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Abstract

The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) occlusion-derived virus (ODV) envelope protein ODV-E56 is essential for oral infection of larvae of *Heliothis virescens*. Bioassays with recombinant clones of AcMNPV lacking a functional *odv-e56* gene showed that ODV-E56 was required for infectivity of both polyhedra and to a lesser extent, purified ODV. However, binding and fusion assays showed that ODV lacking ODV-E56 bound and fused to midgut cells at levels similar to ODV of wild-type virus. Fluorescence microscopy of midguts from larvae inoculated with ODV-E56-positive and -negative viruses that express GFP indicated that ODV-E56 was required for infection of the midgut epithelium. Purified ODV-E56 bound to several proteins in midgut-derived brush border membrane vesicles, but failed to rescue infectivity of ODV-E56-negative viruses *in trans*. These results indicate that ODV-E56 is a *per os* infectivity factor (*pif-5*) required for primary midgut infection at a point before or after virion binding and fusion.

Keywords

Occlusion-derived virus, *Per os* infectivity factor, Baculovirus, *Heliothis virescens*, ODV-e56, Pif-5, *Autographa californica* multiple nucleopolyhedrovirus, Genetics

Disciplines

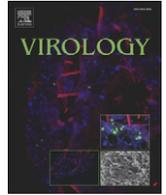
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Comments

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Autographa californica multiple nucleopolyhedrovirus ODV-E56 is a *per os* infectivity factor, but is not essential for binding and fusion of occlusion-derived virus to the host midgut

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ABSTRACT

The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) occlusion-derived virus (ODV) envelope protein ODV-E56 is essential for oral infection of larvae of *Heliothis virescens*. Bioassays with recombinant clones of AcMNPV lacking a functional *odv-e56* gene showed that ODV-E56 was required for infectivity of both polyhedra and to a lesser extent, purified ODV. However, binding and fusion assays showed that ODV lacking ODV-E56 bound and fused to midgut cells at levels similar to ODV of wild-type virus. Fluorescence microscopy of midguts from larvae inoculated with ODV-E56-positive and -negative viruses that express GFP indicated that ODV-E56 was required for infection of the midgut epithelium. Purified ODV-E56 bound to several proteins in midgut-derived brush border membrane vesicles, but failed to rescue infectivity of ODV-E56-negative viruses *in trans*. These results indicate that ODV-E56 is a *per os* infectivity factor (*pif-5*) required for primary midgut infection at a point before or after virion binding and fusion.

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Introduction

Baculoviruses are large dsDNA viruses that have been isolated from the insect orders Lepidoptera, Diptera, and Hymenoptera (Jehle et al., 2006). Baculoviruses have proven useful as protein expression vectors in tissue culture (Kost et al., 2005), potential vectors for gene therapy (Hu, 2006), and as natural insecticides to stem pest outbreaks (Moscardi, 1999). The virus has a bi-phasic life cycle with two distinct forms of virions for within- and between-host viral transmission. Infection begins when the insect ingests the environmentally stable occlusion bodies (OB); polyhedra or granules, composed of the viral protein polyhedrin or granulin respectively. The OBs dissolve in the basic pH of the insect gut, releasing the occlusion-derived virus (ODV), which then bind and fuse to the brush border microvilli of columnar cells in the midgut epithelium (Horton and Burand, 1993). The genus *Alphabaculovirus* includes the lepidopteran-specific nucleopolyhedroviruses (NPV), which package multiple nucleocapsids into a single ODV. The nucleocapsids are transmitted to the nucleus where both viral

replication and assembly occur (Bonning, 2005). The cells first produce budded virus (BV), which buds out from the cell cytoplasm, and infects neighboring cells via endocytosis. ODV are produced in the nucleus late in baculovirus infection and are occluded within the polyhedrin matrix, which is further enveloped. Eventually the host insect dies, infected cells lyse, and polyhedra are released back into the environment (Bonning, 2005).

In contrast to the BV phenotype which has been relatively well characterized, little is known about the ODV beyond characterization of individual ODV proteins and some protein–protein interaction studies (Braunagel et al., 1996; Peng et al., 2010). Over 44 proteins have been identified in ODV, several of which are unique to the ODV envelope (Braunagel et al., 1996; Deng et al., 2007; Fang et al., 2009; Perera et al., 2007; Slack and Arif, 2007). ODV appear to enter the cell via binding followed by fusion of viral and host cell membranes (Horton and Burand, 1993). The majority of baculoviruses infect only a few host species, and the ability of the ODV to bind and fuse to the gut epithelia is a major determinant of virus specificity and host susceptibility (Haas-Stapleton et al., 2003). A core set of 30 genes found in all sequenced baculovirus genomes has been described (Jehle et al., 2006; McCarthy and Theilmann, 2008). An additional 22 genes are conserved among the lepidopteran baculoviruses of *Alphabaculovirus* and *Betabaculovirus*; 19 of which have known functions (Fang et al., 2009; van Oers and Vlak, 2007). Among these 52 lepidopteran baculovirus genes, 14 (including 9 core genes) encode ODV envelope

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proteins or are found within the ODV (excluding polyhedrin). Notably, a class of proteins has been described, the *per os* infectivity factors (PIFs), which include five ODV envelope proteins that play a role in oral infectivity: P74 (*ac138*; *pif-0*), PIF-1 (*ac119*), PIF-2 (*ac22*), PIF-3 (*ac115*), and PIF-4 (*ac96*) (Fang et al., 2009; Haas-Stapleton et al., 2004; Hao et al., 2009; Kikhno et al., 2002; Kuzio et al., 1989; Ohkawa et al., 2005; Pijlman et al., 2003; Yao et al., 2004). The first four PIFs are conserved across the Baculoviridae, and PIF-4 is conserved across the lepidopteran baculoviruses. Deletion of these proteins results in a profound decrease in the oral infectivity of the OB. The impact of deletion of each of these five PIF proteins varies and protein–protein interactions between the ODV proteins are complex (Peng et al., 2010).

We previously conducted a search of five Group I alphabaculoviruses for genes that were undergoing positive selection (Harrison and Bonning, 2004). Two ODV envelope proteins, ODV-E56 and ODV-E18, were identified as undergoing positive selection, whereas, the PIFs P74–PIF-0, PIF-1, PIF-2, PIF-3, and PIF-4 exhibited purifying selection. Genes exhibiting positive, or diversifying, selection have greater amino acid sequence variation, which may lead to different functions that benefit the virus, ultimately leading to fixation of these changes in the population. To determine the role of ODV-E56 in oral infection, we constructed three deletion mutant viruses in which *odv-e56* was disrupted via insertion of a *lacZ* cassette. The deletions did not impact polyhedra formation (Harrison et al., 2010). Initial bioassays of oral infectivity in neonates of the highly susceptible lepidopteran, the tobacco budworm, *Heliothis virescens* revealed a profound loss of oral infectivity for AcIEGFP-e56lacZ(+). The objective of the work described here was to characterize the oral infectivity of ODV-E56 deletion viruses in fourth instar larvae of *H. virescens* to gain insight into the stage at which ODV-E56 is required for oral infection of AcMNPV.

Results

ODV-E56 is essential for the oral infectivity of both ODV and OB. The *odv-e56* ORF was disrupted by insertion of a *lacZ* cassette under the control of the *Drosophila melanogaster* *hsp70* promoter as described in

Harrison et al. (2010) (Fig. 1). Bioassays with fourth-instar *H. virescens* larvae revealed that polyhedra of the construct AcIE1GFP-e56lacZ(+) have significantly lower *per os* infectivity (Table 1). The dose of OB necessary to observe any mortality was five logs higher than that of the other constructs and wild type virus (Fig. 2; Table 1). The LD₅₀ of the parental recombinant AcIE1TV3.EGFP, the revertant AcIE1GFP-e56R, and wild type AcMNPV-C6 OB were not significantly different from one another as determined by probit analysis ($p > 0.05$; LD ratio test) (Robertson and Preisler, 1992). We conducted bioassays with ODV isolated from polyhedra of AcIE1GFP-e56lacZ(+) and AcIE1TV3.EGFP, which also showed a significant difference in infectivity ($p < 0.05$, LD ratio test); the LD₅₀ of the deletion mutant was 200 times greater than that of the *odv-e56* containing virus (Table 1). All larvae co-fed with recombinant, purified His-ODV-E56 and AcIE1GFP-e56lacZ(+), and larvae fed AcIE1GFP-e56lacZ(+) alone survived, while larvae fed with wild type virus died. In contrast to the ODV bioassay results, mortality caused by BV injected into the hemocoel did not differ significantly for any of the viruses tested ($p > 0.05$; LD ratio test; Table 1).

Confirmation of loss of per os infectivity. To confirm that the impairment in infectivity observed with AcIE1GFP-e56lacZ(+) was not due to disruptions in the transcription of neighboring genes, bioassays were set up with *odv-e56* mutants in which *lacZ* had been inserted in either orientation. In bioassays with fourth instar *H. virescens* larvae, the *odv-e56* mutants AcIE1GFP-e56lacZ(–), AcIE1GFP-e56lacZ(+), and Ac69GFP-e56lacZ(+) killed larvae with LD₅₀s ranging from 4.8 to 5.7 × 10⁷ OBs (Table 2). The bioassay results for viruses containing *odv-e56* were not significantly different from one another ($p > 0.05$; LD ratio test; Table 2).

ODV-E56 mutants bound to and fused with the columnar epithelia of H. virescens. To assess the interaction of AcIE1GFP-e56lacZ(+) with the brush border microvilli *in vivo*, binding and fusion studies were conducted as previously described (Ohkawa et al., 2005). *H. virescens* larvae were orally inoculated with excess purified ODV labeled with the fluorescent probe Octadecyl Rhodamine B-chloride (R18). The *odv-e56* mutant virus AcIEGFP-e56lacZ(+) was compared to the wild type virus AcMNPV-C6 (positive control) and a *p74* mutant, AcIEGFP-

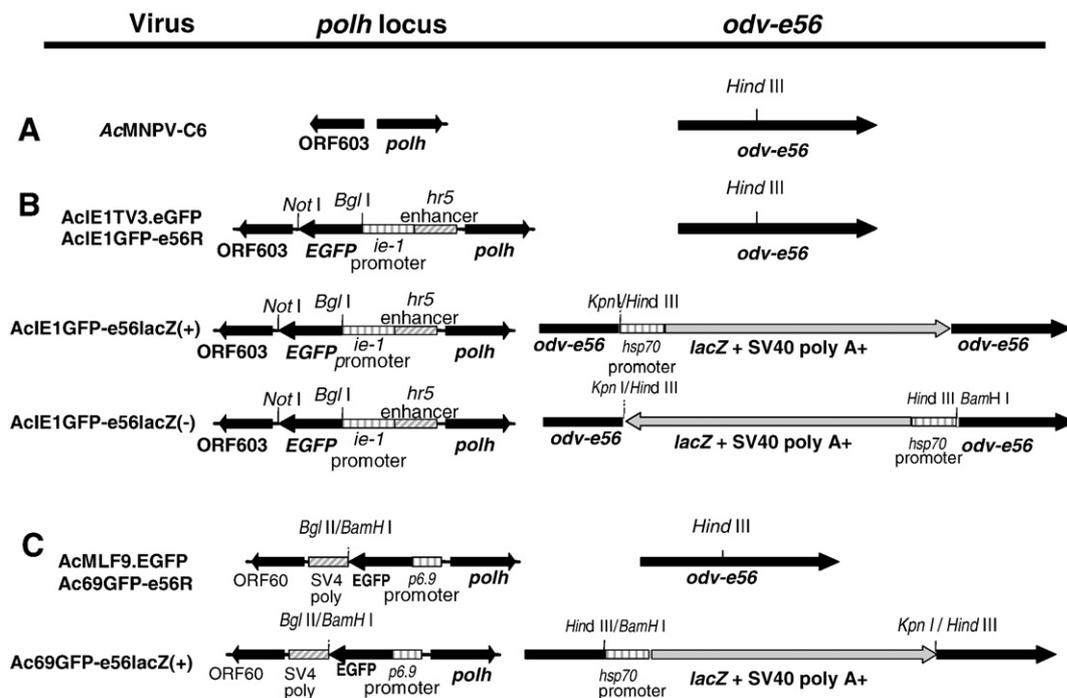


Fig. 1. Recombinant virus constructs. A. Wild-type AcMNPV-C6 virus ORF orientation at the *polh* locus in the left column, the *odv-e56* locus in the right. B. Insertion of EGFP under the control of the *ie-1* promoter (B), or the *p6.9* promoter (C), and disruption of the *odv-e56* locus with the *lacZ* reporter gene under control of the *hsp70* promoter in the positive and negative orientation designated (+) and (–), respectively (A and B from Harrison et al., 2010).

Table 1Dose–response of fourth instar *H. virescens* larvae to OB, ODV, and BV of wild-type and recombinant AcMNPV.

| Treatment | LD ₅₀ ¹ | 95% C.I. ² | | N ³ | Het ⁴ | Slope | LD ratio ⁵ | 95% C.I. | | |
|-----------|-------------------------------|-------------------------|-----------------------|-----------------------|------------------|-------|-----------------------|----------|-------|-------|
| | | Lower | Upper | | | | | Lower | Upper | |
| OB | AcMNPV-C6 | 155.1a | 124.3 | 203.4 | 345 | 0.46 | 1.582 | 1.267 | 0.915 | 1.756 |
| | AcIE1TV3.EGFP | 196.6a | 144.9 | 281.4 | 395 | 1.108 | 1.612 | 1 | – | – |
| ODV | AcIE1GFP-odve56lacZ(+) | 4.9 × 10 ⁷ b | 1.9 × 10 ⁷ | 7.1 × 10 ⁸ | 311 | 0.55 | 1.157 | 0 | 0 | 0 |
| | AcIE1GFP-odve56R | 117.8a | 98.53 | 142.7 | 480 | 0.53 | 1.721 | 1.325 | 0.953 | 2.404 |
| | AcMNPV-C6 | n/d ⁶ | n/d | n/d | n/d | n/d | n/d | n/d | n/d | n/d |
| | AcIE1TV3.EGFP | 11.79a | 6.489 | 20.711 | 315 | 1.14 | 1.534 | 1 | – | – |
| BV | AcIE1GFP-odve56lacZ(+) | 2425b | 1467 | 4192 | 287 | 1.18 | 1.206 | 0.004 | 0.003 | 0.007 |
| | AcIE1GFP-odve56R | n/d | n/d | n/d | n/d | n/d | n/d | n/d | n/d | n/d |
| | AcMNPV-C6 | 0.191a | 0.139 | 0.269 | 377 | 0.61 | 1.038 | 1.193 | 0.742 | 1.918 |
| | AcIE1TV3.EGFP | 0.227a | 0.104 | 0.716 | 408 | 2.25 | 0.971 | 1 | – | – |
| BV | AcIE1GFP-odve56lacZ(+) | 0.278a | 0.214 | 0.362 | 352 | 0.65 | 1.364 | 0.819 | 0.531 | 1.263 |
| | AcIE1GFP-odve56R | n/d | n/d | n/d | n/d | n/d | n/d | n/d | n/d | n/d |

¹ LD₅₀, the lethal dose to kill 50% of the insects was calculated using the software package POLO-Plus (LeOra Software). LD₅₀ values with different letters within each treatment type (OB, ODV or BV) are significantly different from each other.

² 95% confidence interval, upper and lower bounds as calculated by POLO-Plus.

³ N, sample size.

⁴ Het, heterogeneity.

⁵ LD ratio test, The LD ratio test was performed to compare treatments to the AcIE1TV3.EGFP virus, and LD Ratios with confidence intervals that include 1.0 indicate the LD₅₀ of the viruses compared are not significantly different from one another ($p > 0.05$) (Robertson and Preisler, 1992).

⁶ n/d, not determined.

p74lacZ(+) virus (negative control; Fig. 3). AcIE1GFP-e56LacZ(+) bound and fused to midgut cells at levels similar to the wild type virus, AcMNPV-C6. In contrast, the p74 mutant virus, AcIEGFP-p74lacZ(+), exhibited significantly reduced binding and fusion, consistent with previously published observations (Haas-Stapleton et al., 2004).

Fluorescence microscopy of infected midguts at 24 hpi. We used fluorescence microscopy to address whether *odv-e56* mutant viruses were able to enter midgut cells and establish productive infections. Infection with the AcIE1TV3.EGFP virus produced observable fluorescent foci at a dose of 1×10^7 , whereas no EGFP fluorescence was detected in the negative control sections infected with wild type AcMNPV-C6, or in the *odv-e56* mutant virus AcIE1GFP-e56lacZ(+) virus (data not shown). Fig. 4 shows the bright field, FITC and merged FITC and DAPI images from midguts infected with the six virus constructs at a dose of 1.5×10^{10} polyhedra. At this higher dose, many foci were seen in sections of midguts from larvae infected with the ODV-E56-positive viruses (Fig. 4A,D). The viruses expressing EGFP from the late *p6.9* promoter produced brighter, more distinct and easily visible foci than the viruses expressing EGFP from the weaker *ie-1* promoter. No foci were observed with viruses lacking ODV-E56,

in the AcIE1GFP-e56lacZ(+), AcIE1GFP-e56lacZ(–), and Ac69GFP-e56lacZ(+) infected gut tissues (Fig. 4B,C,E).

Interaction of recombinant ODV-E56 with BBMV proteins. Full length ODV-E56 with an N-terminal polyhistidine tag (His-ODV-E56) was expressed in *E. coli*, and purified under native or denaturing conditions. The polyhistidine tag is estimated to add 6 kDa to the size of the protein, and the predicted molecular mass of AcMNPV ODV-E56 is 40.8 kDa. However, on SDS-PAGE gels of protein purified under native conditions, a protein of 66 kDa was detected. A single 66 kDa protein was detected by western blot with the anti-ODV-E56 and anti-His antisera (not shown). Under denaturing conditions a predominant 45 kDa band was observed, along with minor 40 and 66 kDa bands (Fig. 5A). All three of these bands were detected by both the anti-E56 antibody (data not shown) and the anti-his antibody, indicating that the 40 kDa product of His-ODV-E56 was not degraded at the N-terminus. ODV-E56 has previously been observed to migrate at mobilities that deviate from the predicted molecular weight (Braunagel et al., 1996; Theilmann et al., 1996).

Full-length ODV-E56, both native and denatured, bound to a high molecular weight protein band from BBMV preparations separated by SDS-PAGE and immobilized on PVDF membrane, when incubated under basic conditions (Fig. 5B). The identity of this protein is unknown. Far western blots with the peptides GBP3.1 and C6 (Liu et al., 2010) under the same conditions did not result in any binding (not shown).

Feeding of larvae with recombinant, native His-ODV-E56 and either polyhedra or ODV of AcIE1GFP-e56lacZ(+) did not restore the oral infectivity of the *odv-e56* mutant virus (data not shown).

Discussion

The lack of ODV-E56 in the AcMNPV virion had a profound impact on the oral infectivity of both polyhedra and ODV towards fourth-instar *H. virescens*. This result is in agreement with our earlier studies showing loss of oral infectivity of *odv-e56* mutants in neonate *H. virescens* (Harrison et al., 2010). The degree of impairment in oral infectivity observed with the elimination of ODV-E56 protein expression is similar to that observed for deletion of the other PIFs (Haas-Stapleton et al., 2004; Ohkawa et al., 2005; Zhang et al., 2005). The impairment in oral infectivity observed for ODV-E56 deletion mutants, concurrent with the lack of an observable impact on BV infectivity, places ODV-E56 within the family of *per os* infectivity factors along with *p74* (*pif-0*), *pif-1*, *pif-2*,

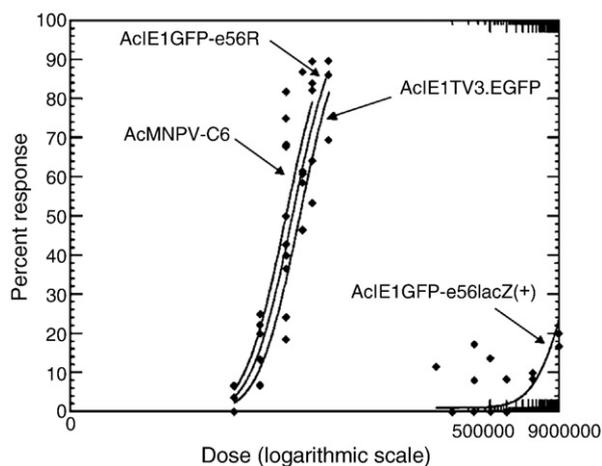


Fig. 2. Deletion of *odv-e56* impairs infectivity of occlusion bodies (OB). Log-transformed mortality dose–response curves calculated by POLO-Plus (LeOra software). The mortality curves from left to right are for AcMNPV-C6, AcIE1GFP-e56R (revertant virus), AcIE1TV3.EGFP, and AcIE1GFP-e56lacZ(+) as indicated.

Table 2
Oral infectivity of recombinant AcMNPV OB against fourth instar larvae of *H. virescens*.

| Treatment | LD ₅₀ ¹ | 95% C.I. ² | | N ³ | Het ⁴ | Slope | LD ratio ⁵ | 95% C.I. | |
|---------------------|-------------------------------|-----------------------|-----------------------|----------------|------------------|--------|-----------------------|----------|-------|
| | | Lower | Upper | | | | | Lower | Upper |
| AcIE1TV3.EGFP | 196.509a | 144.907 | 281.404 | 395 | 1.108 | 1.612 | 1 | – | – |
| AcIE1GFP-e56lacZ(+) | 4.9 × 10 ⁷ b | 1.9 × 10 ⁷ | 7.1 × 10 ⁸ | 311 | 0.55 | 1.157 | 0.000 | 0.000 | 0.000 |
| AcIE1GFP-e56lacZ(–) | 4.8 × 10 ⁷ b | 1.9 × 10 ⁶ | 1.6 × 10 ⁸ | 305 | 0.52 | 0.53 | 0.000 | 0.000 | 0.000 |
| AcIE1GFP-e56R | 117.776a | 98.53 | 142.67 | 480 | 0.53 | 1.721 | 1.325 | 0.953 | 2.404 |
| AcMLF9.EGFP | 118.562a | 76.226 | 183.639 | 207 | 2.15 | 2.151 | 1.303 | 0.957 | 1.774 |
| Ac69GFP-e56lacZ(+) | 5.7 × 10 ⁷ b | 1.0 × 10 ⁷ | 1.8 × 10 ⁸ | 295 | 0.36 | 0.0516 | 0.000 | 0.000 | 0.000 |
| Ac69GFP-e56R | 121.297a | 53.656 | 256.343 | 200 | 4.53 | 2.045 | 1.602 | 0.87 | 2.952 |

¹ The LD₅₀ for each virus treatment was calculated using the software package POLO-Plus (LeOra Software). LD₅₀ values with different letters are significantly different, $p < 0.05$.

² The lower and upper bounds of the 95% confidence interval (C.I.).

³ N, sample size.

⁴ Het, heterogeneity.

⁵ The LD ratio test was performed to compare treatments to the AcIE1TV3.EGFP virus, and LD Ratios with confidence intervals that include 1.0 indicate the LD₅₀ of the viruses compared are not significantly different from one another ($p < 0.05$) (Robertson and Preisler, 1992).

pif-3, and *pif-4* (*ac96*). We propose to designate *odv-e56* as *per os infectivity factor-5*, or *pif-5*.

The impact of *odv-e56* deletion on oral infectivity differed depending on whether polyhedra or purified ODV was used as the inoculum. While the *odv-e56* mutant ODV were two orders of magnitude less infectious than wild type virus ODV, the polyhedra of the *odv-e56* mutants were five orders of magnitude less infectious than polyhedral of wild type virus. This trend is similar to that observed for *ac150*, for which elimination of expression resulted in a modest (6- to 18-fold) reduction in the virulence of polyhedra against larvae, but had no effect on the virulence of purified ODV (Zhang et al., 2005). The difference in OB and ODV virulence detected with the *odv-e56* mutants could be explained by postulating a role for ODV-E56 in the efficient occlusion of virions during polyhedron assembly or their release from polyhedra after solubilization of the polyhedrin matrix. Cross-sections of polyhedra examined by transmission electron microscopy did not reveal any obvious differences in numbers of ODV between *odv-e56* mutants and *odv-e56*-positive virus, at least none of a magnitude that could account for the difference in virulence of OB and ODV in bioassays (Harrison et al., 2010). It also may be possible that the difference in virulence between OB and ODV is an artifact of the process for purification of ODV.

Unlike *p74*, *pif-1*, and *pif-2* mutant viruses, the *odv-e56* mutant AcIE1GFP-e56lacZ(+) did not exhibit any impediment in binding and

fusion to midgut cells *in vivo*. Mutation of *pif-3* (*ac115*) also resulted in a profound impairment in oral infectivity with no accompanying effect on binding and fusion (Ohkawa et al., 2005). Yeast two-hybrid analysis of ODV protein interactions indicates that ODV-E56 interacts with PIF-3 in a reciprocal manner (Peng et al., 2010). PIF-3 is thought to have a role downstream of binding and fusion. Similarly ODV-E56 may mediate necessary interactions for viral infection of the cell after fusion of the viral and cellular membranes.

As observed in previous studies, we found that ODV-E56 migrated in SDS-PAGE gels at a size greater than the predicted molecular weight. Although putative conserved N-glycosylation sites have been identified in the amino acid sequence, treatment with N-glycosidases failed to detect N-glycosylation of ODV-E56 (Braunagel et al., 1996). In addition, the antibodies used to detect ODV-E56 both targeted the N-terminus (the polyhistidine tag and the N-terminal 135 aa of ODV-E56), so potential N-terminal cleavage products of ODV-E56 may not be detected. P74, the only PIF shown to be exposed on the surface of the ODV envelope thus far, has been shown to undergo protein cleavage by insect midgut trypsin (Faulkner et al., 1997; Slack et al., 2008). Analysis of the amino acid sequence with the TMHMM software (<http://www.cbs.dtu.dk/services/TMHMM/>) indicated a putative transmembrane domain in the C-terminal conserved hydrophobic domain reported by Theilmann et al. (1996), with the portion of the protein N-terminal to this domain predicted to be “outside”. It is possible that this portion of ODV-E56 binds to the unidentified protein from preparations of BBMV (Fig. 5). Despite the strong binding of both native and denatured ODV-E56 to gut BBMV, co-feeding with expressed ODV-E56 did not rescue the *odv-e56* deletion viruses as was shown for P74 (Yao et al., 2004). In its individual form, ODV-E56 may not be stable in the gut long enough to achieve rescue, or, alternatively, may have the wrong conformation or be incorrectly folded due to expression in *E. coli*. Alternatively, if ODV-E56 mediates intracellular processes, oral inoculation may not allow for functional entry of the protein into the cell.

We currently lack understanding of how ODV proteins work to mediate primary infection of the midgut. More information is needed on how the PIFs interact with each other and with host proteins. This study contributes to our understanding of the mechanism of midgut infection and lays the groundwork for continued studies into the molecular basis of baculovirus virulence.

Materials and methods

Virus construction, amplification, and purification. The construction of the ODV-E56 deletion mutants has been described in Harrison et al. (2010) (Fig. 1). Briefly, transfer vectors in which *odv-e56* was disrupted with an *hsp70-lacZ* expression cassette in each orientation were created in a plasmid containing the 7.1 kb *Clal*-F fragment of

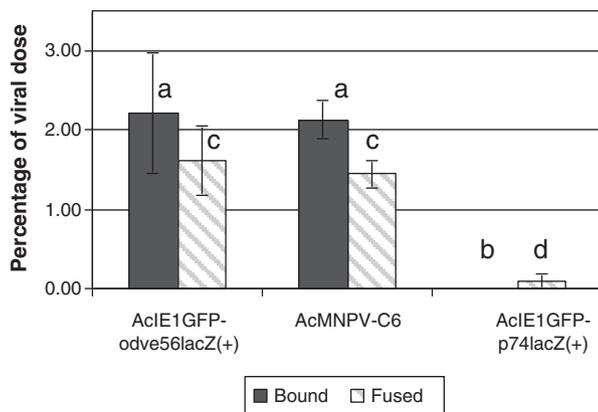


Fig. 3. Deletion of *odv-e56* does not impact ODV binding and fusion to *H. virescens* midgut epithelial cells. Sucrose gradient-purified ODV were labeled with R-18 at levels not exceeding 1×10^6 FLU/ μ g ODV and orally inoculated into newly molted fourth instar larvae (2.6 μ g AcIE1GFP-e56lacZ(+), 1.8 μ g AcMNPV, and 2.2 μ g AcIE1GFP-p74lacZ(+)) per larva). Each column represents the mean of six larvae with standard error bars. Solid bars indicate the percent of the ODV that bound to the midgut, and striped bars indicate the amount of ODV that fused. Treatments were significantly different ($p < 0.05$; One way ANOVA) for both binding and fusion, with a 5% LSD of 0.84% and 0.54% respectively, as indicated by a,b for binding and c,d for fusion.

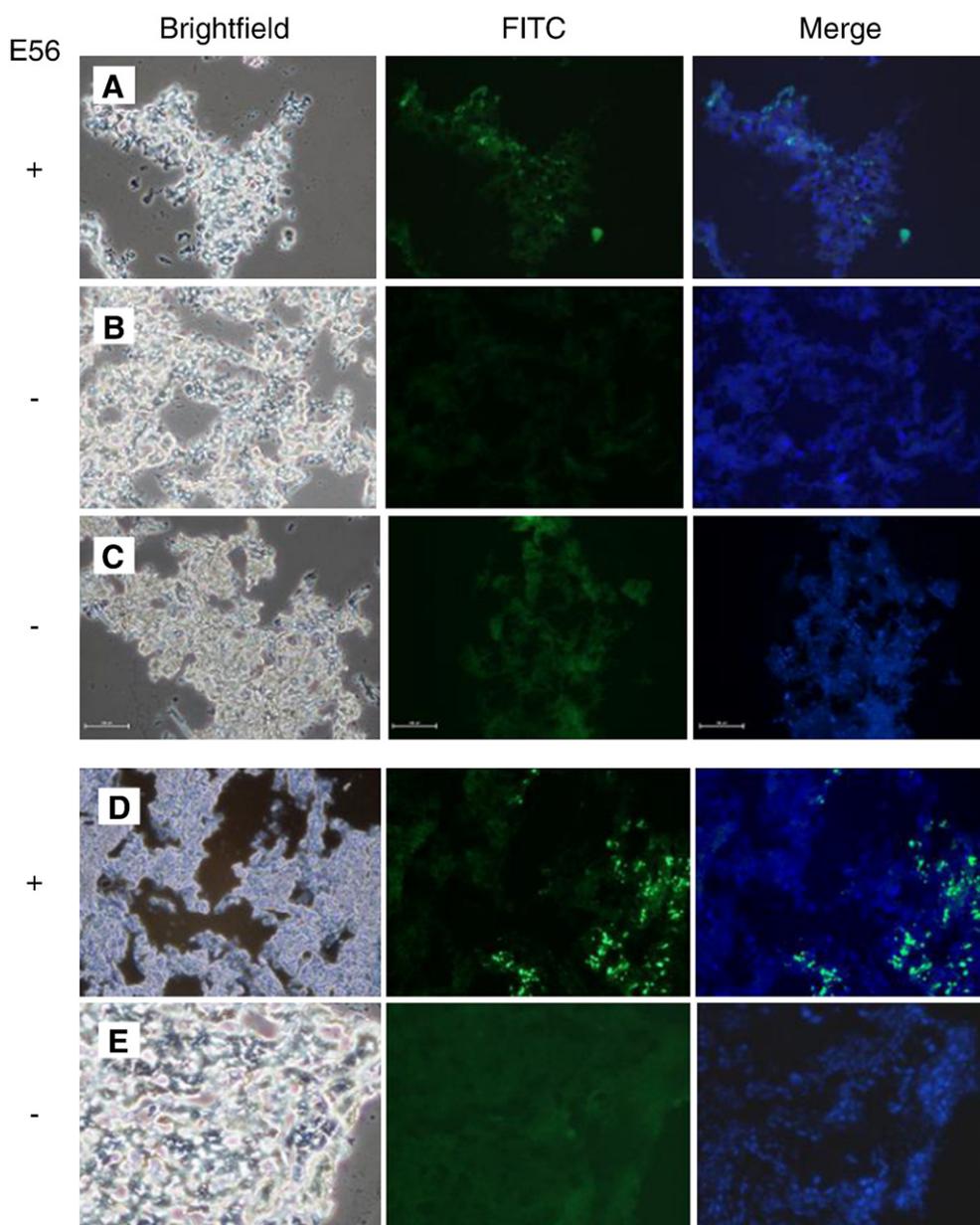


Fig. 4. ODV-E56 is required for expression of baculovirus genes in *H. virescens* gut epithelial cells. Fourth instar *H. virescens* were orally inoculated with 1.5×10^{10} polyhedra; midguts were dissected at 24 hpi and cryosectioned for fluorescence microscopy. Nuclei are stained with DAPI (blue), viral foci are green due to EGFP expression which is indicative of viral expression. This figure contains representative images from 6 to 10 cryosections from three to six insects infected with: A. AcIE1TV3.EGFP, B. AcIE1GFP-e56lacZ(+), C. AcIE1GFP-e56lacZ(-), D. AcMLV9.EGFP, and E. AcP69GFP-e56lacZ(+). The left hand column of panels shows the brightfield images, the middle column the FITC filter to detect EGFP expressing viral foci, and the right hand column the overlay of the DAPI and FITC images. The presence or absence of ODV-E56 in the viruses is indicated at left. Bars indicate 100 μ m.

AcMNPV-C6. The (–) designated virus contains the cassette in the reverse orientation relative to the ORF, while the (+) construct is in the same orientation as the ORF. This plasmid was then co-transfected with either the recombinant virus AcIE1TV3.EGFP which contains EGFP at the *polh* locus under control of the *immediate early-1* (*ie-1*) gene promoter, or AcMLF9.EGFP, which contains EGFP, also at the *polh* locus, under control of the late *p6.9* gene promoter, into Sf-9 cells using Cellfectin (Invitrogen). Several rounds of plaque purification were carried out to isolate LacZ-positive and occlusion-positive plaques. To ensure that any phenotypic differences were due only to inactivation of *odv-e56*, revertant viruses were created using transfer vectors with uninterrupted *odv-e56* and selecting for LacZ-negative/occlusion-positive plaques. In theory, a short peptide of the first part of the *odv-e56* orf could be transcribed and translated in the disruption mutants, but this potential protein was not detected by western blots of infected Sf9 cells using anti-E56 antibody that

recognizes the N-terminus (Harrison et al., 2010). The *p74* mutant virus AcIEGFP-p74-lacZ(+) was created in the same manner using an initial plasmid containing the *HindIII*-Q fragment of AcMNPV-C6. The *hsp70-lacZ* expression cassette was inserted into the *SacII* site of the *p74* ORF, and the resulting transfer vector was co-transfected with AcIE1TV3.EGFP to make a virus with an inactivated *p74* gene. The structure of all viruses was confirmed by restriction endonuclease digests and PCR, and virus constructs are shown in Fig. 1.

BV preparation. BV was amplified using Sf21 cell culture. Cells (1×10^6) were incubated with virus constructs, and cells harvested at 48 hpi. Cells were pelleted at $420 \times g$ for 10 min and the supernatant containing the BV transferred to a new tube. BV was quantified by plaque assay using standard methods (King and Possee, 1992).

Polyhedra preparation. Each of the recombinant viruses was amplified in *H. virescens* to produce polyhedral stocks. BV was injected into fifth instar larvae via the proleg, and polyhedra were

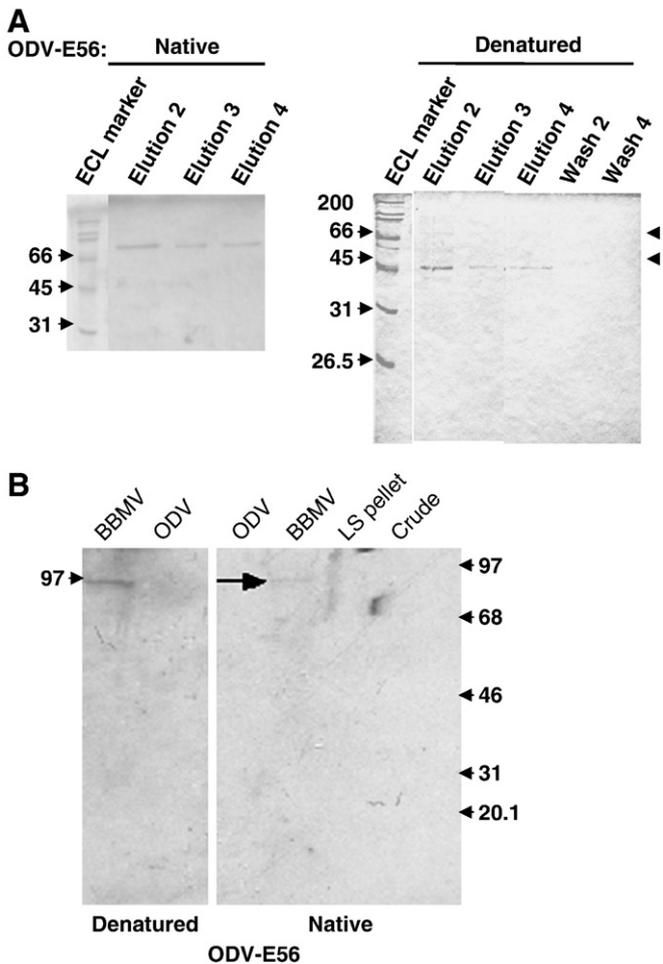


Fig. 5. Purification of recombinant ODV-E56 and binding to *H. virescens* gut BBMVs proteins. **A.** Nickel column purification of recombinant ODV-E56 under native and denaturing conditions. Proteins were run on a 12% SDS polyacrylamide gel and stained with Coomassie Blue. ECL markers, a LacZ positive control for nickel column purification, and selected fractions following elution for native and denaturing protein purification are shown. ODV-E56 was observed at 66 kDa, when purified under native conditions and at 45 kDa when purified under denaturing conditions (arrow heads). **B.** Far-western blot to show binding of ODV-E56 to *H. virescens* gut BBMVs proteins. BBMV, ODV proteins (ODV), low speed cell pellet (LS pellet) from BBMV preparation (cellular debris), and crude midgut homogenate (Crude) from midguts for BBMV isolation are shown. Membranes were incubated with denatured His-ODV-E56 or native His-ODV-E56, as indicated, and detected using anti-His antibody. ODV-E56 purified under both native and denaturing conditions bound to a 97 kDa protein in BBMV samples (black arrows).

isolated from the cadavers using standard methods and stored at 4 °C (O'Reilly et al., 1992). Polyhedra were subsequently purified by centrifugation through a 25% w/w sucrose in PBS cushion, and then further purified in 45–65% w/w sucrose in PBS linear sucrose gradients created using Gradient Master (BioComp). The banded polyhedra were collected using a needle and syringe and washed in PBS. Purified polyhedra were resuspended in small volumes of sterile water with 0.1% sodium azide and quantified using a hemocytometer.

ODV preparation. ODV was purified using sucrose purified polyhedra which were solubilized with 0.1 M NaCO₃ and 0.1 M NaCl for 10 min on ice in amber microcentrifuge tubes to protect ODV from light. The alkaline solution was neutralized by addition of 1/10 volume of 1 M Tris pH 7.3 then placed in an orbital shaker at room temperature at 150 rpm for 1 h. Virus suspensions were briefly centrifuged at 420 × g for 5 min. The supernatants were immediately loaded onto 63%/57%/35%/25% sucrose (w/v in TE buffer) step gradients in Beckman Ultra Clear tubes. Samples were overlaid with mineral oil, balanced, and spun for 1 h at 90,000 × g in an SW28 rotor

at 4 °C. White ODV bands (observed at the interfaces between 57% and 63%, and 35% and 57% interfaces) were harvested by puncturing the side of the tube with an 18-gauge needle with a glass syringe. Samples were diluted with water to twice the volume, and pelleted by centrifugation at 90,000 × g for 30 min. ODV pellets were resuspended in small volumes of PBS, stored in amber microfuge tubes at 4 °C for no more than 1 week. ODV were quantified by Bradford assay using the BioRad Protein Detection system.

Insects and bioassays. *H. virescens* larvae (BioServ) were reared on artificial diet (Southland Products, Inc.) at 28 °C with 12-h day/night cycles. Fourth-instar larvae were used for all experiments into the nature of ODV-E56. For OB bioassays and microscopy work, third instar larvae showing head capsule slippage were starved overnight, and then fed the respective dose of polyhedra on a small diet cube. After 24 h, larvae that did not consume the entire cube were discarded, and diet was provided to those that remained. ODV bioassays were conducted by starving the larvae overnight followed by inoculation via droplet feeding with a total volume of 1 to 2 μl. Only insects that consumed the entire dose of virus were returned to diet. For BV bioassays, fourth instar larvae were injected via the proleg using a microapplicator (Burkhard) with a 32-gauge sharp needle affixed to a 1 ml syringe. For binding and fusion assays, ODV were administered orally using a 32-gauge blunt needle inserted into the midgut (Li et al., 2008; Sparks et al., 2008). Dose–response bioassays were performed in triplicate on sample sizes of 15–30 insects per dose, for at least five doses of virus. After infection, insects were inspected twice daily for death, and death by nuclear polyhedrosis confirmed by light microscopy. Data were analyzed using Polo Plus version 2.0 (LeOr software), and statistical significance determined by the LD ratio test (Robertson and Preisler).

BBMV purification. Midguts were collected from newly molted fourth instar *H. virescens* dissected in ice-cold MET buffer (300 mM mannitol, 2 mM EDTA, 17 mM Tris, pH 8.0). The midguts were rinsed free of the peritrophic membranes, placed in small aliquots of buffer, flash frozen using liquid nitrogen, and stored at –80 °C. Brush border membrane vesicles were prepared in accordance to a modified method of Wolfersberger et al. (1987). Briefly, midguts were quickly thawed in a water bath, mixed with nine times their wet weight of MET buffer, and homogenized using a Wheaton homogenizer for nine strokes at speed 3. An equal volume of 24 mM MgCl₂ was added and mixed three times. A small sample of the crude homogenate was taken, and the remaining sample spun at 4500 rpm for 15 min in an SW28 Beckman rotor. The supernatant was transferred to a fresh tube and spun at 15000 rpm for 45–60 min. The supernatant was removed, and the pellet resuspended in equal volumes of MET and MgCl₂; and the high and low speed centrifugation repeated as above. The final supernatant was decanted, and the final BBMV pellet resuspended in 100–300 μl of 0.5 × MET buffer in water, aliquoted, and flash frozen in liquid nitrogen. Samples of crude homogenate, cell pellet, and supernatant were assayed for protein concentration and leucine aminopeptidase activity. Protein concentrations were determined using BioRad Protein Detection system. For leucine aminopeptidase activity, 50 μl of 50 mM NaPO₄ buffer pH 7.2 and 2 μl sample were placed in a 96 well plate. At a ratio of 43.5 μl of 50 mM NaPO₄ buffer to 6.5 μl fresh 24 mM L-leucine *p*-nitroanilide (Sigma), 50 μl of this solution was added to the sample wells, and the plates immediately read at 412 nm for 5 min using a Vmax spectrophotometer (Molecular Devices). Readings were normalized to protein concentrations, and preparations exhibiting at least an eight-fold increase in aminopeptidase activity relative to the original crude homogenate were used for further assays.

Octadecyl rhodamine B-chloride (R18) labeling/binding and fusion studies. ODV were labeled with octadecyl rhodamine B chloride (R18) using a modified method of Ohkawa et al. (2005). ODV were prepared as described above, with the addition of 10 μl of R18 dye to ODV from 1 × 10¹⁰ polyhedra. After centrifugation on sucrose density gradients,

the labeled ODV appeared as a major band, magenta in color, in the gradient between the 35% and 57% steps and a minor band between 57% and 63%. The bands were collected and diluted 2× with water. The ODV were pelleted, and then resuspended in minimal amounts of PBS (100–200 µl). ODV were quantified using the BioRad Protein Detection System, and the fluorescence units determined using a spectrophotometer with an excitation 556 nm and emission 583 nm. Only virus labeled in the range of 5×10^5 to 2×10^6 fluorescence units per microgram (FLU/µg) were used for further study (as determined by Ohkawa et al., 2005). Newly molted fourth instars were orally inoculated using a 32-gauge blunt needle in doses ranging 2–6 µg of virus within 15 min of the molt. Insects were dissected 1 hpi in low light conditions in Separation Buffer (SB) (100 mM NaCO₃, 100 mM KCl, 100 mM EGTA, pH 9.5). Midguts were cut longitudinally and rinsed free of peritrophic matrix, then placed in 6-well dissection dishes containing 200 µl of SB on ice for 15 min. After this incubation, the midgut epithelia were separated from the basement membrane using forceps and a dissecting probe. The gut and buffer were transferred to a microfuge tube containing 800 µl of SB, and flash frozen in liquid nitrogen. Tubes were stored at –80 °C until fluorescence was determined. Samples were thawed and added to cuvettes containing 1 ml of SB. The midgut epithelial cells were dispersed by pipetting the solution up and down six times. Fluorescence was determined using a spectrophotometer, and normalized to background and volume. This measurement in relative fluorescent units (FLU) represented the total amount of viral fusion. After the first measurement was taken, Triton X-100 was added to 1% of the total volume, to disrupt any remaining dimers of R18, and the samples read again. This measurement, corrected for volume, represented the total amount of R18 bound to the gut. The data are presented as the percentage of virus bound and fused based on the fluorescence of the dose of virus given to the insect, normalized for any background fluorescence. The data presented represent the mean of six individual insects per treatment group and were analyzed by one-way ANOVA and Student's *t*-test.

Microscopy. Newly molted fourth instar *H. virescens* were fed a solution of polyhedra on a small diet cube. Insect midguts were dissected out at 24 hpi in 1% formalin in Neutral Buffer, rinsed free of the peritrophic matrix, and fixed for 1–2 h in 500 µl of formalin buffer. Midguts were washed in PBS for 5 min, excess PBS allowed to drip off, then embedded in Tissue-Tek Optimal Cutting Temperature (O.C.T.) compound (Ted-Pella, Inc.) in the chamber of a Universal cryostat. Guts were cryo-sectioned at 20 µm thick and mounted onto Probe-On Plus slides (Fisher Scientific). Sections were air-dried for 1 h, and circled with a PAP-pen, to reduce the amount of reagent required for the following steps. Sections were washed in PBS to remove excess O.C.T., then incubated in 3% Triton X-100 in PBS for 30 min to solubilize membranes. Slides were washed in PBS, and then incubated with 40–120 µl of DAPI (Invitrogen) in water (7 µl of DAPI to 1 ml total) for 30 min in a covered container. Slides were then washed two times in PBS, overlaid with Fluorogel (E.M.T.) followed by a cover slip, and sealed with finger nail polish. Slides were stored in the dark at 4 °C until image capture. Fluorescence of infected tissues was captured on a Zeiss Axioplan II microscope with an Axiocam MRC color camera and FITC (green), and DAPI (blue) filters. Images were captured using Axiovision Imaging software (Carl Zeiss Inc., Thornwood, NY), with an exposure time of 1 s used for all images. Cryo-sectioning of midgut tissue into approximately 20-µm-thick sections was found to reduce the gut auto-fluorescence, and fixation of the tissues in formalin containing buffer had no effect on detection of foci of EGFP expression. Samples were examined from 6 to 10 cross-sections from three to four insects per treatment

Recombinant ODV-E56 protein purification. The *odv-e56* gene was PCR amplified from AcMNPV C6 genomic DNA (Primers: F: GCAGG-TACCGATGAGTTTTTTTCA, R: CGCAAGCTTTTATCGAGGGCCG), cloned into pBADhisC (Invitrogen), and transformed into Top-10 *E. coli*

(Invitrogen). A 500 µl of overnight cultures was re-inoculated into 50 ml Low-salt Luria Broth (LB) medium + ampicillin (50 µg/ml) and incubated at 37 °C according to the manufacturer's protocol. When the cultures reached an OD₅₉₅ of 0.4, they were induced with 0.004% arabinose for 12–24 h at 30 °C in an orbital shaker at 150 rpm. The polyhistidine-tagged protein was batch-purified using Ni-NTA agarose under both native and denaturing conditions following the manufacturer's instructions (Qiagen), and then desalted and concentrated with a Microcon YM-30 centrifugal filter. The resulting protein was analyzed by SDS-PAGE and western blot to confirm expression of polyhistidine-tagged ODV-E56 using anti-polyhistidine (Sigma) and anti-ODV-E56 sera (kindly provided by Max Summers and Sharon Braunagel, Texas A&M University).

To determine whether His-ODV-E56 could rescue the ODV-E56-negative phenotype *in trans*, co-feeding experiments were conducted with His-ODV-E56 protein (100 or 200 µg) and ODV (0.006 or 0.06 µg) or polyhedra of AcIE1GFP-e56lacZ(+). Elution fractions of native His-ODV-E56, which showed no evidence of contamination with bacterial proteins on SDS-PAGE were pooled for these bioassays. Co-feeding experiments were conducted with 15 larvae per treatment in each of two replicate experiments. Control treatments were ODV or polyhedra of AcIE1GFP-e56lacZ(+) alone (negative control), and wild type virus (positive control). Bioassays were conducted, and data analyzed as described above.

SDS-PAGE, western and far-western blots. Bacterial lysates and Ni-NTA purified polyhistidine-tagged ODV-E56 protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-Protean 3 cell (Bio-Rad) following the manufacturer's instructions. Samples were loaded into 12% bis-acrylamide gels with 4% stacking gels, along with unstained broad range molecular weight markers (Bio-Rad) and prestained All Blue Broad Range markers (Bio-Rad). The proteins were visualized using Coomassie blue stain. Gels used for western blots were transferred to PVDF membrane (Hyclone) using a semi-wet transfer XCell II Mini-blot module (Invitrogen) for 2 h at 25 V. Membranes were blocked overnight in PBS, 4% milk, 0.1% Tween, washed in PBS 0.1% Tween three times for 5 min with agitation, then incubated with mouse anti-His antibody (Sigma) or rabbit anti-ODV-E56 (Braunagel et al., 1996). Blots were washed as before, then probed with secondary antibody of goat anti-mouse-HRP (Sigma) or goat anti-rabbit-HRP (Sigma). The ECL ladder (GE Healthcare) was incubated with S protein-HRP for the final 30 min of the secondary antibody incubation. Bound antibody and weight markers were visualized using the Amersham ECL Plus Western Blotting Detection system (GE Healthcare). Fluorescent western blot signals were detected using a Typhoon 9410 Variable Mode Imager set at an excitation wavelength of 457 nm and emission wavelength of 532 nm. Far-westerns were performed as above with the addition of incubation of native or denatured ODV-E56 protein in sodium phosphate buffer pH 9.0 for 1 h at room temperature or TBS pH8.0 at 4 °C for 1 h. Control far western blots were incubated with an aphid gut binding peptide, GBP3.1 and a control peptide C6 (Liu et al., 2010). The membranes were then washed three times with TBS-0.1% Tween. ODV-E56 was detected using anti-His or anti-ODV-E56 antibody as previously described.

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