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

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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 of these genes experimentally on the insecticidal phenotype, a bacmid-based recombination system was constructed to delete selected genes from a SeMNPV isolate, VT-SeAL1, designated as SeBacAL1. All of the knockout viruses were viable 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. *Se5* is an attractive candidate gene for further studies, as it affects both biological parameters of this important biocontrol virus.

1. Introduction

Baculoviruses are a large group of insect-specific DNA viruses (van Oers and 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, 2013). 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 and 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 and 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 and 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

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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 75,006 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 and 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.

2. Materials and methods

2.1. 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 28 °C 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).

2.2. 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 and 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 37 °C. The restriction enzyme was heat inactivated for 20 min at 65 °C. Then, 1 μg of bacmid cloning vector BAC-*Bsu*-*Mau*BI was digested with 10 U of *Mau*BI for 3 h at 37 °C. The vector was dephosphorylated using 1 U alkaline phosphatase (Promega) for 1 h at 37 °C and gel purified with GFX Gel Band Purification Kit (GE Healthcare). Ligation was performed overnight at 4 °C 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 37 °C and spread on agar plates containing kanamycin. A SeMNPV bacmid with the correct restriction profile was selected and designated as SeBacAL1.

2.3. 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 5́ 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), 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 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 and Hope, 2006). To confirm the deletion of the ORFs, *Bgl*II restriction endonuclease analysis of the bacmid DNA and PCR amplifications using primers targeting the flanking regions of the deleted genes (Table 1) were performed. Bacmids with the predicted *Bgl*II restriction profiles and yielding PCR amplicons with the correct size were selected. Once the correct knockout was selected, bacmid DNA extraction was performed and electroporated into DH10β (Invitrogen).

2.4. 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 using M13F-R and M13F-GentaR primers (Table 1).

2.5. Transfection of SeMNPV bacmids

Se301 cells were seeded in a six-well tissue culture plate (Greiner Bio-One) at a confluency of 5×10^5 cells/well. Transfec-

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	28,565–28,614
SeORF28-KO-R	TCACTCCGAGTACATTATTCGAAGTTCATTTTCAAACTTATCCAAATCGTCCTCTAGATGCATGCTCG	Se28 deletion from SeMNPV bacmid; reverse primer	29,088–29,137
SeORF76-KO-F	ATGGAATCGATAACACTCGGTTGTTCGGTGTGCATGTCCGAAGTCTGAATGCTCGGATCCACTAGTAACG	Se76 deletion from SeMNPV bacmid; forward primer	74,519–74,568
SeORF76-KO-R	TTAAAATTTAGCTTTTTTAAAAATGGCAATAGTGTTAGACGACGTCGATGCCTCTAGATGCATGCTCG	Se4 deletion from SeMNPV bacmid; reverse primer	75,876–75,925
SeORF87-KO-F	TACAATATTAAAACGTTGCCGGCAAATTGGGTTTTGATTCTAATTTGAGAGCTCGGATCCACTAGTAACG	Se87 deletion from SeMNPV bacmid; forward primer	84,058–84,107
SeORF87-KO-R	TCAATGTCGATGTGTGTTAATTCGGTTGCGACCAACGATGTCGCCAAACCCCTCTAGATGCATGCTCG	Se87 deletion from SeMNPV bacmid; reverse primer	84,766–84,815
SeORF129-KO-F	ATGATGAGCTTTGCGAGTTTTTTACTAGTGCTCATTTGTTCGGCGTGATCGCTCGGATCCACTAGTAACG	Se129 deletion from SeMNPV bacmid; forward primer	122,957–123,001
SeORF129-KO-R	CTATACGATATTGCCAATACTGTCGTCGTTGTCGTCGTCGTCTTTGTTCTCCTCTAGATGCATGCTCG	Se129 deletion from SeMNPV bacmid; reverse primer	123,747–123,796
SeORF4-F	GG*TCTAGA*GCGTACACAAAAGCAAAAAA	Se4 insertion into SeBacAL1Δ4; forward primer	5478–5497
SeORF4-R	GG*GGTACC*GAAACACTCATATAGAAAGC	Se4 insertion into SeBacAL1Δ4; reverse primer	3159–3178
SeORF5-F	GG*CTCGAG*GAATGATGACCAACTTTTTTG	Se5 insertion into SeBacAL1Δ5; forward primer	5968–5988
SeORF5-R	GG*AAGCTT*ATATGTACACAATAAAATTCAAAG	Se5 insertion into SeBacAL1Δ5; reverse primer	7768–7791
SeORF28-F	GG*TCTAGA*TTTCAACGTATTGCCTACGC	Se28 insertion into SeBacAL1Δ28; forward primer	28,436–28,455
SeORF28-R	GG*GGTACC*GAAAAAAGCGTGGTTTCCAA	Se28 insertion into SeBacAL1Δ28; reverse primer	29,138–29,157
SeORF76-F	GG*TCTAGA*TATCATGTACTACCTATCAT	Se76 insertion into SeBacAL1Δ76; forward primer	74,368–74,387
SeORF76-R	GG*GGTACC*TTGCAAATAAAATACAGTTTAC	Se76 insertion into SeBacAL1Δ76; reverse primer	75,929–75,950
SeORF87-F	GG*TCTAGA*TACAACGTTTTGCGCATTCG	Se87 insertion into SeBacAL1Δ87; forward primer	84,942–84,961
SeORF87-R	GG*GGTACC*CTGTGAATCAAATGTGAATC	Se87 insertion into SeBacAL1Δ87; reverse primer	84,037–84,056
SeORF129-F	GG*TCTAGA*CAAGAAACTGCCATTTTATA	Se129 insertion into SeBacAL1Δ129; forward primer	122,806–122,825
SeORF129-R	GG*GGTACC*TTTAATGTCGGCTCGGATCA	Se129 insertion into SeBacAL1Δ129; reverse primer	123,860–123,879
M13F	CCCAGTCACGACGTTGTAAAACG	Check transposition in *att*Tn7 site. Forward primer	
M13R	AGCGGATAACAATTTCACACAGG	Check transposition in *att*Tn7 site. Reverse primer	
GentaR	AGCCACCTACTCCCAACATC	Check transposition in *att*Tn7 site.	
SeORF4-CON-F	CCTAAAACTAATATCACATT	Check Se4 deletion from SeMNPV bacmid; forward primer	3122–3141
SeORF4-CON-R	GGCGCCCAAGCCTTACAAAC	Check Se4 deletion from SeMNPV bacmid; reverse primer	5410–5429
SeORF5-CON-F	GCGGAATATATAACGACGTC	Check Se5 deletion from SeMNPV bacmid; forward primer	6103–6122
SeORF5-CON-R	GTACACAATAAAATTCACAA	Check Se5 deletion from SeMNPV bacmid; reverse primer	7784–7803
SeORF28-CON-F	CGACATATAAATGATTGTTG	Check Se28 deletion from SeMNPV bacmid; forward primer	28,640–28,659
SeORF28-CON-R	GCAACTCATCATTCGCAAAC	Check Se28 deletion from SeMNPV bacmid; reverse primer	29,361–29,380
SeORF76-CON-F	CCAATTGTTAGGCAGGCAAC	Check Se76 deletion from SeMNPV bacmid; forward primer	74,378–74,397
SeORF76-CON-R	CGTCGAAATGATTATAATAA	Check Se76 deletion from SeMNPV bacmid; reverse primer	75,897–75,916
SeORF87-CON-F	CGCTTCATCGATATTATAGG	Check Se87 deletion from SeMNPV bacmid; forward primer	83,837–83,856
SeORF87-CON-R	CGAGAGAAAATTAAAAATAA	Check Se87 deletion from SeMNPV bacmid; reverse primer	84,773–84,792
SeORF129-CON-F	GCATACATATTGTAAATAAG	Check Se129 deletion from SeMNPV bacmid; forward primer	122,754–122,773
SeORF129-CON-R	CGACTTTTTCGTCGACAGCT	Check Se129 deletion from SeMNPV bacmid; reverse primer	123,781–123,800

tion 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. OBs were purified from infected larvae following the protocol by Muñoz et al. (2001).

2.6. Bioassays

The insecticidal properties of the different knockout and repair viruses were assessed in terms of mean lethal concentration (LC_{50}) and mean time to death (MTD), as an expression of pathogenicity and virulence (Bernal et al., 2013; Cory et al., 1997), respectively, in *per os* insect bioassays (Hughes and Wood, 1981). Mean lethal concentration (LC_{50}) bioassays with OBs from the knockout and the repair viruses were performed using the droplet feeding method (Hughes et al., 1986). For this, groups of 24 *S. exigua* second instars were starved for 12 h 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 °C 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 25 °C 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).

2.7. Electron microscopy

To investigate whether the observed differences in LC_{50} were due to differences in OB size or ODV content, electron microscopy was performed. 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 ODV were counted for cross-sections of 30 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).

2.8. 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).

3. Results

3.1. 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 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.

3.2. 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 h post-inoculation (hpi). All different viruses tested could be classified into three groups according to their speed of kill (Fig. 1). 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).

3.3. Electron microscopy

Scanning electron microscopy (SEM) pictures showed OBs diameters between 0.88 and 1.04 μm, values were statistically similar between vSeBacAL1, vSeBacAL1Δ5 and vSeBacAL1Δ5repair ($p > 0.05$) (Fig. 2). Transmission electron microscopy (TEM) observations revealed that the number of ODVs was statistically similar for vSeBacAL1, vSeBacAL1Δ5 and vSeBacAL1Δ5repair OBs ($p > 0.05$) (Fig. 3).

3.4. 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 C phosphorylation sites (aa 114–116, 152–154, 363–365, 480–482 and 491–

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	1.63×10^4	1	–	–
SeAL1	1.12×10^4	1.45	0.99	2.12
SeBacAL1Δ4	6.09×10^4	**0.26**	**0.18**	**0.38**
SeBacAL1Δ5	1.60×10^5	**0.10**	**0.06**	**0.16**
SeBacAL1Δ28	1.09×10^4	**1.48**	**1.05**	**2.10**
SeBacAL1Δ76	3.68×10^4	**0.44**	**0.31**	**0.62**
SeBacAL1Δ87	2.14×10^4	0.76	0.54	1.07
SeBacAL1Δ129	2.96×10^4	**0.55**	**0.39**	**0.77**
SeBacAL1Δ4repair	2.30×10^4	0.71	0.49	1.01
SeBacAL1Δ5repair	2.38×10^4	0.68	0.46	1.09
SeBacAL1Δ28repair	1.97×10^4	0.83	0.57	1.21
SeBacAL1Δ76repair	1.91×10^4	0.85	0.60	1.22
SeBacAL1Δ87repair	2.31×10^4	0.71	0.49	1.01
SeBacAL1Δ129repair	1.55×10^4	1.05	0.74	1.48

Probit analysis was performed using the PoloPlus program. A test for non-parallelism was not significant ($\chi^2 = 9.40$, df = 13, $P = 0.742$). Regressions were fitted with a common slope (±SE) of 1.289 ± 0.308.
*Figures in bold indicate significance, that is, a relative potency significantly lower or higher than that of SeBacAL1.

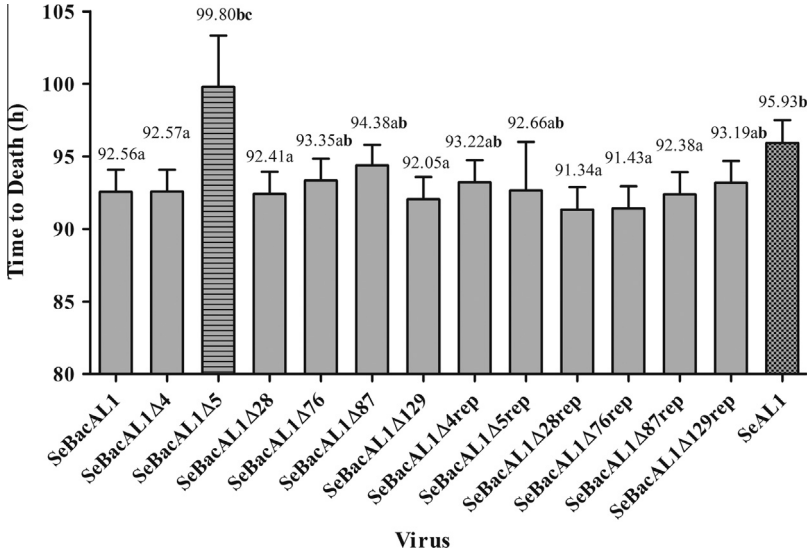

Fig. 1. Mean-time-to-death (MTD) values of vSeBacAL1 and the different gene knockout and repair viruses in second instar *S. exigua*. MTD values were estimated by Weibull survival analysis. Bars labelled with the same letter did not differ significantly ($p > 0.05$).

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.

4. 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 and 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, virulence or other biological traits. 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 and Franklin, 2012).

The pathogenicity of the bacmid-derived virus vSeBacAL1 was similar to that of the wildtype isolate SeAL1. The time to death of vSeBacAL1 was somewhat shorter (3 h) as compared to SeAL1, which may relate to the insertion of the bacmid cloning vector in the Se27–Se28 intergenic region. Deletion of *se4* decreased OB pathogenicity by almost 4-fold compared with vSeBacAL1 (Table 2), but had no effect on speed of kill (Fig. 1). *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.,

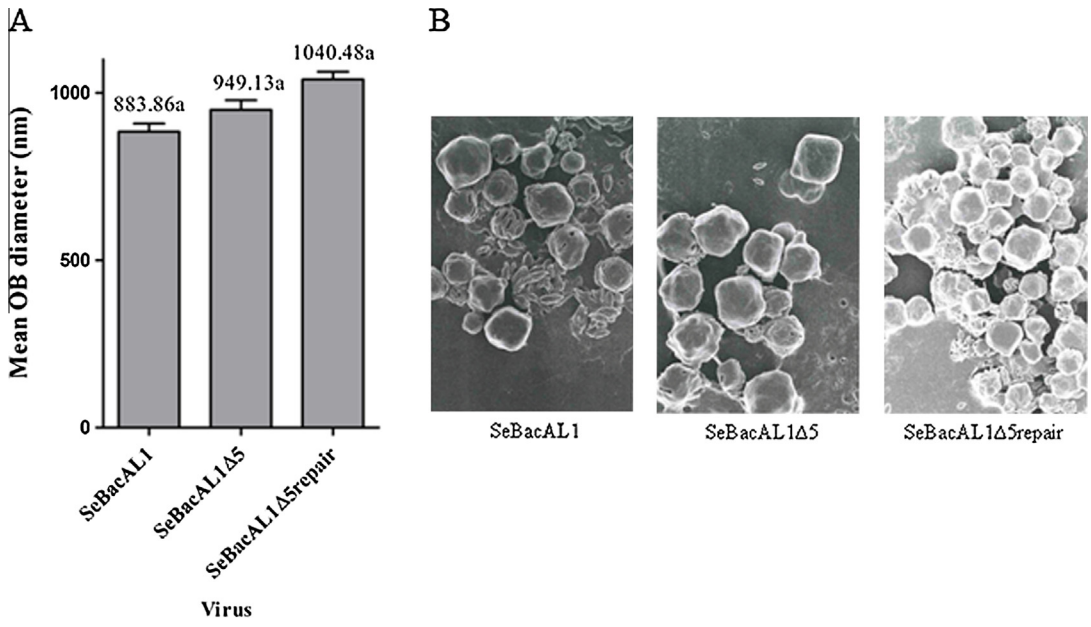

Fig. 2. (A) Mean OB diameter of SeBacAL1, SeBacAL1Δ5 and SeBacAL1Δ5repair as determined by scanning electron microscopy (SEM). Error bars indicate standard error of the mean. Bars labelled with the same letter did not differ significantly ($p > 0.05$). (B) SEM pictures of SeBacAL1, SeBacAL1Δ5 and SeBacAL1Δ5repair.

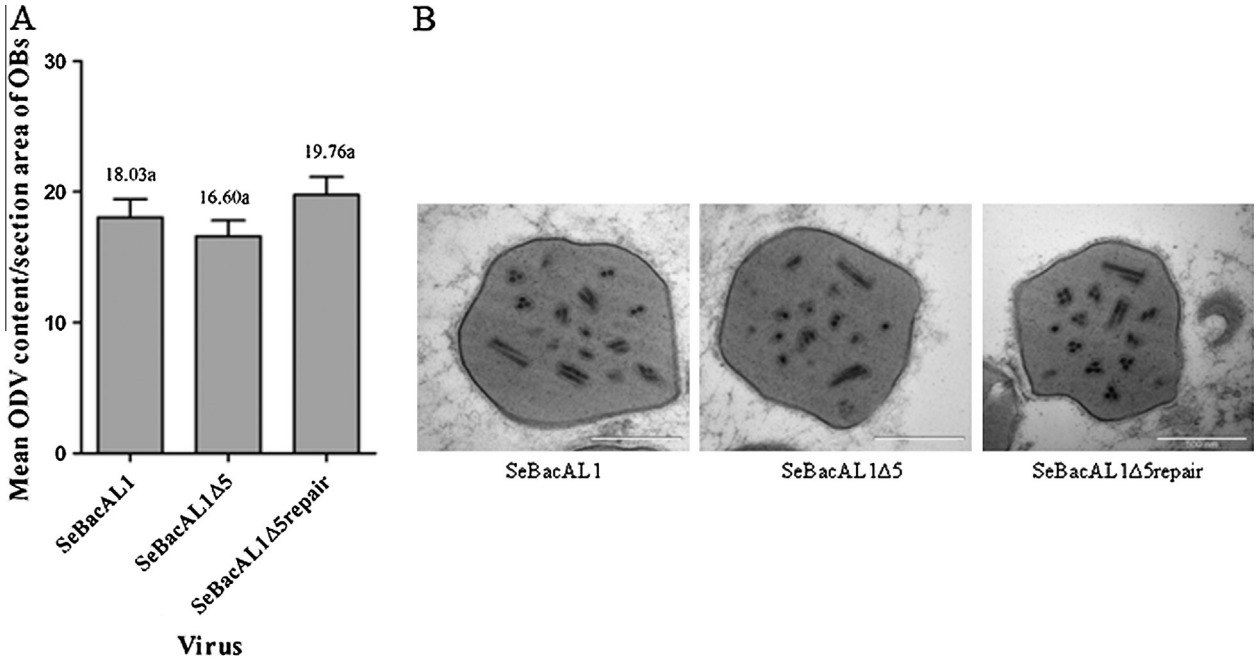

Fig. 3. (A) Mean ODV content within OBs of vSeBacAL1, vSeBacAL1Δ5 and vSeBacAL1Δ5repair as determined by transmission electron microscopy (TEM) analysis. Error bars indicate standard error of the mean. Bars labelled with the same letter did not differ significantly ($p > 0.05$). (B) TEM pictures of vSeBacAL1, vSeBacAL1Δ5 and vSeBacAL1Δ5repair.

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. 1). Differences in the pathogenicity and virulence 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. The reason for the reduced ODV infectivity remains unclear, but warrants further studies on the specific mode

of action of *Se5*. One experiment that should be addressed is to study the number of nucleocapsids per ODV, because there could still be fewer copies of DNA (number of nucleocapsids) per ODV even when the size of the OBs and the number of ODV/OB are the same.

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 and 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 12–39 (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 insect 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 and 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 and 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.

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 and 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.

5. Conclusions

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 mean lethal concentration and mean time to death, although it is conceivable that these properties are linked in this case. The other genes deserve further investigation as their differential effects on virulence and pathogenicity are not connected.

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