Genetic baculovirus determinants for pathogenicity, virulence and transmission

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Genetic baculovirus determinants for pathogenicity, virulence and transmission

Amaya Serrano

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Chapter 1

General introduction and thesis outline

BACULOVIRUSES

Structure and infection cycle

The family *Baculoviridae* is a group of arthropod-specific viruses with double-stranded, circular, supercoiled DNA genomes, with sizes varying from about 80 to over 180 kb encoding between 90 and 180 genes (Rohrmann, 2013b; Van Oers & Vlak, 2007). Baculovirus infections have been reported in more than 600 insect species, including members of the orders Lepidoptera, Hymenoptera and Diptera (Herniou *et al*., 2003). Based on phylogenetic studies, genome composition and morphological characteristics, the family *Baculoviridae* is divided in four genera: *Alphabaculovirus* (lepidopteran-specific nucleopolyhedroviruses), *Betabaculovirus* (lepidopteran-specific granuloviruses), *Gammabaculovirus* (hymenopteran-specific nucleopolyhedroviruses) and *Deltabaculovirus* (dipteran-specific nucleopolyhedroviruses) (Jehle *et al*., 2006) (Fig. 1).

The genome of baculoviruses is packaged in rod-shaped nucleocapsids varying in

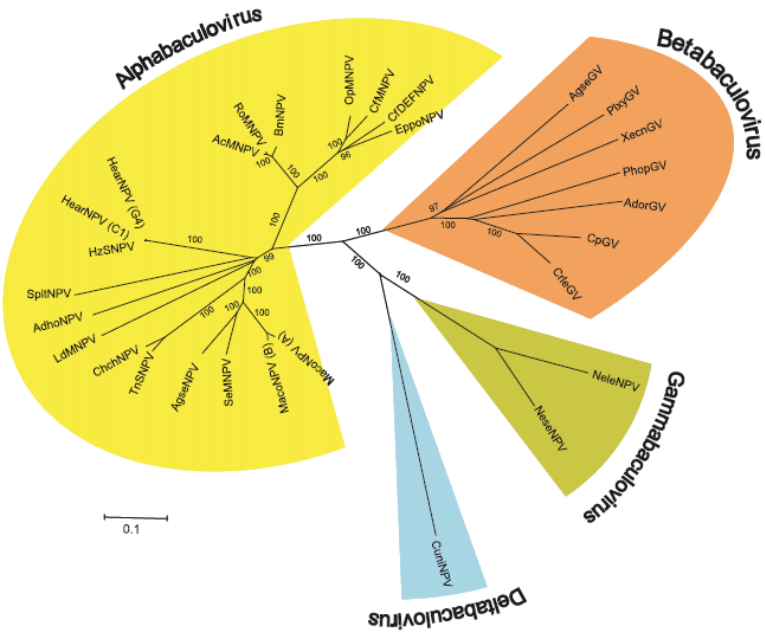

Figure 1. Neighbour-joining tree of the amino acid alignment of 29 baculovirus core genes of 29 sequenced baculovirus genomes. (Adapted from Jehle *et al*. 2006).

length (230–385 nm) and diameter (40–60 nm) (Ackermann & Smirnoff, 1983; Federici, 1986). For most baculoviruses, two types of virions are commonly produced in infected cells or tissues: occlusion derived virions (ODVs) and budded virions (BVs). Those two types of virions differ in the origin and composition of their envelopes and their roles in the virus life cycle, but have identical genomes (Rohrmann, 2013a). ODVs are occluded in a paracrystalline proteinaceous matrix, forming so-called occlusion bodies (OBs), and they are in charge of initiating the infection in insects, more specifically the midgut epithelium cells (Jehle *et al*., 2006). OBs are responsible for the horizontal spread of the virus in insect populations. The BVs are produced after initial infection of cells and they are in charge of cell-to-cell systemic spread to other tissues of the larvae. BVs consist of single-enveloped nucleocapsids that bud from the membrane of infected cells. ODVs may contain single (SNPV) or multiple (MNPV) nucleocapsids depending on the species, and acquire their envelopes in the nucleus by *de novo* assembly. The OBs vary in size from 0.5 to 2 μm (Adams & McClintock, 1991), the protein matrix is made of polyhedrin for the genera *Alphabaculovirus, Gammabaculovirus* and *Deltabaculovirus* and granulin for the genus *Betabaculovirus* (Fig. 2). OBs are highly stable and can resist extreme environmental conditions, allowing the ODVs to remain infectious for long periods of time.

The group *Alphabaculovirus* can be divided in two phylogenetic clades, group I and group II NPVs (Herniou & Jehle, 2007) (Fig. 1). Group I NPVs use the GP64 protein as BV envelope fusion protein, whereas group II NPVs, as well as the beta- and deltabaculoviruses, use the F protein as the envelope fusion protein (Rohrmann, 2013b).

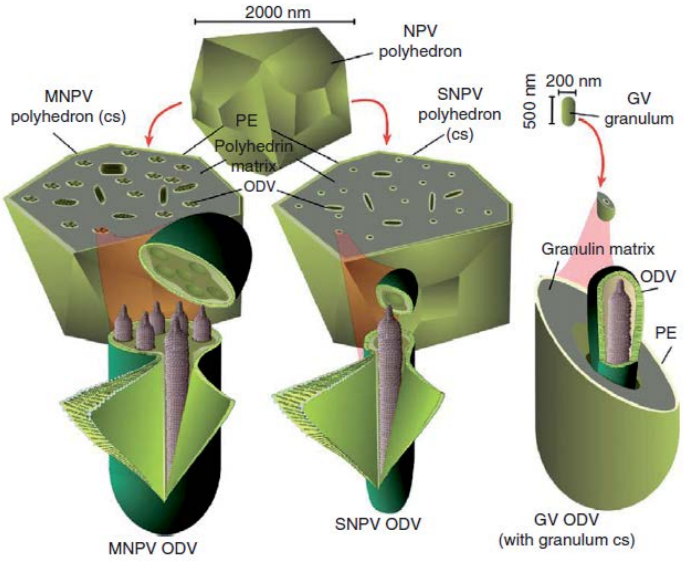

Figure 2. Major occlusion-derived virions (ODVs) forms. Nucleopolyhedroviruses (NPV) are divided into multiple nucleopolyhedroviruses (MNPV) and single nucleopolyhedroviruses (SNPV). The granulovirus (GV) ODV is represented as partially encapsulated (Adapted from Slack & Arif, 2006).

Transmission

Baculoviruses are transmitted to insects via the oral route mediated by OBs. When insects ingest OB-contaminated food, the OBs reach the midgut. The alkaline pH of the insect midgut dissolves the OBs and releases the ODVs. ODVs fuse with midgut epithelial cells and release the nucleocapsids into the cytosol (Adams & McClintock, 1991; Haas-Stapleton *et al.*, 2004). In this primary infection the nucleocapsids travel to the nucleus, where the viral DNA is released and DNA replication starts. After assembly of the BVs at the basolateral side of the midgut epithelial cells, they are released either directly or via the trachea into the hemocele cavity infecting susceptible tissues and organs of the caterpillar to establish a secondary infection. Infected insect midgut cells are sloughed off regularly (Engelhard & Volkman, 1995) and the midgut is regenerated. In the last stage of the secondary infection, ODVs are produced in the nucleus of the infected cells, where they are occluded into OBs, which are then released into the environment from the host by virus-induced tissue

liquefaction and cuticle rupture after death (Slack & Arif, 2006).

Baculoviruses can be transmitted horizontally, from one insect to another, as well as vertically, from parents to offspring. Horizontal transmission is an effective mechanism of virus persistence, when the host density is high. However, baculovirus persistence is difficult to explain when the host density is low or when the insects are highly mobile (Burden *et al.*, 2003) or spatially separated. The role of vertical transmission is more complex. The existence of persistent baculovirus infections were reported after spontaneous outbreaks of baculovirus infections in laboratory-controlled insect rearing (Rohrmann, 2013a), or also after induction with stress factors such as high temperature (Khurad *et al.*, 2004) or irradiation (Jurkovíčová, 1979). Those spontaneous outbreaks occur more commonly in fourth and fifth instar larvae, although they can happen in all instar stages (Khurad *et al.*, 2004). Molecular evidence indicates that baculovirus can persist in adult insects that were sublethally infected as larvae (Burden *et al.*, 2002; Cabodevilla *et al.*, 2011a), and are hence capable of transmitting the infection to their progeny (Kukan, 1999). Vertical transmission includes transovum transmission with viruses on the egg surface, transovarian transmission from within the eggs, either as virus or DNA, to passage as a latent infection (Cory & Myers, 2003). The sublethal infection of the host can lead to diverse effects like reduction in fecundity and fertility, altered host development time, lower pupal and adult body weight, and altered pre-oviposition period (Burden *et al.*, 2002; Myers *et al.*, 2000; Vilaplana *et al.*, 2010). The benefit for the virus is the availability for long periods of time and many generations in the environment (Cabodevilla *et al.*, 2011b). Persistent baculovirus infections have been reported in a large number of lepidopteran species, and it has been proposed as a strategy for the viral survival in the host, even when the host density is low (Cory & Myers, 2003). However, little is known about the molecular mechanism that allows the establishment of a persistent baculovirus infection in the host.

BACULOVIRUS APPLICATIONS

Pest control

Baculoviruses play an important role in controlling the size of insect populations. These viruses have been widely applied for the last six decades as biocontrol agents against forest and agricultural pests due to their efficacy, high specifity and safety for non-target organisms (Moscardi, 1999). The interest in developing baculovirus as biocontrol agents is growing due to the development of insect resistance to most common insecticides (Moulton *et al*., 2002), to the overcoming of (engineered) insect resistance in crops and the presence of less toxic chemical residues in food and water (Garrido *et al*., 2004). However, there are also several drawbacks that limit the use of baculovirus, like their slow speed of action relative to chemicals and the limitations on large scale production. These factors made the engineering of baculoviruses an important field of research for the last 25 years.

The speed of action of baculoviruses is relatively slow (days or weeks) compared with most chemical insecticides (<day). Reducing the time to kill is a major research area and it has involved the expression of foreign genes such as insect-specific toxins derived from the scorpion *Androctonus australis* (Maeda *et al*., 1991). The deletion of some genes has also been proven to affect the time to kill. The ecdystroid UDP-glucosyltransferase (*egt*) gene from *Autographa californica* MNPV (AcMNPV), for example, prevents infected insect from moulting, and its deletion reduces the speed of kill with respect to wild-type (wt) viruses (O'Reilly & Miller, 1991). *Spodoptera exigua* MNPV (SeMNPV), a baculovirus with a narrow host range, kills larvae faster than AcMNPV, which has a wider host range. Identification of genes responsible for the increased virulence may also help reducing the speed of kill.

Heterologous gene expression

Baculoviruses are widely applied in biotechnology as vectors to produce recombinant proteins in insect cells due to the high level of very late gene expression (Van Oers,

2011). The advantage of using insect cells is that they provide the proper post-translational modifications, e.g. glycosylation or phosphorylation that make them suitable for vaccine production, and the inherent safety of the production process. The majority of the commercially available baculovirus insect cell expression systems are based on the baculovirus type species, *Autographa californica* MNPV (AcMNPV). The very late *polyhedrin* and *p10* genes have an extremely high transcriptional activity (about 25% total mRNA of infected cells) and are not required for BV production which make their promoters available for baculovirus-mediated high level foreign gene expression (Condreay & Kost, 2007). In the baculovirus insect cell expression system, the *polyhedrin* or *p10* gene-coding sequence is replaced by the gene of interest and the expression of this foreign gene is driven by the very late *polyhedrin* or *p10* promoter. However, there are other expression vectors available using promoters that are less transcriptionally active or are active earlier in the infection process (Condreay & Kost, 2007).

Upscaling

A limiting factor for the use of baculoviruses for biocontrol is the large-scale production of OBs. Currently, this is being done *in vivo*, in insects reared on artificial diets or under field conditions (Moscardi, 1999). This process is labour intensive and very low cost-competitive with chemical insecticides (Fuxa, 1991). Production of baculovirus in insect cells bioreactors would facilitate the selection of baculovirus clonal isolates with improved insecticidal properties (Moscardi, 1999). The *in vitro* production of baculoviruses for biocontrol, however, has some drawbacks as well. Baculovirus replication in cell culture leads to generation of genetic alterations after few passages (Krell, 1996). Upon SeMNPV replication in cell culture, deletion mutants that have lost the ability to liquefy infected larvae (Dai *et al*., 2000), or even completely lack oral infectivity *in vivo* (Heldens *et al*., 1996), are generated. Another problem is the generation of defective interfering particles (DIPs) and few polyhedra

(FP) mutants. DIPs are viral deletion mutants that arise during serial passage of viral replication in cells and interfere with the growth of virus with complete genomes (Bangham & Kirkwood, 1990). DIPs are often enriched in *oris* (origins of replication) and have a smaller size, which leads to faster replication in cell culture at the expense of the intact virus (Lee & Krell, 1994). DIPs need the assistance of a helper virus for the supply of replication factors such as DNA polymerases and helicases, and thus will lose their replication advantage in low MOI infections. FP mutants are defective in a 25-KDa protein, due to an insertion of a host transposable element into the *fp25* gene (Bull *et al*., 2003). FP mutants produce low yields of occlusion bodies in cell culture, however, they are being selected because a favoured production of BV (Harrison & Summers, 1995). Together, these phenomena are a major concern regarding the large-scale production of baculovirus in insect cell bioreactors.

Gene therapy

The baculovirus-expression system is also being used as a vector for gene delivery into mammalian cells. Although baculoviruses cannot replicate in mammalian cells, they can enter the nucleus of a broad spectrum of mammalian cell types (Condreay & Kost, 2007). By using a mammalian promoter in a baculovirus vector, transient expression of several foreign gene products has been observed in different human cell lines (Condreay *et al*., 1999).

GENOTYPIC DIVERSITY OF BACULOVIRUSES

Baculoviruses display interesting differences in host range. Some of them are host mono-specific, like *S. exigua* MNPV (SeMNPV), but others have a broader host spectrum, e.g. AcMNPV can infect at least 39 lepidopteran species belonging to 15 different families (Gröner, 1986). A high heterogeneity within a single baculovirus species can exist, indicated by the presence of different genotypic variants within a single NPV field isolate (Lee & Miller, 1978; Muñoz *et al*., 1998; Simón *et al*., 2004a).

The existence of genotypic variants within an isolate is usually indicated by the presence of submolar bands in restriction endonuclease digestion analysis of viral DNA. Those genotypic variants can have different modifications like deletions, insertions, or duplications in the viral genome (Caballero & Muñoz, 2001; Muñoz *et al*., 1998).

The genotypes present in a single baculovirus isolate can be purified *in vitro*, i.e. by plaque purification in cell culture (Croizier & Ribeiro, 1992; Maruniak *et al*., 1994; Simón *et al*., 2004a), or *in vivo*, using larvae (Muñoz *et al*., 1999; Muñoz *et al*., 1998; Smith & Crook, 1988). The isolation of individual genotypes has facilitated the evaluation of their biological activity and has revealed marked differences in pathogenicity (indicated by different lethal concentrations), and in virulence (with differences in time to death) (Lynn *et al*., 1993; Simón *et al*., 2004a).

A Nicaraguan isolate of *Spodoptera frugiperda* MNPV (SfMNPV-NIC) is composed of nine distinct genotypes (Fig. 3) of which eight contain a deletion. Three of them are not infectious *per os*, and the remaining genotypes present a higher mean lethal concentration (LC_{50}) compared to the complete genotype, as well as differences in their speed of kill (Simón *et al*., 2004a).

Plaque purified genotypes from *Lymantria dispar* MNPV (LdMNPV) show great differences in lethal concentrations, ranging from $2.2x10^4$ OBs/ml to $6.5x10^5$, and in virulence, with mean time to death values ranging from 9.8 to 18.7 days post-inoculation (Lynn *et al*., 1993). The study of the different genotypes may help select baculovirus strains with improved insecticidal properties.

The analysis of genotypes with different genomic deletions has led to the identification of important genes for virus transmission. For instance, some deletion genotypes were unable to infect insects *per os*, although they were as pathogenic as the wild-type virus by injection (Kikhno *et al*., 2002; Pijlman *et al*., 2003a). These genotypes lacked a specific gene(s) called *per os* infectivity factor (*pif*), which are ODV-specific structural proteins required for initiation of infection in the midgut epithelial cells.

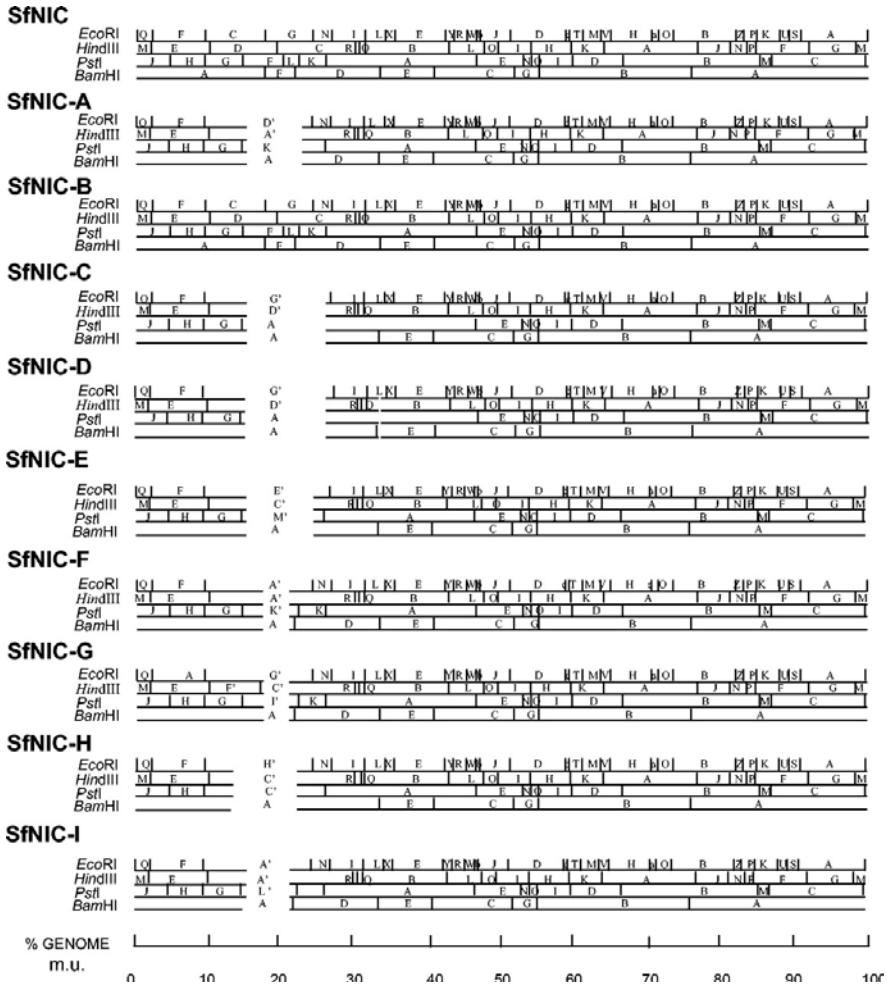

Figure 3. Physical map of SfNIC isolate and nine genotypic variants. (Adapted from Simón *et al*., 2004a).

Also, analysis of fast-killing deletion genotypes present in SfMNPV populations has identified the ecdystroid UDP-glucosyltransferase (*egt*) gene, whose deletion is responsible for the fast-killing phenotype of this genotype (Harrison *et al*., 2008). Maintenance of such deletion genotypes, which may lack genes essential for virus replication or transmission, in the population, is due to co-occlusion with complete genotypes, which provide the essential infection factors upon host cell co-infection (López-Ferber *et al*., 2003). Co-infection of an insect cell by two different genotypes in vivo occurs at a high frequency (Bull *et al*., 2001). The packaging of multiple genomes within an OB allows the exchange of genetic material, via viral

recombination, leading to a high genotypic heterogeneity. The mechanism for generating and maintaining such genotypic diversity is still not clear, a reason why additional studies are needed.

The cloning of genotypes present in a single isolate of baculovirus can be difficult to achieve. *In vivo* cloning methods do not allow selection of genotypes that lack *pif* genes (essential for oral infectivity), and *in vitro* cloning methods may select genotypes with a better replication efficiency in cell culture. Also, passage in cell culture may lead to the generation of deleted genotypes that are not present in the natural baculovirus isolate. Another method for cloning genotypes is by direct cloning of full length baculovirus genomes in *E. coli*. The entire baculovirus genome can be cloned into an 8.5 kb bacterial element with a mini-F replicon, a Tn7 transposition site and an antibiotic selection marker (bacmid). The bacmid is maintained in *E. coli* as a single-copy plasmid and the infectious virus can be generated in insect cell lines after transfection with bacmid DNA. The advantage of the bacmid systems is that it is possible to clone all genotypes present in the wild-type population, including those lacking essential genes for replication, since replication is driven by the mini-F replicon. Understanding the generation and maintenance of such genotypic variability, which is important for virus transmission, gives clues on the ecology and evolution of baculoviruses.

Spodoptera exigua

S. exigua, also known as the beet armyworm (Hübner) (Insecta: Lepidoptera: Noctuidae), originates from Southeast Asia. It was first discovered in North America in 1876, but now it is found in all tropical and subtropical areas of the world (Capinera, 2001) (Fig. 4).

It is an important agricultural and horticultural insect pest since it has an extremely

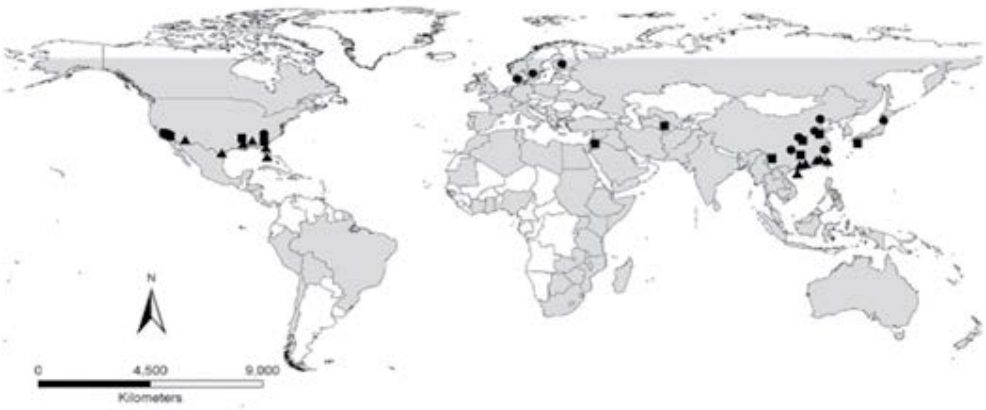

Figure 4. Geographic distribution of *S. exigua* (gray background in the map) in the world. Adapted from (Zheng *et al.*, 2011).

wide host range of more than 200 plant species belonging to over 40 different plant families (Brown & Dewhurst, 1975). It affects plants such as tomato, sweet pepper, asparagus, cabbage, wheat, and chrysanthemum. Generally, *S. exigua* undergoes five larval instars, although sometimes a sixth instar has been reported (Fig. 5A). The metamorphosis, the pupal stage, takes six to seven days in warm weather, and pupation usually occurs in the soil (Belda, 1994). Moths are usually mottled grey and brown, and they may live 10-20 days (Smits *et al.*, 1987) (Fig 5B). Female moths produce on average 500-600 eggs, in batches of 150 eggs maximum, which they usually overlay with hairs and scales (Belda, 1994; Smits *et al.*, 1987).

S. exigua is considered one of the major crop pests of greenhouses in The Netherlands and southern Spain. Larvae of *S. exigua* feed on leaves and fruits and are considered serious defoliators, causing extensive economical loses. Under the optimal temperature conditions of greenhouses, complete development of a generation can take as few as 20 days (Belda, 1994), and it can rapidly colonize the entire greenhouse given the highly mobility of this species.

Control of the beet armyworm has mainly been accomplished with the intensive use of chemical insecticides (Belda, 1994). However, *S. exigua* has developed tolerance and resistance to most commonly used chemical insecticides, like pyrethroids (Moulton

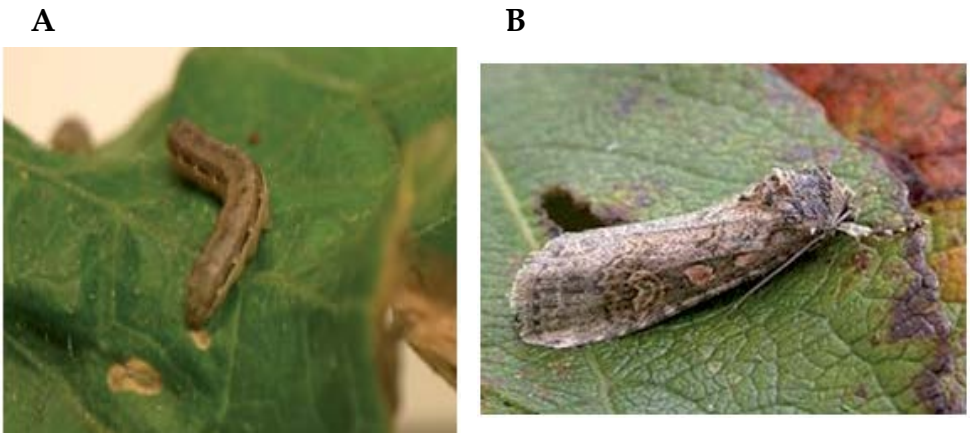

Figure 5. (A) *S. exigua* fifth instar larvae; (B) Moth of S. exigua (photo credit: Ian Kimber, www.ukmoths.org.uk).

et al., 2002). Additionally, the repeated use of chemical insecticides does not allow implementation of biological control programs that target other major greenhouses pests, for which effective biocontrol agents have been implemented (Caballero *et al*., 2009). For that reason the development of safe and effective biological control methods for *S. exigua* larvae meets a growing interest.

Spodoptera exigua MULTIPLE NUCLEOPOLYHEDROVIRUS (SeMNPV)

SeMNPV constitutes an appealing bioinsecticide given its monospecificity for *S. exigua* larvae and the high insecticidal performance for its homologous host in comparison to other baculoviruses (Smits & Vlak, 1988). SeMNPV belongs to group II alphabaculoviruses, its genome size is 135 kbp and it encodes 139 open reading frames (ORFs) (Fig. 6).

SeMNPV has been isolated from many different geographical regions of the world like California, Florida, The Netherlands, Japan, Thailand and Spain (Caballero *et al*., 1992a; Gelernter & Federici, 1986a; Hara *et al*., 1995; Kondo *et al*., 1994; Muñoz *et al*., 1998; Murillo *et al*., 2001; Vlak *et al*., 1981). Three SeMNPV isolates have been developed as commercial bioinsecticides. The SeMNPV-US1 isolate from California

(USA) has been commercialized as Spexit® by Andermatt Biocontrol (Grossdietwil, Switzerland). The SeMNPV-US2 isolate from Florida (USA) has been commercialized as Spod-X® by Certis (Columbia, USA) and used in The Netherlands and Thailand. The SeMPNV-SP2 isolate from southern Spain has been commercialized as a product containing a mixture of genotypes with the name of Virex® by Biocolor Ltd. (Almería, Spain), and it is extensively being used in the greenhouses of Spain.

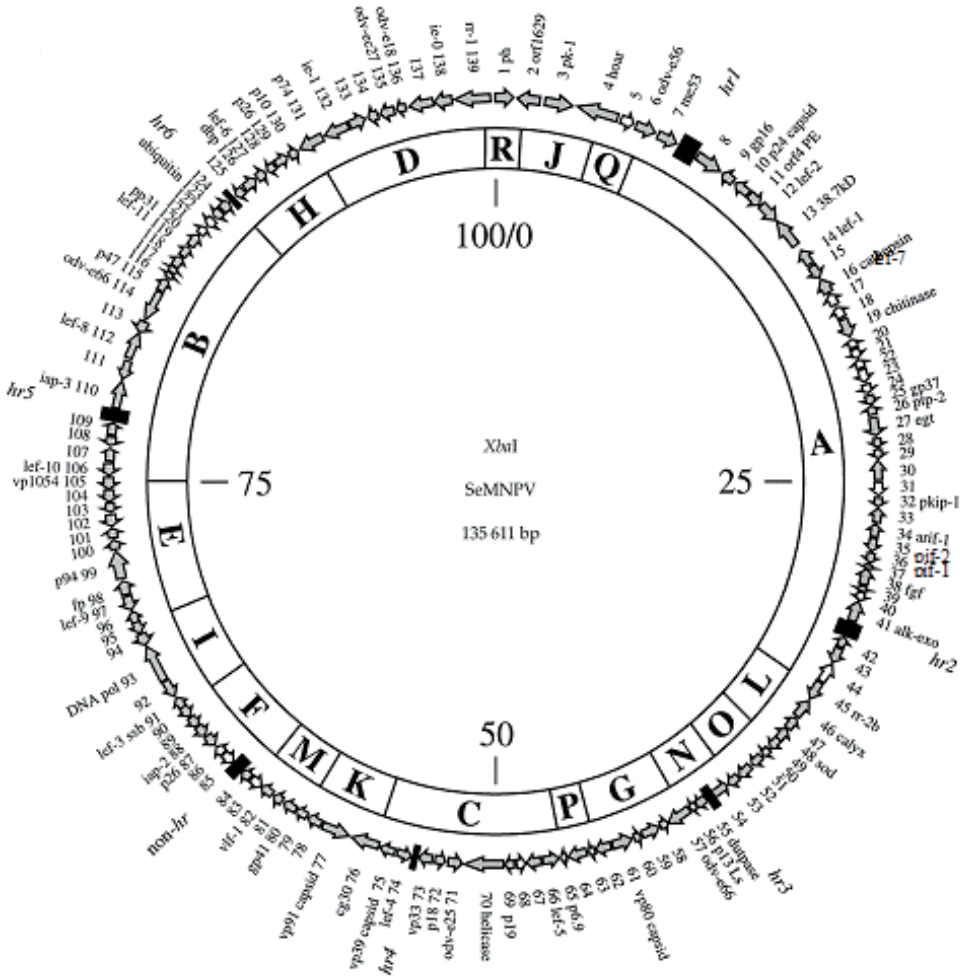

Figure 6. Circular map of the SeMNPV genomic organization. Sites for restriction enzyme *Xba*I are shown. The positions of the 139 ORFs identified are indicated by arrows that also represent the direction of transcription. The scale on the inner circle is in map units. (Adapted from IJkel *et al*., 1999).

SeMNPV isolates frequently comprise mixtures of different genotypes, identified by the presence of submolar bands on restriction endonuclease digestion profiles of the viral DNA (Muñoz *et al*., 1999). An SeMNPV hypervariable genomic region has been located between ORFs 17 to 39, in the *Xba*I-A fragment (Fig. 5). Most genotypic variations occur in this region. Isolation of individual genotypes by *in vivo* and *in vitro* cloning methods has allowed the study of different insecticidal properties of the genotypic variants (Dai *et al*., 2000; Muñoz *et al*., 1998 and 1999). The SeMNPV-US2 isolate, the active ingredient of the Spod-X®, comprises a mixture of genotypes, one of which is considered a parasitic genotype, since it reduces the insecticidal activity of the product (Muñoz *et al*., 1998). A detailed study on the genotypic composition of the different SeMNPV isolates seems to be necessary to understand the maintenance of such "defective" genotypes, which appear to remain stable in the viral population.

Figure 7. *S. exigua* larvae dead by SeMNPV.

SCOPE OF THE THESIS

Baculoviruses are natural mortality factors of insect populations and in the last decades, they have been used as agents for the control of insect pests in agricultural crops and forest trees. Along with the main use of baculovirus as biological control agents, they have been widely used in the applied biomedical industry as vectors

to produce recombinant proteins, and as gene delivery vectors in mammalian cells. Wild-type baculovirus populations are composed of a mixture of different genotypes that appear to play an important role in the biological performance of the virus. Detailed understanding of the genetic and phenotypic diversity within and between baculovirus populations can facilitate the selection of highly insecticidal strains for their development as commercial products. The main objective of this thesis is to determine the genomic factors that determine the insecticidal properties of the *S. exigua* MNPV baculovirus, namely, pathogenicity, virulence and transmission of this virus in *S. exigua* populations. This is of crucial importance to design more efficient active compounds in order to both, reduce costs and enhance the sustainability of SeMNPV-based control programs.

DNA sequencing technologies have facilitated the identification of virus genes that may play an important role in the insecticidal performance of SeMNPV. In a previous study, sequencing and comparison of seven different SeMNPV genotypes with different insecticidal properties, identified several ORFs as genes likely involved in such insecticidal traits: Se004, Se005, Se028, Se076, Se087 and Se129 (Thézé *et al.*, 2014). In **Chapter 2** a bacmid-based recombination system was developed to delete the individual ORFs previously identified. Their individual role in the insecticidal properties of the SeMNPV was studied by bioassays in *S. exigua* larvae.

Studies on the ecology of SeMNPV have revealed that some genotypes are specifically associated with covert infections of the virus that survive based on vertical transmission, whereas others are associated with horizontal transmission of the virus. By comparison of horizontal and vertically transmitted isolates, Thézé *et al.*, (2014) identified three ORFs that may be involved in the vertical transmission of the virus: Se005, Se096 and Se099. **In Chapter 3** the bacmid-based recombination system was used to delete the respective ORFs and test their role in vertical transmission. *S. exigua* larvae were sublethally infected and covert infections of SeMNPV were detected (or not) in moths by quantitative-PCR.

Different isolates of SeMNPV and SfMNPV have similar population structures, with the presence of different genotypes with deletions in the same genomic region encoding several ORFs. Since the SeMNPV population structure is stable upon passaging in *S. exigua*, the questions is what the role of these deleted genotypes is. In **Chapter 4** the genotypic structure of SeUS2, SeUS1, and SfNIC isolates was compared to determine the evolutionary or ecological mechanisms of these genotypically similar population structures. The isolation of a deleted genotype and functional complementation studies with different mixtures of a deleted and a complete genotype, demonstrated the interaction between genotypes in a natural viral population.

The SeMNPV-US1 isolate, which is also composed of several genotypes, contains a genotype with a large deletion of up to ~25 kb, in a hypervariable region of the SeMNPV genome. This genotype was selected for in insect cell culture and enabled the virus to replicate in these cultured cells. In **Chapter 5** the sequencing of a natural deletion genotype from the SeUS1 population, SeBac72, led to the identification of ORFs affected by this deletion. By differential deletion of individual ORFs from the complete genome of SeUS1, isolated as the bacmid SeBac10, the responsible gene that prevents successful viral spread of SeMNPV in cell culture was identified.

Finally in **Chapter 6**, the overall results of the previous experimental chapters were discussed, and different recommendations and future research will be discussed as well.

Chapter 2

Identification of *Spodoptera exigua* nucleopolyhedrovirus genes involved in pathogenicity and virulence

Adapted from:
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Abstract

Genome sequence analysis of seven different *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) isolates that differed in insecticidal phenotype permitted the identification of genes likely to be involved in pathogenicity of occlusion bodies (OBs) and speed of kill (virulence) of this virus: *se4* (*hoar*), *se5* (unknown function), *se28* (unknown function), *se76* (*cg30*), *se87* (*p26*) and *se129* (*p26*). To study the role these genes experimentally on insecticidal phenotype, a bacmid-based recombination system was constructed to delete selected genes from a SeMNPV isolate, VT-SeAL1, designated as SeBacAL1. None of the knockout viruses were lethal and the repair viruses behaved like the wild-type control, vSeBacAL1. Deletion of *se4*, *se5*, *se76* and *se129* resulted in decreased OB pathogenicity compared to vSeBacAL1 OBs. In contrast, deletion of *se87* did not significantly affect OB pathogenicity, whereas deletion of *se28* resulted in significantly increased OB pathogenicity. Deletion of *se4*, *se28*, *se76, se87* and *se129* did not affect speed of kill compared to the bacmid vSeBacAL1, whereas speed of kill was significantly extended following deletion of *se5* and in the wild-type isolate (SeAL1), compared to that of the bacmid. Therefore, biological assays confirmed that several genes had effects on virus insecticidal phenotype. *Se*5 is an attractive candidate gene for further studies, as it affects both biological parameters of this important biocontrol virus.

Introduction

Baculoviruses are a large group of arthropod-specific DNA viruses (van Oers & Vlak, 2007) with a double-stranded, circular genome varying in size from 80 to over 180 kb and encoding 90-180 open reading frames (ORFs) (Rohrmann, 2013b). Baculoviruses are used as biological control agents of insect pests (Eberle *et al.*, 2012), as protein expression systems of foreign genes in insect cells (Condreay & Kost, 2007), and more recently as potential viral vectors for gene delivery (Hitchman *et al.*, 2011). The family *Baculoviridae* is divided into four genera: *Alphabaculovirus* (lepidopteran nucleopolyhedroviruses), *Betabaculovirus* (lepidopteran granuloviruses), *Gammabaculovirus* (hymenopteran nucleopolyhedroviruses) and *Deltabaculovirus* (dipteran nucleopolyhedroviruses) (Herniou & Jehle, 2007; Jehle *et al.*, 2006). Comparison of all baculovirus genomes sequenced to date has resulted in the identification of 37 core genes (Garavaglia *et al.*, 2012), which seems to encode key factors for crucial processes such as infection, viral DNA replication and virion assembly.

Baculovirus isolates show a high degree of genetic heterogeneity. The genotypic variation in baculovirus populations has been associated with differences in phenotypic traits such as pathogenicity, virulence and occlusion body (OB) productivity (Erlandson, 2009). A comparison of the genomes of phenotypically distinct virus strains can be used to identify genes involved in these traits (Allen & Little, 2009), and guide the selection of strains or development of recombinant viruses with improved insecticidal properties as compared to the wild-type parental viruses.

To this end, Thézé *et al.* (2014), sequenced and compared the whole genome sequence of seven biologically distinct *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) isolates from Europe (named VT-SeAL1, VT-SeAL2, VT-SeOx4, HT-SeG24, HT-SeG25, HT-SeG26 and HT-SeSP2) that differed in transmission strategy and in related phenotypic traits. Two of these strains, HT-SeG25 and HT-SeG26,

were associated with horizontally transmitted infections, having been isolated from individuals infected during epizootics in natural field populations of *S. exigua*. These strains were the most pathogenic, in terms of concentration-mortality metrics, and their genomes shared a 6 bp deletion in *p26* (*ac136*). A unique characteristic of the SeMNPV genome, in contrast with those of all the other baculoviruses already sequenced, is that SeMNPV carries two copies of *p26*, named *se87* and *se129*. The function of P26 is presently unknown, but its conserved sequence, in almost all *Alphabaculovirus* genomes analyzed to date suggests an important role in baculovirus biology (Simón *et al.*, 2008). The single-nucleotide polymorphism (SNP) differences detected between HT-SeG25 and HT-SeG26 pointed out another gene, *se5*, as a correlate in OB pathogenicity. *Se5*, of unknown function, has an early promoter with a TATA sequence and a CAKT start site 20-40 nt downstream (IJkel *et al.*, 1999). Another isolate, SeOx4, was the fastest killing isolate and had a 4 bp deletion in *se28*, which encodes a putative protein of 190 amino acids of unknown function. An early promoter element TATA was identified 74 nt upstream the start codon followed by a CAKT element 31 nt downstream the TATA box (IJkel *et al.*, 1999). Furthermore, a SNP mutation was detected at position 75006 in the RING finger of *cg30* (*se76*). SE76 encodes for a protein of 468 amino acids with two functional motifs, a RING finger and a leucine zipper (Ishihara *et al.*, 2013; Passarelli & Miller, 1994). Mutants lacking *cg30* in *Bombyx mori* NPV produced fewer budded viruses and released lower number of occlusion bodies into the hemolymph of infected larvae, and reduced the speed of kill (Ishihara *et al.*, 2013).

In the present study we developed a bacmid-based recombination system to examine the influence of the genes identified by Thézé *et al*. (2014) on aspects of the insecticidal phenotype of SeMNPV. The results provide the basis for the development of improved biological insecticides based on novel recombinant baculoviruses.

Materials and methods

Insects, cell lines and viruses. *S. exigua* larvae were obtained from a laboratory

colony, maintained at constant environmental conditions (25 ± 1° C, 50% ± 5% RH and a photoperiod of 16:8 h light: dark) and reared on a wheat germ-based semisynthetic diet (Greene *et al.*, 1976). *S. exigua* Se301 cells, kindly provided by S. Herrero (Universidad de Valencia, Spain), were maintained at 28ºC in HyClone Insect Cell Culture Media CCM3 supplemented with 5% fetal bovine serum (Thermo Scientific). The SeMNPV isolate used in this study was VT-SeAL1, one of the isolates sequenced by Thézé *et al*. (2014), which originated from the progeny of field-collected moths that produced progeny that subsequently died from virus infection during laboratory rearing. The isolate, which was associated with a vertically-transmitted infection, was amplified by inoculating *S. exigua* fourth instars from the laboratory colony using the droplet feeding method (Hughes *et al.*, 1986).

Construction of SeMNPV bacmid. Empirical analysis of VT-SeAL1 DNA with restriction enzymes identified a single *Mau*BI site. The bacmid cloning vector BAC-*Bsu*36I (Pijlman *et al.*, 2002) was modified by adding a *Mau*BI restriction site using a oligonucleotide linker and was designated as BAC-*Bsu*-*Mau*BI. VT-SeAL1 DNA for direct cloning was purified by CsCl gradient centrifugation (King & Possee, 1992). A 2 µg sample of viral SeAL1 DNA was linearized by digestion with 10 U *Mau*BI (Thermo Scientific) for 16 h at 37ºC. The restriction enzyme was heat inactivated for 20 min at 65ºC. Then, 1 µg of bacmid cloning vector BAC-*Bsu*-*Mau*BI was digested with 10 U of *Mau*BI for 3 h at 37ºC. The vector was dephosphorylated using 1 U alkaline phosphatase (Promega) for 1 h at 37ºC and gel purified with GFX Gel Band Purification Kit (GE Healthcare). Ligation was performed overnight at 4ºC with approximately 500 ng linearized SeAL1 DNA and 25 ng linearized vector DNA using T4 DNA ligase (Promega). Electrocompetent *E. coli* DH10β cells (Invitrogen) were transformed with 3 µl ligation mix at 1.8 kV using a Bio-Rad Gene Pulser. The transformed cells were recovered in SOC medium for 1 h at 37º C and spread on agar plates containing kanamycin. A SeMNPV bacmid with the correct restriction profile

was selected and designated as SeBacAL1.

Generation of knockout bacmids. For deletion mutagenesis of genes *se4*, *se5*, *se28*, *se76*, *se87* and *se129* from SeBacAL1, 68- to 70-bp primers were designed with 50-bp 5´ends within the deletion target region on the SeMNPV genome (Table 1). The 3' ends of the primers anneal to the chloramphenicol gene flanked by mutant *LoxP* sites (Suzuki *et al.*, 2005) using a mutant lox sequence, which was amplified from pCRTopo-lox-cat-lox (Marek *et al.*, 2011). PCR on pCRTopo-lox-cat-lox was performed using Phusion Polymerase (Thermo Scientific) according to the manufacturer, giving a product of 1170 bp. SeBacAL1 DNA was cloned into electrocompetent MW003 cells (Westenberg *et al.*, 2010), and selected on LB-plates with streptomycin and kanamycin for 2 d at 32°C. Single colonies were picked and used to inoculate 1 ml SOB-medium at 32°C. This culture was used to inoculate 10 ml SOB-medium, incubated at 32°C and cells were harvested when the OD_{600} value reached 0.6. The culture was then split in two and 5 ml were induced for 10 min at 42°C. After incubation, the cells were washed twice with ice-cold 10% glycerol. Finally, the cells were suspended in 100 µl of 10% glycerol and stored at -80°C. The next day cells were electroporated with 150 ng of the PCR product from pCRTopo-lox-cat-lox. The cells were recovered in 1ml SOB-medium and incubated for 3 hours at 32°C. Subsequently, both induced and non-induced cells were plated out on LB plates, supplemented with 50 µg/ml kanamycin and 50 µg/ml chloramphenicol. The plates were incubated for 48 h at 32°C. Finally, single colonies were picked to analyze if recombination had occurred (Dolphin & Hope, 2006). To confirm the deletion of the ORFs, restriction endonuclease analysis of the bacmid DNA and PCR amplifications were performed. Once the correct knockout was selected, bacmid DNA extraction was performed and electroporated into DH10β (Invitrogen).

Generation of repair bacmids. The ORF coding regions were amplified using

Phusion Polymerase (Thermo Scientific) with primers including their own promoter and containing *XbaI* and *KpnI* restriction sites (Table 1). The resulting fragments were cloned into a CloneJET PCR Cloning Kit (Thermo Scientific), sequenced and cloned as *XbaI*/*KpnI* fragments into a pFastBacΔAcPpol. The protocol from the Bac-to-Bac manual (Invitrogen) was followed to transpose the ORFs from pFastBacORF into the *att*Tn7 transposon integration site of SeBacAL1 to generate the repair bacmids. To confirm the correct transposition into the *att*Tn7 site, PCR amplifications were performed.

Transfection of SeMNPV bacmids. Se301 cells were seeded in a six-well tissue culture plate (Greiner Bio-One) at a confluency of $5x10^5$ cells/well. Transfection was performed with 1 µg SeBacAL1ΔORF or SeBacAL1ΔORFrepair using 10 µl lipofectin (Invitrogen). As a positive control, 1 µg SeMNPV-AL1 DNA was transfected. At 7 d post-transfection, OBs were formed by cells transfected with SeMNPV-AL1 and the bacmids. Cells were harvested at 14 d post-transfection. For OB amplification, fourth-instar *S. exigua* from the laboratory colony were inoculated by the droplet feeding method.

Bioassays. Bioassays with OBs from the knockout and the repair viruses were performed using the droplet feeding method. For this, groups of 24 *S. exigua* second instars were starved overnight and then allowed to drink from an aqueous suspension containing 10% sucrose, 0.001% Fluorella blue, and OBs at one of five different concentrations (2.45×10^5, 8.1×10^4, 2.7×10^4, 9×10^3, 3×10^3 OBs/ml). Control larvae drank a solution of sucrose and Fluorella blue without OBs. Larvae that ingested the suspension within 10 min were transferred individually to 24-well tissue culture plates with semi-synthetic diet. Each bioassay was performed three times. Inoculated insects were incubated at 25 ^oC and mortality was recorded daily until larvae died from polyhedrosis disease or pupated. Virus-induced mortality

was subjected to probit analysis using the Polo-PC program (LeOra Software, 1987). Mean time to death (MTD) was calculated using groups of 24 *S. exigua* second instars, that had been inoculated with the LC_{90} concentration of each virus (1.71×10^5 OBs/ml for SeBacAL1, 1.11×10^5 OBs/ml for SeAL1, 5.73×10^5 OBs/ml for SeBacAL1Δ4, 1.31×10^6 OBs/ml for SeBacAL1Δ5, 7.75×10^4 OBs/ml for SeBacAL1Δ28, 2.78×10^5 OBs/ml for SeBacAL1Δ76, 1.61×10^5 OBs/ml for SeBacAL1Δ87, 2.27×10^5 OBs/ml for SeBacAL1Δ129, 2.03×10^5 OBs/ml for SeBacAL1Δ4repair, 2.49×10^5 for SeBacAL1Δ5repair, 2.35×10^5 OBs/ml for SeBacAL1Δ28repair, 1.57×10^5 OBs/ml for SeBacAL1Δ76repair, 1.95×10^5 OBs/ml for SeBacAL1Δ87repair, and 1.07×10^5 OBs/ml for SeBacAL1Δ129repair), as estimated in the previous bioassay. Inoculated larvae were reared individually at 25ºC and mortality was recorded at 8 h intervals until death or pupation. The whole experiment was performed three times. Time mortality data were subjected to Weibull survival analysis using the Generalized Linear Interactive Modeling (GLIM) program (Crawley, 1993).

Infectivity of ODVs. The infectivity of ODVs isolated from SeBacAL1, SeBacAL1Δ5 and SeBacAL1Δ5repair OBs was determined by end point dilution assays using Se301 insect cells. For this, ODVs were released from samples of $5x10^8$ OBs in a volume of 500 µl by incubation with 0.1 M Na_2CO_3 at 28°C for 30 minutes. The resulting suspension was passed through a 0.45 µm filter and serially diluted 1:5 in CCM3 medium + 5% FBS. Se301 infected cells were titrated in a 60-well microtiter-dish. Titers were analyzed using the Spearman-Karber method to estimate 50% tissue culture infectious dose ($TCID_{50}$). The $TCID_{50}$ values were converted to infectious units per $5x10^8$ OBs. The results were normalized by logarithmic transformation and compared by one-way ANOVA in SPSS v21 (IBM SPSS Version 21.0. Armonk, NY: IBM Corp). The experiment was performed three times.

Electron microscopy. Scanning electron microscopy (SEM) was used to determine

OB diameter of SeBacAL1, SeBacAL1Δ5 and SeBacAL1Δ5repair. OBs were fixed in 12.5% glutaraldehyde in 0.1 M Sörenson phosphate buffer, pH 7.2, and dehydrated in series of ethanol solutions. A total of 30 OBs were analyzed for each virus. The numbers of ODVs occluded within OBs of SeBacAL1, SeBacAL1Δ5 and SeBacAL1Δ5repair was determined by examination of OB sections by transmission electron microscopy (TEM) as described by Hikke *et al.* (2014) and analyzed via a JEOL JEM 1011 (JEOL, MA, USA). The numbers of ODVs was counted for 30 cross-sections of randomly selected OBs. The mean size of OBs and mean numbers of ODVs were normally distributed and were compared by one-way ANOVA in SPSS v21 (IBM SPSS Version 21.0. Armonk, NY: IBM Corp).

Gene and protein sequence analysis. DNA and protein homologs of *se5* were searched in the updated GenBank/EMBL databases using BLAST (Altschul *et al.*, 1990). PSIPRED was used to predict protein secondary structure (McGuffin *et al.*, 2000) and PROSITE was used to search for protein domains (Hulo *et al.*, 2008). Cellular location was predicted with TargetP 1.1 (Emanuelsson *et al.*, 2000) and transmembrane domains were detected with TMHMM v2.0 (Jones, 2007).

Results

Generation of SeBacAL1 and knockout mutants. To determine the effects of removing genes *se4*, *se5*, *se28*, *se76*, *se87* and *se129* individually on OB pathogenicity and speed of kill of SeMNPV, a SeMNPV bacmid using the VT-SeAL1 isolate genome was constructed (SeBacAL1) (Fig. 1A). This genotype was picked up from a colony collapse of a laboratory rearing of *S. exigua*. A BAC-*Bsu*36I vector was inserted at position 28454 nt within the VT-SeAL1 genome, an intergenic region located between *se27* (*egt*) and *se28*. Both genes have been previously found to be non-essential for virus replication *in vivo* and in cell culture (Muñoz *et al.*, 1998; Serrano *et al.*, 2013). SeBacAL1Δ4, SeBacAL1Δ5, SeBacAL1Δ28, SeBacAL1Δ76, SeBacAL1Δ87 and SeBacAL1Δ129 knockout bacmids were then constructed by deleting *se4*, *se5*, *se28*,

se76, *se87* and *se129*, respectively, via lambda Red recombination (Westenberg *et al.*, 2010). A chloramphenicol acetyltransferase (*cat*) gene was inserted in replacement of these genes to facilitate antibiotic selection in *E. coli*. As controls, knockout-repair bacmids were constructed by re-inserting each gene under the control of their native promoters into the mini-*att*Tn7 attachment site within the bacmid. The resulting repair-knockouts viruses were designated as SeBacAL1Δ4repair, SeBacAL1Δ5repair, SeBacAL1Δ28repair, SeBacAL1Δ76repair, SeBacAL1Δ87repair

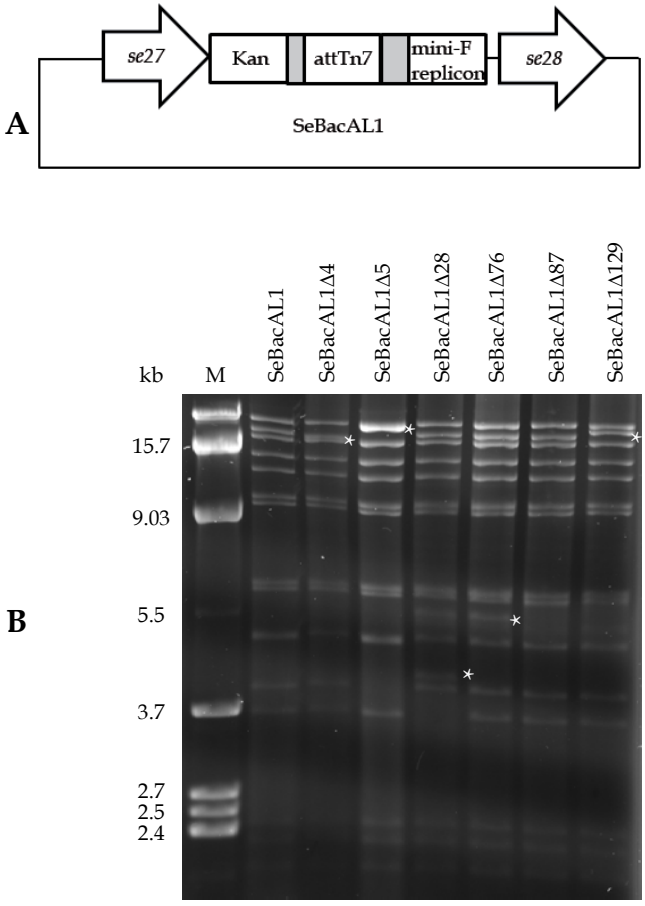


Figure 1. (A) Schematic overview of the SeBacAL1 (not to scale) and position of the bacterial insert and the relevant SeMNPV ORFs. **(B)** Restriction endonuclease profiles for *Bgl*II of the DNAs of SeBacAL1 and the knockout viruses, vSeBacAL1Δ4, vSeBacAL1Δ5, vSeBacAL1Δ28, vSeBacAL1Δ76, vSeBacAL1Δ87, and vSeBacAL1Δ129. Lane M contains a λ *Bam*HI/*Hin*dIII/*Eco*RI DNA size marker. The asterisks indicate the restriction polymorphisms of each knockout.

and SeBacAL1Δ129repair. To check the identity of the knockouts viruses and their repairs, restriction endonuclease analysis (Fig. 1B) and PCR analysis (data not shown) were performed. OB production was achieved in Se301, which were transfected with bacmid DNA from the different SeMNPV constructs. OBs were subsequently amplified *in vivo* by feeding *S. exigua* fourth instars via the droplet feeding method.

Pathogenicity of gene knockout viruses. Mean lethal concentrations (LC_{50}) values of each of the knockout and repair bacmids were estimated in *S. exigua* second instars by droplet feeding method (Table 2). The wild-type isolate VT-SeAL1 was

Table 2. 50% lethal concentration (LC_{50}) values of vSeBacAL1 and the different knockout and repair bacmids in *Spodoptera exigua* second instars.

Virus	LC_{50}(OBs/ml)	Relative Potency	95% confidence limits	
			Lower	Upper
SeBacAL1	$1.63x10^4$	1	-	-
SeAL1	$1.12x10^4$	1.45	0.99	2.12
SeBacAL1Δ4	$6.09x10^4$	**0.26**	**0.18**	**0.38**
SeBacAL1Δ5	$1.60x10^5$	**0.10**	**0.06**	**0.16**
SeBacAL1Δ28	$1.09x10^4$	**1.48**	**1.05**	**2.10**
SeBacAL1Δ76	$3.68x10^4$	**0.44**	**0.31**	**0.62**
SeBacAL1Δ87	$2.14x10^4$	0.76	0.54	1.07
SeBacAL1Δ129	$2.96x10^4$	**0.55**	**0.39**	**0.77**
SeBacAL1Δ4repair	$2.30x10^4$	0.71	0.49	1.01
SeBacAL1Δ5repair	$2.38x10^4$	0.68	0.46	1.09
SeBacAL1Δ28repair	$1.97x10^4$	0.83	0.57	1.21
SeBacAL1Δ76repair	$1.91x10^4$	0.85	0.60	1.22
SeBacAL1Δ87repair	$2.31x10^4$	0.71	0.49	1.01
SeBacAL1Δ129repair	$1.55x10^4$	1.05	0.74	1.48

Probit analysis was performed using the PoloPlus program. A test for non-parallelism was not significant (χ^2=9.40, df=13, P=0.742). Regressions were fitted with a common slope (±SE) of 1.289 ± 0.308

as pathogenic as the vSeBacAL1, as indicated by the overlap of the 95% confidence limits. The recombinant virus vSeBacAL1Δ28 was significantly more pathogenic than the reference vSeBacAL1, as judged by the relative potency 95% confidence limits.

In contrast, most of the recombinant viruses were significantly less pathogenic than vSeBacAL1, namely: vSeBacALΔ4 by 3.8-fold, vSeBacAL1Δ5 by 10-fold, vSeBacAL1Δ76 by 2.3-fold and vSeBacAL1Δ129 by 1.8-fold. vSeBacAL1Δ87 was the only recombinant with an LC_{50} value statistically similar to that of control virus OBs (Table 2). OBs of all repair viruses were as pathogenic as the parental vSeBacAL1 OBs, as indicated by the overlap of the 95% fiducial limits of the relative potency values.

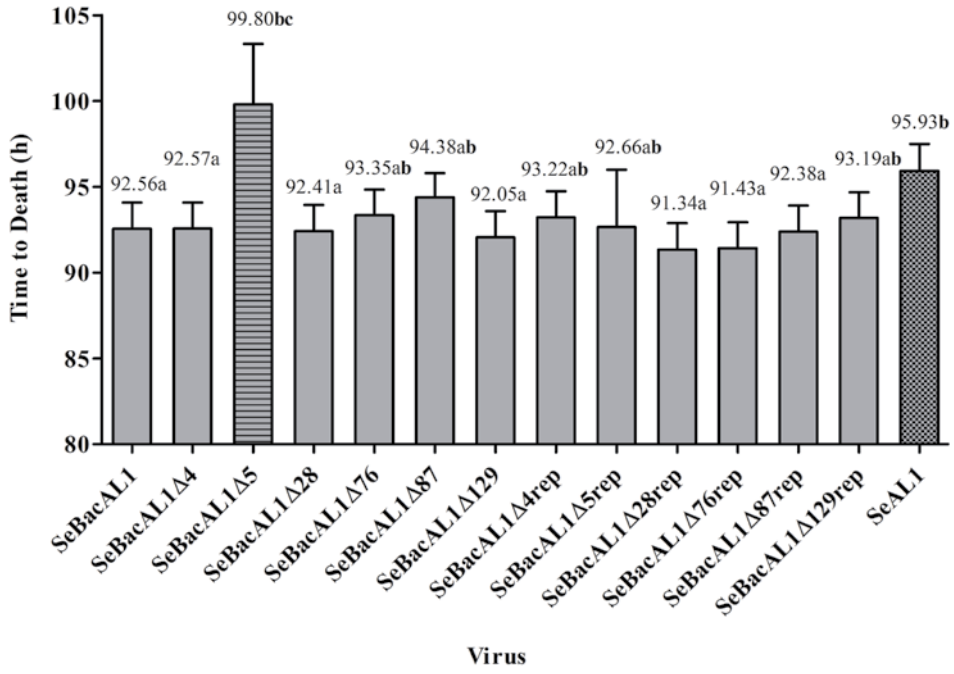


Figure 2. Mean-time-to-death (MTD) values of vSeBacAL1 and the different gene knockout and repair viruses in second instar *S. exigua*. MTD values were estimated by Weibull survival analysis. Bars labelled with the same letter did not differ significantly (p>0.05).

Mean-time-to-death of gene knockout viruses. Mean-time-to-death (MTD) values of the different viruses in second instars ranged between 91.3 and 99.8 hours post-inoculation (hpi). All different viruses tested could be classified into three groups according to their speed of kill (Fig. 2). vSeBacAL1Δ4, vSeBacAL1Δ28, vSeBacAL1Δ76, vSeBacAL1Δ87, vSeBacAL1Δ129, and all six repair viruses were as virulent as vSeBacAL1, and their MTDs ranged, from 91.3 to 94.4 hpi (group a). The wild type virus, VT-SeAL1, ranked second (group b) and the slowest killing isolate was vSeBacAL1Δ5, with MTD 99.8 hpi (group c).

ODV infectivity. To investigate whether the specific infectivity of ODVs was affected by the deletion of *se5*, the infectivity of vSeBacAL1, vSeBacAL1Δ5 and vSeBacAL1Δ5repair ODVs was compared via an end-point dilution assay. ODVs

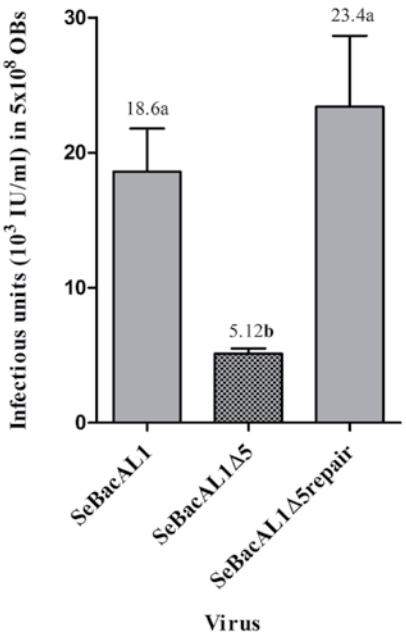
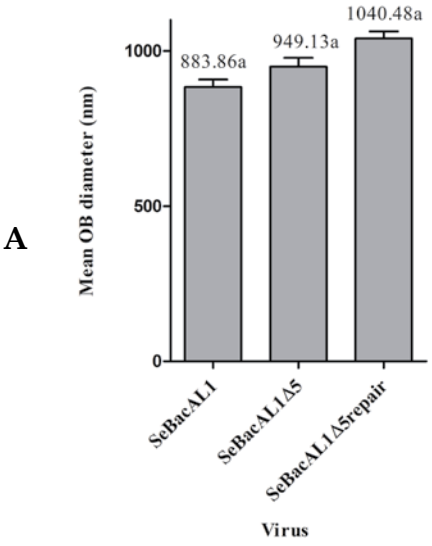


Figure 3. ODV content in 5x108 OBs of vSeBacAL1, vSeBacAL1Δ5 and vSeBacAL1Δ5repair. ODV titers were calculated by end-point dilution. Error bars indicate standard error of the mean. Bars labelled with the same letter did not differ significantly (p>0.05).

were isolated from $5x10^8$ OBs of each virus and were titrated on Se301 cells. The titer of infectious units of ODVs released from vSeBacAL1Δ5 OBs was markedly lower than those of the vSeBacAL1 or vSeBacAL1Δ5repair viruses ($p< 0.05$) (Fig. 3). Scanning electron microscopy (SEM) pictures showed OBs diameters between 0.88



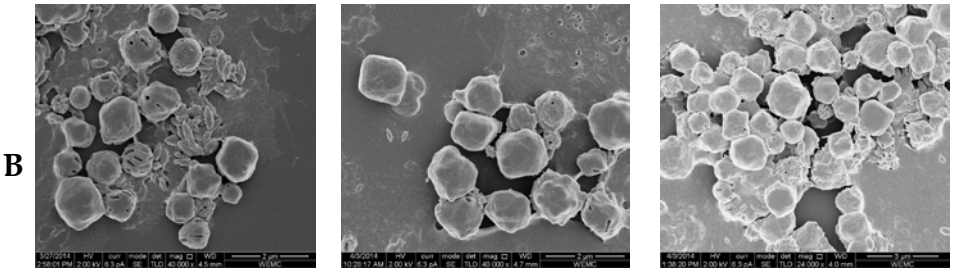
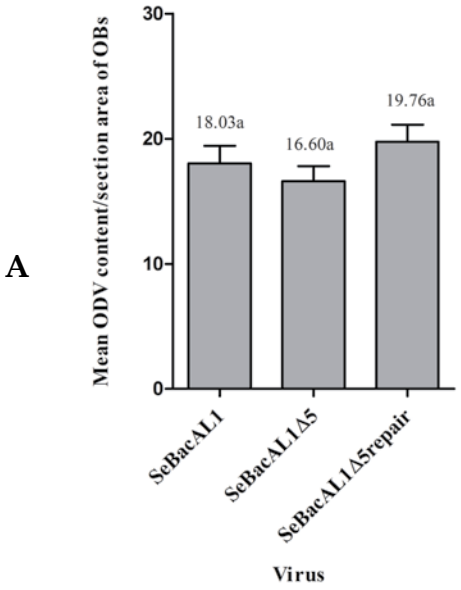


Figure 4. (A) Mean OBs size of SeBacAL1, SeBacAL1Δ5 and SeBacAL1Δ5repair as determined by scanning electron microscopy (SEM). Error bars indicate standard error of the mean. Bars labelled with the same letter did not differ significantly ($p>0.05$). **(B)** SEM pictures of SeBacAL1, SeBacAL1Δ5 and SeBacAL1Δ5repair

to 1.04 µm, values were statistically similar between vSeBacAL1, vSeBacAL1Δ5 and vSeBacAL1Δ5repair ($p>0.05$) (Fig. 4A) (Fig. 4B). Transmission electron microscopy (TEM) observations revealed that the number of ODVs were statistically similar for vSeBacAL1, vSeBacAL1Δ5 and vSeBacAL1Δ5repair OBs ($p>0.05$) (Fig. 5A) (Fig. 5B).

Sequence analysis of *se5*. Sequence analysis revealed that *se5* was located between nucleotides 6164 and 7694 in the positive strand of VT-SeAL1. SE5 is a protein of 513 amino acids (aa) with a predicted molecular weight of 59.24 kDa present in all sequenced SeMNPV genotypes. The *se5* gene is a homologue of *S. litura* MNPV ORF6, an ORF of unknown function. Secondary structure prediction with PSIPRED revealed 15 helices and 6 β-sheets. The PROSITE predicted some functional motifs in SE5 such as a tyrosine kinase phosphorylation site (aa 57-64), a cAMP- and cGMP-dependent protein kinase phosphorylation site (aa 95-98), five protein kinase



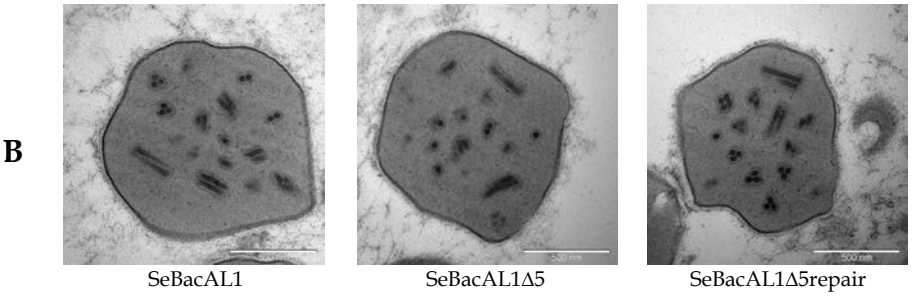


Figure 5. (A) Mean ODVs content within OBs of vSeBacAL1, vSeBacAL1Δ5 and vSeBacAL1Δ5repair as determined by transmission electron microscopy (TEM) analysis. Error bars indicate standard error of the mean. Bars labelled with the same letter did not differ significantly ($p>0.05$). **(B)** TEM pictures of vSeBacAL1, vSeBacAL1Δ5 and vSeBacAL1Δ5repair.

C phosphorylation sites (aa 114-116, 152-154, 363-365, 480-482 and 491-493), nine casein kinase II phosphorylated sites (aa 162-165, 239-242, 255-258, 257-260, 268-271, 377-380, 502-505, 503-506 and 509-512) and two N-myristoylation sites (aa 330-335 and 409-414). No signal peptide or transmembrane domains were detected in the putative protein.

Discussion

After a genomic comparison of SeMNPV isolates displaying a distinct pathogenicity and virulence pattern (Thézé *et al.*, 2014), six genes, *se4, se5, se28, se76, se87* and *se129*, were selected for further investigation. This was done by constructing individual gene knockout mutant and repair viruses, and studying two main aspects of their insecticidal phenotype: OB pathogenicity and speed of kill. These six genes are not part of the set of 37 baculovirus core genes shared by all known members of the *Baculoviridae* family sequenced to date (Garavaglia *et al.*, 2012). Core genes are thought to be involved in fundamental processes such as DNA replication, gene transcription, nucleocapsid assembly or virion formation (van Oers & Vlak, 2007). Despite the high number of conserved genes, the total number of genes present in baculoviruses collectively is remarkable (>1000). Some of these genes are unique to a virus species and are believed to contribute to the specific phenotype of each baculovirus. Others may be involved in pathogenicity and virulence, or to biological traits to be involved. Viruses undergo evolution by gene loss or gain, gene exchange and by accumulation of point mutations that can lead to specialization in gene function (Garavaglia *et al.*, 2012). Evolutionary variation in entomopathogenic populations could benefit selection of variants with an enhanced level of a particular trait, that can be useful when these pathogens are used as a microbial control agent (Cory & Franklin, 2012).

Deletion of *se4* decreased OB pathogenicity by almost 4-fold compared with vSeBacAL1 (Table 2), but had no effect on speed of kill (Fig. 2). *Se4* is a homolog of

the *hoar* gene, which is found in other group II *alphabaculoviruses*. This gene, which is under the control of an early promoter (IJkel *et al.*, 1999), has an unusual codon bias and displays genetic variability suggesting frequent mutations in this locus (Le *et al.*, 1997). It has been suggested that *hoar* may interfere with host defenses and the high mutation rate could be an adaptation that favors infection across different host species (Le *et al.*, 1997). Additionally HOAR has been found in the ODV membrane in *Helicoverpa armigera* NPV (Hou *et al.*, 2013), and in midgut cells of *Mamestra configurata* larvae during infection by *M. configurata* NPV-A (Donly *et al.*, 2014). As a result, HOAR has been hypothesized to be a regulatory protein that allows adaptation to different insect hosts, and that seems to play a role in the primary infection of host midgut cells (Donly *et al.*, 2014). In view of our observations, *se4* may enhance the ODV efficiency of entry in the midgut.

Deletion of *se5*, a gene of unknown function, resulted in a 10-fold decrease in OB pathogenicity (Table 2), whereas time to death of infected insects was increased by 7 h compared to the parental SeBacAL1 (Fig. 2). In addition, the infectivity of ODVs in cell culture was between three and four times lower. Differences in the infectious activity of ODVs by end-point dilution may be the consequence of less ODVs occluded within SeBacAL1Δ5 OBs, or OBs of smaller size. However, there were not significant differences observed in the number of virions occluded within OBs, or in the size of the OBs. Analysis of the putative SE5 protein did not indicate the presence of signal peptide or transmembrane domains, suggesting that it might be an intracellular protein. Two *N*-myristoylation sites were detected in SE5 that may be involved with weak and reversible protein-membrane and protein-protein interactions (Murray *et al.*, 1997; Peitzsch & McLaughlin, 1993). Several phosphorylation sites were also identified. Post-translational phosphorylation affects many cellular signaling pathways, including metabolism, growth, differentiation and membrane transport (Blom *et al.*, 1999). Although protein analysis provided no further clues about the putative function of *se5*, but bioassays indicated an important role of this gene in

pathogenicity and virulence requiring further studies.

Deletion of *se28* (vSeBacAL1Δ28) resulted in a slight increase in OB pathogenicity compared to vSeBacAL1 OBs. *Se28* encodes for a putative protein of 190 aa with homologs only in some group II *alphabaculoviruses*, but not in those of group I. *Se28* deletion did not significantly affect the speed of kill and protein sequence analysis failed to detect conserved domains or motifs that could provide hints concerning its function. Moreover, *se28* is located in a hypervariable region of the SeMNPV genome; genotypes have been identified in natural SeMNPV populations with deletions encompassing ORFs 12 to 39 (Dai *et al.*, 2000; Muñoz *et al.*, 1998), indicating that this gene is unlikely to be essential for viral replication . A homolog of *se28* (*Maco40*) was expressed in midgut cells of *Mamestra configurata* larvae after infection with MacoNPV-A, suggesting that *se28* might have a role in the early stages of infection (Donly *et al.*, 2014).

Deletion of *se76* (vSeBacAL1Δ76) resulted in a 2.3-fold increase in LC_{50} value, indicating reduced OB pathogenicity (Table 2). *Se76* encodes a homolog of CG30. The *cg30* gene is present in almost all *alphabaculoviruses,* and contains a RING finger motif and a leucine zipper motif (Thiem & Miller, 1989). *cg30* may be an ubiquitin ligase that catalyzes the ubiquitination and destruction of cellular p53 (Imai *et al.*, 2003). *Autographa californica* MNPV (AcMNPV) lacking *cg30* was found to replicate in a similar fashion as wild-type virus in cell culture or in insects (Passarelli & Miller, 1994). A *cg30* knockout mutant of *Bombyx mori* NPV, however, produced fewer budded virions (BVs) and OBs and took 24 h longer than wild-type BmNPV to kill infected insects (Ishihara *et al.*, 2013). Speed of kill was not affected in case of vSeBacAL1Δ76, in contrast to the observations of Ishihara *et al.* (2013), perhaps due to the different virus-host systems employed. Another member of the RING finger gene family, *ie-2*, displays different phenotypes upon infection in different host cells (Prikhod'ko *et al.*, 1999). Indeed, the ability of *ie-2* to trans-stimulate viral DNA replication is dependent on the cell line used. Due to its DNA binding motifs

it is likely that CG30 binds to viral genomic DNA and may play a role in regulation of viral and/or host gene expression, but the consequences for pathogenicity remain elusive.

Both *se87* and *se129* encode for the double copy of P26 in SeMNPV (IJkel *et al*., 1999). The *p26* gene is not essential for virus replication in cell culture (Goenka & Weaver, 2008). A *p26* knockout in SfMNPV did not affect OB infectivity or speed of kill in larvae (Simón *et al*., 2008). Our results on the *se87* knockout support previous observations on *p26*; deletion had no significant effect on LC_{50} value, whereas *se129* knockout resulted in a 1.8-fold reduction in OB pathogenicity. Deletion of *se87* and se129 resulted in similar speed of kill compared to the parental bacmid virus. The role of these two copies of P26 remains of interest and a double-knockout (*se87* and *se129*) might provide further information on their role in pathogenicity and/or virulence.

In conclusion, the genes studied, *se4, se5, se28, se76* and *se129* have an effect on insecticidal properties of SeMNPV, as hypothesized by Thézé *et al.* (2014). *Se5* is the most promising gene for further studies as it affects both pathogenicity and virulence, although it is conceivable that these properties are linked in this case. The other genes deserve further investigation as their effect on virulence and pathogenicity are not connected.

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Supplementary information

Table 1. Name and sequence of the primers used for the construction of SeBacAL1 knockout and repair bacmids.

Primer	Sequence	Amplification purpose	Annealing position in SeAl1
SeORF4-KO-F	TACAATTTAATTTTTTTAGATGGTGGTTGTGATGATTCGTCGTCGTGATCGCTCGGATCCACTAGTAACG	Se4 deletion from SeMNPV bacmid; forward primer	3187-3236
SeORF4-KO-R	CAAATGGTGTGTTCGACGCCGTTCGTGCACGTCGGAGATGTTGATCTCAACCTCTAGATGCATGCTCG	Se4 deletion from SeMNPV bacmid; reverse primer	5284-5333
SeORF5-KO-F	ATGGTTAACGATTCCAGAAACACTGATATCATCGACGCTGTCGTCTGAGCGCTCGGATCCACTAGTAACG	Se5 deletion from SeMNPV bacmid; forward primer	6165-6214
SeORF5-KO-R	TTATGCATCAGCTGTTGTTTGATCTTCGTCATCGGTGGTTTCTCCGTCGCCCTCTAGATGCATGCTCG	Se5 deletion from SeMNPV bacmid; reverse primer	7645-7694
SeORF28-KO-F	ATGGCCACGATCAGAAATAAAAGCTTGTTGCGCAGTCTCGAACACTGACGGCTCGGATCCACTAGTAACG	Se28 deletion from SeMNPV bacmid; forward primer	28565-28614
SeORF28-KO-R	TCACTCCGAGTACATTATTCGAAGTTCATTTTCAAACTTATCCAAATCGTCCTCTAGATGCATGCTCG	Se28 deletion from SeMNPV bacmid; reverse primer	29088-29137
SeORF76-KO-F	ATGGAATCGATAACACTCGGTTGTTCGGTGTGCATGTCCGAAGTCTGAATGCTCGGATCCACTAGTAACG	Se76 deletion from SeMNPV bacmid; forward primer	74519-74568
SeORF76-KO-R	TTAAAATTTAGCTTTTTTAAAAATGGCAATAGTGTTAGACGACGTCGATGCCTCTAGATGCATGCTCG	Se4 deletion from SeMNPV bacmid; reverse primer	75876-75925
SeORF87-KO-F	TACAATATTAAAACGTTGCCGGCAAATTGGGTTTTGATTCTAATTTGAGAGCTCGGATCCACTAGTAACG	Se87 deletion from SeMNPV bacmid; forward primer	84058-84107
SeORF87-KO-R	TCAATGTCGATGTGTGTTAATTCGGTTGCGACCAACGATGTCGCCAAACCCCTCTAGATGCATGCTCG	Se87 deletion from SeMNPV bacmid; reverse primer	84766-84815
SeORF129-KO-F	ATGATGAGCTTTGCGAGTTTTTTACTAGTGCTCATTTGTTCGGCGTGATCGCTCGGATCCACTAGTAACG	Se129 deletion from SeMNPV bacmid; forward primer	122957-123001
SeORF129-KO-R	CTATACGATATTGCCAATACTGTCGTCGTTGTCGTCGTCGTCTTTGTTCTCCTCTAGATGCATGCTCG	Se129 deletion from SeMNPV bacmid; reverse primer	123747-123796
SeORF4-F	GGTCTAGAGCGTACACAAAAGCAAAAAA	Se4 insertion into SeBacAL1Δ4; forward primer	5478-5497
SeORF4-R	GGGGTACCGAAACACTCATATAGAAAGC	Se4 insertion into SeBacAL1Δ4; reverse primer	3159-3178
SeORF5-F	GGCTCGAGGAATGATGACCAACTTTTTTG	Se5 insertion into SeBacAL1Δ5; forward primer	5968-5988
SeORF5-R	GGAAGCTTATATGTACACAATAAAATTCAAAG	Se5 insertion into SeBacAL1Δ5; reverse primer	7768-7791

SeORF28-F	GGTCTAGATTTCAACGTATTGCC-TACGC	Se28 insertion into SeBacAL1Δ28; forward primer	28436-28455
SeORF28-R	GGGGTACCGAAAAAAGCGT-GGTTTCCAA	Se28 insertion into SeBacAL1Δ28; reverse primer	29138-29157
SeORF76-F	GGTCTAGATATCATGTACTACCTAT-CAT	Se76 insertion into SeBacAL1Δ76; forward primer	74368-74387
SeORF76-R	GGGGTACCTTGCAAATAAAATA-CAGTTTAC	Se76 insertion into SeBacAL1Δ76; reverse primer	75929-75950
SeORF87-F	GGTCTAGATACAACGTTTTGCG-CATTCG	Se87 insertion into SeBacAL1Δ87; forward primer	84942-84961
SeORF87-R	GGGGTACCCTGTGAATCAAATGT-GAATC	Se87 insertion into SeBacAL1Δ87; reverse primer	84037-84056
SeORF129-F	GGTCTAGACAAGAAACTGC-CATTTTATA	Se129 insertion into SeBacAL1Δ129; forward primer	122806-122825
SeORF129-R	GGGGTACCTTTAATGTCGGCTCG-GATCA	Se129 insertion into SeBacAL1Δ129; reverse primer	123860-123879

Chapter 3

Analysis of genes potentially involved in vertical transmission of *Spodoptera exigua* multiple nucleopolyhedrovirus

Adapted from:
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Abstract

Baculoviruses can be transmitted horizontally from insect to insect, or vertically, from parents to offspring. Analysis of the whole genome sequence of seven different *Spodoptera exigua* nucleopolyhedrovirus (SeMPNV) genotypes associated with vertical (VT-SeAL1, VT-SeAL1, VT-SeOx4) or horizontal transmission strategies (HT-SeG24, HT-SeG25, HT-SeG26 and HT-SeSP2A), revealed that the vertically transmitted genotypes shared the same mutations in three genes that were different from those of the horizontally transmitted isolates (Thézé *et al*., 2014). Those three genes were *se5*, *se96* and *se99*. A bacmid-based recombination system was used to successfully generate single 'knockout' viruses from the parent genotype, VT-SeAL1. Fourth instar *S. exigua* larvae were infected with a 40% lethal concentration (LC_{40}) of the different 'knockout' viruses; a concentration that in case of VT-SeAL1 produced a high prevalence of sublethally infected adults. Analysis of the prevalence of infection in the adults, inoculated at the larval stage with the different viral treatments showed no significant differences in the prevalence of sublethal infections in any treatment, as compared to the wild type control. This observation was supported by the near-equal amounts of virus estimated to be present in infected adults. The results in this study indicate that *se5*, *se96* and *se99* alone did not affect the prevalence of sublethal infections in adults. To what extent the transmission of SeMNPV was affected, remains to be determined. Improving our knowledge on the genetic mechanisms associated with vertical transmission of baculoviruses may contribute to the development of optimized strategies for the use of virus-based insecticides.

Introduction

Baculoviruses are arthropod-specific viruses with double-stranded, circular, supercoiled genomes varying in size from about 80 to over 180 kb, encoding between 90 and 180 genes (Rohrmann, 2013b). Baculoviruses are characterized by the production of two types of virions; budded viruses (BVs), that mediate the cell-to-cell spread of the infection within the insect, and the occlusion derived viruses (ODVs), involved in the horizontal transmission of the virus from insect to insect (Slack & Arif, 2006). ODVs are surrounded by a proteinaceous matrix forming an occlusion body (OB), which protects the virions from environmental decay and enhances their persistence in the field. Baculoviruses can be transmitted horizontally, from insect to insect, when a susceptible caterpillar ingests OBs persisting in the environment (Cory & Myers, 2003). Horizontal transmission of the virus is more prevalent when the host population densities are high. At low population densities or with highly mobile host, other mechanisms of transmissions may dominate (Burden *et al.*, 2002). Indeed, baculoviruses have been found to be vertically transmitted, from parents to offspring (Burden *et al.*, 2002; Vilaplana *et al.*, 2010; Cabodevilla *et al.*, 2011a; 2011b; Virto *et al.*, 2013). Vertical transmission can be due to surface contamination of the eggs (transovum transmission), virus passing within the egg (transovarial transmission), or as a latent infection (Cory & Myers, 2003).

Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) has been isolated in many regions of the world. A number of genotypes have been isolated from OBs present in the soil of Almerian greenhouses (Murillo *et al.*, 2007). Because OBs in the soil can only be transmitted when they are being eaten by susceptible hosts (Fuxa & Richter, 2001), the genotypes found in the soil are expected to be predominantly transmitted horizontally. On the other hand, insect colonies frequently succumb to spontaneous nucleopolyhedrosis under controlled conditions in the laboratories (Burden *et al.*, 2002; Cory *et al.*, 1997) as a consequence of sublethal infections (Murillo *et al.*, 2011). Genotypes isolated from diseased laboratory colonies are expected to be

predominantly transmitted vertically.

Thézé *et al*. (2014) identified and compared the whole genome sequence of seven different SeMPNV isolates associated with vertical (VT-SeAL1, VT-SeAL2, VT-SeOx4) or horizontal transmission strategies (HT-SeG24, HT-SeG25, HT-SeG26 and HT-SeSP2A). Vertically transmitted genotypes shared the same mutations in three genes that were different from those of the horizontally transmitted isolates. The genome of three vertically transmitted isolates shared a deletion of 3 bp at 1382 nt in open reading frame (ORF) 5 (*se5*), a 3 bp deletion at 114 nt in ORF96 (*se96*), and a deletion of 9 bp at 54 nt in ORF99 (*se99*). The *se5* gene, of unknown function, has a homolog in *S. litura* MNPV. The *se96* gene is homologous to *ac150*, which is considered to be a *per os* infectivity factor that mediates, but is not essential for oral infection (Zhang *et al*., 2005). The *se99* gene is a homolog of *ac134 (*also called *p94),* a non-essential gene for AcMNPV, which harbours a non-hr origin of replication (*ori*) (Pijlman *et al*., 2003b). One could hypothesize that one or more of these three genes are involved in sublethal infections and vertical transmission, but experimental evidence is lacking.

Vertical transmission can influence the effectiveness of baculoviruses in biological control programs against agricultural or forest pests, in particular when inoculative strategies are employed (Kukan, 1999). However, little is known about the molecular mechanisms that determine different transmission routes. In the present study, we examined the different ORFs selected by Thézé *et al.* (2014) in order to determine their potential role vertical transmission of SeMNPV. For this purpose, a bacmid-based recombination system was used to delete the respective ORFs associated with vertical transmission of the SeAL1 genotype and the occurrence of these viruses in the abdomens of male and female moths upon infection was measured. The results indicate that *Se5*, *Se96* and *Se99* alone did not affect the degree of sublethal infections and that this was reflected in the near equal concentrations of wild-type and "knockout" viruses in the abdomens of infected adult moths. A putative role of

any of these genes in vertical transmission, if at all, is predicted to occur beyond this stage, possibly in the process of transmission to offspring.

Material and methods

Insect and viruses. *S. exigua* larvae were obtained from Andermatt Biocontrol AG (Grossdietwil, Switzerland), maintained at constant environmental conditions (25 ± 1° C, 50% ± 5% RH, and a photoperiod of 16:8 h light: dark) and reared on a wheatgerm-based semisynthetic diet (Greene *et al.*, 1976). The SeMNPV genotype used in this study was SeAL1, one of the genotypes sequenced by Thezé *et al.* (2014), which originated from the progeny of field-collected moths that produced progeny that subsequently died from virus infection during laboratory rearing, and was known to be capable of being vertically transmitted. This virus was selected for the construction of a SeMNPV bacmid. The complete genotype was cloned into bacmid cloning vector BAC-*Bsu*36I (Pijlman *et al.*, 2002), that was modified by adding a *Mau*BI restriction site and designated as BAC-*Bsu*-*Mau*BI (Serrano *et al.*, submitted).

Generation of knockout bacmids. For deletion mutagenesis of *se5*, *se96* and *se99* from SeBacAL1, 68- to 70-bp primers were designed with 50-bp 5´ends within the deletion target region on the SeMNPV genome (Table 1). The 3' ends of the primers anneal to chloramphenicol resistance gene flanked by mutant *LoxP* sites (Suzuki *et al.*, 2005), which was amplified from pCRTopo-lox-cat-lox (Marek *et al.*, 2011). PCR on pCRTopo-lox-cat-lox was performed using Phusion Polymerase (Thermo Scientific) according to the manufacturer, giving a product of 1170 bp. SeBacAL1 DNA was cloned into electro-competent MW003 cells (Westenberg *et al.*, 2010), and selected on LB-plates with streptomycin and kanamycin for 2 days at 32°C. Single colonies were picked and grown ON in 1 ml SOB-medium at 32°C. The ON-culture was used to inoculate 10 ml SOB-medium, incubated at 32°C and cells were harvested when an OD_{600} of 0.6 was reached. The culture was then split into two and 5 ml were induced for 10 min at 42°C. After incubation, the cells were washed

twice with ice-cold 10% glycerol. Finally, the cells were resuspended in 100 µl 10% glycerol and stored at -80°C. The next day, cells were electroporated with 150 ng of the PCR product amplified from pCRTopo-lox-cat-lox. The cells were recovered in 1 ml SOB-medium and incubated for 3 h at 32°C. Subsequently, both induced and non-induced cells were plated out on LB plates, supplemented with 50 µg/ml kanamycin and 50 µg/ml chloramphenicol. The plates were incubated for 48 h at 32°C. Finally, single colonies were picked to analyze if recombination had occurred (Dolphin & Hope, 2006). To confirm the deletion of the ORFs, restriction endonuclease analysis of the bacmid DNA and PCR amplifications were performed. Once the correct knockout was selected, bacmid DNA extraction was performed and electroporated into DH10β (Invitrogen) for further amplification.

Production of OBs. Fourth-instar *S. exigua* larvae were injected with 10 µl DNA of the knockout and wild type bacmids from a mixture containing 100 µl DNA (50ng/µl) and 50 µl Lipofectin reagent (Invitrogen). Injected larvae were transferred to artificial diet and reared individually at 25°C. Virus-induced mortality was recorded daily. OBs were extracted from NPV-killed larvae by homogenization in water and purified by filtration and differential centrifugation as described (Muñoz & Caballero, 2000). These OBs were, together with SeAL1, used for bioassays.

Bioassay. To determine the effect of the different ORFs on vertical transmission of SeMNPV, bioassays with OBs from the knockout viruses were performed using the droplet feeding method (Hughes *et al.*, 1986). For this, fourth instar *S. exigua* were starved for 12 h and then allowed to drink from an aqueous suspension containing 10% sucrose, 0.001% Fluorella blue, and OBs at a concentrations that produced around 40% mortality by each virus: $1x10^4$ OBs/ml for SeAL1, SeBacAL1, SeBacAL1Δ96, and SeBacAL1Δ99; and $1x10^5$ OBs/ml for SeBacAL1Δ5. Control larvae were fed a solution of sucrose and Fluorella blue without OBs. Larvae that

ingested the suspension within 10 min were transferred individually to 24-well tissue culture plates with semi-synthetic diet. Groups of 24 larvae were treated with each concentration. Insects were incubated at 25 ºC and mortality was recorded daily until larvae died or pupated. Adults that survived the infection were individually stored at -80 ºC.

DNA extraction. Total insect DNA was extracted using MasterPure Complete DNA Purification kit (Epicentre Biotechnologies), according to the manufacturer recommended protocol. Abdomens of adults were individually dissected and placed in a 2 ml tube with ceramic beads and 300 µl lysis solution with 1 µl 50 µg/ µl Proteinase K. The tissue was homogenized using MP FastPrep-24 tissue in a cell homogenizer at 4.0 m/s for 20s. The mixture was incubated at 65 °C for 15 min at a constant 1100 rpm orbital agitation. A 150 µl volume of the sample was then treated with RNase for 30 min at 37°C. Protein precipitation reagent was added to lyse the sample and the debris was pelleted by centrifugation at 10,000 *g* for 15 min. Cold isopropanol was used to precipitate nucleic acids. Samples were washed twice with 70% ethanol, resuspended in 20 µl sterile Mili-Q water and stored at -20°C. Blank extraction sample containing only water were processed in parallel to control for cross-contamination during extraction.

Detection of sublethal infections. In order to estimate the prevalence of latent infections, quantitative PCR (Q-PCR) based on SYBR fluorescence was performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) in 96-well reaction plate. Specific primers were designed to amplify a 149 bp region of the SeMNPV *DNA polymerase* gene (Table 1) based on the complete genome sequence of the SeAL1 genotype (GenBank accession number: HG425343). Amplifications were performed in a total reaction of 10 µl containing 5 µl SYBR green Premix *Ex Taq* (2x) (TaKaRa Bio Inc., Shiga, Japan), 0.4 µl of both forward and reverse primers (10 pmol/µl) and

1 µl 50 ng/µl DNA. Four non-template reactions were included in each run and a standard curve was included in triplicate to determine the efficiency of each reaction. For the standard curve, SeAL1 DNA was extracted from OBs, purified through CsCl gradients, quantified using a spectrophotometer (Eppendorf BioPhotometer plus) and then serially diluted in sterile MilliQ water to the following concentrations: 6.25 x 10^{-4}, 1.25 x 10^{-4}, 2.5 x 10^{-5}, 5 x 10^{-6} and 1 x 10^{-6} µg/µl. All reactions were performed in triplicate. The Q-PCR temperature and time conditions included an initial denaturation step at 95 °C for 30s, followed by 45 amplification cycles comprising three steps: 95°C for 5s, 60°C for 15s and a final cycle for the dissociation curve consisting in 95°C for 15s, 60°C for 15s and 95°C for 15s. Data acquisition and analysis was performed with CFX Manager Software 3.1 (Bio-Rad).

Statistical analysis. The frequencies of insects that tested positive for sublethal infection by Q-PCR were not normally distributed and were analyzed by non-parametric Kruskal-Wallis test in SPSS (IBM).

Results

Construction of 'knockout' viruses. To determine the effects of removing *se5*, *se96* and *se99* individually on the transmission of SeMNPV, a SeMNPV bacmid with the VT-SeAL1 isolate genome was used (SeBacAL1) (Serrano *et al.*, submitted). SeBacAL1Δ5, SeBacAL1Δ96 and SeBacAL1Δ99 'knockout' bacmids were then constructed by deleting *se5*, *se96* and *se99*, respectively. In their place, a chloramphenicol acetyltransferase (*cat*) gene was inserted to facilitate antibiotic selection in *E. coli*. To identify the knocked out loci, restriction endonuclease analysis (Fig. 1) and PCR on the flanking sequences of the deleted gene (data not shown) were performed. To test the transfectivity of these 'knockout' and control bacmids, these bacmids were directly injected into fourth instar *S. exigua* larvae together with lipofectin transfection reagent. All larvae sucumbed to lethal polyhedrosis disease indicating that the deletions were non-essential. OBs were harvested and tested for

the presence of the correct virus by PCR. These OBs were propagated in fourth instar *S. exigua* and used for further experiments.

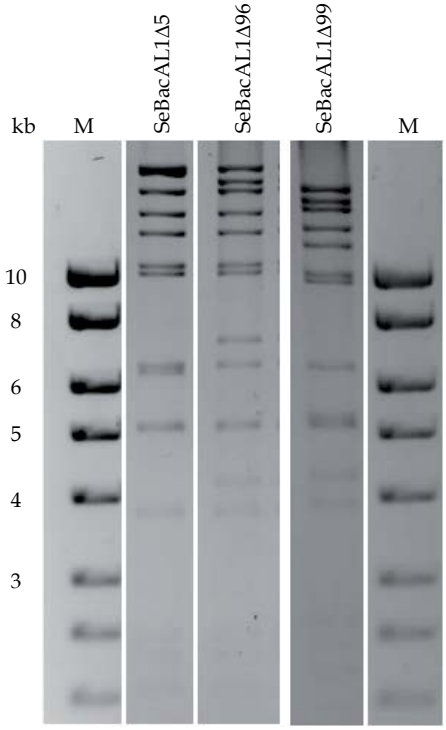

Figure 1. Restriction endonuclease profiles with *Bgl*II of the DNAs of SeBacAL1 and the recombinant knockouts SeBacAL1Δ5, SeBacAL1Δ96 and SeBacAL1Δ99. Lane M contains a 1 kb Marker-Ladder from Stratagene. The asterisks indicate the restriction polymorphisms of each knockout.

Detection of sublethal infections in adult *S. exigua*. To detect the prevalence of sublethal infection of all the knockout viruses in adults, *S. exigua* fourth instar larvae were infected with the 40% lethal concentration (LC_{40}) of the SeAL1 genotype, SeBacAL1 bacmid and all the 'knockout' viruses. As expected, mortality ranged from 33.3 to 45.5%, whereas no mortality was observed in the control mock-infected larvae (Table 2). In a previous study, a similar concentration of SeAL1 OBs resulted

Table 2. Percentage of *S. exigua* larval mortality due to nucleopolyhedrovirus disease and number of surviving pupae in vertical transmission assays.

Virus	OB concentration (OBs/ml)	No. of virus-killed larvae (% mortality)	Total no. of infected larvae	No. of pupae
Control	0	0	24	21
SeAL1	$1x10^4$	8 (33.3)	24	15
SeBacAL1	$1x10^4$	9 (39.1)	23	11
SeBacAL1Δ5	$1x10^5$	6 (35.3)	17	8
SeBacAL1Δ96	$1x10^4$	9 (37.5)	24	13
SeBacAL1Δ99	$1x10^4$	10 (45.5)	22	11

in a prevalence of sublethal infections in adult moths as high as 60%, as indicated by the number of Q-PCR positive adult survivors (Cabodevilla *et al.*, 2011b).

All the emerged adults from the different viral treatments were subjected to Q-PCR analysis using primers designed to target the highly conserved viral DNA polymerase gene (Table 1).

None of the control adults gave Q-PCR positive results, indicating that the laboratory insect population is free of virus (Table 3). Adults infected with the wild-type virus, VT-AL1, and the wild-type bacmid, SeBacAL1, showed 90 and 62.5% prevalence of sublethal infections, respectively, indicating that the LC_{40} resulted in a high prevalence of sublethally infected adults (Table 3). Statistical analysis of the prevalence of infection in the adults inoculated with the different viral treatments, including *se5*, *se96* and *se99*, showed no significant differences in any treatment, as compared to SeBacAL1 ($P>0.05$) (Table 3). Given the absence of significant differences between the different knockouts in the prevalence of virus infection allowed the use of SeBacAL1 bacmid as the only control, there was no need to test 'repair' viruses.

Table 3. Prevalence of sublethal infection in *S. exigua* adults that survived OB treatments and average viral load in the virus-positive adults. Values labelled with the same letter did not differ significantly (p>0.05).

Virus	No. of NPV-positive adults (%)	Average viral load (µg/µl)	No. adults analyzed
Control	0 (0) a	-	11
SeAL1	9 (90) a	3.75×10^{-6}	10
SeBacAL1	5 (62.5) a	2.29×10^{-6}	8
SeBacAL1Δ5	3 (50) a	8.45×10^{-6}	6
SeBacAL1Δ96	8 (72.7) a	2.27×10^{-6}	11
SeBacAL1Δ99	10 (90.9) a	1.07×10^{-5}	11

Discussion

The development of highly sensitive molecular techniques has allowed detection of baculovirus sublethal infections as well as studies on their vertical transmission to assess the role of this spread strategy on the population dynamics of their hosts. Baculoviruses can establish sublethal infections to transmit the virus from parents to offspring. However, little is known about the molecular mechanisms that regulate the formation and persistence of sublethal infections. In a previous study, several SeMNPV genotypes differed in their ability to persist as sublethal infections in the adult stage, indicating that certain genotypes are better adapted to vertical transmission than others (Cabodevilla *et al.*, 2011a). The VT-SeAL1 genotype produced 100% sublethal infection in adults that survived an OB treatment in the larval stage, whereas genotype HT-SeG25 only produced 16% sublethal infection among survivors of a similar treatment (Cabodevilla *et al.*, 2011a). The comparison of the whole genome sequence of seven SeMNPV genotypes involved in vertical and horizontal transmission strategies resulted in the identification of three candidate

genes as potentially being involved in the transmission strategy of SeMNPV: *se5*, *se96* and *se99* (Thézé *et al.*, 2014).

Se5 is an early gene of unknown function and it is homolog to the ORF6 of *Spodoptera litura* MNPV (*Spln6*). Unfortunately, *Spln6* has not been characterized and its hypothetical function is unknown. SE5 is 509 aa in length and has a protein size of 59.24 kDa. As discussed in the previous chapter (Serrano *et al.*, submitted), deletion of *se5* decreased the pathogenicity of SeBacAL1 by almost 10-fold, and the mean time to death was significantly increased by almost 7 h. Although *se5* appears to have an important role in the viral infectivity of SeMNPV (Serrano *et al.*, submitted), it did not affect the virulence of the virus, since its deletion did not result in a change in the prevalence of sublethal infections in adults, as compared to the wild-type (wt) bacmid SeBacAL1 (Table 3).

SE96 is 118 aa long and has a protein molecular size of 12.67 kDa. *Se96*, a late expressed gene, is a homolog of *ac150*, which belongs to the 11K gene family encoding products with a hydrophobic N-terminal domain, and a core "C6" motif, and whose functions frequently involve interaction with chitin, although no functions have been assigned yet (Dall *et al.*, 2001). Deletion of *ac150* alone did not affect the viral pathogenicity in *Trichoplusia ni* or *Heliothis virescens* larvae (Lapointe *et al.*, 2004). However, when it was deleted together with *ac145*, another member of the 11K gene family, the pathogenicity decreased drastically in *T. ni* larvae, but not in *H. virsecens* larvae. Intrahemocoelic injection of budded virus from the double mutant recovered the pathogenicity as compared to the wt virus, indicating that *ac150* might have a role in the primary infection of AcMNPV, in a host dependent manner (Lapointe *et al.*, 2004). In a different study, deletion of *ac150* significantly decreased the pathogenicity of AcMNPV, with a higher LD_{50} compared with the wild-type virus in *S. exigua*, *H. virescens* and *T. ni* larvae (Zhang *et al.*, 2005). However, deletion of *ac150* resulted in an identical pathogenicity compared to that of wild-type AcMNPV after intrahemocoelic injection of BV in fourth instar *S. exigua*, *H. virescens* and *T.*

ni. Both experiments indicated that *ac150* can be considered a *per os* infection factor that mediates, but is not essential for, oral infection (Zhang *et al*., 2005). In our study, the same OB concentration was administered for SeBacAL1 and the knockout of *se96*, and both concentrations produced similar mortalities, in contrast to what was observed by Zhang *et al*. (2005). The conserved viral motif "C6" is different in *ac150* than in *se96*, they do not group together in a phylogenetic tree based in the 11K 6C motifs, indicating that those motifs were probably acquired independently (Dall *et al*., 2001). The different C6 motif might be responsible for the observed difference in the pathogenicity between AcMNPV and SeMNPV. Although *se96* is considered to be involved in the oral infection of SeMNPV, its deletion did not affect the prevalence of sublethally infected adults as compared with that of the wild-type virus, SeBacAL1 (Table 3).

SE99 is 716 aa long and has a predicted protein size of 85.65 kDa. *Se99* is a homolog of gene *p94*, which is an early, non-essential gene of unknown function (Friesen & Miller, 1987; Pijlman *et al*., 2003b). *p94* was suggested to have evolved together with the adjacent gene *p35*, which is an inhibitor of apoptosis (Clem *et al*., 1994). Deletion of *p94* from AcMNPV did not affect the pathogenicity of the virus (Clem *et al*., 1994), with similar a LC_{50} value compared with that of wild-type OBs. This seems to be confirmed in this study, where a concentration of $1x10^4$ OBs/ml produced around 40% mortality in both SeBacAL1 and SeBacAL1Δ99. In a detailed study about defective interfering (DI) virus formation, a 2.8 kb fragment that exhibited strong origin of viral replication (*ori*) activity was identified after serial AcMNPV passage in cell culture, (Kool *et al*., 1994). This sequence was designated as non-*hr ori* and is located within the *p94* coding sequence, yet is non-essential (Pijlman *et al*., 2003b). P94 is not present in *Bombyx mori* NPV, however, the BmNPV genome has retained parts of the non-*hr ori*, indicating that this part of *p94* may be important for virus replication (Kool *et al*., 1994), and the generation of DI particles (Pijlman *et al*., 2003b). Although *p94* seems to have an effect on the viral replication, it did not affect

the transmission route, since deletion of *se99* did not affect the prevalence of infected adults as compared to that of the wild-type virus, SeBacAL1.

The results in this study indicate that *se5*, *se96* and *se99* alone did not affect the prevalence of sublethal infections in adults. However, the question remains whether there is an effect of these deletions on the expression level of viral genes or on the efficiency of virus transmission to offspring, and therefore, on the vertical transmissibility of the virus. In the case of genotype SeG25, that showed a low prevalence of vertical transmission, a much lower level of gene expression was found (Cabodevilla *et al.*, 2011a), whereas two other horizontally-transmitted genotypes of SeMNPV did not, compared to viruses showing vertical transmission, such as SeAL1. Therefore it would be of interest to measure the transcription in abdomens of adult moths infected with SeG25 and its deletion mutants, as compared to SeAL1, which adopts predominantly a vertical route of transmission.

Finally, it is now particularly important to analyze virus transmission to offspring to determine whether that is affected by the deletion of any of the three SeMNPV genes, *se5*, *se96* and *se99*.

Vertically transmitted infections may contribute to improve pest control strategies from one generation of insects to the next, by reducing the number of applications of baculovirus-based insecticides required in the field. The genetic factors associated with vertical transmission mechanisms, however, are poorly understood, but merit study as they may contribute to optimize the use of these viruses, and may contribute to an improved understanding of the mechanisms behind the issue of viral latency or sublethal infection in general.

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Supplementary information

Table 1. Primers used for the generation of SeBacAL1 knockout mutants lacking ORFs Se5, Se96, Se99, and for q-PCR amplification to determine prevalence of mutants in vertical transmission assays.

Primer	Sequence	Amplification purpose
SeORF5-KO-F	ATGGTTAACGATTCCAGAAACACTGATATCATCGACGCTGTCGTCTGAGCGCTCGGATCCACTAGTAAC	*Se5* deletion from SeMNPV bacmid; forward primer
SeORF5-KO-R	TTATGCATCAGCTGTTGTTTGATCTTCGTCATCGGTGGTTTCTCCGTCGCCCTCTAGATGCATGCTCG	*Se5* deletion from SeMNPV bacmid; reverse primer
SeORF96-KO-F	ATGAAGTCGTGGATGAAAACGGTCATATTGATTTTGATACTTTACTGAATGCTCGGATCCACTAGTAACG	*Se96* deletion from SeMNPV bacmid; forward primer
SeORF96-KO-R	CATAATTGAAAACAATCTTGAAGTCGTTCTTTTTTCGTTGCCTCAAATCTCCTCTAGATGCATGCTCG	*Se96* deletion from SeMNPV bacmid; reverse primer
SeORF99-KO-F	ATGATTAGAAGAACACTTTTTACAATGGGCATCACCGGCAGCGTCTGAGAGCTCGGATCCACTAGTAA	*Se99* deletion from SeMNPV bacmid; forward primer
SeORF99-KO-R	CAATATTTCAAA-TACATTAATTTCCTTTTCGAATAGTTGATAATGTCTTCCCTCTAGATGCATGCTCG	*Se99* deletion from SeMNPV bacmid; reverse primer
DNApol149-F	CCGCTCGCCAACTACATTAC	DNA polymerase amplification for Q-PCR. Forward primer
DNApol149-R	GAATCCGTGTCGCCGTATATC	DNA polymerase amplification for Q-PCR. Reverse primer

Chapter 4

Analogous population structures in two alphabaculoviruses highlight functional role for deletion mutants

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Abstract

A natural *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) isolate from Florida shares a strikingly similar genotypic composition to that of a natural *Spodoptera frugiperda* MNPV (SfMNPV) isolate from Nicaragua. Both isolates comprise a high proportion of large-deletion genotypes that lack genes that are essential for viral replication or transmission. To determine the likely origin of such genotypically similar population structures, we performed genomic and functional analyses of these genotypes. The homology of nucleotides in the deleted regions was as high as 79%, similar to those of other colinear genomic regions, although some SfMNPV genes were not present in SeMNPV. In addition, no potential consensus sequences were shared between the deletion flanking sequences. These results indicate an evolutionary mechanism that independently generates and sustains deletion mutants within each virus population. Functional analyses using different proportions of complete and deletion genotypes were performed with the two viruses in mixtures of occlusion bodies (OBs) or co-occluded virions. Ratios greater than 3:1 of complete/deletion genotypes resulted in reduced pathogenicity (expressed as median lethal dose), but there were no significant changes in the speed of kill. In contrast, OB yield increased only in the 1:1 mixture. The three phenotypic traits analyzed provide a broader picture of the functional significance of the most extensively deleted SeMNPV genotype and contribute toward the elucidation of the role of such mutants in baculovirus populations.

Introduction

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), and the beet armyworm, *S. exigua* (Hübner) (Lepidoptera: Noctuidae), are polyphagous pests of agricultural crops. *S. frugiperda* is native to the tropical and sub-tropical regions of the Americas, from the United States to Argentina (Sparks, 1979). *S. frugiperda* adults disperse over long distances during the summer months. *S. exigua* originated from the Middle East, but it is now found in tropical and subtropical areas worldwide, including Europe and the Americas (Zheng *et al.*, 2012). The geographical distributions of *S. exigua* and *S. frugiperda* have overlapped in North America for more than a century (Capinera, 1999).

The multiple nucleopolyhedroviruses (genus *Alphabaculovirus*, familia Baculoviridae) isolated from *S. exigua* (SeMNPV) and *S. frugiperda* (SfMNPV) are important species-specific mortality factors, particularly in the high density populations of their respective hosts. Alphabaculoviruses, including SeMNPV, have proven to be effective for use as a base in biological insecticides (Moscardi, 1999). A detailed understanding of the genetic and phenotypic diversity within and between baculovirus populations can facilitate the selection of highly insecticidal strains for development as a base of commercial products (Erlandson, 1990).

The diversity of SfMNPV and SeMNPV has been described both between (Caballero *et al.*, 1992b; Escribano *et al.*, 1999; Murillo *et al.*, 2001) and within populations (Dai *et al.*, 2000; Hara *et al.*, 1995; Maruniak *et al.*, 1984; Muñoz *et al.*, 1998; Simón *et al.*, 2004a). The genomes of SfMNPV and SeMNPV are strikingly similar (Muñoz *et al.*, 1998; Simón *et al.*, 2004b), with 72 to 85% nucleotide homology in colinear regions covering 30% of the genome, and with >85% of genes being shared; this has led researchers to suggest that these two viruses are closely related (Harrison *et al.*, 2008). The large-deletion genotypes present in a Floridan SeMNPV isolate, SeMNPV-US2 (SeUS2), were observed to share parallel genetic and phenotypic characteristics with an SfMNPV isolate from Nicaragua, SfMNPV-NIC (SfNIC) (Simón *et al.*,

2005a). SeMNPV and SfMNPV represent different virus species (Herniou *et al.*, 2011). The genotypic structure was detected later in other geographically distant populations of these viruses. Specifically, a Californian SeMNPV isolate (SeUS1) includes three genotypes with deletions in similar locations and of comparable lengths to those of SeUS2, namely, SeUS1-B (K. Zimmermann, D. Muñoz, and P. Caballero, unpublished data), SeUS1-XD1 (Dai *et al*., 2000), and a 25 kb deletion genotype (Heldens *et al*., 1996) that we designated here as SeUS1-JH (Fig. 1). Five samples from Japan and Thailand show restriction endonuclease profiles (Hara *et al*., 1995) with signs of deletion genotypes, characterized by submolar *Pst*I-C and *Pst*I-D fragments. Finally, in SfMNPV isolates from the United States (Harrison *et al*., 2008) and Colombia (Barrera *et al*., 2011), the presence of genotypes with sizable genomic deletions in the same genomic region has been reported recently. The host ranges of these viruses differ. SeMNPV is not pathogenic for *S. frugiperda* or any other heterologous host, whereas SfMNPV, although it is lethal to *S. exigua*, produces atypical pathogenesis, lack of integument rupture, and no progeny virus in this (*S. exigua*) host. The existence of such similar population structures in distinct viruses that infect different host species raises questions concerning the origins and functions of these genotypes.

In this study, we analyzed the gene content of SeUS2 genotypic variants and compared the genotypic structure of SeUS2, SeUS1, and SfNIC in an attempt to determine the origins of such genotypically similar population structures that would provide clues as to the ecological or evolutionary factors that determine population structure in these viruses. In doing so, we also identify a series of characteristics that can be employed as genotype selection criteria for the development of biological insecticides that are based on these viruses.

Material and methods

Insects, Cell lines and Viruses. *S. exigua* larvae were obtained from a laboratory colony, maintained under constant environmental conditions (25 ± 1° C, 50% ± 5%

RH and a photoperiod of 16 h light and 8 h dark), and reared on a wheat germ-based semisynthetic diet (Greene *et al*., 1976). This colony was known to be free from latent or covert nucelopolyhedrovirus infections (Cabodevilla *et al*., 2011b). *S. exigua* Se301 cells, which were kindly provided by S. Herrero (Universidad de Valencia, Spain) were maintained at 28º ± 0.5 °C in Grace`s insect cell culture medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Scotland, U.K.). The SeMNPV isolate from Florida, SeUS2- wild type (WT), which was used in this study, is the active ingredient of the bioinsecticide Spod-X and was kindly provided by Du Pont Ibérica S.A, Barcelona, Spain. The virus was amplified by infecting *S. exigua* fourth instars from the laboratory colony using the droplet feeding method (Hughes *et al*., 1986). Initially, the SeUS2 genotypic variants SeUS2-A, SeUS2-C, SeUS2-D, SeUS2-E, and SeUS2-F were obtained by *in vivo* cloning (Muñoz *et al*., 1998) in *S. exigua* larvae. SeUS2-E could not be fully isolated, but several samples contained enriched mixtures of this genotype with SeUS2-A as a contaminant, and so these were used to construct the physical map of SeUS2-E (Muñoz *et al*., 1998). Viral occlusion bodies (OBs) were produced by feeding healthy fourth instars with a virus-contaminated diet. OBs were extracted from dead diseased larvae by homogenization in water, and they were purified by filtration and differential centrifugation (Muñoz *et al*., 2001).

Viral DNA purification, restriction endonuclease digestion, cloning and PCR amplification. Viral DNA was extracted from ~10^9 OBs in 300 µl of water by dissolving the polyhedrin matrix with the addition of 100 µl of 0.5 M Na_2CO_3 and 50 µl of 10% (w/v) sodium dodecyl sulfate (SDS) at 60°C for 10 min. Undissolved OBs and other particulates were pelleted by low-speed centrifugation (2,700 x *g* for 5 min). Supernatant containing occlusion-derived virions (ODVs) was recovered and incubated with 500 µg/ml proteinase K at 50°C for 2.5 h. Viral genomic DNA was extracted with phenol and chloroform, precipitated by the addition of 0.1 vol. 3 M sodium acetate (pH 5.2) and 2.5 volumes 96% ethanol, washed with 70% ethanol and

dissolved in 0.1X Tris-EDTA (TE) buffer (10mM Tris-acetate and 1mM EDTA [pH 8.0]). For restriction endonuclease digestion, ~2 µg of viral genomic DNA was treated with 10 U of *Pst*I (Takara, Shiga, Japan); on occasions, it was treated with *Bam*HI, (Amersham) following the manufacturer's recommendation, which in the case of *Bam*HI resulted in cloneable polymorphic fragments for SeUS2-C and SeUS2-D. Electrophoresis was performed in horizontal 1% agarose gels in Tris-acetate-EDTA (TAE) (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.0]), and the DNA fragments were visualized by staining with ethidium bromide. The cloneable SeUS2-C *Pst*I-J fragment, which is characteristic of this genotype and results from the shortening and fusion of SeUS2-A *Pst*I-C and *Pst*I-D fragments, was gel-extracted, purified through GFX™ PCR columns (Amersham Biosciences, Little Chalfont, Bucks, UK) and cloned in pUC19 plasmid (Promega Biotech Iberica, Madrid, Spain) using a DNA ligation kit (LigaFast™, Promega Biotech Iberica). The same procedure was followed using a cloneable 3.1-kb product of *Bam*HI digestion of the characteristic SeUS2-D *Pst*I-C fragment, containing the 2.5-kb insertion. PCR amplifications were performed in a total reaction mixture volume of 50 µl containing 250 µM of each deoxynucleoside triphosphate, 56 pmol of each primer, polymerase buffer and Accuzyme DNA polymerase (Bioline Supply, Segovia, Spain). The following cycling conditions were used: 3 min of initial denaturation at 94°C, 25 amplifications cycle (30s denaturation at 94°C, 1 min annealing at 58°C, and 2 min of extension at 72°C) with a final extension step at 72°C for 10 min.

Nucleotide sequencing. Sequencing was performed in an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit on a 9600 PE model thermocycler. The reaction products were loaded into an automated DNA sequencer ABI PRISM (Sistemas Genómicos, Valencia, Spain).

Determination of the deletion/insertion breakpoints for each variant. For

SeUS2-C, the characteristic SeUS2-C *Pst*I-J fragment was purified from an agarose gel, cloned into pUC19, and sequenced using universal reverse primer. The deletion in SeUS2-E was mapped using the SeUS1-based primers, prE-F01 and prE-R01 (Table 1), designed to target a 2.7-kb fragment around the deletion breaking point. The SeUS2-F deletion was mapped by cloning a 4.8-kb restriction fragment of the SeUS2-F genome obtained with *Pst*I and *Bam*HI and sequencing with universal forward primer. To map the insertion point on SeUS2-D, the SeUS2-D *Pst*I-C fragment containing the insertion was digested with *Bam*HI, cloned into pUC19, and sequenced with primers universal forward, prD13-F02, and prD13-F03 (Table 1). The last two primers were designed from the sequences obtained with primers universal forward and prD13-F02, respectively.

Sequence computer analysis. DNA and protein comparisons with entries in the updated GenBank/EMBL databases were performed with BLASTn, FASTA and BLASTp (Altschul *et al*., 1990; Pearson, 1988). A pairwise alignment of sequences was made using the Needleman-Wunsch global alignment algorithm of the Emboss software (Rice *et al*., 2000).

***In vitro* virus cloning.** For complementation analyses, SeUS2-A and SeUS2-C were cloned *in vitro*. Fifth instars of *S. exigua* from the laboratory colony were inoculated *per os* with SeUS2-WT OBs at a 90% lethal concentration (LC_{90}) ($2.3x10^5$ OBs/ml). The hemolymphs of these insects were extracted at 48 h post infection (h.p.i.), added to 500 µl Grace´s culture medium supplemented with 10 % FBS, filtered throughout a 0.45 µm filter, and used for 10-fold serial dilutions (10^{-1} to 10^{-6}). A 100-µl volume of this mixture was added to Se301 cells at a density of 8×10^5 cells/ml in 96-well tissue culture plates. At 5 days postinfection, the virion-containing supernatants from wells with only one infection plaque were collected and inoculated onto Se301 cells, which were plated at a density of $2x10^6$ cells/ml in 6-well tissue culture

plates and incubated in 2 ml Grace´s insect cell culture medium supplemented with 10% FBS and 2% penicillin-streptomycin (Gibco, Scotland, U.K.). At 10 days post infection the medium and the cells were collected and centrifuged at 2,300 x *g* for 5 min. to separate the OB-containing cells from the budded virus (BV)-containing supernatant. Individual genotypes purified by this procedure were stored at 4ºC. For the production of OBs, fourth-instar *S. exigua* insects from the laboratory colony were injected with 8 µl of each of the BV suspensions obtained *in vitro*. Inoculated larvae were reared individually on a semisynthetic diet until death. Dead larvae were stored at -20ºC. SeUS2-A and SeUS2-C were identified by using restriction endonuclease analysis of viral DNA as described above.

Production of occlusion body mixtures and co-occlusion of genotypes. Two types of virus populations were constructed: i) mixtures of OBs of two genotypes, Se-US2A and Se-US2C and ii) co-occlusion of SeUS2-A and SeUS2C in ODVs within the same OBs, following methods developed previously (Clavijo *et al.*, 2010). For the OB mixtures, suspensions of SeUS2-A OBs or SeUS2-C OBs that had been purified *in vitro* were quantified by titration in an improved Neubauer hemocytometer, diluted in distilled water to a concentration of $1x10^8$ OBs/ml, and mixed in the following proportions: 90% SeUS2-A and 10% SeUS2-C (9A+1C), 75% SeUS2-A and 25% SeUS2-C (3A+1C), 50% SeUS2-A and 50% SeUS2-C (1A+1C), 25% SeUS2-A and 75% SeUS2-C (1A+3C), 10% SeUS2-A and 90% SeUS2-C (1A+9C). These mixtures were then used in bioassays. To produce co-occluded genotypes, 100 µl samples of each of the previous OBs mixtures ($1x10^8$ OBs/ml) were incubated with 1 volume 1M Na_2CO_3 at 50 ºC for 60 min to release ODVs. Eight-microlitre volumes of each ODV suspensions were injected into *S. exigua* fourth instars that were reared individually on semisynthetic diet until death. OBs were extracted from dead larvae, purified as described above, resuspended in 50 µl distilled water, and used in bioassays.

The relative proportions of each genotype in the OB mixtures and the co-occluded

preparations were estimated by semi-quantitative PCR. Primers P1, P2, and P3 (Table 1) were designed to differentiate between SeUS2-A and SeUS2-C, which produced amplicons of 691 and 791, respectively. Amplifications were performed using genomic DNA extracted from OBs. Reactions were stopped at the mid-logarithmic phase of amplification (17 cycles), before the rate of amplification began to decrease. The relative intensities of the two amplicons were compared using the Scion Image PC program (Scion Corporation, Maryland, USA), as described previously (Simon *et al*. 2005b).

Bioassays. Bioassays with OB mixtures and with co-occluded genotypes were carried out using the droplet feeding method (Hughes *et al*., 1986). Second instar *S. exigua* larvae were starved for 12 h and then were allowed to drink from an aqueous suspension containing 10% sucrose, 0.001% Fluorella blue, and OB mixtures or co-occluded genotypes at five different concentrations (2.45×10^5, 8.1×10^4, 2.7×10^4, 9×10^3, 3×10^3 OBs/ml). Control larvae were fed a solution of sucrose and Fluorella blue without OBs. Larvae that ingested the suspension within 10 min were transferred individually to 24-well tissue culture plates and given a semisynthetic diet. Groups of 24 larvae were treated with each concentration. Each assay was performed three times. Insects were incubated at 25 ^{o}C, and mortality was recorded daily until larvae died or pupated. Virus-induced mortality was subjected to probit analysis using the Polo-PC program (LeOra Software, 1987).

Mean time to death (MTD) and OB production were calculated for SeUS2-WT, SeUS2-A, and the following co-occluded genotype mixtures: 9A+1C, 3A+1C and 1A+1C. For this, groups of 24 *S. exigua* second instars were inoculated with an LC_{90} concentration of each inoculum (1.50×10^5 OBs/ml for SeUS2-WT , 1.64×10^5 OBs/ml for SeUS2-A, 2.14×10^5 OBs/ml for 9A+1C, 1.26×10^5 OBs/ml for 3A+1C, and 2.07×10^5 OBs/ml for 1A+1C), which were estimated in the previous bioassay. Inoculated larvae were reared individually at 25^{o}C and mortality was recorded at 8 h intervals

until death or pupation. Cadavers were collected individually and homogenized in 100 µl distilled water. The whole experiment was replicated three times. Time mortality data were subjected to Weibull survival analysis using Generalized Linear Interactive Modeling (GLIM) (Crawley, 1993) software. OB yield per larva was estimated by counting of OBs in a Neubauer hemocytometer. Two OB counts were performed for each larva, and the whole experiment was performed three times with 24 larvae per replicate. Results were normalized by square root transformation and were subjected to analysis of variance (ANOVA) parametric statistics in SPSS v15 (IBM, New York, USA).

Results

Population structure of SeUS2. The large genomic deletions in the SeUS2 genotypic variants SeUS2-C and SeUS2-E were mapped between nucleotides (nt) 16,437 and 39,757 of SeMNPV isolate SeUS1-A (IJkel *et al*., 1999). These deletions encompassed all or part of the *Pst*I-L, D, and C fragments (Fig. 1). Additionally, a 2,535 bp insertion and a 2,639 bp deletion were detected in the SeUS2 genotypic variants SeUS2-D and SeUS2-F, respectively (Fig. 1b). Other SeUS2 genotypes, such as SeUS2-B and SeUS2-H, show small deletions in the homologous region *hr*1 (data not shown). The genomic regions of 19,517 to 39,757 bp in SeUS2-A and of 18,753 to 35,122 bp in

Figure 1. Gene content of SeMNPV and SfMNPV deletion genotypes. (**A**) Schematic representation of the SeMNPV-US1A (SeUS1-A) genome (above). The *Pst*I restriction sites and fragments are represented as light blue bars and letters, respectively. Shaded rectangles correspond to origins of replication within homologous regions (*hrs*) or not (non-hr), and numbers correspond to nucleotide position in kb. ORFs (below) are represented as arrowed bars where size and arrowhead direction indicate their respective length and transcription direction, and colors are according to key panel. Numbers below ORFs indicate their number. (**B**) Linear maps (thick horizontal lines) and corresponding ORFs (colored arrowed bars) of the SeMNPV (Se, above) and SfMNPV (Sf, below) auxiliary gene rich (AGR) regions. Horizontal dashed arrows represent the deletions in the SeUS2, SeUS1-JH and the SfNIC deletion variants. The lengths of the SeUS2-C, SeUS2-E, and SeUS2-F deletions are indicated in bp. The approximate deletion points of the SeUS1 genotypes SeUS1-B (left break point) and SeUS1-JH have also been included. The SeUS2-D insertion point is also represented as a vertical dotted arrow and the insertion as a solid yellow line. Homolog ORFs between SeMNPV and SfMNPV are connected by thin dotted lines.

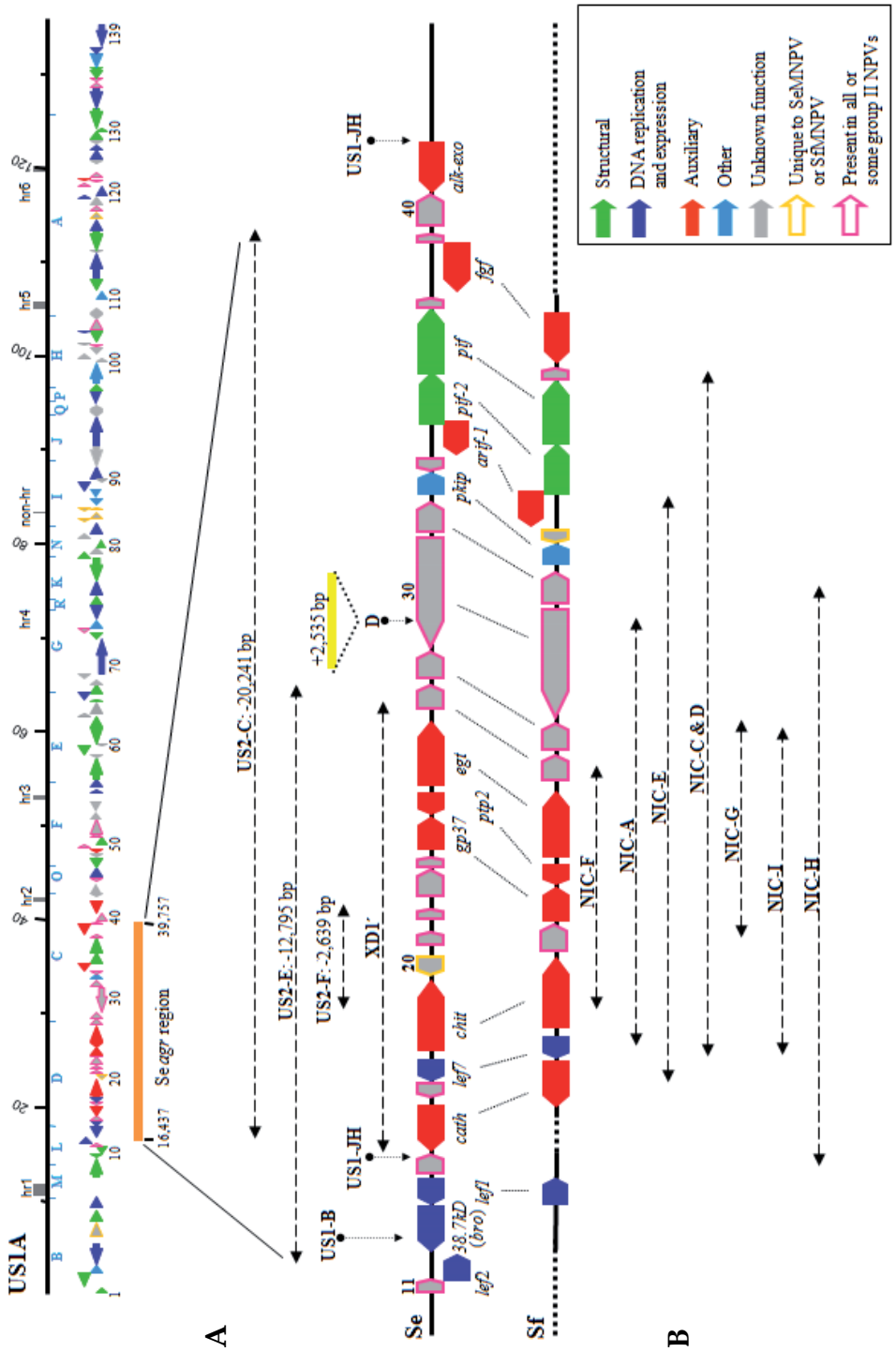
US1A
hr1
hr2
hr3
hr4
non-hr
hr5
hr6
Se agr region
16,437
39,757
US2-C: 20,241 bp
US2-E: 12,795 bp
US2-F: 2,639 bp
+2,535 bp
XD1
US1-JH
US1-B
D
A
Se
Sf
B
alk-exo
fgf
pif
pif-2
arif-1
pktp
egt
ptp2
gp37
chit
lef7
cath
38.7kD (bro)
lef1
lef2
NIC-A
NIC-E
NIC-C & D
NIC-F
NIC-G
NIC-I
NIC-H
Structural
DNA replication and expression
Auxiliary
Other
Unknown function
Unique to SeMNPV or SfMNPV
Present in all or some group II NPVs

Figure 1

SfNIC-B, which encompass the large deletions present in SeUS2-C and SfNIC-C, shared 79% nucleotide sequence identity.

Gene content of SeMNPV deletion genotypes. SeUS2-C has a large deletion of 20,241 bp, representing 14.9% of the genome in relation to that of SeUS1-A, between nt 19,517 and 39,757. Compared with SeUS1-A, SeUS2-C lacked open reading frames (ORFs) 17 to 39, which include the *chitinase*, *gp37*, *ptp-2*, *egt*, *pkip*, *arif-1*, *pif-2, pif,* and *fgf* genes. The left side of the deletion results in a 48% loss in the 5´ coding region of *cathepsin*. The right side of the deletion is 48 bp upstream of the ORF 40 start codon, and this eliminates the promoter elements of this early transcribed gene. No alternative consensus sequences were found in the 100-bp region located to the left of the deletion point. None of the genes in this region are necessary for viral replication, but *pif-1* and *pif-2* are essential for horizontal transmission, specifically during *per os* infection in the insect midgut (Kikhno *et al*., 2002; Pijlman *et al*., 2003a). SeUS2-E has a large deletion of 12,794 bp (between nt 16,437 and 29,230) representing 9.4% of the genome with respect to SeUS1-A. This deletion completely eliminates ORFs 13 to 27, including genes such as *38.7kd*, *lef-1*, *cathepsin*, *chitinase*, *gp37*, *ptp-2*, and *egt*. The left side of this deletion eliminates 258 of the 629 nt that constitute the 3´ end of *lef-2*. Both *lef-1* and *lef-2* are essential genes for viral DNA replication (Lu & Miller, 1995). The right side of this deletion removes most of ORF 28, including the whole promoter region and 510 out of the 572 encoding nucleotides. The remaining parts of *lef-2* (372 bp from the 5´ end) and ORF 28 (63 bp from the 3´ end) fuse in frame and constitute a chimerical protein of 107 aa that has the carboxyl-terminus of ORF 28 and the amino-terminus of *lef-2*.

SeUS2-F had a deletion of 2,639 bp (between nt 21,836 and nt 24,474), representing 1.9% of the genome in relation to SeUS1-A. ORFs 20 to 22, and 1,005 bp at the 3´ end of ORF 19 (*chitinase*) in the left side of the deletion, are absent in the SeUS2-F genome. The right side of the deletion starts 119 bp upstream of the ORF23 starting

codon, but the consensus baculovirus late promoter (GTAAG, at nt 24,579) of this putative gene is conserved.

Table 2. Percentages of nucleotide identity and gaps (italized) existing between the left and right flanking sequences of the deletions in the SeMNPV and SfMNPV deletion genotypes.

		NIC-A		NIC-C		NIC-E		NIC-F		NIC-G		NIC-H		NIC-I	
		left	right	left	right	left	right	left	right	left	right	left	right	left	right
US2-C	left	48.3	38.5	34.2	46.2	38.8	45.9	35.7	46.7	41.9	44.3	36.2	42.6	43.1	43.1
		13.8	*27.7*	*49.3*	*27.7*	*37.3*	*27.9*	*41.4*	*23.3*	*17.7*	*23.0*	*41.4*	*23.0*	*8.6*	*35.4*
	right	37.0	35.3	34.4	35.9	34.8	44.8	39.1	42.2	35.1	35.9	31.6	41.9	44.4	36.1
		7.4	*41.2*	*34.4*	*31.2*	*40.9*	*25.9*	*40.6*	*40.6*	*52.7*	*37.5*	*45.6*	*32.3*	*30.2*	*31.1*
US2-E	left	38.7	40.6	35.1	38.6	41.9	39.7	36.6	44.1	39.1	44.1	31.6	39.0	35.4	31.2
		27.4	*26.6*	*58.4*	*41.4*	*25.8*	*34.9*	*46.5*	*22.0*	*37.7*	*18.6*	*40.4*	*18.6*	*30.8*	*34.4*
	right	**53.6**	42.9	42.9	46.7	**51.9**	38.9	43.5	**63.0**	33.9	42.1	33.9	**53.7**	36.5	32.3
		19.6	*33.3*	*36.5*	*25.0*	*11.1*	*18.5*	*32.3*	*16.7*	*10.7*	*22.8*	*48.2*	*13.0*	*34.9*	*38.7*
US2-F	left	34.4	35.2	41.0	40.6	37.1	**59.3**	37.9	42.6	34.3	32.8	31.0	46.8	32.8	36.8
		32.0	*45.1*	*23.0*	*40.6*	*27.4*	*25.4*	*33.3*	*29.5*	*41.4*	*34.4*	*44.8*	*29.0*	*21.3*	*15.8*
	right	35.5	**54.1**	46.8	42.2	38.1	34.4	37.1	37.5	34.7	41.5	35.7	33.3	33.3	38.6
		29.0	*18.0*	*24.2*	*26.6*	*28.6*	*37.5*	*27.4*	*35.9*	*44.4*	*35.4*	*37.5*	*37.9*	*16.7*	*14.0*
US2-XD1	left	42.4	50.0	45.9	38.3	51.6	35.6	35.9	44.8	43.5	**50.9**	40.7	36.1	41.7	38.7
		43.9	*21.7*	*27.9*	*21.7*	*32.3*	*30.5*	*34.4*	*25.9*	*25.8*	*12.7*	*38.9*	*31.1*	*23.3*	*35.5*
	right	30.3	49.1	42.1	45.0	47.5	47.3	45.2	43.9	44.3	41.3	39.3	39.3	29.0	37.1
		40.9	*8.8*	*12.3*	*18.3*	*26.2*	*14.5*	*25.8*	*19.3*	*42.9*	*33.3*	*41.1*	*27.9*	*43.5*	*32.3*

Bold text indicate nucleotide identities of >50%

Alignment of the deletion flanking regions between the SeMNPV and SfMNPV genotypic variants. To reveal any potential recombination events between SeMNPV and SfMNPV, sequences of ca. 50 bp flanking the deletion breakpoints of the SeMNPV deletion genotypes SeUS2-C, SeUS2-E, and SeUS2-F were aligned with seven of the genotypes present in the SfNIC population (Table 2). A deletion genotype present in the SeMNPV isolate from California, SeUS1-XD1 (Dai *et al.*, 2000), was also included

in the analysis. No significant homologies were detected between the SeUS2 genotypes and those of SfNIC or SeUS1-XD1, even for the genotypic variants with breakpoints on homologous genes, as was the case of the left breakpoints of SeUS2-F and SfNIC-F and of SeUS2C and SfNIC-E, and for the right breakpoints of SeUS1-XD1 and SfNIC-F and of SeUS2E and SfNIC-I. Only seven alignments showed homologies of >50%; the greatest observed homology was 63.3% in the SfNIC-F and SeUS2-E right flanking sequences. However, all variants were aligned with no less than 7.5% gaps. The flanking sequences of the deletions in SeUS2-C, SeUS2-E and, SeUS2-F were analyzed for potential consensus nucleotide features, but none were found.

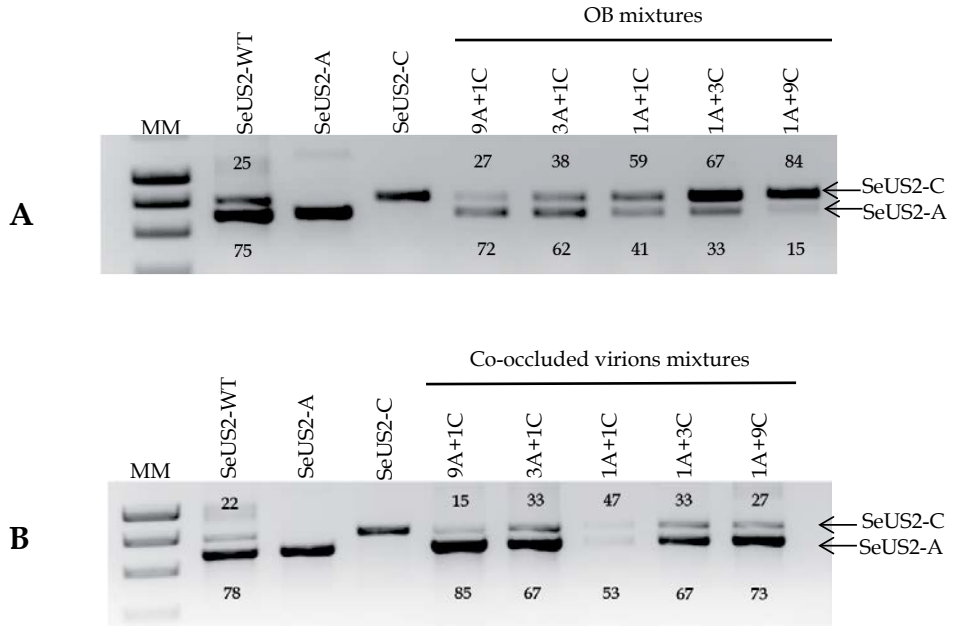

Figure 2. Relative proportions of SeUS2-A and SeUS2-C in mixtures of OBs and occluded virions. Semi-quantitative PCR analysis of specific SeUS2-A and SeUS2-C PCR products in the OBs mixtures (**A**) and co-occlusions (**B**). For amplification, total DNA of the wild-type, pure genotypes SeUS2-A and SeUS2-C, and mixtures of OBs and co-occluded genotypes were used. The figures next to amplicons indicate the relative proportion of each product estimated by densitometric analysis with the Scion Image program. The molecular marker (MM) used was 1 kb ladder from Stratagene.

Relative proportion of SeUS2-A and SeUS2-C in the wild type population and in tissue culture plaques. In the natural SeUS2-WT population, the full-length genome variant, SeUS2A, and the deleted genome variant, SeUS2-C, were present in relative proportions of ~3:1, respectively (Fig. 2). The opposite occurred in tissue culture plaques. SeUS2-C was present in 81% of the 97 plaques obtained following inoculation with hemolymph from SeUS2-WT infected larvae, based on *Pst*I restriction profiles. The remaining 19% of plaques comprised SeUS2-A. The genotypes SeUS2-E or SeUS2-F were not identified in any of the plaques.

Pathogenicity of SeUS2-A and SeUS2-C OB mixtures. The 50% lethal concentration (LC_{50}) of SeUS2-WT (1.69×10^4 OBs/ml) and SeUS2-A (1.31×10^4 OBs/ml) were statistically similar (Table 3). The relative proportions of SeUS2-C in the mixtures were assessed using semi-quantitative PCR and were similar to those predicted from the ratio of OBs in the inoculum: 27% SeUS2-C in the mixture 9A+1C, 38% SeUS2-C in 3A+1C, 59% SeUS2-C in 1A+1C, 67% SeUS2-C in 1A+3C, and 84% SeUS2-C in 1A+9C (Fig. 2A).

Only the 9A+1C OB mixture was as pathogenic as SeUS2-A OBs (Table 3). However, in all other OB mixtures with SeUS2-C OBs in proportions ≥ 25%, LC_{50} values were significantly higher than those of SeUS2-A OBs alone. The relative potencies of the OB mixtures reflected the abundance of SeUS2-A OBs in the inoculum. REN and PCR analysis of the OB samples extracted from larval cadavers confirmed the presence of SeUS2-A alone; SeUS2-C was not detected in the larvae, reflecting the lack of *per os* activity of these OBs (data not shown).

Pathogenicity, virulence, and OB yield of co-occluded SeUS2-A and SeUS2C mixtures. Densitometric analysis of genotypes-specific PCR products indicated that the frequency of SeUS2-C in co-occluded mixed-genotype OBs closely reflected the frequency of this genotype in the ODV inocula used to inject larvae (Fig. 2B).

Co-occluded mixtures for 1A+3C and 1A+9C could not be obtained because the proportion of SeUS2-C in progeny OBs was always markedly lower than those of the ODV mixtures that were used to inject larvae.

The LC_{50}s of the two co-occluded mixtures in which SeUS2-A was the predominant

Table 3. LC_{50} values for *S. exigua* second instars treated with (i) SeUS2-A OBs or SeUS2-WT OBs, (ii) mixtures of SeUS2-A OBs and SeUS2-C OBs, and (iii) OBs comprising co-occluded mixtures of SeUS2-A and SeUS2-C.

Virus	LC_{50} (OBs/ml)			
	Mean	Relative potency	Confidence limits 95%	
			lower	upper
(i) SeUS2-A	1.31×10^4	1	-	-
SeUS2-WT	1.69×10^4	0.77	0.23	1.30
(ii) Mixtures of OBs				
9A+1C	1.54×10^4	0.84	0.56	1.22
3A+1C	2.00×10^4	0.65	0.43	0.98
1A+1C	2.85×10^4	0.46	0.30	0.69
1A+3C	5.80×10^4	0.22	0.13	0.37
1A+9C	1.97×10^5	0.06	0.02	0.15
(iii) Co-occluded genotypes				
9A+1C	1.60×10^4	0.81	0.52	1.26
3A+1C	1.50×10^4	0.86	0.57	1.29
1A+1C	9.30×10^4	0.14	0.08	0.23
1A+3C	Not possible			
1A+9C	Not possible			

Regressions could not be fitted with a common slope; a test for non parallelism was significant (χ^2=40.71, df=9, P<0.001). Relative potency was therefore calculated as the ratio of LC_{50} values of each inoculum and SeUS2-A. As such, relative potencies indicate the relative pathogenicity of each inoculum compared with that of SeUS2-A. The confidence limits refer to relative potency values. Co-occluded mixtures for 1A+3C and 1A+9C OBs could not be produced as the proportion of SeUS2-C was markedly lower than that of the ODV inoculum used to inject larvae

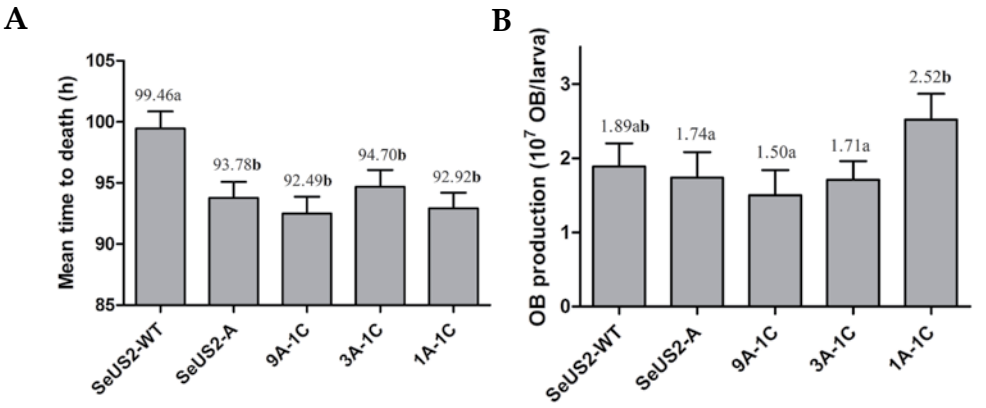

Figure 3. Influence of SeUS2-C on speed of kill and virus yield of occluded genotype mixtures. (A) Mean time to death (MTD) values of SeUS2-WT, SeUS2-A, and co-occluded mixtures 9A+1C, 3A+1C and 1A+1C. Bars labelled with the same letter were significantly similar. (**B**) Occlusion body (OB) yields of SeUS2-WT, SeUS2-A, and co-occluded mixtures 9A+1C, 3A+1C, and 1A+1C. Values above the bars indicate means and those followed by identical letters did not differ significantly for pairwise comparisons (ANOVA, P>0.05).

genotype (9A+1C and 3A+1C) were statistically similar to that of SeUS2-A alone (Table 3). However, the LC_{50} of OBs comprising a mixture of 1A+1C was 7.1-fold higher than that of SeUS2-A alone.

The MTD of the insects infected by SeUS2-A did not differ significantly from those obtained with the co-occluded mixtures involving 9A+1C, 3A+1C, or 1A+1C. In contrast, larvae infected with SeUS2-WT died significantly more slowly than did insects infected by SeUS2-A alone or by any of the co-occluded mixtures (Fig. 3A). The distribution of OB yield values was normalized by transformation and subjected to ANOVA (Fig. 3B). The OB yield values of SeUS2-WT, SeUS2-A, and of the co-occluded mixtures 9A+1C and 3A+1C were statistically similar, with a mean OB production between 1.89 x 10^7 and 1.50 x 10^7 OBs/larva, whereas the co-occluded mixture 1A+1C produced 2.52 x10^7 OBs/larvae and was significantly more productive (in terms of OBs) than were the other inocula tested.

Discussion

Two hypotheses can explain the origin of such similar population structures in SeUS2 and SfNIC: (i) SfNIC genotypes were derived from recombination events occurring between SeMNPV deletion genotypes and SfMNPV, or *vice versa*; or (ii) selection has independently favored the formation and maintenance of deletion genotypes in each population.

The high colinearity and sequence homology between the genomes of SeMNPV and SfMNPV suggest a recent common ancestor for these viruses. Interestingly, both viruses are capable of replication in *S. exigua* when inoculated singly and, although SeMNPV cannot replicate alone in *S. frugiperda,* SfMNPV can assist SeMNPV replication in *S. frugiperda* larvae, although the molecular basis for this helper function is unknown (Simón *et al.*, 2004b).

It seems highly unlikely that the deletion genotypes present in the American SeMNPV and SfMNPV populations could have originated from Asian SeMNPV populations following the introduction and spread of Asian *S. exigua* biotypes in the New World (Capinera, 1999). Specifically, two pieces of evidence suggest that this is not the case. First, none of the SfNIC deletion genotypes share homologous flanking sequences around the deletion breakpoints of SeUS2-C, SeUS2-E, SeUS2-F or SeUS1-XD1. Second, SfNIC deletion genotypes could not have originated from SeUS2-C or SeUS2-E because the US2 variants lack genes that are present in SfNIC. Theoretically, based on genetic content alone, SfNIC-F and SfNIC-I could have been generated by recombination with SeUS2-F, and SfNIC-H by recombination with SeUS1-XD1, but their flanking sequences around each deleted region could only have been generated if they had undergone additional recombination events involving identical crossovers; however, this is extremely unlikely. Similarly, the possibility that New World SeMNPV deletion genotypes were derived from SfMNPV populations is inconsistent with the presence of similar SeMNPV genotypes in isolates from Asia (Hara *et al.*, 1995), a region where SfMNPV is not found.

This leaves the independent generation of similar population structures in SeMNPV and SfMNPV as the only viable hypothesis. Genetic and functional analyses suggest that deletion variants may have arisen by selection due to their important roles in the survival and/or transmissibility of the viral population.

Genetically, SeMNPV and SfMNPV deletion regions are auxiliary gene-rich regions that contain genes that are not essential for virus replication but which reduce dependence on the host cell machinery or increase virus fitness in other ways (O'Reilly, 1997). Deletion affected two essential genes for viral replication, both of which are late expression factors (*lef-1* and *lef-2*), only in the genotype SeUS2-E. Variants lacking genes that are essential for replication or transmission are maintained in the population through complementation with complete genotypes in multiply infected cells (Turner & Chao, 1999). This is possible in baculoviruses because, on average, each cell is infected by four budded virions, each carrying a single genome (Bull *et al.*, 2001; Simón *et al.*, 2006). Complementation was observed in SfMNPV genotypes lacking *per os* infection factor (*pif-1, pif-2*) genes, which are required for ODV entry into midgut epithelial cells. As such, SfNIC-C and SfNIC-D are not infectious when administered *per os*, and the same situation is observed in SeUS2-C, which has a peroral non-infectious phenotype.

In tissue culture cells, SeUS2-C appeared to have a replicative advantage over the complete genotype, as indicated by the 1:3 SeUS2-A:SeUS2-C ratio of plaques resulting after infection with an SeUS2wt population, which comprised the opposite proportion of these two genotypes.

The ability of a virus population to produce co-occluded genotypes was dependent on the prevalence of both deletion and complete genotypes in the SeMNPV population. When the ratio of complete/deletion genotypes in the injected inoculum was 1:3 or 1:9, the replication rate of the deletion genotype was too low to produce OBs with a frequency similar to those of co-occluded genotypes; the complete genotype invariably dominated. This differed from the situation in SfNIC genotypes, in which

OBs comprising ratios up to 1:9 could be produced by injection of the appropriate inoculum (López-Ferber *et al*., 2003), suggesting species-specific differences in the interactions among genotypes in their respective insect hosts.

In SfMNPV, co-occluded mixtures of complete and deletion variants at a ratio of 3:1 enhanced the pathogenicity of the mixed genotype OBs with respect to that of the complete genotype alone, seemingly due to the dilution effect of the deletion genotype on the concentration of PIF-1 in the ODV envelope (Clavijo *et al*., 2009).

No such interaction was observed between SeUS2-A and SeUS2-C. SeUS2-C genotype was previously classified, in a study performed prior to the development of co-occlusion techiniques, as a defective parasitic genotype because it reduced the pathogenicity of OB mixtures comprising SeUS2-A OBs and ≥80% of SeUS2-C OBs (Muñoz *et al*., 1998). In the present study, the presence of SeUS2-C did not significantly affect OB pathogenicity when co-occluded mixtures were tested at ratios of 9:1 or 3:1 of SeUS2-A/SeUS2-C (Table 3), whereas the pathogenicity of OBs comprising a 1:1 ratio was severely compromised.

SeUS2-C and SfNIC-C both appear to play important roles in determining the pathogenicity of the *Spodoptera* virus population. Intriguingly, the prevalence of deletion variants in natural populations is maintained at a remarkably similar and highly stable frequency, representing approximately 25% of the population genotypes in SeUS2-WT (Muñoz & Caballero, 2000, this study) and 25% of the genotypes in SfNIC (Lopez-Ferber *et al*., 2003; Simón *et al*., 2006). Heterogeneous host susceptibility has been recently demonstrated both to increase prevalence of mixed-genotype baculovirus infections (van der Werf *et al*., 2011) and to mediate the selection of viral genotypes (Hitchman *et al*., 2007). Host ecology has also been observed to determine the relative fitness levels of genotypes in mixed-genotype infections (Hodgson *et al*., 2004).

The presence of genotypic diversity within ODVs and the occlusion of multiple genotypes into each OB is a natural phenomenon in multicapsid

nucleopolyhedroviruses that favors the transmission of virus diversity but also allows deletion variants to survive in the population (Clavijo *et al*., 2010).

Functional complementation of co-occluded viral mixtures allowed us to examine the consequences of SeUS2-A-SeUS2-C interactions on two additional correlates of virus transmissibility. Speed of kill (virulence) did not vary among co-occluded genotype mixtures, but was slower for SeUS2-WT, indicating that additional genotypes present in the natural population may be capable of modulating this phenotype. Indeed, genotype SeUS2-D, which has an insertion in the auxiliary gene rich region, was identified as a slow-killing variant in the population (Muñoz *et al*., 2000). As for OB production, all co-occluded mixtures showed OB yields/larvae similar to that of the wild-type population, except the mixture comprising 50% SeUS2-C (1A-1C). Larvae infected by this mixture produced a significantly higher number of OBs, which suggest that SeUS2-C may have a role in promoting virus transmissibility via increased OB production. The genetic basis for this is presently unclear, but it may be related to the absence of one or more of the genes of unknown function that are present in the SeUS2-C deleted region. One such gene is *se28*, the homolog of which is partially deleted in SfMNPV (*sf27*) in a fast-killing variant of this virus (SfMNPV-3AP2), although its influence on OB production has not been determined (Harrison *et al*., 2008)

Although SfNIC and SeUS2 populations are clearly similar in terms of genotypic structure and diversity, the functional importance of their deletion variants appear to encompass different aspects of virus transmissiblity in each virus population, ranging from OB pathogenicity effects (López-Ferber *et al*., 2003) and speed-of-kill characterisitics (Harrison *et al*., 2008) to the quantities of OBs produced in each infected insect (as in this study). The three phenotypic traits analyzed here provide a broader picture of the functional significance of the defective genotype SeUS2-C. Previous studies demonstrated that the deletion variants SeUS2-C and SeUS2-E, could invade a deletion variant-free SeMNPV population and become as abundant

as in their original population after only four serial passages in larvae (Muñoz & Caballero, 2000). Studies on SeUS2-E were hindered because this genotype lacks the essential *lef-1* and *lef-2* genes, making its purification impossible in cell culture. Future cotransfections with plasmids containing these factors may allow us to clone this genotype. Although the function of SeUS2-E remains veiled, it is clear that defective genotypes tend to persist in baculovirus populations because of their functional importance in key processes, particularly in transmission.

In conclusion, genomic and functional analysis of deletion genotypes present in SeMNPV and SfMNPV isolates indicate that the existence of remarkably similar population structures in distinct baculovirus species from different hosts clearly points to a shared evolutionary mechanism that generates and sustains deletion mutants within each virus population independently, rather than using a recent exchange of deletion variant genotypes between these viruses.

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Supplementary information

Table 1. Name and sequence of the primers used to map the deletion break point for SeUS2-E and for the quantification of the SeUS2-C and SeUS2-A genotypes.

Primer name	Sequence	**Genotype**	**Use**
prE-F01	5´-TGTTACTTGCGTGGCTATCG-3´	SeUS2-E	Mapping
prE-R01	5´-GTGACACGCTTCGATCTTGA-3´		Mapping
P1	5´-CACGTAGCGCAACAAATCCTC-3´	SeUS2-C	Quantification
P2	5´-CGAGGACGAGTAGAGTGTTG-3´	SeUS2-C & SeUS2-A	Quantification
P3	5´-CAGTCACCTTCGCCCACAGC-3´	SeUS2-A	Quantification

Chapter 5

The baculovirus SeMNPV ORF28 region is a key regulator of viral spread

Adapted from:
Amaya Serrano, Sarah Nadif, Delia Muñoz, Monique M. van Oers, Primitivo Caballero, Just M. Vlak and Gorben P. Pijlman. The baculovirus SeMNPV ORF28 region is a key regulator of viral spread, *manuscript in preparation.*

Abstract

The US1 natural isolate of the *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) is composed of several genotypic variants, most of which carry deletions of variable size in the ORF15-41 region. From this isolate, bacmids containing the complete SeMNPV genome (SeBac10) and a natural genotypic variant with a deletion (SeBac72) were generated. SeBac72 displayed a much more efficient viral spread in *S. exigua* cells as compared to SeBac10. We hypothesized that one or more gene(s) in the deleted region of SeBac72 severely affected viral spread of SeBac10 in cell culture. Complete genome sequencing of SeBac72 revealed a 9.5 kb deletion spanning ORF16-28. SeBac10-derived bacmids with combined or individual ORF16-28 knockouts were constructed to assess viral spread in Se301 and SeUCR cells. SeMNPV ORF28 (*se28*) was identified as the responsible gene preventing successful spread of genome-length SeMNPV in cultured cells, which explains why Se28-deleted viral genomes are preferentially selected in cell culture. Unexpectedly, *se28*, when reinserted into SeBac72, could not rescue the inhibition of viral spread, and gene silencing of *se28* did not enhance the spread of SeBac10. This suggested that not the transcript or translated product, but rather the DNA sequence and/or the sequence topology of *se28* determine the viral spread. Sequence analysis revealed that the entire SeMNPV ORF27-30 region was collinear with the AcMNPV ORF15-18 region and shares an overall 43% identity. Despite extensive similarity at the DNA level between *se28* and *ac16* (DA26), their amino acid sequences were non-homologous and clearly separate the group I from the group II NPVs. Both the *se28*/*ac16* genes are part of hypervariable regions associated with insertions/deletions. Overall, we identify the *se28* region as a key regulator of viral spread in a region that may drive the genotypic variation in natural baculovirus isolates.

Introduction

The family *Baculoviridae* groups arthropod-specific viruses with double-stranded, circular, supercoiled genomes varying in size from about 80 to over 180 kb, encoding between 90 and 180 genes (Rohrmann, 2013b). Baculoviruses have long been used as biocontrol agents of natural insect populations in many agricultural crops and in forestry (van Oers & Vlak, 2007). The application of baculoviruses as bioinsecticides is increasing due to development of insect resistance to common chemical pesticides and societal demands for the use of safer pesticides and less-toxic chemical residues in the food chain (Moulton *et al.*, 2002). All products currently on the market are produced *in vivo* (Moscardi, 1999). Production of baculoviruses *in vitro*, in bioreactors with cultured insect cells, is theoretically attractive because such systems require selection of insect cell clones supporting high baculovirus yields. These systems can be well controlled, allow rapid scale up and may be cost competitive (Moscardi, 1999). However, the baculovirus genome is prone to genetic alterations (mainly deletions) which are favorable for virus amplification in cell culture (Kool *et al.*, 1991; Pijlman *et al.*, 2001). The deleted genes are obviously non-essential for *in vitro* amplification but may well negatively affect infectivity *in vivo* and hamper the utilization of insect cell bioreactors for the production of effective baculovirus-based bioinsecticides (Dai *et al.*, 2000; Pijlman *et al.*, 2003b; Pijlman *et al.*, 2001).

The beet armyworm *Spodoptera exigua* is an important agricultural insect pest that is distributed worldwide and causes significant economic losses. *Spodoptera exigua* nucleopolyhedrovirus (SeMNPV) is an attractive bioinsecticide since it is monospecific to the beet armyworm and is relatively virulent compared to other baculoviruses (Smits & Vlak, 1988). SeMNPV has been isolated from several different geographical regions in the world (Caballero *et al.*, 1992a; Gelernter & Federici, 1986a; Hara *et al.*, 1995; Kolodny-Hirsch *et al.*, 1993; Kondo *et al.*, 1994; Vlak *et al.*, 1981).

Natural NPV populations are genetically heterogeneous and have been found to contain a mixture of several distinct genotypes; complete genotypes as well

as genotypes carrying different deletions (Simón *et al.*, 2004a). Evolutionary mechanisms independently generate and sustain these deletion genotypes within the viral population, indicating that this heterogeneity is important for virus survival (Serrano *et al.*, 2013). Muñoz *et al.* (1998) characterized a natural SeMNPV isolate from Florida (SeUS2) that contains at least seven genotypic variants. The genotypic variants had alterations, insertions or deletions, within the same region of the SeMNPV genome, predominantly between 16437 nt and 39757 nt (ORFs 12 to 40), indicating that this part of the genome is hypervariable (Muñoz *et al.*, 1998). Similarly, a Californian isolate of SeMNPV (SeUS1) also comprises a mixture of genotypes with varying deletions (Caballero *et al.*, 1992b; Dai *et al.*, 2000). The *PstI* digestion of the SeUS1 isolate showed several submolar bands, and PCR amplification using primers annealing to open reading frame (ORF)14 and ORF28, resulted in at least five products (Dai *et al.*, 2000), suggesting that SeUS1 is composed of at least five deleted genotypes in this hypervariable locus alone. When this SeMNPV was grown in Se301 insect cell culture, viruses with large deletions were quickly selected for (Heldens *et al.*, 1996). While the deletion mutants were replication competent in cell culture, they lost infectivity *in vivo*. The deletions were as large as ~25 kbp, located approximately between 18500 nt to 40500 nt, and affected ORF 15 to 41. Later, it became clear that the deletion included essential genes required for oral infectivity, the so-called *per os* infectivity factors pif-1 (*se35*) and pif-2 (*se36*) (Kikhno *et al.*, 2002; Pijlman *et al.*, 2003a).

To repair the loss of *in vivo* infectivity in a practical manner, alternating rounds of infection with the SeUS1 isolate between Se301 cell culture and *S. exigua* larvae yielded a SeMNPV variant, called SeXD1, which retained its biological activity *in vivo* and displayed an enhanced speed of kill of *S. exigua* larvae. SeXD1 appeared to be also a natural genotype within the SeUS1 population and had a somewhat smaller deletion of 10.5 kb, from 18513 to 29106 nt comprising ORF 15 to 28 (Dai *et al.*, 2000). The deleted region included the *chitinase*, *cathepsin*, *gp37*, *ptp-2* and *egt*

genes and nine others (Dai *et al.*, 2000).

These findings clearly illustrate that cell culture production of complete, genome-length SeMNPV with retained bioactivity *in vivo* is cumbersome. However, the causal mechanism for rapid generation and/or selection of viruses carrying large deletions in the ORF15 to 41 region in cell culture remains unknown. To obtain better insight in the mechanism behind SeMNPV genotypic variation, viral DNA of the SeUS1 isolate was used for the isolation of complete and deleted viral genotypes via bacmid cloning in *E. coli,* as described previously (Pijlman *et al.*, 2002). Bacmids containing the complete SeMNPV genome (SeBac10) and a genotypic variant (SeBac72) were generated and their replication in cell culture was studied. Significant differences in amplification between both bacmids in cell culture led to the identification of the ORF28 gene as a regulator of virus spread in *S. exigua* cells. Our results provide an explanation for the selective advantage of deleted genotypes that are naturally present in baculovirus populations.

Materials and methods

Cell lines and Viruses. The *S. exigua* cell line Se301 was donated by Dr. T. Kawarabata and was maintained at 28ºC in HyClone Insect Cell Culture Media CCM3 supplemented with 5% fetal bovine serum (FBS) (Thermo Scientific). The SeUCR cell line was donated by Dr. B. A. Federici and maintained at 28ºC in Grace's supplemented medium containing 10% FBS (Thermo Scientific) (Gelernter & Federici, 1986b). The generation of SeMNPV bacmids used in this study was described before (Pijlman *et al.*, 2002).

Complete genome sequencing of SeBac72. The genome sequence of SeBac72 was determined by Roche 454 Next Generation Sequencing (NGS) (KeyGene). The samples were *de novo* assembled using Newbler (454 runAssembly software) using default parameters.

Generation of SeMNPV gene knockouts by recombination in *E. coli*. For deletion

mutagenesis of the desired ORFs of the SeMNPV-US1 bacmid, SeBac10GFP, 68- to 70-bp primers were designed with 50-bp 5´ends flanking the deletion target region on the SeMNPV genome (Table 1). The 3' ends of the primers anneal to chloramphenicol acetyltransferase gene flanked by mutant *LoxP* site (Suzuki *et al.*, 2005) using a mutant lox sequence, which was amplified from pCRTopo-lox-cat-lox (Marek *et al.*, 2011). PCR on pCRTopo-lox-cat-lox was performed using Phusion Polymerase (Thermo Scientific) according to the manufacturer´s protocol, giving a 1170 bp product. The PCR product was purified using the Illustra GFX PCR DNA and gel purification kit (GE Healthcare). Approximately 150 ng PCR product was used for homologous recombination in MW003 cells (Westenberg *et al.*, 2010) containing SeBac10GFP. Homologous recombination was performed as follows. SeBac10GFP DNA was transformed into electrocompetent MW003 cells, and selected on LB-plates with streptomycin and kanamycin for 2 days at 32°C. A single colony was picked and grown ON in 1ml SOB-medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl and 10 mM $MgCl_2$) at 32°C. The ON-culture was used to inoculate 10 ml SOB-medium. The culture was incubated at 32°C until cells reached OD600. Next, the culture was split in two and 5 ml were induced for 10 min at 42°C. After incubation, cells were washed twice with 10% ice-cold glycerol. Cells were then resuspended in 100 µl 10% glycerol and stored at -80°C. The next day cells were electroporated with 150 ng of the PCR product, recovered in 1 ml SOB-medium and incubated for 3 h at 32°C. Subsequently, both induced and non-induced cells were plated on LB plates, supplemented with 50 µg/ml kanamycin and 50 µg/ml chloramphenicol. The plates were incubated for 48 h at 32°C. Finally, single colonies were picked to analyze if recombination had occurred (Dolphin & Hope, 2006) by restriction endonuclease analysis of the bacmid DNA and PCR amplification. Bacmid DNA was electroporated in DH10β cells. For deletion mutagenesis of *gp37*, *ptp-2*, *egt* and ORF28 in the SeMNPV-US1 bacmid SeBac10GFP, the same cloning strategy previously described was followed. Replacement was confirmed by restriction

endonuclease analysis and by PCR with primers SeORF25-F-CON and SeORF28-R-CON, designed outside the regions to be deleted (Table 1).

Transfection of SeMNPV bacmids. Se301 cells were seeded in a six-well tissue culture plate (Greiner Bio-One) at a confluency of $5x10^5$ cells. Transfection was performed with 1 µg recombinant bacmid DNA using 10 µl Lipofectin transfection reagent (Invitrogen). Six days after transfection, GFP expression was checked under a fluorescence microscope Zeiss Axio Observer Z1.

Expression of *se28* in AcBacmid. To express *se28* in a heterologous virus, the bacmid AcΔccΔp10 from *Autographa californica* MNPV was used (Metz *et al.*, 2011). This bacmid contains a deletion of the promoters and large parts of the coding sequences of *cathepsin* and *chitinase* (Kaba *et al.*, 2004). Furthermore, the *p10* promoter and ORF are deleted by replacement with a zeocin resistance marker by Lambda Red recombination (Datsenko & Wanner, 2000). Modified *LoxP* sequences flanking the zeocin resistance marker were used for subsequent removal of the resistance marker by Cre recombinase (Suzuki *et al.*, 2005) using a mutant *lox* sequence. *Se28* was cloned into the pFastBac derivative pDEST8 plasmid (Invitrogen) fused with mCherry (pDESTmChSe28) and *se28* with mCherry and with 2A ribosomal skipping element from Foot-and-Mouth disease virus (FMDV) (pDESTmCh2ASe28). The pDEST8 constructs were transposed into the *att*Tn7 transposon integration site from the AcΔccΔp10 using the Bac-to-Bac baculovirus expression system resulting in AcBac-mChSe28 and AcBac-mCh2ASe28. The transposition was checked by PCR using M13 and M13F-Genta primers (Table 1). As a control, we used AcBac-mCh. Transfection of AcBac-mCh, AcBac-mChSe28 and AcBac-mCh2ASe28 was performed in Se301 cells using Lipofectin as a transfection reagent.

Transient expression. *Se28* was cloned into pIB-V5-His-GW plasmid (OpIE2

promoter) for transient expression in Se301 insect cells. *Se28* was cloned fused to mCh (with or without FMDV-2A) resulting in pIB-mChSe28 and pIB-mCh2ASe28. Forty-eight hours post-transfection, mCherry expression was checked under fluorescence microscope Zeiss Axio Observer Z1.

Expression of Se28 in SeBac72. *Se28* was amplified using Phusion Polymerase (Thermo Scientific) with primers including its own promoter and containing a *KpnI* restriction site (Table 1). The resulting fragment was cloned into a CloneJET PCR Cloning Kit (Thermo Scientific), sequenced and cloned as *KpnI* fragment into a pFastBacSepolGFP. The protocol from the Bac-to-Bac manual (Invitrogen) was followed to transpose the *se28* from pFastBacSe28 into the *att*Tn7 transposon integration site of SeBac72GFP to generate the repair bacmid, SeBac72GFPSe28.

Gene and protein sequence analysis. DNA and protein homologs of *se28* were searched in the updated GenBank/EMBL databases using BLAST (Altschul *et al.*, 1990). PSIPRED was used to predict protein secondary structure (McGuffin *et al.*, 2000) and PROSITE to search for protein domains (Hulo *et al.*, 2008). Transmembrane domains were detected with TMHMM v2.0 (Jones, 2007). For the Bayesian phylogeny of baculoviruses based on *lef-8* gene, *lef-8* sequences of all baculoviruses that have *se28* or *Da26* genes were included. Sequences were translated in frame to proteins and aligned using MAFFT version 7 (Katoh *et al.*, 2005). Protein alignment was converted back into corresponding codon alignment using PAL2NAL (Suyama *et al.*, 2006). Gblock was used for trimming sequences to select conserved domains (Talavera & Castresana, 2007). Bayesian inference was conducted using MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003), using the GTR + I + G model (default settings, six million generations, burn-in of 25%). A pairwise alignment of SeMNPV ORF27 to 30 (26928 to 32669 nt) and AcMNPV ORF15 to 18 (11426 to 15459 nt) was made using the Needleman-Wunsch global alignment algorithm in Emboss software (Rice

et al., 2000).

Results

Analysis of cloned SeMNPV bacmids. During the direct cloning of SeUS1 as a bacmid (Pijlman *et al.*, 2002), several complete bacmid clones that retained the predicted restriction profile were selected, as well as bacmids with apparent deletions in the *Pst*I-D fragment (Fig. 1A). Clone 10 (SeBac10) was identified as the full-length bacmid. However, SeBac72 was identified as a bacmid with a ~9 kbp deletion in the *Pst*I-D restriction fragment (Fig. 1A), which probably represents a natural genotype of the wt SeUS1 isolate, since deletions in this region have often been reported (Dai *et al.*, 2000; Heldens *et al.*, 1996).

In order to visualize putative differences in viral replication and spread in cell culture, a pFastBac vector expressing GFP under control of the *polh* promoter was transposed into the *att*Tn7 transposon integration site of the different bacmids. After transfection of the bacmids in *S. exigua* Se301 cells, it was observed that vSeBac72GFP readily replicated in cell culture and initiated viral spread in cell culture much faster as compared to the complete bacmid vSeBac10GFP, which mostly remained confined to a single cell or small group of neighbouring cells (Fig. 1B). Although vSeBac10GFP did not efficiently spread from cell to cell, the initially transfected cell did express GFP from the very late polyhedrin promoter, suggesting that baculovirus DNA replication was taking place and (very) late gene expression was not impaired.

In previous studies with SeUS1 it was shown that a deletion of ~25 kbp of the viral genome was selected for within the first passage in cell culture (Heldens *et al.*, 1996) and that transfection of vSeBac10 in cell culture yielded a ~16 kbp deletion in the region ORF17-35 before the virus could be further amplified (Pijlman *et al.*, 2002). Together with the current observations, this suggested that the efficiently amplifying vSeBac72 lacked important sequences on a ~9 bkp deleted fragment that may explain why the complete, genome-length SeBac10 failed to spread in cell culture.

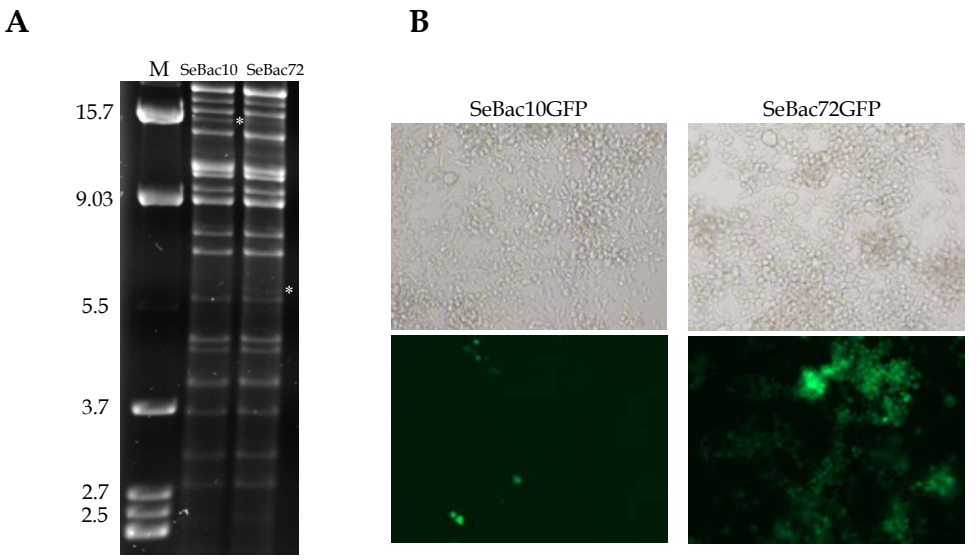


Figure 1. Characterization of genome-length SeMNPV bacmid SeBac10 and deletion mutant SeBac72 (**A**) *Pst*I restriction endonuclease analysis of genomic DNAs from SeBac10 and SeBac72. Asterisks indicate the *PstI*-D diagnostic restriction fragment in SeBac10 and the truncated variant in SeBac72. Lane M contains a λ *Bam*HI/*Hin*dIII/*Eco*RI DNA size marker. (**B**) Se301 insect cells transfected with bacmid DNA from SeBac10GFP and SeBac72GFP. Bright field (upper row) and fluorescence field (lower row). Pictures were taken 6 days post transfection.

Complete genome sequencing of SeBac72 identifies a 9.5 kbp deletion spanning multiple ORFs. To initiate the identification of the gene(s) responsible for preventing viral spread by the complete vSeBac10, the complete genome sequence of SeBac72 was determined by Roche 454 next generation sequencing (NGS). The sequence reads (31876) were assembled into 5 large contigs with an average coverage of 97.1% per contig, which could be joined based upon the published SeMNPV sequence (Genbank accession number NC_002169) with confidence to yield the complete SeBac72 sequence. Upon comparison of the SeBac72 sequence with the published SeUS1 sequence (IJkel *et al.*, 1999), it was found that SeBac72 contained a deletion of ~9.5 kbp, located from nt 19172 to 28718 (Fig. 2A) spanning ORF16 to ORF27. A small fragment from the downstream ORF30 was found inserted in reverse orientation at the junction site, suggesting that the deletion was most likely the

result of a non-homologous recombination event. The deletion affected genes such as *cathepsin*, *chitinase*, *gp37*, *ptp-2*, *egt* and some ORFs with unknown function, as well as the putative promoter region of ORF28. By revisiting the PCR results of Dai *et al*. (2000), it became clear that the sequence of the 1.9 kbp PCR product in that paper (Fig. 2B) corresponded to the sequence of the deletion junction now identified in SeBac72 by NGS. We therefore concluded that SeBac72 contains the viral genome of a naturally occurring SeUS1 genotype, which is closely related to SeXD1 but was not characterized in detail at that time.

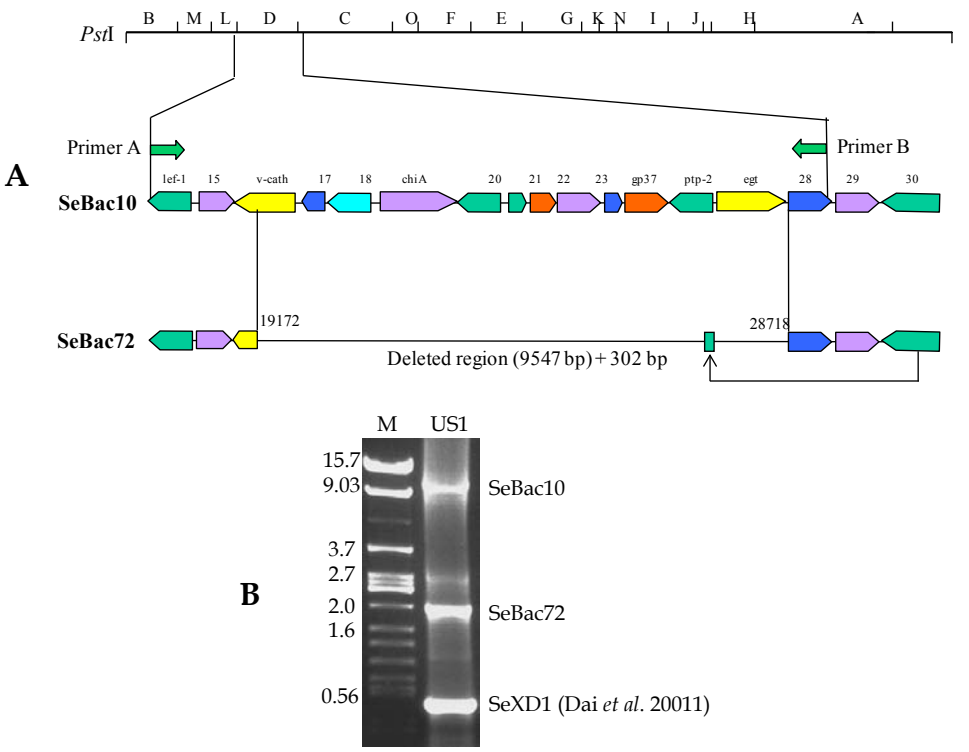


Figure 2. Sequence analysis of SeBac72 (**A**) The top line represents the physical map of the wt SeMNPV-US1 genome, *Pst*I restriction endonuclease sites are represented below the genome line. The coloured arrow bars show the organization of ORFs in the deleted region in SeBac72. ORF numbering is according to the published SeMNPV-US1 genome (IJkel *et al*., 1999). The thick arrows above indicate the position of primers A and B to distinguish genotypes by PCR. The length of deletion is indicated in bp. (**B**) PCR analysis of genotypic variants present in the SeMNPV-US1 isolate. The primers A and B correspond to nt 17874 to 17904 and 29135 to 29163, respectively. The arrow indicates the 1,9 kbp amplicon corresponding to the deletion in SeBac72. Lane M contains a λ DNA size marker *Bam*HI/*Hin*dIII/*Eco*RI (sizes in kbp).

Mapping SeMNPV gene(s) preventing virus spread in cell culture. Based on the observed failure of vSeBac10 to spread in cell culture, we hypothesized that one or more gene(s) in the SeBac72 deleted region (*se16-se28*) was responsible for this phenotype. To identify this gene(s) we first constructed three different multiple-gene bacmid knockouts by lambda red recombination using SeBac10 as a backbone: SeBac10GFPΔ16-19 (deleting ORFs from 16 to 19), SeBac10GFPΔ20-24 (deleting ORFs from 20 to 24) and SeBac10GFPΔ25-28 (deleting ORFs from 25 to 28) (Fig. 3A). The deletion of the desired region was made by introduction of a chloramphenicol resistance gene (*cat*) through homologous recombination in *E. coli* as described (Pijlman *et al*., 2002). Replacement was confirmed by restriction endonuclease analysis (Fig. 3B) and PCR with specific primers designed outside of the regions to be deleted (data not shown). Bacmid DNA was used for transfection of Se301 insect cells using Lipofectin. Six days post transfection the cells were observed with fluorescence microscopy. As expected, vSeBac72GFP transfection showed very efficient replication and spread of the GFP signal in the cell layer (Fig. 3C). In sharp contrast, however, vSeBac10GFP showed a much lower viral spread compared to vSeBac72GFP (Fig. 3C). The knockout vSeBac10GFPΔ25-28 showed a similar spread as vSeBac72GFP, whereas vSeBac10GFPΔ16-19 and vSeBac10GFPΔ20-24 displayed the vSeBac10GFP phenotype (Fig. 3C). This experiment indicated that the gene(s) preventing virus spread of SeBac10 were located within the region spanning ORF25-28.

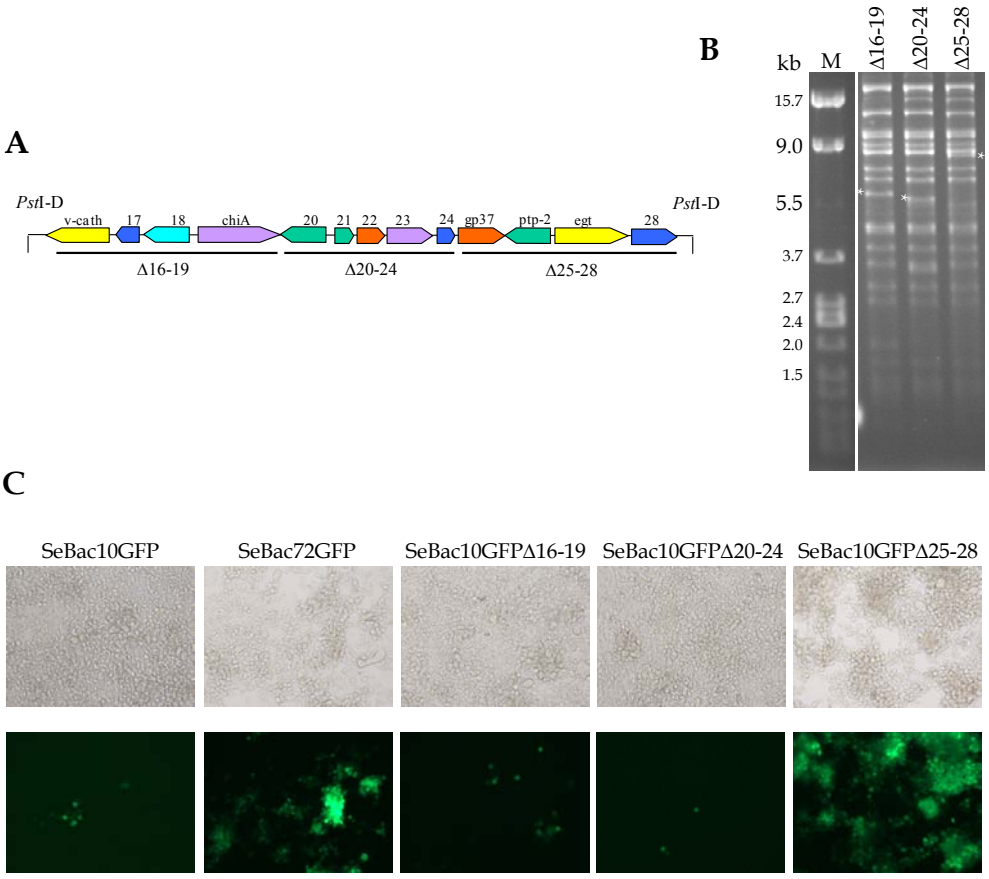


Figure 3. (**A**) Schematic representation of the genes deleted in the multiple knockouts SeBac10GFPΔ16-19, SeBac10GFPΔ20-24 and SeBac10GFPΔ25-28. (**B**) *Pst*I restriction endonuclease analysis of DNA from the multiple knockouts. Lane M contains a λ *Bam*HI/*Hin*dIII/*Eco*RI DNA size marker. (C) Se301 insect cells transfected with DNA from the multiple gene knockouts. Bright field (upper row) and fluorescence field (lower row). Pictures were taken 6 days post-transfection.

ORF28 prevents virus spread of genome-length SeMNPV in cell culture. In order to map the gene(s) within the ORF25-ORF28 region that prevented viral replication in cell culture, we constructed single gene knockouts using the SeBac10 backbone (Fig. 4A). ORF25 is a *gp37* homologue, ORF26 is *ptp-2* homologue, while ORF27 is the *egt* gene and ORF28 is a gene with unknown function. The construction of the single gene knockouts was performed as described above. Replacement was

confirmed by restriction endonuclease analysis (Fig. 4B) and PCR with specific primers designed outside of the regions to be deleted (Fig. 4C). vSeBac10GFPΔgp37, vSeBac10GFPΔptp-2 and vSeBac10GFPΔegt had the same phenotype as vSeBac10GFP but did not rescue efficient viral spread (Fig. 4D). However, the single gene knockout vSeBac10GFPΔ28 did rescue viral spread with the same efficiency

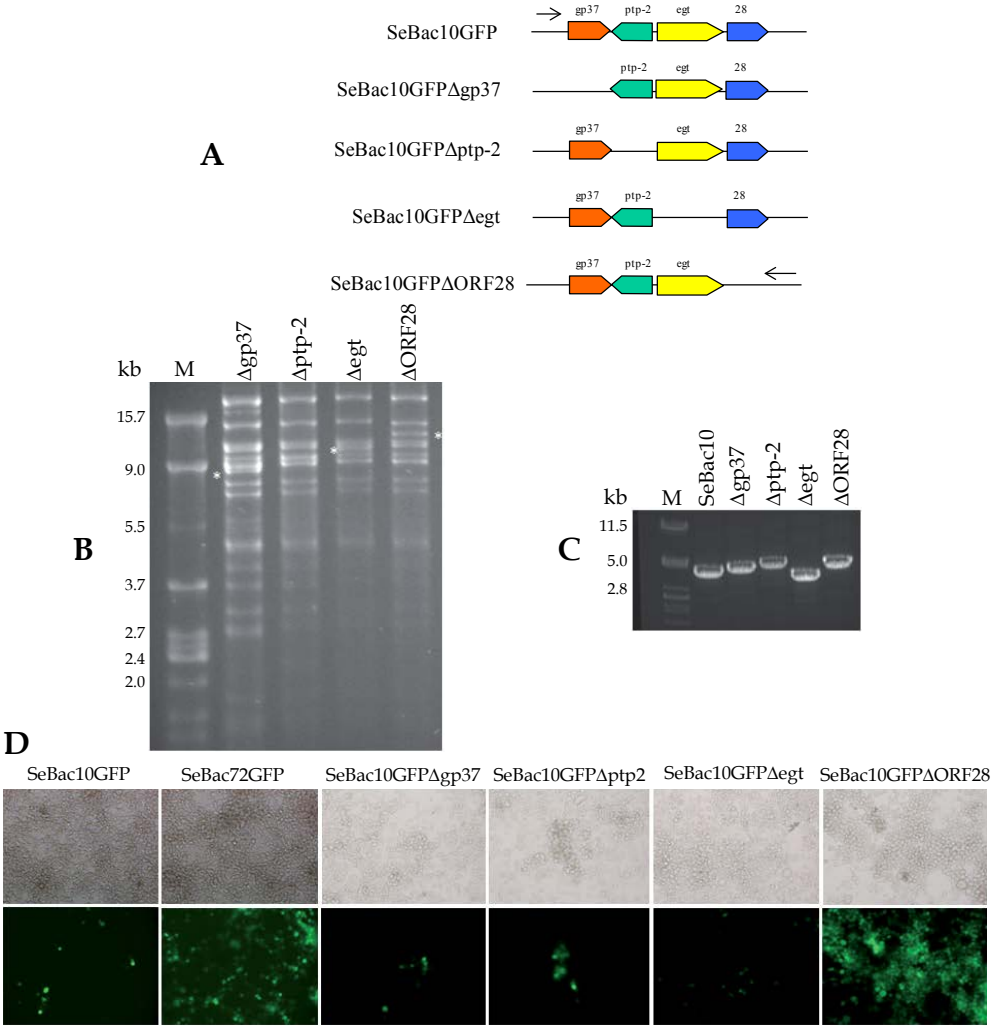


Figure 4. (**A**) Schematic representation of the genes deleted in the single knockouts SeBac10GFPΔgp37, SeBac10GFPΔptp-2, SeBac10GFPΔegt and SeBac10GFPΔOORF28. Arrows indicate the position of the primer for PCR control. (**B**) *Pst*I restriction endonuclease analysis of DNA from the single knockouts. Lane M contains a λ *Bam*HI/*Hin*dIII/*Eco*RI DNA size marker. (**C**) PCR analysis of DNA from the single knockouts. Lane M contains a λ *Pst*I DNA size marker (sizes in kbp). (**D**) Se301 insect cells transfected with DNA from the single gene knockouts.Bright field (upper row) and fluorescence field (lower row). Pictures were taken 6 days post-transfection.

as the positive control vSeBac72GFP (Fig. 4D). *Se28* was therefore identified as the responsible gene that prevents spread of genome-length SeMNPV in cell culture.

In order to confirm that the observation was not an artefact of the cell line used, we transfected SeUCR cells with bacmid DNA from the single knockouts. We observed very similar results as with the Se301 cells: vSeBac10GFPΔgp37, vSeBac10GFPΔptp-2 and vSeBac10GFPΔegt had the same phenotype as vSeBac10GFP (single cell infection), whereas vSeBac10GFPΔ28 showed viral spread with similar efficiency as vSeBac72GFP (Fig. 5). Thus, *se28* prevented the viral spread in both cell lines, indicating that the block of viral spread was independent of the cell line. The overall viral spread in SeUCR was not as efficient as in Se301 cells. This is in agreement with studies by others demonstrating that Se301 cells are more susceptible to SeMNPV infection than SeUCR (Hara *et al.*, 1993). For that reason, further experiments were conducted in Se301 cells.

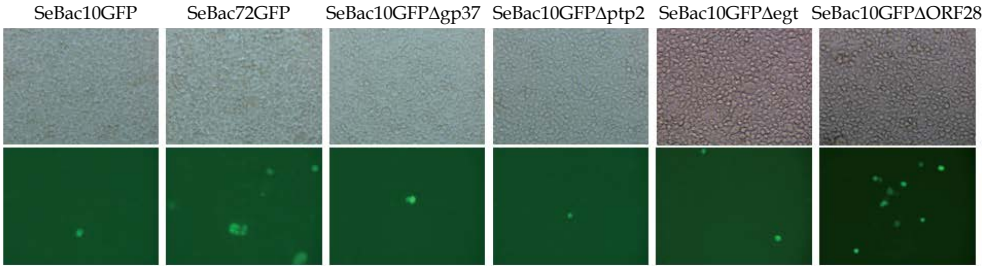


Figure 5. SeUCR insect cells transfected with DNA from the single knockouts SeBac10GFPΔgp37, SeBac10GFPΔptp-2, SeBac10GFPΔegt, and SeBac10GFPΔORF28. Bright field (upper row) and fluorescence field (lower row). Pictures were taken 6 days post-transfection.

Expression of *se28* is not cytotoxic to insect cells. One possible explanation for preventing viral spread by *se28* is that the SE28 protein has a pro-apoptotic function and kills the cells before progeny virus is produced. To study whether *se28* expression was cytotoxic to the insect cells, the gene was initially cloned as a C-terminal fusion to a mCherry marker gene, with/without a Foot-and-Mouth disease virus (FMDV)-2A ribosome skipping element in between to produce nearly

authentic SE28 (Fig. 6A). The expression vector pIB contained an OpMNPV ie2 promoter to drive the expression of the fusion protein in insect cells. Transient expression of both pIB-mChSe28 and pIB-mCh2ASe28 in the Se301 insect cells after

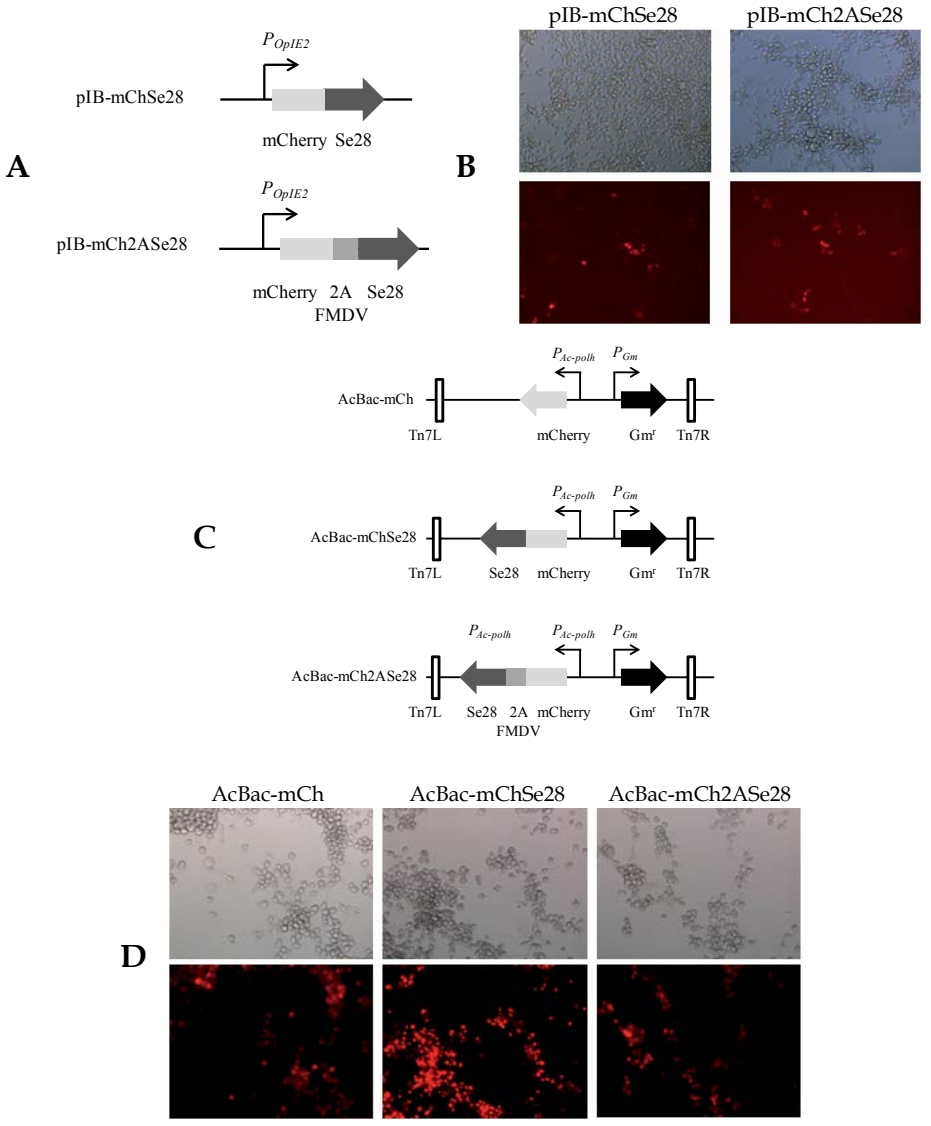


Figure 6. (**A**) Overview of the pIB-DEST expression plasmid containing the OpIE2 promoter, the FMDV autocleaving protease 2A, and the gene Se28. (**B**) Se301 insect cells transfected with pIB-mChSe28 and pIB-mCh2ASe28 for transient expression. Pictures were taken 48 hours post-transfection. (**C**) Overview of the recombinant bacmids of AcMNPV, where Se28 expression is driven under AcMNPV polyhedrin promoter. (**D**) Se301 insect cells transfected with AcBac-mCh, AcBac-mChSe28 and AcBac-mCh2ASe28. Bright field (upper row) and fluorescence field (lower row). Pictures were taken 6 days post-transfection.

48 hours post-transfection showed mCherry expression throughout the cell and no obvious negative effects on cell viability were observed (Fig. 6B).

Expression of *se28* does not block AcMNPV spread in cell culture. In order to determine whether *se28* was also capable of blocking viral spread of a heterologous baculovirus in cell culture, *se28* was expressed from an AcMNPV bacmid. *Se28* was cloned downstream the *polh* promoter in a pDEST8 plasmid (a Gateway-compatible pFastBac-derivative) fused with mCherry (pDESTmChSe28), or separated from mCherry using a FMDV-2A element (pDESTmCh2ASe28). The pDEST constructs were transposed into the *att*Tn7 transposon integration site of an AcMNPV bacmid (AcBac-mChSe28 and AcBac-mCh2ASe28) (Fig. 6C). The transposition was confirmed by PCR using M13F-R and M13F-Genta primers (data not shown). Se301 cells were transfected with bacmid DNA. vAcBac-mCh was used as a negative control. Seven days post-transfection cells were observed with fluorescence microsocopy. vAcBac-mChSe28 and vAcBac-mCh2ASe28 showed the same, very efficient viral replication and spread as the control bacmid vAcBac-mCh (Fig. 6D). Thus, expression of *se28* from a heterologous AcMNPV bacmid in Se301 cells did not prevent virus spread in cell culture, indicating that the block on baculovirus replication is virus-specific. Protein expression of SE28 in vAcBac-mChSe28 and vAcBac-mCh2ASe28 was confirmed by Western blot with antibodies against mCherry (data not-shown).

Gene silencing of *se28* fails to rescue virus spread of genome-length SeMNPV in cell culture. Since deletion of *se28* rescued virus spread of SeBac10 in cell culture, we wished to confirm a role for the SE28 protein by RNA interference (RNAi). We hypothesized that silencing of *se28* would rescue the efficient viral spread of SeBac10 in cell culture. First, Se301 cells were transfected with dsRNA targeting *se28* (specific; dsSe28) or firefly luciferase (unspecific; dsLuc). Next, cells were transfected with bacmids vSeBac10GFP and vSeBac72GFP (positive control) one day post

dsRNA transfection. As a control for the Se28-specific silencing, an infection with an AcMNPV virus expressing mChSe28 (Ac-mChSe28) was included. As expected, silencing with dsSe28 but not with dsFluc substantially decreased the amount of red fluorescence produced by Ac-mChSe28, indicating that the dsRNA-induced silencing of *se28* was efficient and specific (Fig. 7).

Transfection with vSeBac72GFP transfected cells show overall green fluorescence as before, with no difference between cells transfected with dsSe28 and dsLuc (Fig. 7). However, and in contrast to our expectation, transfection with vSeBac10GFP did not show more green fluorescent cells in dsSe28-transfected cells in comparison with dsLuc transfected cells. So, viral spread of vSeBac10 could not be rescued in cells with robust silencing of *se28* expression, but only when the *se28* gene was physically deleted from the viral genome.

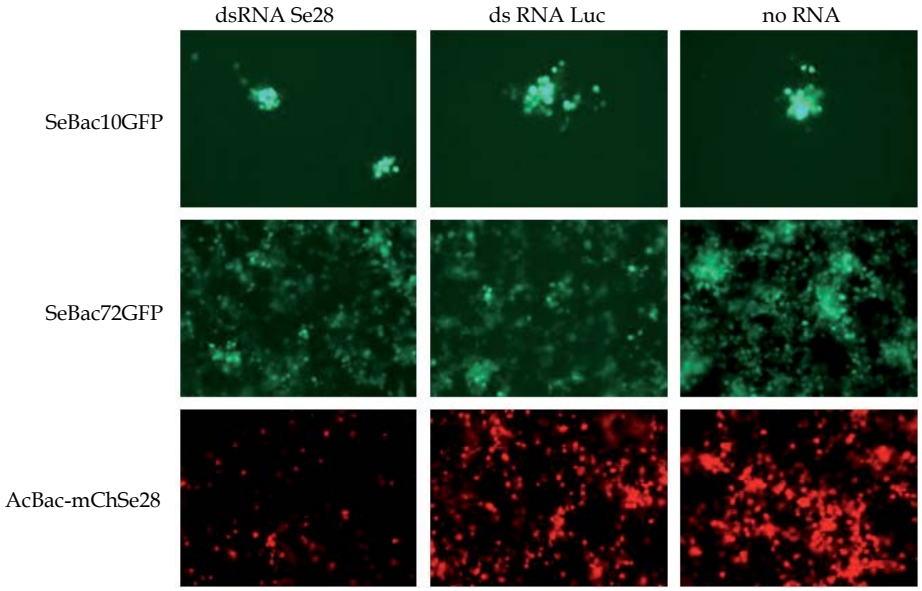


Figure 7. Se301 cells transfected with dsRNA of Se28 (first column), dsRNA of Luc (second column) or not transfected with RNA (third column), and transfected 24 h later with SeBac10GFP (first row), SeBac72GFP (second row), or with AcBac-mChSe28 (third row). Pictures were taken 72 h after first transfection for AcBac-mChSe28, and 144 hrs for SeBac10GFP and SeBac72GFP.

Efficient spread of SeBac72 in cell culture cannot be blocked by *se28* expression from another locus. We showed that deletion of *se28* from SeBac10 rescued viral spread whereas silencing of *se28* via RNAi did not. In order to investigate whether a reintroduction of *se28* under control of its own promoter in SeBac72 could lead to a block in viral spread, *se28* was cloned including its own promoter in a pFB1SeGFP plasmid, which already contained GFP downstream the polyhedrin promoter (Pijlman *et al.*, 2004) to generate pFBGFPSe28. This construct was transposed into the *att*Tn7 transposon integration site of SeBac72 (SeBac72GFPSe28), which is located inside the polyhedrin gene (Pijlman *et al.*, 2002) (Fig. 8A). The transposition was checked by PCR using M13F-R and M13F-Genta primers (data not shown). Se301 cells were transfected with bacmid DNA. Seven days post-transfection, cells were observed with fluorescence microscopy. Rather unexpectedly, however, vSeBac72GFPSe28 showed the same, efficient viral spread as vSeBac72GFP (Fig. 8B). Thus, while deletion of *se28* was required to rescue viral spread of genome-length vSeBac10, *se28* was not able to block the spread of vSeBac72 when expressed from another locus.

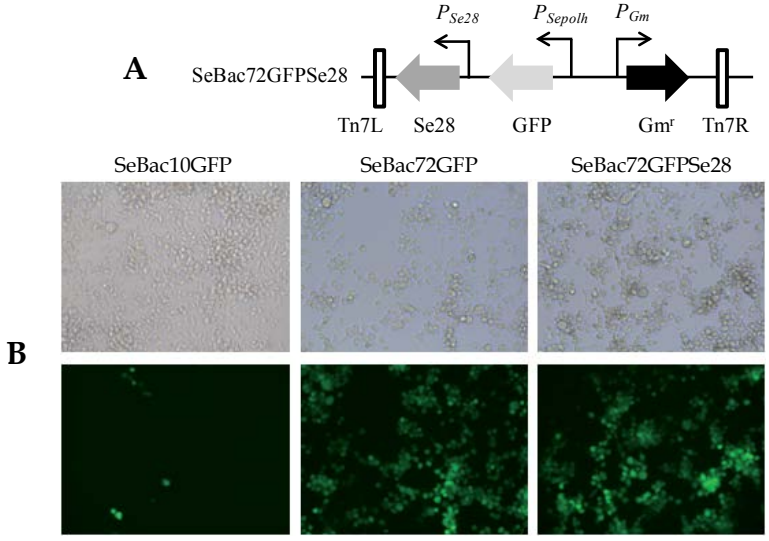
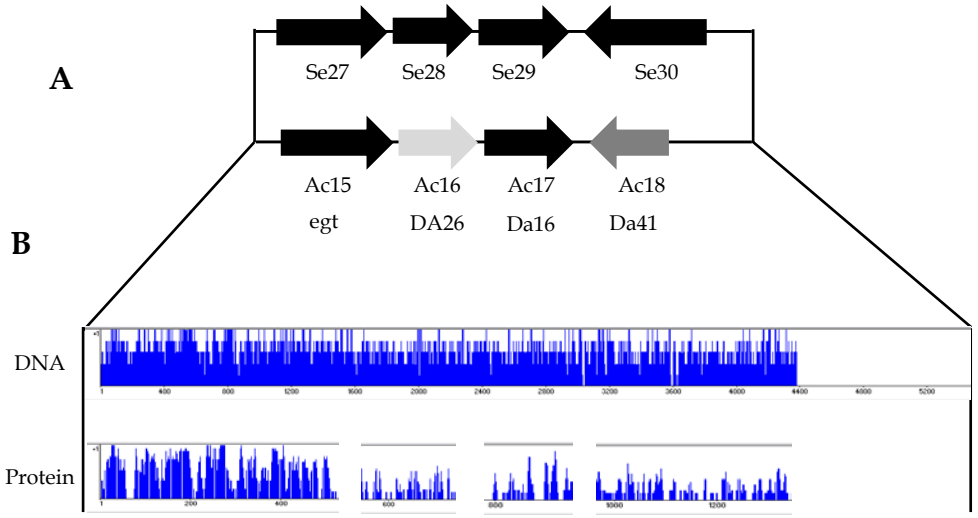


Figure 8. (**A**) Overview of the recombinant bacmid SeBac72GFPSe28, where Se28 expression is driven under its own promoter. (**B**) Se301 insect cells transfected with SeBac10GFP, SeBac72GFP, and SeBac72Se28. Bright field (upper row) and fluorescence field (lower row). Pictures were taken 6 days post-transfection.

***Se28* is located in a hypervariable region that is conserved at the DNA level in group I and II NPVs.** To understand in more detail the surroundings of *se28* in the viral genome, we compared the hypervariable region of SeMNPV with a previously described hypervariable region of AcMNPV (O'Reilly *et al.*, 1990) (Fig. 9A), using a pairwise sequence alignment. DNA sequence alignment between SeUS1 ORF 27 to 30 (26928 to 32669 nt) and AcMNPV ORF 15 to 18 (11426 to 15459 nt) showed an overall 43% identity at nucleotide level with a minimal number of large gaps (Fig. 9B). This suggests that these genomic regions are collinear and are quite conserved. Remarkably, however, only two of the four predicted ORFs in this region are conserved at the amino acid level: *egt* (ac15/se27) and DA16 (ac17/se29). In contrast, *se28* is not a protein homologue of *ac16* (DA26), yet these genes are found at analogous positions in SeMNPV and AcMNPV genomes, and share significant homology at the DNA level (43.7%). The baculovirus bayesian phylogeny based on a *lef-8* gene showed that SE28 is only present in the group II *Alphabaculovirus*, and DA26 is only present in the group I *Alphabaculovirus* (Fig. 9C). In conclusion, the DNA sequence/topology in the *se28* region is likely more important for blocking viral spread than the encoded proteins.



C

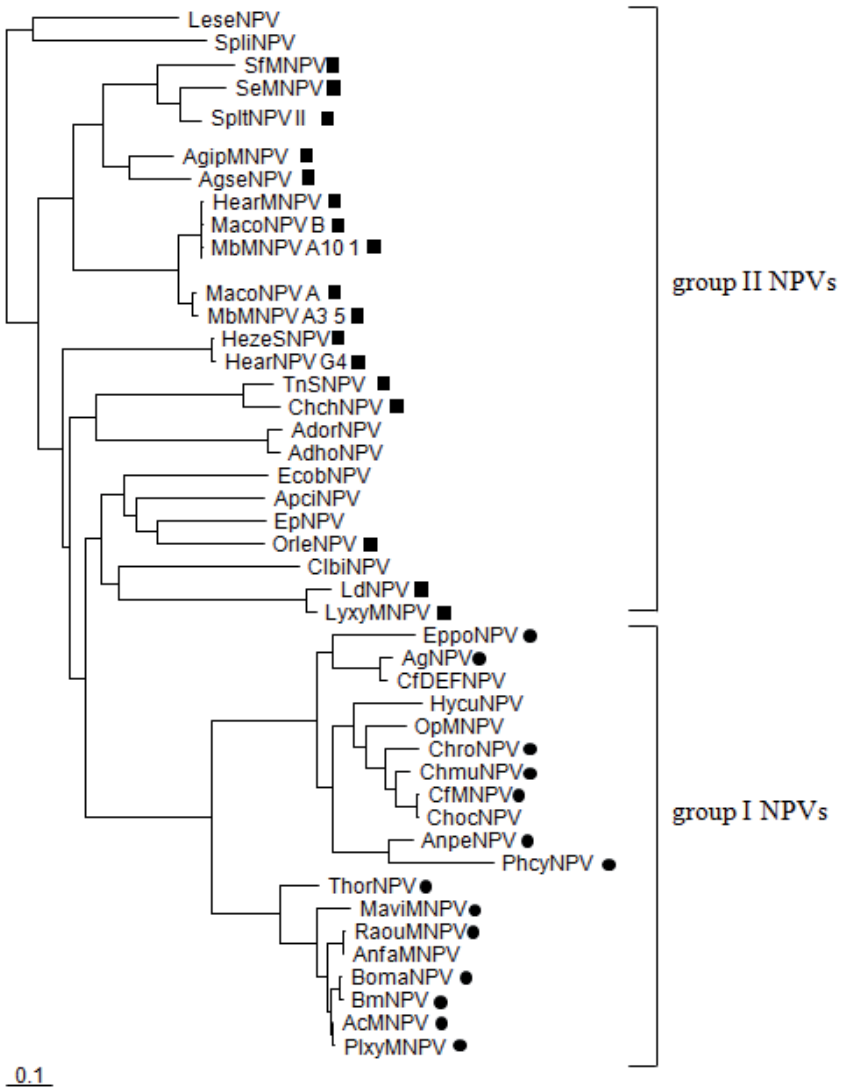


Figure 9. (**A**) Schematic representation of the hypervariable region of SeMNPV (26928 to 32669 nt) and AcMNPV (11426 to 15459 nt). (**B**) DNA and protein alignment of the hypervariable regions of AcMNPV and SeMNPV. (**C**) Bayesian phylogeny of baculovirus based on *lef-8* gene. The bar at the bottom indicates a branch length of 10% distance. Baculoviruses having a *se28* homologue are marked by a black square, while the black dots mark the homologues of *da26*.

Discussion

The SeMNPV natural isolate SeUS1 contains genotypic variants with interesting differences in phenotype. vSeBac72, a bacmid clone of a natural deleted genotype from the SeUS1 isolate, was found to display a much more efficient viral spread in cell culture than vSeBac10, a bacmid containing the complete SeMNPV genome (Pijlman *et al.*, 2002). After 454 high-throughput sequencing of SeBac72, a deletion of 9.5 kb was mapped, which disrupted open reading frames (ORFs) 16 to 28. After sequential knockouts of different ORFs from this region using SeBac10 as backbone, *se28* was identified as the responsible gene that prevents viral replication and spread of the complete, genome size SeMNPV in cell culture. A common feature of baculoviruses is that serial passage in cell culture leads to creation of variants with large deletions, which sometimes act as defective interfering particles (Kool *et al.*, 1991; Lee & Krell, 1994). Heldens *et al*. (1996) reported that in the SeUS1 isolate, however, a deletion of about 25 kbp of the viral genome is rapidly selected within the first passage to enable efficient virus replication in cell culture. Subsequently, Dai *et al*. (2000) selected a natural SeUS1 genotype with an ORF16-28 deletion but with retained bioactivity and cell culture replication. Since we now have found that deletion of *se28* is sufficient and necessary for the replication and spread of SeMNPV in cell culture, it seems likely that *se28* plays an instrumental role in the selection of mutants with large deletions.

Baculoviruses, particularly group II NPVs, can be genetically heterogeneous and often comprise a mixture of different genotypes. The SeMNPV-US2 isolate consists of a mixture of several distinct genotypes (Muñoz *et al.*, 1998). One of those genotypes, SeUS2-C, had a deletion from ORF17 to 39, in the same region of the genome as the deletion in SeBac72. The SeUS2-C genotype was found to have a replicative advantage in cell culture, compared to the complete genotype (Serrano *et al.*, 2013), which is in agreement with the enhanced spread of viral replication of SeBac72. A Nicaraguan isolate of a baculovirus closely related to SeMNPV, *Spodoptera frugiperda*

MNPV (SfMNPV), is composed of nine different genotypes and has a similar population structure as SeMNPV-US2 (Simón *et al.*, 2004a). The genotype SfNIC-C with a deletion of 15kb from ORF 20 to 36, was the most prevalent genotype isolated in cell culture (Simón *et al.*, 2004a). All those deleted genotypes, SeBac72, SeUS2-C and SfNIC-C, appear to have a strong replicative advantage in cell culture as compared to the complete genotype. It has been hypothesized that faster DNA replication, favouring shorter genomes, might be an explanation for the observed replicative advantage of the deleted genotypes (Frank, 2000). However, the single gene knockouts of *gp37*, *ptp-2* and *egt* did not recover the efficient viral spread of SeBac72, whereas the knockout of only *Se28* did. Therefore, it seems unlikely that shorter genomes are the main driving force for a replicative advantage of these deletion mutants in cell culture.

Interestingly, the genetic heterogeneity in the natural genotypes of SeMNPV and SfMNPV map to the same genomic region, showing that this region is intrinsically hypervariable and may play an important role in the generation of genotypes with different biological activity (Muñoz *et al.*, 1998; Simón *et al.*, 2004a). It has been hypothesized that deletion genotypes have arisen by selection due to important roles in survival and/or transmissibility of the virus (Serrano *et al.*, 2013). Cell sloughing, the mechanism in which necrotic epithelial cells from the larvae midgut are discarded, has been described as a key step in the resistance of insects to fatal infection (Hoover *et al.*, 2000). A deleted genotype that can spread the virus a lot faster, may be able to avoid the cell sloughing, but further experimentation is necessary to test this hypothesis. The increased speed of kill of SeXD1, a natural deleted SeMNPV genotype would be in agreement with this model (Dai *et al.*, 2000). When *se28* is present in the viral genome, it efficiently blocks the spread of SeMNPV in cell culture. This leads to wonder why a baculovirus would keep a gene that negatively affects the transmission of the virus. Mathematical models predict that highly virulent parasites will rapidly become extinct following invasion of

a host population due to the disappearance of susceptible hosts (Swinton *et al.*, 1998). This usually does not happen because pathogens have evolved a number of mechanism that ensure persistence like life stages that facilitate long-term survival in the environment (Vilaplana *et al.*, 2010). The presence of a complete genotype that replicates slower might lead to prolonged survival of the host, which in turn may be beneficial for viral transmission. Parasites rely on the host survival for persistence, and it has been suggested that parasites can prevent super infection by more virulent pathogens to prevent killing the host (Vilaplana *et al.*, 2010). It has been observed that upon co-infection of a wild-type (wt) isolate of SeMNPV and a deleted genotype, persistence of the deleted genotype in the viral progeny was observed, and this persistence decreased the pathogenicity of the wt isolate (Muñoz & Caballero, 2000). *Se28* appears to block viral spread, at least in cultured cells, and thus may decrease the overall pathogenicity of the virus *in vivo*, to favour persistence of baculovirus infection in the insect.

In contrast to what we initially expected, *se28* silencing via RNAi did not rescue genome size SeBac10 replication, suggesting that Se28 transcripts or SE28 protein were not involved in blocking viral spread. When *se28* was placed back in SeBac72 ("negative rescue"), the resulting virus could still efficiently replicate and spread in cell culture. Thus, the block in viral spread was not recovered when *se28* was expressed from the polyhedrin locus. When *se28* was placed in *Autographa californica* MNPV (AcMNPV) bacmid, an heterologous virus belonging to the group I of the *Alphabaculovirus* genus, AcBacSe28 could still replicate and spread like the control virus, supporting the view that *se28* is not able to block viral replication or spread of an heterologous virus via transcription or protein products. Together, these results indicate that the regulatory function of *se28* on viral spread is likely dependent on its locus and topology instead of its products (i.e. transcripts, protein).

Sequence alignment of the hypervariable region of SeMNPV with a previously described hypervariable region of AcMNPV (O'Reilly *et al.*, 1990) showed that these

genomic regions are collinear and quite conserved. In this context it is interesting to note that the genomic region of *se28* among *Alphabaculoviruses* is homologous at the DNA level (Fig. 9) but not on the protein level, and that both *se28* in group II and its topological homologue *DA26* in group I *Alphabaculoviruses* are associated with genomic instability. Interestingly, deletion of BmNPV *Bm8*, a homologue of AcMNPV *Da26*, produced an enhanced virus replication and accelerated the speed of kill in insect larvae (Katsuma *et al*., 2012), in agreement to the faster speed of kill of the SeXD-1 genotype (Dai *et al*. , 2000).

We are now further investigating the role of the *se28* sequence in viral spread by mutational analysis of the *se28* region, without affecting the *se28* gene function. Our findings have not only increased our understanding of the role of genotypic variants in baculovirus infection, but they may also be important to establish large-scale bioreactor production of baculoviruses with retained bioactivity *in vivo*.

Acknowledgements

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Supplementary information

Table 1. Name and sequence of the primers used in this study.

Primer	Sequence	Amplification purpose (flanking region)
SeORF16-KO-F	CAACGCCCCAACAACGATCACGG-CAGTGTCGTCGAAATAATATAGTT-GAAGCTCGGATCCACTAGTAACG	Se16-19 deletion from SeMNPV bacmid; forward primer
SeORF19-KO-R	AATAAATGCAATGTATAAA-TAATGATTTTTTTTATTATTATTTA-TAACCTCCTCTAGATGCATGCTCG	Se16-19 deletion from SeM-NPV bacmid; reverse primer
SeORF20-KO-F	TAAAAAAAATCATTATTTATACATT-GCATTTATTTCAAATAAAACATCTT-GCTCGGATCCACTAGTAACG	Se20-24 deletion from SeMNPV bacmid; forward primer
SeORF24-KO-R	CTTATAATGGGTAAAAAAAAAA-ACAAAATTTTATTTAATCAAA-ACTTTATTCCTCTAGATGCATGCTCG	Se20-24 deletion from SeM-NPV bacmid; reverse primer
SeORF25-KO-F	TAAATAAAATTTTGTTTTTTTTTTACC-CATTATAAGTTTAAACACATAAA-GCTCGGATCCACTAGTAACG	Se25-28 deletion from SeMNPV bacmid; forward primer
SeORF28-KO-R	AATTCAAACATAATGAGAATGTGT-GTGTATGTTAAAAGCGTGGTTTCCA-ACCTCTAGATGCATGCTCG	Se25-28 deletion from SeM-NPV bacmid; reverse primer
Segp37-KO-F	TAAATAAAATTTTGTTTTTTTTTTACC-CATTATAAGTTTAAACACATAAA-GCTCGGATCCACTAGTAACG	Segp37 deletion from SeMNPV bacmid; forward primer
Segp37-KO-R	TCAATGATTGTTTCGTCGGC-GACGTAAACTATAATCATCAAACTC-TACGTCCTCTAGATGCATGCTCG	Segp37 deletion from SeM-NPV bacmid; reverse primer
Septp2-KO-F	TCAAAATTCCATTTGTGATTCGGC-CATTTGCAGTTGCCGCAAAAACGA-ATGCTCGGATCCACTAGTAACG	Septp2 deletion from SeMNPV bacmid; forward primer
Septp2-KO-R	ATGCATACTAACGACGACA-ACTTTACGCAACTGTCCAACGGAA-ACTGAGTCCTCTAGATGCATGCTCG	Septp2 deletion from SeM-NPV bacmid; reverse primer
Seegt-KO-F	ATGAACGGTTGCGCTGTCC-TAATTTTATTTTTTGCACTGACCACGT-GATCGCTCGGATCCACTAGTAACG	Seegt deletion from SeMNPV bacmid; forward primer
Seegt-KO-R	TCACACTAAATTAATTCTCAGTAATT-GACGCAGATGGTTCATTACAGT-GACCTCTAGATGCATGCTCG	Seegt deletion from SeMNPV bacmid; reverse primer
SeORF28-KO-F	ATGGCCACGATCAGAAATAAAA-GCTTGTTGCGCAGTCTCGAACACT-GACGGCTCGGATCCACTAGTAACG	Se28 deletion from SeMNPV bacmid; forward primer
SeORF28-KO-R	AATTCAAACATAATGAGAATGTGT-GTGTATGTTAAAAGCGTGGTTTCCA-ACCTCTAGATGCATGCTCG	Se28 deletion from SeMNPV bacmid; reverse primer
M13-F	CCCAGTCACGACGTTGTAAAACG	Check transposition in attTn7 site. Forward primer
M13-R	AGCGGATAACAATTTCACACAGG	Check transposition in attTn7 site. Reverse primer
Genta-R	AGCCACCTACTCCCAACATC	Check transposition in attTn7 site
SeORF25-F-CON	CCAGCAATTGCAAATATCGC	Check construction single knockouts. Forward primer

SeORF28-R-CON	CTACTGCGCAAGTTCGACAT	Check construction single knockouts. Reverse primer

Chapter 6

General discussion

Baculoviruses are arthropod-specific viruses that affect more than 600 lepidopteran, dipteran and hymenopteran insects species (Herniou *et al.*, 2003). Baculoviruses play an important role controlling the size of insect populations, and have been widely applied as biocontrol agents against forest and agricultural insect pests due to their efficacy, high specifity and safety for non-target organisms (Moscardi, 1999). Over the years considerable knowledge has been generated in terms of baculovirus biology, pathology, ecology and genetics. Functional analysis of baculovirus genes and studies on genotypic variation are essential to understand the adaptation of baculovirus isolates towards optimal fitness and transmission. *Spodoptera exigua* MNPV has been long applied as biocontrol agent against larvae of the beet armyworm, *Spodoptera exigua* (Hübner) (Insecta: Lepidoptera: Noctuidae). This doctoral thesis aims to gain further insight in the insecticidal properties of SeMNPV by studying the function of different open reading frames (ORFs) involved in the pathology and transmission of SeMNPV, and by studying the impact of natural genotypic variation in SeMNPV isolates on the ecology of this virus.

SeMNPV genes involved in specific biological properties

The development of bacmid technology, first established for the baculovirus type-species *Autographa californica* MNPV (AcMNPV) to maintain a full-length viral genome as a bacterial artificial chromosome in *E. coli* (Luckow *et al.*, 1993), has enabled numerous studies on the function of specific viral genes by producing specific virus gene knockout viruses. The implementation of a similar strategy for other baculoviruses, e.g. SeMNPV, HaSNPV (Pijlman *et al.*, 2002; Wang *et al.*, 2003), has allowed also studies on the function of baculovirus species-specific genes that are not present in AcMNPV. At the same time the decrease in costs for Sanger sequencing and the concurrent development of next generation sequencing techniques has increased the availability of whole genome sequences of many baculoviruses. This has illuminated detailed information on genetic variation within

and between baculovirus species (Rohrmann, 2013c) and isolates, and provides new information and options to improve the insecticidal properties of SeMNPV by genetic engineering. The genomes of seven different European SeMNPV isolates associated with different routes of transmission and different insecticidal properties, have recently been completely sequenced (Thézé *et al.*, 2014). In chapter 2, a bacmid-based recombination system of SeMNPV isolate VT-SeAL1 (SeBacAL1) was developed to study the potential role of several ORFs selected by Thezé *et al.* (2014) thought to be involved in specific insecticidal properties of SeMNPV. The selected ORFs were *se4, se5, se28, se76, se87* and *se129*. These ORFs do not belong to the 37 baculovirus common genes, known as core genes (Garavaglia *et al.*, 2012). The baculovirus core genes are believed to be involved in essential biological functions such as transcription, virion structure, binding to midgut cells or establishment of infection (Miele *et al.*, 2011). However, there is a much larger number of non-core genes, known as variable or auxiliary genes, that might be responsible for the considerable difference in biological properties of baculovirus species, like host range, virulence, ecological fitness and transmission (van Oers & Vlak, 2007).

Deletion of *se4*, *se5*, *se76* and *se129* clearly reduced the pathogenicity of the SeBacAL1 virus, with an increased lethal dose 50 (LD_{50}) compared with the wild-type (wt) bacmid SeBacAL1. Specifically, a *se5* knockout virus was 10 times less pathogenic than SeBacAL1 and showed a significant reduction in ODV titer in cell culture. It was hypothesized that the differences in infectious ODV titers could be due to differences in the number of ODV per OB, or due to differences in OB sizes. However, TEM and SEM pictures did not show any difference in the number of ODVs per OBs or occlusion bodies size, indicating that *se5* is not required for correct nucleocapsid packaging, ODV formation or OB assembly. Further experiments need to be done to elucidate the function of *se5* to check for a possible effect on the production of BV, such as BV titers and intercellular spread.

The presence of *se28* in the SeMNPV genome seemed to have a negative effect on

the virulence of the virus as compared to the natural SeMNPV genotype SeAL1. Virulence is described as the ability to kill a pest quickly (Cory & Franklin, 2012). The bacmid SeBacAL1 (wt) also killed larvae significantly faster than the natural genotype SeAL1. In the construction of SeBacAL1 a unique *MauB*I restriction site located in the intergenic region between *se27* (*egt*) and *se28* was used. The fastest killing isolate sequenced by Thezé *et al.* (2014), SeOx4, had a frame shift change with a deletion of 4 bp in the *se28* coding sequence and a truncation of 42 aa at the C-terminus of the 190 aa-long authentic protein, also as a result of the frameshift. In fact *se28* was annihilated. In our experiments disruption of the *se27-se28* intergenic region in SeMPNV reduced the time to death (Chapter2). In SeBacAL1 the introduction of the bacmid cloning vector in that specific locus already appeared to affect the speed of kill, which might explain why subsequent deletion of *se28* did not have additional effect on the virulence. The current thinking is that the intergenic region in the SeMNPV genome between *se27* and *se28* is somehow related to virulence.

Transmission of SeMNPV in *S. exigua*

The seven European SeMNPV isolates sequenced by Thezé *et al.* (2014) were shown to be associated in two distinct routes of transmission (Cabodevilla *et al*., 2011b). First, baculoviruses can be transmitted horizontally, from insect to insect, when a susceptible caterpillar ingest an OB available in the environment (Cory & Myers, 2003). Second, they can also be transmitted vertically, from parents to off-spring. Vertical transmission is important when the host density is low and largely dependent on sublethal infection. Baculovirus sublethal infections appear to be common in natural populations of lepidopteran insects and seem to play an important role in virus-host dynamics (Cory & Myers, 2003). Sublethal infections may benefit pest control programs, because insect that do not die due to viral infection have a reduced probability of reproduction and the possibility to transmit the virus to the offspring, which are then more likely to succumb to baculovirus disease following

OBs application than healthy larvae (Cabodevilla *et al.*, 2011b). It has been previously demonstrated that genotypes associated with vertical transmission of the virus have a higher ability of producing sublethal infections than the genotypes associated with horizontal transmission (Cabodevilla *et al.*, 2011a). The three SeMNPV genotypes associated with vertical transmission routes were VT-SeAL1, VT-SeAL2 and VT-SeOx4, and the four genotypes associated with horizontal transmission route were HT-SeG24, HT-SeG25, HT-SeG26 and HT-SeSP2A. Cabodevilla *et al.*, (2011a) found that 100% of larvae subletally-infected with genotypes VT-SeAL1 and VT-SeAL2 developed a persistent viral infection in adults, however, only 15% of larvae subletally-infected with HT-SeG25 showed a persistent viral infection. They hypothesized that this could be due to specialization of the different genotypes in the different transmission routes.

Upon comparison of the whole genome sequences, the vertically transmitted isolates were found to share the same mutations in three genes that were different from those of horizontally transmitted isolates. Those genes were *se5* (unique, unknown function), *se96* (*ac150*-homolog) and *se99* (*ac134*-homolog). In Chapter 3 these three ORFs were deleted one-by-one from SeBacAL1, a bacmid constructed from the vertically transmitted isolate VT-SeAL1. However, single deletion of each ORF did not show any effect on the persistence of sublethal infections in adults, as evidenced from the level of virus in adult abdomens. The (molecular) mechanisms by which the SeAL1 virus persists in the cells of sublethally infected insects still remains enigmatic. The virus might be either present at a low-level infection in reproductive tissue or as an episome in insect cell nuclei. The latter is also the case with herpes viruses and explains the latency phenomenon there. In case the genetic factors that modulate vertical transmission of baculovirus are identified and their mode of action is understood, it may provide new leads to improve the trans-generational biological control of *S. exigua*.

SeMNPV genotypic variability is optimized for maximum likelihood of transmission

The development of the bacmid technology has also allowed the cloning of the different genotypes present in a natural baculovirus isolate, including those that cannot be readily isolated/purified in cell culture. Baculoviruses are highly heterogeneous, usually composed of different genotypic variants within a single NPV field isolate (Lee & Miller, 1978; Muñoz *et al.*, 1998; Simón *et al.*, 2004a). The existence of genotypic variants within an isolate is usually indicated by the presence of submolar bands in restriction endonuclease digestion analysis of viral DNA and those genotypic variants can have different modifications such as deletions, insertions, or duplications in the viral genome (Muñoz *et al.*, 1998). The significance of the maintenance of such genotypic variability on fitness and transmission of the virus at the population level is not yet clear. In Chapter 4 a Florida (US) isolate of SeMNPV (SeUS2) was used to compare for its genotypic structure with that of a Nicaraguan isolate of *S. frugiperda* MNPV (SfNIC), a different group II *Alphabaculovirus* but with similar genotypic structure, high colinearity and DNA sequence homology. It was attempted to determine the origin of such similar genotypic structures. Comparison of the flanking sequences around the breakpoints of the genotypes led to the conclusion that the deleted variants had been generated independently, and subsequently selected for, presumably due to their important roles in the survival and/or transmissibility of the viral population as a whole.

The existence of genotypic variants have been reported in both group I and group II alphabaculoviruses (Lee & Miller, 1978; Muñoz *et al.*, 1998; Simón *et al.*, 2004a). This might indicate that this is a widespread phenomenon with an important role in the transmissibility or the fitness of the baculovirus populations. In Chapter 4 the deleted genotype SeUS2-C was found to be present in a proportion of ~25% in the wild-type population. Interestingly, SeUS2-C seemed to have a strong replicative advantage in cell culture, as indicated by the higher number of plaques obtained with SeUS2-C genotype (81%) than with the complete genotype SeUS2-A (19%). A

similar effect was observed in SfNIC, where the deleted genotype SfNIC-C was the genotype isolated in a higher prevalence in cell culture, although the proportion of SfNIC-C in the wild type virus population is only 22% (López-Ferber *et al.*, 2003).

The differences observed between the prevalence of deleted genotypes *in vitro* and *in vivo* might be due to favored replication in specific tissues. However, the tissue of origin of most insect cell lines is unknown. It has been previously hypothesized that the replicative advantage of the deleted genotypes in cell culture is its faster replication due to a smaller genome, but other factors may also play a role. In SeMNPV the single gene knockouts of *gp37*, *ptp-2* and *egt* did not recover the efficient viral spread of SeBac72, whereas the single knockout of *se28*, a very short ORF with unknown function, did. Therefore, it seems a good possibility that shorter genomes are not the major driving force for a replicative advantage of these deletion genotypes in cell culture, but rather an intergenic region (see below).

Although SeUS2-C displayed a replicative advantage in cell culture, its presence in a high proportion in co-occluded OBs with the complete genotype SeUS2-A negatively affected the pathogenicity of the virus in larvae. The pathogenicity of OBs with a high ratio of deleted genotype SeUS2-C (50%) was severely compromised, with a 10 times lower relative potency as compared with the the wild type SeMNPV isolate (Chapter 4). The SeUS2-C genotype lacks two *per os infection factor* (*pif*) genes, which are required for viral entry into the midgut epithelial cells (Kikhno *et al.*, 2002; Peng, 2012; Pijlman *et al.*, 2003a), which likely explains the decrease in pathogenicity observed. It was impossible to produce OBs with proportions of 75 or 90% of SeUS2-C indicating that selection takes place *in vivo* rather quickly, possibly at the midgut level. In support of this assumption, the SeUS2-C genotype can perfectly replicate after injection in larvae and produces BV that spread the infection from cell to cell. López-Ferber *et al.* (2003) were able to produce co-occluded OBs involving 75% and 90% proportion of the deleted genotype SfNIC-C, a genotype that also lacks the *pif* genes. So, the lack of *pif* genes does not fully explain why it was not possible to

produce OBs with a high proportion (90%) of SeUS2-C genotype co-occluded. This suggests that other selection mechanism(s) may play a role. The reason why it was not possible to achieve co-occluded OBs with high percentage of deleted genotype SeUS2-C still remains unclear. Although the deletion of SeUS2-C and SfNIC-C affects similar ORFs, it seems that the deleted genotypes behave in different ways, depending on the baculovirus species.

Co-infection experiments with SeUS2 and another natural SeMNPV isolate from Spain, SeSP2, also support the importance of deletion genotypes (Muñoz & Caballero, 2000). SeUS2-C invaded and established in the SeSP2 population and became as abundant in the progeny OBs as in their original population after only four serial passages in *S. exigua* larvae (Muñoz & Caballero, 2000), remaining stable at low concentration. When mixed infections at various proportions of OBs of complete and deleted genotypes of SfNIC were subjected to serial passage in insects, the genotype population rapidly converged to a common stable proportion that reflected the natural proportion of each genotype, as is found in wild type isolates. This suggests that the wild type virus population is carefully structured to increase the maximum likelihood of transmission (Simón *et al.*, 2006). An increase of the frequencies of the deleted genotypes was certainly not observed. If the main advantage of the deleted genotypes would have been their faster replication, higher frequencies of the deleted genotypes in the wild-type viral population would be logically expected (Clavijo *et al.*, 2009; Simón *et al.*, 2006). In conclusion, the deleted genotypes appear to have an important role in the transmission of the virus, explaining why they are maintained in the viral populations *in vivo*.

se28 is involved in establishing genotypic variability

In the SeUS2 isolates genomic alterations of the different genotypes all mapped to the same genomic region, designated as a hypervariable region. This hypervariable region maps between nt 16437 and 39757 (Muñoz *et al.*, 1998; Serrano *et al.*, 2013),

affecting genes such as *chitinase*, *cathepsin*, *ptp-2*, *egt* and some genes of unknown function.

The SeMNPV-US1 (SeUS1) isolate has been isolated from California (US) and is also composed of several genotypic variants (Dai *et al.*, 2000). After direct cloning of SeUS1 DNA as a bacmid (Pijlman *et al.*, 2002) it was surprisingly found that the full length genotype SeBac10 could not replicate in cell culture. In order to replicate in cell culture, the SeUS1 isolate first needed to generate large deletions, with sizes up to ~25 Kb in the hypervariable region of SeMNPV genome (Heldens *et al.*, 1996). In sharp contrast, a bacmid derived from another natural genotype of the SeUS1 isolate, the SeBac72, was found to have a much higher replication and virus spread in cell culture. In Chapter 5, it was found that SeBac72 had a deletion of 9.5 kb affecting ORFs 16 to 28. Subsequently, after elimination of the other ORFs *se28* was identified as the gene responsible for the prevention of successful viral replication and spread in cell culture. Deletion of *se28* was found to be both sufficient and essential for the replication and spread of SeMNPV in cell culture. Thus it can be hypothesized that *se28* is largely responsible for the positive selection of natural deleted genotypes, but this should be studied in greater detail in *in vivo* experiments.

Genomic organization of hypervariable regions is conserved among group I and II alphabaculoviruses.

Homologues of *se28* are present in the group II *Alphabaculovirus*, but are not present in group I *Alphabaculovirus*, the group to which AcMNPV belongs (Herniou *et al.*, 2011). However, after DNA sequence alignment of the region encompassing ORFs 27 to 30 of SeMNPV, and ORFs 15 to 18 of AcMNPV, a 43% sequence identity at the DNA level was found in between hypervariable regions previously described for both viruses (O´Reilly *et al.*, 1990). Thus, this region appears to be much better conserved among baculoviruses than previously thought. In *Mamestra configurata* NPV-A, ORF 39 is *egt*, ORF 40 is a homologue of *Se28*, and ORF 41 is a homologue of

AcORF 17, *da16* (Li *et al*., 2002). In *Bombyx mori* NPV ORF 7 is *egt*, ORF 8 is *Da26*, and ORF 9 is homologue of AcORF17, *da16* (Gomi *et al*., 1999). AcMNPV had also been found to create deletions in a hypervariable region of the genome after serial passage in cell culture (Kumar & Miller, 1987), affecting the genes *egt* and *da26*. Deletion of AcMNPV *da26*, which aligns with Se28 at the DNA but not the protein level, does not affect viral growth in cell culture but increases the infectivity of the virus in insects. It has been hypothesized that *da26* is required for infection of specific hosts or tissues (O´Reilly *et al*., 1990).

Deletion of BmNPV *Bm8*, a homologue of *da26*, produced an enhanced virus replication and OB production in the middle silk gland of infected larvae (Katsuma *et al*., 2012). *Bm8* was identified to determine tissue tropism in BmNPV infection in lepidopteran larvae. Katsuma *et al*. (2012) also found that deletion of *Bm8* accelerated the speed of kill in insect larvae, in agreement to what was observed by Dai *et al*. (2000), where the SeXD-1 genotype was found to kill larvae faster. Deletion of *se28* in SeMNPV appears to increase the budded virus (BV) production in cell culture (Chapter 3). An earlier BV production might affect the rate of spread of infection, therefore, increasing the speed of kill. *Se28* appears to be a regulator of virulence in SeMNPV (Chapter 3), a role that was supported by the experiments conducted with SeAL1 genotype. Via the construction of the SeBacAL1 bacmid and its biological testing it was found that the insertion of the bacmid cloning vector in the intergenic region in between intact ORF *se27* and *se28* significantly increased the speed of kill, compared to the wild type isolate SeAL1.

Previous studies showed that deletion of *egt* is responsible for a fast-killing isolate of *S. frugiperda* MNPV (Harrison *et al*., 2008). However, two *egt* deletion mutants of BmNPV did not accelerate the speed to kill in *B. mori* larvae (Katsuma *et al*., 2012), but the deletion of *Bm8* did. Due to the fact that genome organization of the hypervariable regions of SeMNPV and AcMNPV is conserved among baculoviruses, these regions seem to play an important role on tissue tropism infection or an important role in

the viral infection. What remains unexplained at this stage is how a genomic region conserved between group I and II NPV at the nucleotide level can regulate similar phenotypic traits, while the proteins encoded by this region (group I NPVs: DA26, group II NPVs: SE28) share no or very low amino acid homology.

Se28 genomic region as a conserved non-protein-coding genetic element?

Certain process affecting DNA functions such as replication and transcription seem to be related to precisely located non-coding genomics regions that specifically interact with the proteins involved in this process. Recently a conserved non-protein-coding genomic element (CNE) that plays an essential role in baculovirus pathogenesis has been identified (Kikhno, 2014). This element is a sequence of 154-157 bp in length conserved in all the *Alphabaculovirus* genomes sequenced to date and its deletion from an AcMNPV bacmid disables the ability to generate infectious virus particles capable of spreading the infection (Kikhno, 2014). It could be that the genomic region involving *se28*, or homologue genomic region in other alphabaculoviruses, is also a conserved non-protein-coding genomic element that plays a role in the virulence and transmissibility of the *Alphabaculovirus*.

Concluding remarks

This thesis describes the construction a new SeMNPV bacmid SeBacAL1 that allows the functional studies of several ORFs, which led to the identification of *se5* as having an important role on the pathogenicity of the SeMNPV. The study of the genotypic diversity of SeMNPV and SfMNPV isolates has demonstrated that such similar genotypic diversity has been generated independently and is apparently structured to maximize the likelihood of transmission. Finally, the study of a deleted genotype from SeMNPV has allowed the identification of the genomic region of *se28* as the driving force for the selection of deletion genotypes, with an important role in the

virulence and transmission of SeMNPV.

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Summary

Spodoptera exigua MNPV (SeMNPV) only infects larvae of the beet armyworm, *Spodoptera exigua.* SeMNPV natural isolates are comprised of mixtures of related DNA genotypes that are thought to be important for fitness and survival. Amplification of SeMNPV in cultured cells leads to rapid loss of virus infectivity *in vivo* associated with preferential selection of genotypes with large deletions. This thesis focuses on the biological characterization of the baculovirus *Spodoptera exigua* MNPV to gain insight specifically in the role of genotypic diversity and of individual viral genes in pathogenicity, virulence and transmission. Rapid improvements in DNA sequencing technologies and reduced costs have facilitated the identification of virus genes that may play an important role in the SeMNPV infectivity process. In a previous study the sequencing and comparison of seven different SeMNPV isolates with different insecticidal properties, such as virulence and pathogenicity, led to the identification of several ORFs as candidate genes that might be involved in these insecticidal traits: *se4*, *se5*, *se28*, *se76*, *se87* and *se129* (Thézé *et al*. 2014). In Chapter 2 a bacmid-based recombination system was developed using the SeMNPV isolate VT-SeAL1 to delete these ORFs individually previously identified. Deletion of *se4*, *se5*, *se76* and *se129* decreased the pathogenicity of the virus compared to virus derived from the wild-type (wt) bacmid, SeBacAL1. Deletion of *se87* did not change the pathogenicity, whereas deletion of *se28* slightly increased the pathogenicity. As for the speed of kill or virulence, deletion of *se4*, *se28*, *se76, se87* and *se129* did not dramatically change the virulence, although deletion of *se5* delayed the speed of kill with about seven hours. Interestingly, the wild-type virus SeAL1 was also slightly less virulent than the virus derived from SeBacAL1, which may relate to the insertion of the bacmid cloning segment into the SeAL1 genotype in the intergenic region between *se27* and *se28*. Overall, deletion of *se5* displayed the most dramatic effect on the SeMNPV insecticidal properties studied, with a decrease in pathogenicity of almost 10-fold and a delay in time to death of seven hours, as compared to the bacmid SeBacAL1-derived virus. A previous study on the ecology of SeMNPV revealed

that a genotype designated as VT-SeAL1 was found to produce 100% of sublethal infections in adults that survived an OB treatment in the larval stage. Meanwhile, another genotype designated as HT-SeG25 only produced sublethal infections in 16% of the cases. This led to the hypothesis that some SeMNPV genotypes can be associated with a vertical transmission route of the virus (via eggs), whereas others are associated with horizontal transmission of the virus (from larva to larva). By comparison of of the DNA of horizontally transmitted isolates and vertically transmitted isolates, Thézé *et al.* (2014) identified three ORFs that may be involved in the vertical transmission of the virus: *se5*, *se96* and *se99*. In Chapter 3 the bacmid-based recombination system developed in Chapter 2 was used to delete the respective ORFs from the SeMNPV genome and test the consequences for vertical transmission. *S. exigua* larvae were sublethally infected with an LC_{40} and sublethal infections of SeMNPV were detected in adult moths by quantitative-PCR (Q-PCR). This analysis did not detect significantly differences in the number of moths sublethally infected with any of the viruses, which may suggest that they are not involved in vertical transmission at all or only in concert with other ORFs. Different isolates of SeMNPV and a related baculovirus, SfMNPV, have similar genetic population structures. They are characterized by the presence of different genotypes with variable deletions in the same genomic region, roughly from *se12* to *se40* in the SeMNPV and from *sf20* to *sf36* in SfMNPV. These regions encode several important genes such as *cathepsin*, *chitinase*, *gp37*, *ptp2*, *egt*, *pkip*, *arif1*, *pif1*, *pif2* and *fgf*, as well as some open reading frames of unknown function. In Chapter 4 the genotypic structure of SeUS2, SeUS1, and SfNIC isolates is compared to determine the evolutionary and ecological modes of action of these genotypically similar population structures. Sequence alignment of the deletion flanking sequences pointed to an independent evolutionary mechanism that generates and maintains the genotypes in the respective virus. The isolation of a deleted genotype and functional complementation studies with different mixtures of a deleted and a complete genotype, clearly demonstrates the interplay between

genotypes in a natural viral population.

The deleted genotype SeUS2-C appeared to have a replicative advantage in cell culture, since it was present in a 81% of plaques isolated in cell culture after inoculation with hemolymph from SeUS2-WT infected larvae. However, the full-length genotype SeUS2-A was only found to be present in a 19% of plaques. The presence of genotype SeUS2-C in a high concentration in OBs co-occluded with the full length genotype SeUS2-A, severely compromised the pathogenicity of the virus. Interestingly, the prevalence of the SeUS2-C genotype was only about 25% in the wt isolate SeUS2, as compared to the 81% of plaques isolated in cell culture.

It is hypothesized that deleted genotypes have arisen due to their important role(s) in the transmissibility of the virus and that the genotypic diversity in a virus is structured to maximize the likelihood of transmission. The SeMNPV-US1 isolate is also composed of several genotypes, most of which carry deletions of variable size in the *se15-se41* region. In Chapter 5, bacmids containing the complete SeMNPV genome (SeBac10) and a natural genotypic variant with a deletion of about 9.5 kb (SeBac72) and the respective viruses were generated. Virus derived from SeBac72 displayed a much more efficient viral spread in *S. exigua* cells as compared to virus derived from SeBac10 in line with what was found in Chapter 4. Sequencing of SeBac72 led to the observation that genes *se16-se28* were affected by this deletion. By differential deletion of the individual ORFs from the full-length bacmid SeBac10, *se28* was identified as the gene responsible for preventing successful spread of SeMNPV in cell culture. Expression of *se28* in an *Autographa californica* MNPV background, a heterologous virus belonging to group I *Alphabaculovirus,* did not block the viral spread. Strikingly, RNAi silencing of *se28* in the full length genotype SeBac10 did not lead to virus spread in cell culture, suggesting that not the transcript or translated product, but rather the DNA sequence and/or the sequence topology of *se28* determines viral spread. Sequence analysis of the SeMNPV *se27-se30* region with AcMNPV *ac15-ac18* showed a 43% conservation at the DNA level, yet the amino

acid sequences of *se28* and *ac16* (DA26) are non-homologous. Both the *se28*/*ac16* genes are part of hypervariable regions associated with insertions/deletions in both viruses. Overall, the *Se28* region has been identify as a key regulator of viral spread in a region that may drive the genotypic variation in natural baculovirus isolates. In conclusion, the results presented in this thesis furthered our knowledge of genetic and genotypic diversity of SeMNPV, and baculoviruses more general, and may aid the improvement of baculovirus-based biological control strategies in the future.

Baculovirussen worden al vele jaren toegepast bij de biologische bestrijding van plaaginsecten, behorende tot de insectenorden *Lepidoptera*, *Diptera* en *Hymenoptera*, vanwege hun effectiviteit, hoge specificiteit en veiligheid voor niet-doelorganismen. Het kernpolyedervirus (baculovirus) van de floridamot *Spodoptera exigua* (*S. exigua* multicapsid nucleopolyhedrovirus = SeMNPV) infecteert uitsluitend insectenlarven (rupsen). Natuurlijke isolaten van SeMNPV bevatten verschillende, maar verwante DNA genotypen. Verondersteld wordt dat deze variatie belangrijk is voor vitaliteit ('fitness') en overleving van het virus in the natuur. Vermenigvuldiging van SeMNPV in gekweekte cellen leidt tot een snelle afname in infectiositeit van het virus *in vivo* en kenmerkt zich door een voorkeursselectie van genotypen die grote deleties bevatten. Dit proefschrift richt zich op de biologische karakterisering van het baculovirus SeMNPV, vooral om meer inzicht te verkrijgen in de functie van genotypische diversiteit en in de rol van individuele genen betrokken bij pathogenese (ziekteverwekkend vermogen), virulentie (aggressiviteit) en overdracht van dit virus.

De snelle technologische vooruitgang bij het ophelderen van DNA-basenvolgorden en de afnemende kosten hiervan hebben de identificatie van virale genen met een potentieel belangrijke rol in het infectieproces van SeMNPV vergemakkelijkt. In een voorafgaande studie (Thézé *et al*. 2014) werden zeven isolaten van SeMNPV geselecteerd op basis van verschillen in virulentie en pathogeniciteit. Van deze virussen zijn de volledige DNA-basenvolgorden bepaald en met elkaar vergeleken. Deze exercitie heeft geleid tot de identificatie van zes 'open reading frames' (ORFs = genen) die betrokken zouden kunnen zijn bij de verschillen in biologische activiteit. In Hoofdstuk 2 is een bacmide-recombinatiesysteem ontwikkeld voor het SeMNPV-isolaat VT-SeAL1 om de geïdentificeerde ORFs te kunnen verwijderen en het effect hiervan te kunnen bestuderen. Verwijdering van *se4*, *se5*, *se76* en *se129* leidde tot verminderde pathogeniciteit van het virus in vergelijking met het virus verkregen vanuit het wildtype bacmide SeBacAL1. Verwijdering van *se87* had geen effect,

terwijl verwijdering van *se28* in beperkte mate de pathogeniciteit verhoogde. Verwijdering van *se4*, *se28*, *se76, se87* en *se129* had geen invloed op de virulentie, echter verwijdering van *se5* vertraagde de 'speed of kill' met ongeveer zeven uur. Een interessante waarneming was dat het wildtype virus SeAL1 ook minder virulent was dan het virus afgeleid van SeBacAL1. Dit verschil zou het gevolg kunnen zijn van het invoegen van het bacmide-cloningssegment in SeAL1, in de intergene regio tussen *se27* en *se28*. Samengevat, de verwijdering van *se5* had het meest uitgesproken effect op de biologische eigenschappen van SeMNPV: een bijna tienvoudige afname in pathogeniciteit en een vertraagde 'speed of kill' van ongeveer zeven uur in vergelijking met het virus verkregen vanuit het bacmide SeBacAL1.

Een eerdere studie over de ecologie van SeMNPV liet zien dat een genotype, genaamd VT-SeAL1, in 100% van de gevallen sublethale infecties veroorzaakte in volwassen insecten die blootstelling aan polyeders, de verpakkingsmodule van baculovirussen, in het larvale stadium hadden overleefd. Echter, een ander genotype, genaamd HT-SeG25, leidde slechts tot sublethale infecties in 16% van de gevallen. De hypothese was dat sommige genotypes van SeMNPV geassocieerd zijn met verticale overdracht van het virus (via het ei), terwijl andere geassocieerd zijn met horizontale overdracht (van larve naar larve). Door het DNA van deze virusisolaten uit deze verschillende groepen met elkaar te vergelijken, zijn drie ORFs geselecteerd die betrokken zouden kunnen zijn bij verticale virusoverdracht: *se5*, *se96* en *se99* (Thézé *et al.*, 2014). In Hoofdstuk 3 is het bacmide-recombinatiesysteem (ontwikkeld in Hoofdstuk 2) gebruikt om deze ORFs uit het genoom van SeMNPV te verwijderen en het effect hiervan op de verticale virusoverdracht te bestuderen. Larven van *S. exigua* werden geïnfecteerd met een sublethale dosis (LC_{40}), waarna een eventuele sublethale infectie in volwassen insecten (motten) werd gedetecteerd met behulp van een kwantitatieve PCR. Deze experimenten lieten echter geen significante verschillen in aantallen sublethaal geïnfecteerde insecten zien tussen de diverse virussen. Dit kan erop wijzen dat de geselecteerde ORFs niet betrokken zijn

bij verticale virusoverdracht of alleen in combinatie met andere ORFs.

Verschillende isolaten van SeMNPV en een gerelateerd baculovirus, SfMNPV, hebben een overeenkomstige genetische populatiestructuur. Verschillende genotypen zijn hierin aanwezig die variabele deleties bevatten in dezelfde regio op het virale genoom, die loopt van ongeveer *se12* tot *se40* in het SeMNPV-genoom en van ongeveer *sf20* tot *sf36* in het SfMNPV-genoom. Deze regio's coderen voor een aantal belangrijke genen, zoals *cathepsin*, *chitinase*, *gp37*, *ptp2*, *egt*, *pkip*, *arif1*, *pif1*, *pif2* en *fgf*, als ook voor een aantal ORFs met een vooralsnog onbekende functie. In Hoofdstuk 4 is de genotypische populatiestructuur van de isolaten SeUS2, SeUS1 and SfNIC vergeleken, met als doel om de evolutionaire en ecologische functionaliteit van deze populatiestructuren te achterhalen. Een directe vergelijking van de basenvolgorde van de gebieden die de deleties begrenzen, duidde op een onafhankelijk evolutionair mechanisme dat betrokken is bij het tot stand komen en handhaven van de verschillende genotypen in de virusisolaten. De zuivering van genotypen die een deletie bevatten en de functionele complementatie-experimenten met verschillende mengsels van intacte genotypen of die met deleties, lieten zien dat er een wisselwerking bestaat tussen de diverse genotypen in een natuurlijke viruspopulatie. Een van de genotypen met een deletie, SeUS2-C, bleek een selectief voordeel te hebben bij vermeerdering in celkweek. SeUS2-C was aanwezig in 81% van alle plaque-gezuiverde isolaten die vanuit de hemolymph van SeUS2-WT geïnfecteerde larven waren gezuiverd. Het intacte genotype SeUS2-A was slechts aanwezig in 19% van de plaque-gezuiverde isolaten. Een relatief hoge concentratie van genotype SeUS2-C ten opzichte van SeUS2-A in polyeders verminderde de pathogeniciteit van het virus sterk. In het wildtype isolaat SeUS2 was het aandeel van SeUS2-C ongeveer 25%, terwijl het aandeel van SeUS2-C in plaque-gezuiverde isolaten 81% was. De hypothese is nu dat genotypen met deleties bestaan als gevolg van hun belangrijke rol in virusoverdracht en dat genotypische diversiteit zo is gestructureerd dat de kans op virusoverdracht zo groot mogelijk is.

Het SeMNPV-US1 isolaat bevat verschillende genotypen, waarvan de meesten deleties van verschillende grootte bevatten in de regio *se15-se41*. In Hoofdstuk 5 zijn bacmides en virussen gemaakt van het volledige SeMNPV-genoom (SeBac10) en een natuurlijke genotypische variant met een deletie van ongeveer 9.5 kilobasenparen (SeBac72). In overstemming met de bevindingen in Hoofdstuk 4, verspreidde virus, afgeleid van SeBac72, zich veel efficiënter in *S. exigua*-celkweek in vergelijking met virus afgeleid van SeBac10. Het ophelderen van de volledige basenvolgorde van SeBac72 liet zien dat de regio *se16-se28* ontbrak. Door deze ORFs individueel uit te schakelen in SeBac10, kon *se28* worden geïdentificeerd als het gen dat verantwoordelijk is voor het voorkomen van virusverspreiding in celkweek. Het tot expressie brengen van *se28* via *Autographa californica* MNPV (AcMNPV, een baculovirus behorend tot een ander baculovirus-taxon, groep I *Alphabaculovirus*) leidde echter niet tot het voorkomen van virusverspreiding. 'Gene silencing' van *se28* via RNAi leidde ook niet tot verspreiding van het virus, afgeleid van SeBac10, in celkweek. Deze resultaten suggereren dat niet het transcript of het eiwit afgeleid van *se28*, maar wellicht de DNA-basenvolgorde van *se28* bepalend is bij virusvermeerdering. Een vergelijking van de SeMNPV *se27-se30* regio met de AcMNPV *ac15-ac18* regio laat een conservering van 43% op DNA-niveau zien, terwijl de aminozuurvolgorden van *se28* en *ac16* (DA26) geen overeenkomst vertonen. Zowel *se28* als *ac16* zijn onderdeel van een hypervariabel deel in het genoom dat geassocieerd is met deleties en inserties in beide virussen. *Se28* is hier wel geïdentificeerd als een belangrijke regelaar van virusvermeerdering en heeft tevens een mogelijk rol bij het tot stand komen van genotypische variatie in natuurlijke baculovirusisolaten.

De resultaten uit dit proefschrift hebben onze kennis over de genetische en genotypische variatie van SeMNPV en baculovirussen over het algemeen vergroot. De verkregen inzichten zijn waardevol voor de verdere verbetering van biologische bestrijding van plaaginsecten met behulp van baculovirussen.

Los baculovirus se vienen utilizando desde hace tiempo como agentes de control biológico contra lepidópteros, dípteros e himenópteros debido a su eficacia, alta especificidad y seguridad frente a organismos que no son el objeto del tratamiento. El nuclepoliedrovirus múltiple de *Spodoptera exigua* (SeMNPV) tiene un estrecho rango de huésped y solo afecta a larvas de la rosquilla verde, *Spodoptera exigua*. Los aislados naturales del SeMNPV están compuestos de mezclas de genotipos que se creen que son importantes para la supervivencia del virus. La amplificación de SeMNPV en cultivo celular conduce a una rápida perdida de infectividad *in vivo* asociada con la selección preferente de genotipos con grandes delecciones. Esta tesis se centra en la caracterización biológica del baculovirus de *Spodoptera exigua* para mejorar nuestro conocimiento sobre el papel de la diversidad genotípica y sobre la implicación de genes individuales en patogenicidad, virulencia y transmisión.

Avances en la tecnología de secuenciación de ADN y los más bajos costes de la misma han facilitado la identificación de genes virales que pueden jugar un papel importante en el proceso de infectividad del SeMNPV. En un estudio previo la secuenciación y comparación de 7 genotipos diferentes del SeMNPV con diferentes propiedades insecticidas, como la virulencia y la patogenicidad, permitieron la identificación de algunos ORFs como genes candidatos que pueden tener un efecto en esas propiedades insecticidas: *se4*, *se5*, *se28*, *se76*, *se87* y *se129* (Thézé *et al*., 2014). En el Capitulo 2 se construyó un sistema de recombinación basado en bacmidos utilizando el genotipo VT-SeAL1 para deleccionar individualmente los ORFs previamente identificados. La delección de *se4*, *se5*, *se76* y *se129* disminuyó la patogenicidad del virus comparado con el virus derivado del bacmido al insertar aislado silvestre, SeBacAL1. La delección del *se87* no afectó la patogenicidad, mientras que la deleccion de *se28* aumento ligeramente la patogenicidad. La delección individual de los genes *se4*, *se28*, *se76, se87* y *se129* no afectó la virulencia del virus, aunque la delección de *se5* retrasó el tiempo de mortalidad alrededor de 7 horas. Interesantemente, el genotipo silvestre SeAL1 mostró ser ligeramente menos

virulento que el bacmido SeBacAL1, lo cual puede ser debido a la inserción del plásmido de clonaje en la región intergénica del *se27* y *se28* del genoma de SeAL1. En conjunto, la deleccion de *se5* mostró los efectos más significativos en las propiedades insecticidas estudiadas del SeMNPV, con un descenso en la patogenicidad de casi 10 veces y un retraso en el tiempo de mortalidad de 7 horas, comparado con el virus derivado del SeBacAL1.

Un estudio previo sobre la ecología del SeMNPV reveló que el genotipo designado VT-SeAL1 produjo un 100% de infecciones encubiertas en adultos que habían sobrevivido a un tratamiento con OB en el estadio larvario. En cambio, otro genotipo designado HT-SeG25 solo produjo un 16% de infecciones encubiertas. Esto nos llevó a plantear la hipótesis de que algunos genotipos de SeMNPV pueden estar asociados con una ruta de transmisión vertical del virus, mientras otros genotipos pueden estar asociados a una ruta de transmisión horizontal. La comparación de genotipos de transmisión vertical con genotipos de transmisión horizontal, Thézé *et al.* (2014) permitió identificar tres ORFs que pueden estar envueltos en la transmisión vertical del virus: *se5*, *se96* y *se99*. En el Capítulo 3 el sistema de recombinación de bacmidos desarrollado en el Capítulo 2 se utilizó para deleccionar estos ORFs y comprobar el efecto sobre la capacidad de transmisión vertical del virus. Larvas de *Spodoptera exigua* fueron infectadas sub-letalmente con una concentración equivalente a la CL_{40} y las infecciones sub-letales fueron detectadas en adultos mediante PCR cuantitativa (Q-PCR). El análisis no mostró diferencias significativas en el número de adultos que adquirieron una infección persistente con ninguno de los virus, lo que sugiere que dichos genes no están involucrados en la transmisión vertical del virus o, si lo están, lo hacen conjuntamente con otros genes.

Las estructura genotípica de diferentes aislados silvestres de SeMNPV es muy similar a la composición genotípica de una población natural del SfMNPV. Las poblaciones de ambos baculovirus se caracterizan por la presencia de diferentes genotipos con delecciones en la misma región del genoma, la comprendida entre el *se12* y el *se40*

en el caso de SeMNPV, y entre *sf20* y *sf36* en el caso de SfMNPV. En dichas regiones se localizan importantes genes: *cathepsin*, *chitinase*, *gp37*, *ptp2*, *egt*, *pkip*, *arif1*, *pif1*, *pif2* y *fgf*, así como algunas ORFs de función desconocida. En el Capitulo 4 la estructura genotípica de los aislados SeUS2, SeUS1 y SfNIC fue comparada para determinar los mecanismos de acción evolutivos y ecológicos de estas estructuras genotípicas poblacionales tan similares. El alineamiento de las secuencias que flanquean a los puntos de delección de los diferentes genotipos indicó que se trata de un mecanismo de evolución independiente que genera y mantiene los genotipos en las respectivas poblaciones de estos virus. El aislamiento de un genotipo deleccionado y los estudios de complementación con diferentes mezclas de genotipo deleccionado y completo, claramente demostró una interacción entre los genotipos en la población viral. El genotipo deleccionado SeUS2-C tuvo una ventaja replicativa en cultivo celular, ya que estaba presente en un 81% de las placas aisladas en cultivo celular después de la inoculación de hemolinfa extraída de larvas infectadas con SeUS2-WT. Sin embargo, el genotipo completo SeUS2-A solo se encontró presente en un 19% de las placas. La presencia de una alta concentración de SeUS2-C en OBs co-ocluidos con el genotipo completo SeUS2-A comprometió severamente la patogenicidad del virus. Interesantemente, la prevalencia del genotipo SeUS2-C fue solo del 25% en el aislado silvestre SeUS2, comparado con el 81% de placas aisladas en cultivo celular. Se cree que los genotipos deleccionados se han generado debido a funciones importantes en la transmisibilidad del virus y que la diversidad genotípica del virus está estructurada para maximizar la probabilidad de transmisión.

El aislado SeMNPV-US1 está también compuesto por varios genotipos, muchos de los cuales llevan delecciones de tamaño variable en la región del genoma comprendida entre el *se15*y el *se41*. En el Capítulo 5 se generaron bácmidos que contenían el genoma completo de SeMNPV (SeBac10) y un genotipo natural con una deleccion de 9.5Kb (SeBac72). El SeBac72 mostró una proliferación viral más eficiente en células de *S. exigua* que el genotipo completo SeBac10, lo cual concuerda

con lo que se encontró el Capítulo 4. La secuenciación de SeBac72 puso de manifiesto que los genes *se16-se28* estaban afectados por la deleccion. El análisis de diferentes delecciones de ORFs individuales del bácmido SeBac10, permitió identificar al *se28* como el gen responsable de prevenir la proliferación de SeMNPV en cultivo celular. Sin embargo, la expresión de *se28* en un bácmido de *Autographa californica* MNPV, un virus heterólogo que pertenece al grupo I de los *Alphabaculovirus*, no bloqueó la proliferación viral. Sorprendentemente, el silenciamiento de *se28* mediante RNAi en el genotipo completo SeBac10 no condujo a un aumento de la propagación del virus en cultivo celular, sugiriendo que no es el transcrito ni la proteína, sino la secuencia de ADN y/o la secuencia topológica de *se28* lo que determina la proliferación viral. La comparación de la secuencia de la región *se27-se30* del genoma de SeMNPV con la región *ac15-ac18* del genoma de AcMNPV mostró un 43% de homología a nivel de ADN, aunque la secuencia de aminoácidos de *se28* y *ac16* (DA26) no son homólogas. Ambos genes *se28/ac16* son parte de regiones hipervariables asociadas con inserciones/delecciones. En conjunto, la región del gen *se28* ha sido identificada como un regulador clave en la propagación del virus y se cree que puede ser responsable de la variación genotípica encontrada en los aislados naturales de baculovirus.

En conclusión, los resultados presentados en esta tesis profundizan nuestro conocimiento en la diversidad genética y genotípica de SeMNPV, y de los baculovirus en general, y puede ayudar en el futuro a la mejora de las estrategias de control biológico basadas en baculovirus.

1. Se construyo un bacmido usando el aislado silvestre SeAL1, SeBacAL1, para la deleccion de genes individuales del nucleopoliedrovirus múltiple de *Spodoptera exigua*. Curiosamente, el aislado silvestre SeAL1 es significativamente menos virulento que el bacmido SeBacAL1, probablemente debido a la inserción del plásmido de clonaje entre los genes *se27* y *se28.*
2. De todos los genes deleccionados, la deleccion del *se5* fue la que mas afectó la patogenicidad y la virulencia del bacmido SeBacAL1, con un aumento de 10 veces de la concentración letal 50 y un retraso del tiempo de mortalidad de más de 7 horas. *Se5* parece tener un papel importante en la patogenicidad y virulencia del virus, aunque no se pudo determinar su función exacta.
3. La deleccion individual de las pautas de lectura abiertas (ORFs) *se5*, *se96* y *se99* no afecto la prevalencia de infecciones encubiertas en adultos supervivientes a una infección subletal en estadio larvario. Sin embargo, no se puede concluir que estas tres ORFs no actúen juntas formando un complejo.
4. La comparación de las secuencias que flanquean los puntos de deleccion de los diferentes genotipos de SeMNPV y SfMNPV parece indicar un mecanismo independiente que genere y mantenga las estructuras poblacionales tan similares en ambos baculovirus.
5. El genotipo deleccionado SeUS2-C tiene una ventaja replicativa en cultivo celular, indicado por el 81% de placas aisladas en cultivo celular, frente al 19% de placas aisladas del genotipo completo SeUS2-A. Sin embargo, en el aislado silvestre SeUS2, el genotipo SeUS2-C está presente solamente en un 25%.
6. La presencia de una alta concentración del genotipo SeUS2-C en mezclas co-ocluidas con el genotipo SeUS2-A, disminuye drásticamente la patogenicidad del virus. Parece que la diversidad genotípica en SeMNPV está estructurada para aumentar la probabilidad de transmisión viral.
7. La región génica del *se28* se ha identificado como la responsable que previene la proliferación viral del SeMNPV en cultivo celular.

8. *Se28* pertenece a una zona hipervariable del genoma que tiene un 43% de homología a nivel de ADN con otra zona hipervariable del genoma de *Autographa californica* MNPV.

Co-tutelle or dual-degree agreement

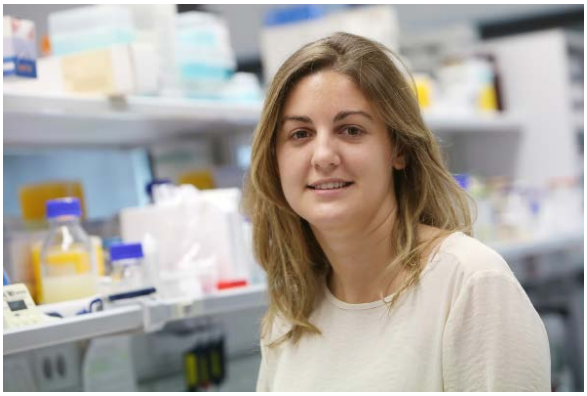
This thesis has been a collaborative venture between the Laboratory of Virology, Wageningen University, Wageningen, The Netherlands and the Catedrático de Departamento de Producción Agraria, Universidad Pública de Navarra, Pamplona, Spain.

By agreement of the two respective universities and signed by the two university boards this thesis by Amaya Serrano is considered a co-tutelle or dual-degree thesis and in fulfilment of the requirements for a degree of doctor for both universities.

About the author

Amaya Serrano García was born on 29th of April 1984, in Pamplona in the province of Navarra, Spain. In 2002 she finished secondary education in Jesuitas, Tudela, and started to study Biology and Biochemistry in Universidad de Navarra, Pamplona. In 2007 she spent ten months in Groningen, The Netherlands, for an Erasmus exchange. After graduating in 2008 she moved to Universidad Pública de Navarra to start a master in Biotechnology. In 2009 she started her master thesis in the labortory of Bioinsecticidas Microbianos, under the supervision of Oihane Simón de Goñi. In her master thesis she worked on the phenotypic effects of a defective genotype in a *Spodoptera exigua* multiple nucleopolyhedrovirus population.

In August 2009 she strated her PhD on genotypic and phenotypic characterization of *Spodoptera exigua* NPV, under the supervision of Delia Muñoz Labiano, of which the results are described in this thesis. In February 2011 she moved by first time to the Laboratory of Virology in Wageningen to continue with part of her PhD under the supervision of Gorben Pijlman. After a very successful collaboration, she strated a double PhD between the Wageningen University and Universidad Pública de Navarra. In August 2012 she received the second best poster presentation award from the Society of Invertebrate Pathology.



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PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Molecular characterization of Spodoptera exigua nucleopolyhedrovirus genotypes and its functional importance in the viral population

Writing of project proposal (4.5 ECTS)

- Molecular characterization of Spodoptera exigua nucleopolyhedrovirus genotypes and its functional importance in the viral population

Post-graduate courses (7.5 ECTS)

- Prácticas de bioinformática básica y anotación de secuencias; Universidad Pública de Navarra (2010)
- Técnicas básicas para la caracterización molecular y biológica de virus entomopatógenos; Universidad Pública de Navarra (2011)
- Técnicas de modificación genética de bacterias; Universidad Pública de Navarra (2012)

Deficiency, refresh, brush-up courses (3 ECTS)

- Certificate in advanced English course (2011, 2012)
- Sharebiotech: confocal microscopy (2012)
- Basic statistics (2014)

Competence strengthening / skills courses (1.5 ECTS)

- How to write a world-class paper; WUR Library (2014)
- Techniques for writing and presenting a scientific paper; Wageningen Graduate Schools (2014)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)

- PE&RC Day (2013)
- PE&RC Weekend (2014)

Discussion groups / local seminars / other scientific meetings (4.5 ECTS)

- Instituto de Agrobiotecnología; monthly meetings (2010-2013)
- Wageningen evolution and ecology seminars (WEES) (2011-2013)
- Escuela de Doctorado de Navarra; Monthly seminars (2013-2014)

International symposia, workshops and conferences (4.5 ECTS)

- Society of Invertebrate Pathogens; Trabzon, Turkey (2010)
- Society of Invertebrate Pathogens; Buenos Aires, Argentina (2012)
- Society of Invertebrate Pathogens; Pittsburgh, USA (2013)

Lecturing / supervision of practical's / tutorials (3 ECTS)

- Plant quality and integrated pest management (2012-2014)
- Molecular virology (2013)

Supervision of MSc student

- Functional analysis of SeMNPV ORF28

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