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The baculovirus Ac108 protein is a *per os* infectivity factor and a component of the ODV entry complex

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Abstract (209 words)

Baculoviruses orally infect insect larvae when these consume viral occlusion bodies (OBs). OBs consist of a crystalline protein matrix in which the infectious virus particles, the occlusion-derived viruses (ODVs), are embedded. The protein matrix dissolves in the alkaline environment of the insect's midgut lumen. The liberated ODVs can then infect midgut endothelial cells through the action of at least nine different ODV-envelope proteins, called *per os* infectivity factors (PIFs). These PIF proteins mediate ODV oral infectivity, but are not involved in the systemic spread of the infection by budded viruses (BVs). Eight of the known PIFs form a multimeric complex, named the ODV entry complex. In this study, we show for *Autographa californica* multiple nucleopolyhedrovirus that mutation of the *ac108* open reading frame abolishes the ODV oral infectivity, while production and infectivity of the BVs remains unaffected. Furthermore, repair of the *ac108* mutant completely recovered oral infectivity. With an HA-tagged repair mutant, we were able to demonstrate by western analysis that the Ac108 protein is a constituent of the ODV entry complex, which formation was abolished in absence of this protein. Based on these results, we conclude that *ac108* encodes a *per os* infectivity factor (PIF9) that is also an essential constituent of the ODV entry complex (Graphical abstract).

Keywords: bm91, sf58, ODV entry complex**,** *per os* infectivity factor, PIF, PIF9

Total number of words in main text: 4332

Introduction

The family *Baculoviridae* harbours a group of arthropod-specific viruses that infect the larval stages of lepidopteran, hymenopteran and dipteran insect species. Baculoviruses have circular double-stranded DNA genomes with 90 -180 open reading frames (ORF). Most baculoviruses have a biphasic replication cycle in which two different virus phenotypes are produced, budded viruses (BVs) and occlusion-derived viruses (ODVs) (see review by [1]). BVs are formed during the replication cycle when a (newly formed) nucleocapsid buds through the plasma membrane of an infected host cell. This phenotype is responsible for systemic spread of the viral infection in the larvae. Later in the replication cycle, ODVs are formed in the nucleus of the infected host cell, when one or more nucleocapsids are enveloped by a membrane that is derived from the inner nuclear membrane [2]. The virions of the ODV phenotype are embedded in a crystalline protein matrix of either polyhedrin or granulin to form occlusion bodies (OBs). This protein matrix protects the ODVs against detrimental influences of the environment after their dispersal.

Insect larvae are orally infected by ODVs when they eat from OB contaminated food-sources. After ingestion of the OBs, the protein matrix dissolves in the highly alkaline milieu of the insect's midgut lumen, liberating the ODVs. The released ODVs infect midgut columnar epithelial cells, which requires a specific set of viral proteins called *per os* infectivity factors (PIFs). These proteins are located in the ODV-envelope and nine different PIFs have been identified to date (reviewed by [3]). Three of these PIFs, PIF0, 1 and 2, are involved in binding of the ODVs to the brush border of the epithelial cells, but the biological role of the other PIFs is still enigmatic [4] [5]. In the prototype baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), eight of these PIFs have been found to form a large complex, named the ODV entry complex [6]. This complex consists of a stable core, formed by PIF1, 2, 3 and 4 and four more loosely associated PIFs: PIF0, 6, 7 and 8 [3, 6-8]. The core complex is regarded as rather stable as it was still detected by western blot analysis

with antiserum against one of its components after partial denaturation of the ODVs [7]. The other components of the entry complex (PIF0, 6, 7 and 8) were only found as part of the entry complex under non-denaturing conditions and are therefore regarded as loosely associated components as these PIFs apparently associate with the core with lower affinity than the core components themselves. All these components of the entry complex have also been identified as interaction partners of PIF1 in a co-immunoprecipitation study [6]. In such a co-immunoprecipitation study, Ac108 was also identified as a possible interaction partner of PIF1, suggesting that this protein might be involved in oral infectivity as well. The *Spodoptera frugiperda* MNPV (SfMNPV) homolog Sf58 has been shown to be essential for ODV oral infectivity, while its homolog in *Bombyx mori* MNPV (Bm91) appeared as non-essential [9, 10]. To determine whether Ac108 is involved in oral infectivity, we constructed and analysed an AcMNPV *ac108* mutant, as well as repaired viruses. The analyses of these viruses revealed that Ac108 is crucial for ODV oral infectivity and that this protein is a component of the ODV entry complex.

Results

Construction of ac108 mutant and repair bacmids

To inactivate the *ac108*-gene in the AcMNPV-bacmid bMON14272 without affecting surrounding genes, we deleted a 12 nt sequence from -8 to +4 relative to the A (+1) of the translational start codon by inserting a chloramphenicol acetyl transferase (*cat*) resistance gene, flanked by modified loxP-sites, via homologous recombination in *E. coli*, as described before [11]. The *cat*-gene was later removed by a CRE-lox reaction, leaving a 100 nt insertion that is composed of fused loxP-sites [12].The insertion and subsequent removal of the *cat*-gene was confirmed by PCR analysis with primers that annealed outside the recombined region (**Fig. 1**). When the wild type AcMNPV bacmid (Wt) was used as template, a PCR-

product of approximately 300 bp was produced. Insertion of the *cat*-gene (of approximately 1200 bp) in the *ac108*-locus (+*cat*) resulted in the formation of a 1500 bp PCR product and after removal of this gene (-*cat*), leaving a 100 bp scar, a 400 bp PCR product was produced.To enable the production of OBs by the resulting *ac108* mutant virus, the function of the polyhedrin (*polh*) gene was restored by Tn7-mediated transposition with a modified pFastBac Dual vector [7] that lacked the p10-promoter and in which the *polh*-ORF was inserted behind its own promoter (**Fig. 2**). The original, non-mutated bacmid was also provided with a *polh*-ORF and is used as the wild type control in this study. The *ac108* mutant bacmid was repaired via Tn7-mediated transposition with the modified pFastBac Dual vector that now also contained the *ac108* promoter and ORF (with or without C-terminal HA-tag). This ORF was inserted between the NcoI and SphI restriction sites, in opposite direction of the *polh*-ORF (**Fig. 2**).

The ac108 mutant virus produces OBs and BVs as wild type.

The constructed *ac108* mutant and repair bacmids were used for transfection of Sf21 cells to produce the modified viruses. For all these viruses, the transfected cells were found to produce OBs as can be seen in light microscopic images taken six days post transfection (**Fig. 3a**). When the OBs were dissolved in alkaline buffer on copper grids for electron microscopic analysis, it was found that the OBs of the *ac108* mutant contained ODVs, just as the repair mutants and the wild type (**Fig. 3b**). Furthermore, the mutant and repair viruses produced similar amounts of OBs as the wild type (Supplementary data). These findings indicate that the Ac108 protein is not crucial for OB-production, nor for embedding of ODVs in the protein matrix of the OBs.

BV production of the mutant and repair viruses was assessed by constructing one-step BV growth curves. For that Sf9-ET cells, which produce green fluorescent protein (GFP) upon baculovirus infection [13], were infected with BVs of the wild type, *ac108* mutant or repair

103 viruses at an MOI of 10, and the BV-titres of the medium were determined at 0, 24, 48 and 72
104 hours post infection (hpi) by end point dilutions assays. The BV-titres were found to increase
105 every 24 hrs with approximately 1-log unit for the *ac108* mutant and repaired viruses, just as
106 observed for the wild type (**Fig. 4**). At 48 and 72 hpi, infection with the *ac108* mutant seemed
107 to result in higher BV titres than infection with the other viruses, but this difference was not
108 statistically significant (one way ANOVA; 48 hpi: $F(3,4)= 2.88$ $p= 0.17$; 72 hpi: $F(3,4)= 1.22$
109 $p= 0.41$). From these results, it was concluded that the mutation in the *ac108*-locus did not
110 affect the production of OBs and BVs.

111 *Inactivation of ac108 affects the oral infectivity of ODVs.*

112 OBs of the mutant and repair viruses were fed by droplet feeding to *Spodoptera exigua* larvae
113 to test the impact of the mutation in the *ac108*-ORF on the oral infectivity of ODVs. The
114 droplet feeding assays were performed with a concentration of 3×10^8 OBs / ml on early L3
115 larvae that had been starved overnight. When OBs of the *ac108* mutant were fed, the larvae
116 did not show any mortality (**Table 1**). However, when the larvae were fed with OBs of the
117 *ac108* repair mutants (with or without HA-tag), they were successfully infected with similar
118 mortality rates as the wild type. These data indicate that *ac108* is essential for the oral
119 infectivity of ODVs in *S. exigua* larvae. In parallel, we tested whether the mutation in the
120 *ac108*-locus affected cell-to-cell transmission in the larvae by injecting BVs into the
121 hemocoel. When the midgut was bypassed in this way, all larvae were infected with 100%
122 mortality. This indicates that, in contrast to the ODVs, the BVs retain their infectivity after
123 mutation of the *ac108*-gene. Hence, the Ac108 protein appears to be essential for primary
124 infection of the midgut epithelial cells by ODVs, but is not required for subsequent systemic
125 infection by BVs.

126

Ac108 is present in BVs and ODVs.

To determine whether Ac108 is a structural component of ODVs, and potentially also of BVs, both virion phenotypes of the HA-tagged repair mutant were fractionated into envelope (E) and nucleocapsid (N) fractions and analysed by western blotting with anti-HA antibodies. With these antibodies, AC108 monomers were detected as 15 kDa polypeptides in the envelope and nucleocapsid fractions of BVs and ODVs. In both types of virus particles, this protein appeared to be enriched in the envelope fraction (**Fig. 5a**). When the fractionation of BVs and ODVs was validated by analyses with antisera against either the major capsid protein VP39, the ODV envelope protein PIF1 or the BV envelope protein GP64, it appeared that the fractionation of the virus particles was not completely efficient (**Figs. 5b-c**). When the fractionated BVs and ODVs were analysed with VP39 antiserum, the 39 kDa monomers were also found in small quantities in the envelope fraction of the ODVs. VP39 was not detected in the BV envelope fraction, as expected (**Fig. 5b**). Furthermore, when the BV fractions were analysed with GP64 antiserum, this BV envelope protein was not only detected in the envelope fraction, but also in the nucleocapsid fraction, although in smaller quantities (**left panel Fig. 5c**). Similarly, analysis of the ODV fractions with PIF1 antiserum not only resulted in the detection of the 60 kDa protein in the envelope fraction, but small traces were also found in the nucleocapsid fraction (**right panel Fig. 5c**). Apparently, the BV and ODV envelopes were not completely separated from the nucleocapsid or these proteins interacted with nucleocapsid proteins, resulting in detection of small amounts of envelope proteins in the nucleocapsid fractions. Based on these results, we concluded that Ac108 is a structural component of BVs and ODVs that is mainly associated with the virus envelope..

Ac108 is a constituent of the ODV entry complex.

The ODV envelope fractions of the *ac108* mutant and the HA-tagged repair viruses were analysed more extensively to determine whether the Ac108 protein is involved in formation of the ODV entry complex. Previous research showed that this complex has a stable core,

153 formed by PIF1-4, that resists partial denaturation in Laemmli buffer, when incubated at 50°C
154 before gel loading [7]. Under these conditions, the core complex was detected as a 170 kDa
155 band by western blot analysis with PIF1 antiserum. When the ODV envelope of the *ac108*
156 mutant and repair viruses were analysed under these conditions, the core complex was found
157 at the expected height for both mutants (**Fig. 6a**). The signal from the complex of the repair
158 mutant was less intense, but the signal from the 60 kDa PIF1 monomers in this lane was also
159 less intense compared to the surrounding lanes, indicating that slightly less protein was
160 loaded. In addition to the 170 kDa complex, smaller complexes (just below 170 kDa) were
161 also found, especially in the repair virus. Incubation at 95°C led to complete dissociation of
162 the core complex as only PIF1 monomers were found. Furthermore, when the repair mutant
163 was analysed with anti-HA antibodies after incubation at 50°C, Ac108 was only found in
164 monomeric form with a mass of 15 kDa (**Fig. 6b**). These results indicate that the core
165 complex can still assemble after mutation of the *ac108*-locus and that the Ac108 protein is not
166 a component of the stable core. When the envelope fraction of the HA-tagged repair virus was
167 analysed under non-denaturing conditions by blue native-PAGE followed by western blot
168 analysis, the ODV entry complex was detected as a 480 kDa band, irrespective whether
169 antisera were used against PIF1, PIF8 or HA, indicating that Ac108 is a constituent of the
170 ODV entry complex (**Fig. 6c**). However when analysing the *ac108* mutant, the 480 kDa
171 protein complex was not detectable with any of these antisera (and neither was a slightly
172 smaller one due to the absence of Ac108), indicating that Ac108 is involved in the formation
173 of the entry complex.
174
175 **Discussion**
176 The results show that the *ac108*-gene of AcMNPV encodes a *per os* infectivity factor, as
177 mutation of this gene abolished ODV oral infectivity, but had no observable effect on the
178 production of BVs and ODVs, nor on the infectivity of BVs. In contrast to all other identified

pif-genes, *ac108* is currently not considered as a core gene as its homologs have only been detected in viruses classified in the genera *Alphabaculovirus*, *Betabaculovirus* and *Gammabaculovirus*, but not in Culex nigripalpus NPV (CuniNPV), the single known member of the genus *Deltabaculovirus* [14]. However, the existence of an *ac108* homolog in CuniNPV cannot be entirely excluded though, as the homolog of another *pif*-gene (*ac110*, encoding PIF7) was initially missed in CuniNPV as well [15].Previous studies on the function of *ac108* homologs in SfMNPV and BmNPV resulted in contradictory observations regarding the involvement of their gene products in ODV oral infectivity. In SfMNPV, the *ac108*-homolog *sf58* was shown to be crucial for oral infectivity, while deletion of *bm91* in BmNPV increased only the time to death, but did not affect the lethality of the OBs [9, 10]. As *ac108* is more closely related to *bm91* (showing 96% amino acid sequence identity), it is remarkable to see that its deletion resulted in a different phenotype than observed after deletion of the homologous gene in BmNPV. On the other hand, OB lethality was also affected after deletion of *sf58* in SfMNPV (only 36% amino acid identity with AcMNPV *ac108*). These conflicting observations might result from the different strategies that were used to delete the *ac108*-homologs, just as observed before with various *pif6* mutants [16, 17]. Another explanation could be that the involvement of Ac108 in ODV oral infectivity is species dependent, as shown with the closely related PIF6 homologs in AcMNPV and BmNPV (93% amino acid identity). In AcMNPV, PIF6 is an ODV-envelope protein that is crucial for oral infectivity, while the homolog in BmNPV, Bm56, is associated with ODV-nucleocapsids and seemed involved in OB-morphogenesis [17, 18]. Apparently, two closely related homologous genes do not necessarily have the same function in different virus species, which could also be the case for *ac108* homologs. Furthermore, some studies indicate that the proteins which are involved in midgut infection could be different for various virus-host combinations, adding an extra layer of complexity. For example,*ac111* in AcMNPV was found to be involved in ODV oral infectivity in *Trichoplusia ni* larvae, but appeared redundant in *S. exigua* [19]. Another

example is a gene that is only present in group II alphabaculoviruses, *ha100* in *Helicoverpa armigera* NPV (HearMNPV), that also appeared to be involved in ODV oral infectivity [20]. In light of these findings, it could be reasoned that the *ac108*-homolog in SfMNPV is apparently more important for ODV oral infectivity in that specific virus-host combination than its homolog in BmNPV. Following this reasoning, it might be interesting to determine whether Ac108 is also crucial for ODV oral infectivity in various other permissive hosts other than the *S. exigua* larvae that were used in this study.

The current study also shows that the Ac108 protein is a structural component of ODVs, just as the previously identified PIFs. Furthermore, homologous proteins have been detected in the ODVs of *Antheraea pernyi* NPV, BmNPV, *Orgyia pseudotsugata* MNPV, SfMNPV and *Spodoptera litura* NPV [9, 10, 21-23]. In contrast to the homologues in these other alphabaculoviruses, Ac108 was also detected in BVs. However, the absence of Ac108 did not result in any observable phenotype regarding the BV infectivity. For PIF4 and PIF6, it was also reported that they were present in ODVs and BVs, and also here without noticeable effects on BV-function after deletion of the corresponding genes [17, 24]. So, whether these PIFs have a functional role in BVs, or whether it is a side effect of the routing that newly formed PIFs follow to reach the inner nuclear membrane is still enigmatic.

In previous studies, conflicting results were obtained regarding the interactions of Ac108 with other PIFs. In yeast-two hybrid and bimolecular fluorescence complementation assays, Ac108 was not found to interact directly with one of the known components of the entry complex, while this protein was identified as a PIF1 interaction partner in a co-immunoprecipitation study with PIF1 antiserum [6, 25]. . The results of this study are in line with the data obtained from the co-immunoprecipitation study and confirm that Ac108 is a component of the ODV entry complex by its detection in the entry complex under non-denaturing conditions. Ac108 was not found in a complex with PIF1 under denaturing conditions, indicating that this

protein associates with the core complex with relatively low affinity, compared to the interactions between the components of the core complex (PIF1-4). We also show that Ac108 is essential for the formation of the ODV entry complex as the complex was not found in the *ac108* mutant. PIF4, of the core complex, and the zinc-finger domain of PIF8, a loosely associated entry complex component, have also been shown to be essential for formation of the ODV entry complex [6, 15]. We conclude that *ac108* encodes a PIF-protein that mediates ODV oral infectivity in AcMNPV by its association with the ODV entry complex and propose an updated model from [3], in which Ac108 is added as a new loosely associated component (**Fig. 7**).

Material and Methods

Insect cells and lepidopteran larvae. TnH5 cells (from *Trichoplusia ni*) and Sf21 cells (from *Spodoptera frugiperda*) were cultured at 27°C in Grace's medium (Thermo Fisher), supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml gentamycin. *Spodoptera exigua* larvae were reared on artificial diet in a climate room of 25°C and 40% humidity and 16:8 hr (light:dark) photoperiod as described before [8].

Construction of recombinant bacmids. The *ac108*-gene of the AcMNPV bacmid bMON14272 (derived from the E2 strain)[26, 27] was inactivated by insertion of the *cat* resistance gene via homologous recombination. The *cat*-gene, flanked by mutated *loxP* sites [12], was amplified by PCR with Phusion polymerase (Thermo Fischer) from the pCR-TOPO-loxLE-cat-loxRE plasmid (kindly provided by L. Galibert, at the time at Généton, France) with primers that have 50 bp overhangs (in italics), homologous to the sequence that flanks the region to be deleted (5'- *GCCGGCGCGACCGCCCGTCGCTCTCGATATAATGTCGGCC GCCGTCGGTT*GCTCGGATCCACTAGTAACG-3' and 5'- *GCAGCGGCATCACGGTGAC GCCCGTCAAGTACAACTATTATTTGTAGTTA*CCTCTAGATGCATGCTCG -3'). These

primers were designed to replace a 12 nt fragment from -8 to +4 relative to the A (+1) of the start codon of *ac108*. The amplicon was purified from gel with the GFX Gel Band Purification kit (GE Healthcare) and introduced by electroporation in *E. coli* MW003 cells (MW001[28] with the *bla* gene removed), which contained the AcMNPV bacmid. λ RED recombination [11] was activated by heat induction at 42°C for 10 min. The transformed bacteria were incubated on LB-agar plates with chloramphenicol (34 µg/ml) and kanamycin (50 µg/ml) for up to two days at 32°C. Colonies were screened by PCR with primers that annealed outside the recombined region to validate insertion of the *cat*-gene (5'- CTTGGTTT AACAAGATCACAACC-3'and 5'- CGCGTCTTAACGGACGG-3'). The inserted *cat* gene was removed by transforming the cells with the temperature sensitive pCRE-TS plasmid [29] to allow a CRE-lox reaction that would leave a 100 bp loxP scar. The cells were incubated on LB-agar with kanamycin (50 µg/ml), ampicillin (100 µg/ml) and IPTG (120 µg/ml) at 32°C. Colonies were transferred to duplicate LB plates, one with kanamycin (50 µg/ml, the other with kanamycin and chloramphenicol (34 µg/ml). The *ac108* mutant bacmid was isolated from a colony growing only in absence of chloramphenicol and introduced by electroporation into DH10ß cells that contained the transposase helper plasmid (pMON7124) [26]. The *polh* locus of the *ac108* mutant bacmid was restored via Tn7-mediated transposition after electroporation with a modified pFastBac Dual vector, from which the p10-promoter was removed and the *polh* ORF was inserted downstream of its native promoter [7]. The non-mutated AcMNPV bacmid, provided equally with the *polh* ORF, was used to generate a bacmid-derived wild type virus.

To construct an *ac108* repair bacmid, the putative promotor (from 150 bp upstream of the ATG start codon) and the *ac108*-ORF were amplified from bMON14272 by PCR with Phusion polymerase (Thermos Fischer) with forward primer 5' – CATCCATGGTTAGTCCG CCCAACACG – 3' and reverse primer 5' – CATGCATGCTTATATTGTTGCATTTCTATT

TCTAATATCATAGTTTTC – 3'. For the HA-tagged repair, the reverse complementary sequence of the HA-tag was added to the reverse primer (shown in italics): 5' – ATGCATGC TTAATT*AGCGTAATCTGGAACATCGTATGGGTA*TATTGTTGCATTTCTATTTCTAATA TCATAGTTTTC – 3'. Purified amplicons were digested with NcoI and SphI restriction enzymes (the recognition sites are underlined) and ligated into the corresponding restriction sites of the modified pFastBacDual vector mentioned above. The resulting vectors, containing both *polh* and *ac108* (with and without HA-tag), were used to repair the *ac108* mutant bacmid via Tn7 mediated transposition.

Production of BVs and isolation of OBs. BV-stocks were generated by transfection of Sf21-cells with the constructed bacmids, using Expres2TR transfection reagent (Expres2ion Biotechnologies). Six days post transfection, passage 1 (P1) BV-stocks were harvested and amplified by an additional infection round in Sf21 cells to generate high titre P2 BV-stocks. OBs were produced in TnH5 cells, by infecting monolayers of these cells with BVs at an MOI of 2. Five to six days post infection, the cells were harvested and pelleted by centrifugation at 4000 x g for 30 minutes. The cells were then lysed by incubation in 0.1% SDS for 1 hr at 37ºC followed by sonication for 1 min at an output of 8 Watt. Cell lysis was verified by light microscopy and the released OBs were washed twice with MilliQ water. The OBs were further purified by ultracentrifugation over a 30 – 60% (w/w) sucrose gradient in a Beckmann SW32 rotor at 90.000 x g for 1 hr at 4°C. The OBs were obtained from the gradient with a Pasteur pipette and pelleted by centrifugation at 4000 x g for 30 min.

Construction of a BV growth-curve. To determine the BV production of the *ac108* mutant and repair viruses, one step growth curves were obtained. For this, Sf21 cells were seeded in a 24-wells plate with 3.0 x 10^5 cells per well and infected *in duplo* with the various types of BVs at an MOI of 10. The cells were incubated for 1 hr at 27ºC and washed once with Grace's medium to remove most of the free BVs. Directly after washing (t=0) and at 24, 48 and 72

hpi, medium samples were taken and analysed for BV-titres by end-point dilution assays (EPDA).

Electron microscopic analysis of OBs. Purified OBs were dissolved in DAS-buffer on carbon coated copper grids and negatively stained with 2% phosphotungstic acid, pH 6.8. The specimens were observed with a JEOL 1400 plus transmission electron microscope.

Bioassays. To determine the oral infectivity of OBs of the *ac108* mutant and repair viruses, early L3 *S. exigua* larvae were starved overnight and then orally inoculated by droplet feeding with 3.0 x 10^8 OBs / ml. The OBs were suspended in a 10% sucrose solution that was coloured blue with Patent Blue V Sodium salt (Fluka). The larvae were allowed to feed on the droplets for 10 min and the blue coloured larvae were then transferred to 12-wells plates, containing blocks of artificial medium, and incubated at 27ºC. Three days post infection, one day before the expected time to death, the larvae were inspected and non-viral deaths were excluded from further analysis. The larvae were scored for liquefaction until pupation. To test the infectivity of BVs, 1 µl normalized BV-stocks (3.5 x 10^7 $TCID_{50}$ / ml) were injected into the hemocoel of L4 *S. exigua* larvae, using an Humapen Luxura insulin pen. From four days post infection, the larvae were scored on a daily basis for liquefaction.

Protein sample preparation, SDS-PAGE and blue native-PAGE. The ODVs of approximately 3.0 x 10^8 OBs were released by incubation in DAS-buffer (0.1 M Na_2CO_3, 166 mM NaCl and 10 mM EDTA, pH 10.5) for 10 min at 37ºC and isolated as previously described . The isolated ODVs were fractionated in an envelope- and a nucleocapsid fraction by overnight incubation in extraction buffer (6.25 mM Tris, 37.5 mM NaCl, 1% Triton X-100, pH 7.2) at 4ºC. The nucleocapsids were then pelleted by centrifugation at 20.800 x g for 20 min and the envelope fraction in the supernatant was collected for further analysis. The nucleocapsids in the pellet were resuspended in extraction buffer and also collected for further analysis. The BVs were pelleted by centrifugation at 20.800 x g for 25 min and fractionated as described

above for the ODVs. Both fractions of the BVs and ODVs were analysed by SDS-PAGE and western blot. The protein samples were incubated in Laemmli buffer at either 50 or 95ºC for 10 min and separated in a 12% SDS-PAGE gel. The proteins were transferred to a PVDF membrane under standard conditions. The envelope fractions of ODVs were also analysed under non-denaturing conditions by blue native-PAGE (BN-PAGE), using the NativePAGE™ Novex® Bis-Tris Gel system (Invitrogen), as previously described [8]. Blotting to a PVDF membrane was in this case performed with NuPAGE® Transfer buffer (Invitrogen) according to the manufacturer's protocol.

Western blot analysis. The blotted PVDF-membranes were analysed with antisera against PIF1 and PIF8 as previously described [6, 7, 30]. In brief: rat anti-PIF1 (1 : 2000 dilution), and rabbit anti-P95 antiserum (PIF8) (1 : 2000 dilution) were used as primary antibodies. Other membranes were incubated with mice anti-VP39 (1 : 1000 dilution; kindly provided by dr. R.M. Kotin and dr. L.E. Volkman), and mice anti-GP64 antisera (1 : 1000 dilution; kindly provided by dr. G.W. Blissard). To detect HA-tagged AC108 in the repair mutant, rat anti-HA antibodies (1 : 2000 dilution, Roche 3F10) were used as primary antibodies. Goat anti-rabbit (1 : 2000 dilution, Dako), goat anti-rat (1 : 2000 dilution, Sigma A8438) and goat anti-mouse (1 : 2000 dilution, Sigma A5153), conjugated to alkaline phosphatase, were used as secondary antibodies to detect the target proteins by conversion of the NBT-BCIP substrate (Sigma) into a blue-purple precipitant in AP-buffer (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM MgCl, pH 10.5).

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448 **Figure legends**

449 **Graphical abstract.** Wild type ODVs (Wt) have an intact ODV entry complex in their
450 envelope and are orally infectious towards insect larvae (left panel). In absence of Ac108 (mut
451 *ac108*), the stable core is still present but nevertheless fails to form an entry complex,
452 affecting the ODV oral infectivity (right panel). The components of the core complex are
453 depicted in yellow and the loosely associated components are depicted in red. PIF7 is depicted
454 in green as its affinity with the complex is currently not known.

455 **Fig. 1** Conformation of insertion and removal of the chloramphenicol acetyl transferase (*cat*)
456 resistance gene in the *ac108*-locus of the AcMNPV bMON14272-bacmid by PCR analysis.
457 Primers were used that annealed outside the recombined region. The addition of double
458 distilled water (MQ) as template served as a negative control for the PCR reaction and did not
459 result in the formation of a PCR-product. Presence of the *cat*-gene is indicated as +*cat* and its
460 absence as –*cat*.

461 **Fig. 2** Schematic overview of the constructed AcMNPV bacmids. The *ac108*-gene was
462 inactivated (mut-*ac108*) by deletion of a 12-nucleotide sequence from -8 to +4 relative to the
463 A (+1) of the translational start codon. After removal of the *cat*-gene, a 100 bp scar was left,
464 which is indicated in dark blue. The polyhedrin gene (*polh*) was restored by Tn7-mediated
465 transposition with a modified pFBD-*polh* vector. The *ac108* mutant bacmid was repaired by
466 transposition with a pFBD-polh vector that also contained the *ac108* putative promotor and
467 ORF (pFBD-*polh*-*ac108*) or with such a vector that contained an HA-tagged *ac108* ORF
468 (pFBD-*polh*-*ac108*HA). This resulted in the *ac108* repair (rep-*ac108*) and HA-tagged *ac108*
469 repair (rep-*ac108*HA) bacmids.

470 **Fig. 3** Light microscopical images of Sf21 cells by six days post transfected with the
471 constructed AcMNPV bacmids. (a) The arrows indicate Sf21 cells with OBs after transfection

of either wild type, *ac108* mutant (mut-*ac108*), *ac108* repair (rep-*ac108*) or HA-tagged *ac108* repair (rep-*ac108*HA) bacmids. The white scale bar corresponds with 10 µm. (b) The OBs were dissolved in alkaline buffer on copper grids, negatively stained and analysed by electron microscopy to validate the presence of ODVs in the OBs of the mutant and repair viruses. The black scale bar corresponds with 100 nm.

Fig. 4 BV-growth curves after infection of Sf21 cells with BVs wild type AcMNPV, the *ac108* mutant (mut-*ac108*), *ac108* repair mutant (rep-*ac108*) or HA-tagged *ac108* repair mutant (rep-*ac108*HA). The BV concentration increased approximately 1-log unit every 24 hrs for all mutants, just as observed with wild type, indicating that AC108 is not involved in BV-production.

Fig. 5 Western blot analysis of fractionated BVs and ODVs of the HA-tagged *ac108* repair mutant. BVs and ODVs were fractionated by incubation in extraction buffer, containing 1% Triton-X100, in an envelope (E) and nucleocapsid (N) fraction and analysed with (a) anti-HA antibodies to detect AC108, (b) antiserum against VP39 as marker for the nucleocapsids, and (c) antiserum against GP64 as marker for the BV-envelope (left panel), and anti-PIF1 antiserum (right panel) as marker for the ODV envelope. AC108 was detected in BVs and ODVs, in which this protein appeared enriched in the envelope fractions.

Fig. 6 Western blot analysis of the ODV-envelope fraction of the *ac108* mutant (mut) and HA-tagged *ac108* repair viruses (rHA) under denaturing and non-denaturing conditions. Under denaturing conditions, the purified proteins were heated at either 50 or 95°C and analysed with antiserum against PIF1 (a) or with anti-HA antibodies (b). The ODV envelope proteins were also analysed under non-denaturing conditions with anti-PIF1 antiserum (left panel of c), anti-PIF8 antiserum (middle panel of c) and anti-HA antibodies (right panel of c).

Fig. 7 An updated model of the ODV entry complex. AC108 is added as a loosely associated component (together with PIF0, 6 and 8), which are depicted in orange. The components of the core complex are depicted in yellow and PIF7 is depicted in green as its association with the complex has only been determined under non-denaturing conditions and not after denaturation. PIF5 is not part of the entry complex and is depicted in white.