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Autographa californica multiple nucleopolyhedrovirus ODV-E56 envelope protein is required for oral infectivity and can be substituted functionally by Rachiplusia ou multiple nucleopolyhedrovirus ODV-E56

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Abstract

he *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) *odv-e56*gene encodes an occlusion-derived virus (ODV)-specific envelope protein, ODV-E56. In a previous analysis, the *odv-e56* gene was found to be under positive selection pressure, suggesting that it may be a determinant of virus host range. To assess the role of ODV-E56 in oral infectivity and host range, we constructed recombinant AcMNPV clones (Ac69GFP-e56lacZ and AcIEGFP-e56lacZ) in which ODV-E56 protein synthesis was eliminated by inserting a β-galactosidase (*lacZ*) expression cassette into the *odv-e56* open reading frame. We also constructed a recombinant virus, Ac69GFP-Roe56, in which the native AcMNPV *odv-e56* coding sequence was replaced with that of *Rachiplusia ou*multiple nucleopolyhedrovirus (RoMNPV), a closely related virus that is significantly more virulent towards some host species than AcMNPV. The *odv-e56* recombinant viruses exhibited no alterations in polyhedron production and morphogenesis or in the production of infectious budded virus in cell culture. In bioassays using three lepidopteran host species, the oral infectivities of the *odv-e56* mutant viruses Ac69GFP-e56lacZ and AcIEGFP-e56lacZ were profoundly impaired compared with those of wild-type and control recombinant viruses. Oral infectivity was restored fully by marker rescue of the *odv-e56* mutant viruses with either the AcMNPV or the RoMNPV*odv-e56* gene. In bioassays using two host species that are more susceptible to RoMNPV than to AcMNPV, Ac69GFP-Roe56 killed larvae with LC_{50} values similar to those of recombinant viruses expressing AcMNPV ODV-E56. This result indicated that replacement of the AcMNPV *odv-e56* gene with the RoMNPV orthologue did not increase virulence against these two species.

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Comments

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Autographa californica multiple nucleopolyhedrovirus ODV-E56 envelope protein is required for oral infectivity and can be substituted functionally by *Rachiplusia ou* multiple nucleopolyhedrovirus ODV-E56

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The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) *odv-e56* gene encodes an occlusion-derived virus (ODV)-specific envelope protein, ODV-E56. In a previous analysis, the *odv-e56* gene was found to be under positive selection pressure, suggesting that it may be a determinant of virus host range. To assess the role of ODV-E56 in oral infectivity and host range, we constructed recombinant AcMNPV clones (Ac69GFP-e56lacZ and AcIEGFP-e56lacZ) in which ODV-E56 protein synthesis was eliminated by inserting a β-galactosidase (*lacZ*) expression cassette into the *odv-e56* open reading frame. We also constructed a recombinant virus, Ac69GFP-Roe56, in which the native AcMNPV *odv-e56* coding sequence was replaced with that of *Rachiplusia ou* multiple nucleopolyhedrovirus (RoMNPV), a closely related virus that is significantly more virulent towards some host species than AcMNPV. The *odv-e56* recombinant viruses exhibited no alterations in polyhedron production and morphogenesis or in the production of infectious budded virus in cell culture. In bioassays using three lepidopteran host species, the oral infectivities of the *odv-e56* mutant viruses Ac69GFP-e56lacZ and AcIEGFP-e56lacZ were profoundly impaired compared with those of wild-type and control recombinant viruses. Oral infectivity was restored fully by marker rescue of the *odv-e56* mutant viruses with either the AcMNPV or the RoMNPV *odv-e56* gene. In bioassays using two host species that are more susceptible to RoMNPV than to AcMNPV, Ac69GFP-Roe56 killed larvae with LC_{50} values similar to those of recombinant viruses expressing AcMNPV ODV-E56. This result indicated that replacement of the AcMNPV *odv-e56* gene with the RoMNPV orthologue did not increase virulence against these two species.

INTRODUCTION

Baculoviruses are rod-shaped, occluded viruses with circular, double-stranded DNA genomes in the family *Baculoviridae* (Bonning, 2005; Jehle *et al.*, 2006; Rohrmann, 2008). These viruses have been identified exclusively in arthropods, with most isolates identified from insects within the order Lepidoptera. A considerable amount of work has been done on baculoviruses due to their potential and realized applications as biopesticides and recombinant protein expression vectors (Kost *et al.*, 2005; Moscardi, 1999; Summers, 2006; van Beek & Davis, 2007). Most studies on baculoviruses have focused on members of the genera *Alphabaculovirus* (lepidopteran nucleopolyhedroviruses, or NPVs) and *Betabaculovirus* (lepidopteran granuloviruses, or GVs). Virion morphogenesis of these baculoviruses, as well as mosquito baculoviruses (genus *Deltabaculovirus*), is distinguished by the production of two physically and biochemically distinct types of infectious virus particles: occlusion-derived virus (ODV) and budded virus (BV) (Braunagel & Summers, 1994; Volkman & Summers, 1977; Volkman *et al.*, 1976). Both ODV and BV contain rod-shaped nucleocapsids that are assembled within the nucleus. ODV nucleocapsids are enveloped within the nucleus and occluded within a matrix of viral protein (polyhedrin or granulin) to form occlusion bodies (OB) or polyhedra/granules. BV nucleocapsids exit the nucleus and acquire an envelope derived from the plasma membrane of the host cell upon budding through the membrane. ODV virions infect the host insect's midgut epithelial cells when OBs are ingested by the host and solubilized in the midgut lumen, releasing the ODV. BV that is assembled during the primary infection of

midgut cells (and also during subsequent secondary infection of other tissues) serves as a vehicle to spread infection to other susceptible tissues in the host (Bonning, 2005; Rohrmann, 2008).

The virulence of alphabaculoviruses against different species within the order Lepidoptera is highly variable. Although some genes that influence baculovirus virulence against individual species have been identified (Chen & Thiem, 1997; Clem & Miller, 1993; Clem *et al.*, 1991; Croizier *et al.*, 1994; Lapointe *et al.*, 2004; Lu & Miller, 1996; Lu *et al.*, 2003; Maeda *et al.*, 1993; Popham *et al.*, 1998), our understanding of the molecular determinants of baculovirus species-specific virulence and host range is incomplete. The accumulation of non-synonymous (amino acid-changing) nucleotide substitutions in specific baculovirus genes may facilitate adaptation to a new host species, or counter host defences to increase virulence against a particular host. Hence, it may be possible to identify candidate baculovirus genes that influence host range and virulence through an examination of non-synonymous (d_N) and synonymous (d_S) substitution rates to identify genes that have undergone positive selection, in which $d_N > d_S$ (Aguileta *et al.*, 2009; Yang, 2007). Selection pressure analysis carried out with gene sequences from other viruses has identified positively selected genes and codon sites within those genes that are involved in host immune recognition, receptor binding, antiviral drug resistance, epidemics and changes in virulence (Banke *et al.*, 2009; Bennett *et al.*, 2003, 2006; Brault *et al.*, 2007; Holmes *et al.*, 2002; Twiddy *et al.*, 2002; Woelk & Holmes, 2001; Woelk *et al.*, 2001; Zlateva *et al.*, 2004). Mutations at a single positively selected site in the helicase gene of West Nile virus were sufficient to increase its virulence against American crows significantly (Brault *et al.*, 2007). Hence, selection pressure analysis can potentially identify differences among highly conserved viral sequences that account for substantial differences in virulence, without prior knowledge of the contributions to pathogenesis by the sequences being analysed.

Selection pressure analysis of sequences from 83 NPV genes using codon-subsitution models revealed nine baculovirus genes predicted to have undergone positive selection (Harrison & Bonning, 2004). One of the genes identified through this analysis was *odv-e56*, a baculovirus core gene found in all baculovirus genomes examined to date (van Oers & Vlak, 2007). The *odv-e56* gene is expressed late during infection and encodes an ODV envelope protein (Braunagel *et al.*, 1996; Theilmann *et al.*, 1996). Five other ODV envelope proteins – P74, PIF-1, PIF-2, PIF-3 and PIF-4 – have been found to be required for oral infectivity of alphabaculovirus ODV (Fang *et al.*, 2009; Faulkner *et al.*, 1997; Kikhno *et al.*, 2002; Kuzio *et al.*, 1989; Ohkawa *et al.*, 2005; Pijlman *et al.*, 2003; Song *et al.*, 2008). These proteins have been referred to as *per os* infectivity factors (PIFs).

In this study, we examined the role of ODV-E56 in oral infectivity with recombinant clones of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) in which expression of ODV-E56 had been eliminated. We also produced a recombinant virus in which the AcMNPV *odv-e56* coding sequence had been replaced with that of *Rachiplusia ou* multiple nucleopolyhedrovirus (RoMNPV) and tested for altered virulence in selected host species. AcMNPV and RoMNPV (which is a variant of *Anagrapha falcifera* multiple nucleopolyhedrovirus) are genetically very similar and have broad, overlapping host ranges (Harrison, 2009b; Harrison & Bonning, 1999; Hostetter & Puttler, 1991). However, individual species within their host ranges are significantly more susceptible to RoMNPV than to AcMNPV (Cardenas *et al.*, 1997; Harrison & Bonning, 1999; Hostetter & Puttler, 1991; Lewis & Johnson, 1982; Vail *et al.*, 1993). Among these species are *Helicoverpa zea*, the corn earworm, and *Ostrinia nubilalis*, the European corn borer (Harrison & Bonning, 1999). The production of BVs and OBs by the recombinant viruses created in this study was measured, and their virulence towards three species of lepidopteran hosts was evaluated to see whether ODV-E56 was required for oral infectivity and whether a recombinant AcMNPV clone expressing the RoMNPV *odv-e56* gene exhibited a higher degree of virulence towards *Helicoverpa zea* and *Ostrinia nubilalis*.

RESULTS

Characteristics of recombinant viruses

Recombinant viruses carrying the *hsp70* promoter–*lacZ* expression cassette in the *odv-e56* open reading frame (ORF) in either orientation were produced successfully by standard co-transfection of parental viral DNA (AcMLF9.EGFP, AcIETV3.EGFP) with transfer vectors carrying the disrupted *odv-e56* ORF (Fig. 1a). Although marker rescue of the *odv-e56* mutant viruses was easily accomplished with a plasmid (pAcClaI-F) carrying the wild-type *odv-e56* sequence, replacement of the native AcMNPV *odv-e56* ORF with the RoMNPV *odv-e56* ORF using an RoMNPV-derived amplimer was inefficient. Only one clone was obtained that contained enough of the RoMNPV *odv-e56* ORF sequence to include all of the amino acid substitutions that distinguish the AcMNPV and RoMNPV gene products (Fig. 1a, b). These differences include 12 amino acid substitutions and an insertion of two additional amino acids near the C terminus of the RoMNPV sequence (Fig. 1b). Two of the substitutions (S97G and I199N) were identified as sites under positive selection pressure (Harrison & Bonning, 2004), whilst a single other substitution (M344I) is located within a conserved hydrophobic region identified in an alignment of five ODV-E56 sequences (Theilmann *et al.*, 1996). No other substitutions occur in a second conserved hydrophobic region identified in the sequence, or in conserved cysteine residues and a putative *N*-glycosylation site (Theilmann *et al.*, 1996). Clones containing *lacZ* in the opposite orientation with respect to *odv-e56* in AcMLF9.EGFP (Ac69GFP-e56lacZ) and in the same

orientation as *odv-e56* in AcIE1TV3.EGFP (AcIE1GFP-e56lacZ) were selected for further study, along with their accompanying marker-rescued viruses (Ac69GFP-e56R and AcIE1GFP-e56R; Fig. 1).

Western blot analysis of ODV protein from polyhedra derived from *Heliothis virescens* larvae revealed that both Ac69GFP-e56lacZ and AcIE1GFP-e56lacZ were missing a protein migrating at 48 kDa that was present in the wild-

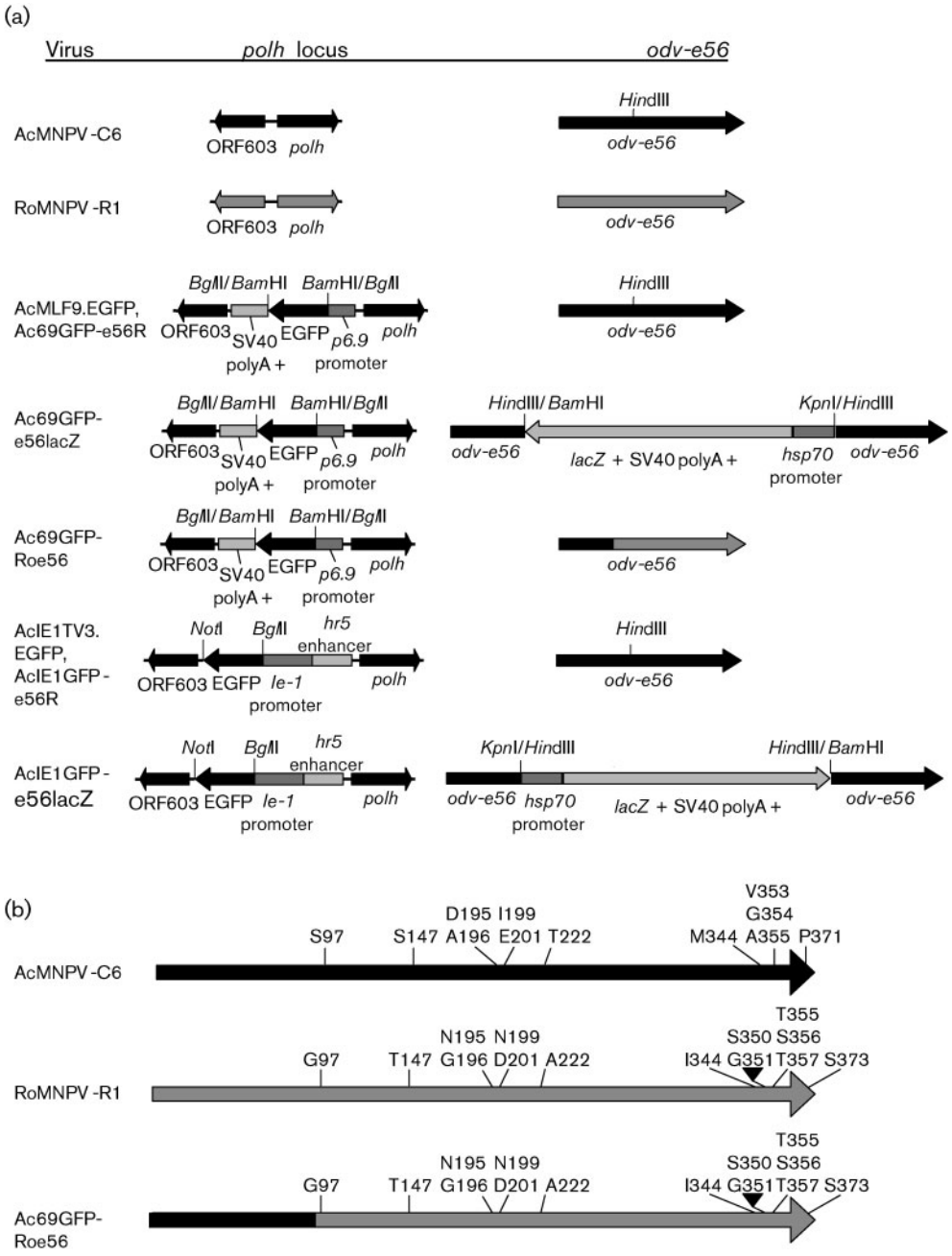

Fig. 1. Construction of recombinant viruses used in this study. (a) Schematic diagram of the *polh* and *odv-e56* loci (centre and right columns, respectively) in wild-type and recombinant viruses used in this study. ORFs are indicated as arrows, with the direction of the arrow indicating the orientation of the ORF. The restriction enzyme sites into which EGFP and *lacZ* reporter genes were inserted are indicated, as are the enhancer/promoter sequences and simian virus 40 (SV40) polyadenylation signal. (b) Schematic diagram of the *odv-e56* ORF in AcMNPV-C6, RoMNPV-R1 and Ac69GFP-Roe56, showing positions of amino acid substitutions in the ODV-E56 sequences of these viruses, as well as two additional amino acids in the RoMNPV sequence (positions 350–351).

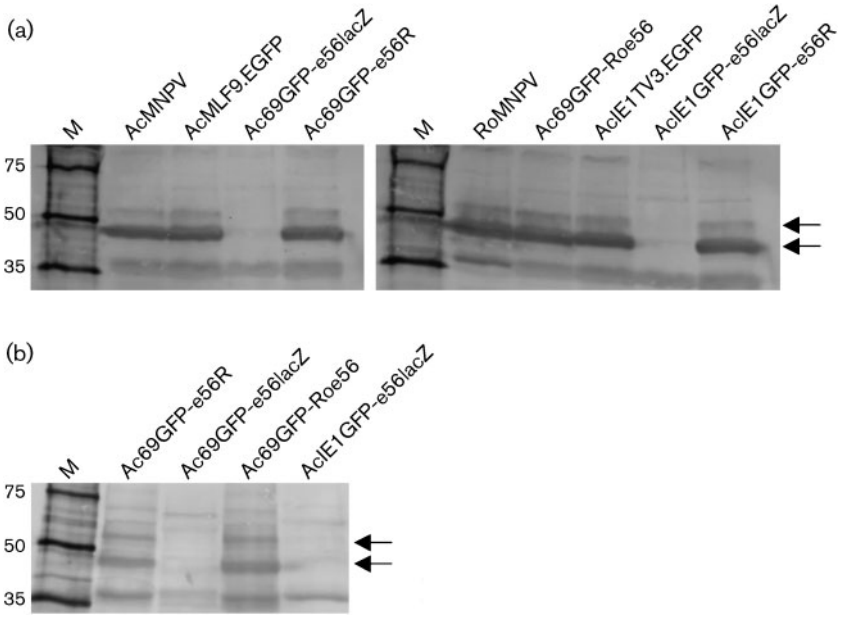

Fig. 2. Western blot analysis of ODV-E56 in samples from (a) total ODV protein derived from virus-killed *Heliothis virescens* larvae and (b) ODV envelope protein from infected *Trichoplusia ni* BTI-TN-5B1-4 cells. The identity of the virus for each protein sample is shown above each lane, and molecular mass marker lanes (M) and sizes (in kDa) of individual markers are indicated on the left of each panel. ODV-E56-specific bands are indicated by arrows.

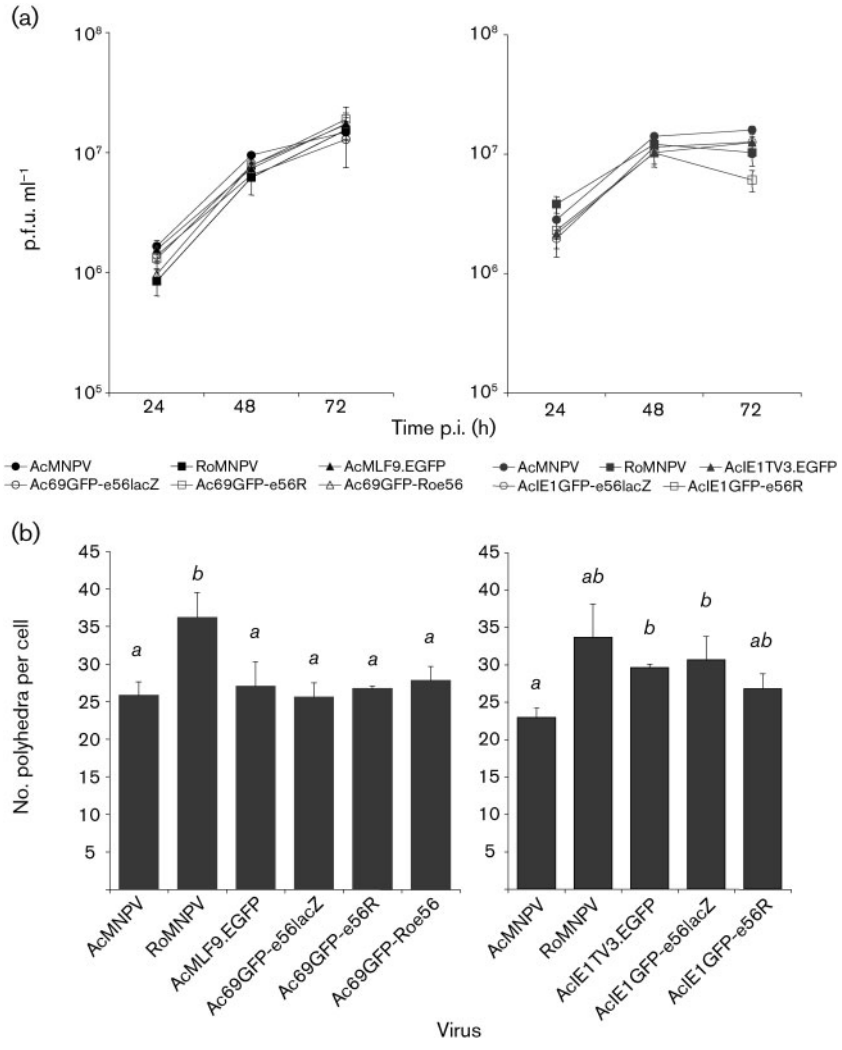

Fig. 3. (a) Budded virus produced by *Sf*9 cells infected with AcMNPV-C6, RoMNPV-R1 and recombinant viruses derived from either AcMLF9.EGFP (left graph) or AcIE1TV3.EGFP (right graph), displayed as mean p.f.u. ml^{-1} of three replicate samples per time point. (b) Polyhedra produced by *Sf*9 cells infected with the same viruses as in (a). Mean numbers of polyhedra per cell from three replicate infections per virus are shown. For both (a) and (b), error bars represent 1 SD. For (b), values with different letters are significantly different at $P<0.05$.

type AcMNPV and in the parental and marker-rescued viruses, indicating that insertion of the *lacZ* expression cassette had eliminated expression of ODV-E56 (Fig. 2a). Whilst the AcMNPV *odv-e56* ORF encodes a protein of approximately 41 kDa, the relative mobility of ODV-E56-specific bands in SDS-PAGE gels of AcMNPV and *Orygia pseudotsugata* multiple nucleopolyhedrovirus has varied from 43 to 56 kDa in previous reports (Braunagel *et al.*, 1996; Theilmann *et al.*, 1996). No bands that could correspond to a partial protein product from the N-terminal part of the *odv-e56* ORF upstream of the *lacZ* insertion were observed in the mutant *odv-e56* virus lanes. A minor band migrating at 58 kDa was also missing from the *lacZ* insertion mutants. The ODV-E56 antibodies also bound to proteins of the same size in RoMNPV and Ac69GFP-Roe56 ODV, indicating that the AcMNPV ODV-E56 antibodies recognized the RoMNPV ODV-E56 protein. The same results were observed with total cellular protein from infected *Sf*9 and BTI-TN-5B1-4 cells and with clones of Ac69GFP-e56lacZ and AcIE1GFP-e56lacZ with the *lacZ* expression cassette inserted in opposite orientations (data not shown).

ODV-E56 was also absent from ODV envelope protein samples prepared from BTI-TN-5B1-4 cells infected with the *odv-e56* mutant viruses Ac69GFP-e56lacZ and AcIE1GFP-e56lacZ (Fig. 2b). The presence of ODV-E56 bands in the Ac69GFP-Roe56 ODV envelope protein sample suggested that the RoMNPV ODV-e56 protein was being assembled into AcMNPV ODV.

Quantification of BV produced by wild-type and recombinant viruses revealed that, whilst there were some small but significant differences between selected pairs of the viruses at some time points (Fig. 3a), eliminating expression of *odv-e56* or replacing AcMNPV *odv-e56* with the RoMNPV orthologue did not significantly affect production of infectious BV observed at 24 h post-infection (p.i.) and afterwards. There also was no effect on production of polyhedra (Fig. 3b). By 96 h p.i., *Sf*9 cell cultures infected with RoMNPV-R1 exhibited a visibly greater degree of cell lysis and number of free-floating polyhedra than cultures infected with wild-type AcMNPV and the AcMNPV recombinants, although there was not a consistently significant difference in numbers of polyhedra produced between these viruses (Fig. 3b). Ultrastructural observations on infected BTI-TN-5B1-4 cells indicated that elimination of ODV-E56 expression or replacement with RoMNPV ODV-E56 also did not appear to affect ODV morphogenesis and assembly into polyhedra (Fig. 4; compare b and d with a, c and f).

Biological activity against larvae

An initial attempt to produce a polyhedron stock for bioassays by orally inoculating fifth-instar *Heliothis virescens* larvae with cell culture-derived polyhedra revealed an impairment in the oral infectivity of the *odv-e56* mutant viruses. Whilst a dose of 1×10^6 polyhedra from wild-type and recombinant viruses carrying an intact *odv-e56* gene was sufficient to kill all larvae in a test population, little to no mortality was observed when larvae were fed this dose of polyhedra from viruses with the *lacZ* cassette inserted in *odv-e56*. To investigate further the biological activity of the recombinant viruses, droplet-feeding bioassays were carried out with polyhedron stocks produced by intrahaemocoelic injection of larvae with BV. Whilst viruses expressing ODV-E56 killed neonate *Heliothis virescens* larvae with LC_{50} values ranging from 0.78×10^5 to 1.30×10^5 polyhedra ml^{-1}, $<50\,\%$ mortality was observed with AcIE1GFP-e56lacZ, even at a dose of 1.5×10^9 polyhedra ml^{-1} (Table 1). Ac69GFP-e56lacZ was able to achieve mortalities $>50\,\%$ in bioassays, with LC_{50} values that were approximately two orders of magnitude higher than those of viruses expressing ODV-E56. In additional bioassay data against fourth-instar larvae, two additional clones of Ac69GFP-e56lacZ and AcIE1GFP-e56lacZ that carried the *lacZ* expression cassette in opposite orienta-

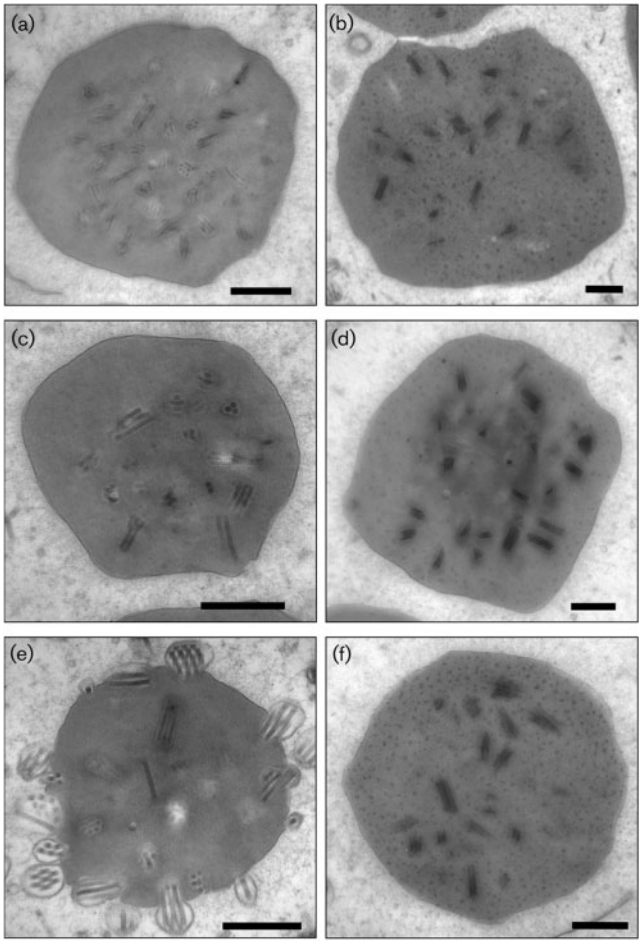

Fig. 4. Electron micrographs of viral occlusions in cells infected by Ac69GFP-e56R (a), Ac69GFP-e56lacZ (b), AcIE1GFP-E56R (c), AcIE1GFP-E56lacZ (d), RoMNPV-R1 (e) and Ac69GFP-RoE56 (f). Cultures were harvested at 72 h p.i. Bars, 500 nm.

Table 1. Dose–mortality response of neonate larvae infected with wild-type and recombinant AcMNPV and RoMNPV

For each host and group of viruses, values with different superscript letters (*a–d*) are significantly different at $P<0.05$. ND, Not determined due to <50 % mortality at the highest dose.

Host/virus	LC_{50}* $\times 10^5$ (95 % CL)	Slope (±SEM)	Heterogeneity
Heliothis virescens			
AcIE1TV3.EGFP	1.20^a (0.85–1.65)	1.95 (±0.331)	0.28
AcIE1GFP-e56lacZ	ND		
AcIE1GFP-e56R	1.30^a (0.95–1.77)	2.00 (±0.312)	0.77
AcMLF9.EGFP	0.89^a (0.63–1.20)	2.10 (±0.293)	0.67
Ac69GFP-e56lacZ	320.0^b (210.2–449.2)	1.96 (±0.409)	0.33
Ac69GFP-e56R	0.84^a (0.35–1.49)†	2.16 (±0.323)	2.44
Ac69GFP-Roe56	0.78^a (0.53–1.06)	2.09 (±0.318)	0.91
Helicoverpa zea			
AcMNPV-C6	3.40^a (2.44–4.98)	1.52 (±0.261)	0.15
RoMNPV-R1	0.54^b (0.38–0.71)	1.90 (±0.303)	0.33
AcMLF9.EGFP	2.90^a (1.90–4.43)	1.25 (±0.189)	0.20
Ac69GFP-e56lacZ	249.5^b (177.1–358.3)	1.60 (±0.227)	0.81
Ac69GFP-e56R	1.52^{cd} (0.50–3.29)	1.18 (±0.188)	1.13
Ac69GFP-Roe56	2.75^{ad} (0.91–7.85)	1.10 (±0.181)	1.38
Ostrinia nubilalis			
AcMNPV-C6	4339.5^a (2289.2–14091.6)	1.58 (±0.257)	1.43
RoMNPV-R1	185.7^b (133.7–337.8)	2.14 (±0.438)	0.10
AcMLF9.EGFP	3259.9^a (1248.7–7362.7)	1.26 (±0.294)	1.56
Ac69GFP-e56lacZ	ND		
Ac69GFP-e56R	2675.5^a (1995.4–3483.0)	2.45 (±0.387)	0.04
Ac69GFP-Roe56	3059.9^a (2023.2–4369.5)	1.71 (±0.323)	0.65

*Values are no. polyhedra ml^{-1}, reported with 95 % confidence limits (CL).
†90 % CL.

tions exhibited levels of impairment in infectivity similar to that observed with the clone of AcIE1GFP-e56lacZ used in bioassays against neonate larvae (data not shown). PCR with *odv-e56* primers on solubilized samples of the bioassay polyhedron stocks produced a faint amplimer for the Ac69GFP-e56lacZ template corresponding to an uninterrupted *odv-e56* ORF, suggesting that the polyhedron stock for this particular clone may carry a minor level of contamination with a virus that expresses ODV-E56. This could explain the difference in dose–mortality characteristics between AcIE1GFP-e56lacZ and Ac69GFP-e56lacZ in bioassays.

Bioassays with AcMNPV-C6 and RoMNPV-R1 against *Helicoverpa zea* and *Ostrinia nubilalis* confirmed previous reports that RoMNPV-R1 is significantly more virulent than AcMNPV against these species (Harrison & Bonning, 1999; Hostetter & Puttler, 1991; Lewis & Johnson, 1982), by magnitudes of approximately 6-fold for *Helicoverpa zea* and approximately 23-fold for *Ostrinia nubilalis* (Table 1). In bioassays with recombinant viruses, Ac69GFP-Roe56 did not kill larvae of these species with significantly lower LC_{50} values than viruses carrying an intact AcMNPV *odv-e56* ORF, suggesting that replacing AcMNPV *odv-e56* with the RoMNPV orthologue was not sufficient to alter virulence against these host species.

DISCUSSION

There are many baculovirus proteins in the ODV envelope, but only a small proportion have been examined with respect to their role in oral infectivity (Braunagel & Summers, 2007; Rohrmann, 2008; Slack & Arif, 2007). The results presented above indicate that ODV-E56 is required for oral infectivity, but not production of infectious BV, much like the PIF proteins P74, PIF-1, PIF-2, PIF-3 and PIF-4. Electron micrographs of *odv*-e56 mutants indicate that ODV-E56 is not required for virion morphogenesis or occlusion assembly. In a previous study, Braunagel *et al.* (1996) generated a recombinant AcMNPV in which the *odv-e56* ORF was replaced with a fusion between the N terminus of ODV-E56 and β-galactosidase. Electron micrographs of cells infected with this virus also did not show any obvious defects in ODV or occlusion body morphogenesis.

The RoMNPV *odv-e56* gene was able to substitute functionally for the AcMNPV orthologue in AcMNPV ODV, as Ac69GFP-Roe56 killed *Heliothis virescens* larvae with LC_{50} values similar to those of AcMNPV viruses with intact *odv-e56* genes. This suggests that the amino acid substitutions encoded by the RoMNPV orthologue affect neither interactions of ODV-E56 with host proteins that may act as a receptor for ODV in midgut cells, nor

interactions with other ODV envelope or capsid proteins that are required for infectivity. Replacing AcMNPV *odv-e56* with the RoMNPV ORF also did not alter biological activity towards two host species that differ in their susceptibility to AcMNPV and RoMNPV. Of the 13 codon positions in *odv-e56* that were identified previously as being under positive selection pressure (Harrison & Bonning, 2004), only two (S97 and I199) differ in the RoMNPV amino acid sequence (Fig. 1b). It may be the case that *odv-e56* has not been under a significant amount of positive selection pressure in the lineage containing AcMNPV and RoMNPV, or has not undergone sufficient changes since the divergence of these NPVs to alter its function. It may be that several genes (perhaps those under positive selection pressure) would need to be swapped between AcMNPV and RoMNPV before host range and species-specific virulence would be altered detectably. Alternatively, ODV-E56 may influence virulence against host species other than *Heliothis zea* and *Ostrinia nubilalis*.

Research is under way to characterize further the role of ODV-E56 as a PIF, including determining whether ODV-E56 is directly required for binding to midgut cells and fusion with the cell membrane.

METHODS

Cells, viruses and insects. The *Spodoptera frugiperda Sf*9 cell line (Vaughn *et al.*, 1977) was maintained in TNM-FH medium (Sigma) supplemented with 10 % fetal bovine serum (Invitrogen), antibiotics (10 U penicillin, 0.1 mg streptomycin and 0.25 μg amphotericin B ml^{-1}; Sigma) and 0.1 % Pluronic F-68 (JRH Biosciences). *Trichoplusia ni* BTI-TN-5B1-4 ('High Five'; Wickham *et al.*, 1992) cells were maintained in Ex-Cell 405 medium (SAFC Biosciences) supplemented with antibiotics as described above.

The wild-type AcMNPV strain C6 (Possee, 1986), RoMNPV strain R1 (Smith & Summers, 1980) and the recombinant viruses described in this study were propagated in *Sf*9 cells and titrated by plaque assay on *Sf*9 cells as described by Summers & Smith (1987). AcBacPAK6 (Kitts & Possee, 1993) was used as the parental virus for the first round of recombinant virus construction in this study.

Eggs of *Heliothis virescens* and *Helicoverpa zea* were obtained from Bio-Serv. Eggs of *Ostrinia nubilalis* were obtained from the USDA-ARS Corn Insects and Crop Genetics Research Unit in Ames, IA, USA. Larvae for all three species were reared at 28 °C and in a 14 : 10 light : dark cycle on species-specific diets obtained from Southland Products.

Construction of recombinant viruses. The plasmid pAcP(+)IE1eGFP, a gift from Dr Don Jarvis (University of Wyoming, Laramie, WY, USA), was constructed by ligating the *Bgl*II–*Not*I fragment from plasmid pEGFP-N1 (Clontech) containing the enhanced green fluorescent protein (EGFP) coding sequence into the corresponding sites in AcMNPV transfer vector pAcP(+)IE1TV3 (Jarvis *et al.*, 1996). To construct transfer vector pAcMLF9.EGFP, a subclone containing the EGFP coding sequence was produced by ligating a *Spe*I–*Sac*II fragment from pAcP(+)IE1eGFP into pBluescript II KS(+) (Stratagene) to produce plasmid pEGFP. A *Bam*HI fragment from this plasmid containing the EGFP coding sequence was subcloned into the *Bgl*II site of AcMNPV transfer vector pAcMLF9 (Harrison & Bonning, 2000; GenBank accession no. EF050536).

Transfer vectors were also constructed to inactivate AcMNPV *odv-e56* by insertion of *Escherichia coli* β-galactosidase (*lacZ*) under the control of the *Drosophila melanogaster hsp70* promoter, an expression cassette originally obtained from plasmid pAcDZ1 (Weyer *et al.*, 1990). The 7.1 kb *Cla*IF fragment of AcMNPV-C6, containing the *odv-e56* gene, was cloned into a modified version of pBluescript II KS(+) from which the restriction sites for *Hin*dIII, *Eco*RV, *Eco*RI, *Pst*I and *Sma*I had been eliminated to produce plasmid pAcClaI-F. A 3.75 kbp *Bam*HI–*Kpn*I fragment containing the *hsp70* promoter–*lacZ* expression cassette was inserted into the *Hin*dIII site of *odv-e56* by blunt-end ligation. Clones from this assembly step with the *hsp70-lacZ* cassette in either orientation were identified and named pAcodve56-hsplacZ(+) and pAcodve56-hsplacZ(−), for clones with *lacZ* oriented in the same or opposite orientation as the *odv-e56* ORF, respectively.

Recombinant viruses were generated by co-transfection of *Sf*9 cells with virus DNA and transfer vector plasmids by liposome-mediated transfection using Cellfectin (Invitrogen) following the manufacturer's instructions, followed by plaque assay of the cell medium 5 days post-transfection. To produce viruses AcMLF9.EGFP and AcIE1TV3.EGFP (Fig. 1), *Sf*9 cells were co-transfected with pAcMLF9.EGFP or pAcP(+)IE1eGFP, respectively, together with AcBacPAK6 DNA that had been linearized by digestion with *Bsu*36I. To produce recombinant viruses with insertionally inactivated *odv-e56* genes, AcMLF9.EGFP and AcIETV3.EGFP viral DNAs were co-transfected with transfer vectors pAcodve56-hsplacZ(−) and pAcodve56-hsplacZ(+) to produce viruses Ac69GFP-e56lacZ and AcIE1GFP-e56lacZ, respectively. To generate marker-rescued versions of the *odv-e56* inactivation mutants, Ac69GFP-e56lacZ and AcIE1GFP-e56lacZ viral DNAs were linearized by digestion with *Bsu*36I, which cuts within the *lacZ* sequence, and co-transfected with pAcClaI-F to make viruses Ac69GFP-e56R and AcIE1GFP-e56R, respectively. To produce recombinant AcMNPV in which the native *odv-e56* coding sequence was replaced with the RoMNPV-R1 coding sequence, *Bsu*36I-linearized Ac69GFP-e56lacZ and AcIE1GFP-e56lacZ DNAs were co-transfected with a PCR amplimer consisting of the RoMNPV-R1 *odv-e56* ORF (nt 126682–127818 of the RoMNPV-R1 genome sequence; Harrison & Bonning, 2003). The single usable clone recovered from these co-transfections was called Ac69GFP-Roe56 (Fig. 1).

Methods for selection and plaque purification of recombinant viruses were followed essentially as described by Summers & Smith (1987). For AcMLF9.EGFP and AcIE1TV3.EGFP, an occlusion-positive/*lacZ*-negative plaque phenotype was selected against an occlusion-negative/*lacZ*-positive background. For Ac69GFP-e56lacZ and AcIE1GFP-e56lacZ, an occlusion-positive/*lacZ*-positive phenotype was selected against an occlusion-positive/*lacZ*-negative background. For Ac69GFP-e56R, AcIE1GFP-e56R and Ac69GFP-Roe56, an occlusion-positive/*lacZ*-negative phenotype was selected against an occlusion-positive/*lacZ*-positive background. Plaque purification was carried out for at least three rounds, or until no plaques with a parental phenotype could be seen on the dish. Recombinant viruses were checked for proper integration of marker genes and the absence of second-site recombination by restriction endonuclease digestion and PCR amplification and sequencing of the *odv-e56* locus.

Western blotting of ODV protein. Monolayer cultures of BTI-TN-5B1-4 cells were infected at an m.o.i. of 1 with wild-type and recombinant viruses. Cells and free-floating polyhedra were collected by centrifugation 5 days p.i. Polyhedra were extracted from cell pellets and purified as described by Braunagel & Summers (1994). Polyhedra for each virus were pelleted and resuspended in 28 ml 0.1 M Na_2CO_3/50 mM NaCl (pH 10.9), then incubated at 37 °C for 1.5 h. Insoluble material was pelleted by centrifugation in a tabletop centrifuge (1320 ***g*** for 10 min) and the supernatant was transferred to a

Beckman SW28 ultracentrifuge tube. The solubilized polyhedron solution was neutralized by the addition of 1 M Tris/HCl (pH 7.6) to a final concentration of 0.1 M. A 3 ml 25 % (w/w) sucrose pad prepared in PBS was layered underneath the solubilized polyhedra, and ODV were pelleted through the sucrose pad by ultracentrifugation (124 060 ***g*** for 75 min). The ODV pellet was resuspended in 150–200 µl 10 mM Tris/HCl (pH 8.5) and quantified by Bradford assay with the Coomassie Plus (Bradford) assay reagent (Thermo Scientific). An envelope protein fraction was prepared from approximately 100–250 µg ODV as described by Braunagel & Summers (1994), except that Triton X-100 at a concentration of 0.1 % was used in place of NP-40 and insoluble material from the extraction was pelleted by brief microcentrifugation prior to loading and centrifugation on a 30–70 % (w/v) continuous glycerol gradient. Envelope protein was collected from the top of the gradient and concentrated using an Amicon Ultra centrifugal filter with a 3 kDa molecular mass cutoff point (Millipore).

Unfractionated ODV protein was prepared from polyhedra extracted from baculovirus-killed *Heliothis virescens* larvae. Larvae that had moulted to fifth instar were inoculated by intrahaemocoelic injection with 5 µl (approx. 50–200 p.f.u.) BV. Virus-killed cadavers were collected at 5–7 days p.i. and stored at −20 °C. Cadavers were homogenized and polyhedra isolated as described previously (Harrison, 2009a). Polyhedron pellets were resuspended in 28 ml 0.1 M Na_2CO_3 and incubated at 37 °C for 15 min. ODV were pelleted through a sucrose pad, resuspended in 10 mM Tris/HCl (pH 8.5) and quantified by Bradford assay as described above.

ODV proteins were separated by SDS-PAGE using a Mini-PROTEAN 3 cell (Bio-Rad) according to the manufacturer's instructions. Approximately 7.5 µg ODV protein samples from polyhedra prepared from *Heliothis virescens* larvae and 15 µg ODV envelope protein from polyhedra isolated from BTI-TN-5B1-4 cells were loaded and run on an SDS-PAGE gel (10 %) along with ECL DualVue Western blotting markers (Amersham/GE Healthcare). Protein was transferred to an Amersham Hybond-P PVDF membrane (GE Healthcare) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer's instructions, and immunoblot analysis was carried out using the Amersham ECL Plus Western blotting detection system (GE Healthcare) with the primary antibody consisting of a 1 : 2000 dilution of rabbit anti-ODV-E56 serum (Braunagel *et al.*, 1996). Fluorescent Western blot signals were visualized with a Typhoon 9410 Variable Mode Imager set at an excitation wavelength of 457 nm and emission wavelength of 532 nm.

Measurement of BV and OB production. Six-well plates were seeded with 0.8×10^6 *Sf*9 cells per well. Three wells per virus were infected with wild-type and recombinant viruses at an m.o.i. of 5. Aliquots of budded virus were harvested from each well at 24, 48 and 72 h p.i. and the cells and free-floating polyhedra of each separate infection were harvested at 96 h p.i. Infectious virus (p.f.u. ml^{-1}) at each time point was quantified by plaque assay. Polyhedra were purified following the procedure of O'Reilly *et al.* (1992) and counted with a Neubauer haemocytometer. The total numbers of polyhedra produced per cell in each well were calculated. Variation in BV and polyhedron production was examined by one-way analysis of variance (ANOVA) with significance evaluated by pairwise two-tailed *t*-test.

Electron microscopy. BTI-TN-5B1-4 cells were seeded into 75 cm^2 flasks at a density of 9×10^6 cells per flask and infected with wild-type and recombinant viruses at an m.o.i. of 5. Cells were harvested at 72 h p.i. and fixed, embedded in resin, sectioned and stained as described previously (Harrison & Summers, 1995), except that resin infiltration was carried out by incubation with a series of increasing concentrations of Spurr's resin in acetone. Stained ultrathin sections were examined with an H-7000 Hitachi electron microscope at 75 kV.

Bioassays. Polyhedron stocks prepared from *Heliothis virescens* cadavers (see above) were used to set up droplet-feeding bioassays as described previously (Hughes & Wood, 1981; Sparks *et al.*, 2008) to assess the biological activity of polyhedra for each virus. Five doses producing a mortality range of 10–90 % were used to infect neonate larvae of *Heliothis virescens*, *Helicoverpa zea* and *Ostrinia nubilalis*. Bioassays were repeated at least twice. Dose–mortality relationships were analysed by probit analysis using Polo Plus version 2.0 (LeOra Software). Comparison of LC_{50} values was carried out as described by Robertson *et al.* (2007).

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