



## Short communication

Effects of *Acp26* on in vitro and in vivo productivity, pathogenesis and virulence of *Autographa californica* multiple nucleopolyhedrovirusOihane Simón<sup>a,b,c</sup>, Trevor Williams<sup>d</sup>, Primitivo Caballero<sup>b,c</sup>, Robert D. Possee<sup>a,\*</sup><sup>a</sup> Centre for Ecology and Hydrology, Mansfield Road, Oxford OX1 3SR, UK<sup>b</sup> Laboratorio de Entomología Agrícola y Patología de Insectos, Universidad Pública de Navarra, 31006 Pamplona, Spain<sup>c</sup> Instituto de Agrobiotecnología, CSIC, Gobierno de Navarra, 31192 Mutilva Baja, Navarra, Spain<sup>d</sup> Instituto de Ecología AC, Apartado Postal 63, Xalapa 91070, Mexico

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## ABSTRACT

Homologs to *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) open reading frame (ORF) 136 or *Acp26* are present within almost all nucleopolyhedroviruses (NPVs). Two copies of the gene are found in some members of group II NPVs, suggesting that it may play an important role in transmission or replication. Phylogenetic analysis revealed that the predicted protein has some similarity with Camelpox virus v-sfn protein, which reduces the virulence of orthopoxviruses in vivo. To investigate the influence of ACP26 on the infectivity and virulence of AcMNPV a bacmid system was used to delete the *Acp26* ORF. The *Acp26*null bacmid was able to generate a transmissible infection in cell culture and larvae, indicating that *Acp26* is not essential for propagation of viral infection in vitro or in vivo. Deletion of *Acp26* from the AcMNPV genome had no apparent effect on timing or production of infectious BV in cell culture or in insect larvae. Additional comparisons of AcMNPV and *Acp26*null bacmid viruses showed that deletion of *Acp26* did not significantly influence the infectivity and virulence of AcMNPV occlusion bodies or the number produced. The *Acp26* may be an auxiliary gene with subtle effects on virus replication and transmission.

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The *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) is the type species of the genus *Alphabaculovirus* (family *Baculoviridae*) and is highly pathogenic to a number of lepidopteran species. The genome of AcMNPV comprises 133,894 base pairs and encodes at least 154 methionine-initiated open reading frames (ORFs) (Ayres et al., 1994). Gene regulation at the transcriptional level can be subdivided into early, late and very late phases. Early genes are recognized and transcribed by host RNA polymerase II whereas late genes are transcribed by a viral-encoded late RNA polymerase (Friesen and Miller, 2001; Guarino et al., 1998).

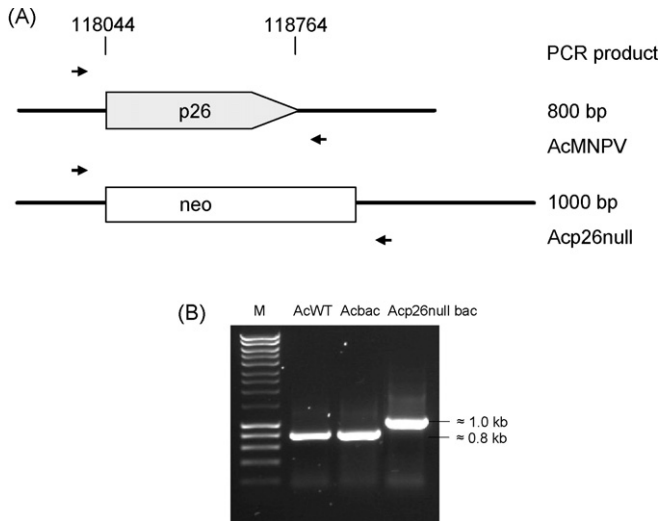
The *p10* gene is one of two baculovirus genes hyperexpressed very late in infection (Rohel et al., 1983; Smith et al., 1983). While mapping *p10* transcription, two additional transcripts were identified in this region (Rankin et al., 1985). One of these coded for a putative 26 kDa protein that was originally designated P26 (Liu et al., 1986) and subsequently open reading frame (ORF) 136 (Ayres et al., 1994). It was shown that *p26* is transcribed by the host RNA polymerase II during the early and late phases of infection, but is

down regulated in the very late phase (Goenka and Weaver, 2008). The P26 occurs primarily in the cytoplasm during transition from the early to the late phase of infection, but is also found in the nucleus during the very late phase (Goenka and Weaver, 2008). It associates with itself, predominantly forming dimers under physiological conditions. Recombinant viruses with partial deletions in *p26* produced plaques containing polyhedra in *Trichoplusia ni* Tn368 and *Spodoptera frugiperda* Sf21 cells and this was considered to be nonessential for viral replication in cultured cells (Rodems and Friesen, 1993). However, the role of P26 in vivo was not determined.

Many NPVs, in both groups I and II, have homologs to AcMNPV *p26*. Two copies of the gene are present in most of the group II NPVs (Ijkel et al., 1999; Jakubowska et al., 2006; Ma et al., 2007; Willis et al., 2005), whereas *p26* has not been reported from granuloviruses or NPVs that infect non-lepidopteran hosts. Besides NPVs, the only other protein reported to have significant similarity (41%) to P26 is an orthopoxvirus protein known as v-sfn (Gubser et al., 2007). Camelpox virus v-sfn protein attenuates the orthopoxvirus infection in vivo but does not affect virus replication or plaque morphology in vitro (Gubser et al., 2007). This observation led us to test the hypothesis that P26 could have a role in mediating NPV infectivity or virulence, thus serving as an auxiliary gene in common with other baculovirus sequences (O'Reilly, 1997). For this, we used a bacmid system to delete *Acp26* from the AcM-

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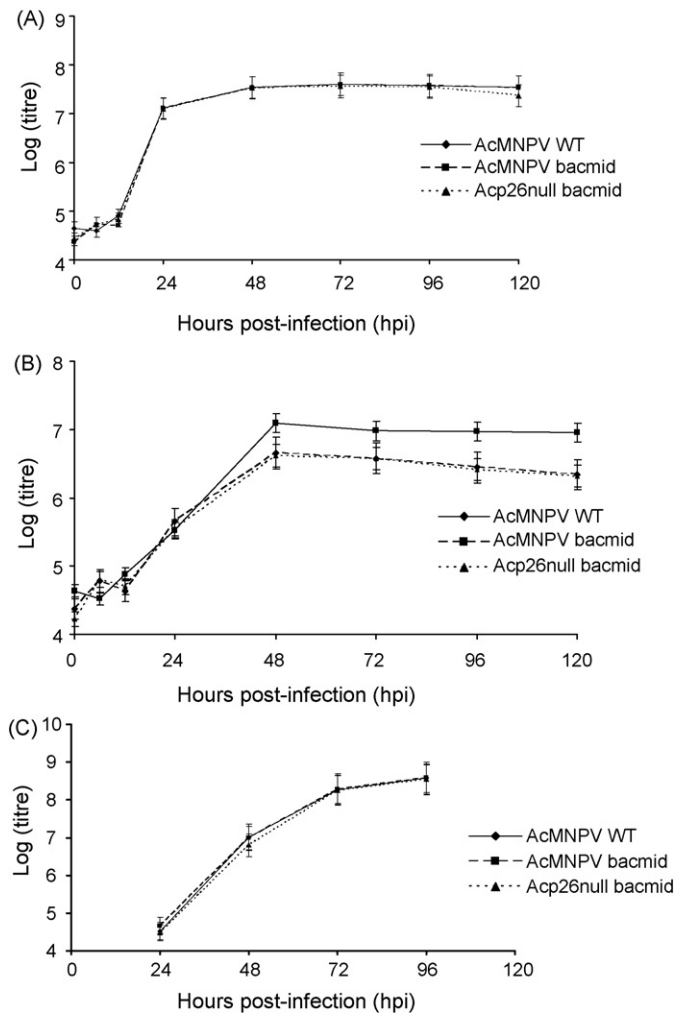
**Fig. 1.** Deletion of *p26* from the AcMNPV genome. (A) Genome organization and coordinates of the AcMNPV *p26* and its replacement with a neomycin (*neo*) cassette. The positions of primers used to characterize the insertion are shown and the predicted sizes of the DNA products derived from PCR amplification of parental (AcMNPV) and recombinant virus (Acp26null) genomic DNA. (B) Agarose gel analysis of the DNA products from PCR analysis of virus DNA.

NPV genome and examine the consequences on virus productivity, pathogenesis and virulence in vitro and in vivo.

The *p26* was deleted from an AcMNPV bacmid containing a bac replicon at the *p10* locus using the Red/ET recombination system (Gene Bridges GmbH) in *Escherichia coli*. A kanamycin resistance gene was amplified from the Tn5-*neo* PCR-template using two sets of overlapping primers (Acp26out.1 [TATACGT-TAAATCAAACGGACGTTTGGACAGCAAGCGAACCGGAATTGC] with Acp26out.2 [CTTATTTAACTATAATATATTGTGTTTCAGAAGAACTCGTC-AAGAAGCG] and Acp26out.3 [GATAATTAATAATTCATTGCAA-GCTATACGTTAAATCAAACGGACGTTTG] with Acp26out.4 [ATATACAAATGATTTGATAATAATTCTTATTTAACTATAATATATTGTG]). These added 50 nucleotides from the 5' and 3' untranslated regions of *p26* to Tn5-*neo*. Electrocompetent *E. coli* cells containing the AcMNPV bacmid and the Red/ET plasmid pSC101-BAD-*gbaA* that had been induced with 0.2% arabinose were transformed with the PCR-amplified *p26*/*neo* cassette. Recombinants were selected as resistant colonies on medium containing chloramphenicol and kanamycin. The selected bacmid (Acp26null) had a deletion in *Acp26* between nucleotides 118,044 and 118,766 (Ayres et al., 1994) confirmed by REN analysis (data not shown) and PCR amplification with primers specific for detection of the predicted recombinant junction regions in Acp26null bacmid (Fig. 1).

Transfection of Sf21 cells (King and Possee, 1992) with CsCl-purified AcMNPV WT, AcMNPV bacmid and Acp26null bacmid DNAs produced budded virus (BV) stocks with similar titers (data not shown). These stocks were amplified and used to examine BV accumulation via plaque assay titration (King and Possee, 1992) at different times after infection of Sf21 and TN368 cells. The titers of infectious BV obtained from the three viruses replicating in Sf21 cells were similar at each time point (Fig. 2A). In TN368 cells, no differences in BV production were observed between the AcMNPV bacmid and Acp26null bacmid at any time point. However the AcMNPV WT produced 2.8 times more BV than the bacmids at 48 hpi, with a maximum difference of four times more BV at 96 and 120 hpi (Fig. 2B).

Production of BV in *T. ni* fourth instars was determined after oral inoculation of insects by the droplet feeding method (Hughes and Wood, 1981) with  $5 \times 10^2$  OB/larva. This dose was estimated to



**Fig. 2.** Budded virus production in cell culture and insect larvae. Sf21 (A) and TN368 (B) cells were inoculated with BV (10 pfu/cell) AcMNPV WT, AcMNPV bacmid or Acp26null bacmid. Medium was harvested at 0, 6, 12, 24, 48, 72, 96 and 120 h post infection and titrated in a plaque assay using Sf21 cells. Each data point represents the average titer derived from four independent infections. (C) Fourth instar *T. ni* larvae were orally inoculated by the droplet feeding method with 500 OBs from AcMNPV WT, AcMNPV bacmid and Acp26null bacmid. Hemolymph was collected between 0 and 120 h p.i. from 10 randomly selected larvae and infectious BV titers were determined in Sf21 cells by plaque assay.

result in 90% mortality. After insects had ingested virus (0 h) they were transferred to diet plugs and incubated at 23 °C. At 6, 12, 24, 48, 72 and 96 hpi hemolymph was extracted from 10 randomly selected larvae infected with each virus, diluted 1:20 in TC100/10% foetal calf serum and sterilized by filtration. By 120 hpi all of the remaining virus-infected larvae had died. Hemolymph was extracted from three groups of larvae in three separate experiments. These samples were diluted and infectious BV was measured by using a plaque assay (King and Possee, 1992). No differences were detected in BV production between the larvae infected with AcMNPV WT, AcMNPV bacmid or Acp26null bacmid at any time point (Fig. 2C). In all cases, BV was not detected prior to 12 hpi but appeared to be in log-phase production at 24 hpi. Unlike the results from virus-infected TN-368 cell (Fig. 2B), AcMNPV did not produce more BV than the two bacmids (Fig. 2C). These results demonstrated that *Acp26* deletion had no discernable effect on BV yields in cultured insect cells or *T. ni* larvae.

The 50% lethal concentration (LC<sub>50</sub>) and mean time to death (MTD) in *T. ni* and *Spodoptera exigua* second instars for AcMNPV

**Table 1**  
Logit regression and time to death analysis for OBs derived from the *Autographa californica* multiple nucleopolyhedrovirus bacmid (AcMNPV bacmid) and corresponding Acp26 deletion mutant bacmid (Acp26null bacmid) compared with the wild-type (AcMNPV WT) isolate in *Trichoplusia ni* second instars

Virus	LC <sub>50</sub> (OBs/ml)	Fiducial limits (95%)		Relative potency	P	MTD (h)	Fiducial limits (95%)	
		Low	High				Low	High
AcMNPV WT	$2.45 \times 10^3$	$2.18 \times 10^3$	$2.85 \times 10^3$	1.0	–	102	98	106
AcMNPV bacmid	$2.14 \times 10^3$	$1.85 \times 10^3$	$2.48 \times 10^3$	1.14	0.217	99	95	103
Acp26null bacmid	$2.10 \times 10^3$	$1.82 \times 10^3$	$2.44 \times 10^3$	1.17	0.164	98	94	102

Logit regressions were fitted in GLIM with a common slope of  $0.7061 \pm 0.018$  (S.E.). A test for non-parallelism was not significant ( $\chi^2 = 0.15$ , d.f. = 2,  $P = 0.93$ ). Relative potencies were calculated as the ratio of effective doses relative to the AcMNPV WT isolate. P values were calculated by t-test of the differences between regression intercepts compared to that of the AcMNPV WT isolate. Mean times to death (MTD) values were estimated by Weibull analysis (Crawley, 1993).

**Table 2**  
Logit regression and time to death analysis for OBs derived from the *Autographa californica* multiple nucleopolyhedrovirus bacmid (AcMNPV bacmid) and corresponding Acp26null bacmid compared with the wild-type (AcMNPV WT) isolate in *Spodoptera exigua* second instars

Virus	LC <sub>50</sub> (OBs/ml)	Fiducial limits (95%)		Relative potency	P	MTD (h)	Fiducial limits (95%)	
		Low	High				Low	High
AcMNPV WT	$3.85 \times 10^5$	$2.96 \times 10^5$	$4.99 \times 10^5$	–	–	122	118	126
AcMNPV bacmid	$3.62 \times 10^5$	$2.76 \times 10^5$	$4.65 \times 10^5$	1.06	0.753	120	116	124
Acp26null bacmid	$4.12 \times 10^5$	$3.18 \times 10^5$	$5.29 \times 10^5$	0.93	0.361	118	114	122

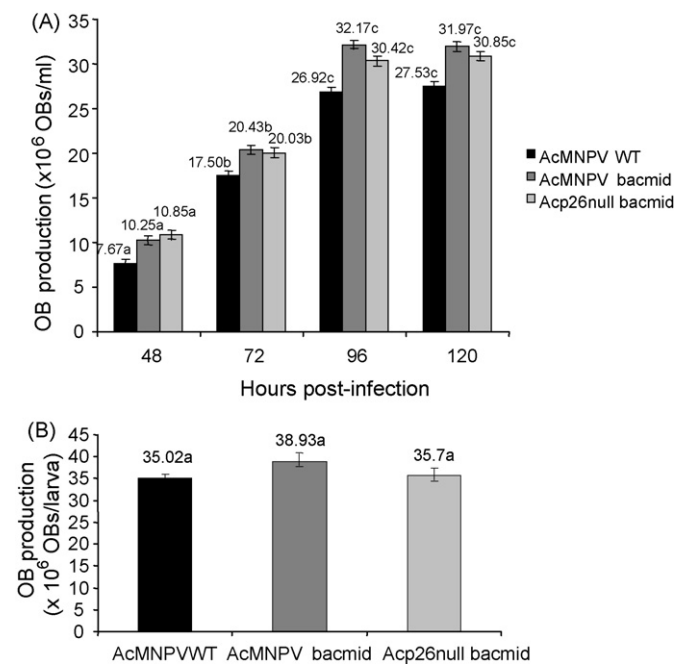
Logit regressions were fitted in GLIM with a common slope of  $0.4772 \pm 0.018$  (S.E.). A test for non-parallelism was not significant ( $\chi^2 = 0.28$ , d.f. = 2,  $P = 0.87$ ).

WT, AcMNPV bacmid and Acp26null bacmid OBs were determined using droplet feeding. The virus concentrations used ranged from 16 to  $10^4$  OBs/ml for *T. ni* and from  $4 \times 10^4$  to  $2.5 \times 10^7$  OBs/ml for *S. exigua*. These concentrations were determined in previous experiments to kill between 5 and 95% of the respective species. Larvae were maintained on diet until death or pupation. Results were analysed with logit regression analysis using the Generalised Linear Interactive Modelling (GLIM) program (Crawley, 1993) and expressed as the LC<sub>50</sub> value. The 95% fiducial limits of the relative potencies, representing the ratio of effective concentrations (Robertson and Preisler, 1992), overlapped broadly in these viruses, indicating no significant differences in the infectivity of OBs in either host species (Tables 1 and 2). In contrast, OBs were approximately 170 times less infective in *S. exigua* larvae compared to *T. ni*. The OB concentration used for time mortality analysis was that estimated to result in ~80% larval deaths, i.e.,  $2 \times 10^3$  OBs/ml for AcMNPV WT, AcMNPV bacmid and Acp26null bacmid in *T. ni* and  $5 \times 10^6$  OBs/ml in *S. exigua*. The time mortality of the three viruses was subjected to Weibull analysis in GLIM. The MTD values of the OBs were lower in *T. ni* larvae but there were no significant differences between the three viruses in either host species. We concluded that Acp26 deletion had no substantial effect on the infectivity or speed of kill of AcMNPV, either in *T. ni* or *S. exigua* larvae.

The number of OBs produced by AcMNPV WT, AcMNPV bacmid and Acp26null bacmid in virus-infected cells were measured in cell culture by direct counting of lysed cells ( $1 \times$  PBS, 10% SDS) in a bacterial counting chamber. The results were subjected to one-way analysis of variance and means were separated by Bonferroni test. No significant differences were observed (Fig. 3A). Similarly, no differences were observed in OB production by the three viruses in *T. ni* larvae (Fig. 3B). OBs were purified from approximately 60 individual larvae (3 groups of 20) for each virus treatment and enumerated with an improved Neubauer counting chamber. The OB counts for each insect were performed three times. When genomic DNA was isolated from  $5 \times 10^8$  OBs for each virus, similar yields were obtained for AcMNPV WT, AcMNPV bacmid and Acp26null bacmid ( $210 \pm 45$ ,  $198 \pm 53$  and  $202 \pm 39$  ng/ $\mu$ l, respectively). Virus DNA was prepared essentially as described by Hunter-Fujita et al. (1998).

The results from this study did not support a role for P26 in AcMNPV replication in vivo or in vivo or in the assembly of either BV

or occluded virus. These observations support the conclusions of Rodems and Friesen (1993), who constructed viruses lacking both *hr5* and parts of *p26*, which replicated normally in Sf21 and TN368 cells. However, in this earlier report, quantitative studies were not performed and the viruses were not tested in insect larvae. Recently, it was shown that Acp26 transcripts accumulated early and late during infection, after which they declined in quantity (Goenka and Weaver, 2008). Immunoblot analysis detected P26 primarily in the soluble cytoplasmic fraction, whereas very weak Acp26 transcript signals were detected in the nuclear fraction only at 48 and 72 hpi. In an RT-PCR based transcriptional analysis of *p26* in vivo in *T. ni* and *S. exigua* larvae, *p26* transcripts were detected in very low amounts during the early phases of infection, increased in the



**Fig. 3.** Production of AcMNPV WT, AcMNPV bacmid and Acp26null bacmid OBs in (A) Sf21 cells (OBs/ml) and (B) *T. ni* larvae (OBs/larva). Figures above columns indicate the value of each column. Values followed by different letters were significantly different (ANOVA, Bonferroni test,  $P < 0.05$ ).

late phase and disappeared in the very late phase. However, consistent with the findings of Goenka and Weaver (2008), the quantity of the transcript generated by *p26* was around 500 times lower than that of *polh* (data not shown). P26 was also observed to form dimers under physiological conditions (Goenka and Weaver, 2008). However, despite the original and detailed nature of these studies on timing of transcription, cellular location and dimeric nature of this protein, it was not possible to identify the role of *p26* in viral replication.

The presence of this gene in most of the NPVs, and the existence of two copies in some group II NPVs, suggests that it is likely to be important in virus transmission or replication. Amino acid sequence analysis revealed that the P26 protein does not have any conserved domains, *N*-glycosylation sites, or signal peptides that could provide clues to its function. However, a degree of sequence similarity (41%) was detected with the schlafen-like protein (*v-slfn*) of the Camelpox virus. The *v-slfn* protein is an orthopoxvirus virulence factor that affects the host immune response to infection. Recombinant vaccinia viruses expressing camelpox virus *v-slfn* were less virulent than control viruses (Gubser et al., 2007). The *v-slfn* protein is an intracellular protein, which attenuates vaccinia virus in vivo but does not affect virus replication or plaque morphology in vitro (Gubser et al., 2007). Other orthopoxvirus proteins have also been found to decrease viral virulence in one model but not others (Alcamí and Smith, 2002; Staib et al., 2005; Tschärke et al., 2002).

Two copies of *p26* are present in *S. frugiperda* multiple NPV (SfM-NPV). An SfMNPV bacmid was constructed in which *p26-b* (ORF131) was disrupted due to the presence of a single *Ascl* restriction site used in the construction of the bacmid (O. Simón, unpublished). We found no differences between the biological activity of OBs originating from SfMNPV wild-type virus and those of the SfM-NPV bacmid. This observation supports the results of the study in AcMNPV that deletion of *Acp26* has no substantial effects on the insecticidal properties of these viruses. However, the SfMNPV has two copies of this gene: Sf86 and Sf131. Studies with Sf86 deletion mutants are required to determine the possible function of this second copy. It seems that *Acp26* and its homologs do not emulate the *v-slfn* protein or other orthopoxvirus proteins that affect virus virulence differently in vivo or in vitro.

The function of *p26* homologs in NPVs remains unclear. Its presence in almost all NPVs and the presence of two copies in some of the group II NPVs suggest that this gene could be important in NPV infection. The present study indicates that this is an auxiliary gene that does not influence key aspects of virus transmission such as BV or OB production, infectivity or speed-of-kill.

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