Baculovirus *per os* infectivity – a complex matter

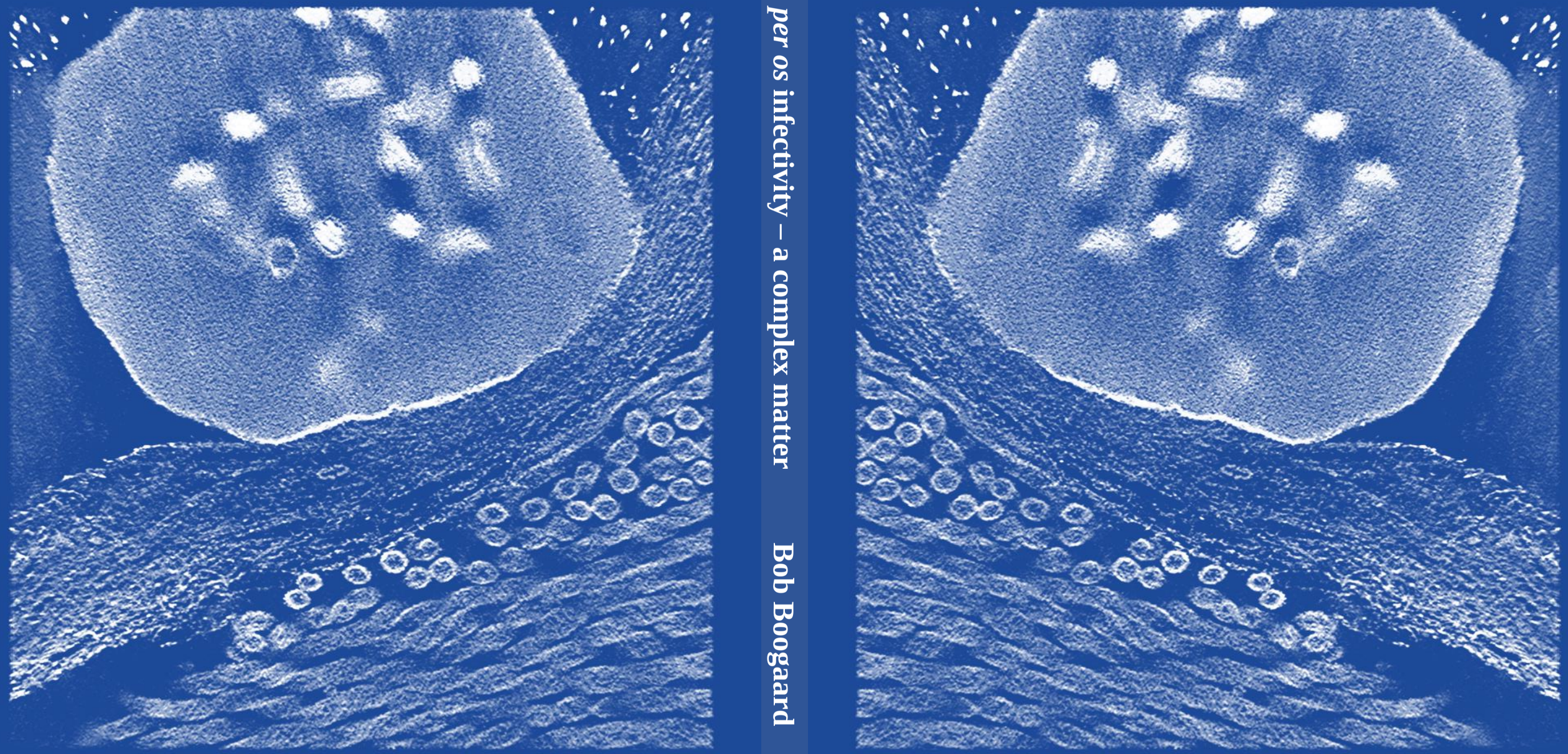
Bob Boogaard

2020

Baculovirus *per os* infectivity

–

a complex matter



Bob Boogaard

Propositions

1. Baculovirus *per os* infectivity factors act according to the slogan of the three musketeers: all for one, one for all (this thesis).

2. The use of only fluorescence dequenching assays to study membrane fusion leads to premature conclusions on virus entry (this thesis).

3. The origin of virus-like sequences, identified by metagenomic sequencing, should be elucidated before the claim of a newly identified virus can be deposited.

4. The use of recombinant DNA technology to increase herbicide resistance in soya bean plants is immoral.

5. The value of fundamental scientific research without direct societal relevance is underestimated.

6. The broadcast of the new “Dancing with the Stars” series increases the horizontal transmission of the dance-virus rather than its vertical transmission to the next generation.

Propositions belonging to the thesis, entitled

Baculovirus *per os* infectivity – a complex matter

Bob Boogaard
Wageningen, 15 January 2020

Baculovirus *per os* infectivity factors

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This research was conducted under the auspices of the Graduate School for Production Ecology and Resource Conservation.

Baculovirus *per os* infectivity factors

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Bob Boogaard

Thesis

submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus,

Prof. Dr A.P.J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Wednesday 15 January 2020

at 4 p.m. in the Aula.

Bob Boogaard
Baculovirus *per os* infectivity – a complex matter, 157 pages

PhD thesis, Wageningen University, Wageningen, The Netherlands (2020)
With references, with summaries in English and Dutch

ISBN: 978-94-6395-213-2
DOI: https://doi.org/10.18174/506926

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Chapter 1

General introduction and thesis outline

Introduction

Baculoviruses are insect specific viruses that are used in agriculture as biological insecticide against insect pests (see review by Szewczyk *et al.*, 2009). For example, the Helicoverpa armigera nucleopolyhedrovirus (HearNPV) is used in the cotton fields in China and India against the cotton bollworm (*Helicoverpa armigera*) (Mettenmeyer, 2002). In Brazil, soybean plants are protected against the velvet bean caterpillar (*Anticarsia gemmatalis*) with the Anticarsia gemmatalis multiple nucleopolyhedrovirus (AgMNPV) (Moscardi and Sosa-Gomez, 1992). The major advantage of using baculoviruses in agricultural pest management is the small ecological footprint as most baculoviruses infect only one or a few related insect species. The pest insects are infected at their larval stages when consuming virus contaminated plant material, after which the ingested viruses infect the epithelial cells in the larval midgut. The primary infection of the midgut is one of the host range determinants of baculoviruses (Haas-Stapleton *et al.*, 2005) and is the first crucial step of the replication cycle. Despite the broad scale application of these viruses as biological insecticide, the underlaying mechanism of the midgut infection process is still enigmatic. The research presented in this thesis therefore studies the viral proteins that are essential for the virus entry into midgut epithelial cells.

The baculovirus infection cycle

Baculoviruses infect insect larvae via the oral route when a larva consumes food sources, which are contaminated with viral occlusion bodies (OBs). OBs consist of a protein matrix of crystallized polyhedrin or granulin, in which the virus particles are embedded (**Fig. 1**). The protein matrix protects the virus particles after the dispersal of OBs into the environment. These virus particles are called: occlusion derived viruses (ODVs). Upon ingestion of the OBs by an insect larva, the protein matrix dissolves in the highly alkaline environment of the midgut, resulting in release of the ODVs (**Fig. 2a and b**). The ODVs pass the peritrophic membrane that separates the gut contents from the epithelium

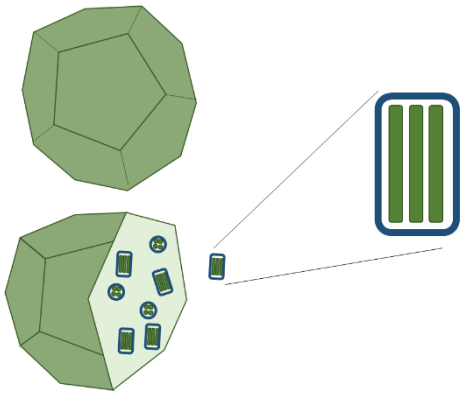

Figure 1: Baculovirus occlusion bodies with embedded virions. The occlusion bodies consist of crystalized polyhedrin or granulin, in which the occlusion derived viruses (ODVs) are embedded. The ODVs contain multiple nucleocapsids (in green) and is surrounded by an envelope that is derived from the host cell inner nuclear membrane (in blue).

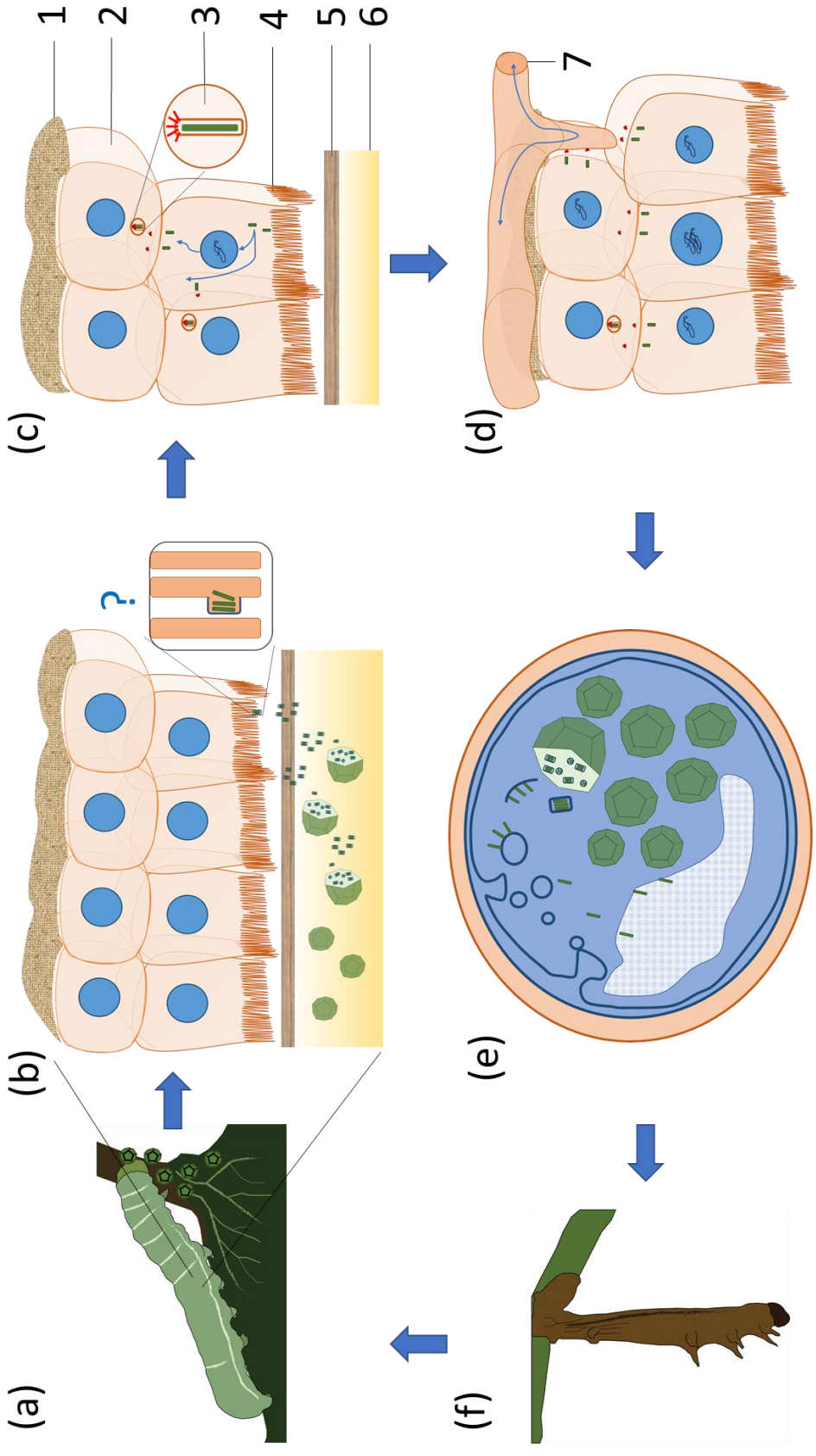

Figure 2: The baculovirus infection cycle. A lepidopteran larva ingests OB contaminated plants (a). The OBs dissolve in the alkaline midgut, resulting in release of the ODVs that infect midgut epithelial cells (b). The parental nucleocapsids migrate towards the nucleus for the first round of replication, or directly bud through the plasma membrane that contain the viral fusion protein (GP64 or F-protein; depicted as red spikes) in the form of a budded virus (c). The budded virus infect surrounding columnar or regenerative midgut cells and form infection foci. From the midgut, the infection is spread to other larval tissues via the trachea (d). At the end of the replication cycle, the nucleocapsids remain inside the nucleus and are enveloped by a membrane that is derived from the inner nuclear membrane to form new ODVs (e). These are occluded in the protein matrix. In the end, the larva dies and liquefies and the OBs are dispersed into the environment for the next infection round (f). 1. Basal lamina; 2. Regenerative midgut cell; 3. Budded virus; 4. Epithelial midgut cell; 5. Peritrophic membrane; 6. Midgut lumen; 7.Trachea.

(Hegedus *et al.,* 2009), and infect the midgut epithelium cells (**Fig 2b**). Mainly the epithelial cells in the anterior region of the midgut are infected by the ODVs (Javed *et al.,* 2016).

The ODVs bind and fuse with the microvilli at the apical side of the epithelium cells, followed by release of the nucleocapsids into the cytosol (Kawanishi *et al.,* 1972, Granados *et al.,* 1981, Horton and Burand, 1993). At the start of this PhD research, eight different ODV envelope proteins were known to be crucial for midgut infection (Faulkner *et al.,* 1997, Kikhno *et al.,* 2002, Pijlman *et al.,* 2003, Ohkawa *et al.,* 2005, Fang *et al.,* 2009, Sparks *et al.,* 2011a, Nie *et al.,* 2012, Zhu *et al.,* 2013). These proteins are called *per os* infectivity factors or PIFs. Three PIFs had been found to be important for the binding of ODVs to the midgut epithelium (Haas-Stapleton *et al.,* 2004, Ohkawa *et al.,* 2005) and the biological role of the other PIFs in midgut entry was unknown. Six of the PIFs had been shown to participate in the formation of a multimeric complex (Peng *et al.,* 2010, 2012), which in this thesis is named the "ODV entry complex". This thesis focuses on the involvement of this protein complex in primary infection of the midgut cells (**Fig. 2b**). The biochemical and functional characteristics of the PIFs and their involvement in formation of the ODV entry will be further introduced in Chapter 2.

After the ODV envelope has fused with the microvillar plasma membrane, the parental nucleocapsids are released and migrate to the nucleus for the first round of replication and production of progeny capsids (**Fig. 2c**). The infected midgut cells subsequently produce a second virion phenotype, the budded viruses (BVs). These are formed when the progeny nucleocapsids bud through the host cell plasma membrane that contains the BV fusion protein (either GP64 or the F-protein) (**Fig. 2c**). BVs infect the adjacent midgut cells, which ultimately leads to the formation of infection foci in the midgut epithelium (Engelhard *et al.,* 1994, Flipsen *et al.,* 1995, Javed *et al.,* 2016). There is experimental evidence that suggests that some parental nucleocapsids directly migrate to the underlaying regenerative cells as those were found infected before virus replication in the epithelial cells became apparent (Flipsen *et al.,* 1995). The infection of the regenerative cells could be important to prevent clearing of the infection by sloughing of the infected epithelial cells (Flipsen *et al.,* 1993). The direct transfer of some parental nucleocapsids might be mediated by the early expression of the BV fusion protein GP64 (Washburn *et al.,* 2003), which is also required for the formation of the infection foci (Javed *et al.,* 2016).

The virus infection is systemically spread from the midgut to other larval tissues by BVs via trachea and the hemolymph (**Fig. 2d**) (Engelhard *et al.,* 1994, Chapter 4 Thesis J.T.M. Flipsen,

1995), where the infected cells also start to produce BVs. Later in the infection cycle, the progeny virus production is switched from BVs to ODVs, in which the nucleocapsids are retained inside the host cell nucleus and are enveloped by a PIF-containing membrane that is derived from the inner nuclear membrane (**Fig. 2e**) (Williams and Faulkner, 1997). The ODVs are embedded in crystallized polyhedrin or granulin to form new OBs for the next infection round. The OBs are dispersed into the environment after liquefaction of the larva by the activity of viral cathepsin and chitinase (**Fig. 2f**) (Hawtin *et al.,* 1997).

As previously mentioned, this PhD thesis aims to study how PIF proteins, and the multimeric complex they form, achieve entry into the midgut epithelial cells, to initiate a new infection round in insect larvae. PIF proteins are highly conserved among the baculoviruses and some are even found in related insect viruses, suggesting that this entry mechanism has already evolved millions of years ago (Thézé *et al.,* 2011). Furthermore, the PIFs operate under highly alkaline conditions, in contrast to other known viral entry proteins that act under acidic or neutral conditions. The exceptional conditions for virus entry and the formation of a multimeric complex suggest that we are dealing with an unknown entry pathway, of which the molecular mechanism has yet to be elucidated.

Outline of the thesis

At the start of this thesis research, eight PIFs had been identified, of which six were known to be present in what we now call the ODV entry complex. The aim of this thesis was to determine the composition of the baculovirus entry complex in more detail and to understand the requirements for complex formation. Furthermore, the first studies were conducted to understand the biological function of complex formation for the entry into midgut cells. For this, a series of *pif* mutant viruses were generated for the prototype alphabaculovirus Autographa californica multiple nucleopolyhedrovirus (AcMNPV) and analysed for their ability to assemble the complex and to orally infect *Spodoptera exigua* larvae. For some mutants, fluorescently labelled ODVs were generated and their infection properties were monitored on isolated larval midgut cells by confocal microscopy.

Most data on PIF proteins, described in earlier literature, were obtained not knowing that these proteins form a complex. **Chapter 2** therefore reviews PIF literature and the function of PIF proteins in view of their presence in the complex.

When the first *pif* mutant viruses were generated for our initial studies on the ODV entry complex, it was found that besides the PIF protein targeted by mutagenesis, also the non-targeted PIFs were not detectable when the OBs were produced in *S. exigua* larvae. However, the non-targeted PIFs were still found when the mutant viruses were produced in cultured insect cells. It turned out that the co-occluded host proteases in larval-derived OBs, which have been described by Eppstein and Thoma (1975), Payne and Kalmakoff (1978) and Wood (1980), severely affected the integrity of the PIFs in these mutant viruses. **Chapter 3** describes how these proteases affect the PIFs and the complex when the viruses are produced by their (natural) caterpillar host. For this, OBs of bacmid derived AcMNPV and various *pif* deletion mutants are produced in *S. exigua* larvae and cultured insect cells, and the individual PIFs and the complex are compared by western blot analyses with antisera against various PIF proteins.

Earlier proteomic analysis (Peng *et al.,* 2012) identified the AcMNPV protein AC108 as a putative PIF1 interaction partner. In **Chapter 4**, AC108 is studied for its function in oral infectivity and presence in the entry complex by using *ac108* deletion and repair viruses. The function of AC108 in binding to and entry into midgut epithelium cells is subsequently studied using fluorescently labelled ODVs of the *ac108* mutant and wild type virus. Interactions of ODVs with isolated midgut epithelium cells are followed as function of time by life imaging with a confocal microscope (**Chapter 5**). This chapter also reports observations with fluorescent ODVs of a *pif3* deletion mutant that is still able to bind and fuse with the epithelium cells according to literature, but nevertheless fails to establish infection in the midgut (Ohkawa *et al.,* 2005).

PIF1 and PIF2 are essential for formation of the entry complex and are involved in interaction with the midgut epithelium of the host (Ohkawa *et al.,* 2005, Peng *et al.,* 2010). To determine whether these PIFs interact with the midgut directly or whether ODV binding is mediated via the interaction with other PIFs, a series of *pif1* and *pif2* truncation mutant viruses are analysed for oral infectivity and complex formation (**Chapter 6**). In addition, the requirements for complex formation are analysed by coimmunoprecipitation studies with the truncation mutants and various *pif* deletion mutant viruses.

PIF5 is the only PIF that is not part of the ODV entry complex (Peng *et al.,* 2012), while being important for the oral infectivity (Harrison *et al.,* 2010, Sparks *et al.,* 2011). Furthermore, some non-PIF proteins are also known to influence the infectivity of ODVs, although more subtle (Xiang *et al.,* 2011, Sparks *et al.,* 2011b, Li *et al.,* 2018, Lapointe *et al.,* 2004, Zhang *et al.,*

2005). To determine how the ODV entry complex relates to the other ODV envelope proteins, a proteomic analysis is performed after immunoprecipitation of three different components of the entry complex (PIF6, 8 and 9), and PIF5 (**Chapter 7**).

In **Chapter 8**, the results are discussed in a broader perspective. The complexity of midgut infection by ODVs is discussed and the entry complex is compared with two other known viral entry complexes. Furthermore, a model of how PIFs mediate infection of the midgut epithelial cells is proposed.

Chapter 2

An advanced view on baculovirus *per os* infectivity factors

Bob Boogaard, Monique M. van Oers, Jan W. M. van Lent

Abstract

Baculoviruses are arthropod-specific large DNA viruses that orally infect the larvae of more than six hundred different insect species of the orders Lepidoptera, Hymenoptera and Diptera. Insect larvae become infected when they eat a food source that is contaminated with viral occlusion bodies (OBs). These OBs contain occlusion-derived viruses (ODVs), which are released upon ingestion of the OBs and infect the endothelial midgut cells. At least nine different ODV envelope proteins are essential for this oral infectivity and these are denoted as *per os* infectivity factors (PIFs). Seven of these PIFs form a complex, consisting of PIF1, 2, 3 and 4 that form a stable core complex, and PIF0 (P74), PIF6 and PIF8 (P95) that associate with this complex with lower affinity than the core components. The existence of a PIF complex and the fact that the *pif* genes are conserved in baculovirus genomes suggests that PIF-proteins cooperatively mediate oral infectivity rather than as individual functional entities. This review therefore discusses the knowledge obtained for individual PIFs in light of their relation with other members of the PIF complex.

This chapter has been published in modified form as:
Boogaard, B., van Oers, M. M., & van Lent, J. W. M. (2018). An advanced view on baculovirus *per os* infectivity factors. *Insects*, *9*, 84.

Introduction

The *Baculoviridae* form a family of arthropod-specific large double stranded DNA viruses that infect the larvae of more than six hundred different insect species (Williams *et al.,* 2017). The family is divided into four genera, which reflects the co-evolution of these viruses and the insects they infect. Viruses classified in the genera *Alphabaculovirus* and *Betabaculoviruses* infect the larvae of lepidopteran insect species. *Gammabaculoviruses* infect larvae of hymenopteran insect species and the single known virus classified as *Deltabaculovirus* infects *Culex* mosquito larvae (Reviewed by Williams *et al.,* 2017). The majority of baculoviruses that infect lepidopteran larvae have narrow host ranges, i.e. a specific baculovirus infects the larval stage of only one or a few host species. Thus, several baculoviruses have been successfully used as biological control agents for particular species of Lepidoptera that are economic pests in agriculture and forestry (Reviewed by Szewczyk *et al.,*2009). For example, Anticarsia gemmatalis multiple nucleopolyhedrovirus (AgMNPV) is used to protect soybean plants against the velvet bean *(Anticarsia gemmatalis)* caterpillar in Brazil (Moscardi *et al.,* 2007).

The name baculovirus derives from the latin word baculum, which refers to the rod-shaped morphology of their nucleocapsids. All baculoviruses have large, circular double stranded DNA genomes that range from 80-180 kbp in size and encode 90-180 genes (Williams *et al.,* 2007). Baculoviruses form two different virion phenotypes, the budded viruses (BVs) and the occlusion-derived viruses (ODVs). The BV virion type is responsible for systemic spread of the infection within the larvae. Each BV consists of a single nucleocapsid surrounded by an envelope derived from the plasma membrane of a host cell. These BVs display a fusion protein to allow cell entry (either GP64 or F protein). The ODVs in contrast are formed in the nuclei of infected cells and consist either of a single (SNPV) or multiple (MNPV) nucleocapsids. ODVs are surrounded by an envelope that is derived from the inner nucleus membrane and contains a number of proteins encoded by baculovirus genes (see below). ODVs are also embedded in a crystalline matrix, forming occlusion bodies (OBs). The OBs produced by Alpha-, Gamma-, and Deltabaculoviruses are composed of a viral protein named polyhedrin, while the OBs produced by Betabaculoviruses are comprised of a different viral protein, named granulin. The structure of the OBs protects the ODVs against detrimental influences from the environment. ODVs are responsible for the horizontal transmission of the virus. When insect larvae eat OB-contaminated food, the OBs disintegrate in the highly alkaline milieu of the larval midgut, which releases ODVs into the gut lumen. ODVs then bind to the microvilli of midgut columnar cells and the viral envelope fuses with the cell membrane, releasing nucleocapsids into the cell.

This process of virus entry is mediated by a specific set of proteins in the ODV envelope, the so-called *per os* infectivity factors (PIFs). For Autographa californica multiple nucleopolyhedrovirus (AcMNPV), Peng and co-workers showed that PIFs form a large protein complex, suggesting that these proteins mediate midgut entry of baculovirus ODVs in a cooperative manner (Peng *et al.*, 2010, 2012). In this review, we summarize current knowledge of PIF-protein structure and function.

Definition and general features of PIFs

Per os infectivity factors are proteins that are essential for primary infection of the midgut columnar cells as deletions of *pif*-genes inhibit the oral infectivity of ODVs. Using AcMNPV, nine ODV-envelope proteins have been identified as PIF (**Table 1**). Since PIFs are not needed for cell to cell spread of the virus within the insect, the infectivity of BVs is not affected after deletion of a *pif*-gene. As a consequence, *pif* deletion mutants show wildtype characteristics in cultured insect cells. Larvae, however, can only be infected with such mutants when the midgut is bypassed by injecting BVs into the hemocoel (Faulkner *et al.*, 1997, Kikhno *et al.*, 2002, Pijlman *et al.*, 2003). All known PIF proteins identified to date are encoded by baculovirus core genes, i.e. they are present and highly conserved in all sequenced baculovirus genomes across the four genera in the family *Baculoviridae* (Javed *et al.*, 2017, Wang *et al.*, 2017) Some PIFs are also found in other invertebrate DNA viruses like nudiviruses, bracoviruses, hytrosaviruses and nimaviruses (Reviewed by Wang *et al.*, 2017). This high level of conservation suggests that PIFs mediate an ancient virus entry pathway.

A feature shared by all PIFs is the presence of highly conserved cysteine residues (**Table 1**). This high-level of cysteine conservation indicates that sulfate bridges are important for the structural and functional properties of PIFs. *In silico* analysis, using the Phobius algorithm

Table 1. Identified PIFs in AcMNPV

PIF	ORF	Size (kDa)	Nr. Of Cys.	Reference
PIF0	*ac138*	74	6	Faulkner *et al.* 1997
PIF1	*ac119*	60	24	Kikhno *et al.*, 2002
PIF2	*ac22*	44	14	Pijlman *et al.*, 2003
PIF3	*ac115*	23	12	Ohkawa *et al.*, 2005
PIF4	*ac96*	20	2	Fang *et al.*, 2009
PIF5	*ac148*	56	6	Harrison *et al.*, 2010
PIF6	*ac68*	16	1	Nie *et al.*, 2012
PIF7	*ac110*	7	1	Liu *et al.*, 2016
PIF8	*ac83*	96	13	Zhu *et al.*, 2013

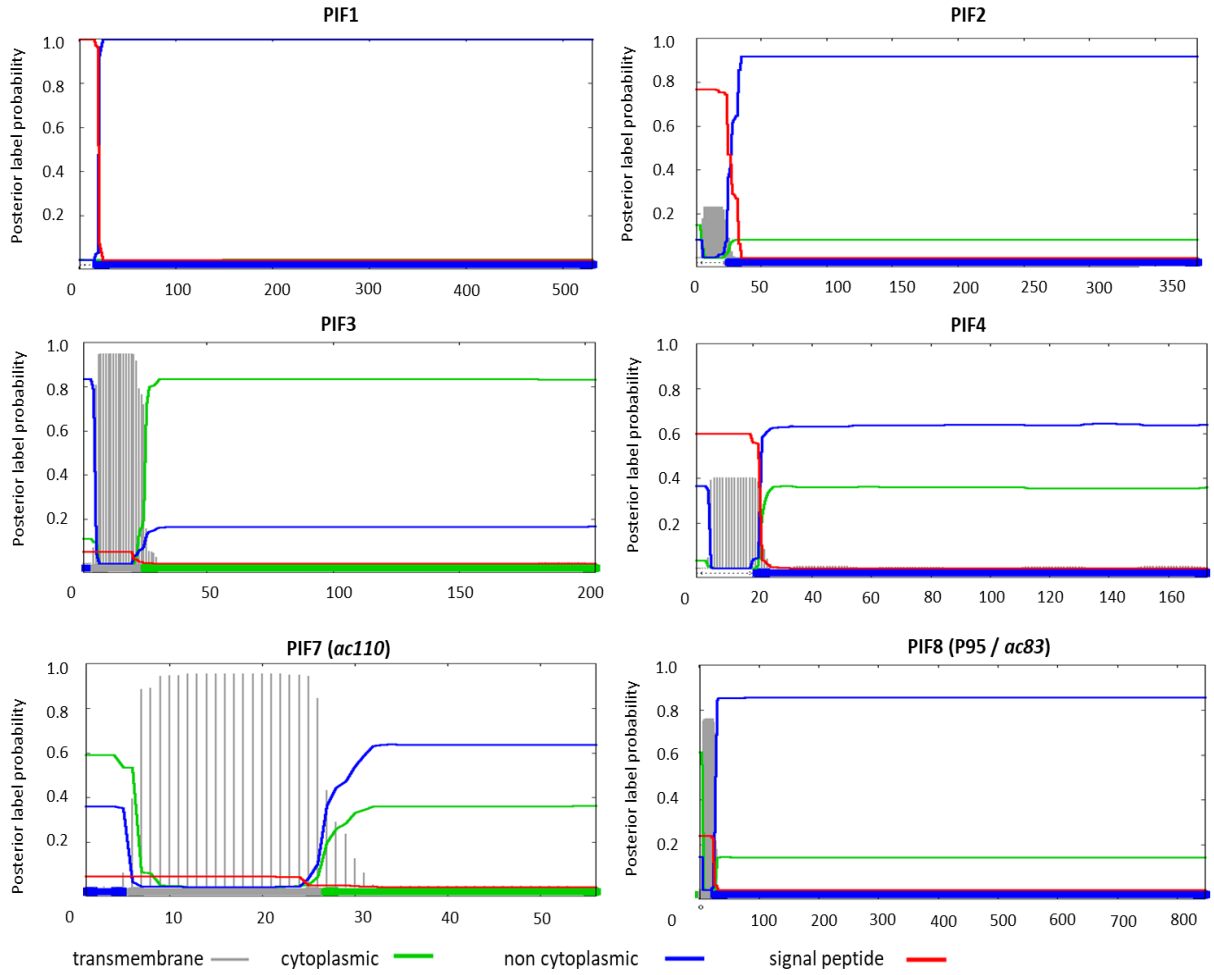

Figure 1. Prediction of the presence of transmembrane domains and signal peptides in *per os* infectivity factors (PIFs) and orientation of these proteins in an infected host-cell with the Phobius algorithm (Käll *et al.*, 2004)**.** On the *y*-axis: the probability for the presence of transmembrane domains (grey), cytoplasmic (green) or non-cytoplasmic (blue) orientation of the amino acid sequence and presence of a signal peptide (red). The final result of the prediction is summarized by the bar at the bottom with the indicated colours. The *x*-axis shows the number of the amino acids of that specific sequence. PIF1, PIF2, PIF3, PIF4, PIF7 (*ac110*) and PIF8 (P95/*ac83*) are predicted to have an N-terminal signal peptide or transmembrane domain.

(Käll *et al.*, 2004) to find transmembrane domains and signal peptides, revealed N-terminal signal peptides and/or transmembrane helices in six out of the nine PIFs (PIF1 to 4, PIF7 and 8) (**Fig. 1**). For ODV-E66, another ODV-envelope protein, such an N-terminal domain was shown to be important for its translocation to the inner nuclear membrane (Braunagel *et al.*, 2004). This domain was therefore called the inner nuclear membrane sorting motif (INM-SM) and consists of a hydrophobic domain of about 18 amino acids followed by at least one positively charged amino acid within 4-8 amino acids from the C-terminal end of the hydrophobic sequence. The predicted N-terminal transmembrane domain of the abovementioned PIFs also meets these criteria and the importance of this domain for routing to the inner nuclear membrane has been confirmed for PIF3 and PIF8 (**Fig. 2**) (Li *et al.*, 2007, Zhu

AcMNPV (E2)

ODV-E66: MSIVLIIVIVVIFLICFLYLSNSNN**K**NDAN**K**NNAFIDLNP
PIF1: MHFAIILLFLLVIIAIVYTYVDLIDV*HH*EEV**R**YPITVFDN
PIF2: MYRVLIFFLFVFYIVYQPFYQAYL*H*IG*H*AQQDYNDTLD
PIF3: MLNFWQILILLVIILIVYMYTF**K**FVQ**K**FILQDAYMHINEL
PIF4: MLSIMLAIVFVLFVLIYLIISI**K**N*HH*PFLH**R**IETLIQDFN
PIF7: MKYFLSATFLIIVFMYLMYFCISIVVNNA*H*VQQDLFY*H*YN
PIF8: MMSGVMLLMLAIFLIIAFTLMYLAIYFEFDETTFT**K**RLGV

Figure 2: Comparison of the N-terminal transmembrane domains of PIF1, PIF2, PIF3, PIF4, PIF7 and PIF8 with the inner-nuclear membrane sorting motif of ODV-E66. In red: the hydrophobic region; in bold and underlined: the positively charged amino acids lysine (K) and arginine (R); in italic and underlined the amino acid histidine (H) which is only positively charged when protonated.

et al., 2013). Similar N-terminal regions were not predicted for PIF0, PIF5 and PIF6 (**Fig. 3**). These proteins rather have hydrophobic domains at their C-termini, suggesting an alternative way for routing to the inner nuclear membrane and the ODV-envelope.

The AcMNPV PIF-complex

As shown by Peng *et al.*, 2010, 2012, AcMNPV forms a macromolecular complex in the ODV envelope. So far, seven out of nine PIF proteins have been identified as components of this complex. This so-called ODV entry complex consists of a stable core, formed by PIF1, 2, 3 and 4, to which PIF0, 8 are more loosely associated. PIF6 is also a loosely associated component of this complex, as will be shown in **Chapter 3.** The core of the complex is characterised as stable,

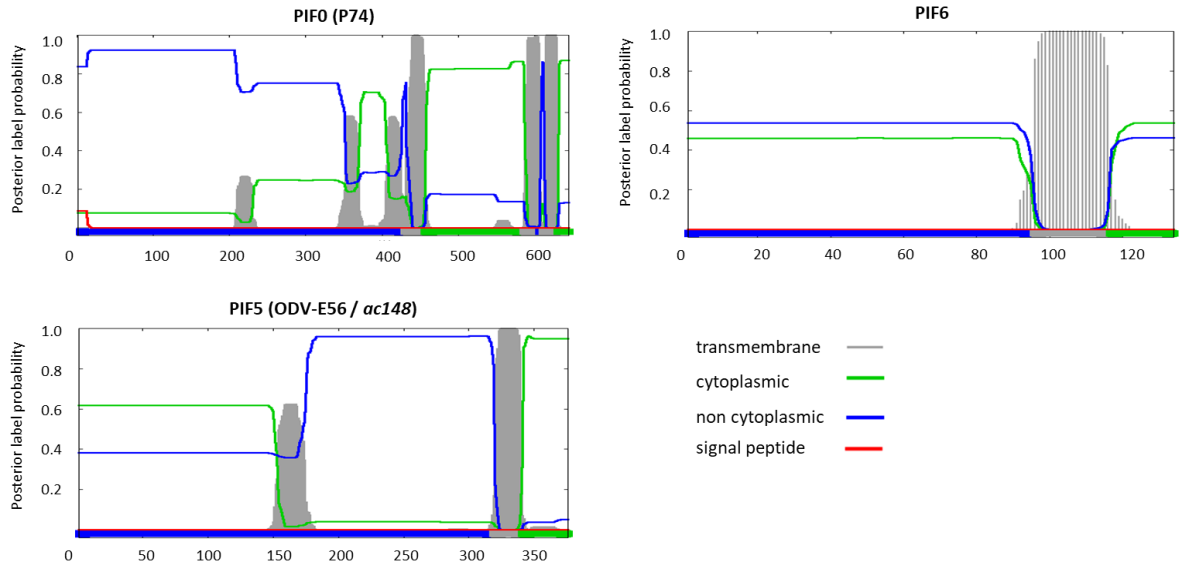

Figure 3: Prediction of the presence of transmembrane domains and signal peptides in PIF0, PIF5 and PIF6 and orientation of these proteins in an infected host-cell with the Phobius algorithm (Käll et al., 2004)**.** On the y-axis: the probability for the presence of transmembrane domains (grey), cytoplasmic (green) or non-cytoplasmic (blue) orientation of the amino acid sequence and presence of a signal peptide (red). The final result of the prediction is summarized by the bar at the bottom with the indicated colours. The x-axis shows the number of the amino acids of that specific sequence.

since it appears as a 170 kDa band in western blot analysis when ODVs were partially denatured by SDS and reducing agents and incubation at 50°C (**Fig. 4a**). When under these conditions the temperature was increased to 95°C, the core complex completely dissociated into PIF monomers (Peng *et al.*, 2010). PIF1, 2 and 3 are essential for formation of the core complex, since the complex was not detected when any of the corresponding genes was deleted (Peng *et al.*, 2010). After deletion of *pif4*, however, a smaller complex consisting of PIF1, 2 and 3 was found by western blot analysis (Peng *et al.* , 2012).

When ODVs were analysed under non-denaturing conditions by blue native-PAGE, the entry complex had an apparent molecular mass of 480 kDa (**Fig. 4b, left panel**). This large multi-molecular complex contains, besides the components of the core (PIF1, 2, 3 and 4), also PIF0, 6 and 8 (Peng *et al.*, 2012, **Chapter 3**). ODV-envelope protein AC110 was recently assigned as PIF7 (Liu *et al.*, 2016). To find out whether this PIF is also a component of the large entry complex, we analysed a virus with an HA-tagged PIF7 in the ODV-envelope under non-denaturing conditions as described above. With anti-HA antibodies, we were able to detect the same 480 kDa band as found with PIF1 antiserum (**Fig. 4b, right panel**). This indicates that PIF7 is a component of the entry complex, which brings us to a total amount of eight complex-associated PIFs (**Fig. 5**). Apparently, PIF0, 6, 8 (and maybe also PIF7) associated to the core

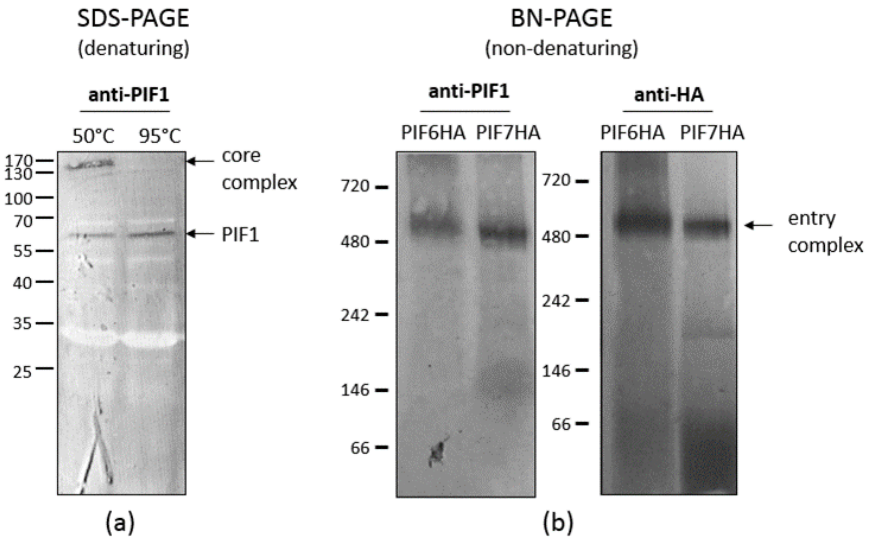

Figure 4: Detection of the stable core and the entry complex as a whole. When isolated ODVs were analysed under denaturing conditions by incubation in Laemmli buffer at 50 °C, the core complex was found as a 170 kDa band by western blot with PIF1 antiserum. Under these conditions, also PIF1 monomers were detected at 60 kDa. When the ODVs were incubated at 95°C, the core complex dissociated and only PIF1 monomers were found (a). When isolated ODVs were analysed under non-denaturing conditions (b), the entry complex was detected as a band of approximately 480 kDa with antiserum against PIF1 (left panel) and with anti-HA antibodies against HA-tagged PIF6 (right panel). When analysing ODVs that contain HA-tagged PIF7 with anti-HA antibodies, the entry complex was also found, indicating that PIF7 is part of this complex (right panel).

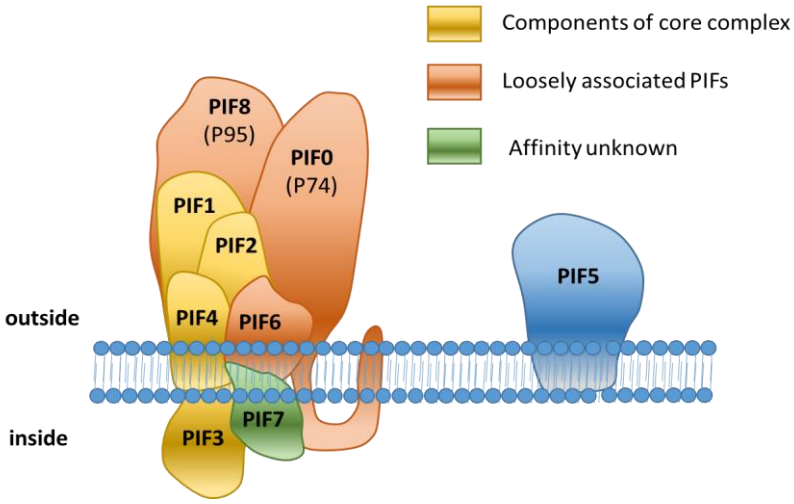

Figure 5: Model of the ODV entry complex. In yellow, the components of the core complex (PIF1, 2, 3 and 4); and in red, the PIFs that associate to the entry complex with lower affinity (PIF0, 6 and 8). PIF7 is depicted in green as this protein was detected in the entry complex under non-denaturing conditions but the affinity of this PIF for the complex is not known. PIF5 does not make part of the entry complex and is depicted in blue.

with lower affinity as these PIFs dissociated after (partial) denaturation by treatment with SDS and reducing agents, in contrast to the core components. Only PIF5 appears not to be present in the entry complex and this was confirmed by co-immune precipitations with PIF1 antiserum, in which this PIF was not found as interaction partner of PIF1 (Peng *et al.*, 2012). This raises the question whether PIF5 functions independently from the entry complex during primary infection of the midgut. This co-immunoprecipitation study however, resulted in co-precipitation of PIF2, 3, 4, 0, 6 and 8, providing additional evidence for their presence in the entry complex. PIF7 was not found as an interacting partner in this study. AC5 and AC108 also precipitated with PIF1 (Peng *et al.*, 2012). AC5 has recently been shown to associate with OBs and it seems not to be involved in oral infectivity or formation of the entry complex (Wang *et al.*, 2018). This finding seems not so surprising considering the fact that *ac5* homologs are present only in a subset of the Alphabaculoviruses, in contrast to other *pif* genes. Whether AC108 is involved in either oral infectivity or complex formation still remains to be established.

PIFs mediate viral entry under alkaline conditions in the midgut.

Depending on the insect species, the pH in the larval midgut varies between 10 and 12, which makes it probably the most alkaline environment in nature (Terra and Ferreira, 1994). Orally ingested OBs dissolve in this alkaline environment, releasing ODVs in the midgut lumen. The ODVs then first encounter the peritrophic membrane. This membrane is composed of chitin, mucopolysaccharides and proteins, and mechanically protects the epithelial midgut cells from

the food bolus in the gut lumen (reviewed by Hegedus *et al.*,2009). Some baculoviruses encode metalloproteinases, called enhancins, which degrade the mucin component of the peritrophic membrane (Wang and Granados, 1997, Slavicek, 2012, Toprak *et al.*, 2012). However, most baculoviruses, including AcMNPV, do not encode such enhancins, while their ODVs still pass the peritrophic membrane. Hence, it is unclear to which extent the peritrophic membrane is a barrier for ODVs to reach the midgut epithelial cells and whether PIFs are involved in passing this structure as recognized by Rohrmann *et al.*, 2013. PIF8 (P95) is so far the only PIF for which experimental data support a role in assisting ODVs in passing the peritrophic membrane (see section PIF8 below).

Once ODVs have passed the peritrophic membrane, they infect midgut columnar cells via microvilli that form so-called brush borders at the luminal side of these cells. Electron microscopic analysis of the brush border of *Trichuplusia ni* larvae showed numerous nucleocapsids inside microvilli within four hours of inoculation (Kawanishi *et al.*, 1972, Granados and Lawler, 1981). Furthermore, virus-binding assays revealed that ODVs bind to the brush borders in a saturable manner and that binding was significantly reduced upon protease pre-treatment of the brush borders (Horton and Burand, 1993). These findings suggest that the ODVs bind to an as yet unknown proteinaceous receptor on the microvilli. The subsequent fusion between the ODV-envelope and the microvillar cell membrane has been demonstrated by octadecyl rhodamine (R18) de-quenching assays (**Fig. 6**). In this assay,

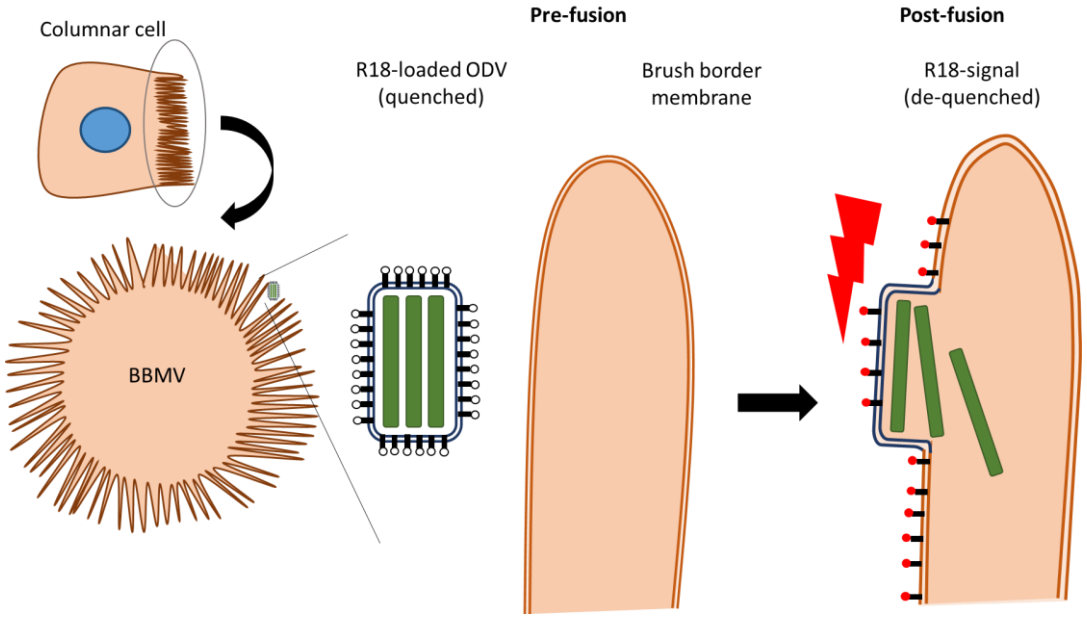

Figure 6: Schematic representation of the R18 de-quenching assay. The fluorescent activity of R18 is initially quenched in the ODV envelope because of the high concentration. Upon membrane fusion, R18 dilutes and gives a fluorescent signal.

isolated brush border membrane vesicles (BBMV), an *in vitro* model for the columnar cell's brush border, were mixed with ODVs that were loaded with high concentrations of the fluorescent dye R18. Due to the high concentration of R18 in the ODV-envelope, the fluorescence of the dye is quenched. However, upon fusion of the envelope with the BBMV, R18 dilutes in the fused membranes which results in de-quenching of the fluorescent signal that then can be recorded (Hoekstra *et al.*, 1984). This approach revealed that the fusion between ODVs and BBMV's occurs at 4 and 27 °C, and in a pH-range of 4-11 (Horton and Burand, 1993). Binding and fusion was most efficient at 27 °C under alkaline conditions. Therefore fusion via the endocytic pathway, as shown for BVs, is less likely as this pathway takes place under acidic conditions (Horton and Burand, 1993, Blissard and Wenz, 1992, Monsma and Blissard, 1995). Microvilli seem to be suitable structures for membrane fusion, as their cylindrical shape and narrow diameter facilitates interactions between membranes. The curvatures create disorder in the membrane, which may facilitate intermixing of (in this case viral and host) membranes during fusion (Wilson and Snell, 1998). In addition, the microvilli provide a scaffold for adhesion molecules in which polarized actin filaments create micro-domains that can concentrate adhesion and fusion complexes at the tip of the microvilli (Wilson and Snell, 1998). On the side of the virus, PIFs play an essential role in virus entry into the midgut epithelial cells. These proteins are implicated in binding, membrane fusion and further downstream processes like intracellular transport of the nucleocapsids to the nucleus. In the following sections, PIF functions will be discussed.

PIFs forming the stable core of the ODV entry complex

PIF1 and 2 mediate binding to the columnar cell microvilli.

PIF1, 2, 3 and 4 form the stable core in the ODV entry complex. Functionally, PIF1 and PIF2 appear to be involved in binding of ODVs with the microvilli of columnar cells, as has been demonstrated by Ohkawa *et al.*, 2005 with R18 de-quenching assays (the technique is explained above). These assays showed that absence of either PIF1 or PIF2 in the ODV envelope resulted in a threefold decrease in ODV binding compared to wild type. However, once these crippled ODVs had bound, at least half of them were able to fuse with the epithelial membrane, similar as for the wild type virus. This indicates that the absence of either PIF1 or 2 affects ODV binding but not fusion. Live imaging of ODVs with GFP-tagged nucleocapsids also showed that at least a fraction of the ODVs of *pif1*- and *pif2* deletion mutants were still able to bind to the microvilli of freshly isolated epithelial cells (Mu *et al.*, 2014). The finding that PIF1 and

PIF2 are involved in ODV binding is in accordance with predictions of the Phobius algorithm, which indicate that the topology of these PIFs in non-cytoplasmic (**Fig. 1**). In this orientation, these PIFs point out from the ODV envelope after virion envelopment, according to the model of Shi *et al.,* 2015, making them available for interaction with host cell receptors. However, as PIF1 and PIF2 are essential for formation of the stable core and therefore the ODV entry complex (Peng *et al.,* 2010), the observed phenotypes in absence of these PIFs may (at least in part) result from lost interactions with other components of the entry complex. So whether PIF1 and PIF2 are involved in ODV-binding via direct interactions with host factors or indirect by facilitating formation of the entry complex or may be even both is not clear.

PIF3 mediate oral infectivity after binding and fusion.

PIF3, the third component that is essential for formation of the core complex (Peng *et al.,* 2010), is not involved in ODV binding and fusion as was determined by R18 de-quenching assays (Ohkawa *et al.,* 2005). It was proposed that PIF3 is involved in oral infectivity in a process downstream from binding and fusion, for example by aiding translocation of the nucleocapsids to the nucleus. This is supported by predictions of the Phobius algorithm, which indicate that PIF3 is cytoplasmic oriented in the host cell and thus is probably directed towards the nucleocapsids and would therefore not be available to interact with a host receptor (**Fig. 1**). Furthermore, Song *et al.,* 2012 reported the presence of low levels of PIF3 in the nucleocapsid fraction of HearMNPV ODVs, suggesting an interaction with the nucleocapsid. The finding that *pif3*-deletion does not affect ODV binding and fusion, implies that binding and fusion may also occur in absence of the entry complex as its stable core cannot be formed in absence of PIF3 (Peng *et al.,* 2010).

Predictions of the Phobius algorithm also indicate that PIF3 has an N-terminal transmembrane domain (**Fig. 1**). Analysis of viruses with PIF3 truncations- and amino acid substitutions revealed that the N-terminal transmembrane domain is crucial for routing to the inner nuclear membrane, but that the final incorporation into the ODV-envelope is mediated by other parts of PIF3 (Li *et al.,* 2007). Two PIF3 truncation mutants, in which either twenty or forty amino acids were deleted downstream from the N-terminal transmembrane domain, were routed to the inner nuclear membrane, but were not incorporated into the ODV-envelope. The ODVs of these mutant viruses were not orally infectious and are as such phenotypically the same as a *pif3* deletion mutant. Similar results were found when the C-terminal forty amino acids were removed. Two mutants, however, a twenty amino acid C-terminal truncation mutant and a

mutant in which the cysteine residue C164 was replaced by a glycine, still showed a low-level of oral infectivity while these mutated forms were also not detected in the ODV envelope by western blot analysis. Of all mutants, the C162G substitution mutant is particularly interesting as the oral infectivity was severely affected, while this form of PIF3 was still incorporated into the ODV-envelope. The C162G mutant is the only PIF3 mutant where loss of oral infectivity can be directly related to the mutation itself, and not to side-effects as affected routing. This study demonstrates that it can be difficult to analyse PIF-proteins functions by mutagenesis as this might affect routing to the ODV envelope.

The role of PIF1-3 in host-range determination

PIFs that mediate ODV-binding seem to be host-range determinants, as resistance of *Spodoptera frugiperda* larvae to AcMNPV could partially be explained by differences in efficiencies in ODV-binding to the midgut, when compared to ODVs of Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) (Haas-Stapleton *et al.,* 2005). This was further supported by an experiment, in which either PIF1 or 2 of Helicoverpa armigera nucleopolyhedrovirus (HearNPV) were replaced by their homologs from Spodoptera litura nucleopolyhedrovirus (SpltNPV). The resulting HearMNPV hybrids with the single *pif*-gene replacements were not orally infectious for *Helicoverpa armigera* larvae (Song *et al.,* 2016). In contrast, a hybrid HearNPV, in which PIF3 was substituted for the SpltNPV homologue from, was able to infect *H. armigera* larvae. PIF3 is not involved in ODV binding and apparently, the biological function of PIF3 is (to a certain extend) inter exchangeable between these viruses (Ohkawa *et al.,* 2005). On the other hand, PIF1 and 2 seemed to mediate ODV-binding species specifically. In these experiments, it has not been analysed whether the hybrid virus was able to form a complex. So it remains to be established whether the failing binding properties of these PIFs affected oral infectivity or whether these PIFs are not compatible with the remaining native PIFs to form a functional entry complex.

PIF4 provides proteolytic resistance to the stable core.

PIF4 was identified when an AcMNPV *ac96* deletion mutant was not able to orally infect *Trichuplusia ni* larvae (Fang *et al.,* 2009). Similar results were obtained with HearMNPV and Bombyx mori NPV (BmNPV) after deletion of the *ac96* homologs *ha85* and *bm79* (Huang *et al.,* 2012, Dong *et al.,* 2014). In AcMNPV, PIF4 was found in the ODV and BV envelopes by western blot analysis (Fang *et al.,* 2009), but this was not supported by mass spectrometry

analysis of the virus particles (Braunagel *et al.*, 2003, Liu *et al.*, 2008, Wang *et al.*, 2010). However, PIF4 was detected by mass spectrometry after immune precipitation of PIF1, indicating that the complexity of the protein samples affected detection of this protein when analysing whole virus particles (Peng *et al.*, 2012). Nevertheless, the *pif4* deletion mutant of AcMNPV showed wild type levels of BV production and mortality in injection assays (Fang *et al.*, 2009). In ODVs of AcMNPV, PIF4 is essential for oral infectivity and is part of the core complex (Peng *et al.*, 2012). However, unlike *pif1*, *2* and *3*, deletion of the *pif4*-gene (*ac96*) did not affect interactions between the other components of the core complex as a smaller stable complex made-up of PIF1, 2 and 3 was found in the *pif4* deletion mutant.

This smaller complex was only found in ODVs that were released from OBs obtained from cell culture infections (C-OBs) and not in ODVs from larval-derived OBs (L-OBs) (**Chapter 3**). When analysing ODVs from L-OBs, the smaller complex appeared to be degraded by host-derived proteases that were co-occluded in the OBs. When these proteases were inactivated by heat treatment of L-OBs prior to their dissolution in alkaline buffer and isolation of the ODVs, the smaller complex was found again, like in ODVs from C-OBs. Apparently, the presence of PIF4 in the stable core provides the core complex resistance against proteolytic degradation by alkaline proteases.

Such a property of PIF4 would also explain the, at the time contradictory, results obtained earlier with HearNPV, where no smaller complex with PIF1, 2 and 3 was found after deletion of the *pif4*-homolog *ha85* (Huang *et al.*, 2012). As these analyses were performed with larval-derived ODVs, the smaller complex could have been degraded by host-derived proteases. Another contradicting finding was that HA85 was not detected in the stable core by western blot and that immunoprecipitation of HearMNPV PIF1 resulted in co-precipitation of PIF0, PIF2 and PIF3, but not HA85 (Huang *et al.*, 2012). To which extend the experimental conditions or the presence of host-derived proteases complicated these analyses is not clear. A yeast-two-hybrid assay nevertheless identified interactions between PIF4-PIF0, PIF4-PIF1, PIF4-PIF2 and PIF4-PIF3 (Huang *et al.*, 2012). Similar interactions of PIF4 with PIF1, PIF2 and PIF3 were also identified in BmNPV by bimolecular fluorescence protein complementation assays (BIFC) and co-immunoprecipitation studies after overexpression of these PIFs (Dong *et al.*, 2014).

In conclusion, there is strong evidence that in addition to AcMNPV, other baculovirus species form a core complex with PIF1, PIF2, PIF3 and possibly also PIF4. However, the exact nature

of the protein-protein interactions needs further validation under native conditions. Although PIF4 in AcMNPV appears not to be important for interactions between the remaining components of the stable core (PIF1-3), this PIF is required for the formation of the entire ODV entry complex as this complex was not detected in the ODV-envelope, released from C-OBs, of the *pif4* deletion mutant with blue native-PAGE (Peng *et al.,* 2012). These results suggest that PIF4 is important for establishing interactions between the stable core and the other associated PIFs and that these interactions are crucial for oral infectivity. The *pif4* deletion mutant has not been tested so-far in R18 de-quenching or live imaging assays.

PIFs that associate with the core complex

PIF0 mediates ODV-binding with the midgut epithelium.

Historically, P74 was the first ODV-envelope protein that was found to be essential for oral infectivity and is now also named PIF0 (Kuzio *et al.,* 1989). This PIF-protein lacks an N-terminal signal sequence for routing to the inner nuclear membrane. Instead, PIF0 has two C-terminal transmembrane domains, which are essential for nuclear translocation of this protein (**Fig. 3**) (Slack *et al.,* 2001, 2010, Yao *et al.,* 2004). The Phobius-algorithm predicts that PIF0 is C-terminally anchored to the ODV envelope and that the N-terminal part is directed outwards from the ODV-envelope, because of its non-cytoplasmic orientation in the host cell. Such a topology is in accordance with results from R18 de-quenching assays and live imaging of fluorescent ODVs that showed that PIF0 is important for ODV binding with midgut epithelial cells (Haas-Stapleton *et al.,* 2004, Mu *et al.,* 2014). When ODVs lack PIF0, one third of these bind to the midgut epithelium, compared to wild type ODVs (Haas-Stapleton *et al.,* 2004). Once bound, about half of the bound ODVs fused with the epithelial membrane, just like wild type ODVs. This suggests that fusion is not affected by the absence of PIF0, as reasoned by Haas-Stapleton *et al.,* 2004. Two independent studies showed that incorporation of PIF0 in the ODV-envelope seems not absolutely required to be able to play a role in oral infectivity (Yao *et al.,* 2004, Slack *et al.,* 2010). The first study showed that co-feeding of purified PIF0-GFP fusion protein along with ODVs of *pif0* deletion mutant, rescued oral infectivity (Yao *et al.,* 2004). Similar results were obtained with purified C-terminal truncated variants of PIF0, lacking the transmembrane domains. The co-feeding of these soluble forms of PIF0 also rescued the oral infectivity of a *pif0* deletion mutant (Slack *et al.,* 2010). Apparently, the C-terminal transmembrane domains of PIF0 are dispensable for oral infectivity, while the rest of the protein mediates oral infectivity via interactions with other PIFs and likely also via interactions with

host factors. Co-immunoprecipitation studies demonstrated that PIF0 interacts with PIF1 and PIF3 and further analysis revealed that PIF0 was a component of the entry complex, which was observed only under non-denaturing conditions (Peng *et al.*, 2010, 2012). Apparently, PIF0 associates with the core with relatively low affinity and might therefore be regarded as a more loosely associated component of the ODV entry complex than PIF1-4.

PIF0 is cleaved by co-occluded proteases and trypsins.

PIF0 is proteolytically cleaved in two separate events, once by a host-derived protease upon OB dissolution in the alkaline environment of the midgut and once by trypsins, present in the gut of the larva (**Fig. 7**). In the first event, PIF0 is cleaved into two fragments of approximately 35 and 40 kDa (Peng *et al.*, 2011). This cleavage was observed with ODVs released from L-OBs, but not with ODVs from C-OBs, indicating that the protease was obtained from the larval host. After cleavage, both fragments remain associated with the core complex as these precipitated together with PIF1 in a co-immunoprecipitation study with PIF1 antiserum (Peng *et al.*, 2011). When the ODV entry complex lacks one of the components of the core complex, the cleaved form of PIF0 was not found, but instead PIF0 was degraded. This suggests that the conformation of the ODV entry complex prevents degradation of PIF0, resulting in cleavage of this PIF (**Chapter 3**). However, the importance of this cleavage event for oral infectivity of ODVs is not clear as a bioassay with *T. ni* larvae showed that L-OBs and C-OBs resulted in a

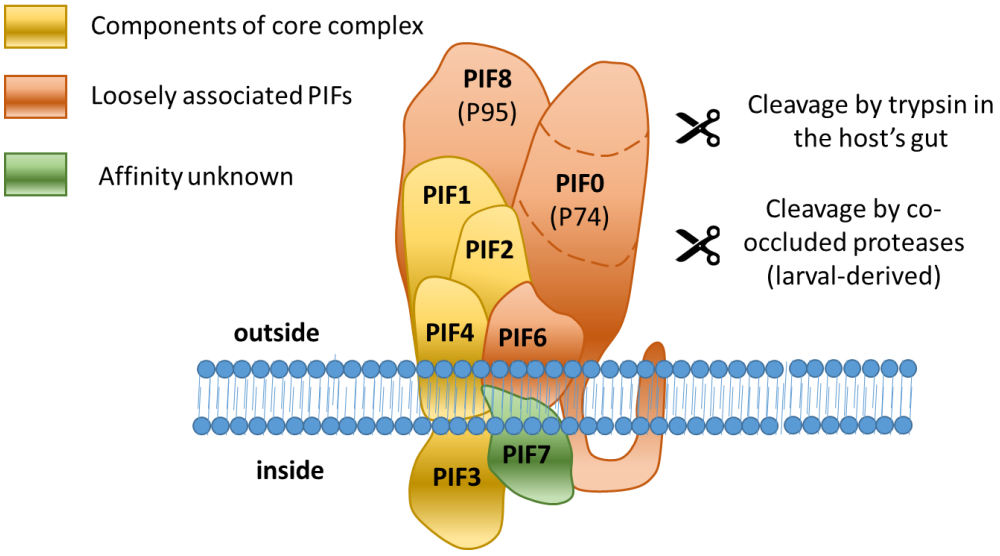

Figure 7: Two step cleavage model of PIF0. PIF0 is first cleaved by co-occluded host-derived proteases when larval-derived OBs dissolves in an alkaline environment. These 35 and 40 kDa fragments remain associated with the core complex. Secondly, a 20 kDa N-terminal fragment is cleaved off by trypsin in the host's gut.

similar level of mortality, although the L-OBs killed these larvae slightly faster than C-OBs (Bonning *et al.*, 1995).

In the second cleavage event, a 20 kDa fragment is cleaved from the N-terminus of PIF0 by trypsins present in the midgut. This cleavage event was observed when purified GFP-tagged PIF0 was incubated with BBMV's made from endothelial cells of third instar *Helicoverpa zea* or fourth instar *T. ni* larvae (Slack *et al.*, 2005, 2008). This trypsin-mediated cleavage appears important for oral infectivity as inhibition of cleavage by soybean trypsin inhibitor or through mutation of conserved arginine residues in the cleavage site affected the infectivity of ODVs (Slack *et al.*, 2008). However, the trypsin inhibitor might inhibit, in addition to the cleavage of PIF0, yet unidentified cleavage events of other ODV-envelope proteins that might also affect oral infectivity of ODVs. Furthermore, the effects of a mutated cleavage site on oral infectivity was determined by feeding isolated OBs to *T.ni* larvae, while the presence of the modified PIF0-molecules was only validated in transfected insect cells and not in the ODVs. So when these mutations would affect proper routing of PIF0, as shown before with some PIF3 mutants, the decreased oral infectivity might be explained by absence of (mutated) PIF0 in the ODV envelope. However, trypsin-mediated cleavage of PIF0 was also observed in wild type ODVs, where this PIF associates with other PIFs in the entry complex. This indicates that this cleavage event also occurs under native conditions and might therefore be important for oral infectivity of ODVs (Slack *et al.*, 2008).

PIF6 is also a component of the ODV entry complex.

The ODV-envelope protein AC68 was identified as PIF6 (Nie *et al.*, 2012), when a *lef3-ac68* double deletion bacmid was repaired with *lef3* and the resulting *ac68* deletion virus displayed the phenotype of the typical *pif* deletion mutant. PIF6 was not only found in the envelope of ODVs, but also in BVs (Nie *et al.*, 2012). Its function in BVs is unknown, since BV production and infectivity were unaffected in absence of this protein. In ODVs, PIF6 precipitated together with PIF1 in a co-immunoprecipitation study with PIF1 antiserum and was identified as part of the entry complex under non-denaturing conditions (Peng *et al.*, 2012, **Chapter 3**). So PIF6 appears to be important for ODV oral infectivity in the context of the entry complex. Predictions with the Phobius algorithm revealed this PIF does not have an INM-SM, but instead has a C-terminal hydrophobic domain and a non-cytoplasmic oriented N-terminus (**Fig. 3**). This indicates that PIF6 is probably directed outwards from the ODV envelope. However, the

biological importance of PIF6 for ODV binding, fusion or for a downstream process as hypothesized for PIF3, has yet to be determined.

The zinc-finger domain of PIF8 is important for oral infectivity.

AC83 (also known as VP91 and P95) has two distinct roles during the infection cycle. Firstly, AC83 is required for nucleocapsid formation and is therefore essential for the production of BVs and ODVs (Zhu *et al.,* 2013). Secondly, AC83 is involved in oral infectivity. The protein has a zinc-finger (ZF) domain (also addressed as chitin-binding domain (CBD)) and mutants that lack this domain show the phenotype of a *pif* deletion mutant. Although this protein has multiple roles in the infection cycle, it is considered to be a PIF and is now named PIF8. Oral infectivity of ODVs of such a mutant was partially rescued by feeding OBs to larvae together with calcofluor white, an agent known to damage the peritrophic membrane (Zhu *et al.*, 2013). A similar effect was not noted for viruses with a deletion of *p74*, *pif1*, *2* or *3* (Song *et al.*, 2008). These data suggest that PIF8 binds to the chitin component of the peritrophic membrane. Affinity for chitin might also be advantageous for binding to the brush borders of epithelial cells as brush border microvilli produce chitin for the peritrophic membrane (Rohrmann, 2013). Possible interaction of PIF8 with host factors is also supported by the Phobius algorithm, which indicates that the topology of this protein is non-cytoplasmic, so is directed outwards from the ODV envelope (**Fig. 1**).

In the ODV envelope, PIF8 associates with the ODV entry complex with lower affinity than the components of the stable core, as this PIF was only found in the complex under non-denaturing conditions (Peng *et al.,* 2012). As this complex was not found in the mutant virus with the truncated form of PIF8, that lacks the ZF-domain, it was hypothesized that this domain is involved in formation of the entry complex by recruiting other PIFs (Javed *et al.*, 2017). However, this hypothesis was not experimentally supported as it was not determined whether the other PIFs (and the truncated PIF8 itself) were indeed absent in the envelope of mutant ODVs.

PIF5 might operate independent from the entry complex.

ODV-E56 (AC148) has been established as PIF5 on the basis that deletion of the corresponding gene significantly impaired oral infectivity of ODVs (Harrison *et al.*, 2010, Sparks *et al.,* 2011a). However, low-level mortality has consistently been observed after deletion of this gene, suggesting that PIF5 is important but not crucial for oral infectivity, in contrast to the other

PIFs. PIF5 has a C-terminal hydrophobic domain for translocation to the ODV envelope (**Fig. 3**) (Braunagel *et al.*, 1996). When this domain was substituted for β-galactosidase, PIF5 translocated to the virogenic stroma and finally ended up in the nucleocapsids, instead of in the ODV envelope (Braunagel *et al.*, 1996). PIF5 was not detected in the entry complex and did not co-immune precipitate with PIF1 (Peng *et al.*, 2012). However, in a yeast-two-hybrid analysis PIF5 reciprocally interacted with PIF3, a component of the core complex (Peng *et al.*, 2010b). Whether this interaction also occurs in the ODV envelope and in the presence of all other PIFs has yet to be determined. Like PIF3, PIF5 seems also not to be involved in ODV binding and fusion (Sparks *et al.*, 2011a). It could therefore be speculated that PIF5 mediates oral infectivity after binding and fusion, may be in association with PIF3. At this moment, the post-fusion configuration of PIF-proteins is entirely enigmatic though. It is presently also unknown whether the entry complex remains intact, falls apart, or even changes its composition after ODV binding and fusion.

ODV-E66 can retrospectively also be assigned as a PIF.

ODV-E66 (*ac46*) is important but not crucial for midgut infection as deletion of this gene, like with PIF5, only impairs oral infectivity of ODVs (Sparks *et al.*, 2011b, Xiang *et al.*, 2011). This protein mediates oral infectivity possibly by (co-) facilitating ODV-binding with the midgut epithelium as peptide-derivatives of ODV-E66 compete with ODVs for binding with BBMVs (Sparks *et al.*, 2011b). These results are in accordance with predictions of Phobius-software

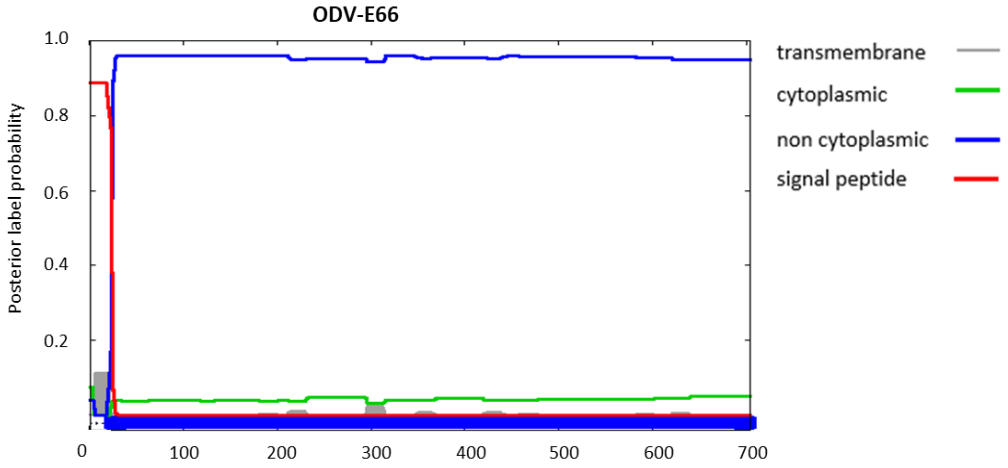

Figure 8: Prediction the presence of a transmembrane domain and signal peptides in ODV-E66 and orientation of this protein in an infected host-cell with the Phobius algorithm (Käll et al., 2004)**.** On the y-axis: the probability for the presence of transmembrane domains (grey), cytoplasmic (green) or non-cytoplasmic (blue) orientation of the sequence and a signal peptide (red). The final result of the prediction is summarized by the bar at the bottom with the indicated colours. The x-axis shows the number of the amino acids of that specific sequence.

that indicates that ODV-E66 is N-terminally anchored in the ODV envelope by its INM-SM and that the rest of the protein is directed outwards from the ODV envelope, because of its non-cytoplasmic topology in the host cell, enabling interactions with host factors (**Fig. 8**). Two studies indicated that ODV-E66 interacts with other components of the ODV entry complex. In a yeast-two-hybrid analysis, ODV-E66 reciprocally interacted with PIF2 and PIF3 (Peng *et al.*, 2010b). Furthermore, in co-immunoprecipitation studies and bimolecular fluorescence protein complementation assays with BmNPV, ODV-E66 interacted with the PIF4 homolog BM79 (Dong *et al.*, 2014). In light of this, it is interesting to determine whether ODV-E66 associates with the ODV entry complex. As ODV-E66 appears to be important (but not crucial) for oral infectivity by (co-) facilitating ODV-binding with the midgut epithelium, the protein can retrospectively be assigned as a PIF.

Perspectives and conclusions

The infection cycle of baculoviruses starts with infection of midgut epithelial cells. To mediate this infection, the ODV envelope contains an array of at least nine different PIFs and possibly also ODV-E66. The genes that encode these proteins are highly conserved among all baculoviruses and, with exception of *ac110* (encoding PIF7), in other large, nuclear replicating invertebrate DNA viruses (Reviewed by Wang *et al.*, 2017). The fact that the *pif*-genes are found in a large group of evolutionary related invertebrate DNA viruses that have co-evolved with their hosts for millions of years, suggest that PIF-mediated entry is a commonly used and ancient entry mechanism (Thézé *et al.*, 2011). The number of PIFs seems to increase gradually during evolution, from PIF0-PIF3 in the *Nimaviridae* to all nine in the *Baculoviridae*, suggesting that PIF-mediated entry became more and more advanced over time (Wang *et al.*, 2017). As said, ten different proteins are known to be involved in midgut infection by baculovirus ODVs, but there are maybe even more. A good candidate is AC108 that precipitated together with PIF1 (Peng *et al.*, 2012).

Furthermore, eight out of nine PIFs form a large complex in the ODV envelope, suggesting that PIFs cooperatively mediate cell entry in a network of protein-protein interactions. This finding complicates the interpretation of studies with *pif*-deletion mutants as the observed phenotypes concerning ODV binding and fusion cannot be directly attributed to absence of a PIF-protein itself, but may be a consequence of indirect effects because of lost protein-protein interactions. Hence, making deletion mutants is a good approach to identify PIFs, but not to elucidate the molecular mechanism of PIF-mediated cell entry or to identify the biological importance of a

single PIF in that process. Now that we know that there are many PIF-proteins of which at least eight form a complex, the questions that emerge are: what is the role of the ODV entry complex during midgut infection and does the various PIFs have different functions?

In absence of PIF3, a crucial protein for complex formation, ODVs are still able to bind and fuse with the plasma membrane of columnar cell brush borders, but nevertheless fail to infect the larval midgut. This suggests that the entry complex itself is not crucial for binding and fusion, but probably mediates infection by serving as a scaffold for proteins that mediate binding and fusion (PIF0, 1 2 and maybe also ODV-E66), and proteins involved in downstream processes (Such as PIF3 and PIF5). However, what these downstream processes are and what other function(s) PIFs have besides mediating binding and fusion during midgut infection is not clear.

Normally, the midgut epithelium digests and absorbs nutrients. Viruses may exploit this physiology for entry and passage of the midgut barrier. For example, the Jujonia coenia densovirus is transported across the midgut epithelium of *S. frugiperda* larvae via transcytosis (Wang *et al.*, 2013), which is normally used to transport large unprocessed proteins (Casartelli *et al.*, 2005,). Furthermore, infection with this densovirus altered the structural and functional properties of the epithelial tissue is such a way that it negatively affects its gate function. However, how infection with baculoviruses affects the midgut physiology and how PIFs are involved in this is unexplored. These insights might be important to define what other processes besides ODV binding and fusion are required for successful infection of the midgut epithelium.

Acknowledgements

This work was supported by grant 824.14.16 of the Netherlands Organization for Scientific Research (NWO).

Chapter 3

Baculoviruses require an intact ODV entry complex to resist proteolytic degradation of *per os* infectivity factors by co-occluded proteases from the larval host

Bob Boogaard, Jan W. M. van Lent, David A. Theilmann, Martin A. Erlandson, Monique M. van Oers

Abstract

Baculoviruses orally infect caterpillars in the form of occlusion-derived viruses (ODVs). The ODV-envelope contains a number of proteins which are essential for oral infectivity, called *per os* infectivity factors (PIFs). Most of these PIFs are involved in the formation of an ODV-entry complex that consists of a stable core, formed by PIF1, PIF2, PIF3 and PIF4, and the more loosely associated PIFs PIF0, 7 and 8. PIF6 was in this study also identified as a loosely associated component of the entry complex. PIF1, PIF2 and PIF3 are essential for formation of the stable core, whereas deletion of the *pif4* gene results in the formation of a smaller complex. PIF0 is not needed for formation of the stable core. We show here in larva-derived ODVs of the Autographa californica multicapsid nucleopolyhedrovirus that PIF-proteins are degraded by host-derived proteases after deletion of a single *pif*-gene. Constituents of the stable core-complex appeared to be more resistant to proteases as part of the complex than as monomer, as in ODVs of a *pif0* deletion mutant only the stable core was found but no PIF monomers. When the stable core lacks PIF4, it lost its proteolytic resistance as the resulting smaller core complex was degraded in a *pif4* deletion mutant. The core was also sensitized to proteolytic degradation in absence of PIF6. We conclude from these results that an intact entry-complex in the ODV-envelope is prerequisite for proteolytic resistance of PIF-proteins under the alkaline conditions of the larval midgut.

This chapter has been published as

Boogaard, B., Van Lent, J. W. M., Theilmann, D. A., Erlandson, M. A., & van Oers, M. M. (2017). Baculoviruses require an intact ODV entry-complex to resist proteolytic degradation of *per os* infectivity factors by co-occluded proteases from the larval host. *Journal of General Virology*, *98*, 3101-3110.

Introduction

Baculoviruses infect the larval stages of insects in the orders Lepidoptera, Hymenoptera and Diptera (Jehle *et al.,* 2006). The name baculovirus refers to the bacillus-shaped nucleocapsid, in which a large circular, double-stranded DNA genome is packaged. The genome sizes range from 80-180 kbp, encoding 90-180 genes (Williams *et al.,* 2017). Baculoviruses have a biphasic replication cycle in which two virion-types are produced, first the budded virus particles (BVs) and later in infection occlusion-derived virus particles (ODVs). BVs consist of a single nucleocapsid which is enveloped by a membrane derived from the plasma membrane of the host cell. BVs establish systemic infection within the larvae after a primary infection. ODVs are of two types, single- or multiple nucleocapsids in single capsid- (SNPVs) and multicapsid nucleopolyhedroviruses (MNPVs), within an envelope derived from the inner nuclear membrane of the host cell. ODVs are occluded in a crystalline protein-matrix of polyhedrin, forming occlusion bodies (OBs). When larvae consume OB-contaminated plant material, ODVs establish the primary infection in the insect. The OBs dissolve in the alkaline environment of the insect midgut to release the ODVs. The released ODVs then enter insect midgut epithelial cells by means of the activity of *per os* infectivity factors (PIF-proteins) which are present in the ODV-envelope. So far, nine different PIF-proteins have been identified: P74 or PIF0 (encoded by the Autographa californica (Ac) MNPV gene *ac138*) (Faulkner *et al.,* 1997), PIF1 (*ac119*) (Kikhno *et al.,* 2002), PIF2 (*ac22*) (Pijlman *et al.,* 2003), PIF3 (*ac115*) (Ohkawa *et al.,* 2005), PIF4 (*ac96*) (Fang *et al.,* 2009), PIF5 (ODV-E56/*ac148*) (Harrison *et al.,* 2010), PIF6 (*ac68*) (Nie *et al.,* 2012), PIF7 (*ac110*) (Liu *et al.,* 2016) and PIF8 (*ac83*) (Zhu *et al.,* 2013). Most of these PIFs have been shown to be part of an entry complex in the ODV-envelope that consists of a stable core complex and some more loosely associated components. The stable core complex is formed by PIF1, PIF2, PIF3 and PIF4 and resists treatment with Laemmli buffer containing 2% SDS and 5% 2-mercaptoethanol at 50°C for SDS-PAGE analysis (Peng *et al.,* 2010). Of these components, PIF1, PIF2 and PIF3 appeared essential for formation of the stable core, while deletion of *pif4* resulted in the formation of a smaller complex of only PIF1, PIF2 and PIF3 (Peng *et al.,* 2012). PIF0, 7 and 8 have been shown to associate with the core-complex in a more loose way, as these components can only be detected as part of the entry complex under non-denaturing conditions (Peng *et al.,* 2012, **Chapter 2**). PIF0 appeared not essential to form the stable core complex as this complex can be formed by a *pif0* deletion mutant (Peng *et al.,* 2010, 2012). PIF5 has not been found in association with

the entry complex (Peng *et al.*, 2012). Whether the other identified PIFs are part of the entry complex is currently not known.

Earlier studies reported proteolytic activity from co-occluded alkaline proteases in larva-derived OBs (L-OBs) for various baculovirus-host combinations (Eppstein and Thoma, 1975, Summers and Smith, 1975, Tweeten *et al.*, 1978, Payne and Kalmakoff, 1978, Wood, 1980). The biological significance of this proteolytic activity is poorly understood, but it has been suggested that it may be important for optimal release of ODVs from the protein matrix of occlusion bodies, as significant amounts of the matrix protein polyhedrin was still associated with the ODVs after OB dissolution in alkaline buffer when the these proteases were inhibited (Wood, 1980). Although degradation of the matrix protein was most prominent, peptide analysis showed that proteins of the ODVs were also degraded during treatment with alkaline buffer (Wood, 1980). Furthermore, it has been shown that PIF0 is cleaved into two fragments by an endogenous co-occluded protease when ODVs were released from OBs that were produced in *Spodoptera exigua* larvae (Peng *et al.*, 2011). PIF0 has not been found to be cleaved when the ODVs were isolated from cell culture-derived OBs (C-OBs), suggesting that the co-occluded protease was obtained from the larval host (Peng *et al.*, 2011). As well, cleavage of PIF0 was not observed when the L-OBs were heated prior to the release of the ODVs as a consequence of heat-inactivation of these proteases. These data suggest that the presence of co-occluded proteases in larva-derived OBs have significant effects on PIF-proteins as shown with PIF0.

Here, we study how co-occluded proteases affect PIF-proteins by comparing isolated cell-derived and larva-derived ODVs (C-ODVs and L-ODVs respectively) in SDS-PAGE analysis. By using wildtype and various *pif* deletion mutants for this analysis, we were able to compare the proteolytic sensitivity of PIF-proteins as part of the stable core-complex with monomeric PIF-proteins. This study revealed that deletion of only a single *pif*-gene resulted in enhanced proteolytic degradation of the remaining PIFs, regardless of whether the missing PIF is member of the stable core or a loosely associated component of the entry complex. So the entire entry complex, comprised of the stable core complex and loosely associated PIFs, needs to be intact to resist the proteolytic activity of co-occluded proteases from the larval host.

Results

The PIF-core complex is affected by co-occluded proteases from the host.

Peng *et al.*, 2010 showed that the PIF-core-complex appeared as a 170 kDa band in western blot analysis with PIF1 antiserum, when a protein sample of isolated C-ODVs (released from cell-culture derived OBs) was heated at 50°C, instead of the 95°C normally used for complete denaturation. On the other hand, the complex completely dissociates after denaturation at 95°C. Here, we compared the stability of the core-complex in presence and absence of lepidopteran proteases. Therefore, we analysed the complex of wildtype AcMNPV in C-ODVs and L-ODVs (released from OBs produced by cultured insect cells (C) or *S. exigua* larvae (L)) after heating at 50, 60, 75 and 95°C by western blot analysis with PIF1 antiserum (**Fig. 1**). In C-ODVs, the 170 kDa core-complex and the PIF1 monomers were found after heating at 50, 60 and 75°C, while only PIF1 monomers were found when the sample was heated at 95°C (**Fig. 1**). These results indicate that the complex is very stable and only dissociates after heating at temperatures above 75°C. However, with L-ODVs, degradation products of the core complex between 120 and 70 kDa were detected when the sample was heated at 50°C and monomers were not found, probably because of the proteolytic activity of co-occluded proteases present in L-OBs that are activated during ODV-release in alkaline solution (**Fig. 1**). When the protein sample was heated at 60 or 75°C, the 170 kDa complex was found again, as were the PIF1 monomers. Possibly,

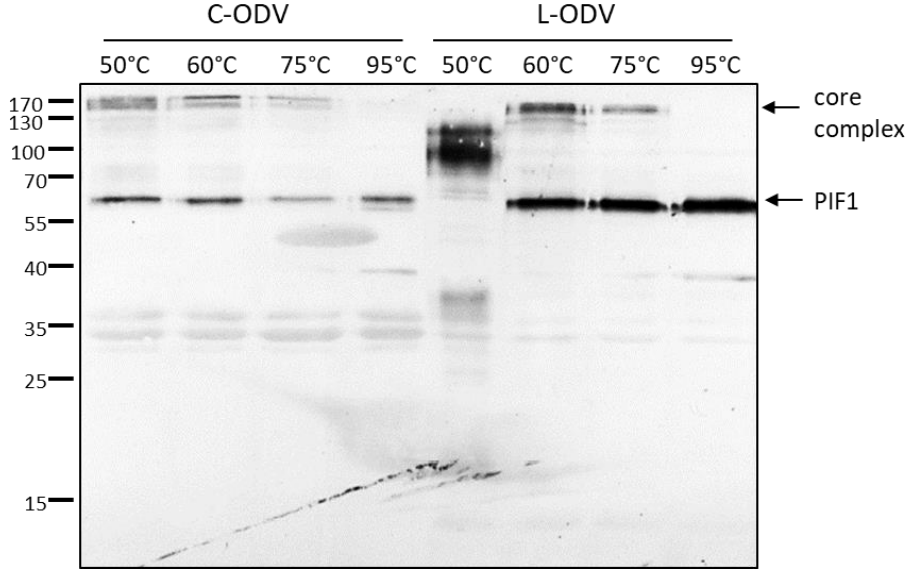

Figure 1: Temperature stability of the PIF core-complex and PIF1 monomers for wildtype AcMNPV, derived from cultured insect cells (C) and infected larvae (L). Wildtype ODVs were released from C-OBs (designated as C-ODV) and L-OBs (designated as L-ODV). The ODVs were purified and heated in Laemmli buffer at 50°C, 60°C, 75°C and 95°C for 5 min and separated by SDS-PAGE. Western blot analysis was performed with anti-PIF1 antiserum to detect the 170 kDa core-complex and 60 kDa PIF1 monomers.

the higher incubation temperatures sufficiently inactivated the proteases in the L-ODV protein sample. As the complex of C-ODVs and L-ODVs were more comparable when heated during sample preparation at temperatures above 50°C, all further analyses were conducted at 60, 75 and 95°C.

PIF1 is more resistant to proteolytic degradation as part of the core-complex than as monomer.

To assess how proteases affect PIF-proteins during OB-dissolution in a *pif*-deletion mutant that still contains the 170 kDa core-complex in the ODV-envelop, C-ODVs and L-ODVs of a *pif0* deletion mutant were subjected to western blot analysis with PIF1 antiserum (**Fig. 2**). In C-ODVs, the complex and PIF1 monomers were found when heated at 60°C and 75°C, and the complex was fully dissociated into monomers when completely denatured at 95°C (**Fig. 2a**), just as observed with the wildtype virus. In contrast, in L-ODVs of a *pif0* deletion mutant, only the core-complex was found when heated at 60 and 75°C but not the PIF1 monomers (**Fig. 2b).** Even after complete dissociation of the core-complex at 95°C, no PIF1 monomers were found. As it was suspected that the co-occluded proteases degraded the PIF1 monomers, the proteases were inactivated by heat-treatment of L-OBs at 80°C for 40 minutes prior to ODV release in alkaline buffer. The PIF1 monomers were indeed detected again after heat treatment of L-OBs,

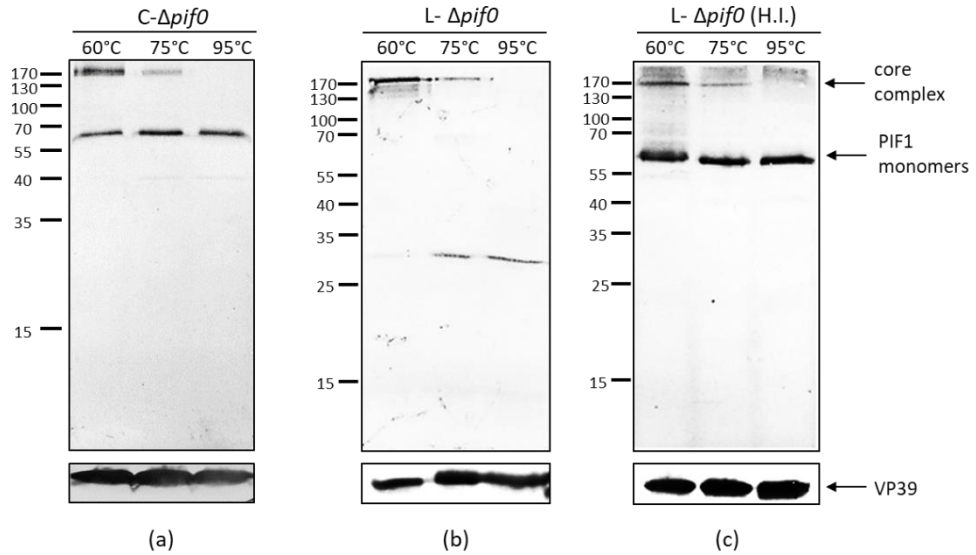

Figure 2: Western blot analysis of the PIF core-complex and PIF1 monomers in ODVs of the pif0 deletion mutant, derived from cultured insect cells (C) and infected larvae (L). ODVs of the *pif0* deletion mutant (Δ*pif0*) were released from C-OBs (a), L-OBs (b) or heat-inactivated L-OBs (c). For heat-inactivation (H.I.), the L-OBs were heated at 80°C for 40 min to inactivate co-occluded proteases, prior to dissolution in alkaline DAS-buffer. After purification of the ODVs, the samples were heated in Laemmli buffer at 60°C, 75°C or 95°C for 5 min and separated by SDS-PAGE. Western blot analysis was performed with antiserum against PIF1. Detection of capsid protein VP39 with VP39 antiserum was used as loading control (lower panels).

further supporting the involvement of proteases in the degradation of PIF1 (**Fig. 2c**). After deletion of *pif0*, PIF1 is apparently more sensitive in monomeric form to the co-occluded proteases than as part of the stable core complex, which is still formed in this deletion mutant.

PIF4 is important for the proteolytic resistance of the stable core-complex.

Peng *et al.*, 2012 showed that deletion of *pif4* resulted in the formation of a smaller core-complex of approximately 150 kDa, instead of the 170 kDa core-complex of the wildtype virus. To determine the impact of the co-occluded proteases on this smaller complex, C-ODVs and L-ODVs of a *pif4* deletion mutant were analysed by western blot analysis with antiserum against PIF1. When C-ODVs of a *pif4* deletion mutant were analysed, the approximately 150 kDa complex and PIF1 monomers were detected with expected sizes when heated at 60°C (**Fig. 3a**). However, in L-ODVs, neither the smaller sized complex nor the PIF1 monomers were found after heating at 60°C (**Fig. 3b, left panel**). Only when the proteases associated with L-OBs were heat inactivated prior to L-OB dissolution in DAS-buffer, the ~150 kDa complex and PIF1 monomers were detected (**Fig. 3b, right panel**). Furthermore, when C-ODVs of a *pif4* deletion mutant were analysed after heating at 75°C, only PIF1 monomers were found and not the ~150 kDa complex. This was also observed in L-ODVs isolated from heat treated OBs. Thus the smaller complex that lacks PIF4 completely dissociates into monomers when heated

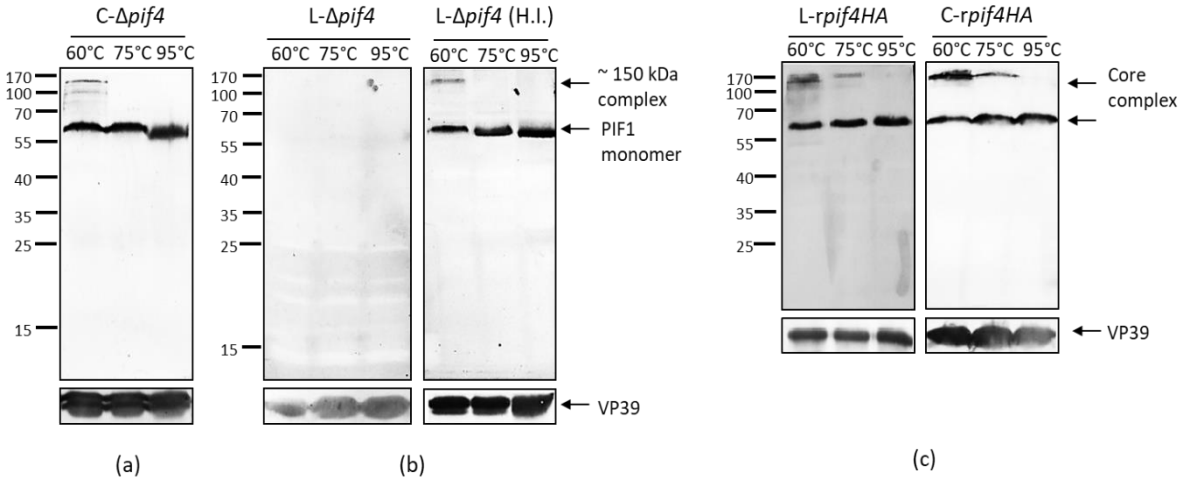

Figure 3: Western blot analysis of the PIF core-complex and PIF1 monomers in ODVs of the *pif4* deletion and repair mutants. ODVs of the pif4 deletion mutant (Δ*pif4*) were released from either C-OBs (a) or L-OBs with and without heat inactivation (b) for analysis with SDS-PAGE. The same analysis was performed with ODVs of the *pif4* repair mutant (r*pif4*HA), released from C-OBs or L-OBs without heat treatment (c). For all samples, ODVs were purified, heated in Laemmli buffer at 60°C, 75°C or 95°C for 5 min and separated by SDS-PAGE. Western blot analysis was performed with PIF1 antiserum. Detection of capsid protein VP39 with VP39 antiserum was used as loading control (lower panels). The arrows indicate the full-sized 170 kDa complex, 150 kDa complex in the *pif4* deletion- and repair mutant, and monomers of PIF1 and VP39.

at 75°C. However, when the deletion mutant was repaired with a HA-tagged *pif4* gene, the 170 kDa complex and PIF1 monomers were detected again in the ODVs after heating at 60 and 75°C, even without prior heat inactivation of the co-occluded proteases in L-OBs (**Fig. 3c**). Apparently, the smaller complex is degraded more easily by these proteases than the ~170 kDa complex that still contains PIF4. These observations indicate that PIF4 is an important stabilizing factor of the core-complex to resist proteolytic activity of host derived co-occluded proteases.

PIF0, PIF2 and PIF3 are also degraded by co-occluded proteases in L-ODVs of various pif-deletion mutants.

The results above show that PIF1 monomers are prone to proteolytic degradation by co-occluded proteases in L-ODVs after deletion of *pif0*- or *pif4*. To determine whether PIF1 monomers are also degraded in other *pif*-deletion mutants, ODVs of *pif1*-, *pif2*-, and *pif3* deletion mutants were analysed for the presence of PIF1 monomers. As this research question only relates to PIF-protein monomers, all ODV-samples were fully denatured by heating at 95°C and analysed by western blot analysis with antiserum against PIF1. In C-ODVs, PIF1 monomers were found in all *pif* deletion mutants, except for the *pif1* deletion mutant, while PIF1 monomers in L-ODVs were only detected in wildtype and the *pif4* repair mutant (**Fig. 4a and b**). However with ODVs released from heat-treated L-OBs, the PIF1 monomers were found again in all *pif* deletion mutants, except the *pif1* deletion mutant (**Fig. 4c**). Also some non-specific bands of unknown origin were found of approximately 50 kDa, which are unlikely

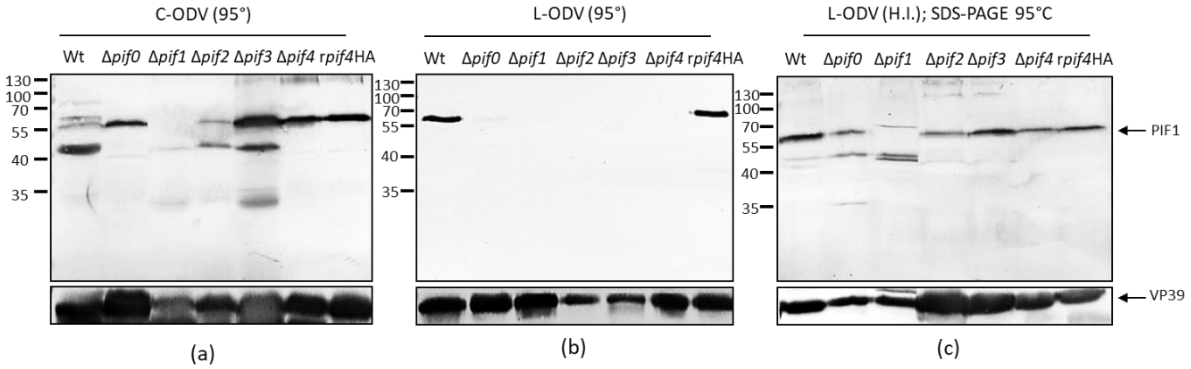

Figure 4: Western blot analysis of PIF1 monomers in ODVs of various *pif* deletion mutants, derived from cultured insect cells (C) and infected larvae (L). ODVs were purified from a wildtype (Wt) construct, Δ*pif0*-, Δ*pif1*-, Δ*pif2*-, Δ*pif3*-, Δ*pif4* mutants and the *pif4* repair mutant (r*pif4*HA), heated in Laemmli buffer at 95°C for 5 min and separated by SDS-PAGE for western blot analysis with PIF1 antiserum. The ODVs were released from C-OBs (a), L-OBs (b) or heat-inactivated L-OBs (c). In the analysis with C-ODVs and heat inactivated L-ODVs, some nonspecific bands were also detected in some samples but are from unknown origin. Detection of capsid protein VP39 with VP39 antiserum was used as loading control (lower panel).

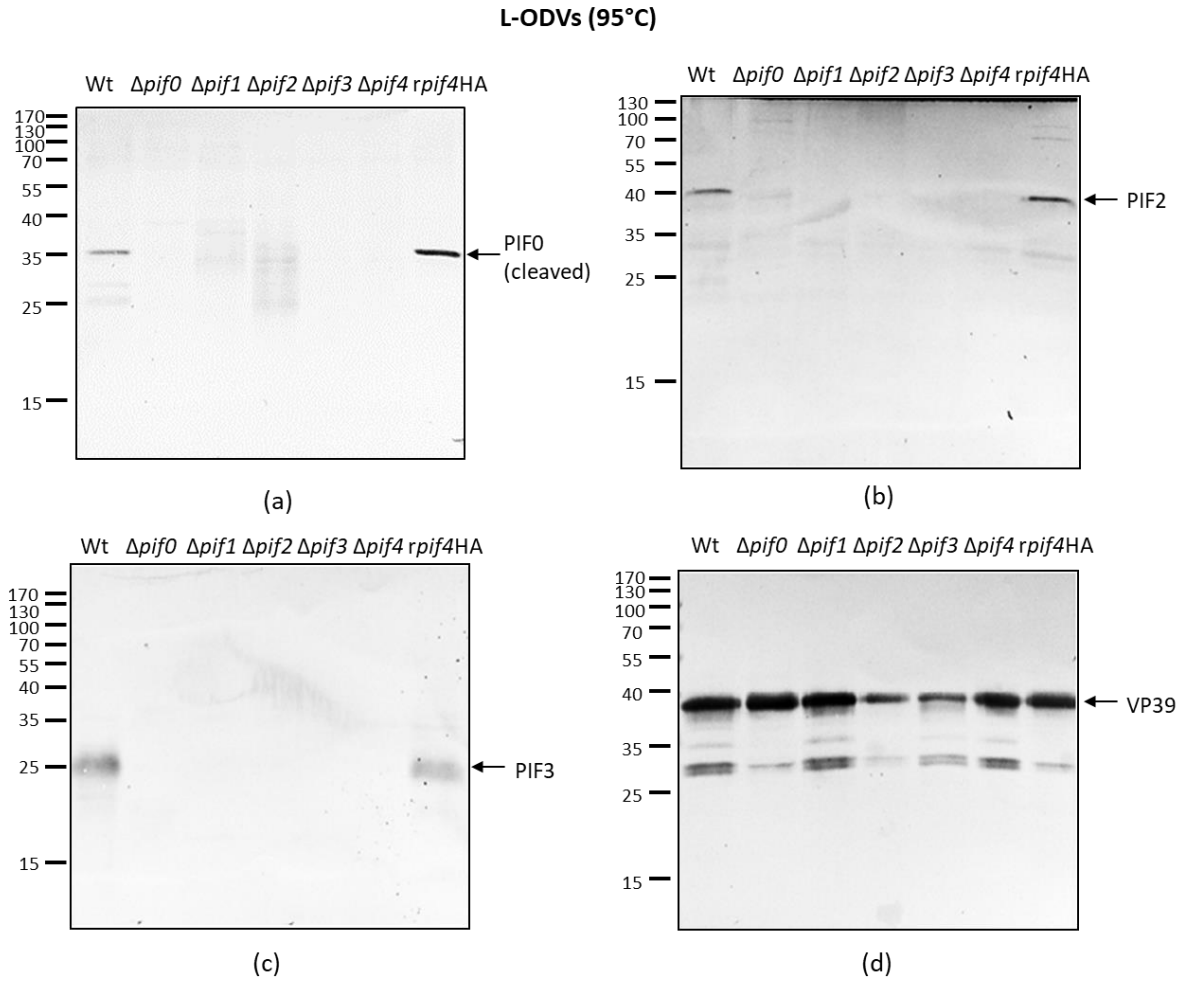

Figure 5: Western blot analysis of PIF-protein monomers on ODVs of various *pif* deletion mutants, released from L-OBs. ODVs were purified from L-OBs without prior heat treatment of a wildtype (Wt) construct, Δ*pif0*-, Δ*pif1*-, Δ*pif2*-, Δ*pif3*-, Δ*pif4* mutants and the *pif4* repair mutant (r*pif4*HA), heated in Laemmli buffer at 95°C for 5 min and separated by SDS-PAGE for western blot analysis with antiserum against PIF0 (a), PIF2 (b) and PIF3 (c). Detection of the major capsid protein VP39 with VP39 antiserum was used as loading control (d).

PIF1 degradation products as these bands were also detected in the *pif1* deletion mutant. The L-ODVs released from L-OBs without prior heat treatment were also analysed with antiserum against PIF0, PIF2 and PIF3 to assess whether these PIFs are degraded as well (**Fig. 5**). Just as previously observed with PIF1, these PIFs were only found in L-ODVs of wildtype and *pif4* repair viruses and not in the *pif* deletion mutants, indicating that these PIFs are degraded as well. In ODVs of wildtype and the *pif4* repair mutant, PIF2 and PIF3 were detected at the expected sizes of 40 and 25 kDa respectively. PIF0 was detected at 35 kDa rather than 74 kDa due its cleavage by co-occluded host derived proteases as observed previously by Peng *et al.*, 2011. These data indicate that not only PIF1, but also PIF0, PIF2 and PIF3 are proteolytically degraded by host derived proteases in the various *pif*-deletion mutants.

PIF6 is a loosely associated component of the entry complex.

Prior research identified the protein AC68 as a PIF-protein (denoted PIF6) and as a possible interaction partner of PIF1 (Nie *et al.*, 2012, Peng *et al.*, 2012). Here the envelope proteins of a HA-tagged *pif6*-repair mutant were analysed under denaturing and non-denaturing conditions to determine whether PIF6 is part of the core-complex or is a relatively loosely associated component of the entry complex similar to PIF0. Under denaturing conditions, when samples were heated at 60°C, anti-HA antibodies detected PIF6 only as 15 kDa monomers and not in the complex (**Fig. 6a, right panel**). However, when the blot was treated with antiserum against PIF1, the ~170 kDa complex and PIF1 monomers were found as expected (**Fig. 6a, left panel**). PIF6 therefore does not appear to be part of the 170 kDa core-complex. When the ODV-envelope proteins of the HA-tagged *pif6* repair mutant were analysed under non-denaturing conditions, a double band was detected with antiserum against PIF1 and antibodies against the HA-tag just below the 480 kDa marker, similar to what was observed with wildtype virus and

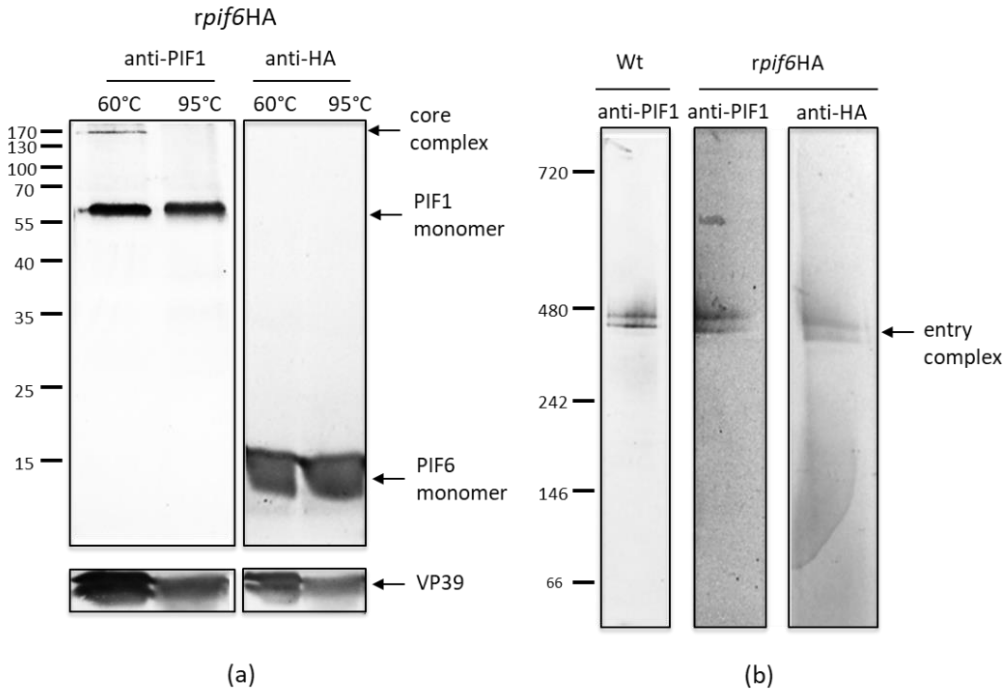

Figure 6: Western blot analysis of the PIF complex, PIF1- and PIF6 monomers under denaturing and non-denaturing conditions using a HA-tagged *pif6* repair virus. ODV envelope proteins of the *pif6* repair mutant (r*pif6*HA) were purified from ODVs by incubation in extraction buffer, containing 0.5% Triton, and analysed under denaturing (a) or non-denaturing conditions (b). For analysis under denaturing conditions, a sample of purified envelope proteins was heated in Laemmli buffer at 60°C or 95°C for 5 min and separated by SDS-PAGE for western blot analysis with antiserum against PIF1 (left panel of a) and the HA-tag of PIF6 (right panel of a). Detection of the capsid protein VP39 with anti-VP39 antiserum was used as loading control (lower panel of a). For analysis under non-denaturing conditions, a sample of ODV envelope proteins was separated on a 4-16% Bis-Tris gradient gel for western blot analysis with antibodies against PIF1 and the HA-tag of PIF6 (b). The same analysis was done with the envelope proteins of wildtype (Wt) ODVs as positive control.

anti-PIF1 antiserum in blue native-PAGE gels (**Fig. 6b**). These results show that PIF6 is also a loosely associated component of the entry complex.

PIF6 is important for the proteolytic resistance of the core-complex against co-occluded proteases.

As the PIF6 protein appears to be a loosely associated component of the entry complex, L-ODVs of a *pif6* deletion mutant were analysed by SDS-PAGE and western blot analysis with and without prior heat treatment of L-OBs, to determine whether the presence of PIF6 is important for the proteolytic resistance of the core-complex and PIF monomers. When the co-occluded proteases in the L-OBs were inactivated by heat prior to dissolution, the core complex and PIF1 monomers were found after heating of the isolated ODVs at 60°C with antiserum against PIF1 (**Fig. 7a**). After heating at 95°C, the complex was fully dissociated and only PIF1 monomers were found in the western blot analysis. When analysing L-ODVs without prior heat treatment, the anti-PIF1 antibodies only detected a pattern of degradation products below the 170 kDa marker when heated at 60°C, and the PIF1 monomers were absent (**Fig. 7b**). When the complex was completely dissociated by heating at 95°C, the PIF1 monomers were not detected either. However, when L-ODVs of the *pif6*-repair mutant were analysed, the core-complex (together with degradation products) and PIF1 monomers were detected at 60°C, and

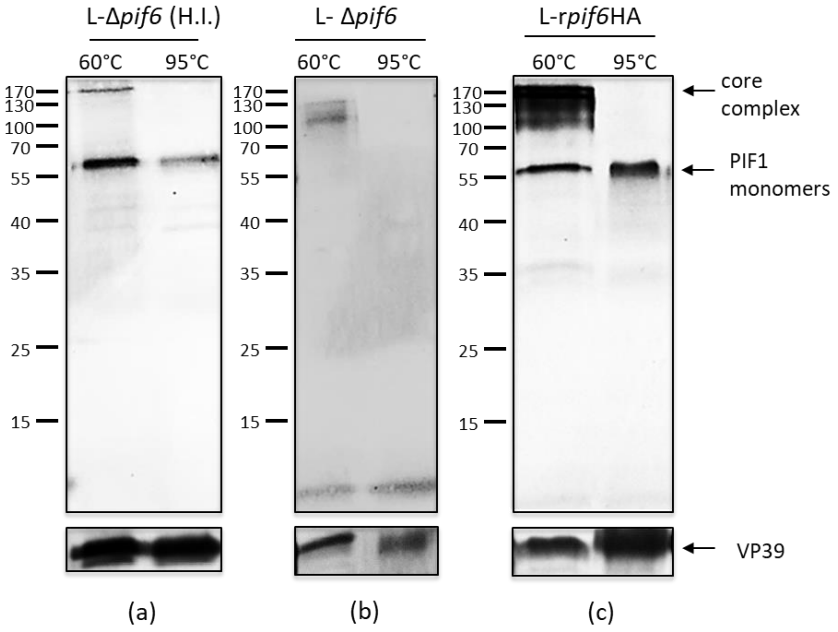

Figure 7: Western blot analysis of the PIF complex, PIF1- and PIF6 monomers in ODVs of the *pif6* deletion and pif6 repair mutants. ODVs of the *pif6* deletion mutant (Δ*pif6*) were purified from L-OBs with (a) and without (b) heat inactivation (H.I.) and heated in Laemmli buffer at 60°C or 95°C for 5 min and separated by SDS-PAGE. Western blot analysis was performed with PIF1 antiserum in. L-ODVs of the *pif6* repair mutant without heat inactivation were also analysed with antiserum against PIF1 (c). Detection of capsid protein VP39 with anti-VP39 antiserum was used as loading control (lower panels).

only PIF1 monomers were detected upon heating at 95°C (**Fig. 7c**). These results indicate that PIF6 is important for the proteolytic resistance of the core-complex, despite being a loosely associated component of the entry complex.

Discussion

Various studies have shown that the proteins in ODVs released from L-OBs are affected by co-occluded host-derived proteases (Summers and Smith, 1975, Wood, 1980, Peng *et al.*, 2011). Here we show that the baculovirus ODV-entry complex, consisting of a stable core-complex and at least four different loosely associated PIFs (PIF0, PIF7, PIF8 and now also PIF6), needs to be completely intact to resist proteolytic activity of these proteases during L-OB dissolution under alkaline conditions *in vitro*. Even in wildtype L-ODVs with an intact entry complex, we observed that the stable core-complex was largely degraded after treatment in Laemmli buffer at 50°C, whereas the core remained largely intact after heating at 60°C or 75°C. This observation can be explained by dissociation of the loosely associated components of the entry-complex from the stable core due to the presence of SDS in the Laemmli buffer for SDS-PAGE analysis, leaving the core-complex more exposed to proteases. It also implies that the co-occluded proteases are still present in the protein sample after L-OB dissolution and that these are still active in SDS-containing Laemmli buffer at elevated temperatures. This is in accordance with previous reports about co-occluded proteases in various other baculoviruses, which showed that the proteolytic activity of the proteases was mainly associated with the virus particles (Payne and Kalmakoff, 1978), and that the proteases are thermostable and compatible with detergents as SDS and reducing agents (Summers and Smith, 1975, Payne and Kalmakoff, 1978, Tweeten et al., 1978). As such, when the isolated L-ODVs were incubated in Laemmli buffer at 50°C, the proteases were likely still active and degraded the exposed core-complex and dissociated PIFs. In contrast, when the L-ODVs were heated at 60°C or 75°C, the proteases appeared sufficiently inactivated as both the core-complex and PIF monomers were found again, as they were with C-ODVs. Consequently, to assess only the effects of the co-occluded proteases on PIFs during OB-dissolution in DAS-buffer (mimicking the alkaline environment of the larval midgut), we heated the protein samples at 60°C or higher to prevent further degradation during SDS-PAGE sample preparation. Although these experimental conditions are not biologically relevant, they can nevertheless be used to assess the importance of the different components of the entry complex with regard to the proteolytic activity of co-occluded proteases during OB dissolution in the larval midgut. The presented results provide some

insight in the properties of the co-occluded proteases in L-OBs, but further characterisation of these proteases is still necessary to unravel the biological consequences and importance of their activities.

Analysis of a *pif0* deletion mutant initially suggested that PIFs in the core-complex are more resistant to co-occluded proteases than as monomer, as only the stable core-complex and not the PIF1 monomers were found in ODVs without prior heat treatment of the L-OBs. In contrast, the stable core-complex was found degraded in the *pif4* and *pif6* deletion mutants, indicating that the core-complex requires these components to maintain proteolytic resistance. It was previously shown that deletion of *pif4* resulted in the formation of a smaller core-complex of PIF1, PIF2 and PIF3 (Peng *et al.*, 2012). Apparently, PIF4 is not essential for forming the stable core, but it does seem important for the resistance of the core-complex against the proteolytic activity of co-occluded proteases. In contrast to PIF4, PIF6 proved not to be part of the core-complex but was identified as a loosely associated component of the entry complex. Despite this more loose association with the core-complex, PIF6 appeared to be more important for the proteolytic stability of the core-complex than PIF0. Apparently, the importance of the various PIF-proteins for the proteolytic resistance of the core-complex can differ independently of whether the missing PIF-protein is a core component or a loosely associated component of the complex. To understand this aspect better, further information is needed about the protein-protein interactions in the ODV entry complex and the stoichiometry of the individual components.

The importance of an intact entry complex in the ODV-envelope for the proteolytic resistance of PIF-proteins can also be nicely illustrated by the current observations on PIF0 monomers. PIF0 has previously been shown to be cleaved into two fragments of approximately 35 kDa and 37 kDa in L-ODVs (Peng *et al.*, 2011). However, current study showed that deletion of any *pif*-gene affected proper cleavage of PIF0 and resulted in proteolytic degradation of this PIF-protein instead. Apparently, the protein-protein interactions in the entry complex ensures that PIF0 is cleaved only at the directed cleavage site and prevent total degradation, but when a PIF-protein is missing due to deletion of a *pif* gene, co-occluded proteases are able to degrade PIF0 along with the other PIF-proteins.

The observations presented in this study indicate that deletion of a *pif*-gene affects the structure of the entry-complex which consequently leads to degradation of the remaining PIF-proteins by most likely co-occluded alkaline proteases during OB-dissolution. This finding conflicts

with previous observations on binding and fusion properties of *pif1*-, *pif2*- and *pif3* deletion mutants of AcMNPV with midgut epithelial cells in *Heliothis virescens* larvae (Ohkawa *et al.*, 2005). These authors showed that deletion of *pif1* or *pif2* compromised binding and fusion of the L-ODVs with epithelial cells in the larval midgut, while the *pif3* deletion mutant was still functional in terms of binding and fusion. In addition to PIF1 and PIF2, PIF0 has also been shown to be crucial for binding and fusion of ODVs to epithelial midgut cells (Haas-Stapleton *et al.*, 2004). However, our study indicates that deletion of *pif3* results in proteolytic degradation of PIF0, PIF1 and PIF2 under alkaline conditions, which possibly affects the binding and fusion capacities of ODVs in the larval midgut. Furthermore, PIF3 has been shown to be essential for formation of the stable core complex and was also shown to interact with PIF0 in AcMNPV (Peng *et al.*, 2010). A recent yeast-two-hybrid study suggests that the interaction between PIF3 and PIF0 is indirect (Zheng *et al.*, 2017), but this needs to be validated by further experiments. Nevertheless, deletion of *pif3* results in the inability to form the entry complex as a consequence of lost protein-protein interactions between the PIFs known to be involved in binding and fusion, and might additionally be degraded by co-occluded proteases. Consequently, it can be hard to draw firm conclusions about the involvement of a single PIF during the early stages of oral infection by making *pif* deletion mutants as the remaining PIFs in the ODV-envelope are severely affected by loss of protein-protein interactions and presence of host derived co-occluded proteases.

Conclusions

This study demonstrates the importance of an intact ODV entry complex to resist proteolytic activity of host-derived proteases that are co-occluded in L-OBs, as upon deletion of a *pif* gene the remaining PIFs are more sensitive to proteolytic degradation. This observation could be biologically relevant as in the alkaline environment of the midgut, the co-occluded proteases can be activated as did the alkaline buffer in our *in vitro* experiments. But even when the co-occluded proteases are not activated in the larval midgut, proteolytic resistance is still important as the alkaline proteases normally present in the midgut lumen can supplement the co-occluded proteases. Thus, proteolytic resistance against proteases can be considered as an important selective pressure, explaining the high level of conservation of PIF-proteins among the different baculoviruses in order to maintain complex formation and preserve oral infectivity.

Material and methods

Culture of insect cells and lepidopteran larvae. Sf21 cells were cultured in T25 flasks with Graces medium (Fisher N.V.), supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml gentamycin. Sf21 suspension cultures were grown in Sf900II medium (Fisher N.V.) supplemented with 5% FBS and 50 µg/ml gentamycin. *Spodoptera exigua* larvae were reared on artificial diet in a 26°C climate room with 40% humidity and a 16:8 h (light:dark) photoperiod.

Bacmids. The AcMNPV bacmid bMON14272 was derived from the Bac-to-Bac system (Invitrogen; Luckow *et al.,* 1993). The construction of the bacmids with the individual *pif*-gene deletions (del-*pif0*, del-*pif1*, del-*pif2* and del-*pif4*) has been described previously (Peng *et al.,* 2010, 2011, 2012). The *pif3* deletion bacmid was kindly provided by Dr Xinwen Chen of the Wuhan Insitute of Virology and has been described before in Li *et al.,* 2007. The construction of the HA-tagged *pif4* repair bacmid has also been described previously (Fang *et al.,* 2009, Peng *et al.,* 2012). The bacmids of the *ac68* (*pif6*) deletion mutant and the corresponding HA-tagged repair construct has also been described previously (Nie *et al.,* 2012). In all deletion and repair bacmids, the polyhedrin gene was restored to enable the resulting virus to form OBs. The polyhedrin (*polh*) gene was restored by using the Bac-to-Bac transposition protocol with a pFastBacDual vector (Invitrogen) from which the p10 promotor was deleted and the ORF of the *polh* gene was inserted behind the polyhedrin promotor as previously described (Peng *et al.,* 2010). The *pif* ORFs needed to repair the deletion bacmids were introduced simultaneously with the *polh* gene and were placed under control of their native promoters.

Virus proliferation and purification. Sf21-cells were transfected with the bacmids using Expres2TR reagent (Expres2ion Biotechnologies) to generate first generation BV-stocks. Subsequently, the obtained BV-stocks were amplified in Sf21 cells by an additional round of infection at low MOI. Cell-derived OBs (C-OBs) were generated by infecting a 80 ml suspension cultures of Sf21-cells at 1.5 x 10^6 cells/ml with 1 ml of amplified BV-stock (approximate MOI of 1) and incubated for a week. After a week of incubation, the cells were pelleted by centrifugation at 4000 x g for 30 min. The cells were suspended in 0.1% SDS dissolved in demi water and incubated for 2h at 37°C with gentle agitation. Then the cells were sonicated for 1 min at 7 Watt. Total cell lysis was verified by light microscopy and the OBs were pelleted by centrifugation at 4000 x g for 30 min. Larva-derived OBs (L-OBs) were obtained by injection of 1 µl of the amplified BV-stock into the haemocoel of L4 *Spodoptera*

exigua larvae, using an Humapen Luxura insulin pen (Lilly) ($TCID_{50}$ ~$5x10^7$ – $1x10^8$ per ml). Three to four days post injection, the liquefied larvae were collected, homogenized in sterilized water and filtered through two layers of cheese cloths. The debris in the flow through was pelleted by centrifugation at 500 x g for 10 min and the L-OBs in the supernatant were subsequently harvested by centrifugation at 4000 x g for 30 min. The pellet of the first centrifugation step, containing the cell debris, was washed multiple times with water followed by centrifugation to further purify the OBs. Finally, the purified OBs were washed twice in sterilized water. Both C-OBs and L-OBs were further purified by centrifugation over a 30%-60% (w/w) sucrose gradient in a Beckmann SW32 rotor at 90,000 x g for 1h at 4°C. The band with OBs was withdrawn from the gradient with a Pasteur pipette and subsequently pelleted by centrifugation at 4000 x g for 30 min.

SDS-PAGE and Western blot analysis. ODVs were released from approximately 3.0 x 10^8 OBs by treatment with DAS buffer (0.1M Na_2CO_3, 166mM NaCl and 10mM EDTA, pH 10.5) for 10 minutes at 37°C. When endogenous alkaline proteases needed to be inactivated, L-OBs were heated at 80°C for 40 min prior to dissolution in alkaline DAS-buffer. After verification of OB dissolution by light microscopy, the ODV suspension was neutralized with 1/10 volume of 0.5M Tris-HCl pH 7.5. Non-dissolved debris was removed by centrifugation at 1500 x g for 2 min and the ODVs in the supernatant were pelleted by centrifugation at 20,800 x g for 25 min at 4°C. The ODVs were resuspended overnight with gentle rotation at 4°C in 0.1x TE buffer, supplemented with Complete Protease Inhibitor Cocktail (Roche). Then 4x Laemmli buffer was added to the ODV-suspension which was then heated at 50°C, 60°C, 75°C or 95°C for 5 minutes. The samples were separated in a 12% SDS-PAGE gel and blotted onto a PVDF membrane (Milipore Immobilon-P). Western blot analyses with antisera against various PIF-proteins were performed as previously described in Peng *et al.*, 2010. In brief, anti-PIF0 antiserum (1:1000 dilution), produced in rabbit, and anti-PIF1 (1:2000 dilution), anti-PIF2 (1:1000 dilution) and anti-PIF3 antisera (1:1000 dilution), all produced in rats, were used as primary antibodies. To detect the HA-tagged PIF6 in the *pif6*-repair mutant, anti-HA antibodies were used as primary antibody (1:2000; Roche 3F10). Detection of capsid protein VP39 with anti-VP39 antibodies (1:1000), produced in mice, was used as loading control. Goat anti-rabbit (1:2000 dilution; Dako), goat anti-rat (1:2000 dilution; Sigma A8438) and goat anti-mouse antibodies (1:2000 dilution; Sigma A5153), conjugated to alkaline phosphatase, were used as secondary antibodies to detect the proteins of interest by conversion of NBT-BCIP substrate

(Sigma) in a blue-purple coloured precipitant in AP-buffer (0.1M Tris-HCl, 0.1M NaCl, 5mM MgCl, pH 10.5)

Blue-native PAGE. ODVs of 3.0 x 10^8 OBs were isolated as described above. The ODV pellet was resuspended in 100 µl extraction buffer (6.25 mM Tris, 37.5 mM NaCl, 0.5% Triton-X100, pH 7.2) and incubated overnight at 4°C with gentle rotation to extract the ODV envelope proteins. The envelope fraction was separated from the nucleocapsids by centrifugation at 16,000 x g for 30 min at 4°C. The supernatant containing the envelope fraction, was collected and mixed with 4x BN-PAGE sample buffer (200mM Bis-Tris, 64mM HCl, 200mM NaCl, 40% glycerol, 0,004% Ponceau S, pH 7.2). The protein samples were supplemented with 5% Coomassie G-250 to a final concentration of 0.1%. Electrophoresis of 4-16% Bis-Tris gradient gels was performed as described in the manual for the NativePAGE™ Novex® Bis-Tris Gel system (Invitrogen). NativeMark™ unstained protein ladder (Invitrogen LC0725) was also stained with Coomassie and used as marker. Blotting to a PVDF membrane was performed with NuPAGE® Transfer buffer (Invitrogen) according to the manufacturer's protocol.

Acknowledgements

This work was supported by grant 824.14.16 of the Netherlands Organization for Scientific Research (NWO). Els Roode and Hanke Bloksma are acknowledged for rearing the insects, culturing cells and their technical assistance in the lab.

Chapter 4

The baculovirus AC108 protein is a *per os* infectivity factor and a component of the ODV entry complex

Bob Boogaard, Felix Evers, Jan W. M. van Lent, Monique M. van Oers

Abstract

Baculoviruses orally infect insect larvae when they 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 then infect midgut epithelial 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 the 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 blot 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.

This chapter has been published as:
Boogaard, B., Evers, F., van Lent, J. W. M., & van Oers, M. M. (2019). The baculovirus AC108 protein is a *per os* infectivity factor and a component of the ODV entry complex. *Journal of General Virology*, *100*, 669-678.

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 Williams *et al.*, 2017). 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 (Shi *et al.*, 2015). 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 in **Chapter 2**). 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 (Haas-Stapleton *et al.*, 2004, Ohkawa *et al.*, 2005). 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. 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 (Peng *et al.*, 2010, 2012, Javed *et al.*, 2017, **Chapter 2**, **Chapter 3**). 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 (Peng *et al.*, 2010). 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 (Peng *et al.*, 2012).

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* NPV (Bm91) appeared as non-essential (Simon *et al.*, 2012, Tang *et al.*, 2013). 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 (Datsenko and Wanner, 2000). The *cat*-gene was later removed by a CRE-lox reaction, leaving a 100 nt insertion that is composed of fused loxP-sites (Suzuki *et al.*, 2005). The insertion and subsequent removal of the *cat*-gene was confirmed by PCR analysis with primers that annealed

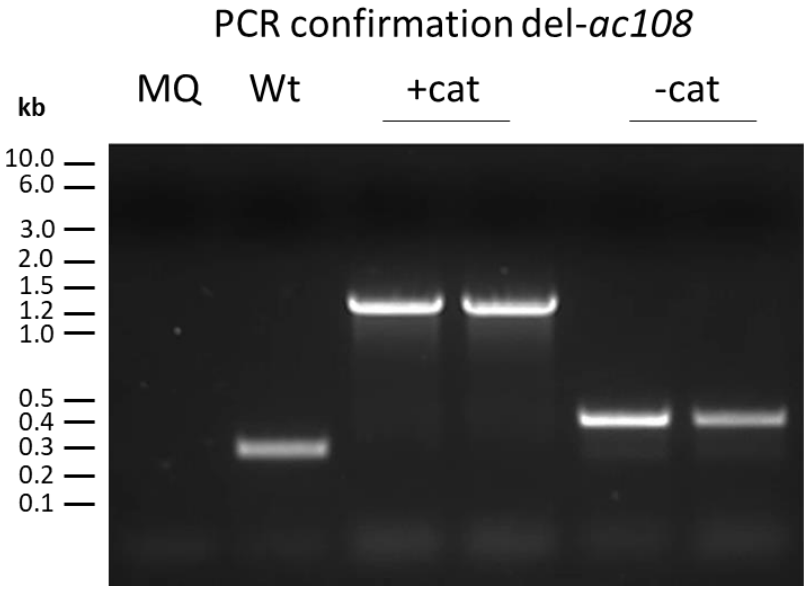

Figure 1: Conformation of insertion and removal of the chloramphenicol acetyl transferase (*cat*) resistance gene in the *ac108*-locus of the AcMNPV bMON14272-bacmid by PCR analysis. Primers were used that annealed outside the recombined region. The addition of double distilled water (MQ) as template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

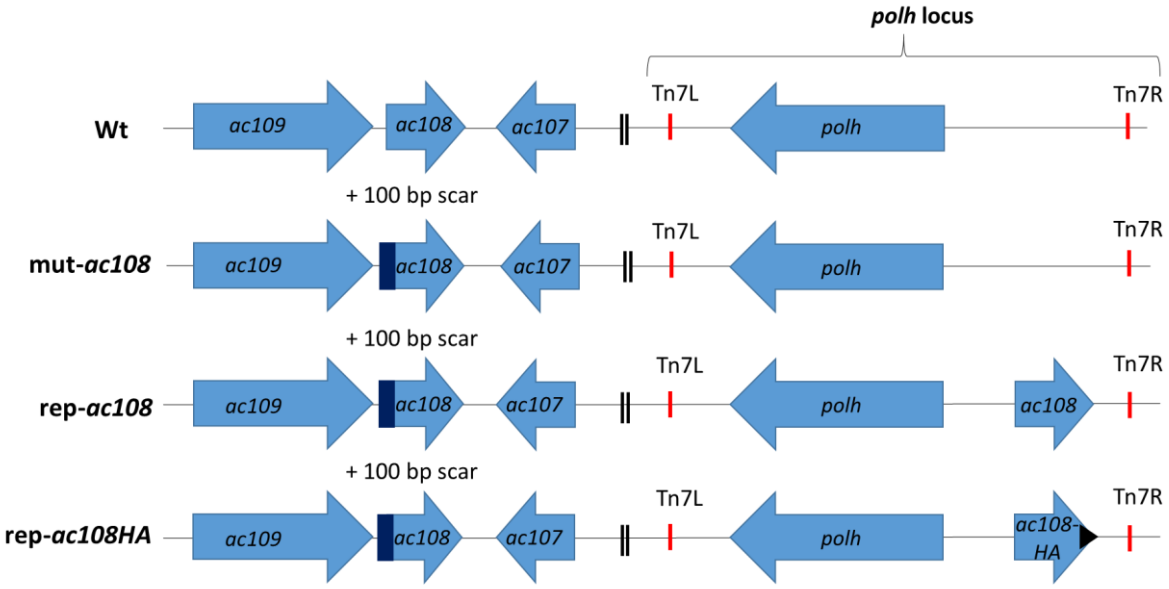

Figure 2: Schematic overview of the constructed AcMNPV bacmids. The ac108-gene was inactivated (mut-*ac108*) by deletion of a 12-nucleotide sequence from -8 to +4 relative to the A (+1) of the translational start codon. After removal of the cat-gene, a 100 bp scar was left, which is indicated in dark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

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 from that lacked the p10-promoter and in which the *polh*-ORF was inserted behind its own promoter (Peng *et al.*, 2010) (**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 the transfection of Sf21 cells to produce the modified viruses. For all these viruses, the transfected cells were found to

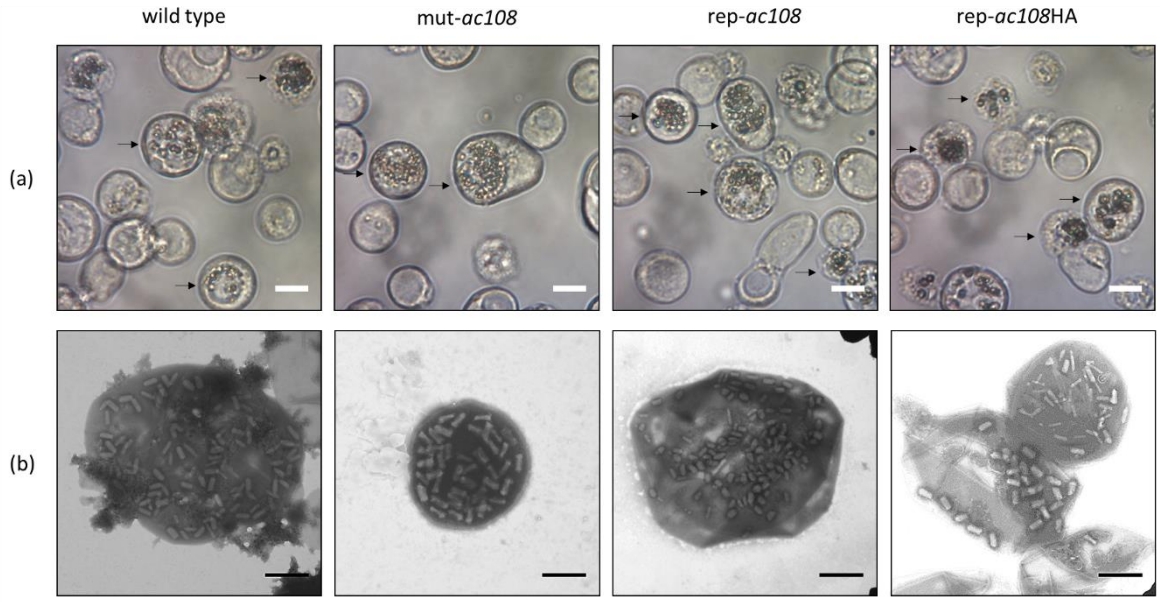

Figure 3: Microscopic analysis of OBs produced by the mutant viruses. (a) Sf21 cells at six days post transfection with the constructed bacmids of either wild type, *ac108* mutant (mut-*ac108*), *ac108* repair (rep-*ac108*) or HA-tagged *ac108* repair (rep-*ac108*HA) viruses. The black arrows indicate the OB containing cells and the white scale bar corresponds with 10 µm. (b) Electron microscopic analysis of the OBs to validate the presence of ODVs.

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 (Hopkins and Esposito, 2009), were infected with BVs of the wild type, *ac108* mutant or repair viruses at an MOI of 10, and the BV-titres of the medium were determined at 0, 24, 48 and 72 hours post infection (hpi) by end point dilutions assays. The BV-titres were found to increase every 24 hrs with approximately 1-log unit for the *ac108* mutant and repaired viruses, just as observed with wild type (**Fig. 4**). At 48 and 72 hpi, infection with the *ac108* mutant seemed to result in higher BV titres than infection with the other viruses, but this difference was not statistically significant (one way ANOVA; 48 hpi: $F(3,4)= 2.88$ $p= 0.17$; 72 hpi: $F(3,4)= 1.22$ $p= 0.41$). From these results, it was concluded that the mutation in the *ac108*-locus did not affect the production of OBs and BVs.

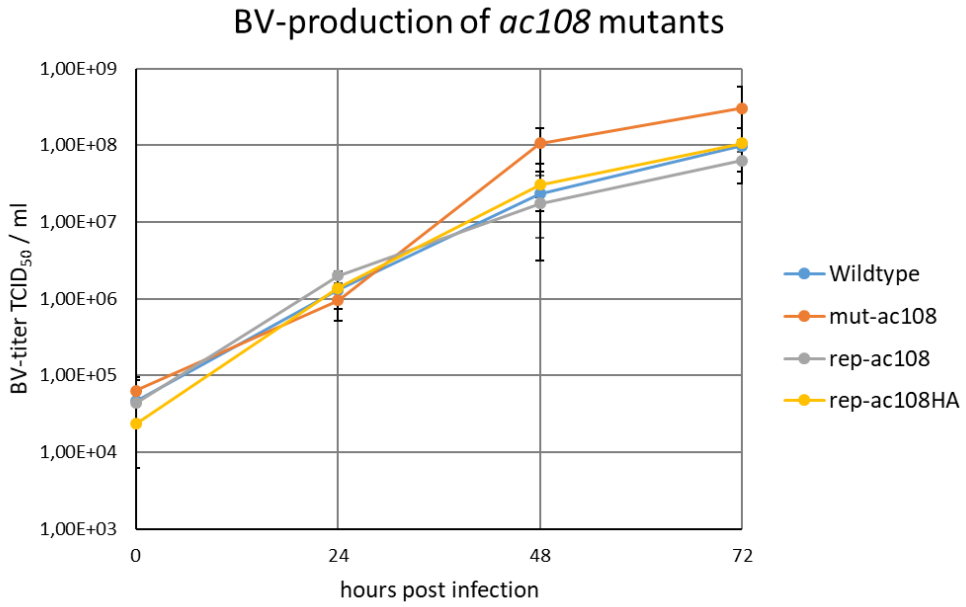

Figure 4: BV-growth curves after infection of Sf21 cells with BVs wild type AcMNPV, the *ac108* mutant (mut-*ac108*), *ac108* repair mutant (rep-*ac108*) or HA-tagged *ac108* repair mutant (rep-*ac108*HA). The BV concentration increased approximately 1-log unit every 24 hrs for all mutants, just as observed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

Inactivation of ac108 affects the oral infectivity of ODVs.

OBs of the mutant and repair viruses were fed by droplet feeding to *Spodoptera exigua* larvae to test the impact of the mutation in the *ac108*-ORF on the oral infectivity of ODVs. The droplet feeding assays were performed with a concentration of 3 x 10^8 OBs / ml on early L3 larvae that had been starved overnight. When OBs of the *ac108* mutant were fed, the larvae did not show any mortality (**Table 1**). However, when the larvae were fed with OBs of the *ac108* repair mutants (with or without HA-tag), they were successfully infected with similar mortality rates as the wild type. These data indicate that *ac108* is essential for the oral infectivity of ODVs in *S. exigua* larvae.

In parallel, we tested whether the mutation in the *ac108*-locus affected cell-to-cell transmission in the larvae by injecting BVs into the hemocoel. When the midgut was bypassed in this way, all larvae were infected with 100% mortality. This indicates that, in contrast to the ODVs, the

Table 1. Mortality after infection with *ac108* mutant and repair viruses

Droplet feeding assays (3.0 x 10^8 OBs / ml)				**Injection of BVs** (3.5 x 10^7 $TCID_{50}$ / ml)		
mock	0/12	0/12	0/11	mock	0/24	0/12
mut-*ac108*	0/12	0/15	0/12	mut-*ac108*	24/24	12/12
rep-*ac108*	11/12	17/17	11/12	rep-*ac108*	24/24	12/12
rep-*ac108*HA	10/12	19/19	12/12	rep-*ac108*HA	24/24	12/12
wild type	8/12	12/15	11/12	wild type	n.d.	n.d.

BVs retain their infectivity after mutation of the *ac108*-gene. Hence, the AC108 protein appears to be essential for primary infection of the midgut epithelial cells by ODVs, but is not required for subsequent systemic infection by BVs.

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

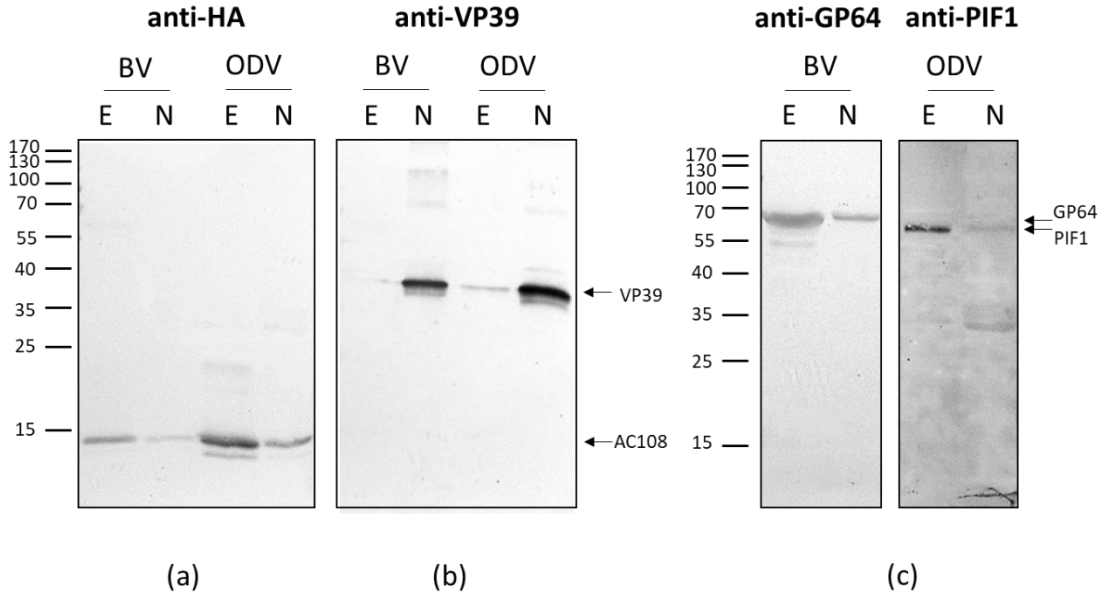

Figure 5: Western blot analysis of fractionated BVs and ODVs of the HA-tagged ac108 repair mutant. BVs and ODVs were fractionated by incubation in extraction buffer, containing 1% Triton-X100, in an envelope (E) and nucleocapsid (N) fraction and analysed with (a) anti-HA antibodies to detect AC108, (b) antiserum against VP39 as marker for the nucleocapsids, and (c) antiserum against GP64 as marker for the BV-envelope (left panel), and 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.

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, formed by PIF1-4, that resists partial denaturation in Laemmli buffer, when incubated at 50°C before gel loading (Peng *et al.*, 2010). Under these conditions, the core complex was detected as a 170 kDa band by western blot analysis with PIF1 antiserum. When the ODV envelope of the *ac108* mutant and repair viruses were analysed under these conditions, the core complex was found at the expected height for both mutants (**Fig. 6a**). The signal from the complex of the repair mutant was less intense, but the signal from the 60 kDa PIF1 monomers in this lane was also less

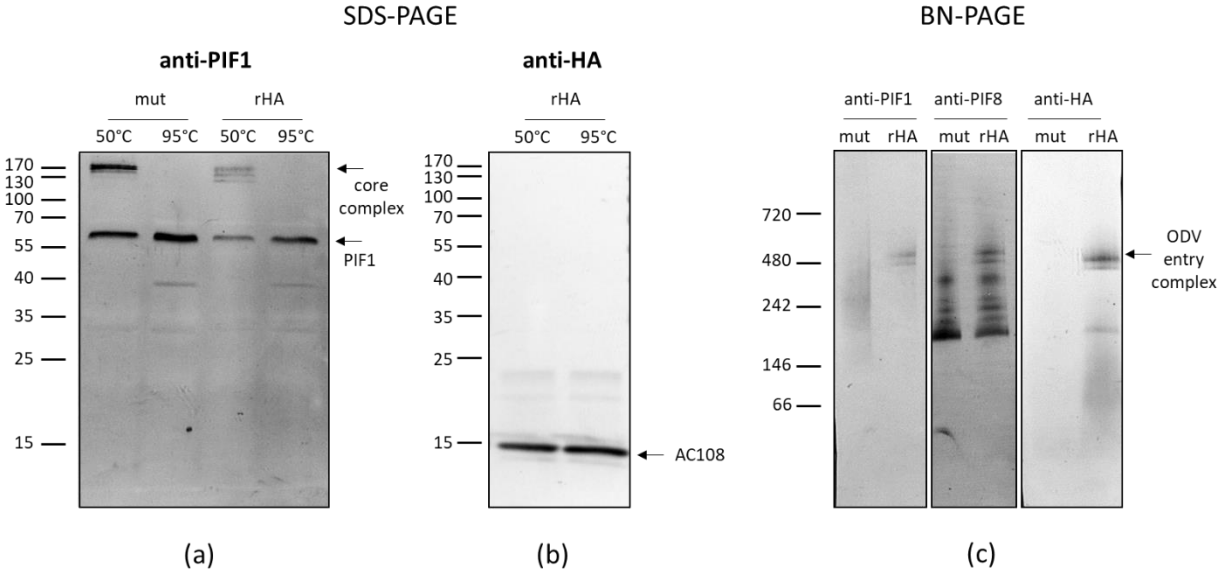

Figure 6: Western blot analysis of the ODV-envelope fraction of the *ac108* mutant (mut) and HA-tagged *ac108* repair viruses (rHA) under denaturing and non-denaturing conditions. Under denaturing conditions, the purified proteins were heated at either 50 or 95°C and analysed with antiserum against PIF1 (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).

intense compared to the surrounding lanes, indicating that slightly less protein was loaded. In addition to the 170 kDa complex, smaller complexes (just below 170 kDa) were also found, especially in the repair virus. Incubation at 95°C led to complete dissociation of the core complex as only PIF1 monomers were found. Furthermore, when the repair mutant was analysed with anti-HA antibodies after incubation at 50°C, AC108 was only found in monomeric form with a mass of 15 kDa (**Fig. 6b**). These results indicate that the core complex can still assemble after mutation of the *ac108*-locus and that the AC108 protein is not a component of the stable core. When the envelope fraction of the HA-tagged repair virus was analysed under non-denaturing conditions by blue native-PAGE followed by western blot analysis, the ODV entry complex was detected as a 480 kDa band, irrespective whether antisera were used against PIF1, PIF8 or HA, indicating that AC108 is a constituent of the ODV entry complex (**Fig. 6c**). However when analysing the *ac108* mutant, the 480 kDa protein complex was not detectable with any of these antisera (and neither was a slightly smaller one due to the absence of AC108), indicating that AC108 is involved in the formation of the entry complex.

Discussion

The results show that the *ac108*-gene of AcMNPV encodes a *per os* infectivity factor, as mutation of this gene abolished ODV oral infectivity, but had no observable effect on the 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* (Garavaglia *et al.,* 2012). 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 (Javed *et al.,* 2017). 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 (Simon *et al.,* 2012, Tang *et al.,* 2013). 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 (Li *et al.*, 2008, Nie *et al.*, 2012). 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 (Nie *et al.*, 2012, Xu *et al.*, 2008). Apparently, two closely related homologous genes do not necessarily have the same function in different viruses, 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* (Li *et al.*, 2018). 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 (Luo *et al.*, 2011). 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 (Shi *et al.*, 2007), BmNPV (Tang *et al.*, 2013), *Orgyia pseudotsugata* MNPV (Ahrens *et al.*, 1997), SfMNPV (Simon *et al.*, 2012) and *Spodoptera litura* NPV (Chen *et al.*, 2006). 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 (Fang *et al.*, 2009, Nie *et al.*, 2012). 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

(Zheng *et al.*, 2017), while this protein was identified as a PIF1 interaction partner in a co-immunoprecipitation study with PIF1 antiserum (Peng *et al.*, 2012). 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 (Peng *et al.*, 2012, Javed *et al.*, 2017).

We conclude that *ac108* encodes a PIF-protein that mediates ODV oral infectivity in AcMNPV by its more loose association with the ODV entry complex and propose an updated model from **Chapter 2**, in which AC108, designated as PIF9, is added as a new loosely associated

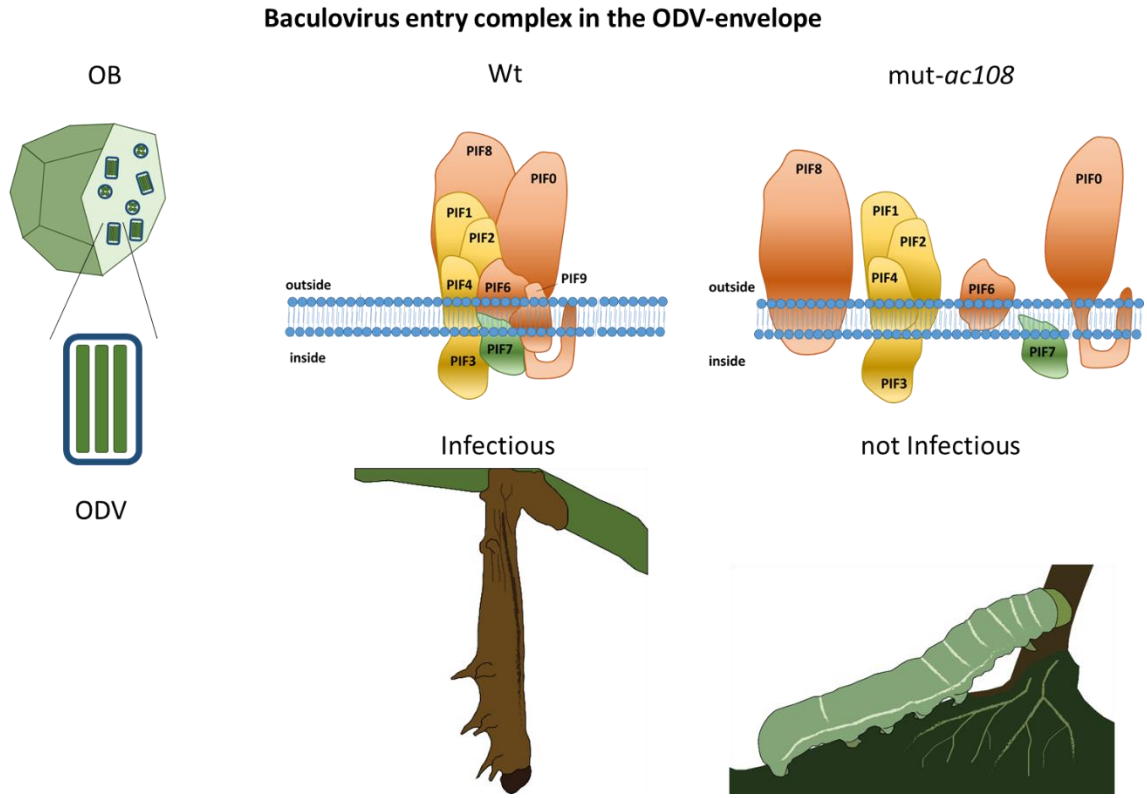

Figure 7: Updated model of the ODV entry complex. Wild type ODVs (Wt) have an intact ODV entry complex in their envelope, containing PIF9, and are orally infectious towards insect larvae (left panel). In absence of PIF9 (mut *ac108*), the stable core is still present but nevertheless fails to form an entry complex, affecting the ODV oral infectivity (right panel). The components of the core complex are depicted in yellow and the loosely associated components are depicted in red. PIF7 is depicted in green as its affinity with the complex is currently not known.

component (**Fig. 7**). In absence of PIF9, the stable core can still be formed but not the whole entry complex.

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 in **Chapter 3**.

Construction of recombinant bacmids. The *ac108*-gene of the AcMNPV bacmid bMON14272 (derived from the E2 strain) was inactivated by insertion of the *cat* resistance gene via homologous recombination. The *cat*-gene, flanked by mutated *loxP* sites (Suzuki *et al.*, 2005), 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'-*GCCGGCGCGACCGCCCGTCGCTCTCGATATAATGTCGGCCGCCGTC GGTT*GCTCGGATCCACTAGTAACG-3' and 5'- *GCAGCGGCATCACGGTGACGCCCGT CAAGTACAACTATTATTTGTAGTTA*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 described in Westenberg *et al.*, 2010 with the *bla* gene removed), which contained the AcMNPV bacmid. λ RED recombination (Datsenko and Wanner, 2000) 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'- CTTGGTTTAACAAGATCACAACC-3' and 5'- CGCGTCTTAACGGACGG-3'). The inserted *cat* gene was removed by transforming the cells with the temperature sensitive pCRE-TS plasmid (described in Ros *et al.*, 2015) 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) (Luckow *et al.,* 1993). 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 (Peng *et al.*, 2010). 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*ATTGTTGCATTTCTATTTCTAATA 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 Milli Q 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 in **Chapter 3**. 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 (Russel and Rohrmann, 1997, Peng *et al.*, 2010, 2012). 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).

Acknowledgements

This research was funded by the Netherlands Organization for Scientific Research (NWO), grant number 824.14.16. Felix Evers was involved in this project as a MSc. student and is acknowledged for the construction of the *ac108* deletion and repair bacmids and the performance of the initial experiments. I like to thank Els Roode for rearing the insects and culturing the insect cells.

Supplementary data

TnH5 cells were infected with 100µl of a P1 BV-stocks to produce OBs at small scale for initial analyses. Six days post infection, the OBs were isolated and counted. The different mutants were found to produce similar amount of OBs as wild type.

Viruses (bacmid-derived)	Number of OBs
Wild type AcMNPV	1.6×10^7
mut-*ac108*	3.0×10^7
rep-*ac108*	2.56×10^7
rep-*ac108*HA	2.67×10^7

This finding was not cell type dependent as similar results were also obtained when OBs were produced in either Sf9 or Sf21 cells by infecting these cells with P2 BV-stocks with an MOI of 2.

Viruses (bacmid-derived)	Sf9	Sf21	TnH5
Wild type AcMNPV	0.98×10^8	0.84×10^8	1.9×10^8
mut-*ac108*	0.90×10^8	0.42×10^8	2.5×10^8
rep-*ac108*HA	0.98×10^8	0.84×10^8	1.9×10^8

Chapter 5

Functional analysis of the baculovirus *per os* infectivity factors 3 and 9 by imaging the interaction between fluorescently labelled virions and isolated midgut cells

Bob Boogaard, Jan W. M. van Lent, Monique M. van Oers

Abstract

The envelope of the baculovirus occlusion derived viruses (ODVs) contains ten different viral proteins, which are classified as *per os* infectivity factors (PIFs). These proteins are crucial for midgut infection of insect larvae. All these PIFs, with exception of PIF5, are required to form an ODV entry complex. Here, the involvement of PIF3 and 9 in midgut infection is examined in greater detail. PIF3 is known to be dispensable for binding and fusion of ODVs to the brush borders of the epithelial cells, but midgut infection nevertheless fails in absence of PIF3. Where the infection process is halted with the *pif3* deletion mutant virus is currently unknown. PIF9 has only recently been identified and the biological role of this PIF in midgut infection is unknown. Here, the biological role of these PIFs in midgut infection was examined by monitoring the fate of fluorescently labelled ODVs of the Autographa californica multiple nucleopolyhedrovirus, that lack either PIF3 or 9. Isolated midgut cells from *Spodoptera exigua* larvae were inoculated with the fluorescent mutant ODVs and examined by confocal microscopy. This revealed that in absence of either PIF3 or PIF9, the ODVs bound to the brush borders, but the nucleocapsids failed to enter the cell.

Introduction

The *Baculoviridae* is a family of arthropod-specific viruses with large circular DNA genomes of 80 - 180 kbp, encoding 90 - 180 genes (Williams *et al.*, 2017). This virus family is divided into four genera. The *Alphabaculoviruses* and *Betabaculoviruses* infect the larvae of lepidopteran insect species, the *Gammabaculoviruses* infect hymenopteran larvae and the single known member of the genus *Deltabaculovirus* infects the larvae of *Culex* mosquitoes (Jehle *et al.*, 2006). The larvae become orally infected when they consume food sources that are contaminated with occlusion bodies (OBs). These OBs consist of a protein matrix, in which the infectious occlusion derived viruses (ODVs) are embedded. When the OBs are ingested, the protein matrix dissolves in the alkaline environment of the larval midgut lumen and the ODVs are released. These viruses then infect the midgut epithelial cells, in which the ODV-envelope binds and fuses with the plasma membrane of the microvilli of these cells, thus releasing the nucleocapsids into the cells (Kawanishi *et al.*, 1972, Granados *et al.*, 1981). After primary infection of the midgut epithelium, the virus spreads systemically to other tissues by means of a different virus phenotype, the budded viruses (BVs).

Infection of the midgut epithelium cells by ODVs requires at least ten different ODV-envelope proteins: the *per os* infectivity factors or PIFs (Faulkner *et al.*, 1997, Kikhno *et al.*, 2002, Pijlman *et al.*, 2003, Ohkawa *et al.*, 2005, Fang *et al.*, 2009, Sparks *et al.*, 2011a, Nie *et al.*, 2012, Zhu *et al.*, 2013, Javed *et al.*, 2017, Liu *et al.*, 2016, Wang *et al.*, 2019, **Chapter 4**). Each of these proteins is essential for infection of the midgut. Deletion of any of the *pif*-genes abolished the oral infectivity of the ODVs without affecting their formation or embedding into OBs. The production and infectivity of BVs remain unaffected as well. All PIFs, except PIF5, participate in the formation of a large complex, called the ODV entry complex. PIF1 to 4 form the stable core of this complex, which even resists treatment with SDS and ß-mercaptoethanol. PIF0 and PIF6 to 9 dissociate from the complex in the presence of such denaturing chemicals, indicative for a lower binding affinity (Peng *et al.*, 2010, 2012, Wang *et al.*, 2019, **Chapter 2, 3, 4**). PIF5 apparently promotes ODV infectivity independently from the complex.

Infection of the midgut is initiated by the binding of ODVs to the epithelial cell brush border, followed by fusion of the ODV envelope with the plasma membrane. The nucleocapsids are then released into the cytoplasm, which possibly requires a downstream process that has yet to be characterised (Ohkawa *et al.*, 2005). The initial steps of midgut infection has been studied by fluorescence dequenching assays with R18-loaded ODVs and brush border membrane

vesicles. This showed that various mutant viruses, lacking either PIF0, 1, 2 or 3, can be phenotypically discriminated in terms of their ability to bind and fuse with prepared brush border vesicles. PIF0, 1 and 2 were shown to be important for the binding of ODVs to the vesicles (Haas-Stapleton *et al.*, 2004, Yao *et al.*, 2004, Ohkawa *et al.*, 2005), while PIF3 was dispensable for ODV binding and fusion (Ohkawa *et al.*, 2005). This indicated that PIF3 is involved in another event during infection of the midgut, which takes place downstream from binding and fusion.

These findings indirectly imply that the binding and fusion of the ODV envelope with the cellular plasma membrane does not require the presence of the entry complex, as the core (and thereby also the entry complex) fails to form in absence of PIF3 (Peng *et al.*, 2010). Furthermore, the entry complex also fails to assemble in absence of PIF9, which just has recently been identified (**Chapter 4**). However, the role of this new PIF in infection of the midgut remains to be established. To gain more insight in the biological function of PIF3 and PIF9 during midgut infection, we visualized the interaction of fluorescently labelled ODVs, in absence of either PIF3 or 9, with the brush border of isolated midgut cells using confocal microscopy imaging, as previously described by Mu *et al.*, 2014. This was done with the alphabaculovirus Autographa californica multiple nucleopolyhedrovirus (AcMNPV) and midgut cells isolated from *Spodoptera exigua* larvae. The current study confirms that ODVs that lack PIF3 are able to bind to the brush borders of midgut epithelial cells, but that the nucleocapsids failed to enter the cells. Similar results were obtained for the *pif9* mutant virus.

Results

Construction of recombinant bacmids to produce fluorescently labelled mutant ODVs that lacks PIF3 or 9.

The *pif3* gene in the AcMNPV bacmid bMON14272 was deleted via insertion of a chloramphenicol acetyltransferase (*cat*) resistance gene into its open reading frame (ORF) via λ-red mediated homologous recombination as described before (Datsenko *et al.*, 2000, Li *et al.*, 2007). The *cat*-amplicon with the homologous arms was produced by PCR by using the same primers as in Li *et al.*, 2007. The insertion of the *cat*-gene was confirmed by PCR with primers that anneal outside the recombined region. When the wild type bacmid was used as template, a PCR product was produced of 500 bp (**Fig. 1**). However, a 1500 bp PCR product was produced after homologous recombination, indicating that the *cat*-gene was successfully inserted into the

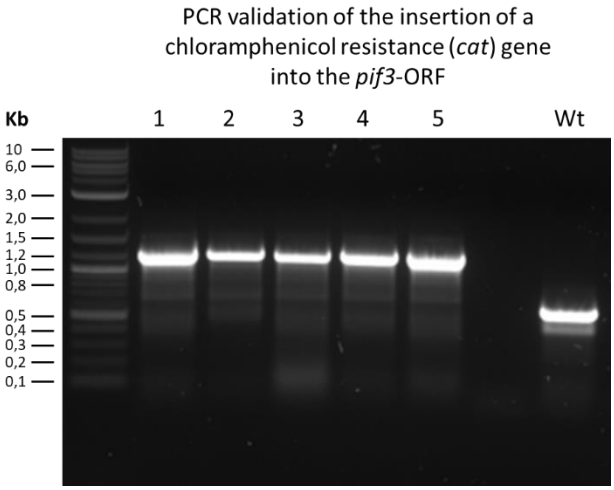

Figure 1: Confirmation of the insertion of the chloramphenicol acetyl transferase (*cat*) resistance gene into the *pif3* locus by PCR. Insertion of the *cat*-gene resulted in the formation of an amplicon 1500 bp (clone 1-5), while without insertion a 500 bp amplicon was produced (Wt).

ORF of *pif3*. The *cat*-gene was found inserted in all five clones and clone 1 was used for further studies. The *pif3* deletion bacmid was used for site directed transposition using a modified pFastBac Dual vector that contains the polyhedrin (*polh*) gene under the polyhedrin promoter, and the ORF of *vp39* fused to the 3'end of the *egfp*-ORF, expressed under the p10 promoter (pFastBac-*polh-egfp:vp39*) (**Fig. 2**). An *ac108* mutant bacmid, previously described in **Chapter 4**, was also transposed with this pFastBac-*polh-egfp*:*vp39* vector to obtain mut-*pif9* (**Fig. 2**). In this way, the resulting del-*pif3* and mut-*pif9* viruses are able to produce OBs and fluorescent

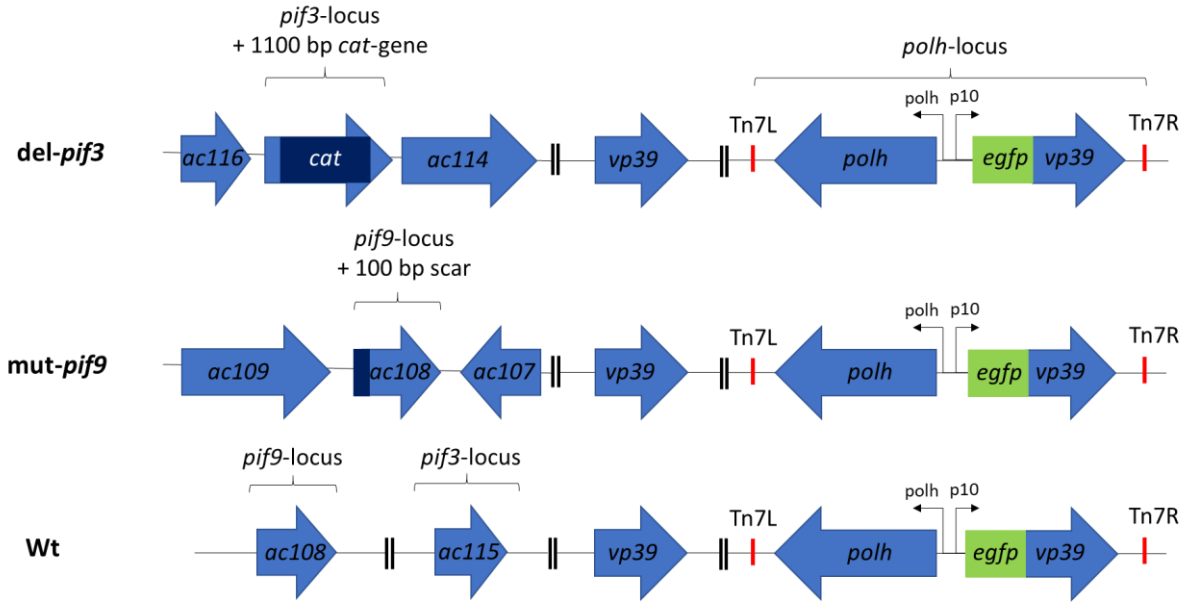

Figure 2: Overview of the constructed bacmids. The *pif3* deletion and *pif9* mutant bacmids (the latter is described in Chapter 4) were transposed with a pFastbac-*polh-egfp*:*vp39* vector to enable the mutant viruses to produce OBs and fluorescently labelled nucleocapsids. The fluorescent wild type virus was previously described in Mu *et al.*, 2014 and is used in this study as wild type control.

nucleocapsids. The fluorescent "wild type" (Wt) virus used in this study was previously described in Mu *et al.*, 2014.

Western blot analysis of the mutant ODVs envelope and nucleocapsid fractions

To confirm the absence of PIF3 in the ODV envelope after *pif3* deletion, as well as the incorporation of the eGFP:VP39 fusion protein into the nucleocapsids, the ODVs of the wt and mutant viruses were fractionated and analysed by western blotting. The envelope fractions were analysed with PIF3 antiserum and the nucleocapsid fractions with antiserum against the major capsid protein VP39. These analyses showed that PIF3 monomers were found at the expected height (corresponding to 27 kDa) in the envelope fractions of the wt and mut-*pif9* virus, but not in the del-*pif3* virus (**Fig. 3a**). When the nucleocapsid fractions of these three viruses were analysed with antiserum against VP39, the capsid protein was detected as a 39 kDa protein and the eGFP:VP39 fusion protein was detected just below the 70 kDa marker, as expected, although in smaller quantities than the native VP39 protein (**Fig. 3b**). This indicated that the fusion protein was incorporated into the nucleocapsids of the wt and mutant viruses.

The infectivity of the fluorescent viruses for S. exigua larvae

The infectivity of the fluorescent viruses was tested in *S. exigua* L4 larvae via intra-hemocoelic injection of BVs and by oral inoculation of early L3 larvae with OBs. The oral infectivity of the *pif3* deletion and *pif9* mutant ODVs were determined in separate bioassays, in which the

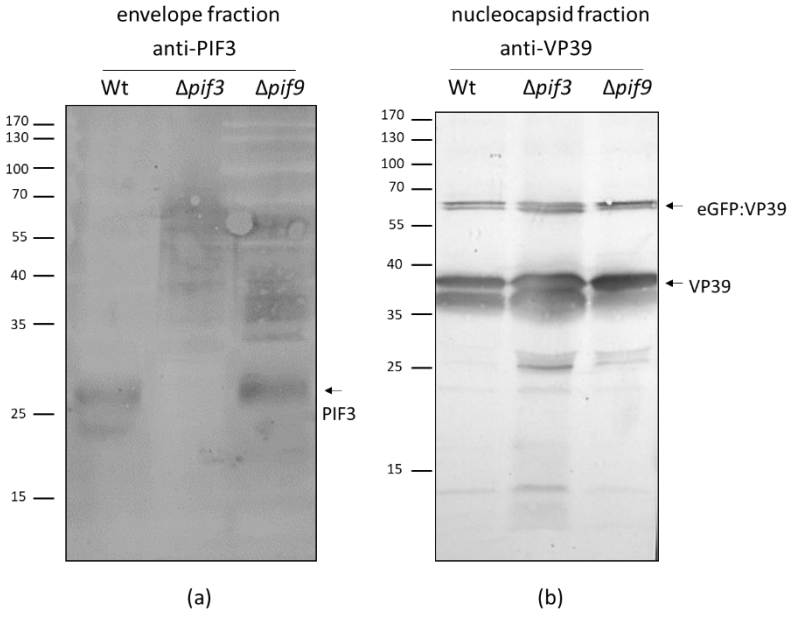

Figure 3: Western blot analysis of the ODV-envelope and nucleocapsid fractions of wild type, *pif3* deletion mutant and *pif9* mutant viruses. The envelope fractions were analysed with antiserum against PIF3 (a) and the nucleocapsid fractions with VP39 antiserum (b).

Table 1: Mortality of *S. exigua* larvae after injection with BVs or oral inoculation with OBs

Injection with BVs	**3.5×10^7 $TCID_{50}$ / ml**		
mock	0/12		
del-*pif3*-*egfp:vp39*	11/12		
mut-*pif9*-*egfp:vp39*	11/12		
Wt-*egfp:vp39*	12/12		
Oral infectivity 3.0×10^8 OBs / ml	**Exp. 1**		**Exp. 2**
mock	0/14		0/12
del-*pif3*-*egfp:vp39*	0/13		0/11
Wt-*egfp:vp39*	12/13		11/11
Oral infectivity 3.0×10^8 OBs / ml	**Exp. 1**	**Exp.2**	**Exp. 3**
mock	0/12	0/14	0/12
mut-*pif9*-*egfp:vp39*	0/15	0/12	0/12
Wt-*egfp:vp39*	12/15	12/13	10/12

pif3 deletion mutant was tested in duplicate and the *pif9* mutant in triplicate, because additional information is available on the function of PIF3 in literature, whereas PIF9 just has been identified (**Table 1**). When the larvae were injected with Wt or one of the mutant BVs stocks, fluorescence was observed in at least 11 out of 12 larvae for each virus at 3 dpi, in contrast to the mock injected larvae (**Fig. 4a**). This indicates that the eGFP:VP39 fusion protein is produced and that the Wt and *pif* mutant viruses replicate in the larvae when bypassing the midgut. When the larvae were orally inoculated with OBs, only the larvae that were fed with the wt virus showed fluorescence (**Fig. 4b**). These observations correspond with the mortality rates, in which ODV inoculation only caused significant mortality for the Wt virus (**Table 1**). These results show that the fluorescently labelled *pif* mutant viruses have the same phenotype as previously described for the non-fluorescent mutant viruses (Li *et al.*, 2007, Boogaard *et al.*, 2019).

Wild type ODVs enter the midgut cell within 2 – 6 minutes after the start of observation.

To visualize the interaction between (mutant) ODVs and the epithelial cell brush border, isolated midgut cells of *S. exigua* larvae were incubated with fluorescently labelled ODVs and examined by confocal microscopy to monitor the location of the ODVs in relation to time. These analyses were first performed for Wt ODVs to validate whether cell entry can be observed under the applied experimental conditions. Twenty one Wt ODVs were followed over

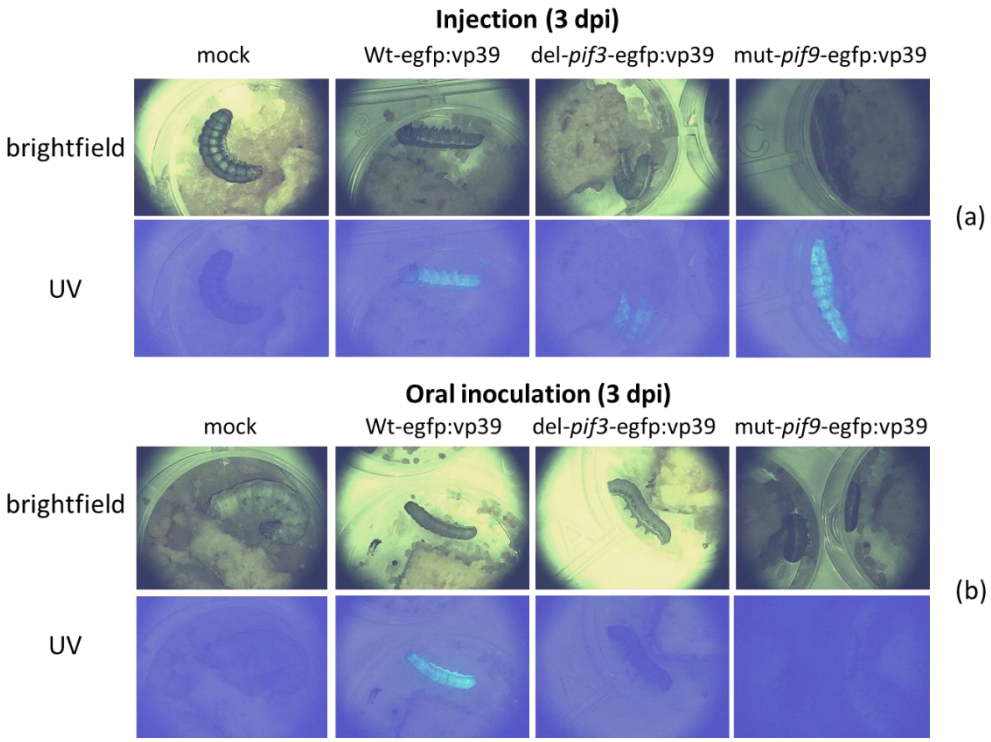

Figure 4: Validation of viral replication of eGFP-tagged AcMNPV mutants in *S. exigua* larvae. The larvae were examined under UV-light to observe green fluorescence after injection with BVs (a) or oral inoculation with OBs (b). Green fluorescence is indicative for viral replication.

time and these interacted with eight separate midgut cells (**Table 2**). These ODVs were detected in the brush border for 2 to 6 minutes before they disappeared from the cell surface (**Fig. 5**). About half of the ODVs disappeared within 2-4 minutes after the start of the time lapse experiment. As the ODVs under study could also not be found in the surrounding focal planes, it was concluded that the nucleocapsids had entered the midgut cell.

Table 2: Overview of the observations of fluorescent ODVs interacting with the brush border of primary midgut cells.

Virus	Nr. of observations	Nr. of observed cells	Average observation time before disappearance (min.)
Wild type	21	8	4.5 ± 2
del-*pif3*	7	4	-
mut-*pif9*	14	4	-

Mutant ODVs that lack either PIF3 or 9 bind to the brush border, but fail to enter the cell.

The experiment was repeated with mutant ODVs, for which seven ODVs of the *pif3* deletion mutant and fourteen of the *pif9* mutant were analysed, each on four distinct midgut cells (**Table 2**). The ODVs of both mutants were detected at the cell surface until the end of the

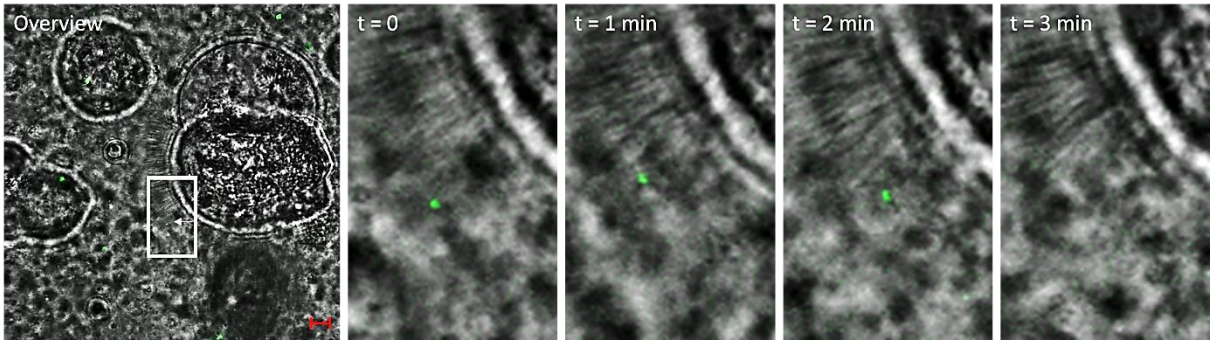

Figure 5: Time lapse analysis of one fluorescently labelled wild type ODV on the brush border of an isolated midgut cell. The red scale bar corresponds with 5 µm. In this case, the ODV disappeared within 3 minutes after the start of the observation.

measurements, even after continuous excitation with the laser for 20 minutes of the eGFP:VP39 fusion protein (**Fig. 6**). These observations show that in absence of PIF3 or PIF9 the ODVs are still able to bind to the brush borders of midgut epithelial cells, but the nucleocapsids fail to enter the cell.

Discussion

Baculovirus ODVs infect the midgut epithelium of insect larvae via direct membrane fusion with the plasma membrane of the host cell brush border (Kawanishi *et al.*, 1972, Horton *et al.*, 1993). After the ODV envelope has fused with the plasma membrane, the nucleocapsids enter the cell and are transported through the microvilli and the rest of the cytoplasm to the nucleus for replication (Kawanashi *et al.*, 1972, Tanada *et al.*, 1975, Granados *et al.*, 1981). The ODV-envelope contains at least ten different proteins that are crucial for midgut infection, PIF0 to 9, of which nine (all except PIF5) form the ODV entry complex (Peng *et al.*, 2010, 2012, **Chapter 2, 3, 4**). The formation of this complex is abolished when one of these components is absent.

Although the complex cannot be formed in absence of one of the PIFs (Peng *et al.*, 2010, 2012, **Chapter 4**), two different phenotypes regarding binding and fusion have been observed with R18 dequenching assays when using various mutant ODVs. It had been found that PIF0, 1 and 2 are important for ODV binding (Haas-Stapleton *et al.*, 2004, Yao *et al.*, 2004), while PIF3 is dispensable for binding and fusion (Ohkawa *et al.*, 2005). The ODVs of the *pif3* deletion mutant nevertheless failed to establish an midgut infection. PIF9 has been identified only recently (**Chapter 4**). However, no data on the biological role of this protein has been reported. This study aims to complement the existing knowledge on the function of PIF3 in midgut infection and to obtain the first data on the biological function of PIF9. Therefore, fluorescently labelled ODVs of AcMNPV that lack either PIF3 or 9 were generated and followed over time in the

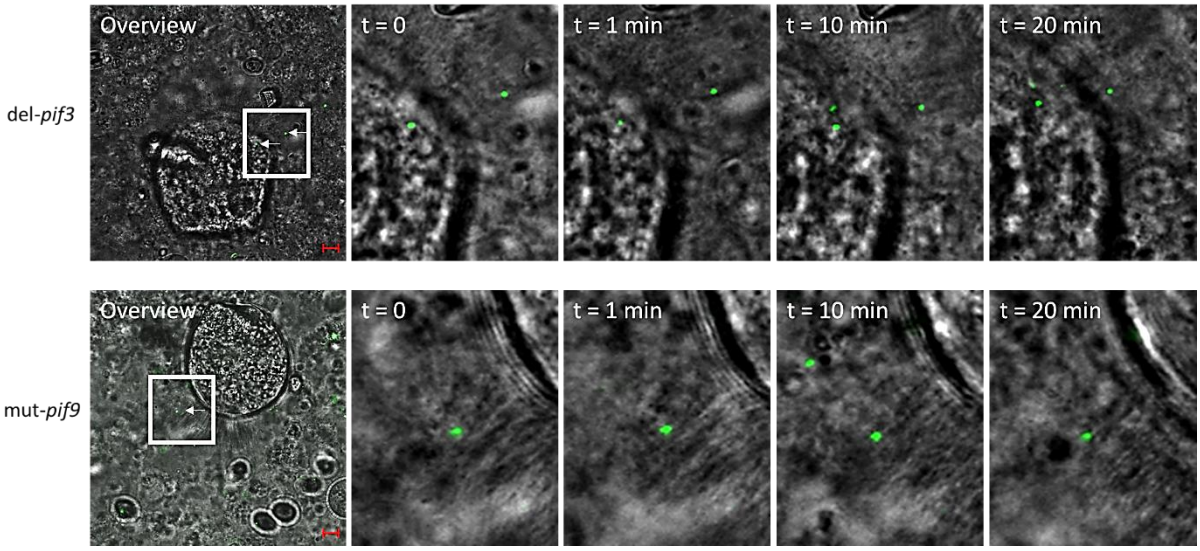

Figure 6: Time lapse analysis of one (representative) fluorescently labelled ODV of a *pif3* deletion mutant and a *pif9* mutant virus on the brush border of an isolated midgut cell. The red scale bar corresponds with 5 µm. The fluorescent mutant ODVs remain attached to the brush borders to the end of the observations.

brush borders of isolated midgut cells from *S. exigua* larvae with a confocal microscope, analogous to the assay that had been described in Mu *et al.*, 2014.

Wild type ODVs were detected on the brush border for 2 to 6 minutes before they disappeared from the cell surface, while in absence of PIF3 or 9 the ODVs remain detectable until the end of the measurements (**Fig. 5-6**). Hence, the disappearance of wild type ODVs could not be explained by fading of the fluorescent signal by continuous excitation of the eGFP:VP39 fusion protein with the laser. As the wild type ODVs were also not found in the flanking sections, it was concluded that the fluorescent signal disappeared by decomposition of the grouped nucleocapsids of the multiple capsid ODV upon cell entry, as was observed before in electron microscopy studies (Kawanashi *et al.*, 1972, Granados *et al.*, 1981). It took longer for the Wt ODVs to enter the cells under the present experimental conditions than previously described in Mu *et al.*, 2014. This might be explained by the usage of ODVs that were released from cell culture-derived OBs (C-ODVs) instead of OBs from infected larvae (L-ODVs), as used in the other study. Infection with C-ODVs might initially be hampered by remnant polyhedrin that sticks to the virus envelope, which is degraded by co-occluded host proteases in L-ODV preparations (Harrap *et al.*, 1977, Payne and Kalmakoff, 1977, Wood *et al.*, 1980). However, as the remaining PIF-proteins in *pif* mutants are highly sensitive to degradation by such co-occluded proteases during OB-dissolution (**Chapter 2**), we decided to use cell-culture derived viruses for the experiments. The remnant polyhedrin on the C-ODVs was probably degraded by (membrane associated) digestive enzymes of the primary midgut cell (Javed *et al.*, 2019) when the virus and cell suspensions were mixed, enabling the ODVs to enter the cell after a

few minutes. Whether the PIFs in the mutant viruses underwent a certain level of degradation under the conditions used was not determined. The proteolytic activity cannot be completely inhibited, e.g. by adding a cocktail of protease inhibitors, as this hampers the infection by ODVs (Slack *et al.*, 2008). PIF0 has been shown to be the cleaved by host trypsins in the gut and by co-occluded proteases (Slack *et al.*, 2008, Peng *et al.*, 2011), but the importance of these cleavage events for ODV infectivity needs further studies.

Our observations show that in absence of PIF3, the ODVs bind to the brush border (**Fig. 6**), just as indicated before by the R18 dequenching assays (Ohkawa *et al.*, 2005). Whether the ODV-envelope of this mutant also fused with the brush border plasma membrane could not be confirmed with the currently used assay. Our observations nevertheless show that the nucleocapsids stayed together and failed to enter the cell. This indicates that PIF3 is indeed required for the translocation of the nucleocapsids into the host cell as proposed by others (Ohkawa *et al.*, 2005). However, it has been observed with mutants of other viruses that R18-dequenching also takes place after hemifusion of the lipid membranes, in which only the outer leaflets of the bilayers fuse (Kemble *et al.*, 1994, Laliberte *et al.*, 2011). It could therefore also be reasoned that in absence of PIF3, only the outer leaflets fuse and that this PIF is required to overcome this hemifusion intermediate state (**Fig. 7**). This might explain why the R18-dequenching assays with the *pif3* deletion mutant suggested that membrane fusion took place, while the infection of the midgut epithelium failed.

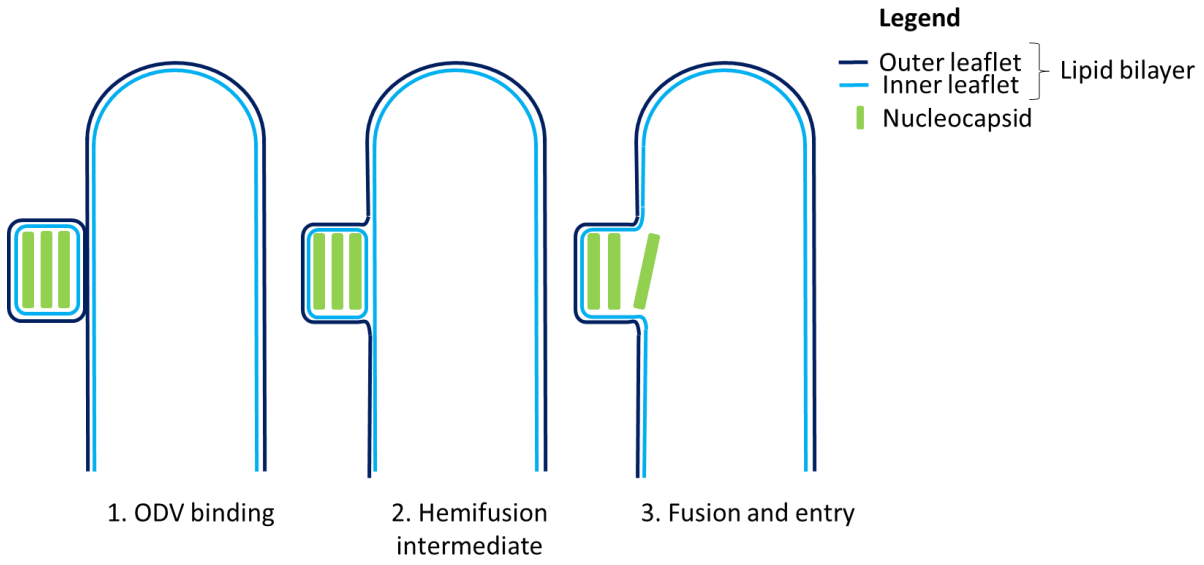

Figure 7: Model for the membrane fusion event. During primary infection of the midgut epithelium, the ODV first binds to the microvilli (1) and membrane fusion occurs via the formation of a hemifusion intermediate (2). It is hypothesized that in absence of PIF3, membrane fusion is stuck in this intermediate state and prevents entry of the nucleocapsids into the host cell.

ODVs that lack PIF9 also bound to the epithelial cell brush borders. Whether the binding of these mutant ODVs is indeed not affected needs to be validated quantitatively by R18 dequenching assays, as the live imaging assay is biased for ODVs that bind to the brush borders; i.e. non-interacting ODVs were not analysed in the time lapse experiments. However, as we regularly encountered bound ODVs, it is reasoned that binding is not significantly affected by the absence of PIF9. Whether this PIF is involved in membrane fusion needs also to be determined by R18-dequenching assays. Our observations nevertheless show that midgut entry of the nucleocapsids failed in absence of PIF9, as shown previously for PIF1, 2 (Mu *et al.,* 2014), and now also for PIF3.

Despite the fact that both mutants are unable to form the ODV entry complex, the mutant ODVs were nevertheless found to be able bind to the brush border of the isolated midgut cells. Formation of the entry complex is apparently not important for the ODV binding properties as long as PIF0, 1 and 2 are present in the ODV envelope. It was also observed for both mutants that the entry of the nucleocapsids was abolished. The exact reason for this is unfortunately unclear. It could be concluded from these observations that formation of the entry complex is crucial for the early stages of midgut infection, either for completion of membrane fusion at the epithelial cell brush border or for the translocation of the nucleocapsids into the cytosol.

Materials and methods

Insect cells and lepidopteran larvae. TnH5 (from *Trichoplusia ni*) and Sf21 cells (from *Spodoptera frugiperda*) were cultured in Grace's medium (Thermo Fisher), supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml gentamycin (Gibco, Life technologies) at 27°C. *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 (**Chapter 3**).

Construction of recombinant bacmids. The ORF of *pif3* of the AcMNPV bacmid bMON14272 (derived from the E2 strain) (Luckow *et al.,* 1993, Smith and Summers, 1978) was deleted by insertion of the chloramphenicol acetyl transferase (*cat*) resistance gene via homologous recombination, as described in Li *et al.,* 2007. The *cat*-gene 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 in the *pif3*-ORF that flanks the sequence that needs to be deleted. (5-*CACGGGGATCGTGCGAGCCAGTGCCGTTTGGCGAAATAGCATTTTTTTCAT* GCTCGGATCCACTAGTAACG -3' and (5'-*TACACGTTTAAATTTGTTCAAAAATTTATTTT*

*GCAAGATGCATACATGCA*CCTCTAGATGCATGCTCG -3'). The amplicon was purified from an agarose gel with the GFX Gel Band Purification kit (GE Healthcare) and electroporated into *E. coli* MW003 cells (MW001 (Westenberg *et al.,* 2010) with the *bla* gene removed), carrying the AcMNPV bacmid. The expression of the λ RED for recombination (Datsenko and Wanner, 2000) was activated in these bacterial cells by heat induction at 42°C for 10 min. The transformed bacteria were incubated on LB-agar plates in the presence of 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 into the *pif3*-ORF (5'-CAGTTGTAAACGCGTCTGTAC -3'and 5'- CATGTTGAAC TTTTGGCAAATACTTATTC-3'). The *pif3* deletion bacmid was isolated, by alkaline lysis of the bacterial cells and subsequent precipitation in isopropanol, and electroporated into DH10ß cells that contained the transposase helper plasmid (pMON7124) (Luckow *et al.,* 1993). Construction of the *pif9* mutant bacmid is described in Boogaard *et al.,* 2019. Both bacmids were transposed with a pFastBac Dual vector of the Bac-to-Bac system with the *polh*-gene under the *polh*-promoter and the ORF of *egfp* fused to the 5'end of the *vp39*-ORF under control of the p10 promoter (pFastbac-*polh-egfp:vp39*) as described in Mu *et al.,* 2014. These genes were transposed via Tn7 mediated transposition into the *polh*-locus of the bacmids to enable the resulting viruses to produce OBs and fluorescently labelled nucleocapsids.

Production of the mutant viruses. First generation BV-stocks (P1) of the *pif3* and *pif9* mutants were generated by transfection of Sf21 cells with the bacmids, using Expres2TR transfection reagent (Expres2ion Biotechnologies). Six days post transfection, the BV stocks were amplified by an additional infection round in Sf21 cells, generating P2 BV-stocks. OBs were produced by infection of TnH5 cells with BVs at an MOI of 2 and isolated as described before (**Chapter 4**). In brief, the infected cells were harvested at six days post infection by centrifugation at 4000 x g for 30 min and lysed by incubation in 0.1% SDS for 2 hrs. at 37°C with gentle agitation and subsequent sonication for 1 minute at an intensity of 8 Watt on ice. Complete lysis was verified by light microscopy and the cells were sonicated once more when needed. When all the cells had lysed, the OBs were pelleted by centrifugation at 4000 x g for 30 min. and washed three times with sterile double distilled water.

SDS-PAGE and western blot analysis. The ODVs of the *pif3* and *pif9* mutants were isolated, fractionated and analysed by SDS-PAGE and western blotting as described previously in Chapter 3, 4. The envelope fraction was analysed with rat-derived PIF3 antiserum (described in Peng *et al.,* 2010, diluted 1:1000) and the nucleocapsid fraction with antiserum against the

major capsid protein VP39, derived from mice (diluted 1:1000). Goat anti-rat (1:2000, Sigma A8438) and goat anti-mouse (1:2000, Sigma A5153), conjugated to alkaline phosphatase, were used as secondary antibodies to detect the target proteins via the conversion of the NBT-BCIP substrate (Roche) in a purple precipitant in AP-buffer (0.1 M Tris-HCl, 0.1 M NaCl and 5 mM MgCl, pH 10.5).

Bioassays in S. exigua larvae. The oral infectivity of the OBs of the fluorescent *pif3* and *pif9* mutant viruses was tested by oral inoculation of starved early L3 *S. exigua* larvae in droplet feeding assays. Fluorescent wt virus (previously described in Mu *et al.,* 2014) was used as positive control. The OBs were suspended in 10% (w/v) sucrose at a concentration of 3.0 x 10^8 OBs / ml and the suspension was coloured blue with Patent Blue V Sodium salt (Fluka). The larvae were allowed to feed on the OB-sucrose solutions for 10 minutes, transferred to 12-wells plates with artificial medium in each well and incubated at 27°C. One day prior to the expected time of death (3 dpi), the larvae were scored for green fluorescence under UV light (indicative for viral replication) and incidental non-viral deaths were excluded from further analyses. The infectivity of the BVs of the fluorescent *pif3* and *pif9* mutant viruses was determined by injection of 1 µl BV-stock (3,5 x 10^7 $TCID_{50}$ / ml) into the hemocoel of L4 larvae, using an Humapen Luxura insulin pen. These larvae were also scored for green fluorescence at 3 dpi as described above. For both assays, the number of liquefied larvae were scored on a daily basis.

Isolation of the midgut cells from S. exigua larvae. The midguts of twenty *S. exigua* L5 instar larvae were dissected by cutting of the larva's head and tail, after which the midgut was pulled out and the peritrophic membrane was removed. The midguts were collected in Graces medium, supplemented with 50 µg/ml gentamycin, washed twice (also in Graces medium) and incubated in Graces medium, containing 2 mg/ml collagenase from *Clostridium histolyticum* (Sigma, blend type H), for 90 min. at 27°C under vigorous shaking at 850 rpm. The cells were separated from the remaining midgut tissues by filtering through a 200 µm meshwork of nylon, fixed at the bottom of a microtube. The primary midgut cells were collected and pelleted by centrifugation at 200 x g for 10 min, and subsequently washed three times with Grace's medium. After the last washing step, the cells were suspended in pre-cooled EDTA-free DAS buffer (0.1 M Na_2CO_3, 166 mM NaCl, pH 10.5) and kept on ice till further use.

Time-lapse analysis of fluorescent ODVs on the brush border of primary midgut cells. The interaction between the fluorescent ODVs and the brush borders of the isolated midgut cells was visualized by constructing a z-stack in a time lapse experiment with a confocal microscope

(Zeiss LSM-510). The ODVs were isolated in slightly a modified way from what was described in Chapter 3. The ODVs were released by a 10 min incubation in EDTA-free DAS-buffer and the OB membrane was disrupted by two sonication bursts. The ODVs were incubated with the isolated midgut cells under alkaline conditions in EDTA-free DAS-buffer on ice for 15 min to allow these to bind to the brush borders prior to imaging. The cell-virus suspension was examined for midgut cells with (bound) ODVs in the brush border by detection of GFP fluorescence by confocal microscopy, using a 63x oil objective lens under excitation with an Argon laser. This interaction was then analysed by construction of a z-stack with 600-700 nm optical slices (15-20 pcs) in a time lapse experiment for 20 min. It turned out to be crucial not to add too much ODVs to be able to clearly discriminate individual ODVs over time. The images were analysed by using Zen (blue) 2.3 lite software.

Acknowledgements

We like to thank Dr Norbert de Ruijter (Wageningen University and Research) for his technical assistance with the confocal microscope and Els Roode for the rearing of the *S. exigua* larvae. We also acknowledge Felix Evers for construction of the bacmid for the generation of fluorescent mut-*pif9* ODVs, and Wendy van Elst and Priyanka Thaoroijam for their assistance with midgut cell isolation and assay optimization. This work was supported by grant 824.14.16 of the Netherlands Organization for Scientific Research (NWO).

Chapter 6

The C-termini of baculovirus *per os* infectivity factors 1 and 2 mediate ODV oral infectivity by facilitating the binding of PIF0 and PIF8 to the core of the entry complex.

Bob Boogaard, Jan W. M. van Lent, Monique M. van Oers

Abstract

Oral infection of caterpillars by baculoviruses is initiated by occlusion-derived virus particles (ODVs) that infect midgut epithelium cells. The ODV envelope therefore contains at least ten different proteins, which are called *per os* infectivity factors (PIFs). Nine of these PIFs form the so-called ODV entry complex that consists of a stable core formed by PIF1, 2, 3 and 4, to which the other PIFs (PIF0, 6, 7, 8 and 9) bind with lower affinity. PIF1 and 2 are not only essential for complex formation, but also mediate ODV-binding to the epithelial cell brush border, probably via the C-termini. To study the involvement of these PIFs during midgut infection in greater detail, we assessed the oral infectivity and the ability to form the complex of a series of PIF1 and PIF2 C-terminal truncation mutants of Autographa californica multiple nucleopolyhedrovirus (AcMNPV). Limited truncation of either PIF1 or 2 already severely impaired the ODV oral infectivity, but did not affect formation of the core complex. However, the entry complex as a whole was not assembled by these mutants as PIF0 and 8 failed to bind to the core. This suggests that the interactions between PIF1 and 2 in the core and PIF0 and PIF8 are important for the ODV infectivity and that complex formation complicates the determination of the exact roles of PIF1 and 2 during midgut infection. We also showed that the presence of PIF0, 6 and the ZF-domain of PIF8 are crucial for formation of the entry complex.

Introduction

Baculoviruses are double stranded DNA viruses that orally infect the larvae of lepidopteran, hymenopteran and dipteran insect species when they eat plant material that is contaminated with viral occlusion bodies (OBs) (Williams *et al.,* 2017). The protein matrix of the OBs dissolves in the highly alkaline environment of the midgut lumen, resulting in the release of occlusion derived viruses (ODVs). The ODVs then bind and fuse with the microvilli of midgut epithelial cells to initiate a primary infection of the larval midgut. The infected epithelial cells produce a second type of virus particles, the budded viruses (BVs), which systemically spread the infection to other tissues in the larva.

The entry mechanism of ODVs into midgut epithelial cells is poorly understood due to its complexity as it involves at least ten different viral proteins. As each of these proteins is of specific importance for the virus oral infectivity, they are called *per os* infectivity factors (PIFs). In the Autographa californica multiple nucleopolyhedrovirus (AcMNPV), the ten PIFs and their specific genes are: PIF0 (or P74, encoded by the AcMNPV gene *ac138*) (Faulkner *et al.*, 1997), PIF1 (*ac119*) (Kikhno *et al.*, 2002), PIF2 (*ac22*) (Pijlman *et al.*, 2003), PIF3 (*ac115*) (Ohkawa *et al.,* 2005), PIF4 (*ac96*) (Fang *et al.*, 2009), PIF5 (ODV-E56/*ac148*) (Sparks *et al.,* 2011a), PIF6 (*ac68*) (Nie *et al.*, 2012), PIF7 (*ac110*) (Liu *et al.*, 2016), PIF8 (P95/*ac83*) (Zhu *et al.*, 2013) and PIF9 (*ac108*) (Wang *et al.,* 2019, **Chapter 4**). When OBs of a *pif* deletion mutant are orally administered to larvae, the infection fails. However, in cultured insect cells the deletion of a *pif* gene does not affect the production of ODVs or OBs, nor the production and infectivity of BVs. PIF8 is the single exception as this protein is besides crucial for oral infectivity also involved in nucleocapsid assembly (Zhu *et al.*, 2013, Javed *et al.*, 2017). These two functions are performed by two separate domains, of which the zinc-finger (ZF) domain is crucial for oral infectivity (Javed *et al.*, 2017).

Nine of the ten PIF proteins form a large complex in the ODV envelope. This so-called ODV entry complex consists of a stable core, formed by PIF1-4 (Peng *et al.,* 2010, 2012), and five other PIFs (PIF0 and PIF6-9) that bind to the complex with lower affinity (Peng *et al.,* 2012, Wang *et al.,* 2019, **Chapter 2, 3, 4**). PIF5 is not a component of the entry complex (Peng *et al.,* 2012). PIF1-3 are essential for formation of the stable core and still interact with each other in absence of PIF4 (Peng *et al.,* 2010, 2012). Furthermore, the core complex appears to be highly stable, since it remained intact when isolated ODVs were partially denatured by treatment with 8% SDS and 5% 2-mercaptoethanol and heating up to 75°C (Peng *et al.,* 2010, **Chapter 3**).

The complete entry complex however, can only be found under non-denaturing conditions as the more loosely associated components dissociate from the core upon treatment with SDS. Therefore, PIF0, 6, 7, 8 and 9 were only detected as part of the entry complex by blue native-PAGE analysis and by mass spectrometry as co-immune precipitants of PIF1 (Peng *et al.*, 2012, Wang *et al.*, 2019, **Chapter 2, 3, 4**). Further examination of the entry complex under non-denaturing conditions also showed that PIF4, the ZF-domain of PIF8 and PIF9 are essential for formation of the complex (Peng *et al.*, 2012, Javed *et al.*, 2017, Wang *et al.*, 2019, **Chapter 4**).

PIF0, 1 and 2 have been shown to be required for the binding of ODVs to the brush border of the midgut epithelial cells (Haas-Stapleton *et al.*, 2004, Ohkawa *et al.*, 2005, Mu *et al.*, 2014) and were indeed indicated to point outwards from the ODV envelope in immunogold labelling analyses (Peng *et al.*, 2010). This corresponds with *in silico* data, in which these proteins are predicted to point outwards from the ODV envelope, making these available for interaction with the larval host in the midgut (**Chapter 2**). PIF0 was predicted to be inserted in the ODV envelope via its C-terminal transmembrane domains, so that the N-terminal parts of this protein point outwards. PIF1 and 2 are predicted to be N-terminally inserted in the ODV envelope, with their C-termini sticking out.

In addition to being important for ODV binding, PIF1 and 2 are essential for formation of the entry complex. This raised the question on whether these PIFs are directly involved in ODV-binding via the exposed C-termini, or indirectly by the binding of other PIF proteins to these C-termini. Most baculoviruses have a narrow host range and the ODV binding capacity is one of the host range determinants, as the resistance of *Spodoptera frugiperda* larvae to oral infection with AcMNPV was attributed to aberrant ODV binding when compared to the Spodoptera frugiperda multiple nucleopolyhedrovirus (Haas-Stapleton *et al.*, 2005). The amino acid sequences of PIF1 and PIF2 of these two alphaviruses are highly similar, but show (slightly) more variation at the C-terminal ends than at the N-terminal parts (**See alignments and truncation sites in Supplemental data Fig. S1 and S2**). It was therefore hypothesized that the more variable C-terminal parts of PIF1 and 2 are involved in ODV binding and the more conserved N-terminal parts in complex formation. This hypothesis was tested by the construction a series of AcMNPV PIF1 and PIF2 C-terminal truncation mutant viruses, of which the ODVs were analysed for the presence of the core and entry complex and for oral infectivity. This revealed that upon truncation of one of these PIFs, the two loosely associated components PIF0 and 8 failed to bind to the core and oral infectivity was abolished. The involvement of PIF0, 4, 6 and PIF8 in complex formation were also studied.

Results

Construction of PIF1 and PIF2 C-terminal truncation mutants of AcMNPV

To determine the involvement of the C-terminal parts of PIF1 and PIF2 in oral infectivity and complex formation, a series of recombinant AcMNPV viruses with C-terminally truncated versions of either PIF1 or PIF2 were generated. These viruses were constructed by repairing *pif1* and *pif2* deletion bacmids via Tn7-mediated transposition using modified pFastBacDual (pFBDΔ*p10-polh*) vectors that contain full or shorter versions of the *pif1* or *pif2* open reading frames (ORFs) under control of their endogenous promoters, as previously described in Peng *et al.*, 2010. The AcMNPV PIF1 protein that consists of 530 amino acids was step-wise truncated from the C-terminus until amino acid V459, F423 and A314, respectively (**Fig. 1**). For PIF2, that has 382 amino acids in the wild type virus, the C-terminal truncation mutants N358 and P351 were constructed (**Fig. 2**). The truncation sites were chosen based on the presence of conserved amino acid motifs in the C-terminus. The pFBD-vectors also contained the polyhedrin (*polh*) ORF downstream of its own promoter to enable the mutant viruses to form OBs (**See alignments and truncation sites in Supplemental data Fig. S1 and S2**).

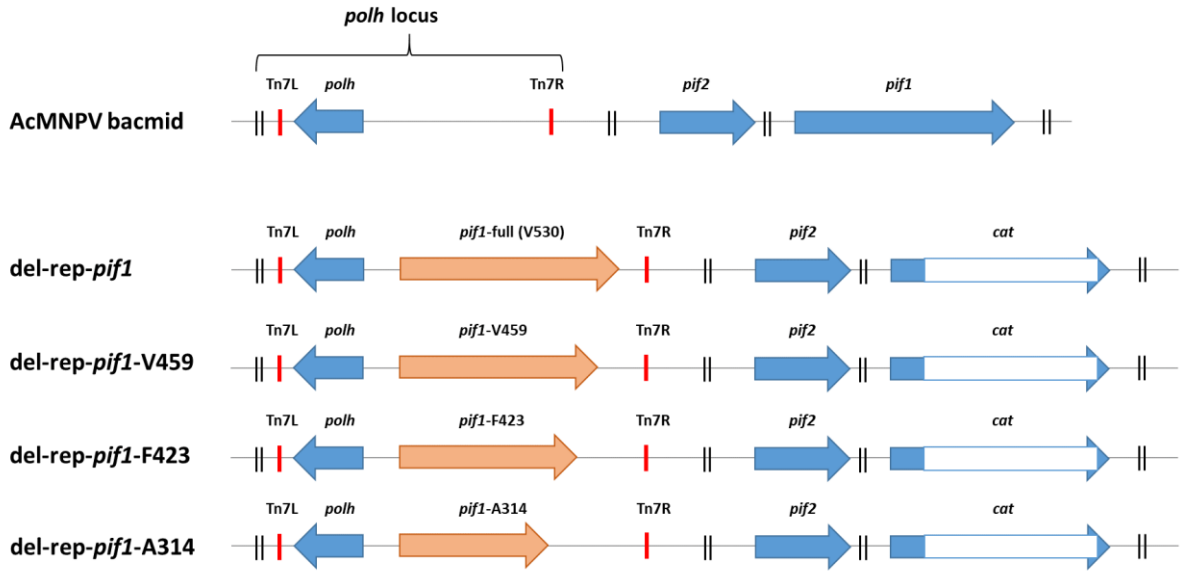

Figure 1: Overview of constructed bacmids of the *pif1* truncation mutants. The *pif1* deletion bacmids (described in Peng *et al.*, 2010) were repaired with shorter versions of the respective open reading frames (ORF) via Tn7 mediated transposition into the polyhedrin (*polh*) locus. The bacmids also contain the *polh* ORF under its own promoter to enable the resulting viruses to produce OBs. The directions of the ORFs in the genome are indicated by the arrows. The bacmids of the *pif1* truncation mutants and the del-rep-*pif1* repair mutant, containing the full-length ORF. In the del-rep-*pif1*-V459, the deletion bacmid was repaired with the *pif1* ORF that was truncated at the 3'end until the codon that corresponds with Valine 459. The bacmids del-rep-*pif1*-F423 and –A314 contain the shortened ORF that corresponds with Fenylalanine 423 and Alanine 314, respectively.

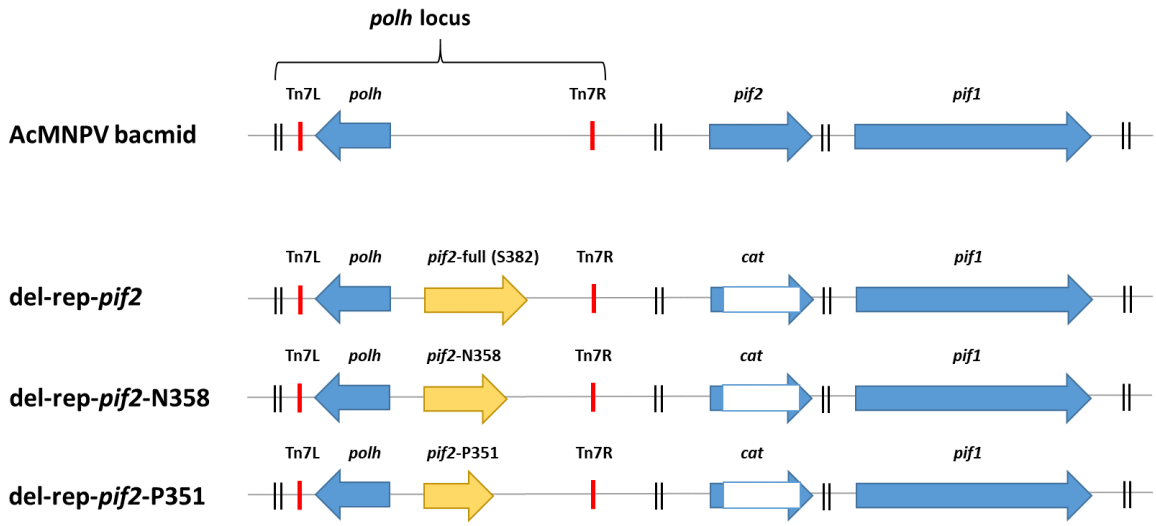

Figure 2: The bacmids of the *pif2* truncation mutants. Del-rep-*pif2* bacmid is a repair mutant, containing the full-length ORF, while the del-rep-*pif2*-N358 and -P351 bacmids contain 3'end truncated versions of the *pif2* ORF, corresponding with Asparagine 358 and Proline 351 respectively.

C-terminal truncations of PIF1 and PIF2 impair oral infectivity in Spodoptera exigua larvae.

Oral infectivity of the PIF1 and PIF2 truncation mutants was tested by oral inoculation of early L3 *S. exigua* larvae with a sucrose solution containing 3 x 10^8 OBs / ml. This showed that the full-length repair viruses were able to infect the larvae, resulting in their liquefaction, while the oral infectivity of all the truncation mutants was severely affected (**Table 1**). Truncation mutants *pif1*-V459 and *pif1*-A314 showed no mortality whereas with *pif1*-F423, a single larva out of 24 died in experiment 3 (**Table 1**). Inoculation with *pif2*-N358 showed low a level of mortality in all three bioassays and *pif2*-P351 resulted in a single larval death. To confirm that the liquefied larvae indeed had been infected by the truncation mutant under study, the larvae were analysed by PCR with various primer pairs, in which the reverse primer annealed either at or downstream of one of the truncation sites (**See Supplementary data Fig. S3 and S4**). This showed that the larvae inoculated with the *pif1*-F423 and *pif2*-P351 mutant viruses were

Table 1: Mortality of *S. exigua* larvae after oral inoculation with OBs

Virus (3.0 x 10^8 OBs / ml)	**Exp. 1**	**Exp. 2**	**Exp. 3**
mock	0/24	0/24	0/24
rep-*pif1*	20/22	23/24	21/21
-V549	0/24	0/24	0/24
-F423	0/23	0/24	1/24
-A314	0/24	0/24	0/24
rep-*pif2*	21/24	22/24	22/23
-N358	1/23	3/24	3/24
-P351	0/24	0/24	1/24

indeed infected by the corresponding mutant virus. However, the larvae that died from inoculation with *pif2*-P358 showed to be infected by the repair or wild type virus. The virus stock that was used to inoculate the larvae was also checked and did not contain wild type or repair virus. These findings confirm that the larvae were indeed incidentally infected by the corresponding truncation mutants, but the C-terminal parts of PIF1 and 2 are nevertheless important for ODV oral infectivity.

PIF1 and PIF2 C-terminal truncation mutants form the stable core complex.

To test whether the truncated PIF1 and PIF2 proteins are routed to the ODV envelope and still able to form the stable core complex, the envelope fractions of the truncation mutants were analysed by SDS-PAGE and western blotting with antisera against either PIF1 or PIF2. It was previously shown that the core could be detected as a 170 kDa band with these antisera when a sample of ODV envelope proteins was incubated in Laemmli buffer at 50°C, instead of 95°C (Peng *et al.,* 2010). When the ODV envelope fractions of the PIF1 truncation mutants were analysed with PIF1 antiserum under these conditions, the core was found for the truncation mutants *pif1*-A459 and *pif1*-F423, but not for the *pif1*-A314 mutant (**Fig. 3**). In the latter, the PIF1 antiserum only found the truncated PIF1 monomers between 35 and 40 kDa heights. The 40 kDa band was more prominent after partial denaturation at 50ºC and the 35 kDa band after complete denaturation at 95ºC. This indicates that this longer C-terminal truncation affected the protein conformation of the half-life of PIF1. The core complex was also found in

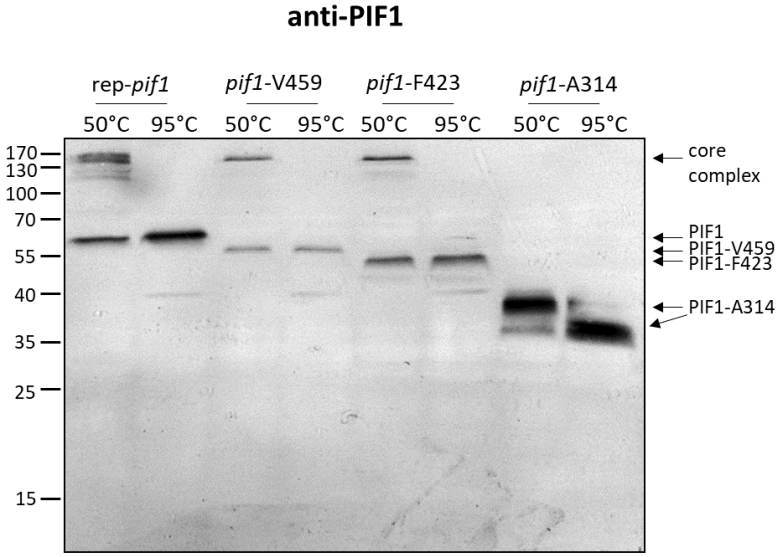

Figure 3: Western blot analysis of the ODV envelope fractions of the *pif1* repair and truncation mutants with PIF1 antiserum. The ODV envelope proteins were extracted by incubation in extraction buffer, containing 0,5% Triton-X100, incubated in Laemmli buffer at either 50 or 95°C for 10 min. and separated in SDS-PAGE gels. The core complex and PIF monomers are indicated by arrows.

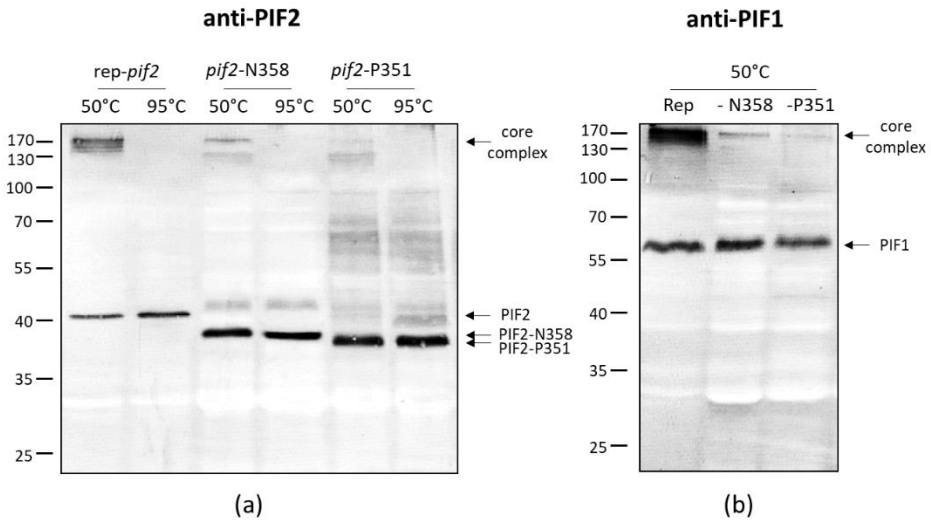

Figure 4: Western blot analysis of the ODV envelope fractions of the *pif2* truncation and repair mutants. For these analyses, antiserum against PIF2 was used (a). The presence of the core complex was validated with PIF1 antiserum (b).

both *pif2* truncation mutants under these conditions by using either PIF1- or PIF2 antiserum, although in lower quantities than in the repair mutant for unknown reasons (**Fig. 4**).

When the envelope proteins were incubated at 95°C, the core complex dissociated completely and only PIF1 or PIF2 monomers were found. In the full-length repair mutants, the monomers were found at the expected heights in the gel, PIF1 at 60 kDa and PIF2 at 40 kDa height. After truncation, monomers were found with lower mass as expected, corresponding with the extend of the truncation, i.e. the larger the truncation, the smaller the monomers. These analyses showed that the C-terminal truncated forms of PIF1 and PIF2 were properly targeted to the ODV envelope and that all mutants except *pif1*-A314 were able to form the core complex, although in smaller quantities after truncation of PIF2.

PIF1- and PIF2 truncation mutants lack the ODV entry complex.

The ODV envelope proteins were also analysed under non-denaturing conditions by blue-native PAGE to determine whether the PIF1 and PIF2 truncation mutants were still able to form the ODV entry complex. Previous studies showed that the entry complex was found as a double band running at roughly the same height as the 480 kDa marker, when using antiserum against one of its components (Peng *et al.*, 2012). When using antisera against one of the core components (PIF1 and PIF2), or the loosely associated component (PIF8), the ODV entry complex was detected in the *pif1*- and *pif2* full length repair viruses, but not in any of the truncation mutants (**Fig. 5**). Analysis with PIF8 antiserum was also used as loading control by the detection of PIF8 dimers between the 242 and 146 kDa markers, as reported before (Wang

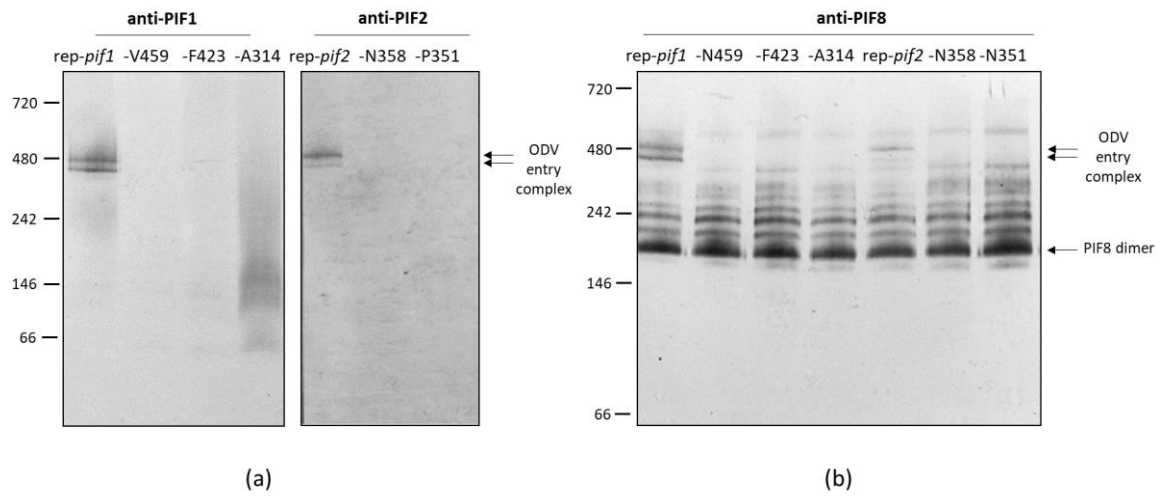

Figure 5: Blue native-PAGE and western blot analysis of the ODV envelope fraction of the pif1 and pif2 repair and truncation mutants. The ODV envelope proteins were separated under non-denaturing conditions in a BN-PAGE gel and analysed by western blotting with antiserum against PIF1 or 2 (in the core) (a) and antiserum against the loosely associated protein PIF8 (b). The entry complex and the PIF8 dimers (used as loading control) are indicated by arrows.

et al., 2019) (**Fig. 5b**). The core was not detected by this method in any of the truncation mutants. These results indicate that the C-termini of PIF1 and PIF2 are essential for formation of the entry complex.

C-terminal truncation of PIF1 or PIF2 affects the interaction with PIF0 and PIF8.

To determine why the formation of the ODV entry complex was abolished after C-terminal truncation of either PIF1 or PIF2, while the core was still present, a co-immunoprecipitation study was performed with the PIF1 antiserum on ODV envelope fractions of the *pif1*-F423 and *pif2*-P351 truncation mutants. The *pif1* repair virus was used as positive control and the *pif1* deletion virus as a negative control for the precipitation of PIF1. The immuno-precipitations were first analysed by western blot with antisera against PIF1, to verify that this protein indeed precipitated; then against PIF2 to confirm the interaction of PIF1 with PIF2 in the core complex; and finally with antisera against PIF0 and PIF8 to analyse the interaction with these two largest (loosely associated) components of the entry complex (**Fig. 6**). PIF1 was found to be precipitated from ODV envelope fractions of the *pif1* repair mutant and the *pif2*-P351 truncation mutant, but not from the *pif1* deletion mutant. After immunoprecipitation, PIF1 was found to run higher than in the input sample, probably due to the presence of large amounts of immunoglobulin that also eluted from the beads. Pre immune serum failed to precipitate PIF1, indicating that the PIF1 antiserum specifically precipitated its target protein (**Fig. 6a**). Unfortunately, precipitation of the truncated PIF1-F423 could not be confirmed directly as this

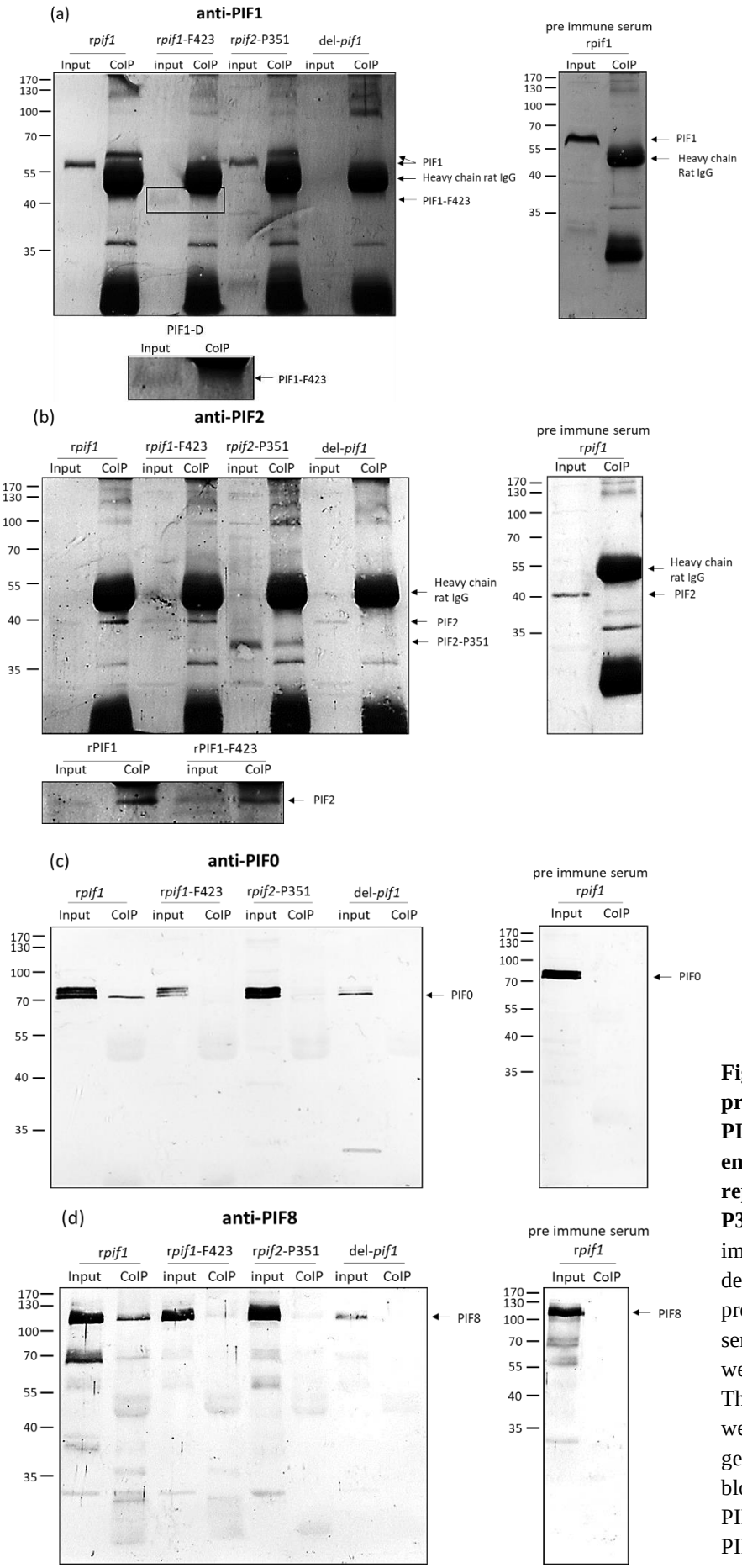

Figure 6: Coimmuno-precipitation analysis with PIF1 antiserum on the ODV envelope proteins of the *pif1* repair, *pif1*-F423 and *pif2*-P351 truncation mutants. An immunoprecipitation on a *pif1* deletion mutant and a precipitation with pre-immune serum on the *pif1* repair virus were used as negative controls. The input and eluted proteins were separated in SDS-PAGE gels and analysed on western blots with antiserum against PIF1 (a), PIF2 (b), PIF0 (c) and PIF8 (d).

protein had approximately the same migration rate as the rat immunoglobulin heavy chain (**Fig. 6a**), which was also recognized by the secondary antibodies during western blot analysis. PIF2 co-precipitated with PIF1 in the *pif1* repair mutant, also after its truncation (see the *pif2*-P351 truncation mutant), and with the *pif1*-F423 truncation mutant, but not in the *pif1* deletion mutant or after usage of pre immune serum. This indicates, although indirectly, that the shorter version of PIF1 (F423) was also precipitated by the PIF1 antiserum and confirmed that the interaction between PIF1 and PIF2 remains intact after C-terminal truncation of one of these PIFs (**Fig. 6b**). On the contrary, the loosely associated PIF0 and PIF8 only co-precipitated in the *pif1* repair virus, so with full length PIF1, but not with any of the truncated versions (**Fig. 6c, d**). PIF0 was detected as multiple bands between 70 and approximately 85 kDa in the input samples, suggesting that this protein is post-translationally modified and occurs in multiple forms in the ODV envelope. These forms are separated in these analyses, because of the relatively long running times that we used to separate the immunoglobulins from the target proteins. However, after immunoprecipitation of PIF1, only a 70 kDa band was found. Whether the other forms of PIF0 are below detection level or whether the complex contain only one form of this protein was not further analysed. The results indicate that the C-termini of PIF1 and PIF2 are essential for formation of the ODV entry complex by facilitating the interaction between these PIFs present in the stable core and at least two of the loosely associated PIFs, namely PIF0 and PIF8.

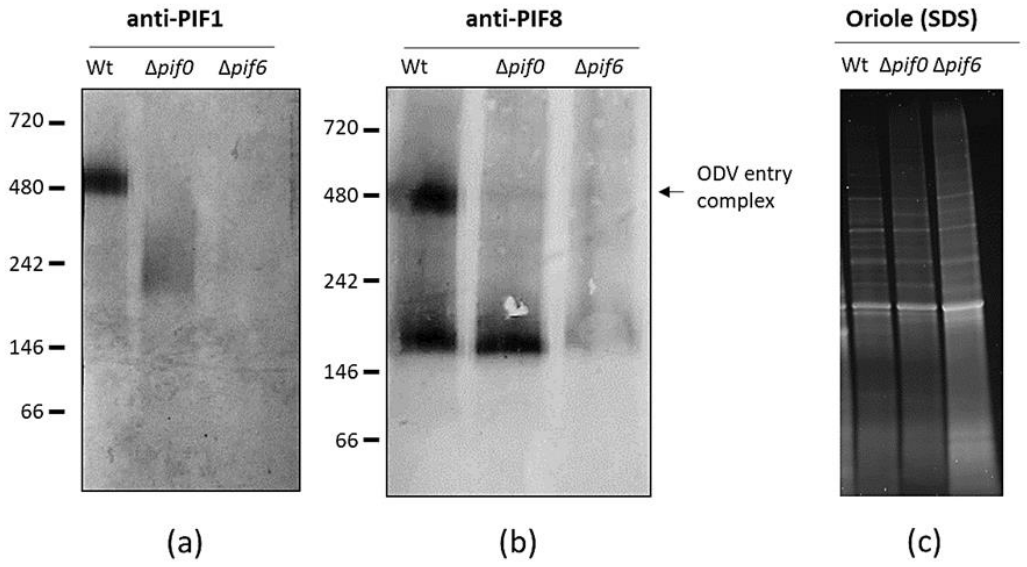

Figure 7: Blue native-PAGE analysis of the ODV envelope fractions of the *pif0* and *pif6* deletion mutants. The ODV envelope proteins were separated in a BN-PAGE gel and analysed by western blotting with antiserum against PIF1 (a) and PIF8 (b). The entry complex is indicated by the arrows. Part of the ODV envelope fractions were incubated at 95°C, separated in SDS-PAGE gels and stained with Oriole as loading control (c).

PIF0, PIF4 and PIF6 facilitate the interaction between PIF1 in the stable core and PIF8.

To determine whether the loosely associated PIFs only need an intact core complex for binding or whether this also requires the presence of other loosely associated PIFs, the ODV-envelope fractions of *pif0*- and *pif6* deletion mutants were analysed by blue-native PAGE and by co-immunoprecipitation with PIF1 antiserum. The entry complex was detected in wild type AcMNPV with antisera against PIF1 and PIF8, but not in absence of either PIF0 or 6 (**Fig. 7**). In the *pif0* deletion mutant, PIF1 containing complex(es) was found around 242 kDa height with very low resolution. This might be the core complex as described in Wang *et al.*, 2019. These data show that PIF0 and PIF6 are crucial for formation of the entry complex.

For the co-immunoprecipitation study with PIF1 antiserum, a *pif4* deletion mutant was also included as an earlier study had shown that a smaller complex is formed by PIF1 to 3 in absence of PIF4, but nevertheless failed to form the entry complex (Peng *et al.*, 2012). Pre-immune serum was used as negative control for the precipitation of PIF1 and the co-precipitation of PIF2, PIF0 and PIF8 (**Fig. 8**). Western blot analysis with PIF1 antiserum showed that the target protein indeed precipitated in wild type and *pif4*, *pif0* and *pif6* deletion mutants and was not found precipitated by the pre-immune serum (**Fig. 8a**). In the *pif4*- and *pif6* deletion mutants, PIF2 and PIF0 were found precipitated with PIF1, but not PIF8. (**Fig. 8b**-**d**). In the *pif0* deletion mutant, only PIF2 precipitated with PIF1 (**Fig. 8b**). These analyses indicate that the binding of PIF0 to the core is not dependent on the presence of PIF4 or the binding of PIF6, and possibly PIF8 (as this PIF failed to bind the complex in all these mutant viruses). However, the binding of PIF8 to the stable core requires the presence of PIF4 in the core and at least the binding of PIF0 and 6.

The zinc finger domain of PIF8 interacts with PIF0 and PIF1.

A working model has been proposed for the function of PIF8, in which the zinc finger (ZF-) domain recruits and promotes assembly of PIF proteins in the ODV envelope to form the entry complex (Javed *et al.*, 2017). To validate the presence of the PIFs in the ODV envelope of a PIF8ΔZF-HA mutant (lacking this domain), the ODV-envelope fractions of this mutant and a *pif8*-HA repair virus were analysed by western blotting with anti-HA antibodies and antisera against either PIF0 or PIF1. When using anti-HA antibodies, PIF8 and the ΔZF mutant were detected as >100 and 70 kDa bands respectively, implying their presence in the ODV-envelope (**Fig. 9a**). Some smaller bands were also detected (below the 40 and 35 kDa marker), which were previously detected by the PIF8 antiserum as well (**Fig. 4**). That these bands are now also

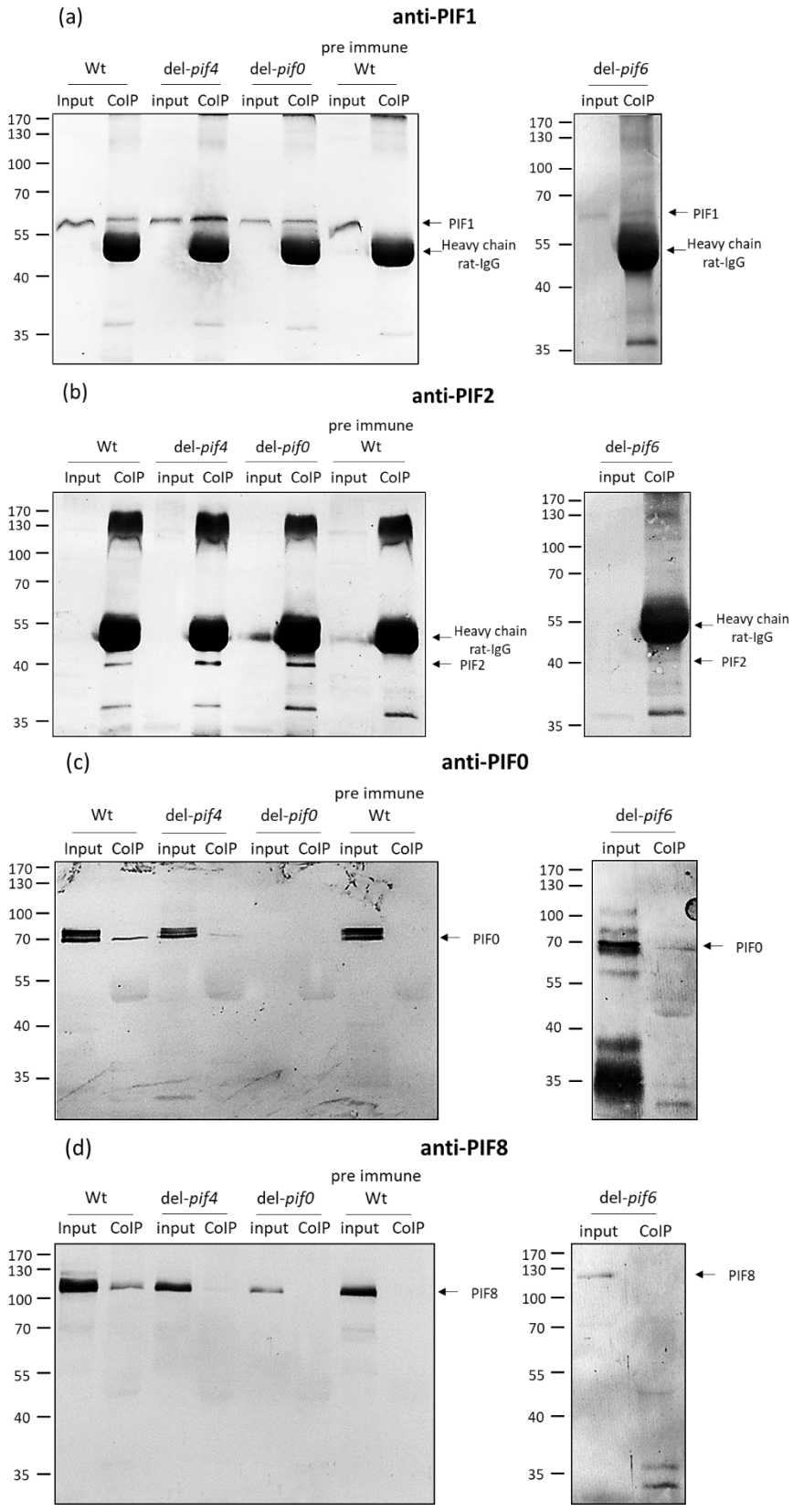

Figure 8: A co-immunoprecipitation analysis with PIF1 antiserum on the ODV envelope proteins of *pif0*, *pif4* and *pif6* deletion mutants. Precipitation with pre immune serum was used as negative control. The input and eluted proteins were separated in SDS-PAGE gels and analysed by western blot with antiserum against PIF1 (a), PIF2 (b), PIF0 (c) and PIF8 (d). PIF2 was only detected in the concentrated protein sample after elution from the anti-HA beads and not in the diluted input sample (b).

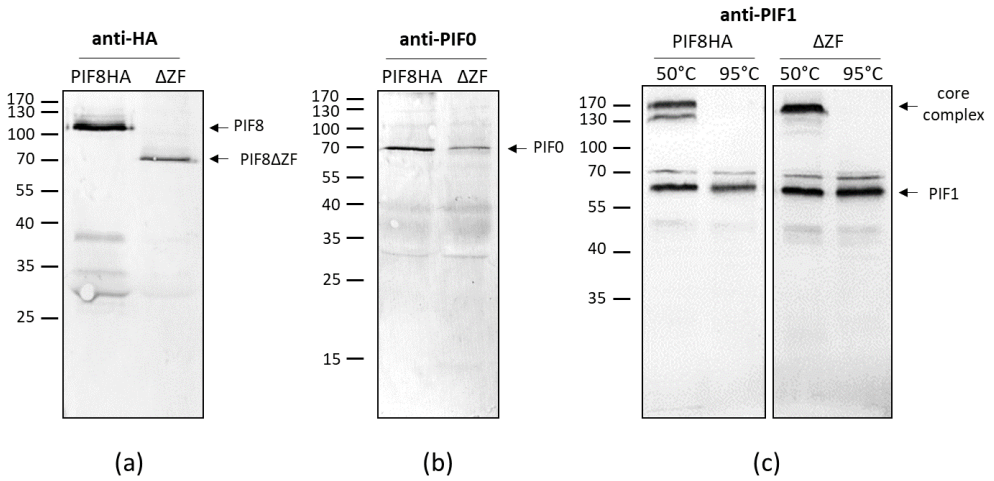

Figure 9: Western blot analysis of the ODV envelope fractions of an HA-tagged PIF8 repair mutant and an HA-tagged PIF8 mutant that lacks the zinc-finger (ZF-) domain with antibodies against the HA-tag (a), PIF0 antiserum (b) and PIF1 antiserum (c). Part of the ODV envelope fractions were incubated at 50°C for detection of the 170 kDa core complex.

found with anti-HA antibodies suggests that these bands are specific for PIF8. These lower bands were also found by others (personal communication Theilmann). With antisera against either PIF0 or PIF1, 70 and 60 kDa bands were detected respectively, confirming the presence of these PIFs in the ODV-envelope of both the repair and ZF-mutant virus (**Fig. 9b, c**). Furthermore, when the protein sample was incubated at 50°C (instead of 95°C) and analysed with PIF1 antiserum, the core complex was found, indicating that in addition to PIF1, also PIF2, 3 and 4 were present in the ODV envelope in these mutants.

As the ZF-domain of PIF8 was shown to be essential for formation of the entry complex (Javed *et al.*, 2017), the importance of this domain for the interaction with other PIFs was studied by a co-immunoprecipitation study with anti-HA magnetic beads. The eluted proteins were analysed by western blotting with anti-HA antibodies and confirmed the precipitation of the HA-tagged PIF8 itself (**Fig. 10a**). The blots were also analysed with antisera against PIF0 or PIF1, to validate the interaction of PIF8 (either direct or indirect) with another loosely associated PIF (PIF0) and the stable core respectively. This showed that PIF0 and 1 co-precipitated with PIF8, but not after removal of the ZF-domain. This indicates that the ZF-domain of PIF8 is important for the interaction with other PIFs of the entry complex (**Fig. 10b and c**). These results also demonstrate that the ZF-domain of PIF8 is not involved in recruitment of the PIFs to the ODV envelope and confirm the importance of this domain for complex assembly.

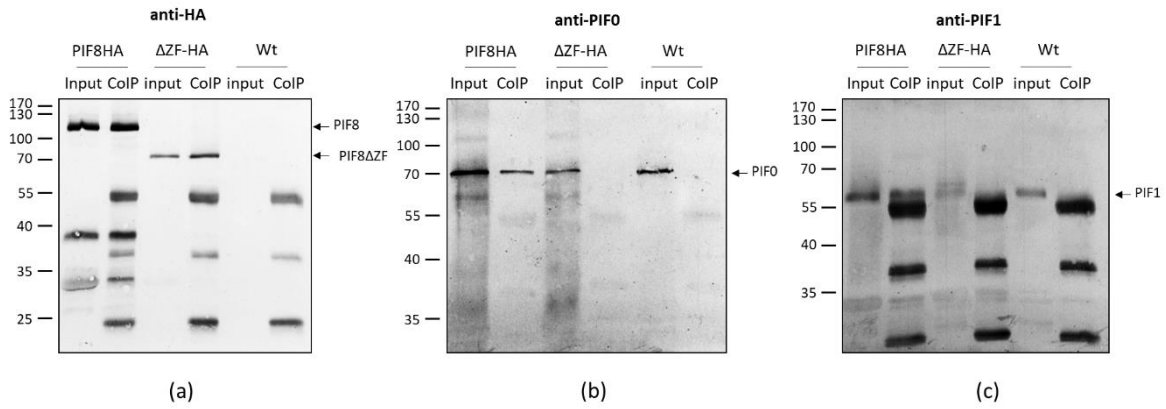

Figure 10: A co-immunoprecipitation study with anti-HA magnetic beads on the ODV envelope proteins of HA-tagged PIF8 and PIF8ΔZF mutant. The input and eluted proteins were analysed by SDS-PAGE and western blot with anti-HA antibodies (a), PIF0 antiserum (b) and antiserum against PIF1 (c). The ODV envelope of wild type AcMNPV (no HA-tag) was used as a negative control.

Discussion

Infection of larval epithelial midgut cells by baculovirus ODVs is initiated by at least ten different PIF-proteins, of which nine (all except PIF5) form an ODV entry complex, located in the ODV envelope. This complex consists of a stable core formed by PIF1 to 4 and five loosely associated PIFs, i.e. PIF0 (P74) and PIF6 to 9 (Peng *et al.*, 2010, 2012, Wang *et al.*, 2019, **Chapter 2, 3, 4**). That all these PIFs form a complex suggests that midgut infection requires a close collaboration between these proteins in a network of protein-protein interactions. This retrospectively complicates the interpretation of the results of earlier studies towards the functional role of individual PIFs using *pif* deletion mutants, as the function of non-targeted PIFs might be affected indirectly in these mutants. Previous studies with *pif1* and *pif2* deletion mutants showed that these particular PIFs are important for the binding of ODVs to the midgut epithelium (Ohkawa *et al.*, 2005). Later, these PIFs were also shown to be essential for the formation of the stable core of the entry complex (Peng *et al.*, 2010). That PIF1 and 2 could be involved in interaction with the host is in accordance with *in silico* data that indicated that the C-termini of these PIFs point outwards from the ODV envelope (**Chapter 2**). However, whether these PIFs are directly involved in ODV binding or whether the binding was affected due to an inability to form the complex was not known.

To elaborate more on the involvement of PIF1 and 2 in midgut infection, we constructed a series of recombinant AcMNPVs, in which these PIFs were C-terminally truncated, and were analysed for oral infectivity and the capacity to form the core and entry complex. This showed that even limited truncation of PIF1 or 2 severely affected the oral infectivity of ODVs and that formation of the entry complex was abolished in all mutants, despite the fact that these truncated

versions were still able to form the core-complex, except *pif1*-A314 (**Table 1, Fig. 4-5**). Immunoprecipitation studies showed that the truncations affected the binding of the loosely associated PIF0 and 8 (and possibly also the binding of other PIFs) to the core complex (**Fig. 6**). Since the *pif1*-A314 mutant was also defective in formation of the core complex, A314 is located in a domain that is important for the interaction with PIF2, 3 and 4. How far this domain extends N-terminally is not known. The observations suggest that the truncated parts of the C-terminal ends of PIF1 and 2 are important for formation of the entry complex by facilitating the interaction between the stable core and the loosely associated PIF0 and 8. PIF4 appeared not to be crucial for the binding of PIF0 to the core, as in a *pif4* deletion mutant, in which a smaller core complex is formed by PIF1 to 3 (Peng *et al.*, 2012), PIF0 was still found to be associated (**Fig. 8**). That the C-termini of PIF1 and 2 are crucial for the binding of PIF0 and 8 to the core suggests that these PIFs are indirectly involved in the interaction with the host. As PIF0 has also been shown to be important for ODV binding (Haas-Stapleton *et al.*, 2004), it could be reasoned that the interaction between PIF0, 1 and 2 is important for the binding properties of the ODVs. This would imply that the ODV binding to the epithelium is affected after truncation of PIF1 or 2, while the ODVs of the *pif4* deletion mutant would still be able to bind. Alternatively, if PIF1 and 2 are directly involved in ODV binding, the midgut interacting sections might only be exposed in the right conformation when the entry complex is formed. It even seems likely that amino acids from a couple of complex components should be in a proper mutual conformation to make binding happen.

To determine whether the loosely associated PIFs bind to the core independently from each other or whether this requires the presence of the other PIFs as well, we also analysed the binding of PIF0 and 8 to the core in a *pif6* deletion mutant by immunoprecipitation of PIF1. This showed that PIF6 is redundant for the binding of PIF0, just as observed with PIF4 (**Fig. 8c**), while PIF8 was not able bind to the core in absence of PIF6. PIF0 possibly associates only when PIF1-4 are bound together in the stable core, as previous protein interaction studies with yeast two-hybrid (Y2H) and bimolecular fluorescence complementation assays indicated that PIF0 does not directly bind to one of the core components in monomeric form (Peng *et al.*, 2010b, Zheng *et al.*, 2017). The association of PIF8 on the other hand did not only require the presence of a core complex, but also the presence of PIF0 and 6 (**Fig. 8d**). Our results show that PIF0 and 6 are essential for formation of the entry complex and that the ZF-domain of PIF8 is essential for association with the complex, just as shown in a Y2H study (Zheng *et al.*, 2017).

Although the formation of the entry complex was abolished after C-terminal truncation of PIF1 or 2, low mortality levels were observed after oral inoculation of *S. exigua* larvae with OBs of some of the mutant viruses. This has also been observed in studies with other *pif* mutant viruses, which are now known to be defective in formation of the entry complex (Pijlman *et al.,* 2003, Ohkawa *et al.,* 2005, Zhu *et al.,* 2013, Dong *et al.,* 2014, Wang *et al.,* 2019). The oral infectivity of the entry complex-defective PIF8 ΔZF-mutant of AcMNPV was even shown to be partially rescued when the larvae were first treated with calcofluor white, an agent that damages the peritrophic membrane (Zhu *et al.,* 2013). This has not been observed when ODVs lack either PIF0, 1, 2, 3 or 7 (Song *et al.,* 2008, Liu *et al.,* 2016). Whether the treatment with calcofluor white also rescues the oral infectivity of *pif4*-, *pif5*-, *pif6* and *pif9* deletion mutant viruses remains to be established. Apparently, the ODVs of complex-defective mutants can still be infectious at a low level, indicating that the remaining PIFs, although in suboptimal configuration, remain their functionality to some extent. Another explanation could be that the entry complex is not fully responsible for midgut cell entry and that other ODV envelope proteins can partially substitute the function of this complex when it is dissociated. PIF5 for example, which is not a constituent of the ODV entry complex (Peng *et al.,* 2012), is also known to be important for the infectivity of ODVs via an unknown mechanism (Harrison *et al.,* 2010, Sparks *et al.,* 2011a). Furthermore, at least four other ODV envelope proteins, AC46 (ODV-E66) (Sparks *et al.,* 2011b, Xiang *et al.,* 2011), AC111 (Li *et al.,* 2018), AC145 and AC150 (Lapointe *et al.,* 2004, Zhang *et al.,* 2005), have also been shown to be involved in ODV oral infectivity. The viral glycoprotein GP37, that was previously known to be involved in passage of the peritrophic membrane, was recently also reported to have fusogenic properties (Liu *et al.,* 2019). These observations suggests that midgut infection is more complex than initially thought and that the ODVs apparently require more factors than only the complexed PIFs to be fully infectious.

Material and methods

Virus, insect cells and Lepidopteran larvae. The *pif1*- and *pif2* deletion bacmids have previously been described by Peng *et al.,* 2010 and have the AcMNPV bacmid bMON14272 as backbone. The HA-tagged PIF8 and the truncation mutant bacmid have been described in Javed *et al.,* 2017. Sf21 cells were cultured in T25 flasks with Graces medium (Fisher N.V.), supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml gentamycin. Sf21 suspension cultures were grown in Sf900II medium (Fisher N.V.) supplemented with 5% FBS and

50 µg/ml gentamycin. *Spodoptera exigua* larvae were reared on artificial diet in a 26°C climate room with 40% humidity and a 16:8 h (light:dark) photoperiod.

Construction of recombinant bacmids. The recombinant bacmids were constructed via the Bac-to-Bac system (Invitrogen) with *pif1*- and *pif2* deletion bacmids that were partially or completely repaired with the respective genes. In the truncation mutants, the *pif1*- or *pif2* deletion bacmids were repaired with truncated versions that lacked increasing portions of the 3'end of the coding sequence. To this end, the required parts of the ORFs proceeded by the putative promoters (150bp upstream of the ATG start codon) were amplified by PCR with Phusion polymerase (Invitrogen). The used primers are listed in **Table 2** with underlined the NcoI and SphI restriction sites introduced for cloning purposes. The amplicons were cloned into a pJET vector (ThermoFisher) for sequence confirmation. The inserts were then re-cloned via the NcoI and SphI restriction sites into a modified pFastBacDual vector (Invitrogen). In this vector, the *p10* promoter was deleted and the ORF of the polyhedrin (*polh*) gene was inserted downstream of the *polh* promoter as described by Peng *et al.,* 2010 (pFBDΔp10-polh). The resulting plasmids were used to construct the recombinant bacmids via Tn7-mediated transposition, using the Bac-to-Bac system (Invitrogen).

Virus proliferation and purification. Sf21-cells were transfected with the constructed bacmids by using Expres2TR (Expres2ion Biotechnologies) as transfection reagent to generate BV-stocks of the recombinant viruses that were amplified by an additional infection round in Sf21 cells. OBs were produced by infection of 80 ml suspension cultures with a density of 1.5 x 10^6 Sf21 cells/ml with a m.o.i. of 5 $TCID_{50}$ units/cell. After a week of incubation at 27°C, the cells were pelleted by centrifugation at 4000 x g for 30 min and lysed by incubation in 0.1% SDS for 2h at 37°C with gentle agitation. For complete lysis, the cells were sonicated for 1 min at

Table 2: Primers used to amplify 3'truncated versions of *pif1* and *pif2*.

Primer name	Sequence (underlined: NcoI and SphI restriction sites)
Ac-*pif1*-Fw	CATCCATGGAATTGCAAACAAAACCACGTAC
Ac-*pif1*-FULL-Rv	CTAGCATGCTTATACAGAGTAGTTGGGGTAAGTTTC
Ac-*pif1*-V459-Rv	CTAGCATGCTTATACACACCACGAGTTGGTG
Ac-*pif1*-F423-Rv	CTAGCATGCTTAGAAGCATACCGGCTGAGC
Ac-*pif1*-A314-Rv	CTAGCATGCTTAAGCGTTCGCGACGG
Ac-*pif2*-Fw	GAGCCATGGCGCGTATCTGCTGTCTATCAC
Ac-*pif2*-Full-Rv	GAGGCATGCTTAAGATAACTGCCCTCTGACG
Ac-pif2-N358-Rv	GAGGCATGCTTAGTTGATGCCGTTGCGC
Ac-pif2-P351-Rv	GAGGCATGCTTAAGGGTAGGAGCCGGG

7 Watt as verified by light microscopy. The OBs were pelleted by centrifugation at 4000 x g for 30 min and washed three times with Milli Q water. The OBs were further purified by centrifugation 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 band with OBs was withdrawn from the gradient with a Pasteur pipette and subsequently pelleted by centrifugation at 4000 x g for 30 min.

SDS-PAGE and Blue native-PAGE analysis. ODVs of approximately 3.0 x 10^8 OBs were isolated and fractionated as previously described (**Chapter 4**). In brief, the ODVs were released from the OBs by treatment with alkaline DAS buffer (0.1 M Na_2CO_3, 166 mM NaCl and 10 mM EDTA, pH 10.5) to dissolve the polyhedrin matrix and pelleted by centrifugation at 20,800 x g for 25 min at 4°C. The ODVs were then fractionated by resuspension in 50-100µl extraction buffer (6.25 mM Tris, 37.5 mM NaCl, 0.5% Triton-X100, pH 7.2) and incubated overnight at 4°C with gentle rotation. Next day, the nucleocapsids were pelleted by centrifugation at 20,800 x g for 20 min at 4°C and the envelope proteins in the supernatant was split and mixed with either 4x Laemmli buffer for SDS-PAGE analysis or 4x BN-PAGE sample buffer (200 mM Bis-Tris, 64 mM HCl, 200 mM NaCl, 40% glycerol, 0,004% Ponceau S, pH 7.2), supplemented with 5% Coomassie G250 in 1 : 50 ratio, for BN-PAGE analysis. Prior to SDS-PAGE analysis, the protein samples were heated at 50 or 95°C for 5 minutes and separated in a 12% SDS-PAGE gel. For BN-PAGE, the proteins were separated in 4-16% Bis-Tris gradient gels as described in the manual for the NativePAGE™ Novex® Bis-Tris Gel system (Invitrogen). NativeMark™ unstained protein ladder (Invitrogen LC0725) was stained with Coomassie and used as marker. Blotting to a PVDF membrane was performed with NuPAGE® Transfer buffer (Invitrogen) according to the manufacturer's protocol.

Co-immunoprecipitation. For CoIP analysis, ODVs were released from 5 x 10^8 OBs and purified and fractionated as described above. However, instead of using the extraction buffer, the 500 µl IP buffer (25 mM Tris, 150 mM NaCl, pH 7.2) containing 0.5% Triton X-100, was used to fractionate the ODVs by a 2 hr incubation at 4°C with gentle rotation. In parallel, 30 µl of PIF1 antiserum was mixed with 40 µl bed-volume of Protein G agarose beads (Pierce) in 500 µl IP buffer and incubated at 4°C for 2 hrs. After incubation, the Protein G agarose-antibody complex was collected by centrifugation at 1000 x g for 2 min and washed with 1ml IP buffer. The ODV envelope protein suspension was centrifuged at 20,800 x g for 25 min to pellet the nucleocapsids and 450 µl supernatant was mixed with the Protein G agarose-antibody complex and incubated overnight at 4°C with gentle rotation. For the HA-tagged PIF8 viruses,

the ODV envelope was isolated as described above and incubated overnight with anti-HA magnetic beads in IP-buffer (Invitrogen) at 4°C. The remaining 50 µl of the supernatant was kept as input control. Next day, the agarose or magnetic beads were collected and washed for 3 times with 1 ml IP buffer. The captured proteins were eluted in 100µl Laemmli buffer by heating at 95°C for 10 min. The samples were separated in 12% SDS-PAGE gels and analysed by western analysis.

Western blot analysis. Western blot analysis with antisera against various PIF-proteins were used as previously described in Peng *et al.*, 2010. In brief, anti-PIF0- (1:1000 dilution) and anti-PIF8 antibodies (1:2000 dilution), both produced in rabbit, and anti-HA (1:2000 dilution, Roche 3F10), anti-PIF1 (1:2000 dilution) and anti-PIF2 (1:1000 dilution) produced in rats, were used as primary antibodies. Goat anti-rabbit (1:2000 dilution; Dako) and goat anti-rat (1:2000 dilution; Sigma A8438), conjugated to alkaline phosphatase, were used as secondary antibodies for detection of the proteins of interest by conversion of NBT-BCIP substrate in a blue-purple coloured precipitant in AP-buffer (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM MgCl, pH 10.5)

Bio-assays. S. exigua larvae were orally inoculated with isolated OBs of the recombinant viruses to determine their oral infectivity. To this end, the OBs were suspended in 10% sucrose at a concentration of 3.0 x 10^8 OBs / ml and fed to starved early L3 *S. exigua* larvae in a droplet feeding assay. The 10% sucrose solution was coloured blue with Patent Blue V Sodium salt (Fluka) to be able to determine whether the larvae were properly inoculated by looking at their colour. The larvae were allowed to feed on the OB-containing sucrose suspension for 10 min and were then transferred to a well in a 12-wells plate with a block of artificial diet, and incubated at 27°C. As the larvae were expected to die at the 4^{th} dpi, the larvae were checked 3 dpi to exclude the larvae that died for other reasons than viral infection. The liquefied larvae were analysed by PCR to validate whether the larva died due to infection with the mutant virus. Therefore, the cadavers were suspended in sterile Milli Q, treated with alkaline (DAS) buffer to dissolve the OBs, and the released ODVs were incubated at 95°C for 10 min to release the viral DNA. The viral DNA was analysed by PCR with GoTaq polymerase (Promega), according to the manufacturers protocol with the primers enlisted in **Table 2**.

Acknowledgements

We like to thank Fabiola D. Ortega Murillo and Alexander Sminia, who participated in this project as MSc. students, for the construction of the bacmids to generate the truncation mutants and initial protein and functional analyses. Els Roode and Hanke Bloksma are acknowledged

for technical assistance in the lab and for insect rearing and cell culturing. This work was supported by grant 824.14.16 of the Netherlands Organization for Scientific Research (NWO).

Supplementary data

Multiple alignment of PIF1

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CLUSTAL O(1.2.4) multiple sequence alignment


AcMNPV          MHFAIILLFLLVIIAIVYTYVDLIDVHHEEVRYPITVFDNTRAPLIEPPSEIVIEGNAHE      60
BmNPV           MHFAIILLFLLVIIAIVYTYVNLIDVHHEEVRYPIAVFDNTGVPLIEPPSEIIIEGNTHE      60
HearMNPV        MYLIVAAVLLIFIIVVLYNYVSLLTFAQDETIFPLERFNNVGVPLITPPTEIVIEGNEHE      60
SeMNPV          MHNIVLIVLLVILIAVIYNNVTLLQYVQQDFIPIVQTFDNSNVPLIEPPTEIIIEGNQYE      60
SfMNPV          MYNILLIVLLIFIILIVYNNITLLQYVQQDYIPVLTRFDNTHVPLIEPPTEIVIEGNTHE      60
                *:  :  ::*:.:* ::*. : *:   :::    :  *:*  .*** **:**:**** :*

AcMNPV          CHKTLTPCFTHGDCDLCREGLANCQLFDEDTIVKMRGD-DGQEHETLIRAGEAYCLALDR      119
BmNPV           CHKTLTPCSTHSDCNLCREGLANCQLFDEDTIVKMRGD-DGQEQEKLIRAGEAYCLALDR      119
HearMNPV        CHKQLTPCQTHLDCDICREGLANCQYFDEKAIITITDAETNTEQTFTIEAGESYCLALDR      120
SeMNPV          CHKQLTPCSTHQDCDICREGLANCQYFDDKTLITITHD-DGEKQEFTIEPGESYCMALDR      119
SfMNPV          CHKTLTPCSTHMDCDVCREGLANCQYFENKTIITITDE-DNVERQFTIEPGESYCMALDR      119
                *** **** ** **::********* *::.:::.:     . ::   *. **:**:****

AcMNPV          ERARSCNPNTGVWLLAETETGFALLCNCLRPGLVTQLNMYEDCNVPVGCAPHGRIDNINS      179
BmNPV           ERARSCNPNTGVWLLAETETGFALLCSCLRPGLVTQLNMYEDCNVPVGCAPHGRIDDINS      179
HearMNPV        ERARSCNPHTGVWILAESPVGYSLLCSCLTPGLVTQLSLYNDCDVPVGCQPHGNIVSIYE      180
SeMNPV          ERARSCNPNTGVWILAESPVGYSLLCSCLTPGLVTQLSLYHDCDIPVGCQPHGQIVSINE      179
SfMNPV          ERARSCNPNTGVWILAQSPVGFSLLCSCLTPGLVTQLSLYHDCDIPIGCQPHGNIISINE      179
                ********:****:**:: .*::***.** *******.:*.**::*:** ***.* .* .

AcMNPV          ASIRCVCDDGYVSDYNADTETPYCRPRTVRDVMYDESFFPRAPCADGQVRLDHPALNDFY      239
BmNPV           ASIRCVCDDGYVSDYNADTETPYCRPRTVRDVMHDESFFPRAPCADGQVRLDHPGLNDFY      239
HearMNPV        SPMRCACDTGFVPDFNVETQTPFCRSRRVRDMIENTDAFPVAPCERGYIRLDHTGLKPFY      240
SeMNPV          RPMRCSCEVGYVADFNADTQTPFCRTRRIRDVIQNSEFFPHAPCPLGYIPIEHPGLDPEY      239
SfMNPV          RPMRCSCEVGYVADFNTETQTPYCRTRRIRDVIQNPDFFPLAPCSWPYIPIEHPGLDPAY      239
                  :** *: *:* *:*.:*:**:** * :**:: : . ** ***    : ::* .*.  *

AcMNPV          RRHFRLEDICVIDPCSVDPISGQRTSGRLFHQPTVN-GV-GINGCNCPADDGLLPVFNRH      297
BmNPV           RRHFRLEDICVIDPCSVDPISGQRTRGRLFHHPIDK-GVNGINGCNCPVSDLLLPVFNRH      298
HearMNPV        NTNIGIPDLCVIDPCSVDPISGERHSGYLQTYRLNFE---DYHFCVCPIADGLFGVYNDQ      297
SeMNPV          LRMTNARNVCVIDPCSVDPVSGQRHEGYLVSYTYEDR---ERHFCNCPVDDGLFGVYSDR      296
SfMNPV          LQSTNARNACVIDPCTVDPITGQQVVGWLVTRYLNDEDKDTQFFCNCSAGHNLFGVYNDQ      299
                      : ******:***::*::  * *               * *   . *: *:. :
                                                           ↓
AcMNPV          TADTGMVRQSDRTVANACLQPFNVHMLSLRHVDYKFFWGRSDHTEFADADMVFQANVNQL      357
BmNPV           TAETGMIRQSDRTVANACLRPFSVHMLRLRRVDYKYFWGRRDHTEFSDADVVFQANANQL      358
HearMNPV        ---LNMIRPSPRQVTNACLKPFNVHISALRRIDYKFFWAHTDRTR-SDEDVIAAVQESQL      353
SeMNPV          ---PTMVRPSSERLVNACLKPFNVHISAIPVIEYKWFWGHIDLFV-SDDDIVAFVDRNRV      352
SfMNPV          ---PNMIRPSAEKLVNACIQPFNVHVAQLPAIEYKWFWGQRNLYT-SDDDVVATVRPDQI      355
                     *:* * . :.***::**.**:  :  ::**:**.: :    :* *::  .  .::

AcMNPV          SHERYRAILYPLLESHPDVTEIVTVNMGVMKISVSYDTTLKNILLPSSVFRLFR--FKES      415
BmNPV           SHERYQAILYPLLGS----TEIVPAGTGVMKISVSYDTTLKDMRLPFSIFRIFR--LKES      412
HearMNPV        SSPRYRRMLFNYFTPHPAANIPS--DHRIFKLSTSYSMLYTLNSNGAIGQNLYTRYRAIS      411
SeMNPV          SSSRYHRMMFPILKPHPHIPWDISGILLIMKFSSAFTPVFDV--PPT-MQNTYTRYYLQ-      408
SfMNPV          SSPRYRRMLFTYLTPHPFFPESV--NFMVMKFSTAYTPIFNE--ADRNYHNLFTHYYELN      411
                *  **: :::  :               ::*:* ::            . :
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The multiple alignments continues on the next page.

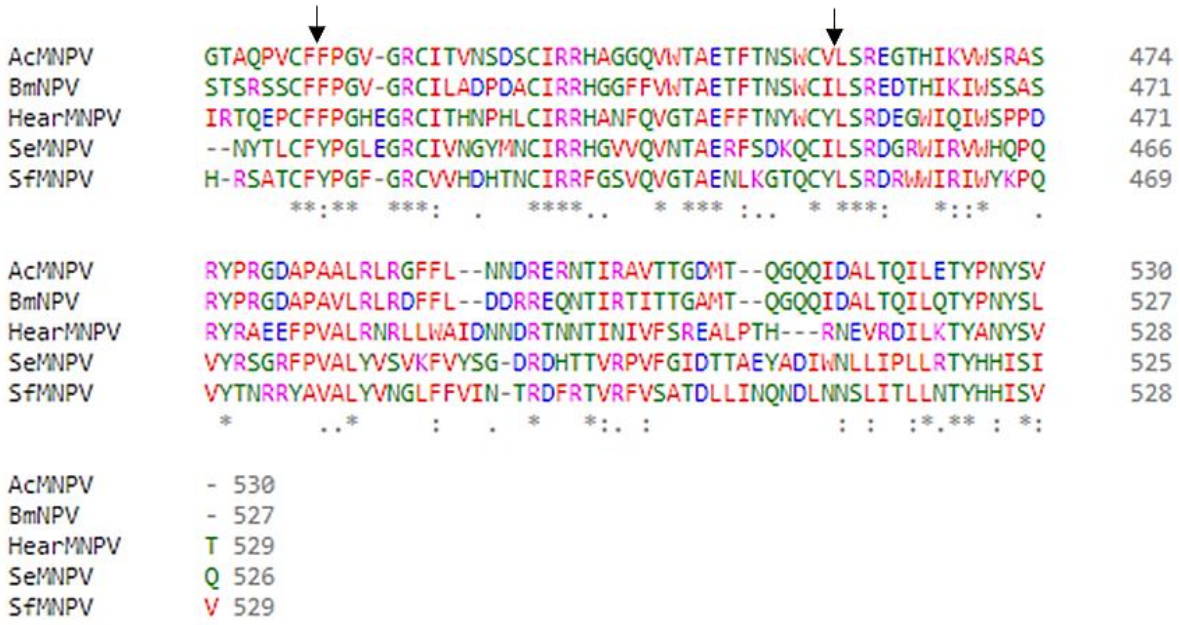

Fig. S1 Multiple alignment of PIF from various alphabaculoviruses. PIF1 of AcMNPV E2 strain (Genbank AIU56976.1) was aligned with its homologues in BmNPV (NP_047516.1), HearMNPV (ACH88565.1), SeMNPV (NP_037796.1) and SfMNPV (YP_001036327.1). The asterisks (*) indicate the completely conserved amino acid residues, the colon (:) indicates the conservation of amino acid residues with strong similarity in properties (scoring > 0.5 in Gonnet PAM 250 matrix) and the dots (.) indicate the conservation of residues of amino acids with weak similarity in properties (scoring < 0.5 in Gonnet PAM 250 matrix). These alignments show that the C-terminal part of PIF1 is less conserved than the N-terminal part.

Multiple alignment PIF2

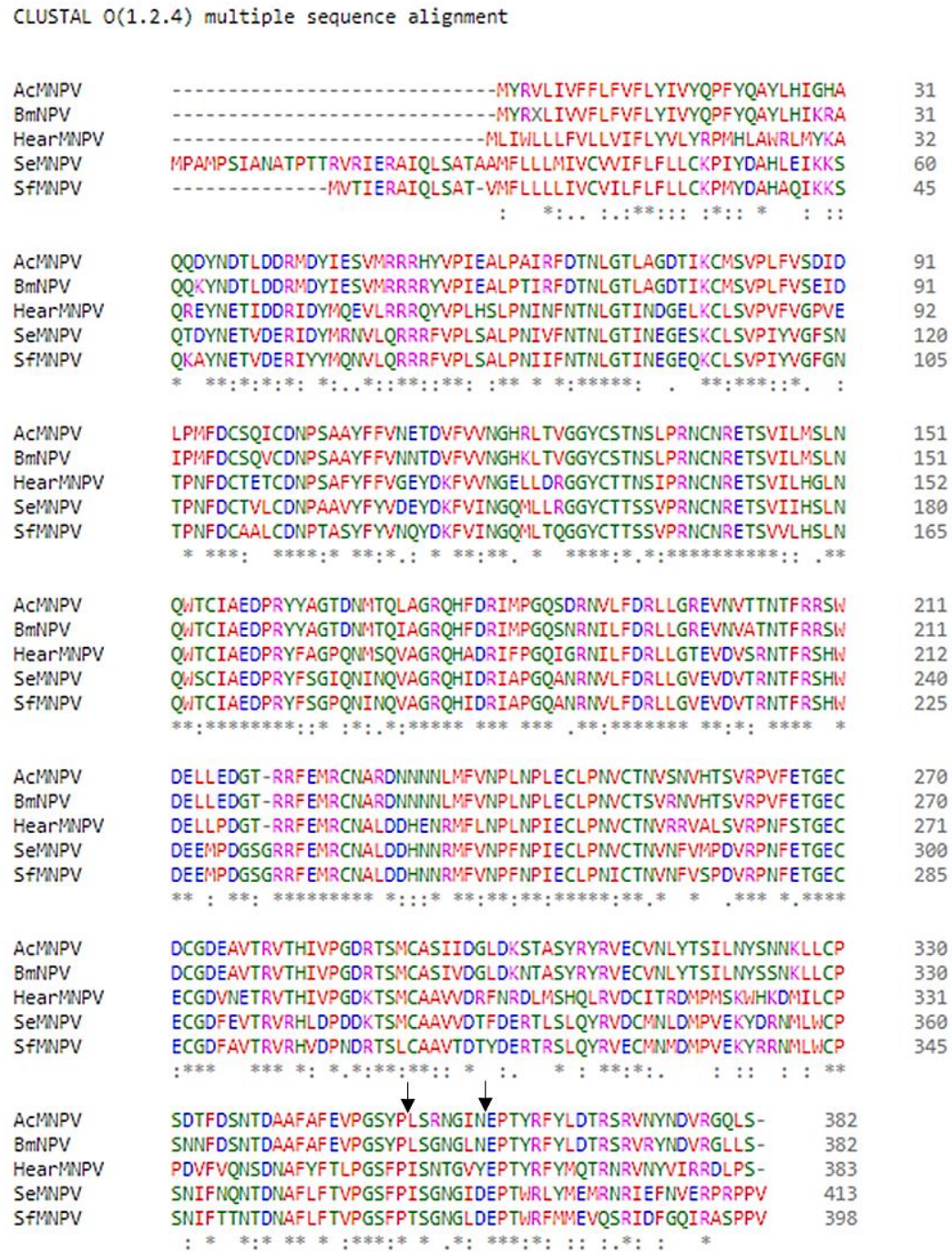

Fig. S2 Multiple alignment of PIF from various alphabaculoviruses. PIF2 of AcMNPV E2 strain (Genbank AIU57013.1) was aligned with its homologues in BmNPV (AIS92745.1), HearMNPV (QBM79106.1), SeMNPV (CDG72376.1) and SfMNPV (YP_001036326.1). The asterisks (*) indicate the completely conserved amino acid residues, the colon (:) indicates the conservation of amino acid residues with strong similarity in properties (scoring > 0.5 in Gonnet PAM 250 matrix) and the dots (.) indicate the conservation of residues of amino acids with weak similarity in properties (scoring < 0.5 in Gonnet PAM 250 matrix). These alignments show that the C-terminal part of PIF2 is less conserved than the N-terminal part.

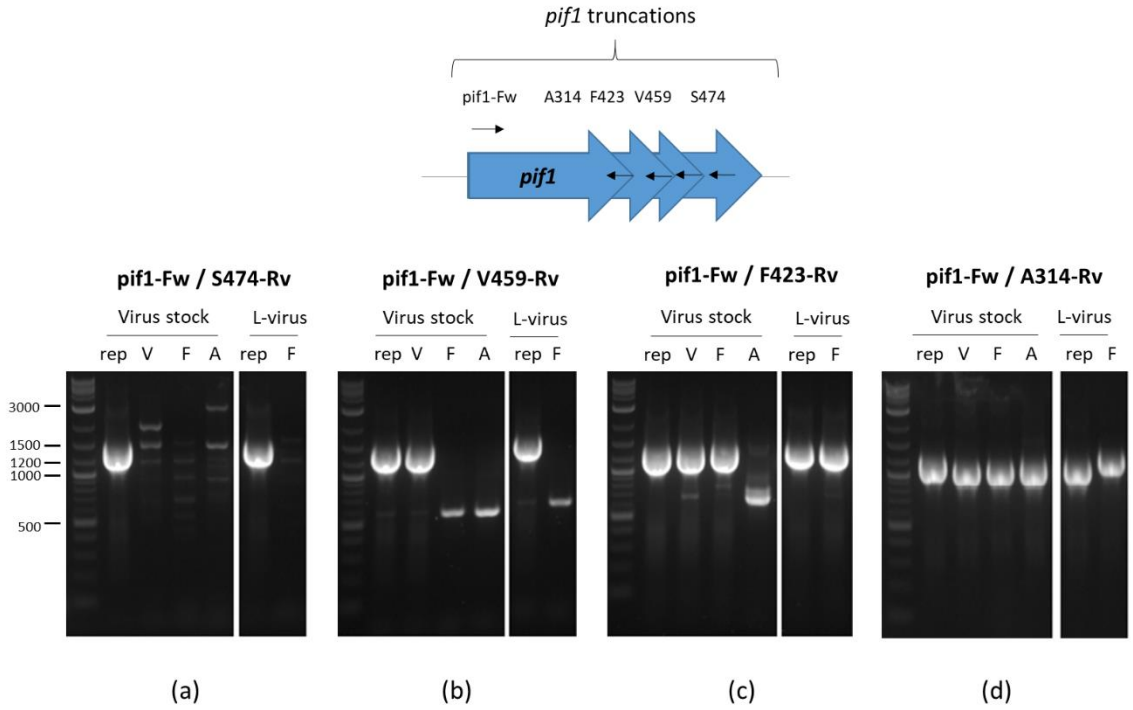

Fig. S3: PCR analysis of ODV-derived bacmid from the *pif1* virus stocks that were used to inoculate the larvae (virus stock, left panels) and the bacmids that were retrieved from infected larvae (L-virus, right panels) from the bioassay. On top, a schematic overview of the annealing sites of the various primers. The mutants are indicated by the amino acid at which the PIF1 protein was truncated (V, F or A). This analysis was performed with four different primer sets in which the reverse primer was varied: S474-Rv (a), V459-Rv (b), F423-Rv (c) and A314-Rv (d).

When using the reverse primer *pif1*-S474-Rv that annealed downstream from the first truncation site, no specific amplicon was produced for any of the truncation mutants, as expected. This was first shown for the virus stocks that were used to inoculate the larvae, indicating that these stocks did not contain wild type or full length repair viruses (a). PCR amplification on the virus that was retrieved from the single liquefied larva (L-virus) after inoculation with the *pif1*-F423 truncation mutant, only produced an amplicon with reverse primers that annealed at the respective truncation sites or more upstream (F423-Rv and A314-Rv) (c, d), but not with primers that annealed downstream of the truncation sites (S474-Rv and V459-Rv) (a, b). This confirms that the liquefied larvae was indeed infected by the *pif1*-F423 truncation mutant.

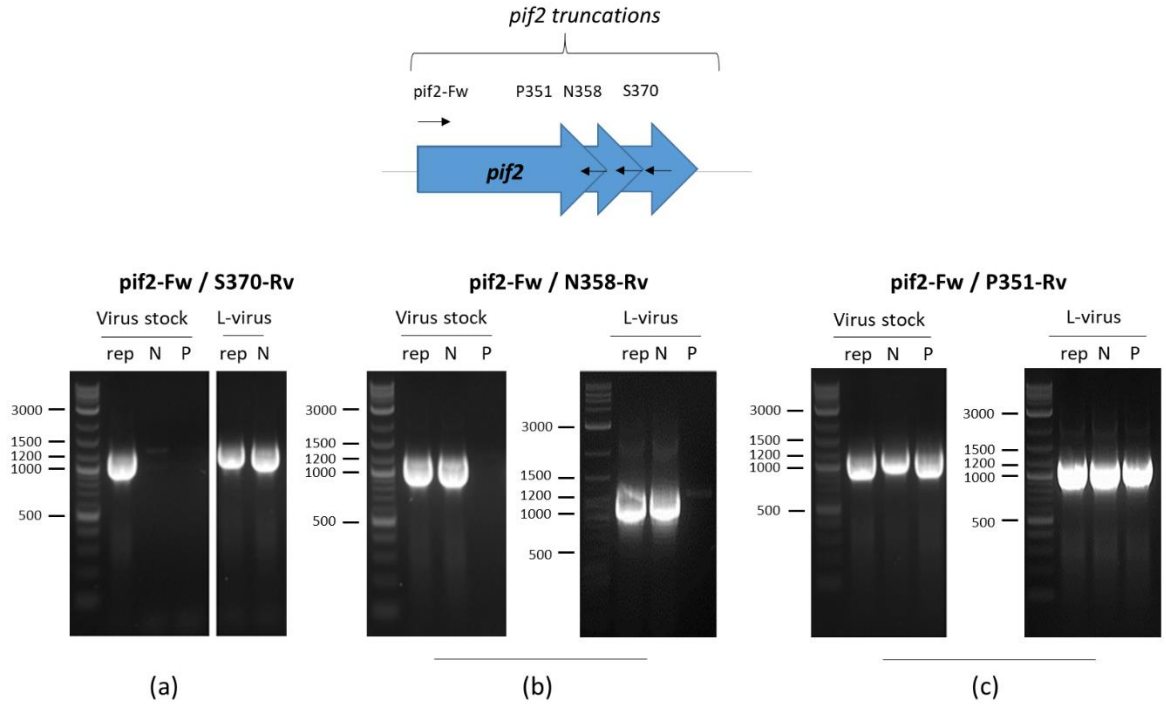

Fig. S4: PCR analysis of ODV derived bacmid from the *pif2* virus stocks that was used to inoculate the larvae (virus stock, left panels) and the bacmid that was retrieved from infected larvae (L-virus, right panels) of the bioassay. On top, a schematic overview of the annealing sites of the various primers. The mutants are indicated by the amino acid at which the PIF2 protein was truncated (N or P). This analysis was performed with four different primer sets in which the reverse primer (Rv) was varied: S370-Rv (a), N358-Rv (b), P351-Rv (c).

When using the reverse primer *pif2*-S370-Rv that annealed downstream from the first truncation site, no specific amplicon was produced for any of the truncation mutants of the virus stocks, indicating that these are clean. However, an amplicon with this reverse primer was produced with the virus that was retrieved from the larvae that were inoculated with the *pif2*-N358 truncation mutants, indicating that those larvae were infected by wild type or full repair virus. PCR analysis on the virus that was retrieved from the single liquefied larva that was inoculated with the *pif2*-P351 only produced an amplicon when using the P351-Rv primer (c) and not with N358-Rv (b). This confirms that the liquefied larvae was indeed infected by the *pif1*-F423 truncation mutant.

Chapter 7

Reconstruction of the interactome of the baculovirus *per os* infectivity factors

Bob Boogaard, Jan W. M. van Lent, Monique M. van Oers

Abstract

The baculovirus occlusion-derived virus (ODV) phenotype infects the midgut epithelium of insect larvae. The ODV-envelope contains at least ten different proteins that are required for this oral infection and are named *per os* infectivity factors (PIFs). Nine of these PIFs, PIF0 to 4 and PIF6 to 9, form the ODV entry complex. PIF5 is the only PIF that is not a constituent of this complex, suggesting that the entry complex is not the only determinant of ODV *per os* infectivity. Moreover, there are also other ODV-envelope proteins that influence the infectivity of the ODVs, suggesting that the entry complex function in relation with other proteins. We reconstructed a PIF interactome for the Autographa californica multiple nucleopolyhedrovirus by identification of the interaction partners of PIF5, 6, 8 and 9 using immunoprecipitation of these PIFs followed by mass spectrometry analysis. Our analysis revealed that the PIFs interact not only with other PIF-proteins, but also with other ODV proteins that are involved in nucleocapsid assembly, virion production and OB-formation. Especially PIF5 and 9 were found to have many non-PIF interaction partners. Furthermore, PIF0 and 1 were the only components of the complex that co-precipitated with PIF5, suggesting that these two PIFs also occur outside the complex. These results confirm the idea that the entry complex acts in concert with other viral proteins.

Introduction

The *Baculoviridae* is a family of large DNA viruses that infect their insect hosts at the larval stage via the oral route (reviewed by Williams *et al.,* 2017, Harrison *et al.,* 2019). Two different virion phenotypes are produced during the replication cycle: budded viruses (BVs) and occlusion-derived viruses (ODVs). The BVs are formed when the newly formed nucleocapsids migrate towards the host cell plasma membrane and bud off to spread the viral infection systemically within a host. The ODVs are formed later in the replication cycle, when the nucleocapsids are retained inside the nucleus and are enveloped by derivatives of the (inner) nuclear membrane (Braunagel and Summers, 2007, Shi *et al.,* 2015). In all viruses, except the ones belonging to the genus *Betabaculovirus*, the ODVs are embedded in a matrix of crystallized polyhedrin protein, which results in the formation of polyhedral-shaped occlusion bodies (OBs) in the nucleus. Hence, the name nucleopolyhedrovirus (NPVs). In betabaculoviruses, the ODVs are embedded in a protein matrix of granulin. The protein matrix of the OBs protects the ODVs outside the insect host upon dispersal of the virus into the environment. The ODVs are responsible for the horizontal transmission of the virus from one larva to the next.

Upon ingestion of contaminated plant material, the OBs dissolve in the highly alkaline environment of the midgut lumen. The released ODVs pass the peritrophic membrane and infect the midgut epithelial cells by direct fusion of the viral envelope with the plasma membrane (Horton and Burrand, 1993) of the microvilli by which the nucleocapsids are deposited into the host cell. The ODV-envelope contains at least ten different proteins that are crucial for the infection of the midgut epithelium, the so-called *per os* infectivity factors (PIFs). Per definition, these proteins are only involved in the primary infection of the midgut epithelium by ODVs and not in systemic spread of the virus infection by BVs (Faulkner *et al.,* 1997, Kikhno *et al.,* 2002, Pijlman *et al.,* 2003, Ohkawa *et al.,* 2005, Fang *et al.,* 2009, Sparks *et al.,* 2011, Nie *et al.,* 2012, Zhu *et al.,* 2013, Javed *et al.,* 2017, Liu *et al.,* 2016, Wang *et al.,* 2019, **Chapter 4**).

Nine PIFs form a large complex in the ODV-envelope, the so-called ODV entry complex, that consists of a stable core, formed by PIF1 to 4, to which PIF0 and PIF6 to 9 associate more loosely (Peng *et al.,* 2010, 2012, **Chapter 2, 3, 4**). PIF5 is the only PIF that is not a constituent of this complex (Peng *et al.,* 2012). The core is very stable as it was still found in western blots after partial denaturation of ODVs in SDS-solution at 75ºC (**Chapter 3**). The loosely associated components apparently dissociated under these conditions. The complete entry complex can

only be observed under non-denaturing conditions and is detected as a 480 kDa band in blue native-PAGE gels (Peng *et al.,* 2012).

ODVs do not only rely on the entry complex to be fully infectious. For example, the oral infectivity of mutant ODVs that lack PIF5 is significantly reduced, while the entry complex is still formed (Harrison *et al.,* 2010, Sparks *et al.,* 2011a, Peng *et al.,* 2012). Furthermore, the entry complex is important, but not indispensable for midgut infection as various *pif* mutants were incidentally able to infect the larvae (Ohkawa *et al.,* 2005, Fang *et al.,* 2009, Dong *et al.,* 2014, Zhu *et al.,* 2013, Wang *et al.,* 2019), which are now known not be able to form the complex (Peng *et al.,* 2012, Wang *et al.,* 2019, **Chapter 4**). The infectivity of a complex-deficient *pif8* mutant, that lacks the zinc finger (ZF-) domain, was partially rescued after damaging the peritrophic membrane with calcofluor white (Zhu *et al.,* 2013). This suggests that the complex (or the zinc finger) is involved in passing the peritrophic membrane and that the mutant virus is able to infect the midgut cells in absence of the entry complex. Moreover, absence of ODV proteins AC18 (Wang *et al.,* 2007), ODV-E66 (AC46) (Xiang *et al.,* 2011, Sparks *et al.,* 2011b), AC111 (Li *et al.,* 2018), AC114 (Wei *et al.,* 2012), AC124 (Liang *et al.,* 2015), AC145 or AC150 (Lapointe *et al.,* 2004, Zhang *et al.,* 2005) also influences the virus infectivity *in vivo*. Among these, AC111 appeared important for the infection of *Trichuplusia n*i larvae, but was dispensable for infection of *Spodoptera exigua*, adding an extra layer of complexity (Li *et al.,* 2018). These observations indicate that the ODV entry complex is a central player for the initiation of midgut infection, but that other viral proteins are also required for optimal infectivity *in vivo*.

In this chapter, we reconstructed a PIF-interactome using the Autographa californica multiple nucleopolyhedrovirus (AcMNPV) as a model, to better understand the relation between PIFs and other proteins in the ODV. This was done by immunoprecipitation of a panel of HA-tagged PIFs, followed by mass spectrometry to identify the interaction partners of these PIFs

Results and discussion

Selection of PIF proteins for co-immunoprecipitation experiments

It was studied how the AcMNPV ODV entry complex interacts with other ODV proteins. We therefore mapped the proteins that co-immunoprecipitated with a panel of HA-tagged PIFs when using anti-HA magnetic beads, by mass spectrometry. PIF1 of the stable core has been analysed before using a similar strategy (Peng *et al.,* 2012). This resulted in the precipitation of all known components of the complex and a component of the polyhedrin matrix, AC5. In this

study, the loosely associated components entry complex were precipitated as these proteins are probably more likely to interact with other ODV envelope proteins than the PIFs in the stable core. Three loosely associated components, PIF6, 8 and 9, were efficiently immunoprecipitated. PIF0 and PIF7 were excluded from these experiments as PIF0 failed to precipitate efficiently, and PIF7 was not detectable by mass spectrometry despite effective precipitation as shown by western blot analysis (**Supplementary data Fig. S1**). PIF5, the only PIF that is not a constituent of the complex, was also analysed to obtain first data on the interaction partners of this PIF in the ODV envelope.

PIF5 interacts with two components of the entry complex: PIF0 and 1.

PIF0 and 1 co-precipitated with all four target proteins (PIF5, 6, 8 and 9) and were the only components of the entry complex that co-precipitated with PIF5 (**Fig. 1**). In the PIF5 precipitation, PIF0 was 80 times enriched over the negative control (wild type virus without an HA-tagged protein) (t-test, p= 0.003) and PIF1 almost 40 times (t-test, p= 0.0006) (**Supplementary data Fig. S2.1**). The interaction between PIF5 and PIF0 was confirmed by western blot analysis of the co-precipitated proteins with PIF0 antiserum. This was not confirmed for PIF1 as this PIF was hard to detect with PIF1 antiserum in the HA-tagged PIF5

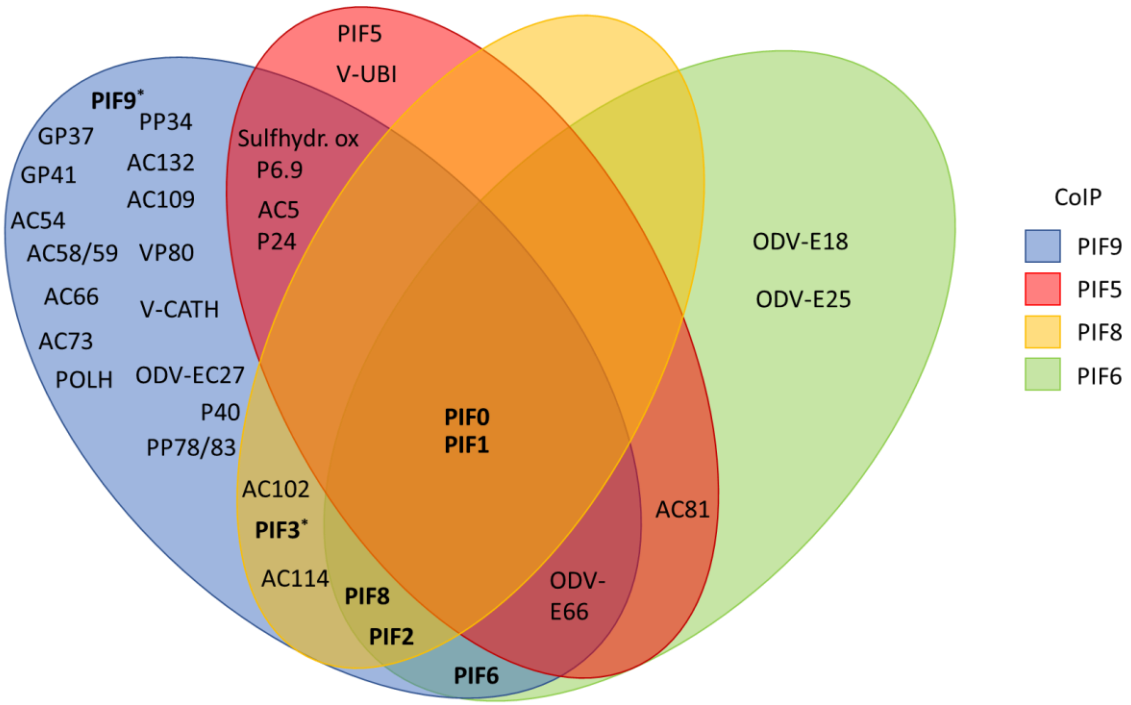

Figure 1: Venn diagram of the identified interaction partners of PIF 9 (in blue), PIF5 (in red), PIF8 (in yellow) and PIF6 (in green). These PIFs were immunoprecipitated in triplicate via the HA-tags with anti-HA magnetic beads and co-precipitated proteins were identified by LC-MS/MS. Wild type ODVs were analysed in parallel as negative control. *PIF3 and 9 were detected by a single peptide after immunoprecipitation of PIF6, for which they were excluded from further statistical analysis.

repair mutant virus (**Supplementary data Fig. S1b**). Furthermore, PIF5 was not enriched after immunoprecipitation of one of the other components of the entry complex (PIF6, 8 or 9) (**Fig. 1**). As immunoprecipitation of PIF5 resulted in the precipitation of only PIF0 and 1, and not of any other component of the entry complex, it is suggested that these two PIFs are not only constituents of the ODV entry complex, but also occur outside the complex. The interaction between PIF5 and PIF0 and 1 had also been detected in in other studies by yeast-two hybrid (Y2H) assays with PIF homologs of *Helicoverpa armigera* NPV (HearNPV) (Peng *et al.,* 2010b). However, PIF5 was not identified as a PIF1 interaction partner in the ODV-envelope of AcMNPV in the earlier co-immunoprecipitation study with PIF1 antiserum (Peng *et al.,* 2012). Maybe the usage of PIF1 antiserum was not suitable for detection of this interaction or this interaction can only be detected in one direction as with the Y2H assays. The Y2H assays also suggested an interaction between PIF5 and PIF3, but our data do not provide any evidence for that.

The biological importance of the putative interaction between PIF0, 1 and 5 is unclear as mutant viruses that lack one of these PIFs have different phenotypes. PIF0 and 1 are involved in the binding of ODVs with the midgut epithelium, while PIF5 appeared dispensable for ODV binding (Haas-Stapleton *et al.,* 2004 Ohkawa *et al.,* 2005, Sparks *et al.,* 2011). This suggests that PIF0 and 1 in addition to ODV binding have other functions as well during midgut infection.

Smaller sized components of the entry complex are more difficult to detect by mass spectrometry.

PIF2 and 8 precipitated together with the components of the entry complex (PIF6, 8 and 9) and not with PIF5 (**Fig. 1**). The smaller sized PIF3 (27 kDa), PIF6 (15 kDa) and PIF9 (17 kDa), were not always detected after immunoprecipitation of one of the target proteins. PIF3 was only detected after precipitation of PIF8 and 9, PIF6 only with PIF9, and PIF9 only after direct precipitation with the anti-HA magnetic beads (**Fig. 1**). This might be due to the fact that less peptides are formed by these smaller PIFs upon trypsin digestion and are therefore more difficult to be detected by mass spectrometry in complex peptide mixtures, especially when these PIFs are present at low quantities (Pers. comm. Sjef Boeren). Precipitation of PIF6 or 9 nevertheless resulted in the precipitation of PIF0, 1, 2 and 8, confirming their presence in the entry complex.

ODV-E66 interacts with PIF6 and 9 of the entry complex and PIF5.

Of the non-PIF proteins, ODV-E66 was identified most often as a PIF interaction partner. This protein was found after immunoprecipitation of three of the four target proteins: PIF5, 6 and 9 (**Fig. 1 and 2**). Other studies also indicated that this protein closely associates with components of the entry complex. Y2H and bimolecular fluorescence protein complementation (BiFC) assays indicated that ODV-E66 interacts with PIF2, 3 and 4 (Peng *et al.*, 2010b, Dong *et al.*, 2014). However, this protein was not identified as a PIF1 interaction partner in an earlier co-immunoprecipitation study (Peng *et al.*, 2012) and appeared not to be a constituent of the ODV entry complex, when the ODV-envelope was analysed under native conditions (Wang *et al.*, 2019). The interaction between ODV-E66 and PIF5 was previously also identified with Y2H assays (Peng *et al.*, 2010b). Our study confirms the close association of ODV-E66 with the PIFs in the ODV envelope and is in accordance with studies that showed that ODV-E66 is indeed important for the oral infectivity of ODVs. This protein is probably involved in ODV binding as peptide derivatives of this protein have affinity for the midgut epithelium (Sparks *et al.*, 2011b, Xiang *et al.*, 2011) (See **table 1** for further information). This protein is was recently also shown to be important for the degradation of the peritrophic membrane (Hou *et al.*, 2019).

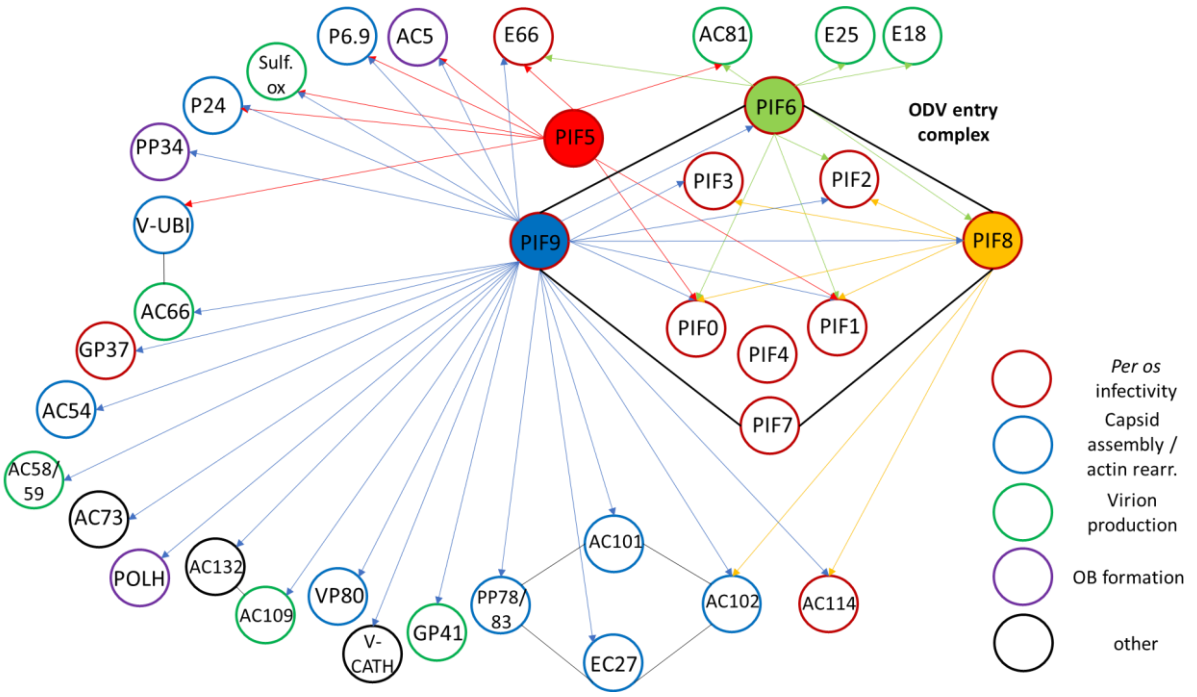

Figure 2: A protein interaction network based on the identified interaction partners after immunoprecipitation of one of the target proteins; PIF5, 6, 8 and 9. The identified proteins were categorized according to their reported function into five different groups: *per os* infectivity, encircled with dark red; nucleocapsid assembly and actin rearrangement, encircled with blue; virion production, encircled with dark green; OB formation, encircled with purple; and rest, encircled with black.

Table 1: Overview of identified PIF interaction partners according to their functions

Protein	AcMNPV ORF	Remarks	References
		***Per os* infectivity**	
ODV-E66	*ac46*	Hyaluronidase activity, peptide derivatives bind midgut epithelium, degrades the peritrophic membrane.	Sparks *et al.*, 2011b, Xiang *et al.*, 2011 Hou *et al.*, 2019
GP37	*ac64*	Polyhedron and BV associated, binds chitin, facilitate PM-passage, fusogenic properties	Vialard *et al.*, 1990, Li *et al.*, 2003, Wang *et al.*, 2010, Liu *et al.*, 2011, Liu *et al.*, 2019
AC114	*ac114*	Associated with BVs and ODVs; involved in embedding of ODVs in protein matrix	Braunagel *et al.*, 2003, Wang *et al.*, 2010, Wei *et al.*, 2012
		Nucleocapsid assembly / actin rearrangement	
PP78/83	*ac9*	Essential gene in Group I and II lepidopteran NPVs, WASP-like protein for actin nuclear assembly in association with Ac101, Ac102 and ODV-EC27	Vialard *et al.*, 1993, Russell *et al.*, 1997, Goley *et al.*, 2006, Ohkawa *et al.*, 2010
V-UBI	*ac35*	Might inhibit degradation of short-lived viral proteins; important for BV production by facilitating nuclear egress of nucleocapsids in association with AC66 and AC141	Haas *et al.*, 1996, Biwas *et al.*, 2018
AC54	*ac54*	Core gene, associated with BVs and ODVs, Nucleocapsid assembly	Olszweski *et al.*, 1997a, Guan *et al.*, 2016
P6.9	*ac100*	Core gene, DNA binding protein, nucleocapsid assembly	Wang *et al.*, 2010b, Liu *et al.*, 2012, Lai *et al.*, 2018
AC101 / C42	*ac101*	Core gene, capsid associated, form a complex with AC102, PP78/83 and ODV-EC27, regulated by miR-3	Braunagel *et al.*, 2001, Garavaglia *et al.*, 2012, Hepp *et al.*, 2018, Jiao *et al.*, 2019
AC102	*ac102*	Associated with the nucleocapsids, involved in nuclearization of G-actin with AC101, PP78/83 and ODV-EC27	Hepp *et al.*, 2018
VP80	*ac104*	Capsid associated; the nucleocapsids stick in the virogenic stroma in absence of Vp80	Braunagel *et al.*, 2003, Marek *et al.*, 2011
P24	*ac129*	Associated with BVs and ODVs, capsid protein	Braunagel *et al.*, 2003, Wang *et al.*, 2010
ODV-EC27	*ac144*	Associated with the nucleocapsids with AC101, AC102 and PP78/83	Braunagel *et al.*, 2001, Hepp *et al.*, 2018

Table 1 (continued)

BV and ODV production			
AC58/59	*ac58*	Non-essential BV and ODV associated protein	Braunagel *et al.*, 2003, Wang *et al.*, 2010
AC73	*ac73*	associated with AcMNPV BVs and ODVs. Important for BV production and infection *in vivo*	Wang *et al.*, 2010, Shao *et al.*, 2019
GP41	*ac80*	Core gene; tegument protein, involved in nuclear egress of nucleocapsids and ODV assembly	Whitford *et al.*, 1992a,b, Olszewski *et al.*, 1997b, Li *et al.*, 2018b
AC81	*ac81*	Core gene, required for nucleocapsid envelopment and embedding of ODVs	Chen *et al.*, 2007, Dong *et al.*, 2016
Sylfhydr ox / P33	*ac92*	Core gene, associated with BVs and ODVs, deletion results in envelopment of single nucleocapsids	Wu *et al.*, 2010, Nie *et al.*, 2011
ODV-E25	*ac94*	Core gene, associated with BVs and ODVs, play a role in the shift from BV to ODV production	Russell *et al.*, 1993, Braunagel *et al.*, 2003, Wang *et al.*, 2010, Chen *et al.*, 2012, 2013, Luo *et al.*, 2013, Rohrmann *et al.*, 2013
AC109	*ac109*	Core gene, BV and ODV associated, various phenotypes observed in virion production with mutants	Braunagel *et al.*, 2003, Fang *et al.*, 2009b, Wang *et al.*, 2010, Lin *et al.*, 2009, Lehiy *et al.*, 2012
ODV-E18	*ac143*	Core gene, BV and ODV associated	Braunagel *et al.*, 2003, Wang *et al.*, 2010
AC66	*ac66*	Core gene, facilitates nuclear egress of nucleocapsids (with V-UBI and AC141), and involved ODV and OB production.	Ke *et al.*, 2008, Biwas *et al.*, 2018
OB formation			
AC5	*ac5*	Component of OB-matrix, unknown function	Wang *et al.*, 2018
POLH	*ac8*	Matrix protein that crystallizes and occludes the ODVs	Ji *et al.*, 2010
PP34	*ac131*	Polyhedron envelope (calyx) protein	Whitt and Manning, 1988, van Lent *et al.*, 1990
Other			
V-CATH	*ac127*	BV associated, involved in liquefaction of the insect host.	Lanier *et al.*, 1996
AC132	*ac132*	Associated with BVs and ODVs, component of nucleocapsids and ODV envelope, involved in nuclear entry of nucleocapsids, might function together with AC109	Braunagel *et al.*, 2003, Wang *et al.*, 2010, Ono *et al.*, 2012, Fang *et al.*, 2016

AC114 and GP37, which are important for oral infectivity, interact with components of the entry complex.

Two other non-PIF proteins that are important for the oral infectivity were also identified as PIF interaction partner. AC114 precipitated together with PIF8 and 9, and glycoprotein GP37 only with PIF9 (**Fig. 1, 2**). The *ac114* gene is highly conserved among alphabaculoviruses and the encoded protein is associated with BVs and ODVs (Braunagel *et al.,* 2003, Wang *et al.,* 2010). This protein is important for the embedding of the ODVs into the polyhedrin matrix, as the OBs contained less ODVs after deletion of *ac114*, which reduced the OB infectivity significantly (Wei *et al.,* 2012). GP37 was probably found because of the presence of polyhedrin after immunoprecipitation of PIF9, as GP37 associates with the polyhedrin matrix of the OBs. GP37 has affinity for chitin and enhances *per os* infectivity by facilitating the passage of ODVs through the peritrophic membrane (Vialard *et al.,* 1990, Li *et al.,* 2003, Liu *et al.,* 2011). A homolog in betabaculoviruses was recently shown to have also fusogenic properties (Liu *et al.,* 2019).

Other proteins that are known to be important for oral infectivity, AC18 (41 kDa), AC111 (8 kDa), AC124 (29 kDa) and the enhancins AC145 (9 kDa) and AC150 (11 kDa) (Wang *et al.,* 2007, Li *et al.,* 2018, Lapointe *et al.,* 2004, Zhang *et al.,* 2005), were not identified as interaction partner of any of the immunoprecipitated PIFs. AC18 was detected after immunoprecipitation of PIF9, but was not significantly enriched compared to the wild type control (**Supplementary data Fig. S2.2**). The other proteins were not detected in any of the protein samples. These proteins were also not found when in a mass spectrometry analysis of the 480 kDa band that is formed by the entry complex in blue native PAGE gels (Wang *et al.,* 2019). However, this does not necessarily mean that they don't interact with PIFs as these proteins might be hard to detect by mass spectrometry due to their small sizes as explained before.

Other proteins involved in nucleocapsid assembly, virion production and OB formation.

Immunoprecipitation of PIF9 resulted in the co-precipitation of eighteen additional ODV proteins which are not described above. These proteins were categorized into four groups according to their reported function: nucleocapsid assembly (and actin rearrangement), virion production, OB formation and others (**Table 1, Fig. 2**). Four of these proteins, sulfhydryl oxidase (P33), P6.9, AC5 and P24, were also found after immunoprecipitation of PIF5 (**Fig. 1, 2**). The identification of PP34 and AC5, both OB components (**Table 1**), might be due to the presence of the polyhedrin matrix protein in the sample after immunoprecipitation of PIF9. Polyhedrin and AC5 were also found previously after immunoprecipitation of PIF1 (Peng *et*

al., 2012). Sulfhydryl oxidase (P33) and P24 have been associated with the ODV entry complex before and are respectively involved in virion production and nucleocapsid assembly (**Table 1**) (Wang et al., 2019). AC73 was also found after immunoprecipitation of PIF9. This proteins only occurs in group I alphabaculoviruses and associates with the nucleocapsids of BVs and ODVs (Shao *et al.,* 2019). The BV production was affected and *in vivo* infectivity was reduced after deletion of the *ac73* gene (Shao *et al.,* 2019). P6.9 is encoded by a core gene and is involved in capsid assembly, possibly because of its DNA binding properties (Wang *et al.,* 2010, Lai *et al.,* 2018). This protein is found associated with PIFs for the first time in this study after precipitation of PIF5 and PIF9. This is quite significant as this protein might be difficult to detect by mass spectrometry due to its small size and repetitive amino acid motif (Garcia-Maruniak *et al.,* 2004). This protein was detected before in the nucleocapsids of ODVs by mass spectrometry (Braunagel et al., 2003). These results indicate that PIF9 also interacts with capsid proteins. This is supported by the finding of all the components of another complex, that is formed at the base of the nucleocapsids by AC101, AC102, PP78/83 and ODV-EC27, and rearrange nuclear actin in the host cell (Hepp *et al.*, 2018) (**Fig. 2**, **Table 1**).

PIF6 interacts with proteins that are essential for virion production.

Immunoprecipitation of PIF6 resulted in the precipitation of AC81, ODV-E18 and ODV-E25, in addition to the larger components of the entry complex (**Fig. 1 and 2**). AC81 was also found after immunoprecipitation of PIF5 (**Fig. 1 and 2**). These proteins seemed to interact specifically with PIF6 as they were not found after immunoprecipitation of PIF8 or 9 and had also not been found after immunoprecipitation of PIF1 before (Peng *et al.*, 2012). These proteins have also been found in the 480 kDa band in blue native-PAGE gels, representing the entry complex (Wang *et al.,* 2019), and are all encoded by core genes and essential for virion production (**Table 1**).

Conclusion

This study shows that the PIFs not only interact with each other in the ODV entry complex, but also with various non-PIF proteins that are present in the ODV envelope, which are important for the formation of virions and OBs. This was especially the case for PIF9, that was found to interact with various components of the nucleocapsids. It was also shown that PIF5 interacts with two components of the entry complex, PIF0 and 1, and not with any other component of the ODV entry complex. This suggests that PIF0 and 1 not only occur in the entry complex, but also outside the complex in interaction with PIF5. The biological relevance of this interaction

with PIF5 for the infectivity of ODVs remains to be established. These findings indicate that, despite formation of the entry complex, the PIFs also function in concert with other ODV proteins. These interactions might be important for the complete infectious potential of ODVs to larval midgut cells.

Materials and methods

Insect cells and viruses. TnH5 (from *Trichoplusia ni*) and Sf21 cells (from *Spodoptera frugiperda*) were cultured in Grace's medium (Thermo Fisher), supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml gentamycin (Gibco, Life technologies) at 27°C. Bacmid derived AcMNPV E2-strain (bMON14272) with a restored *polh* gene was used as wild type in this study. Viruses that encode HA-tagged PIF proteins have the same genetic origin. The HA-tagged PIF5 and 9 viruses have been described before in Peng *et al.,* 2012 and **Chapter 4**. Bacmids for the HA-tagged PIF6 and 8 viruses were kindly provided by dr. D.A. Theilmann of the Summerland Research and Development Centre for Agriculture and Agri-Food, Canada.

Production of the mutant viruses. BV-stocks of the HA-tagged viruses were produced by transfection of Sf21 cells with the respective bacmids using Expres2TR transfection reagent (Expres2ion Biotechnologies), followed by an additional infection round in Sf21 cells as described previously (**Chapter 3, 4**). OBs were produced by infection of TnH5 cells with BVs at an MOI of 2 and isolated as described before (**Chapter 4**). In brief, the infected cells were harvested at six days post infection by centrifugation at 4000 x g for 30 min and lysed by incubation in 0.1% SDS for 2 hrs at 37°C with gentle agitation and subsequent sonication for 1 min with an intensity of 8 Watt on ice. Complete lysis was verified by light microscopy and the cells were sonicated once more when needed. When all the cells were lysed, the OBs were pelleted by centrifugation at 4000 x g for 30 min. and washed three times with sterile double distilled water.

Immunoprecipitation with anti-HA magnetic beads. The ODVs from ~5 x 10^8 OBs of the HA-tagged mutants were isolated and fractionated in triplicate in a slightly modified way from what was described in **Chapter 4**. The ODVs were fractionated by a 2 hr incubation in protein extraction buffer (25mM Tris, 150mM NaCl, 0.5% Triton X-500 pH 7.2), followed by two short sonication bursts. The envelope fractions were incubated overnight with 20 µl anti-HA magnetic beads (Invitrogen) in 200 µl protein extraction buffer at 4º with gentle rotation. Wild type ODVs (without HA-tag) were treated in parallel and were used as a negative control. The

beads were washed for three times with extraction buffer and the captured proteins were eluted from the beads by incubation in Laemmli buffer at 95ºC for 10 min.

SDS-PAGE and western blot analysis. The proteins were loaded on two different Any-Kd TGX precasted protein gels (Biorad). The first gel was used to verify the precipitation of the target protein and the known interaction partners by western blot analysis, with either anti-HA antibodies (Roche 3F10, 1:2000, rat-derived) or antiserum against PIF0, produced in rabbit, 1:1000; PIF1, produced in rats, 1:2000 (Peng *et al.*, 2010) and PIF8, produced in rabbit, 1:2000 (Russell and Rohrmann, 1997). Goat anti-rabbit (1:2000 dilution; Dako) and goat anti-rat (1:2000 dilution; Sigma A8438), conjugated to alkaline phosphatase, were used as secondary antibodies for detection of the proteins of interest by conversion of NBT-BCIP substrate in a blue-purple coloured precipitant in AP-buffer (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM MgCl, pH 10.5). The second gel was run until the loading dye migrated 2.5 cm into the gel and was stained with oriole (Biorad). The proteins in this gel were analysed by mass spectrometry after in gel digestion with trypsin as described in the next section.

In gel digestion and MS/MS sample preparation. The proteins were reduced and alkylated prior to in-gel digestion with trypsin. The proteins were reduced by a 1 hr incubation in 50 mM dithiothreitol (DTT), dissolved in 50 mM ammonium bicarbonate (ABC; Sigma) pH 8.0, and alkylated by incubation in 20 mM acrylamide (Sigma), dissolved in 0.1 M Tris; pH 8.0, for 30 min in the dark. Both treatments were performed at room temperature. The lanes that were loaded with proteins were cut into five slices under UV-light and subsequently into small blocks of approximately 1 cm^2. These blocks were washed with 50 mM ABC buffer and stored overnight at -20ºC to increase the trypsin accessible area. The proteins were digested by overnight incubation with 5 ng/µl bovine sequencing grade trypsin (Roche) in 50 mM ABC at room temperature. The pH was set to 3 by the addition of 10% trifluoro acetic acid and the peptides were purified over C18 µColumns. The peptides were analysed by LC-MS/MS using the LTQ-Orbitrap as described previously in Kariithi *et al.*, 2010.

The raw data were analysed by MaxQuant software (version 1.6.3.4) for (label free) quantification and identification of the peptides by searching against the AcMNPV Uniprot 2017 database (Cox *et al.*, 2008, 2011). A standard contamination database was also used. Modifications that were included for protein quantification were: oxidation (M), acetylation (Protein N-term) and deamination (NQ). The datasets were filtered with Perseus to select only

the proteins that were identified with minimally two peptides (with a minimal peptide length of seven amino acids) of which at least one is unique and one is unmodified (Hubner *et al.*, 2010).

Acknowledgements

We like to thank Dr Sjef W. Boeren of the Laboratory of Biochemistry for his support and mass spectrometry analysis. This work was supported by the Strategic Fund of the graduate school Production and Ecology & Resource Conservation, The Netherlands.

Supplementary data

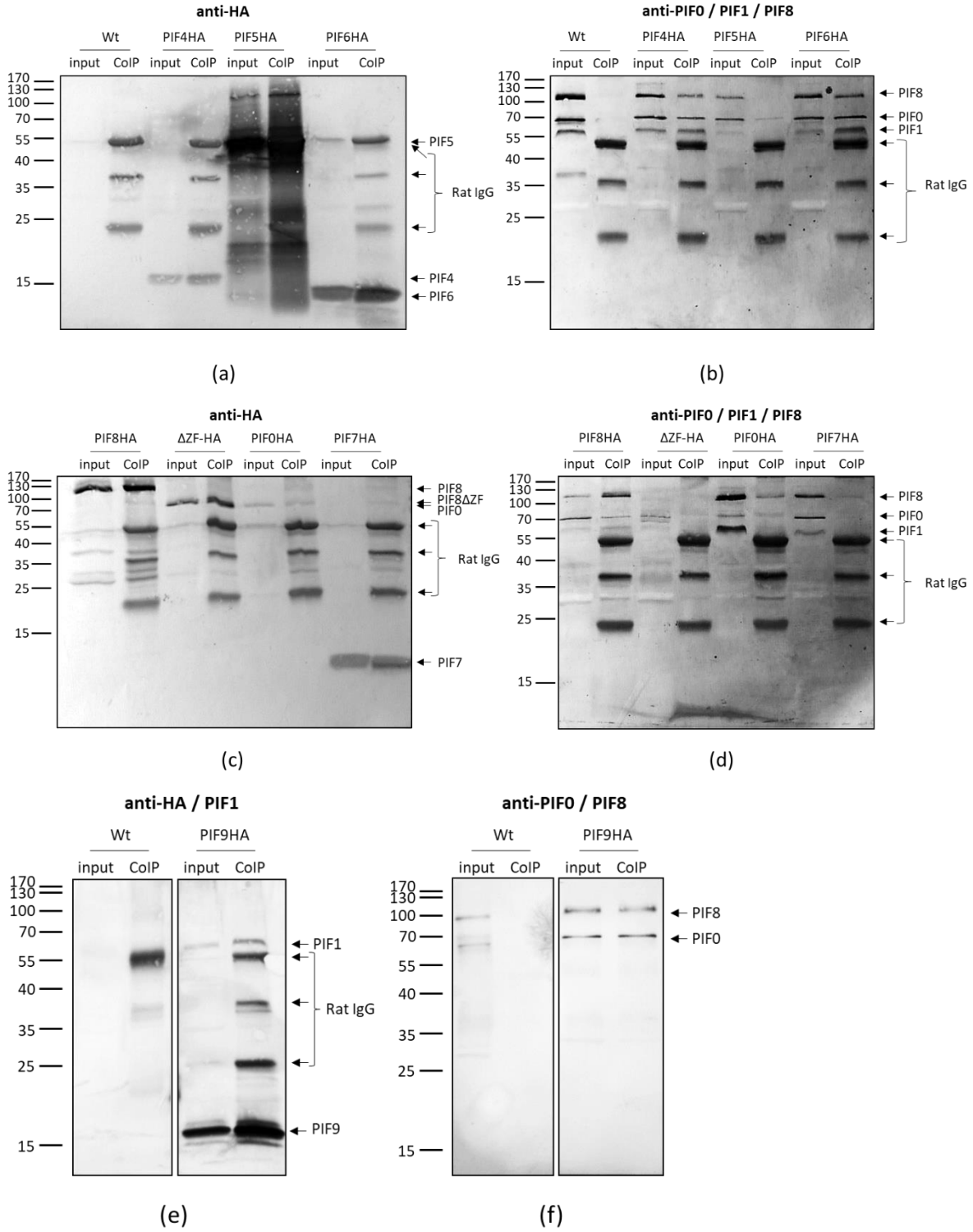

Figure S1: Validation of precipitation of HA-tagged PIF5, 6, 8 and 9 with the anti-HA magnetic beads. Immunoprecipitated proteins were analysed by western blot with anti-HA antibodies in (a), (c) and (e); and antisera against PIF0, 1 or 8 in (b), (d) and (f). The results after immunoprecipitation of HA-tagged PIF4, 5 and 6 are shown in (a) and (b); PIF8, PIF8ΔZF, PIF0 and PIF7 in (c) and (d); and PIF9 in (e) and (f). Immunoprecipitation of PIF0 and PIF7 were excluded from analysis by mass spectrometry as PIF0 failed to precipitate efficiently and PIF4 and 7 appeared not detectable by this method.

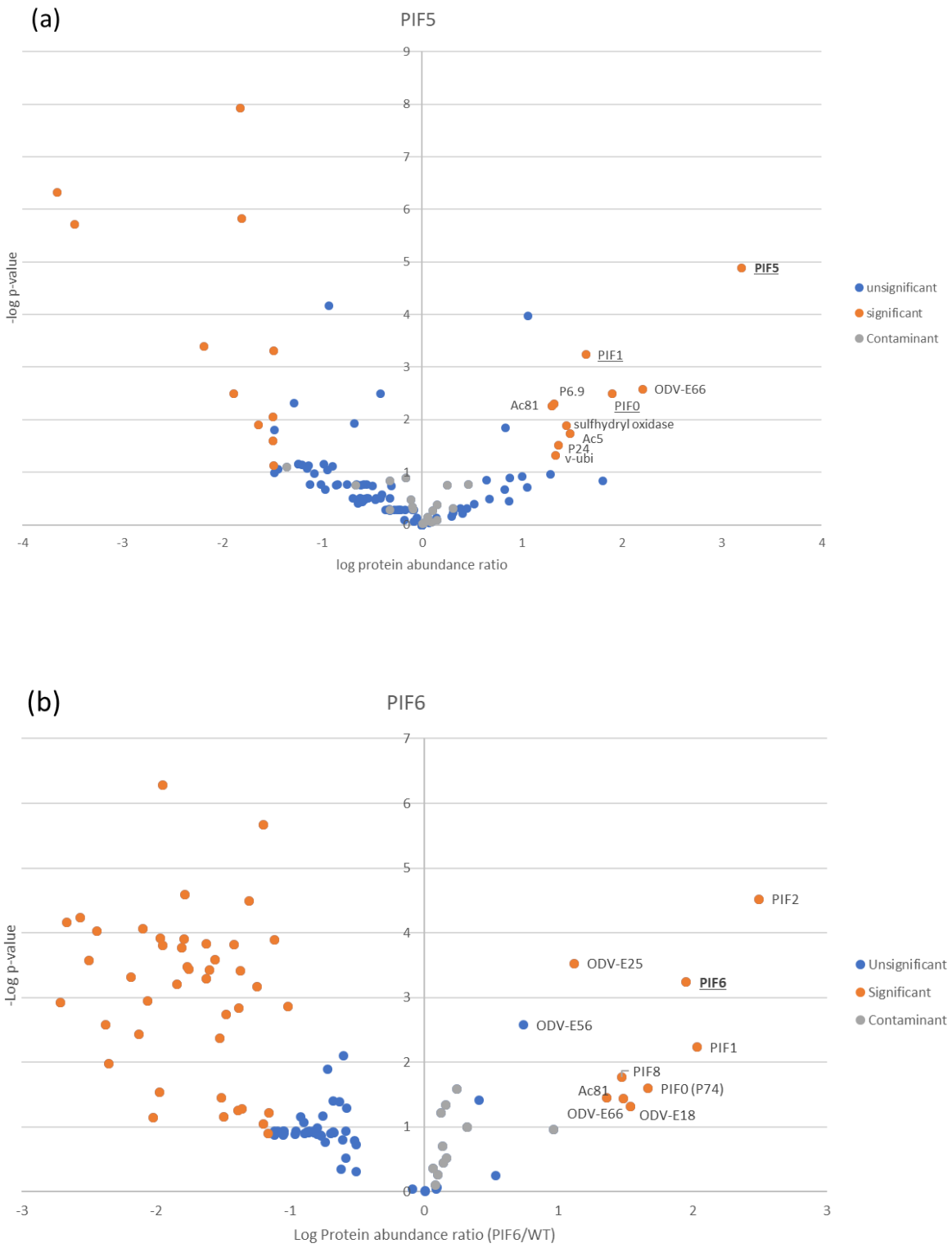

Figure S2.1: Vulcano plots of the identified proteins after immunoprecipitation of PIF5 and PIF6. The x-axis shows the log(10) protein abundance ratio, indicating the enrichment factor over wild type (wt). The Y axis shows the -log p-value of student t-tests of difference in label free quantification (LFQ) values between wild type and after immunoprecipitation of one of the PIFs, indicating the statistical significance of the hits. The hits were considered as significant with log protein abundance ratio > 1.2 over wt (15x enriched) and -log p-values > 1.3 ($p < 0.05$). Significant hits are depicted in orange, non-significant hits in blue and contaminants, as human keratins or immunoglobulins, in grey.

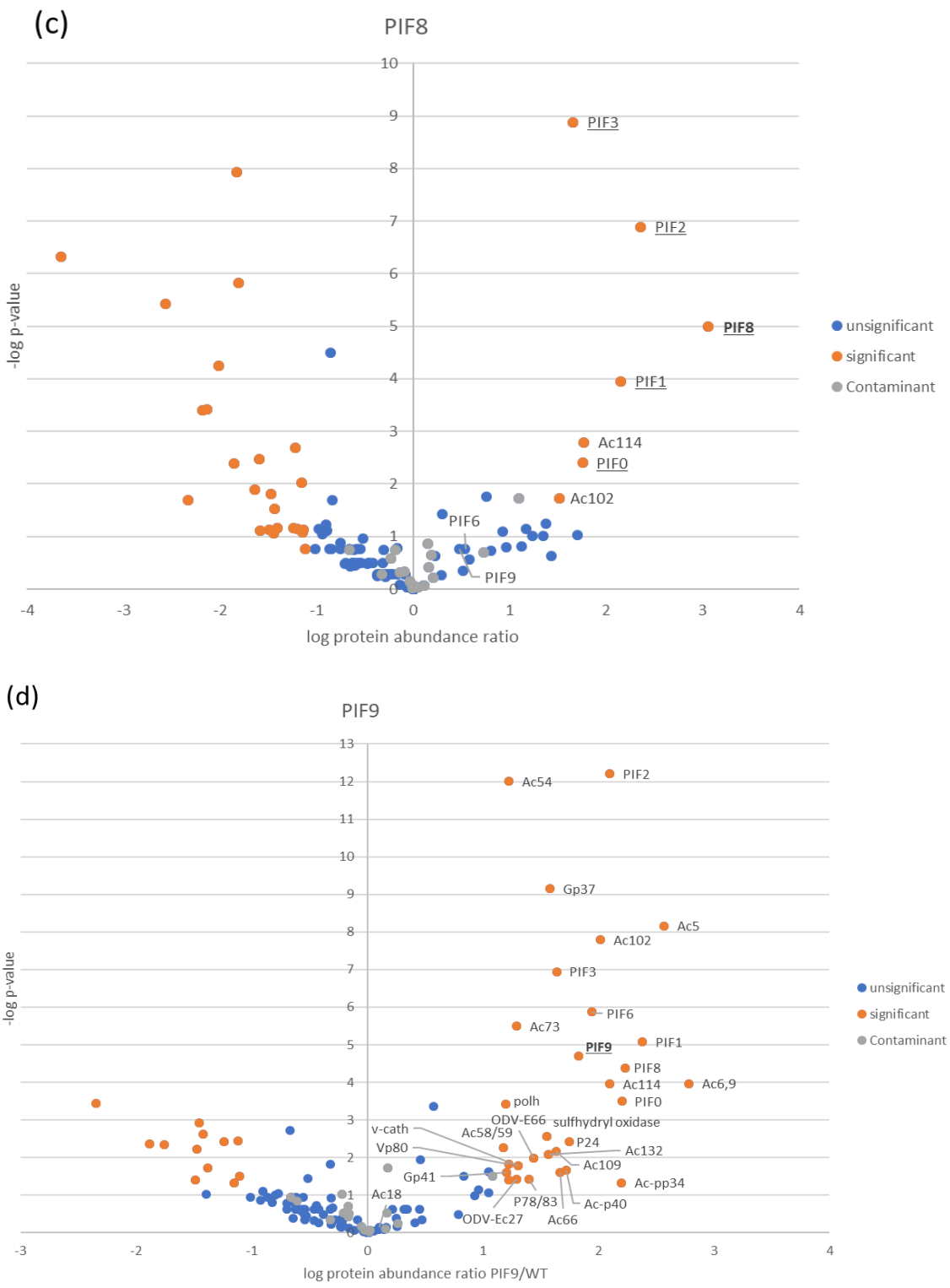

Figure S2.2: Vulcano plots of the identified proteins after immunoprecipitation of PIF8 and PIF9. The x-axis shows the log(10) protein abundance ratio, indicating the enrichment factor over wild type (wt). The Y axis shows the -log p-value of student t-tests of difference in label free quantification (LFQ) values between wild type and after immunoprecipitation of one of the PIFs, indicating the statistical significance of the hits. The hits were considered as significant with log protein abundance ratio > 1.2 over wt (15x enriched) and -log p-values > 1.3 ($p < 0.05$). Significant hits are depicted in orange, non-significant hits in blue and contaminants, as human keratins or immunoglobulins, in grey.

Chapter 8

General discussion

Introduction

The baculovirus occlusion derived viruses (ODVs) infect larval midgut epithelial cells under highly alkaline conditions. For this, the viral envelope contains ten different proteins, called *per os* infectivity factors (PIFs). Nine of these proteins form the ODV entry complex (Peng *et al.*, 2010, 2012, **Chapter 2, 3 and 4**). Per definition, the PIFs are only crucial for the infection of midgut epithelium cells by ODVs and are not involved in cell-to-cell spread of the infection by budded viruses (BVs). Viruses with *pif* gene deletions replicate in cultured insect cells without any noticeable effect on the production of BVs, ODVs and occlusion bodies (OBs). PIF proteins are highly conserved among baculoviruses and some of them also in related invertebrate infecting viruses, suggesting that this mechanism of virus entry into cells underwent millions of years of evolution (Thézé *et al.*, 2011). However, the molecular mechanism of PIF-mediated entry is still elusive to date. In this thesis, the ODV entry complex was biochemically and functionally characterised.

The complexity of the baculovirus entry process in the midgut.

The finding of ten different genes in the baculovirus genome that encode for PIF proteins suggests that the entry of ODVs into larval midgut epithelial cells is a joint effort and that the functioning of a single PIF strongly depends on the interaction with other PIFs. Indeed, nine out of the ten PIFs are crucial for the formation of the ODV entry complex. PIF1 to 4 form the stable core of the entry complex and the five remaining PIFs, i.e. PIF0 and PIF6 to 9, associate more loosely to the complex (Peng *et al.*, 2010, 2012, **Chapter 2, 3 and 4**). However, most studies to unravel the biological function of the PIFs in midgut entry were conducted without the knowledge that these proteins form such a complex. **Chapter 2** of this thesis therefore reviewed the existing literature on PIFs, discussing these proteins in relation to each other, rather than as separate proteins with a similar function in the baculovirus infection cycle. It was concluded that the existence of the entry complex complicates the interpretation of earlier studies with respect to the biological function of each individual PIF. Therefore, it can be questioned whether the observed phenotype with a *pif* deletion mutant is truly the effect of the absence of that specific PIF or an indirect effect due to disturbed protein interactions. The last option is retrospectively more likely.

This idea is supported by the results obtained with PIF1 and PIF2 C-terminal truncation mutant viruses (**Chapter 6**). PIF1 and 2 had been reported as being important for the binding of ODVs to the midgut epithelium (Ohkawa *et al.*, 2005) and for formation of the entry complex (Peng

et al., 2010). As binding to the midgut epithelium is a host range determinant (Haas-Stapleton *et al.,* 2005) and most baculoviruses have a narrow host range, we hypothesized that the highly conserved N-terminal part of PIF1 and 2 is important for complex formation and that the less conserved C-terminal parts of these PIFs interact with the host. By generating PIF1 and PIF2 C-terminal truncation mutant viruses, it was attempted to distinguish these two functions by the identification of a mutant virus that is able to form a complex while lacking oral infectivity. However, small truncations from the C-terminus of one of those PIFs already affected the formation of the entry complex as at least two of the loosely associated components, PIF0 and 8, failed to bind with the stable core of the entry complex. Also, the oral infectivity was severely affected in these mutant viruses (**Chapter 6**). This indicates that PIF1 and 2 mediate ODV infectivity in close association with the other PIFs and in the case that these PIFs are directly involved in host interaction, they are probably only exposed in the right conformation after formation of the complex. These results also demonstrate that it is impossible to study the functionality of individual PIFs by mutagenesis without affecting the formation of the entry complex and thus also the functioning of the remaining PIFs. In addition, we now know that the entry complex also fails to assemble in absence of one of the loosely associated components (Peng *et al.,* 2010, Javed *et al.,* 2017, **Chapter 3 and 6**). Therefore we conclude that these looser PIFs also depend on each other's presence to bind to the complex and probably also for their proper functioning.

Formation of the entry complex is essential to resist the proteolytic activity of host proteases

Although the molecular mechanism of the entry complex and the biological role of individual PIFs are difficult to dissect due to the above discussed reason, the results presented in **Chapter 3** provide a first clue to why this complex is formed. ODVs derived from larvae are, in contrast to ODVs from cultured insect cells (Peng *et al.,* 2011), subjected to the proteolytic activity of alkaline proteases from the host. These host proteases are co-occluded in the OBs (Payne and Kalmakoff, 1977, Wood *et al.,* 1980) and are activated when the OBs are dissolved under alkaline conditions (Peng *et al.,* 2011). In Chapter 3 it was shown that when PIFs of wild type ODVs were analysed under denaturing conditions after dissolution of the OBs in alkaline buffer, PIF monomers were found in both cell culture- and larva-derived virus suspensions. However, when one of the PIFs was absent in any of the *pif* deletion mutants tested and as a consequence the formation of the entry complex was abolished, the monomeric form of the remaining PIFs were only found in cell culture-derived ODVs and not in larva-derived ODVs. These monomeric PIFs were also observed again when the larval OBs were heat-treated to

inactivate the proteases prior to the dissolution in alkaline buffer. These experiments were carried out with a number of *pif* deletion mutant viruses and showed that when PIFs occur in monomeric form, these are prone to proteolytic degradation by host proteases. Furthermore, the PIFs in the stable core (PIF1 to 4) were relatively resistant against these proteases compared to the PIFs in monomeric form. The tight interaction of the PIFs in the complex may be needed to cope with the harsh environment of the larval midgut, in which the virions are heavily exposed to digestive proteases. These results emphasize once again the strong dependency between the PIFs.

How these proteases end up in the OBs and what their functional significance is, is currently unclear. Is has been proposed that these proteases aid in the release of the ODVs by degrading the matrix protein polyhedrin (Wood *et al.,* 1980). The co-occluded proteases have also been shown to cleave PIF0 into two fragments of 35 and 40 kDa, which both remain associated with the complex (Peng *et al.,* 2011). The entry complex also prevents further cleavage of PIF0 as this protein was degraded when complex formation was abolished in mutants with other *pif* gene deletions (**Chapter 3**). The importance of this PIF0 cleavage event for midgut infection is presently enigmatic. PIF0 is cleaved for a second time in the gut by host trypsins, which cleave off a 20 kDa piece from the 35 kDa N-terminal fragment (Slack *et al.*, 2005, 2008). This cleavage event has been shown to be important for the infectivity of ODVs (Slack *et al.*, 2008). These two cleavage events possibly activate PIF0 and maybe indirectly also other PIFs by inducing conformational changes in the entry complex, which are important for ODV entry into epithelial midgut cells.

The importance of entry complex formation for ODV binding and fusion

With respect to binding and fusion, different phenotypes have been observed in R18 dequenching assays with various *pif* deletion mutant viruses (Ohkawa *et al.,* 2005). We now know that these mutants are not able to form the complex (Peng *et al.,* 2010). Absence of either PIF0, 1 or 2 affected ODV binding and not fusion (Haas-Stapleton *et al.*, 2004, Ohkawa *et al.*, 2005), while in absence of PIF3 both binding and fusion were unaffected (Ohkawa *et al.*, 2005). The *pif3* deletion mutant virus nevertheless failed to establish a midgut infection. This suggests that formation of the entry complex is not important for the binding and fusion capacities of ODVs, while this was initially expected to be the main function of the complex. To determine the biological importance of the entry complex, apart from protection of the individual PIFs against proteases as demonstrated in **Chapter 3**, the involvement of PIF3 in midgut infection

was further studied by monitoring fluorescently labelled ODVs of a *pif3* deletion mutant in their interaction with the brush borders of isolated epithelial midgut cells of *S. exigua* larvae (**Chapter 5**). The *pif3* mutant ODVs bound to the brush borders, but the nucleocapsids failed to enter the cell, in contrast to capsids of the wild type (labelled) virus. It has been proposed by others that PIF3 is important for translocation of the nucleocapsids to the microvilli (Ohkawa *et al.,* 2005). This possibility was not excluded by our studies as membrane fusion cannot be observed in the assay with ODVs that have fluorescently labelled capsids. We could only see that the capsids did not move from the place of initial interaction with the brush border. An alternative explanation is that only the outer leaflets of the cellular and viral lipid bilayers fused (a hemifusion state), which also has been shown to result in R18 dequenching with influenza and vaccinia mutant viruses (Kemble *et al.,* 1994, Laliberte *et al.,* 2011). The formation of the entry complex would according to this theory be important to continue to a full membrane fusion stage. Furthermore, ODVs of another complex-deficient mutant virus, that lacks PIF9, showed the same phenotype. However, the fusogenic properties of *pif9* mutant ODVs need to be determined by R18 dequenching assays in future research. These experiments nevertheless show that when formation of the entry complex is abolished, the infection process is halted at an early stage at the brush border of the midgut cell as the nucleocapsids then fail to enter the cell.

Is the entry complex supported by other envelope proteins to achieve optimal ODV infectivity?

Various studies showed that the individual PIFs are important for the primary infection of the larval midgut. This thesis examined the relation between complex formation and oral infectivity, in which it was shown that absence or mutation of a single PIF protein abolished complex formation and infectivity of the ODVs (**Chapter 4, 5, 6**). However, mutant viruses with a C-terminal truncated version of PIF1 or 2, which are deficient in complex formation as well, were incidentally able to infect the larvae (**Chapter 6**). It has also been described for various other *pif* mutant viruses that they caused a low incidence of infection (Ohkawa *et al.,* 2005, Fang *et al.,* 2009, Dong *et al.,* 2014, Zhu *et al.,* 2013 and Wang *et al.,* 2019), while these mutants are now known not to be able to form the entry complex (Peng *et al.,* 2010, 2012, Javed *et al.,* 2017, **Chapter 3, 4, 6**). Furthermore, the infectivity of a complex-deficient *pif8* mutant, that lacks the ZF-domain, can be partially rescued by pre-treatment of the larvae with calcofluor white, an agent that damages the peritrophic membrane (Zhu *et al.,* 2013). This suggests that PIF8 targets the peritrophic membrane and that the remaining PIFs exert some (remnant) biological activity, despite the inability to form the entry complex.

An alternative explanation for these low incidence infections is that other ODV envelope proteins support the ODV entry complex and to a certain extent might compensate for a lacking PIF and the affected complex formation in mutant viruses. PIF5 and ODV-E66 for example are also important for ODV oral infectivity (Harrison *et al.,* 2010, Sparks *et al.,* 2011a, b), but are not associated with the entry complex (Peng *et al.,* 2012, Wang *et al.,* 2019). A significant difference between these proteins and the PIFs in the entry complex is that absence of either PIF5 or ODV-E66 results in a quantifiable increase in the lethal dose (LD_{50}: the required dose to reach 50% mortality) of the mutant ODVs, while absence of one of the constituents of the entry complex has such an impact on the oral infectivity that only a single larva might incidentally be infected at a dose that normally would kill all larvae. In **Chapter 7**, the first data have been obtained that PIF5 and ODV-E66 are interaction partners in the ODV envelope, as immunoprecipitation of PIF5 resulted in the co-precipitation of ODV-E66. This PIF5 pull-down also resulted in the precipitation of PIF0 and 1. That only these two PIFs were found to interact with PIF5 and not any other component of the ODV entry complex, suggests that these PIFs also occur outside the entry complex. These three interaction partners of PIF5 are needed for ODV binding (Haas-Stapleton *et al.,* 2004, Ohkawa *et al.,* 2005, Sparks *et al.,* 2011b). However, the binding and fusion capacities of the ODVs of a *pif5* deletion mutant were unaffected (Sparks *et al.,* 2011a), suggesting that the interactions between PIF5 and ODV-E66, PIF0 or PIF1 are not required for ODV binding. The biological importance of these interactions for the infectivity of ODVs therefore remains unclear.

In addition, there are ODV envelope proteins that are only involved in oral infectivity in specific virus-host combinations, adding an extra layer of complexity. AC111 was found important for the infection of *Trichoplusia ni* larvae, but dispensable for the infection of *Spodoptera exigua* (Li *et al.,* 2018). HA100 in the Helicoverpa armigera NPV is also involved in infectivity and only occurs in group II alphabaculoviruses (Luo *et al.,* 2011). Furthermore, the viral glycoprotein GP37 that is known to be important for passage through the peritrophic membrane, might also be involved in membrane fusion as it was recently shown to have fusogenic properties (Liu *et al.,* 2019). The ODV entry complex remains the main actor of ODV oral infectivity in larvae, but to thoroughly understand the infection process of the larval midgut epithelial cells, it is important to also take the other players and the putatively dual functions of the PIF proteins into account.

Comparison of the ODV entry complex with entry complexes of other viruses

The formation of a protein complex that is crucial for virus entry has also been shown for members of the *Herpesviridae* and *Poxviridae* families. The entry complex in herpesviruses consists of three to six different proteins, depending on the virus species. A heterodimer of glycoproteins gH and gL forms the basis of the entry complex and activates a trimer of gB fusion proteins upon receptor binding. The species specific components of the complex establish tissue tropism by the binding of cell-specific host receptors (reviewed by Sathiyamoorthy *et al.*, 2017). Herpes simplex virus for example adds glycoprotein gD to the complex for receptor binding (Gianni *et al.*, 2009, Atanasiu *et al.*, 2010) and the cytomegalovirus (CMV) adds either gO for the infection of fibroblasts (Vanarsdall *et al.*, 2011, Ciferri *et al.*, 2015, Kabanova *et al.*, 2016) or a UL128-131 tetramer to infect epithelial, endothelial and myeloid cells (Ryckman *et al.*, 2006). The finding of a variable complex composition and tropism among the herpes virus species is quite different from what is known from the baculovirus entry complex. The components of the ODV entry complex are highly conserved among baculovirus species and mediate entry into a single cell type, the columnar epithelial cells in the larval midgut. An intriguing similarity, however, is that both complexes contain a component, the gHgL dimer in herpesviruses and PIF0 in baculoviruses, that was shown to be able to function *in trans* during cell entry in experimental setups (Atanasiu *et al.*, 2010, Yao *et al.*, 2004, Slack *et al.*, 2010).

The entry complex in the poxviruses shows more resemblance with the baculovirus ODV entry complex, especially when looking at the features of its components. This complex is formed by small proteins, nine to eleven in total that range from 4.2 to 44 kDa in size, and that have N- or C-terminal transmembrane domains and conserved cysteine residues (Reviewed in Moss *et al.*, 2016). Like most PIFs among baculoviruses, these proteins are highly conserved among the poxviruses. However, the complex in poxviruses mediates only membrane fusion and is not involved in the attachment of virus particles to the host cell. This provides the opportunity to study the fusogenic properties of the complex and its individual components without the risk of affecting virus attachment. This in contrary to the baculovirus entry complex where some essential components mediate ODV binding (Haas-Stapleton *et al.*, 2004, Ohkawa *et al.*, 2005, Mu *et al.*, 2014). Although the absence of a single component also abolished complex formation in poxviruses, just as observed with the entry complex in baculoviruses (**Chapters 4 and 6**), two stages in the fusion process were distinguished (Laliberte *et al.*, 2011). Eight out of eleven components were crucial for fusion as absence of one of them completely blocked membrane

fusion. In contrast to the poxviruses, no baculovirus mutants have been identified in which fusion was totally blocked, not even for mutant ODVs with diminished binding capacities (Haas-Stapleton *et al.*, 2004, Ohkawa *et al.*, 2005). However, in absence of one of the other three components of the poxvirus entry fusion complex, lipid mixing was still observed, although capsid entry and content mixing failed. A two-step fusion model with a hemifusion intermediate was proposed for poxvirus entry, in which the outer leaflets of the lipid bilayers are apparently still able to mix in absence of the entry complex, but the capsids cannot enter due to the failure to form a fusion pore. This is also suggested for ODV entry in midgut cells, since similar results were obtained with the entry complex deficient mutant virus that lacks PIF3 (Ohkawa *et al.*, 2005, **Chapter 5**). Hemifusion with poxviruses was also observed when the host cell membrane was depleted from cholesterol and upon treatment with inhibitors of tyrosine kinases and actin dynamics (Laliberte *et al.*, 2011). These aspects of virus entry are hard to study with baculovirus ODVs, because of the lack of an *in vitro* cell culture system that models the larval midgut under alkaline conditions.

A model for the infection of midgut cells by ODVs.

This PhD thesis showed that the molecular action of the entry complex during ODV entry into midgut cells is hard to dissect because of the intimate collaboration between the PIFs, in which a small mutation of one of the *pif* genes possibly results in secondary effects due to abolished complex formation (**Chapter 4 and 6**). Furthermore, the infection process in the larval midgut might even be more complicated as additional factors are required for a fully infectious virus (**Chapter 7**). Nevertheless, a model of midgut

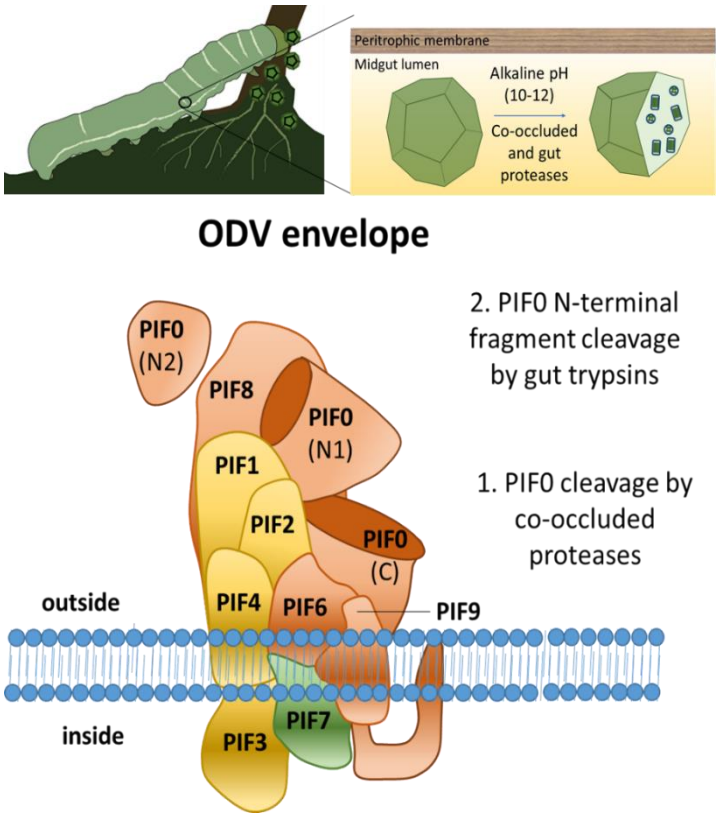

Figure 1: Activation of PIF0 in the midgut by proteolytic cleavage. The ODV entry complex ensures proper cleavage of PIF0 by co-occluded host proteases into two fragments. Both N- and C-terminal fragments remain associated with the complex (Peng *et al.*, 2011). A 20 kDa piece is cleaved off from the N-terminal fragment by trypsins in the gut, generating N1 and N2 (Slack *et al.*, 2008). Whether these fragments remain associated to the complex is unknown.

infection by ODVs will be presented here based on a combination of the experimental results in this thesis and literature data.

In this model, the ODVs are released into the midgut lumen after ingestion of OBs by insect larvae, where these are exposed to digestive enzymes in a highly alkaline environment. Together with the co-occluded proteases in the OBs, the gut proteases facilitate the release of the ODVs by degradation of the polyhedron-shaped protein matrix, in which they are embedded (Payne and Kalmakoff, 1977, Wood *et al.*, 1980). The proteins in the ODV envelope are also subjected to the proteolytic activity by these proteases (Wood *et al.*, 1980), in which the ODV entry complex allows the cleavage of PIF0 into two fragments, but prevents further degradation of PIF0 and degradation of other PIFs (Peng *et al.*, 2011, **Chapter 3**). The two fragments of PIF0 remain associated to the complex (Peng *et al.*, 2011), and the N-terminal fragment is further processed by trypsins by cleaving off a 20 kDa piece from the N-terminal fragment into N1 and N2 (**Fig. 1**) (Slack *et al.*, 2005, 2008). The trypsin-mediated cleavage is important for ODV infectivity (Slack *et al.*, 2008). A two-step cleavage model has been proposed in which PIF0, and may be the associated PIFs as well, are activated for binding and fusion with the epithelial cell brush border (Peng *et al.*, 2011). However, after release of the ODVs into the gut lumen, the virions first need to pass the peritrophic membrane before reaching the epithelial cells. The peritrophic membrane is possibly targeted by PIF8 (**Fig. 2**), as the infectivity of PIF8 mutant ODVs was partially rescued when damaging this membrane with calcofluor white (Zhu *et al.*, 2013). PIF5 interaction partner ODV-E66 has recently also been shown to be involved in this as well (Hou *et al.*, 2019).

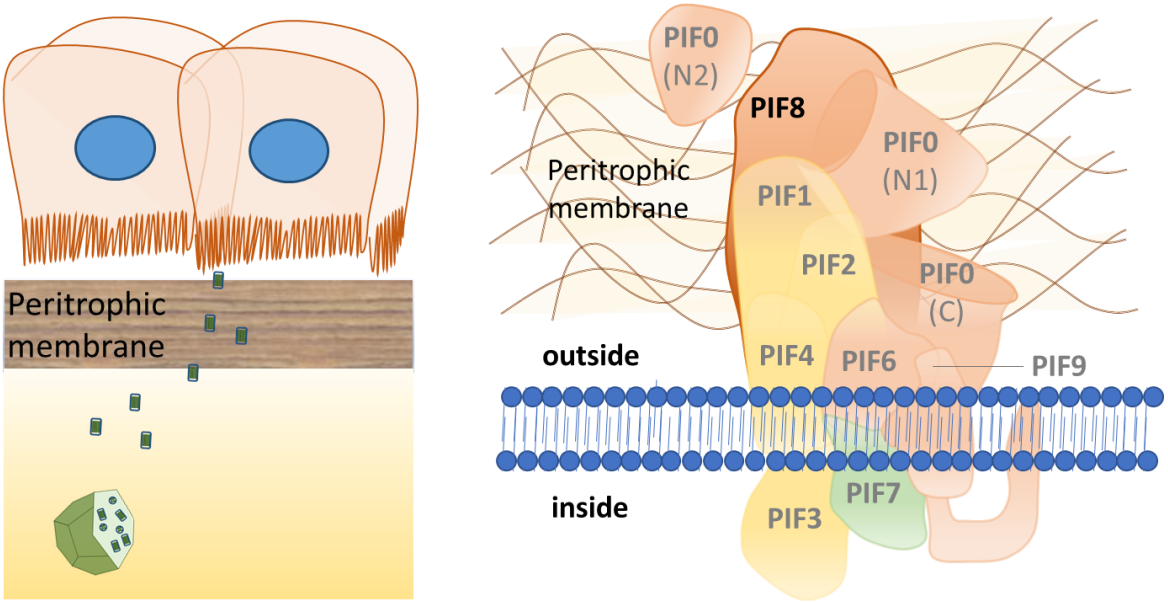

Figure 2: PIF8 is involved in passage of the peritrophic membrane

Once the ODVs have passed the peritrophic membrane, the proteolytically activated PIF0 mediates ODV binding together with PIF1 and 2 via the interaction with an unknown receptor (**Fig. 3a**) (Haas *et al.*, 2004, Ohkawa *et al.*, 2005). Formation of the entry complex is not required for this binding, as bound ODVs were found with two different complex deficient mutants, lacking either PIF3 or PIF9 (**Chapter 5**). The molecular determinants of fusion are rather enigmatic as no *pif* mutants have been identified where fusion was totally blocked, even when binding was affected (Haas-Stapleton *et al.*, 2004, Ohkawa *et al.*, 2005). However, mixing of the outer leaflets of the lipid bilayers might be relatively easy due to the curvature of the microvillus membrane that partially overcomes the repellent nature of biological membranes (Wilson *et al.*, 1998). Furthermore, this hemifusion state also results in a fluorescent signal in R18 dequenching assays with other virus mutants (Kemble *et al.*, 1994, Laliberte *et al.*, 2011), while R18 dequenching was considered as indicative for complete fusion in the previous studies with baculoviruses. PIF3 is important for capsid entry into the midgut cells, either by completion of the fusion process by aiding in the mixing of the inner leaflets of the lipid bilayers, or by supporting the translocation of the nucleocapsids from the ODV to the host cell cytosol (**Fig. 3b**) (Ohkawa *et al.*, 2005, **Chapter 5**). The nucleocapsids of another complex deficient mutant, lacking PIF9, also failed to enter the midgut cell. Therefore, it is proposed for this model that the ODV entry complex mediate midgut infection by providing a scaffold to combine the PIFs that are involved in binding and fusion to facilitate the entry of nucleocapsids into epithelial midgut cells.

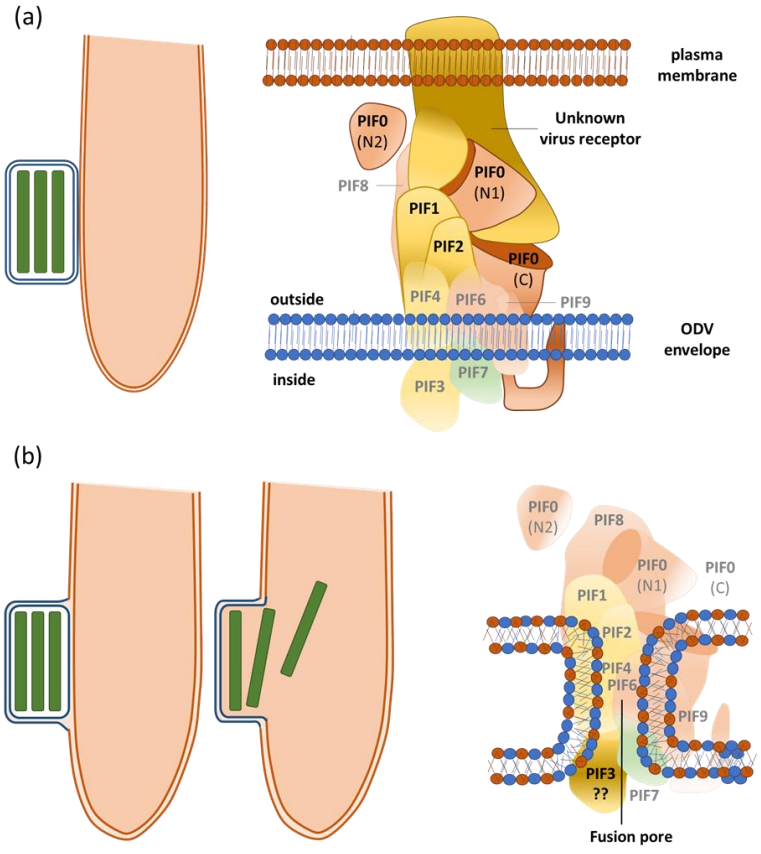

Figure 3: PIF0, 1 and 2 are important for the binding of an unknown receptor in the plasma membrane on the host cell's microvilli (a). PIF3 is important for the entry of the nucleocapsids by either completion of membrane fusion (to pass the hemifusion state) or by translocation of the capsids into the host cell (b).

The infection process in the midgut is probably supported by other ODV envelope proteins, in which PIF5 and ODV-E66 have fine tuning or

regulatory functions during midgut entry. Because of the results from the proteomic study in **Chapter 7**, it is tempting to speculate that these proteins regulate capsid entry by changing the stoichiometry of PIF0 and 1 in the entry complex. To create a fusion pore that is large enough to facilitate the passage of the capsids from the virion to the host cell, fusion events of multiple entry complexes might be required as it has also been shown that clustering of fusion complexes in the vaccinia virus is important for the fusion efficiency (Gray *et al.*, 2018). Once the fusion pore is created, the actin remodelling complex in the base of the nucleocapsids possibly remodels the actin filaments inside the microvillus and clear the road to the nucleus for the first round of replication (Braunagel *et al.*, 2001, Goley *et al.*, 2006, Hepp *et al.*, 2018).

Concluding remarks and future perspectives

The research presented in this thesis provides further information on the characteristics of the ODV entry complex concerning its composition (**Chapter 4**), important protein interactions (**Chapter 6**) and how the complex relates to other viral proteins in the ODV envelope (**Chapter 7**). It was also shown why such a complex is formed (**Chapter 3**) and how complex formation relates to the infectivity of ODVs (**Chapter 5 and 6**). The formation of such a complex complicates the interpretation of earlier research that investigated the biological function of the various PIFs as outlined in **Chapter 2**, since the observed phenotypes are possibly the result of secondary effects instead of being indicative for the function of that particular PIF. Furthermore, the identification of a complex-deficient mutant virus that binds and fuses with the host cell as wild type (Ohkawa *et al.*, 2005), but nevertheless fails to establish midgut infection (The *pif3* deletion mutant), already hinted to the complexity of the infection process in the larval midgut at the start of this research. Our research showed that this complex is indeed not an easy object to study as the PIFs were degraded by host proteases in *pif* mutant viruses (**Chapter 3**) and absence or minor truncation of a single component abolished complex formation (**Chapter 4 and 6**). This indicates that the current experimental approaches are not suitable to dissect the molecular mechanisms of the complex as there is not much room to play around without the risk of introducing secondary effects.

Studying the molecular mechanism of baculovirus oral infectivity without affecting complex formation might be accomplished by the use of nanobodies that inhibit the function of one of the components without the need of mutagenic approaches. Nanobodies are single-domain antigen binding proteins, which are derived from camelid immunoglobulins that only consists of heavy chains. Nanobodies have been used before in the structural and functional

characterisation of fusion proteins in various human viruses as the respiratory syncytial virus and norovirus (Datalle *et al.*, 2016, Palomo *et al.*, 2016, Wu *et al.*, 2017, Rossey *et al.*, 2017, Koromyslova and Hansman, 2017). Nanobodies might also be suitable in the research to the molecular mechanism of the ODV entry complex as well as their small size (15 kDa) allows binding into small cavities in a protein (complex) surface that cannot be reached by conventional antibodies (Reviewed in Desmyter *et al.*, 2015).

Furthermore, the study towards the molecular mechanisms of ODV midgut infection is hampered by the lack of an *in vitro* cell culture system, in which the alkaline conditions of the larval midgut can be simulated. The production of intestinal organoids have been shown to be useful to analyse norovirus and rotavirus infections (Yin *et al.*, 2015, Ettayebi *et al.*, 2016). The development of analogous systems with lepidopteran midgut cells could facilitate further studies to unravel the midgut infection process by ODVs. However, the entry of norovirus and rotavirus could be studied under cell culture conditions, while ODV entry into lepidopteran midgut cells requires highly alkaline conditions (pH 8-10), which are not compatible with conventional cell culturing.

Virus entry does not only require the presence of viral binding and fusion proteins, also the composition of the viral lipid membrane is important for entry into an host cell. The presence of cholesterol in the virus envelope is crucial for membrane fusion for a wide variety of viruses as the influenza virus (Sun and Whittaker, 2003), human herpesvirus 6 (Huang *et al.*, 2006), gastroenteritis virus (Ren *et al.*, 2008), hepatitis C virus (Aizaki *et al.*, 2008) and dengue virus (Carro and Damonte, 2013). The ODV envelope also contain high levels of cholesterol (Braunagel and Summers, 1994). However, the importance of cholesterol for baculovirus ODV entry into midgut cells and its importance for formation of the entry complex is currently unexplored. Experiments with ODVs produced in cholesterol depleted insect cells can complement current knowledge on the requirements for the infection of midgut cells by ODVs.

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List of abbreviations

Viruses

AcMNPV	Autographa californica multiple nucleopolyhedrovirus
AgMNPV	Anticarsia gemmatalis multiple nucleopolyhedrovirus
BmNPV	Bombyx mori nucleopolyhedrovirus
CMV	Cytomegalovirus
CuniNPV	Culex nigripalpus nucleopolyhedrovirus
HearMNPV	Helicoverpa armigera multiple nucleopolyhedrovirus
SfMNPV	Spodoptera frugiperda multiple nucleopolyhedrovirus
SpltNPV	Spodoptera litura nucleopolyhedrovirus

General

ABC	ammonium bicarbonate
AP-buffer	alkaline phosphatase buffer
BBMV	brush border membrane vesicle
BiFC	bimolecular fluorescence protein complementation assay
BN-PAGE	blue native-PAGE
bp	base pair
BV	budded virus
cat	chloramphenicol acetyl transferase resistance gene
CBD	chitin-binding domain
C-OB	cell culture-derived OB
C-ODV	cell culture-derived ODV
CoIP	co-immunoprecipitation
del	deletion
dpi	days post infection
dpt	days post transfection
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
EPDA	end point dilution assay
FBS	fetal bovine serum
GP64	glycoprotein of 64 kDa, BV fusion protein
HA-tag	influenza virus hemagglutinin tag

INM-SM	inner nuclear membrane sorting motif
kDa	kilo dalton
LC-MS/MS	liquid chromatography mass spectrometry
L-OB	larva-derived OB
L-ODV	larva-derived ODV
MNPV	multiple nucleopolyhedrovirus
MOI	multiplicity of infection
mut	mutant
nt	nucleotide
OB	occlusion body
ODV	occlusion-derived virus
ORF	open reading frame
pFBD	pfast bac dual plasmid
PIF	*per os* infectivity factor
Polh	polyhedrin
R18	octadecyl rhodamine
rep	repair
SDS	sodium dodecyl sulphate
SNPV	single nucleopolyhedrovirus
$TCID_{50}$	tissue culture infective dose of 50%
VP39	viral protein of 39 kDa, the baculovirus major capsid protein
Wt	wild type
w/v	weight per volume
w/w	weight per weight
Y2H	yeast-two hybrid assay
ZF-domain	zinc finger domain

Summary

Insect larvae are infected by baculoviruses when they consume plant material that is contaminated with occlusion bodies (OBs). OBs are polyhedral shaped particles of crystallized protein (polyhedrin or granulin), in which the occlusion derived viruses (ODVs) are embedded. Upon ingestion of the OBs, the ODVs are released in the alkaline lumen of the midgut and infect the midgut epithelial cells. To be infectious for these cells under alkaline conditions, the ODV envelope contains a set of proteins, which are called *per os* infectivity factors or PIFs. Eight different PIFs had been identified at the start of this research, of which six were known to participate in the formation of a large complex: the ODV entry complex. Presently, ten different PIFs are known, of which nine form the entry complex. This complex has a stable core that is formed by PIF1 to 4, which even resists treatment with denaturing and reducing agents (SDS and β-mercaptoethanol), and five additional PIFs (PIF0 and PIF6 to 9), which are associated to the complex more loosely. PIF5 is the only PIF-protein that is not a constituent of the entry complex.

Most studies to the functional properties of the PIFs were conducted when it was not known yet that these proteins form a complex. **Chapter 2** therefore reviewed earlier research data concerning these proteins, now taking into account that they are part of the same complex.

Chapter 3 provided a first clue on why complex formation is important for the individual PIFs. Our initial studies with *pif* deletion mutant viruses were hampered by the disappearance of not only the targeted, but also non-targeted PIFs, when the OBs were produced in insect larvae, while the various PIFs were all detectable for the wild type virus. However, those remaining PIFs were found again in these mutant viruses when the larval derived OBs were treated with heat prior to ODV isolation, or when the OBs were produced in cultured insect cells. These observations showed that proteases from the host, which had been reported to be co-occluded in the OBs, are able to degrade PIF proteins when complex formation is interrupted, as was the case in the various *pif* deletion mutant viruses. Analyses of mutant ODVs that lack one of the loosely associated PIFs, PIF0 or PIF6, but are still able to form the stable core, showed that the PIFs in the stable core were slightly more resistant to proteolytic degradation than the same PIFs in monomeric form. However, when the stable core lacks PIF4 and a smaller complex is formed by PIF1, 2 and 3, this resistance was completely lost. It was concluded that complex formation by PIF proteins is required to resist proteolytic degradation by host proteases, which

might be important to cope with the hostile environment in the larval midgut that contains lots of digestive enzymes.

Previous research in our laboratory had identified AC108 as a PIF1 interaction partner in a co-immunoprecipitation study, indicating that this protein might be involved in oral infectivity as well. **Chapter 4** demonstrated that mutation of the *ac108* gene from the AcMNPV genome completely abolished the oral infectivity of ODVs, which was recovered when this gene was repaired. Furthermore, protein analysis of the *ac108* mutant virus and an HA-tagged repair virus showed that AC108 is a loosely associated but nevertheless an essential component of the ODV entry complex. This protein was therefore designated as PIF9.

The biological function of PIF9 was further investigated in **Chapter 5** by the generation of fluorescently labelled ODVs of the *pif9* mutant. The ODVs were made fluorescent by the incorporation of eGFP:VP39 fusion proteins in the nucleocapsids. The fluorescent ODVs were then combined with isolated midgut cells of *Spodoptera exigua* larvae and monitored by confocal microscopy in a time lapse experiment. It was observed that in absence of PIF9, the ODVs were still able to bind to the midgut cell brush border, but that the nucleocapsids failed to enter the cell, in contrast to fluorescent ODVs that have all PIFs. Fluorescent ODVs were also generated for a *pif3* deletion mutant as it had been shown by others with R18 dequenching assays that this (complex deficient) mutant is able to bind and fuse with brush border membrane vesicles as the wild type virus, but nevertheless fails to establish a midgut infection. Our analysis with the confocal microscope showed that this mutant displays the same phenotype as the *pif9* deletion mutant virus: ODV binding occurred, but entry of the nucleocapsids was blocked. As ODVs of the *pif3* deletion mutant had been reported to be fusogenically active in the R18 de-quenching assays, we proposed a two-step fusion process, in which first the outer leaflets of the lipid bilayers fuse and subsequently the inner leaflets. Fusion of the outer leaflets had been shown by others with other mutant viruses to result in R18 dequenching as well and might explain why despite the observed fusogenic activity, infection of the midgut fails with ODVs that lack PIF3. This PIF might therefore instead of important for translocation of the nucleocapsids as proposed by others be important to complete the fusion process by aiding fusion of the inner leaflets of the lipid bilayers.

PIF1 and 2 had been reported to be important for ODV binding and were later also shown to be crucial for the formation of the ODV entry complex. It was asked whether these two PIFs are directly or indirectly involved in interaction with the host. As ODV binding is one of the viral

host range determinants and most baculoviruses have a narrow host range, it was hypothesized that the more variable C-terminal parts of PIF1 and 2 are important for host interaction, while the highly conserved N-terminal parts are needed for complex formation. **Chapter 6** aimed to distinguish those two functions by analysing a series of mutant ODVs with C-terminally truncated versions of one of those PIFs. Limited C-terminal truncation of either PIF1 or 2 abolished formation of the entry complex, while the stable core was still being formed. These findings were supported by co-immunoprecipitation studies with PIF1 antiserum. These studies confirmed the interaction between PIF1 and 2 in the core of the truncation mutants, but the interactions between PIF1 and the largest loosely associated PIFs (PIF0 and 8) were lost in those mutants. This shows that the truncated parts of PIF1 and 2 are important for the binding of at least PIF0 and 8 to the stable core of the ODV entry complex. It was also found that in absence of PIF4, 6 or 8, PIF0 was still able to bind the core or the smaller complex formed by PIF1, 2 and 3, while the other loosely associated PIFs requires each other's presence to bind the entry complex. The lost ability to form the entry complex in the truncation mutants was accompanied by a severely hampered oral infectivity in *S. exigua* larvae. However, some larvae incidentally got infected by one of the truncation mutants despite abolished complex formation, which also had been reported by others with various other *pif* mutant viruses. It was therefore speculated that although the ODV entry complex is the main determinant for the infectivity of ODVs, additional factors are present in the ODV that is able to incidentally cause infections in absence of the entry complex.

The context in which the PIFs function was investigated in **Chapter 7** by construction of a so-called PIF-interactome. Three different loosely associated components of the entry complex (PIF6, 8 and 9) were immunoprecipitated and the co-immunoprecipitated proteins from the ODV envelope were identified by mass spectrometry. These experiments were also performed for PIF5, the only PIF that is not part of the entry complex. These analyses revealed that the PIFs interact with a plethora of viral proteins with a wide variety of functions, from nucleocapsid assembly to OB formation. Immunoprecipitation of PIF5 also resulted in the co-precipitation of two components of the entry complex, PIF0 and 1. This indicated that PIF0 and 1 are not only present in the ODV envelope as part of the entry complex, but also occur outside the complex where these proteins interact with PIF5. ODV-E66 was also identified as a PIF5 interaction partner and this protein was also found after the immunoprecipitation of PIF6 and 9. These findings are in line with other studies that showed that ODV-E66 is important for the ODV oral infectivity and that this protein interact with various PIF proteins. This study shows

that the PIFs not only interact with each other to form the entry complex, but are part of a network of protein interactions in ODVs. The biological significance of these interactions for midgut infection remains enigmatic.

Chapter 8 discussed the findings in a broader perspective and compared the ODV entry complex in baculoviruses with two other known viral entry complexes that occur in members of the *Herpesviridae* and *Poxviridae*. Also an infection model was presented, in which, based on the studies of this PhD thesis and literature data, the roles of the PIFs were integrated. At last, possible directions for future research were discussed.

Samenvatting

Insectenlarven worden door baculovirussen geïnfecteerd als ze met polyeders besmet bladmateriaal eten. Polyeders zijn door het virus gevormde deeltjes die bestaan uit een veelvlakvormige matrix van gekristalliseerd eiwit (polyhedrine of granuline) waarin de infectieuze virusdeeltjes zijn ingebed. De polyeders vallen na consumptie uiteen in het alkalische milieu van de middendarm van de insectenlarf, waardoor de virusdeeltjes vrijkomen en vervolgens de darmepitheelcellen infecteren. Om deze cellen te kunnen infecteren bevat het lipide membraan van de virusdeeltjes een specifieke groep eiwitten genaamd *per os* infectiefactoren (PIFs). Aan het begin van dit onderzoek waren er acht verschillende PIFs bekend, waarvan er zes betrokken zijn bij de vorming van een eiwitcomplex: het PIF-complex. Inmiddels zijn er tien verschillende PIFs bekend, waarvan er negen deel uitmaken van dit complex. Vier PIFs, PIF1, 2, 3 en 4, vormen een stabiele kern van dit complex dat bestand is tegen behandeling met denaturerende en reducerende stoffen. De overige vijf PIFs (PIF0, PIF6, 7, 8 en 9) binden met lagere affiniteit aan het complex en kunnen alleen onder niet-denaturerende omstandigheden in het complex gevonden worden. PIF5 is het enige PIF-eiwit dat geen deel uitmaakt van het complex.

De meeste studies naar de biologische functies van de verschillende PIFs bij de infectie van het darmepitheel waren uitgevoerd toen het nog niet bekend was dat ze samen een complex vormen. Deze studies worden daarom in **hoofdstuk 2** besproken met in ogenschouw neming van het feit dat dat de PIFs deel uitmaken van hetzelfde eiwitcomplex.

De experimenten die in **hoofdstuk 3** beschreven staan gaven voor het eerst een idee waarom de PIFs eigenlijk een complex vormen in het ODV-membraan. Uit eiwitanalyses van mutantvirussen met een deletie van één van de *pif*-genen bleek namelijk dat niet alleen dat betreffende PIF-eiwit afwezig was, maar dat ook de andere PIF-eiwitten, waarvan het gen intact is, niet gedetecteerd konden worden, als het virus was geproduceerd in insectenlarven. De overige PIFs werden echter wel gevonden als dezelfde virusmutanten waren geproduceerd in gekweekte insectencellen of als de in larven-geproduceerde polyeders waren verhit vóórdat de ingebedde virusdeeltjes gezuiverd werden met behulp van een alkalische buffer. Deze observaties wijzen erop dat proteasen van de gastheer, die ook worden ingebed in de eiwitmatrix van de polyeders, in staat zijn om individuele PIF-eiwitten af te breken als het complex niet gevormd wordt. Uit analyses van virusmutanten waarin de stabiele kern van het PIF-complex nog gevormd werd, bijvoorbeeld in de afwezigheid van één van de losse

componenten PIF0 of PIF6, bleek dat de PIFs als onderdeel van de stabiele kern minder snel werden afgebroken dan dezelfde PIF in niet-geassocieerde vorm. Dit effect was echter verdwenen wanneer PIF1, 2 en 3 samen een kleinere kern vormen in afwezigheid van PIF4. Op basis van deze observaties werd geconcludeerd dat de PIF-eiwitten een complex vormen om bestand te zijn tegen de proteolytische activiteit van gastheerproteasen, wat mogelijk belangrijk is voor behoud van de biologische activiteit van de virusdeeltjes in de middendarm alwaar veel digestieve proteasen aanwezig zijn.

Voorgaand onderzoek in dit laboratorium identificeerde het AC108-eiwit als interactiepartner van PIF1. Dit duidde er op dat dit virale eiwit mogelijk ook betrokken is bij de perorale infectie van de gastheer door het virus. **Hoofdstuk 4** liet zien dat een virus met een mutatie van het *ac108*-gen niet meer infectieus was via de orale route. Perorale infectie was echter weer volledig hersteld als dit gen gerepareerd was. Uit eiwitanalyses bleek dat dit eiwit een essentieel component is van het PIF-complex; d.w.z. dat in afwezigheid van AC108 het PIF-complex niet gevormd werd. Na aanleiding van deze experimenten werd AC108 aangeduid als PIF9.

De biologische functie van PIF9 werd verder onderzocht in **hoofdstuk 5** met fluorescerende virusdeeltjes van de *pif9* virusmutant. De virusdeeltjes werden fluorescerend gemaakt door het inbouwen van een eGFP:VP39 fusie-eiwit in de capsiden van het virus. De fluorescerende virusdeeltjes werden vervolgens gemengd met geïsoleerde epitheelcellen uit de middendarm van de gastheer (*Spodoptera exigua* larven) en geobserveerd met een confocale laserfluorescentie microscoop. Hieruit bleek dat in afwezigheid van PIF9 de virusdeeltjes nog wel in staat waren om aan de microvilli van de darmepitheelcellen te binden, maar dat de capsiden de cel niet konden binnendringen in tegenstelling tot die van het wildtype virus. Dit experiment werd ook uitgevoerd met virusmutanten waarin het PIF3-eiwit ontbrak. Ondanks dat het PIF-complex ook in deze mutant niet gevormd werd, bleek uit eerdere studies naar membraanfusie waarin het membraan van de virusdeeltjes zijn geladen met een fluorescerende stof (R18), dat deze virusmutant desondanks instaat is het virale membraan te kunnen laten fuseren met het membraan van de gastheercel. Echter vond geen infectie van het darmepitheel van de gastheer plaats. Uit onze microscopische analyses met fluorescerende capsiden bleek dat deze virusmutant hetzelfde fenotype had als de hiervoor beschreven *pif9* virusmutant: wel binding aan de microvilli maar geen binnendringing van de capsiden. De uitkomsten van deze twee studies ondersteunen een model waarin fusieproces bestaat uit twee stappen, waarbij eerst de buitenlagen van de twee membranen fuseren en daarna de binnenlagen. Hieruit werd

geconcludeerd dat PIF3 mogelijk belangrijk is voor het fuseren van de binnenlagen van de membranen tijdens de infectie van de darmepitheelcellen.

PIF1 en 2 zijn belangrijk voor de bindingscapaciteit van de virusdeeltjes aan de darmepitheelcellen van de gastheer en bleken later ook essentieel voor de vorming van het PIF-complex. Dit leidde tot de vraag of deze twee PIF-eiwitten direct of indirect met de gastheercellen interacteren. Omdat de bindingscapaciteit van de virusdeeltjes medebepalend is of het virus wel of niet bepaalde insectensoorten kan infecteren en de meeste baculovirussen slechts één of enkele insectensoorten kunnen infecteren werd verondersteld de meer variabele C-terminale delen van PIF1 en 2 betrokken zijn bij gastheerinteractie en dat de sterk geconserveerde N-terminale delen betrokken zijn bij de vorming van het PIF-complex. In **hoofdstuk 6** werd deze hypothese getoetst door een panel virusmutanten te analyseren die C-terminaal verkortte PIF1- of 2-eiwitten bevatten. Het ontbreken van zeer kleine delen van de C-terminale uiteinden van PIF1 of 2 resulteerde al in een deficiëntie om het PIF-complex te vormen. De stabiele kern werd echter nog wel gevormd in deze virusmutanten. Deze bevindingen werden ondersteund door co-immunoprecipitatie studies met antiserum tegen PIF1, waaruit bleek dat na afknotting van PIF1 of 2 deze twee PIFs nog aan elkaar konden binden, maar dat de losse componenten van het PIF-complex (PIF0 en 8) niet meer direct of indirect aan PIF1 konden binden. Blijkbaar zijn de afgeknotte C-terminale uiteinden van PIF1 en 2 belangrijk voor de binding van tenminste twee losse componenten van het PIF-complex, PIF0 en 8, aan de stabiele kern van het PIF-complex. Deze studies lieten ook zien dat in afwezigheid van PIF4, 6 of 8, PIF0 zich onafhankelijk aan de stabiele kern kan binden, terwijl de andere losse componenten van het PIF-complex elkaars aanwezigheid nodig hebben om het complex te vormen. De deficiëntie om het PIF-complex te vormen na afknotting van PIF1 of 2 ging gepaard met een sterke afname van de perorale infectie van *S. exigua* larven. Enkele larven bleken echter geïnfecteerd te zijn door de virusmutant, wat ook beschreven is in de literatuur voor diverse andere baculovirus *pif*-mutanten. Op basis hiervan werd gespeculeerd dat naast het PIF-complex ook andere virale factoren betrokken zijn bij de infectie van de middendarmcellen.

Om te bepalen in welk onderling verband de PIF-eiwitten functioneren in het virale membraan is er in **hoofdstuk 7** een zogenaamde “PIF-interactome” gereconstrueerd, waarin de interactiepartners van verschillende PIFs in kaart zijn gebracht. Hiervoor zijn drie losse componenten van het PIF-complex, PIF6, 8 en 9, geprecipiteerd en zijn de interactiepartners van deze eiwitten geïdentificeerd met massaspectrometrie. Deze analyses waren ook uitgevoerd

na precipitatie van PIF5, het enige PIF-eiwit dat geen deel uitmaakt van het complex. PIF0 en 1 werden als enige twee componenten van het PIF-complex geïdentificeerd als interactiepartner van dit eitwit, wat erop duidt dat deze twee PIFs in het viraal membraan ook buiten het PIF-complex voorkomen en dan aan PIF5 binden. ODV-E66 bindt ook aan PIF5 en dit eiwit werd ook gevonden na precipitatie van PIF6 en 9. Deze resultaten komen overeen met studies van anderen waaruit bleek dat ODV-E66 belangrijk is voor de perorale infectie en hierbij interacteert met diverse PIF-eiwitten. Daarnaast bleek uit deze analyses dat de verschillende PIF-eiwitten, met name PIF9, interacteren met vele andere virale (niet-PIF) eiwitten in het membraan van de virusdeeltjes. Deze eiwitten hebben uiteenlopende functies: van vorming van de capsiden tot inbedding van de virusdeeltjes in de eiwitmatrix van de polyeders. De biologische functie van deze interacties zijn echter nog onduidelijk.

De resultaten van dit onderzoek werden in een bredere context bediscussieerd in **hoofdstuk 8**, waarin het PIF-complex van baculovirussen werd vergeleken met twee andere virale eiwitcomplexen in virussen die behoren tot de *Herpesviridae* en *Poxviridae* en essentieel zijn voor de infectie van gastheercellen. Daarnaast werd er een infectiemodel gepresenteerd, waarin de resultaten van onze studie en uit de literatuur geïntegreerd werden. Tot slotte werden mogelijkheden voor verder onderzoek voorgesteld en bediscussieerd.

Acknowledgements - Dankwoord

In maart 2015 begon dit avontuur dat na ruim 4.5 jaar hard werken resulteert in de totstandkoming van dit proefschrift. Deze tijd is ongelofelijk snel voorbij gegaan en ik had deze ervaring voor geen goud willen missen. Nu rest het mij om alle mensen die in deze periode een belangrijke rol hebben gespeeld te bedanken.

Allereerst wil ik Monique en Jan bedanken voor het gegeven vertrouwen en de vrijheid die jullie mij gaven om naar eigen inzicht invulling aan dit onderzoek te geven. Ik weet nog dat ik bij jullie op sollicitatiegesprek kwam en daardoor mijn interesse in het project verder aanwakkerde en dat ik na afloop dacht: hieraan wil ik werken. Gelukkig dachten jullie er ook zo over ondanks dat ik geen enkele virologische ervaring had en begon ik een ander halve maand later aan mijn PhD-project over het baculovirus *per os* infectie complex.

Monique, ik wil jou in het bijzonder bedanken voor jouw aanstekelijke enthousiasme en inspiratie, en dat je deur altijd openstond als ik even iets wilde weten. Het tempo waarin je mijn stukken nakeek is zeer bewonderenswaardig en zorgde voor een vloeiend verloop van het publicatieproces van de verschillende artikelen. Daarnaast bracht je mij altijd in contact met de juiste mensen als ik specifieke expertise nodig had.

Jan, afgelopen jaren hebben we intensief samengewerkt, waarbij je mij hebt geleerd om kritisch te denken en vooral naar simpele oplossingen te zoeken. Daarnaast bewonder ik jouw taalgevoel, waarvan ik tijdens het schrijven van de verschillende stukken bijzonder veel van heb opgestoken. Ook denk ik met veel plezier terug aan de practica van het vak Fundamentals and Applied Virology, die we gegeven hebben met Dick Lohuis en wijlen Hanke Bloksma. Dat waren gezellige perioden waarvoor ik het andere werk graag even aan de kant zette. Naast het harde werken en de wetenschappelijke discussies denk ik ook met veel plezier terug aan de gezellige lunches (en natuurlijk de ambachtelijke frietjes) en onze discussies over de actualiteit waarbij we de controversiële onderwerpen niet uit de weg gingen. Kortom, jij hebt mijn PhD-periode kleur gegeven. Bedankt daarvoor!

Vera, hartelijk bedankt voor de raad en daad die je mij afgelopen jaren hebt gegeven. Ik bewonder je scherpe oog voor detail dat goed van pas kwam als we de presentaties gingen oefenen voor de SIP-meeting. Ook aan het geven van de practica pestmanagement denk ik met plezier terug.

Els, jij staat altijd voor iedereen klaar en zorgde ervoor dat alles op het lab ordelijk verliep. Daarnaast zorgde je voor de reagentia en het kweken van de cellen en insecten. Zonder jouw ondersteuning was dit werk dan ook niet mogelijk geweest! Ook was je altijd in voor een praatje en zorgde je voor een goede sfeer op het lab. Bedankt voor de fijne samenwerking.

Han, you were my buddy in the lab and helped me a lot when I started my research at virology. You were always there when I had a question, even when it was already late. It was a pleasure to work with you and I wish you all the best in your future career!

Simone: it was a pleasure to work with you in the lab, especially during the practicals of Fundamentals and Applied Virology and with the organization of the international dinner. You have a great attitude and I will never forget the radio-show “Danske Bingo” that you like to listen in the lab. As I don’t speak Danish, you always explained me the jokes the Disc-jockeys made so I can appreciate the Danish sense of humor. Also the trip to Valencia for the SIP-meeting was a lot of fun. I also want to thank you for being my Paranymph. Tak igen for alt og held og lykke med resten af din ph.d.!

I also like to thank my students, Kevin, Coen, Fabiola, Alexander, Maarten, Felix, Wendy, Jorn, Priyanka and Justin, who participated in my PhD-project for their efforts. It was a pleasure to supervise you. I have learned a lot from being your supervisor and this work would not be possible without your accomplishments! I hope you have enjoyed it as much as I did and I wish you all the best for your future careers.

It is very important for me to work in a vivid environment and my time at virology would not be the same without the people who together shape the virology department: Andre, Christina, Corien, Corinne, Dick, Fengqiao, Gabriela, Giel, Gorben, Haidong, Irene Meki, Irene van Grindsven, Jelke, Just, Magda, Marcel, Marleen, Mia, René, Richard, Sandra and Tessy. Thanks a lot for the nice coffee breaks (with lots of pie) and the fun we have had during the international dinners and Labtrips!

Het laatste hoofdstuk van dit proefschrift met de proteomics analyse was niet mogelijk geweest zonder Sjef Boeren van het Laboratorium voor Biochemie. Sjef, hartstikke bedankt voor het meedenken met dit experiment, de massa spectrometrie metingen en de analyses.

Ik wil graag Claudius van de Vijver en Lennart Suselbeek van de PE&RC graduate school bedanken voor de leerzame PE&RC dagen en weekenden. Ik vond het altijd leuk om hierbij aanwezig te zijn. Ook wil ik de PE&RC graduate school bedanken voor het beschikbaar

stellen van strategische middelen wat mij instaat stelde extra onderzoek te doen in samenwerking met het Laboratorium voor Biochemie.

I also want to thank the members of the virus division of the Society of Invertebrate Pathology (SIP) for giving me the opportunity to share my thoughts and ideas with international experts. I was able to attend at four of those meetings, of which the first was in Tours in 2016. Each year I looked forward to attend these meetings and it was an honour to serve as student representative for two years. I especially want to thank Prof. Dr Zhihong Hu (Rose), Dr Manli Wang, Dr David Theilmann and Dr Martin Erlandson for their interest and fresh views on my work, which really helped my work forward.

Tot slotte wil ik mijn steun en toeverlaat bedanken: Basia, toen ik begon aan dit traject mijn vriendin en nu mijn vrouw. Jij hebt mij altijd gesteund als ik het druk had en in het weekend nog moest werken. Daarbovenop heb je ook nog veel geregeld voor onze bruiloft (wat een onvergetelijk dag was) en heb je de verbouwing van ons eerste eigen huis in goede banen geleid. Daarom mag je niet aan mijn zijde ontbreken als paranymph bij de verdediging van dit proefschrift. Schat, bedankt voor de steun van afgelopen jaren en ik vind het geweldig de rest van mijn leven met je te mogen delen.

Bob Boogaard

Vlaardingen

29 oktober 2019

About the author

Bob Boogaard was born on April the 3rd 1987 in Zeist. After finishing primary and secondary school, he studied Biomedical Sciences at the VU University in Amsterdam and obtained his BSc-degree in 2012 by completing his thesis entitled: "The effects of small molecule TMC207 on mortality and ATP homeostasis in *Mycobacterium bovis* BCG". He continued his studies with a Master in Biomolecular Sciences, also at the VU University, in which he gained more fundamental knowledge on molecular mechanisms underlying health and disease states. In this track, he followed two internships, in which he studied paracetamol-induced starvation responses in yeast cells, at the Molecular Toxicology department of the VU, and the constitutive signaling activity of adhesion-GPCR GPR64 at the department of Medicinal Chemistry at Leiden University. In 2015, he started his PhD-project on the molecular action of a protein complex that is formed by baculovirus *per os* infectivity factors (PIFs), which is essential for the infection of moths and butterflies via the oral route. In this project, he studied the composition of this complex and obtained more insight on the protein interactions that are required to form such a complex. He also studied with which other viral proteins the PIFs interact in the virus envelope and performed functional studies with fluorescently labeled *pif* mutant viruses on isolated midgut cells. In addition to doing research, he was also involved in education as PGO-tutor in the course Cell Biology and Health and as practical assistant in the course Fundamental and Applied Virology. Furthermore, he supervised ten thesis students, who participated in his research. Bob was also a student representative of the virus division of the Society of Invertebrate Pathology (SIP), in which he organized and moderated student meetings during the annual SIP-meetings. After finishing this PhD-study, he will start as a postdoctoral researcher in the group of Prof. Dr Ron Fouchier at the Erasmus MC to study the aero stability of various influenza viruses under supervision of Dr Sander Herfst.

Account

Boogaard, B., Van Lent, J. W. M., Theilmann, D. A., Erlandson, M. A., & van Oers, M. M. (2017). Baculoviruses require an intact ODV entry-complex to resist proteolytic degradation of *per os* infectivity factors by co-occluded proteases from the larval host. *Journal of General Virology*, 98, 3101-3110.

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PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)
- An advanced view on baculovirus per os infectivity factors

Post-graduate courses (3.7 ECTS)
- Advanced proteomics; VLAG (2015)
- Advanced statistics course design of experiments; PE&RC (2017)
- Basic transmission electron microscopy; WUR (2019)

Invited review of (unpublished) journal manuscript (2 ECTS)
- Applied and Environmental Microbiology: second type of resistance against CpGV
- Journal of General Virology: microscopic examination of AcMNPV infection in the larval midgut

Deficiency, refresh, brush-up courses (2.3 ECTS)
- Molecular virology; Virology (2015)
- Better Safe Than Sorry (BSTS); Jakob Laegdsmand (2015)

Competence strengthening / skills courses (4 ECTS)
- Competence assessment; WGS (2016)
- Essentials of writing and presenting; WGS (2016)
- Scientific writing; WGS (2017)
- Scientific artwork, vector graphics and images; WUR (2018)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.7 ECTS)
- PE&RC First years weekend (2015)
- PE&RC Day (2015-2017)
- PE&RC Midterm weekend (2017)

Discussion groups / local seminars / other scientific meetings (4.5 ECTS)
- Wageningen evolution and ecology seminars (2015-2018)
- Dutch annual virology symposium (2015-2019)

International symposia, workshops and conferences (15 ECTS)
- Viruses: host pathogen interactions; poster presentation (2016)
- Society of invertebrate pathology; oral and poster presentations (2016-2019)
- European congress of virology; poster presentation (2019)

Lecturing / supervision of practicals / tutorials (4.5 ECTS)
- Cell biology and health; PGO (2016-2018)
- Fundamental and applied virology (2016-2019)

Supervision of MSc students
- Construction and characterisation of PIF truncation mutant viruses
- Identification of a putative new PIF protein in AcMNPV
- Monitoring of fluorescent virus particles on isolated midgut cells

The research described in this thesis was financially supported by The Netherlands Organisation of Scientific Research (NWO), grant number: 824.14.16, and by strategic means of the Graduate School for Production Ecology and Resource Conservation (PE&RC).

Cover design: Electron microscopic picture of a baculovirus occlusion body in the midgut of a *Spodoptera exigua* larva, made by Dr Jan W. M. van Lent at the Wageningen Electron Microscopy Center.

Printed by GVO drukkers & vormgevers, Ede, The Netherlands