Effects of Ac150 on virulence and pathogenesis of *Autographa californica multiple nucleopolyhedrovirus* in noctuid hosts

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*Ac150* is expressed late during infection of cultured lepidopteran insect cells by *Autographa californica multiple nucleopolyhedrovirus*. The *Ac150* gene product is predicted to have a molecular mass of 11 161 Da and consists of a hydrophobic N terminus and a single ‘peritrophin-A-like’ domain, connected by a short region of charged amino acids. An *Ac150* deletion mutant and its parental wild-type virus were compared for differences in virulence by both oral and intrahaemocoelic routes of infection. It was found that the mutant was significantly less virulent in larvae of all three host species tested (*Heliothis virescens*, *Spodoptera exigua* and *Trichoplusia ni*) when occlusions were administered orally, but not when isolated occlusion-derived virus (ODV) was administered orally or budded virus was administered intrahaemocoelically. ODV yields were the same from equal numbers of mutant and wild-type occlusions, and nucleocapsid-distribution frequencies within the two ODV populations were the same, eliminating these features as explanations for the observed differences in virulence. Comparison of pathogenesis, as revealed by *lacZ* expression from identical reporter-gene cassettes in the mutant and wild-type virus, indicated that the mutant was less efficient at establishing primary infection in midgut cells; otherwise, it exhibited infection kinetics identical to those of wild-type virus. *Ac150*, therefore, can be considered a *per os* infection factor that mediates, but is not essential for, oral infection.

**INTRODUCTION**

*Autographa californica multiple nucleopolyhedrovirus* (AcMNPV), the type species of the genus *Nucleopolyhedrovirus*, family *Baculoviridae*, fatally infects the larvae of more than 30 species of Lepidoptera (Adams & McClintock, 1991; Granados & Williams, 1986). Completion of the infection cycle *in vivo* requires two genetically identical, but morphologically distinct, viral phenotypes, each of which performs a specific role during infection. Occlusion-derived virus (ODV) is embedded within a crystalline matrix of polyhedrin protein that forms an occlusion, a polyhedral structure that dissolves upon exposure to the highly alkaline juices within the host’s midgut lumen. ODV is a specialist that only infects columnar epithelial cells of the midgut. The ODV-infected midgut cells produce budded virus (BV), which, in turn, infects tracheolar cells servicing the infected midgut epithelium (Engelhard *et al.*, 1994; Washburn *et al.*, 1995). Tracheolar cells also produce BV, a generalist that spreads infection to other tissues throughout the insect until death and liquefaction ensue, the hallmarks of polyhedrosis disease.

During the early stages of AcMNPV pathogenesis in penultimate larvae of the permissive hosts, *Trichoplusia ni*, *Spodoptera exigua* and *Heliothis virescens*, secondary infection by BV of even a single tracheolar cell leads to overwhelming infection and death (Engelhard & Volkman, 1995; Washburn *et al.*, 1995; Zhang *et al.*, 2004). Host larvae, however, can clear primary infection by sloughing ODV-infected midgut cells, a defensive response that varies qualitatively among host species and temporally within instars of a single species (Inoue & Miyagawa, 1978; Briese, 1986; Keddie *et al.*, 1989; Engelhard & Volkman, 1995; Washburn *et al.*, 1995, 1998, 1999, 2003). If a host can eliminate ODV-infected midgut cells prior to BV transmission to secondary targets, systemic infection fails and the insect survives. The ability to slough infected cells increases as larvae age and this response is an important component of developmental resistance (Engelhard & Volkman, 1995; Washburn *et al.*, 1998). It is not surprising, therefore, that selection has favoured an AcMNPV infection strategy that incorporates both the timely onset of primary midgut
infection and the rapid transmission of BV to nearby tracheolar cells. Two classes of viral factors impact these events and contribute to virulence of per os infection without affecting virulence of BV.

The so-called pif (per os infectivity factor) genes of AcMNPV and their homologues are representative of the first class of factors. The pif genes are essential for establishing midgut infection and are highly conserved among all sequenced baculoviruses. Moreover, their absence is inconsequential to BV infectivity. AcMNPV p74, the founding member of this class, was described over a decade ago (Kuzio et al., 1989). Two more genes were identified subsequently in Spodoptera littoralis NPV and S. exigua NPV; these were SiNPV ORF 7 (pif) and SeNPV ORF 35 (pif-2), homologues of Ac119 and Ac022, respectively (Kikhno et al., 2002; Pijlman et al., 2003). AcMNPV p74 and pif encode ODV structural proteins and AcNPV P74 is involved in the specific binding of ODV to midgut cells (Haas-Stapleton et al., 2004). The functions of PIF and PIF-2 are still unknown.

Members of the second class of factors promote rapid transmission of BV to tracheolar cells and thereby enhance virulence of infection initiated per os. This class of factors is diverse and includes PE38 (Milks et al., 2003) and GP64 expressed early, prior to virus replication (Washburn et al., 2003; Zhang et al., 2004). Such factors are of interest because, whilst not essential for in vivo infection, they ‘fine-tune’ virulence in host insects and their effects may vary among susceptible species.

Recently, Lapointe et al. (2004) reported that two members of the ‘11K gene family’, Ac145 and Ac150, enhance virulence of AcMNPV occlusions without affecting BV infectivity. The ‘11K genes’ are predicted to encode small proteins of 90–110 aa that contain hydrophobic N termini and single copies of the so-called ‘C6 motif’ or ‘peritrophin-A domain’, thought to bind chitin (Dall et al., 2001; Tellam et al., 1999). The C6 motif also occurs within proteins encoded by diverse species within the ecdysozoan clade. Such proteins include various chitinases, mucins, peritrophins and other proteins incorporated within peritrophic membranes lining the guts of caterpillars and basal laminae of insect tracheae (Dall et al., 2001). Between the hydrophobic N terminus and the peritrophin-A domain, Ac150 also encodes a short stretch of basic and then acidic amino acids, with an RGD sequence separating the two. This is of note because RGD is an integrin-binding domain, and integrins make transmembrane connections to the cytoskeleton and may activate cellular signalling pathways (Hynes, 2002).

All baculovirus species infecting lepidopteran or hymenopteran larvae that have been sequenced to date contain one or more of the 11K homologues, and the apparent affinity of the proteins for chitin suggests a role during primary infection, possibly at the peritrophic-membrane interface. Lapointe et al. (2004), however, were unable to demonstrate chitin-binding activity for either Ac150 or Ac145, nor were they able to show that the absence of Ac150 alone had any adverse effect on virulence. The latter was a surprising result because deletion of Ac145 alone, or together with Ac150, reduced virulence in orally infected H. virescens larvae by 6- and 39-fold, respectively. Moreover, Ohkawa (1997) found that deletion of the Bombyx mori NPV homologue of Ac150, BmNPV ORF 126, reduced virulence in orally infected B. mori larvae. Our long-term interest in baculovirus pathogenesis in vivo led us to revisit the question of a possible role for Ac150 in oral infection. We generated an Ac150 deletion mutant, AcΔ150, in which the hsp70/lacZ reporter cassette was inserted into the Ac150 ORF. In comparative bioassays with wild-type occlusions, we found that virulence of AcΔ150 occlusions was decreased significantly in larvae of all three species tested (H. virescens, T. ni and S. exigua). Comparison of pathogenesis revealed that the only discernible role of Ac150 was to enhance establishment of primary midgut-cell infection, rather than to facilitate rapid transmission of BV. In this regard, Ac150 is in the same class as the pif genes.

**METHODS**

**Plasmid construction.** PCR was used to amplify the 5’ and 3’ regions of Ac150, using the AcMNPV EcoRI B fragment (which contains Ac150 and adjacent genes) as template. The 5’ 502 bp region of Ac150 was amplified by using primer pairs 150A (5’-TAATT-AAAACCTGCGCTGA-3’) and 150B (5’-ACTGAACCCGCTCGTGG-3’), ensuring that Ac149 was left intact. The 3’ 508 bp region of Ac150 was amplified by using primers 150C (5’-ATGGTACCAAAATAAATATTTATGATTAAATGATATTTATTATATATATTATTATATTATTATATTATTATATTATATTATTATATT-3’) and 150D (5’-ACTGTGAAACCGAGTTC-3’), the former made longer by the addition of a Kmbl site for use in determining fragment orientation within transfer plasmids (Fig. 1a) and also to make the annealing temperature similar to that of 150D. pBSKS+ (Stratagene) was cut with XbaI, then blunted by Klenow digestion and ligated to the 5’ terminus of Ac150. The correctly oriented clone, designated p150–5’–BSKS, was digested with BamH1/XbaI and ligated to an hsp70/lacZ cassette (cleaved from pAcDZ1 by using the same enzymes; Zuidema et al., 1990) to form p150–5’–hsp70/lacZ–BSKS. This second plasmid was cut with Smal and blunt-ligated to the 3’ terminus of Ac150, generating the final transfer plasmid, p150–3’–hsp70/lacZ–3’–BSKS, in which 60 % of the coding region of Ac150 was deleted (Fig. 1b). DNA sequencing of p150–5’–hsp70/lacZ–3’–BSKS showed that the inserted sequence was oriented correctly (data not shown).

**Construction and genetic analysis of AcΔ150 and AcΔ150R.** An AcMNPV Ac150 deletion mutant was constructed by cotransfecting Spodoptera frugiperda (SF-9) cells with p150–5’–hsp70/lacZ–3’–BSKS and wild-type AcMNPV DNA. The deletion mutant, AcΔ150, was identified by lacZ expression in infected SF-9 cells and isolated after four rounds of plaque purification. Various restriction endonucleases were used to digest AcΔ150 DNA prior to its analysis by 0.7 % agarose-gel electrophoresis and ethidium bromide staining; the restriction profiles indicated that the deletion mutant was constructed properly (data not shown). To further ensure the integrity of the DNA backbone of AcΔ150, we constructed a revertant, AcΔ150R, by co-transfecting SF-9 cells with AcΔ150 genomic DNA and a plasmid containing the AcMNPV EcoRI B fragment. AcΔ150R, lacking lacZ expression, was isolated after three rounds of plaque purification. AcΔ150R and wild-type AcMNPV DNAs were digested with various restriction endonucleases and compared by

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agarose-gel electrophoresis as above; the restriction profiles were indistinguishable (data not shown). The genetic integrities of AcD150 and AcD150R were further examined by PCR, with the products again analysed by agarose-gel electrophoresis and ethidium bromide staining (Fig. 1c). Primers 150E and 150F, designed to bind to the viral genomic sequences flanking Ac150 (Fig. 1b), were 5' - ATATCTTGTACTAGTGTCGGGGCGC-3' and 5' - CACAGTCGCCAGATTTGTTTGCCTCG-3', respectively. Primers lacZ1 and 2, designed to bind to the 5' and 3' regions of hsp70/lacZ (Fig. 1b), were 5' - CACAATAACCAGTTTGTTTTGGGATTCTAG-3' and 5' - CAGTTGGTCTGGTGTCAAAAATAATAATAA-3', respectively. All sequencing data confirmed the correct genetic construction of AcD150 and AcD150R (data not shown).

Virus preparation. Four viruses were used in the experiments described in this report: AcD150 and AcD150R (described above), AcMNPV-hsp70/lacZ (Engelhard et al., 1994) and AcMNPV E2, the parental wild-type virus (Smith & Summers, 1978). AcMNPV-hsp70/lacZ BV and ODV both have wild-type virulence levels in vivo (Engelhard et al., 1994; Washburn et al., 1995). Occlusion populations of each virus were generated from infected Sf-9 cells, harvested at 5 days post-infection and partially purified by sucrose-gradient centrifugation (Summers & Smith, 1987). Occlusions were suspended in a neutrally buoyant solution of glycerine and water (3 : 2, v/v) and quantified by using a haemocytometer (Washburn et al., 1995). ODV used in bioassays was liberated from occlusions by exposure to dilute alkaline saline and neutralized with 1 M Tris buffer. Undissolved occlusions and empty calyces were removed by pelleting at 2000 g for 10 min; subsequently, ODV in the supernatant was banded by density-equilibrium centrifugation on continuous 25–59% sucrose gradients for 1 h at 90 000 g. The resulting ODV bands were harvested and pooled, diluted 1 : 3 in PBS and pelleted at 90 000 g for 30 min. ODV pellets were collected in a minimal volume of PBS and aliquots of the two ODVs were quantified.

Fig. 1. Construction and confirmation of AcΔ150 and AcΔ150R. (a) Schematic diagram of the AcMNPV ORF150 region in AcMNPV wild-type DNA. Flanking ORFs are indicated, as are the orientations and approximate locations of primers used to construct p150-5'-hsp70/lacZ-150-3'-BSKS. (b) Schematic diagram of p150-5'-hsp70/lacZ-150-3'-BSKS. The hsp70/lacZ cassette is flanked by the 5' and 3' termini of the Ac150 ORF. The orientations and approximate locations of primers used to confirm the genetic integrities of AcΔ150 and AcΔ150R are indicated. (c) PCR and agarose-gel electrophoresis analysis of AcΔ150 and AcΔ150R. PCR products of the expected size were generated when using 150E and lacZ1 primers (lane 2) or 150F and lacZ2 primers (lane 3) with AcΔ150 DNA. No reaction products were generated when these primers were used with AcΔ150R DNA (lanes 4 and 5). When primers 150E and 150F were used in conjunction with AcΔ150R DNA, the PCR products generated were of the expected size (lane 6) and identical to those generated with wild-type E2 DNA (lane 7). The positions of molecular size markers (in kb) are indicated to the left of the blot.
by using a BSA protein assay (Pierce) (Haas-Stampleton et al., 2004). To stabilize ODV, we added BSA to a final concentration of 10 μg ml⁻¹ and dispensed small aliquots, which were stored at −20 °C until use. For bioassays, ODV inocula were thawed and diluted to the appropriate concentration in PBS immediately before use. BV was harvested at 72 h post-infection from the supernatant of SF-9 cells infected with each of the viruses and titrated by immunoplaque assay on SF-9 cells (Volkman & Goldsmith, 1982). For bioassays, BV stocks were diluted to the appropriate concentration with PBS and BSA (10 μg ml⁻¹, final concentration). Stocks of all viruses were stored at 4 °C in the dark until use.

**ODV content and nucleocapsid-packaging characteristics.** To compare ODV content of occlusions and nucleocapsid-packaging characteristics of AcΔ150, Ac150R and AcMNPV wild-type viruses, BV was harvested from 1.8 × 10⁶ occlusions of each virus and equal volumes were subjected to density-equilibrium centrifugation as described above. The banding patterns of each virus were compared by visual inspection and photographed prior to fractionation with PBS and BSA (10 μg ml⁻¹, final concentration). Stocks of all viruses were stored at 4 °C in the dark until use.

**Insects and virus inoculation.** For all experiments, we used fourth-instar larvae of *H. virescens, T. ni* or *S. exigua* reared from eggs provided by the USDA Western Cotton Research Laboratory, Phoenix, AZ, USA. All larvae were reared in groups at 28 ± 2 °C on a modified wheatgerm diet (Stoneville) until the onset of quiescence at the end of the third instar, indicative that larvae are preparing to moult to the fourth instar. For some experiments, large numbers of quiescent third instars were held between 4 and 15 °C until sufficient insects of the same developmental stage were available for testing (Washburn et al., 1995). Each larva was inoculated individually with occlusions, ODV or BV in 0.5–1.5 μl aliquots, using a microapplicator (Burkhard) fitted with a blunt- or sharp-tipped 32-gauge needle (for oral and intrahecmocoelic inoculations, respectively) mounted on a 1 ml tuberculin syringe (for details, see Washburn et al. (1995)). For one experiment, suspensions of AcΔ150 and AcMNPV wild-type occlusions additionally contain 1 % M2R dissolved in DMSO or just DMSO for control inocula (see Washburn et al., 1998).

Occlusions and ODV were administered orally by inserting the blunt-tipped needle through the mouth until the tip was well within the midgut lumen. BV was injected into the haemocoel by inserting the sharp-tipped needle through the planta of one of the prolegs, as described previously (Washburn et al., 1995). Larvae were inoculated orally within 15 min after moultling to the fourth instar (i.e. newly moulted larvae or 4th h after the moult (4–16 h)). For all BV inoculations, we used fourth-instar larvae 24 ± 6 h post-moult. After inoculation, test larvae were maintained individually in 25 ml plastic cups containing diet ad libitum in a growth chamber at 28 ± 2 °C.

**Bioassays and time-course experiments.** Bioassays were performed to compare the virulence of AcΔ150 and Ac150R occlusions, ODV and BV relative to those of AcMNPV wild-type in *H. virescens, T. ni* and *S. exigua*. For these and all additional assays described below, individual larvae were inoculated with varying dosages of inoculum (n = 22–32 larvae per dosage) administered orally or intrahecmocoelically as described above. All larvae were maintained until pupation or death from polyhedrosis disease, and baculovirus-induced mortality was confirmed by microscopic examination (400 ×) of cadaver tissues for occlusions. For each of the three species, we established the oral dose–mortality relationships for AcΔ150 by inoculating 4th larval with various occlusion numbers. The dose–mortality relationship for each species was quantified by the method of least squares and regression equations were used to calculate the LD₅₀ for each species. These values were compared with the LD₅₀ of 4th larvae inoculated with wild-type AcMNPV occlusions. A minimum of five assays was used to calculate the wild-type LD₅₀ for each species.

M2R is a still-borne-derived optical brightener known to bind chitin and damage the peritrophic membrane (Wang & Granados, 2000). To determine whether M2R affected the virulence of AcΔ150, 4² and 4¹⁶ T. ni were inoculated orally with 50 and 10 occlusions of AcΔ150 or AcMNPV wild-type virus, respectively, in the presence or absence of 1 % M2R. These dosages were predicted to generate final mortalities of between 30 and 50 %, levels sufficiently low to quantify M2R mortality enhancement, if present, for both developmental cohorts. Additional bioassays were conducted to compare the virulence of AcΔ150 and AcMNPV wild-type ODV in *H. virescens* and *T. ni*. In these experiments, identical dosages of between 0.1 and 100 pg of either AcΔ150 or wild-type ODV were administered orally to larval cohorts of each species.

To evaluate the effects of deleting Ac150 on pathogenesis in vivo, we conducted a time-course experiment using 4² *S. exigua* inoculated with occlusions of either AcΔ150 or AcMNPV-hsp70/λacZ. In this experiment, we used a dosage for each virus (determined from bioassays described above) that yielded final mortalities of ~85 %. At 4 h intervals during the first 24 h post-inoculation (p.i.), cohorts of between 26 and 32 larvae from each viral treatment were dissected and their midguts and associated tissues were removed. These tissues were processed to elucidate the blue β-galactosidase reporter signal and examined using stereo (10 ×) and/or compound microscopy (100–480 ×) in order to quantify infection foci and identify infected cell types (Engelhard et al., 1994; Washburn et al., 1995, 2003). For each host species, an additional cohort of 32 insects was inoculated orally with AcΔ150 or AcMNPV-hsp70/λacZ to confirm that the dosages used yielded the same final mortality.

**RESULTS**

**Bioassays**

In bioassays, we found that the virulence of AcΔ150 BV was identical to that of wild-type AcMNPV BV following intrahecmocoelic injection into fourth-instar *H. virescens, T. ni* and *S. exigua*. In all three hosts, a dosage of 1 p.f.u. resulted in final larval mortalities of between 52 and 86 %, depending on the host species (Table 1). Similarly, oral administration of 10 occlusions of either AcΔ150R or AcMNPV wild-type virus to 4th larvae yielded comparable levels of mortality in each of the hosts (Table 2), providing biological evidence that the genetic backbone of AcΔ150 was wild-type. In sharp contrast, significant differences in the virulence of occlusions of the AcΔ150 deletion mutant compared with wild-type virus were observed in all three host
species (Fig. 2). For each species, significantly more occlusions were required per LD$_{50}$ for Ac$_{150}$ compared with wild-type virus, but the amount varied among hosts. For 40-inoculated S. exigua and H. virescens, the Ac$_{150}$ dosages required were 4·1- and 5·6-fold greater than for wild-type, respectively, and for T. ni, the dosage was 18-fold greater (Fig. 2). These results indicated that Ac$_{150}$ indeed had an enhancing effect on per os infection and that the effect was host-specific.

**Time-course experiments**

In order to determine how the loss of Ac150 impacted on virulence of orally inoculated AcMNPV, we conducted a time-course experiment in which we used the lacZ reporter signal as a marker of infection to compare early viral pathogenesis of Ac$_{150}$ with AcMNPV-hsp70/lacZ in 40-inoculated S. exigua. In doing so, we defined several critical benchmarks of infection for each virus including: (i) the temporal onset of primary infection of midgut cells; (ii) the rate of primary infection of midgut cells; (iii) the number of infection foci generated per occlusion; (iv) the number of foci required to achieve comparable mortality; and (v) the rate at which primary cellular targets transmitted BV to secondary targets within the tracheal epidermis. In S. exigua inoculated with either Ac$_{150}$ or AcMNPV-hsp70/lacZ, lacZ expression (indicative of infection) was first observed at 8 h p.i. (Fig. 3). For both viral treatments, the proportion of LacZ-positive larvae increased rapidly between 8 and 16 h p.i., and by 16 h p.i. the proportions of larvae expressing LacZ were roughly equivalent to the final mortality determined in the companion Ac$_{150}$ and AcMNPV-hsp70/lacZ bioassays (~85 %) (Fig. 3a). Thus, the rate and timing of primary infection by Ac$_{150}$ were the same as for the control virus. Similarly, we detected no significant differences in either the numbers of foci at any time point during the first 24 h p.i. (analysis not shown) or in the number of foci required to generate the same final mortality (Fig. 3b). Notably, however, five times as many occlusions of Ac$_{150}$ were administered as AcMNPV-hsp70/lacZ, suggesting that control ODV was 5-fold more efficient at establishing primary foci than the deletion mutant. As with primary infection, the onset and rate of secondary infection of the tracheal epidermis of host larvae were identical for both viruses. Tracheal cells infected by the ODVs of Ac$_{150}$ and AcMNPV-hsp70/lacZ were first observed at low frequencies at 8 h p.i. and increased linearly with identical slopes until 24 h p.i., when sampling was curtailed (Fig. 3c).

**Table 2.** Mortalities (%) of H. virescens, T. ni and S. exigua inoculated orally as newly moulted fourth instars with 10 occlusions of either AcMNPV E2 or Ac$_{150}$R

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<th>H. virescens</th>
<th>T. ni</th>
<th>S. exigua</th>
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<tr>
<td>AcMNPV E2</td>
<td>48</td>
<td>39</td>
<td>57</td>
</tr>
<tr>
<td>Ac$_{150}$R</td>
<td>50</td>
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Each cohort contained 20–32 larvae.
As a consequence, cohorts of *S. exigua* larvae became systemically and fatally infected at exactly the same rate by the mutant and control viruses (Fig. 3d). Thus, the only significant difference between early viral pathogenesis of AcΔ150 and AcMNPV-hsp70/lacZ in *S. exigua* larvae was in the number of primary foci generated by each occlusion. Finally, in a second set of time-course experiments, we quantified the mean time to death for *H. virescens* larvae challenged orally or intrahaemocoelically with occlusions or BV, respectively, of AcΔ150 or AcMNPV wild-type, using dosages that yielded a final mortality of ~85% for each treatment. By both routes of introduction, the mean time to death by polyhedrosis disease (~100 h) was statistically the same for cohorts challenged with either AcΔ150 or AcMNPV E2 (data not shown).

**ODV content and nucleocapsid-packaging characteristics among AcΔ150, AcΔ150R and AcMNPV E2**

In order to determine whether there were differences in ODV content per occlusion or in nucleocapsid distribution among ODV populations that could account for differences in virulence, we banded ODV isolated from equal numbers of occlusions of AcΔ150, AcΔ150R and wild-type AcMNPV on matching sucrose-density gradients. In this experiment, no obvious differences in ODV concentration or in banding patterns were observed (Fig. 4a). Further analysis by density-gradient fractionation and $A_{254}$ measurement also failed to reveal any differences either in the amounts of ODV or in the relative proportion of each ODV band (Fig. 4b). Thus, the virion populations within occlusions of both the deletion mutant and its revertant were the same as wild-type virus with regard to ODV concentration and nucleocapsid distribution.

**Effects of M2R on virulence of AcΔ150 and AcMNPV wild-type administered orally**

Another possible explanation for the reduced number of primary foci by AcΔ150 could be that Ac150 enhances ODV contact with primary target cells, possibly by facilitating virion passage across the peritrophic membrane. We conducted an experiment, therefore, to see whether we could increase AcΔ150 oral virulence to wild-type levels by physically damaging the peritrophic membrane with M2R. M2R is a chemical with chitin-binding properties that has been shown to dissociate proteins from lepidopteran peritrophic membranes, presumably by competing with the chitin-binding domains of the proteins (Wang & Granados, 2000). We reasoned that if Ac150 acted in a manner similar to M2R, and if AcΔ150 occlusions were administered at fivefold the dosage of wild-type occlusions, then M2R should enhance infectivity of the AcΔ150 occlusions to a
greater degree than the wild-type. We orally inoculated 4\(^0\) and 4\(^{16}\) larvae of *T. ni* (the host exhibiting the greatest resistance to fatal infection in the absence of Ac150) with 50 occlusions of Ac150 or 10 occlusions of AcMNPV wild-type in the presence or absence of 1% M2R (Washburn *et al.*, 1998). M2R addition to the inoculum yielded no significant change in mortality in the 4\(^0\) cohorts challenged with either virus (Fig. 5). In the 4\(^{16}\) cohorts, *T. ni* larvae exhibited similar levels of developmental resistance to both viruses in the absence of M2R, whilst in the presence of M2R, mortality levels were rescued to 4\(^{0}\) levels. Thus, although M2R enhanced mortality significantly in the 4\(^{16}\) cohorts, the level of enhancement was the same for mutant and wild-type, even though the mutant virus was present at fivefold the concentration (Fig. 5). Hence, in both *T. ni* developmental cohorts, disruption of the physical integrity of the peritrophic membrane by M2R failed to rescue the reduction in oral virulence when Ac150 was deleted, suggesting that the mode of action of Ac150 was different from that of M2R.

### Comparison of virulence of Ac\(^{Δ150}\) and AcMNPV wild-type ODV administered orally

Lapointe *et al.* (2004) reported that Ac150 was associated with occluded and pre-occluded virus late and very late during infection. To determine whether Ac150 activity tracked with the ODV particle, we isolated the ODV from wild-type and Ac\(^{Δ150}\) occlusions and compared their virulence in 4\(^0\) *H. virescens* and 4\(^{16}\) *T. ni*. In both host species, there were no significant differences in the dose–mortality relationships of the deletion mutant and wild-type virus (Fig. 6). Thus, whilst occlusions of Ac\(^{Δ150}\) were 5-6- and 18-fold less virulent than occlusions of wild-type virus in *H. virescens* and *T. ni*, respectively, the purified ODVs of both exhibited identical virulence following oral administration.

### DISCUSSION

Our results showed unequivocally that Ac150 enhanced oral infection; from 4- to 18-fold more Ac\(^{Δ150}\) occlusions than wild-type occlusions were required to achieve 50% mortality, depending on the host species. No differences were found in ODV content per occlusion, nor in nucleocapsid distribution among the ODV populations, that could explain the observed differences in virulence. In time-course experiments using *λacZ* expression to elucidate the
Interestingly, whilst the occlusions of AcΔ150 were less infectious orally than wild-type occlusions, the isolated ODVs had the same infectivity. These results suggested that exposure to dilute alkaline saline inactivated Ac150 or that it was lost during ODV purification, or both. The lack of Ac150 activity associated with purified ODV is consistent with the findings of Braunagel et al. (2003), who found no evidence of Ac150 in AcMNPV ODV by using multiple analytical approaches.

To test whether Ac150 facilitated passage of ODV across the peritrophic membrane (which could explain the reduced efficiency of primary infection), we inoculated AcΔ150 occlusions in the presence of the stilbene-derived optical brightener, M2R, which is known to release proteins from the peritrophic membrane and cause holes to form (Wang & Granados, 2000). In our experiments, addition of 1% M2R failed to enhance mortality levels generated by the deletion mutant to expected levels if Ac150 worked by a similar mechanism to M2R. This result was consistent with the lack of chitin-binding activity reported by Lapointe et al. (2004).

It is possible that Ac150 has a role in signalling. Integrins are known to propagate signalling when bound by a ligand, and Ac150 has an RGD integrin-binding motif in the middle of a cluster of charged amino acids. Alternatively, microvilli of midgut cells are coated heavily with glycosylated proteins and the peritrophin-A domain of Ac150 may bind one of these. A number of membrane-bound receptors for growth factors and cytokines are glycosylated, and evidence indicates that oligosaccharide moieties are crucial for the functions of some of those receptors (Takahashi et al., 2004). Whether or not Ac150 binds to midgut cells at all, however, remains to be determined.

**REFERENCES**


In vivo effects of Ac150


