino acid sequence had 42% identity and 59% similarity to AC145.

The C6 motif, also known as chitin binding motif, is present in AC145 and AC150 and was also found in SF138 and SF68, although in the deduced sequence of SF68 there is an additional cysteine between the C_4 and C_5 of the C6 motif (Fig. 1A). Alignment of the C6 motif with the amino acid sequence of SF95 is more complex and the cysteine pattern is not conserved (Fig. 1A). Furthermore, the RGD motif present in some natural variants of BM126 and AC150 (Hao et al., 2009) is not present in SF68 and SF95. No signal peptides were predicted in SF68, SF95, AC145 or AC150, whereas a putative signal peptide was detected in SF138 between 16 and 17 aa following analysis using SIGNALP. TMHMM analysis predicted one transmembrane domain for each AC150-like protein, located between residues 4–26 for SF68, residues 7–24 for SF95 and residues 7–25 for AC150. No transmembrane domains were detected for either SF138 or AC145.

Phylogenetic analysis of all proteins homologous to SF68, SF95 and AC150 revealed that SF95 and its homologs formed a completely different cluster from that of SF68 and AC150. Therefore, it appears unlikely that *sf95* arose by way of a duplication event of the *ac150* gene, but it may be possible that there was an ancestral gene that was duplicated forming two clades, the one comprising the SF68 and AC150-related proteins and the one comprising the SF95-related proteins. Furthermore, only granuloviruses (genus *Betabaculovirus*) and group II nucleopolyhedroviruses (genus *Alphabaculovirus*) were found to encode SF95 homologous proteins. The resulting phylogenetic tree (Fig. 1B) shows only proteins that are closer to SF68 and SF95 (BLAST total score higher than 50) and AC150.

3.3. Reduced DNA infectivity in deletion viruses

Intrahemocelic injection of Sf68null, Sf95null and Sf138null bacmid DNAs resulted in significantly lower mortalities (22–30%) of larvae than those observed after injection of the complete bacmid DNAs ($F_{6,14} = 49.314$, $p < 0.001$). No significant differences were detected in the mean insect mortality following injection with Sfbac, Sf68rep, Sf95rep, or Sf138rep DNA (54–58%) (Fig. 2A). These differences suggest that deletion bacmids are less pathogenic. DNA extracted from OBs obtained after DNA injection was subjected to restriction endonuclease (REN) analysis and PCR, which confirmed that viruses had similar DNA restriction profiles and amplification products to those obtained with the parental bacmid DNAs.

3.4. Viruses did not differ in DNA content per OB

No significant differences were observed in the mean amounts of DNA in OB samples ($F_{6,62} = 1.891$, $P = 0.097$), which varied from 11.5 to 18.0 ng DNA/10^6 OBs among the deletion viruses (Fig. 2B), compared to an average of 16.0 ng DNA/10^6 OBs for the Sfbac virus. Similar quantities were detected in samples of the repair viruses.

3.5. The sf138 gene affects the oral pathogenicity of OBs, whereas sf68 and sf95 affect the speed of kill and OB production

Deletion of *sf138* resulted in a 15-fold increase in the LC_{50} value (1.77×10^6 OBs/ml) of Sf138null OBs compared to Sfbac or Sf138rep OBs (Table 1). In contrast, LC_{50} values obtained for Sf68null (1.56×10^5 OBs/ml), Sf95null (8.84×10^4 OBs/ml) were similar to those of their repair viruses, or the Sfbac parental virus (1.19×10^5 OBs/ml). The 95% confidence levels of the relative potencies, representing the ratio of effective concentrations (Robertson and Preisler, 1992), overlapped broadly in these viruses indicating no significant differences in OB pathogenicity (Table 1).

Deletion of *sf138* did not significantly affect speed of kill of Sf138null compared to parental or repair viruses (Table 1). However, deletion of *sf68* or *sf95* resulted in significant but small increases (9 h) in MTD values compared to those of the parental or the repair viruses (Table 1).

OB production differed significantly between viruses (Fig. 2C; $F_{6,428} = 4.782$, $p < 0.001$). Deletion of *sf68* or *sf95*resulted in 1.6-fold and 1.7-fold increases ($4.27–4.07 \times 10^7$ OBs/larva) in OB production/larva compared to the Sfbac ($2.48 \pm 0.60 \times 10^7$ OBs/larva) or the Sf68rep ($2.58 \pm 0.63 \times 10^7$ OBs/larva) or Sf95rep ($2.51 \pm 0.60 \times 10^7$ OBs/larva) repair viruses. Deletion of *sf138* gene did not significantly affect OB production, as the mean production ($2.46 \pm 0.71 \times 10^7$ OBs/larva) was similar to that of the Sfbac and the repair virus Sf138rep (Fig. 2C).

3.6. Deletion of sf138 affects the infective titer of ODVs

ODV infectious titer was estimated to determine whether it was lower ODV titer that was responsible for the reduced pathogenicity of Sf138null OBs. Significant differences were observed in ODV titers between viruses ($F_{6,34} = 10.623$, $p < 0.001$). Deletion of *sf68* and *sf95* did not significantly influence ODV titer compared to that of the parental or repair viruses (Fig. 2D). In contrast, the ODV titer could not be determined after releasing ODVs from samples of 5×10^8 OBs of the Sf138null virus. The sample was increased to 2.75×10^9 OBs and the exact titer could still not be determined, so only estimation was performed. Sample concentration was not increased further because the efficacy of the alkaline treatment could be compromised by high concentrations of OBs. After converting ODVs titers to infectious units in 5×10^8 OBs, a decrease of more than 100-fold in ODV titers of Sf138null OBs was observed compared to the original and the repair virus ($2.6 \pm 0.3 \times 10^4$ IU/ml) (Fig. 2D).

3.7. Optical brightener did not restore the pathogenicity of Sf138null OBs

As *sf138* deletion resulted in a reduction in OB pathogenicity, we examined the possible involvement of SF138 in disruption of

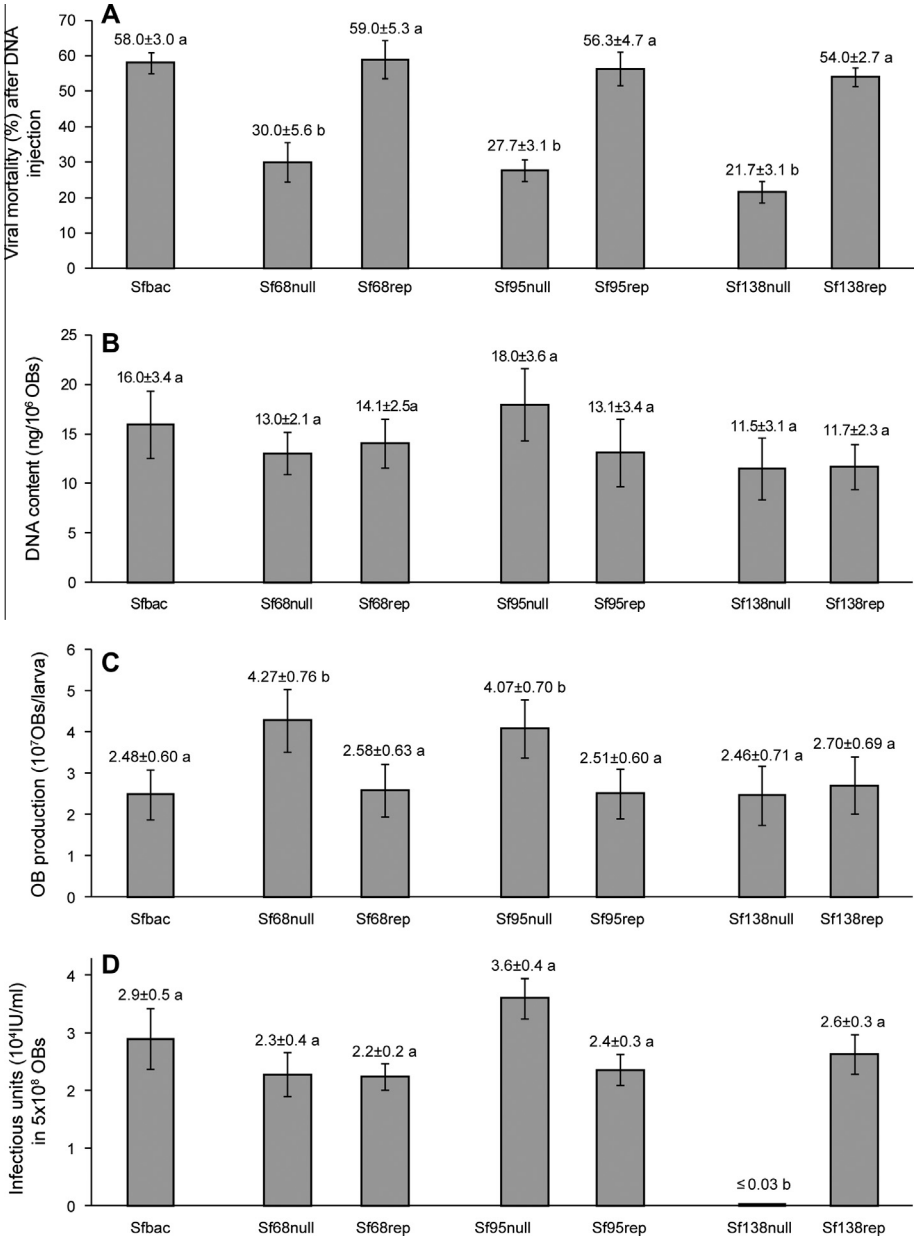

Fig. 2. Effects of gene deletions in the biological activity of the virus. (A) Mean virus-induced mortalities following DNA injection. Values above the columns indicate means and those labeled with different letters are significantly different ($p < 0.05$). Error bars indicate the standard error of the mean. (B) Mean amounts of DNA extracted from samples of 10^6 OBs of Sfbac, Sf68null, Sf95null, Sf138null, Sf68rep, Sf95rep and Sf138rep viruses. Values above columns indicate means. Error bars indicate the standard error of the mean. (C) OB production values in larvae infected with Sfbac, Sf68null, Sf95null, Sf68rep, Sf95rep and Sf138rep viruses. Values above the columns indicate means and those labeled with different letters differed significantly after square root transformation (ANOVA, Tukey test, $p < 0.05$). Error bars indicate the standard error of the mean. D) ODV infectious titers of Sfbac, Sf68null, Sf95null, Sf138null, Sf68rep, Sf95rep and Sf138rep viruses. Sf9 cells were serially infected (1:5, 1:25, 1:125, and 1:625) with ODVs released from OBs. ODV titers (ODV/ml) in 5×10^8 OBs were calculated by end point dilution. Values labeled with different letters differed significantly (ANOVA, Tukey test, $p < 0.05$). Error bars indicate the standard error of the mean.

the peritrophic membrane. The presence of Leucophor AP in mixtures with OBs enhanced OB infectivity of all the viruses tested; a statistically significant ~1.5-fold increase in the prevalence of virus-induced mortality was observed compared to insects inoculated with OBs in the absence of Leucophor AP (Fig. 3; $F_{5,12} = 33.38$, $p < 0.001$). Sf138null OBs alone resulted in 41.7 ± 2.4% mortality that increased to 65.3 ± 3.4% when OBs were inoculated in mixtures with Leucophor AP (Fig. 3). This indicated that reduced OB pathogenicity of Sf138null OBs was unlikely to be related to an interaction with the peritrophic matrix.

4. Discussion

The roles of *sf68*, *sf95* and *sf138* in selected pathogenic characteristics of SfMNPV were studied after construction of three deletion viruses. These genes were selected because of their homology with *ac145* and *ac150*, previously described as genes that affected the oral infectivity of AcMNPV OBs in a host-dependent manner (Lapointe et al., 2004; Zhang et al., 2005). The AC150 protein was described as a non-essential *per os* infection factor (Zhang et al., 2005). However, this protein is dissimilar to

Table 1
LC_{50} and mean time-to-death (MTD) analysis for the Sfbac, Sf68null, Sf95null, Sf138null, Sf68rep, Sf95rep and Sf138rep viruses in second-instar *Spodoptera frugiperda* larvae.

Virus	LC_{50} (OBs/ml)	Relative potency	Fiducial limits (95%)		MTD (h)	Fiducial limits (95%)	
			Low	High		Low	High
Sfbac	1.19×10^5	1	–	–	128	124	132
Sf68null	1.56×10^5	0.762	0.509	1.142	137	134	141
Sf95null	8.84×10^4	1.346	0.847	2.018	137	134	142
Sf138null	1.77×10^6	0.067	0.0045	0.099	133	129	137
Sf68rep	1.20×10^5	0.990	0.668	1.468	129	125	133
Sf95rep	1.08×10^5	1.096	0.733	1.638	128	123	132
Sf138rep	1.14×10^5	1.041	0.699	1.549	130	126	134

Logit regressions were fitted in POLO Plus (LeOra-Software, 1987). A test for non-parallelism was not significant ($\chi^2 = 2.00$; d.f. = 6; $P = 0.919$), so that regressions were fitted with a common slope of 1.00 ± 0.072. Relative potencies were calculated as the ratio of effective concentrations relative to that of Sfbac. Mean time to death (MTD) values were estimated by Weibull survival analysis (Crawley, 1993).

the established *per os* infectivity factors (PIFs) described to date, that are essential for oral infectivity in insect larvae and which form a stable complex on the surface of occlusion-derived viruses (ODVs) (Peng et al., 2012, 2010). In contrast, deletion of *ac150* resulted in a reduction, but not loss, of the pathogenicity of OBs following *per os* inoculation (Zhang et al., 2005).

Most of the experiments of this work have been performed in larvae, given that the Sfbac bacmid is not stable in cell cultures and undesirable recombinations occur after the first passage in cells. However, there are no differences between the Sfbac and the SfMNPV virus in terms of biological activity when infections are performed in larvae (Simón et al., 2008).

The *sf68*, *sf95* and *sf138* genes are not essential for SfMNPV infection, but a two fold decrease in larval mortality was observed after injection of the deletion bacmid DNAs compared to the complete bacmids. Therefore, their role may be important during the virus infection process or systemic spread of infection. The DNA content of deletion virus OBs did not differ significantly from that of the parental or repair viruses, suggesting that the number of nucleocapsids occluded within the OBs was not affected by gene deletions (Beperet et al., 2013), although the distribution of nucleocapsids within the ODVs remains unclear. These data are in agreement with observations made for the deletion mutants of the homologous genes of AcMNPV, *ac145* and *ac150*. OBs from the AcMNPV deletion viruses had similar morphology to that of the complete viruses (Lapointe et al., 2004) and deletion of *ac150* did not affect the ODV content or nucleocapsid distribution within OBs (Zhang et al., 2005).

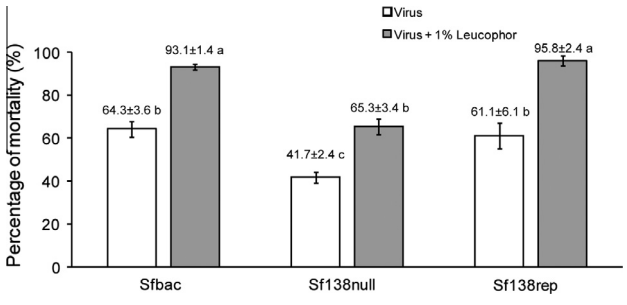

Fig. 3. Effect of addition of Leucophor AP on the pathogenicity of Sfbac, Sf138null and Sf138rep OBs. Percentage of mortality obtained after infection with 5×10^4 OB/ml of each virus, in the presence or absence of 1% Leucophor AP, is shown. Values above the columns indicate means and those labeled with different letters differed significantly (ANOVA, Tukey test, $p < 0.05$). Error bars indicate the standard error of the mean.

Interestingly, deletion of *sf138*, a homolog of *ac145*, resulted in a 15-fold decrease in the oral pathogenicity of OBs, whereas speed-of-kill and OB production were not affected in *S. frugiperda* larvae inoculated with the Sf138null OBs. The effect of the deletion of *ac145* differs depending on the host species infected. Deletion of *ac145* did not alter OB pathogenicity or speed of kill of AcMNPV-infected *Heliothis virescens* (Lapointe et al., 2004). However, in *Trichoplusia ni*, the deletion resulted in reduced OB pathogenicity, but no effect on speed of kill (Lapointe et al., 2004). The decrease in OB pathogenicity observed in the Sf138null virus is likely to be closely related to the markedly reduced infective titer of ODVs in cell culture. This low infective titer is unlikely to be due to differences in the number of nucleocapsids occluded within the OBs, as the DNA content remains stable. Hence, it may be probably related to a lack of ODV infectivity and not to a low viral content within the OBs. The low infectivity of the Sf138null mutant, together with the instability of SfMNPV bacmids in cell cultures, does not allow to perform other experiments in cell cultures, as study the production of BVs. These results, together with the lack of effect of a double *ac145* and *ac150* deletion mutant on BV infectivity (Lapointe et al., 2004), led to the hypothesis that type 145 proteins, including the SF138, may play an important role in the primary infection of the virus, i.e. infection of host midgut cells. Furthermore, these proteins seem to act in a host-dependent manner, as they are more important for infection of *S. frugiperda* and *T. ni* larvae than *H. virescens*. As SfMNPV has a narrow host range, with *S. frugiperda* its only permissive host (Murillo et al., 2003), it was not possible to examine the effect of *sf138* deletion in other insect species.

A chitin-binding motif is present in the predicted protein sequence of both AC145 and SF138. However, the activity of the chitin-binding motif in the *ac145*-like proteins has not been tested. The presence of this motif may suggest a function of these proteins related to the peritrophic matrix, possibly similar to that of baculovirus *enhancins* (Hoover et al., 2010; Slavicek, 2012). The peritrophic matrix is an insect-synthesized barrier composed mainly of chitin and glycoproteins. However, the hypothesized role of SF138 in the disruption of the peritrophic matrix was discounted, as addition of a stilbene optical brightener to Sf138null OB inoculum increased insect mortality for deletion and parental/repair viruses alike.

Deletion of the genes homologous to the *ac150* present in the SfMNPV genome, *sf68* and *sf95*, produced a different effect. Their deletion increased the mean time to death (MTD) and the total production of OBs per larva, without altering the oral infectivity of the viruses. Increases in OB production are often linked to slower speeds-of-kill, because it extends the period during which the virus can replicate and produce new virus particles and OBs prior to host death (Hernández-Crespo et al., 2001; Hodgson et al., 2001; Simón et al., 2008). Regarding speed-of-kill, similar results were observed for BmNPV carrying a deletion in *bm126*, an *ac150*-like gene, in *Bombyx mori* larvae, but not for the deletion of *ac150* in AcMNPV in any of the three host species tested, *S. exigua*, *T. ni* or *H. virescens* (Hao et al., 2009; Lapointe et al., 2004; Zhang et al., 2005). However, the decrease in virulence of the *bm126* deleted virus was not linked to an increase in OB production, as observed with the *sf68* and *sf95* deleted viruses. In fact, the extension of the time-of-kill and concurrent increase in OB production was only observed in one virus carrying a natural variant of the *bm126* (Hao et al., 2009). These results suggest that type 150 genes, including *sf68* and *sf95*, may have an important role in the systemic spread of the infection within the infected insect. A higher speed-of-kill may be linked to a more efficient dissemination of the infection throughout the insect and, consequently a more rapid

establishment of systemic infection, or to a faster production of viral progeny following infection of host cells or more abundant production of budded virions following infection of host cells (Harrison and Bonning, 2001).

Changes in OB pathogenicity were observed for *ac150* mutants in third instar *S. exigua*, *T. ni* and *H. virescens* larvae, but not in neonate larvae (Hao et al., 2009; Lapointe et al., 2004; Zhang et al., 2005), differing from the effects observed for the genes homologous to *ac150* present in SfMNPV and BmNPV.

Furthermore, deletion of *sf68* or *sf95* did not modify the infectious titers of ODVs, as observed for the *ac150* gene (Zhang et al., 2005). It was previously suggested that the similar ODV infectivity found following *ac150* deletion was a consequence of the technical protocol used, as the protein could have been degraded or inactivated during the alkaline treatment performed to release ODVs from OBs in the complete viruses (Zhang et al., 2005). However, as no changes were observed in the pathogenicity of the Sf68null or Sf95null OBs in *S. frugiperda* larvae, no alteration in the infectious titer of ODVs was expected or observed in cell culture assays. These findings tend to abrogate previous suggestions of protein degradation during virion purification or a role for these proteins during primary infection of insect midgut cells.

The deletion of the *sf95* ORF affected the promoter of the *sf96* gene. This gene of unknown function has no homologs in other baculoviruses (Harrison et al., 2008). However, the effects showed after *sf95* deletion are likely to be genuine effect of the deletion of the *sf95* ORF and not the deletion of the *sf96* promoter, as the phenotypic characteristics studied here were similar for the Sf68null and Sf95null viruses, both of them homologs of the *ac150*. To probe this hypothesis, functionality of the *sf96* gene and its implication on the selected characteristics studied here will be studied in the future.

Analysis of the sequences of the type 150 proteins revealed some differences between the SfMNPV proteins and their homologs in AcMNPV and BmNPV. The most important difference is the absence of the C6 motif (a chitin-binding motif) in the SF68 and SF95 predicted proteins. However, it was clear from the present study that the main function of this type of protein does not rely on the activity of this motif, as the functional phenotypes of the SfMNPV proteins, in which the motif is absent, were similar to that of the BM126 protein of BmNPV, in which the motif is present. Moreover, a lack of chitin-binding activity was previously reported for the AC150 protein (Zhang et al., 2005).

Despite the fact that SF95 is less closely related to AC150 than SF68 deletion of the respective genes had similar effects on the phenotypic characteristics of the recombinant viruses, at least in the traits that we measured, suggesting similar functions in the infected host, although additional studies are required to confirm the functional similarity of these proteins.

In general terms the effect of variation in the biological activity of the viruses (pathogenicity, virulence, productivity) arising from the type 150 genes tends to be greater than that of the type 145 genes and depends on the virus-host pathosystem under study. In fact, it is already known that the transcription profile of *ac150* depends on the insect cell line infected (Yamagishi et al., 2003), being more strongly transcribed in *S. frugiperda* Sf9 cells than in *T. ni* High Five cells, and suggesting different effects depending on the host infected. Furthermore, the variation in the biological properties is reflected in the sequences, given that the deletion of the type 145 genes, which is a more robust group in phylogenetic terms (Dall et al., 2001) than the type 150 genes, produces a more uniform effect, affecting mainly OB pathogenicity.

The "11K" protein family, as well as other families of genes with homologs in unrelated taxa of insect viruses, may be important in the process of viral adaptation to the host, in terms of effective utilization of potential hosts and host resources (Dall et al., 2001). As such, the role of the two type 145 and the four type 150 proteins already studied differs depending on the host species. The host-pathogen coevolutionary process involves reciprocal adaptive genetic changes in host and pathogen that oppose one another, such as antiviral mechanisms of the host, and inhibitors of such mechanisms in the virus and similar adaptations designed to overcome barriers to infection or replication in the host (Marques and Carthew, 2007). Hence, the host immune system and virus counter defenses evolve together and constitute a continuously evolving source of diversity (Woolhouse et al., 2002). In this way, viral adaptation to different hosts may lead to progressive divergence (Crill et al., 2000) among homologous genes, promoting changes in different proteins that may result in host-dependent protein effects. As such host specific regulation of viral genes and viral adaptation to host species-specific responses are required to maximize virus transmission and survival. Both these aspects are reflected in the baculovirus "11K" family of proteins.

5. Conclusions

In this study, the *sf68*, *sf95* and *sf138* genes of the Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) have been functionally characterized. These are members of the "11K" family of genes, characterized for having members among baculoviruses and entomopoxviruses and hypothesized to be important in the adaptation to the host. A virus containing a deletion in the *sf138* gene, homologous to *ac145*, was approximately 15-fold less pathogenic than the parental or repair viruses. This was related to a decrease of more than 100-fold in the ODV infective titer observed *in vitro*. Addition of an optical brightener to *sf138*-deleted virus OBs did not result in recovery of the pathogenic activity of OBs indicating that SF138 was unlikely to be involved in degradation of the insect peritrophic matrix. In contrast, viruses carrying a deletion of the *sf68* and *sf95* genes, homologs of *ac150*, were 9 h slower-killing and, produced 1.6–1.7 more OBs per larva. These results, together with previous publications, suggest that *ac145*-like genes may be important during the primary infection of alphabaculoviruses, whereas the *ac150*-like genes may have a role in the systemic spread of the infection.

Competing interests

The authors declare that they have no competing interests.

Authors contributions

Experiments were performed by IB and OS. All authors contributed to the design, analysis and interpretation of the study and to the writing of the manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2015.03.008.

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