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Short Communication

Deletion of *egt* is responsible for the fast-killing phenotype of natural deletion genotypes in a *Spodoptera frugiperda* multiple nucleopolyhedrovirus population

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ABSTRACT

A Nicaraguan population of *Spodoptera frugiperda* multiple nucleopolyhedrovirus, SfMNPV-NIC, includes fast-killing genotypes with deletions in the *egt* region. Four bacmid based recombinants were constructed to determine the role of *egt* in this phenotype. SfdelF bacmid encompassed the deletion found in the NIC-F genotype. Sfdel3AP2 bacmid was constructed using the deletion reported in SfMNPV-3AP2 (Missouri, fast-killing isolate), whereas Sfdelegt and Sfdel27 bacmids lacked the single genes *egt* and the adjacent *sf27* gene, respectively. No significant differences were observed in occlusion body (OB) concentration–mortality metrics (LC_{50} values) among the viruses. Larvae infected by NIC-B (a natural genotype with the largest genome), Sfbac (a bacmid with NIC-B genome) and Sfdel27 survived significantly longer than insects infected by NIC-F, SfdelF, SfMNPV-3AP2, Sfdel3AP2 or Sfdelegt. Fast-killing viruses produced ~6–13-fold fewer OBs/larva compared to other viruses tested. We conclude that deletion/disruption of *egt* is responsible for the fast-killing phenotypes of naturally-occurring genotypes in SfMNPV populations from Missouri and Nicaragua.

Nucleopolyhedrovirus populations are characterized by high levels of genetic heterogeneity (Cory et al., 2005; Harrison et al., 2008; Hodgson et al., 2001; Simón et al., 2004). Certain genotypic variants may also be more prevalent in the population than the associated variants (Erlandson, 2009). The fact that minority genotypes are not eliminated suggests that this heterogeneity is important for virus survival. Moreover, statistical models suggest that, once established in the population, the co-occlusion process allows minority variants to persist over many cycles to insect-to-insect transmission (Bull et al., 2001). Nine distinct genotypes (NIC-A to -I) have been isolated from the Nicaraguan strain of *Spodoptera frugiperda* multiple nucleopolyhedrovirus, SfMNPV-NIC (Simón et al., 2004). The NIC-B genotype with the largest genome, 132,954 bp (Simón et al., 2011), is the predominant genotype in the wild-type population. Genotypes NIC-F and NIC-I have the shortest deletions and were faster-killing in single-genotype infections than the complete NIC-B genotype. NIC-F lacked the *chitinase*, *sf23*, *gp37*, *ptp-2*, *egt* and *sf27* genes. However, variants with larger deletions in the same region, such as NIC-A and NIC-H, killed insects more slowly than NIC-F or NIC-I (Simón et al., 2004, 2005).

Analysis of a SfMNPV isolate from Missouri, USA, revealed the presence of a fast-killing genotype (SfMNPV-3AP2) with a genomic deletion that removed parts of the *egt* and *sf27* genes (Harrison et al., 2008). The *egt* gene encodes the enzyme ecdysteroid UDP glucosyltransferase, that inactivates host ecdysteroids involved in regulating larval development and molting (O'Reilly et al., 1995). Deletion or inactivation of the *egt* gene significantly reduces survival time of infected insects (Cory et al., 2004; Zwart et al., 2009), although this effect may be restricted to late instars in some species (Slavicek et al., 1999). This led Harrison et al. (2008) to postulate that the deletion of most of the *egt* gene in SfMNPV-3AP2 provided a likely explanation for its rapid speed of kill. Our aim in this study was to determine the factor(s) involved in the observed fast-killing phenotypes of NIC-F and SfMNPV-3AP2 OBs via construction of recombinant viruses with modifications in the *egt*/*sf27* region; we then analyzed the consequences of these modifications on OB infectivity and speed of kill phenotype.

PCR and bacmid-based recombination was used to delete regions or specific genes from a complete SfMNPV bacmid (NIC bacmid) that included the NIC-B genotype (Simón et al., 2008a). Four recombinants were constructed: (i) SfdelF bacmid contained a deletion that encompassed the deletion found in NIC-F, located between nt 20,494 and 26,130, (ii) Sfdel3AP2 bacmid lacked the region between nt 24,628 and 26,054, which is the same deletion found in SfMNPV-3AP2, (iii) Sfdelegt bacmid comprised a deletion

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Table 1
Primers used in this study.

Primer	Sequence (position in the genome)	Amplification purpose
SfdelF.1	5′-CCGTCGACAAGGTACCCGTTCCCAA TGGACAGCAAGCGAACCGGAATTGC-3′	Deletion of the NIC-F deleted region from NIC bacmid. Forward primer with 25 nt homologs to Tn5-neo sequence (underlined) and 25 nt homolog to contiguous left region, before NIC-F deletion (nt 20,469–20,493 in NIC-B genome)
SfdelF.2	5′-TCCGAGCATCGCGTTTACGTTTGAA TCAGAAGAACTCGTCAAGAAGGCG-3′	Deletion of the NIC-F deleted region from NIC bacmid. Forward primer with 25 nt homologs to Tn5-neo sequence (underlined) and 25 nt homolog to contiguous right region, after NIC-F deletion (nt 26,131–26,155 in NIC-B genome)
SfdelF.3	5′-TGGGGCGTTTATCCTAGACAATTTC CCGTCGACAAGGTACCCGTTCCCAA-3′	Deletion of the NIC-F deleted region from NIC bacmid. Forward primer with 25 nt homologs to SfdelF.1 (underlined) and 25 nt homolog to contiguous left region, before NIC-F deletion (nt 20,444–20,468 in NIC-B genome)
SfdelF.4	5′-ATATTACTCGACATTGCTTATCTTC TCCGAGCATCGCGTTTACGTTTGAA-3′	Deletion of the NIC-F deleted region from NIC bacmid. Forward primer with 25 nt homologs to SfdelF.2 (underlined) and 25 nt homolog to contiguous right region, before NIC-F deletion (nt 26,156–26,180 in NIC-B genome)
Sfdel3AP2.1	5′-CTGAAAACTTTGAAACTATGGGCGC TGGACAGCAAGCGAACCGGAATTGC-3′	Deletion of the SfMNPV-3AP2 deleted region from NIC bacmid. Forward primer with 25 nt homologs to Tn5-neo sequence (underlined) and 25 nt homolog to contiguous left region, before NIC-3AP2 deletion (nt 24,603–24,627 in NIC-B genome)
Sfdel3AP2.2	5′-TCGACAACAAAGTCTTTTTGTATTT TCAGAAGAACTCGTCAAGAAGGCG-3′	Deletion of the SfMNPV-3AP2 deleted region from NIC bacmid. Forward primer with 25 nt homologs to Tn5-neo sequence (underlined) and 25 nt homolog to contiguous right region, after SfMNPV-3AP2 deletion (nt 26,055–26,079 in NIC-B genome)
Sfdel3AP2.3	5′-CAAATCTCGTCGGGCTACGCTGTGG CTGAAAACTTTGAAACTATGGGCGC-3′	Deletion of the SfMNPV-3AP2 deleted region from NIC bacmid. Forward primer with 25 nt homologs to SfdelF.1 (underlined) and 25 nt homolog to contiguous left region, before SfMNPV-3AP2 deletion (nt 24,578–24,602 in NIC-B genome)
Sfdel3AP2.4	5′-ATCCATACGTACGATAAACAGGTAT TCGACAACAAAGTCTTTTTGTATTTTC-3′	Deletion of the SfMNPV-3AP2 deleted region from NIC bacmid. Forward primer with 25 nt homologs to SfdelF.2 (underlined) and 25 nt homolog to contiguous right region, before SfMNPV-3AP2 deletion (nt 26,080–26,104 in NIC-B genome)
Sfdelegt.1	5′-TTACTGTGTTTGGTCATCTGTCACC TGGACAGCAAGCGAACCGGAATTGC-3′	*egt* Deletion from NIC bacmid. Forward primer with 25 nt homologs to Tn5-neo sequence (underlined) and 25 nt homolog to 3′ untranslated *egt* region (nt 24,031–24,055 in NIC-B genome)
Sfdelegt.2	5′-GATAACATACAATTACATATCCTGT TCAGAAGAACTCGTCAAGAAGGCG-3′	*egt* Deletion from NIC bacmid. Reverse primer with 25 nt homologs to Tn5-neo sequence (underlined) and 25 nt homolog to 5′ untranslated *egt* region (nt 25,633–25,657 in NIC-B genome)
Sfdelegt.3	5′-AAAATAAATTTTTGGGGTCACCTAC TTACTGTGTTTGGTCATCTGTCACC-3′	*egt* Deletion from NIC bacmid. Forward primer with 25 nt homologs to Sfdelegt.1 (underlined) and 25 nt homolog to 3′ untranslated *egt* region (nt 24,006–24,030 in NIC-B genome)
Sfdelegt.4	5′-TTCTAATGAATATTTATTACACATT GATAACATACAATTACATATCCTGT-3′	*egt* Deletion from NIC bacmid. Reverse primer with 25 nt homologs to Sfdelegt.2 (underlined) and 25 nt homolog to 5′ untranslated *egt* region (nt 25,658–25,682 in NIC-B genome)
Sfdel27.1	5′-AAATCGACATCGCGCACTTGCTACC TGGACAGCAAGCGAACCGGAATTGC-3′	*sf27* Deletion from NIC bacmid. Forward primer with 25 nt homologs to Tn5-neo sequence (underlined) and 25 nt homolog to 3′ untranslated *sf27* region (nt 25,795–25,819 in NIC-B genome)
Sfdel27.2	5′-GTAGTCATTGTAAATTACGAAAATA TCAGAAGAACTCGTCAAGAAGGCG-3′	*sf27* Deletion from NIC bacmid. Reverse primer with 25 nt homologs to Tn5-neo sequence (underlined) and 25 nt homolog to 5′ untranslated *sf27* region (nt 26,334–26,358 in NIC-B genome)
Sfdel27.3	5′-GCCGAATCGGTGTTGTTCAGTATTC AAATCGACATCGCGCACTTGCTACC-3′	*sf27* Deletion from SfMNPV bacmid. Forward primer with 25 nt homologs to Sfdel27.1 (underlined) and 25 nt homolog to 3′ untranslated *sf27* region (nt 25,770–25,794 in SfMNPV-B genome)
Sfdel27.4	5′-GTGGCCAGTTGTCGTCGTAGTAGTA GTAGTCATTGTAAATTACGAAAATA-3′	*sf27* Deletion from NIC bacmid. Reverse primer with 25 nt homologs to Sfdel27.2 (underlined) and 25 nt homolog to 5′ untranslated *sf27* region (nt 26,359–26,383 in NIC-B genome)

of the *egt* gene, between nt 24,056 and 25,633, and (iv) Sfdel27 lacked the *sf27* gene found between nt 25,820 and 26,344 of the NIC-B genome (Simón et al., 2011). For the construction of these recombinants a kanamycin resistance gene was amplified from the Tn5-neo template using two set of overlapping primers for each recombinant (Table 1), to add 50 nucleotide terminal sequences corresponding to either the left and right regions of the deletions. The genomic arrangement of all recombinant viruses was verified by sequencing of the PCR products (results not shown).

To produce OBs from Sfbac, SfdelF, Sfdel3AP2, Sfdelegt and Sfdel27 bacmids, *S. frugiperda* fourth instars from a laboratory colony maintained on semisynthetic diet (Greene et al., 1976) were injected with a DNA suspension including bacmid DNAs and lipofectin transfection reagent (Invitrogen) in a 2:1 proportion (Simón et al., 2008a). Injection of these DNAs resulted in 54–65% of larvae succumbing to lethal polyhedrosis disease. Infections were initiated by injection of viral DNAs on three occasions and similar percentages of mortality were observed on each occasion. The insecticidal properties of OBs were determined by insect bioassay in *S. frugiperda* second instars from the laboratory colony following the droplet feeding method (Hughes and Wood, 1986). Larvae were allowed to drink from one of five OB concentrations between 1.2×10^6 and 1.9×10^3 OBs/ml, estimated to kill between 95% and 5% of experimental insects (Simón et al., 2004). Larvae were maintained individually on diet until death or pupation. Bioassays were performed on four occasions using groups of 25 larvae per virus concentration and 25 mock-infected control larvae. Larvae were reared at 26 ± 1 °C and mortality was recorded every 8 h until insects had either died or pupated. Virus induced mortality results were subjected to probit analysis using the Polo-PC program (LeOra Software, 1987). OB pathogenicity was estimated as the 50% lethal concentration (LC_{50}). LC_{50} values of OBs varied from 1.13×10^5 to 2.05×10^5 OBs/ml in second instars but did not differ significantly in any of the viruses assayed as judged by marked overlap of 95% fiducial limits of relative potency values (Table 2).

Time mortality results in droplet-inoculated second instars were subjected to Weibull survival analysis in GLIM 4 (Crawley, 1993). OB concentrations used for the time mortality analyses were those that resulted in ~85% mortality; 3.05×10^6 OBs/ml

Table 2
LC_{50} and relative potencies of OBs of NIC-B, NIC bacmid (Sfbac), NIC-F (SfF), SfdelF bacmid (SfdelF), SfMNPV-3AP2 (Sf3AP2), Sfdel3AP2 bacmid (Sfdel3AP2), Sfdelegt bacmid (Sfdelegt) and Sfdel27 bacmid (Sfdel27) in *Spodoptera frugiperda* second instars.

Viruses	LC_{50} (OBs/ml)	Relative potency	Fiducial limits (95%)		*P*
			Low	High	
NIC-B	1.41×10^5	1	–	–	–
Sfbac	1.39×10^5	1.014	0.618	1.636	>0.05
NIC-F	1.31×10^5	1.075	0.674	1.714	>0.05
SfdelF	1.74×10^5	0.812	0.503	1.313	>0.05
Sf3AP2	1.13×10^5	1.248	0.783	1.989	>0.05
Sfdel3AP2	1.95×10^5	0.722	0.443	1.178	>0.05
Sfdelegt	2.05×10^5	0.689	0.417	1.137	>0.05
Sfdel27	1.55×10^5	0.911	0.574	1.446	>0.05

Fiducial limits refer to relative potency values.
Probit regressions were fitted using the PoloPlus program. A test for non-parallelism ($\chi^2 = 2.07$, *d.f.* = 7, $P = 0.913$) was not significant. Relative potencies were calculated as the ratio of effective concentrations relative to the NIC-B (SfB) virus.

for NIC-B, 3.00×10^6 OBs/ml for Sfbac, 2.60×10^6 OBs/ml for NIC-F, 3.84×10^6 OBs/ml for SfdelF, 2.32×10^6 OBs/ml for SfMNPV-3AP2, 4.60×10^6 OBs/ml for Sfdel3AP2, 5.70×10^6 OBs/ml for Sfdelegt and 2.64×10^6 OBs/ml for Sfdel27 that resulted in comparable mortalities of 85%, 84%, 86%, 81%, 86%, 87%, 89% and 84%, respectively. The experiment was repeated four times and virus induced mortality was recorded every 8 h. Mean time to death (MTD) values of viruses could be divided into two groups (Fig. 1). NIC-B, Sfbac and Sfdel27 bacmid OB-treated larvae survived significantly longer than larvae treated with NIC-F, SfdelF, SfMNPV-3AP2, Sfdel3AP2 and Sfdelegt OBs. The reduction in MTD values in fast-killing viruses was in the range ~42–50 h compared to the slower-killing viruses.

OB production was determined in second instars that died in the time to death experiment (20–23 insects per virus treatment in each repetition, total ~80 larvae per virus treatment). Virus killed insects were individually stored at −20 °C until used for OB counting. Each larva was thawed, homogenized in a volume of 100 µl distilled water and diluted in distilled water. OB counts from each insect were performed in triplicate using a Neubauer hemocytometer. Results were normalized by logarithmic transformation and subjected to analysis of variance and Bonferroni means separation in SPSS v.15 (SPSS Inc., Chicago, IL). OB production of each virus was negatively correlated with speed of kill (Fig. 1). Slower killing viruses NIC-B, Sfbac and Sfdel27 clustered as one set of points with high values of OB production/larva. In contrast, the fast-killing viruses NIC-F, SfdelF, SfMNPV-3AP2, Sfdel3AP2 and Sfdelegt clustered as a separate group with significantly lower values of OB production. Fast-killing viruses produced between 6 and 13-fold fewer OBs per larva.

These results indicate that the deletion found in the naturally fast-killing NIC-F genotype appears to be responsible for the phenotype of this virus. This deletion included genes such as *sf22* (*chitinase*), *sf23* (unknown), *sf24* (*gp37*), *sf25* (*ptp-2*), *sf26* (*egt*), and *sf27* (unknown). In addition, the Sfdel3AP2 bacmid was similar in speed of kill and OB production as the SfMNPV-3AP2 virus, suggesting that the deletion found within this genotype, that included the *sf26* (*egt*) and *sf27* (unknown) genes, may be responsible for the fast-killing phenotype. Finally, the Sfdelegt bacmid was significantly faster-killing and less productive than NIC-B or Sbac, and had a similar speed of kill and OB production phenotype as the NIC-F or SfMNPV-3AP2 viruses indicating that the *egt* deletion is responsible for the fast-killing phenotypes of these viruses. In contrast, the single gene deleted bacmid Sfdel27 has a speed of kill phenotype similar to that of the NIC-B or Sfbac viruses suggesting that this gene has a negligible influence of these phenotypic characteristics in SfMNPV. The function of *sf27* homologs, found only in group II alphabaculoviruses (Harrison et al., 2008; Ijkel et al., 1999) is not known. However, it is expressed early in infection (Ijkel et al., 1999), suggesting a role during viral replication.

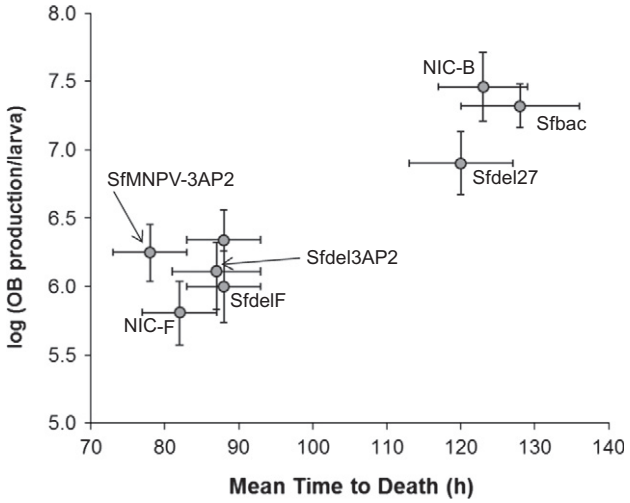

Fig. 1. Mean time to death (h) and log[OB production/larva] characteristics of OBs of NIC-B (SfB), NIC bacmid (Sfbac), NIC-F (SfF), SfdelF bacmid (SfdelF), SfMNPV-3AP2 (Sf3AP2), Sfdel3AP2 bacmid (Sfdel3AP2), Sfdelegt bacmid (Sfdelegt) and Sfdel27 bacmid (Sfdel27) in *Spodoptera frugiperda* inoculated in the second instar. Horizontal and vertical bars indicate SE of each variable.

Partial and complete deletions in the *egt* region occur not only in natural SfMNPV genotypes from Missouri (Harrison et al., 2008) or Nicaragua (Simón et al., 2004, 2005), but also in wild-type SeMNPV isolates (Muñoz et al., 1999). The Nicaraguan SfMNPV isolate is genotypically structured to maximize the likelihood of transmission (Simón et al., 2005, 2008b). It is becoming increasingly clear that minority deletion variants present in NPV populations play a functional role in determining the pathogenicity, virulence and productivity of the population. The bimodal time to death distribution of the NIC population (Simón et al., 2005, 2008b) suggests two conflicting outcomes of mixed infection, as observed for other NPV isolates (Hodgson et al., 2004). As such, time-mortality profiles of NPVs are likely modulated by variation in the speed-of-kill characteristics and relative abundance of the multiple genotypes present within each infected host. In the case of SfMNPV-NIC, these include both slow-killing (NIC-A) and fast-killing (NIC-F) variants, although the relative contribution of each genotype to variables such as survival time and OB production, that can influence transmission of the viral population, remains poorly understood. In the case of certain other deletion variants (NIC-C, NIC-G) the OB pathogenicity phenotype is also markedly

affected. The modification of virus phenotypic characteristics via the production of precise genotype mixtures has clear applications in testing ecological predictions on the role of genetic population structure in virus transmission. Similarly, specific genotype mixture technology has already been used for the development of a virus-based biological insecticide (Vir-ex, Biocolor SA, Spain) that has been commercialized for the control of a greenhouse pest in southern Spain (Caballero et al., 2009).

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