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Determination and analysis of the genome sequence of *Spodoptera littoralis* multiple nucleopolyhedrovirus

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ABSTRACT

The *Spodoptera littoralis* multiple nucleopolyhedrovirus (SpliMNPV), a pathogen of the Egyptian cotton leaf worm *S. littoralis*, was subjected to sequencing of its entire DNA genome and bioassay analysis comparing its virulence to that of other baculoviruses. The annotated SpliMNPV genome of 137,998 bp was found to harbor 132 open reading frames and 15 homologous repeat regions. Four unique genes not present in SpltMNPV were identified, as were 14 genes that were absent or translocated by comparison. Bioassay analysis of experimentally infected *Spodoptera frugiperda* revealed an extended killing time for SpliMNPV as compared to *S. frugiperda* MNPV (SfMNPV), but a level of mortality similar to that caused by infection with SfMNPV and superior to that of *Autographa californica* MNPV (AcMNPV). Although extensive similarity was observed between the genome structure and predicted translation products of SpliMNPV and *Spodoptera litura* MNPV (SpltMNPV), genetic distances between isolates of SpliMNPV and SpltMNPV suggest that they are in fact different species of genus *Alphabaculovirus*.

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1. Introduction

Baculoviruses are large, double-stranded DNA viruses, grouped in family *Baculoviridae*, that cause lethal infection in arthropods and have been used as biological insecticides, for recombinant protein expression, and recently, as mammalian gene transduction vectors (Bonning and Nusawardani, 2007; Grabherr and Ernst, 2010; Hitchman et al., 2011; van Beek and Davis, 2007). Most baculoviruses typically feature a narrow host range both *in vivo* and *in vitro*, proving unable to replicate or cause symptomatic infections when introduced into alternate insect species or cells, often within the same genus (Thiem, 1997). Most known baculoviruses are nucleopolyhedroviruses (NPVs; genus *Alphabaculovirus*) or granuloviruses (GVs; genus *Betabaculovirus*) that infect lepidopteran species (Herniou et al., 2011). During infection of larvae, occlusion bodies (OBs) made up of enveloped virions encased in a crystalline matrix of polyhedrin protein are ingested by the host. Virions are liberated by dissolution of the OBs in the alkaline insect midgut, establishing a primary infection in proximal cells of the midgut. Replication of the virus in these cells produces enveloped, budded virus (BV) which subsequently disseminates the infection throughout the host. Late during infection, expression of viral polyhedrin results in the incorporation of viral particles into OBs, which are then released into the environment following virus-mediated liquefaction of the host (Miller, 1997).

Spodoptera spp. is a genus of generalist crop pests found throughout the Americas, Southeast Asia and countries around the Mediterranean (Ellis, 2004; Meagher et al., 2008). *Spodoptera littoralis*, the Egyptian cotton leafworm, is found in Africa, southern Europe, and the Middle East, where it is a particularly destructive pest of cotton and a wide range of economically important vegetables and ornamentals (Ellis, 2004). The emergence of resistance to the chemical insecticides used to control *S. littoralis* has prompted the development of alternative control measures (Horowitz et al., 1998; Jones et al., 1994). *S. littoralis* multiple nucleopolyhedrovirus (SpliMNPV) is an alphabaculovirus that has been isolated from populations of *S. littoralis* in Egypt, Morocco, France, Israel, the Azores islands, Tunisia, and Turkey (Cherry and Summers, 1985; Croizier et al., 1989; Kislev and Edelman, 1982; Laarif et al., 2011; Martins et al., 2005; Toprak and Gurkan, 2004). The host range of the virus has been shown to be narrow, likely infecting only within *Spodoptera* spp. (Takatsuka et al., 2007; Toprak et al., 2006). Extensive research, including partial sequencing, restriction enzyme

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digestion pattern analysis, and growth characteristics under varied conditions have been carried out to describe the properties and insecticidal activity of SpliMNPV in the laboratory and field, as well as testing different formulations of the virus and effects on non-target species (Kunjeku et al., 1998; Laarif et al., 2011; Seufi, 2008; Toprak et al., 2006, 2007). Commercially available formulations of SpliMNPV for control of *S. littoralis* have been developed (El-Husseini, 2006; Ravensberg, 2011).

Early restriction endonuclease mapping studies revealed that SpliMNPV samples isolated from populations in Israel occurred in two genetically distinct classes, termed SpliMNPV-A (or -T) and SpliMNPV-B (or -D) (Cherry and Summers, 1985; Kislev and Edelman, 1982). Only SpliMNPV-B occurs in virus samples from other countries (Croizier et al., 1986; Martins et al., 2005). NPVs with restriction endonuclease fragment patterns matching those of SpliMNPV-B have been isolated from diseased *S. litura* larvae in Japan (Takatsuka et al., 2003). Partial nucleotide sequences from individual isolates of SpliMNPV-B have been published, including portions of the genomes of isolates SpliMNPV-M2 from Morocco (Croizier et al., 1989) and SpliMNPV-E15 from Egypt (Faktor et al., 1995). In this paper, we present the complete annotated genomic sequence of an Egyptian isolate of SpliMNPV-B and compare conserved features to baculoviruses isolated from other fully sequenced *Spodoptera* species and the prototypic baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV). We also report on the lethality of this isolate, SpliMNPV-AN1956, in neonatal *S. frugiperda* larvae and compare its biological activity to that of *S. frugiperda* MNPV (SfMNPV, strain SfMNPV-3) and AcMNPV (strain L1) in tandem bioassays.

2. Materials and methods

2.1. Viruses, insects, and infections

SpliMNPV-AN1956 was originally isolated by Abul Nasr in 1956 and an extensive study of its growth characteristics is available elsewhere (Abul Nasr, 1956; Grzywacz et al., 1998). *S. frugiperda* eggs and artificial diet (#9772) were commercially obtained from Bio-Serv (Frenchtown, NJ). Larvae were reared in a fashion consistent with previous studies (Harrison et al., 2008).

2.2. DNA isolation and sequencing

Isolation of polyhedra from insect cadavers and subsequent extraction of nucleic acid from OBs has been described (Harrison and Popham, 2008; Harrison et al., 2008). Briefly, 4th instar *S. frugiperda* larvae were fed a mixture of water, sucrose, blue food coloring, and viral OBs. Cadavers were collected in a 50 mL conical tube and approximately 20 mL of cadaver slurry was suspended in 20 mL of PBS and filtered twice through cheesecloth. Half was loaded onto a 40–60% sucrose gradient and centrifuged at 27,000 rpm for 1.5 h to obtain purified OBs which were then aspirated, washed, pelleted, resuspended in ddH$_2$O, and counted. To obtain genomic DNA, the remaining OBs were incubated in 15 mL of 100 mM sodium bicarbonate for 15 min and the undissolved OBs were pelleted by centrifugation at 3000 rpm. Undissolved OBs were subjected to a second treatment in 15 mL of 100 mM sodium bicarbonate and a final centrifugation step removed non-dissolved particles. The two 15 mL aliquots of enveloped virus were underplayed with a sucrose cushion and centrifuged at 27,000 rpm. The pellet containing virus particles was resuspended in ddH$_2$O and incubated at 37 °C in the presence of proteinase K (0.75 mg/mL final concentration) to release viral DNA from particles, and genomic DNA was subsequently phenol:chloroform extracted and resuspended in dH$_2$O following standard protocol (O'Reilly et al., 1993).

Ethanol-precipitated SpliMNPV DNA was pelleted by microcentrifugation and resuspended in distilled deionized H$_2$O. After resuspension, DNA was sheared, size fractionated, and a multiplexed Roche GS FLX Titanium library was prepared for sequencing at the Georgia Genomics Facility (https://gsle.ovpr.uga.edu). Initial sequencing was carried out on an in-house Roche 454 Jr instrument. Reads were sorted by multiplex identifier (MID) tags (SpliMNPV reads were MID tagged with the sequence ACACGACGACT) and *de novo* assembled using Roche's GSAssembler program with default parameters. Three contigs having lengths 104,680 bp, 32,408 bp and 648 bp resulted. The Consed sequence editing utility (Gordon et al., 1998) was used to perform manual adjustments to the assembly; gaps were closed, and a variety of sequencing ambiguities resolved, by PCR amplifying the corresponding genomic regions from viral DNA. PCR amplicon sequencing was performed on an ABI 3130xl Genetic Analyzer instrument using previously described procedures (Harrison and Lynn, 2007). The Lasergene SeqMan NGEN V3.0 (DNAStar) assembler and the SeqManPro V9 sequence editor were used to prepare the finalized contig, which was 137,998 bp in length with an average coverage of 157.09X.

Partial sequence data of the *polh*, *lef-8*, and *lef-9* genes of other SpliMNPV isolates were generated from SpliMNPV OB samples in a USDA Agricultural Research Service insect virus collection. OBs were solubilized as previously described (Rowley et al., 2010), and solubilized material as used as templates for PCR with a set of degenerate primers described by Lange et al. (Harrison and Lynn, 2007; Lange et al., 2004). PCR products were fully sequenced with M13 forward and reverse primers as previously described (Harrison and Lynn, 2007). For some isolates, custom primers were designed for amplification and sequencing of the three loci. Sequence data were assembled into contigs in DNASTAR Lasergene 9 (DNAStar, Madison, WI).

2.3. Genome sequence analysis

ORF identification and construction of the mapped genome was carried out using DNASTAR Lasergene 9 Core Suite software. ORFs that were at least 50 codons in length that did not overlap other ORFs by more than 75 nucleotides and were located outside of homologous repeat (*hr*) regions were selected for further analysis. Sequence similarity of individually translated ORFs to their counterparts, where present in other *Spodoptera* spp. viruses, including SpltMNPV (G2 strain) (GenBank ID: AF325155.1), SeMNPV (GenBank ID: NC_002169.1), SfMNPV (GenBank ID: NC_009011.2), and the prototypic baculovirus AcMNPV (C6 strain) (GenBank ID: L22858.1), was determined at the amino acid sequence level using NCBI blastp with standard settings. Homologous repeat regions were identified using the Tandem Repeats Finder program (Benson, 1999) and by visual examination of intergenic regions in the genome sequence.

Gene parity plots were constructed according to the method of Hu et al. (1998). For SpliMNPV, ORFs were numbered in ascending order beginning with the polyhedrin gene and corresponding ORFs in SpltMNPV, AcMNPV, SeMNPV, and SfMNPV were entered in parallel. Data sets are presented as a line graph in which each point represents an ORF. Deviations from a slope of 1 are indicative of the direction, distance, gaps, presence, and linearity of the ORFs between SpliMNPV and each of the other viruses.

2.4. Phylogeny

Partial sequences from the *polh*, *lef-8*, and *lef-9* genes of group II alphabaculoviruses and a granulovirus were aligned in DNASTAR Lasergene 10 using CLUSTAL W with default parameters (Thompson et al., 1994). Alignments were concatenated with BioEdit (Hall, 1999) and phylogenetic trees inferred from

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