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# The deletion of the *pif* gene improves the biosafety of the baculovirus-based technologies

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## Abstract

Our goal was to improve the biosafety of baculovirus-based technologies by deleting the *pif* (per os infectivity factor) gene from baculovirus expression vectors. Such a deletion would block transmission in nature without disturbing protein production. A *pif* deletion mutant of *Autographa californica* multiple capsid nucleopolyhedrovirus (AcMNPV) was constructed and its infectivity to two host species was tested by oral or intrahemocoelic inoculation. Virus replication after oral inoculation was monitored using PCR. Oral inoculations with a mixture of the wild type and the *pif* deletion viruses were carried out. The *pif* deletion blocked oral infection but it did not hamper infectivity in cell culture. The blockage took place early after inoculation and could not be overcome by mixed inoculations with the wild type. The *cat* gene was inserted under the control of the polyhedrin promoter in the deletion mutant and the wild type CAT yield was measured in *Spodoptera frugiperda* insect cells (Sf9) infected with either recombinant. The *pif* deletion did not hamper CAT production. This deletion significantly improved CAT yields early in the infection. Hence, expression vectors lacking *pif* may produce higher quality protein. The *pif* deletion is a simple measure that dramatically reduces the chances of virus spread or gene transfer in nature.

*Keywords:* Biosafety; Baculovirus; *pif*; Oral infection

## 1. Introduction

Currently, the public and scientific opinion is concerned by the risks associated with the use of genet-

ically engineered organisms (GEOs). In the case of genetic engineered pathogens, the major issues are horizontal gene transfer, infection of non-target hosts and displacement of indigenous pathogen populations (NRC, 2004). The concern triggered by these potential problems has promoted the development of genetic engineering strategies, among others, to obtain the con-

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finement of GEOs, especially of those that can cross or recombine with wild species (Daniell, 2002; Hare and Chua, 2002; NRC, 2004).

Baculoviruses are enveloped, double-stranded DNA viruses which infect specifically arthropods and, among them, especially lepidopteran insects (butterflies and moths). These viruses, in particular *Autographa californica nucleopolyhedrovirus* (AcMNPV), are extensively used for the expression of recombinant proteins in insect cells (King and Possee, 1992; O'Reilly et al., 1992). The baculovirus expression system (BES) provides high expression levels and almost all the post-translational processing events found in mammalian systems (Kidd and Emery, 1993). Recently, another application of baculoviruses as gene delivery vectors is being developed (reviewed by Kost and Condreay, 2002).

From a biosafety point of view, the BES has several attractive features and a perfect safety record. These viruses cannot replicate in vertebrate cells although they can be taken up by them (Kost and Condreay, 2002; Leisy et al., 2003). Furthermore, proteins can be produced without animal supplements as foetal calf serum. Finally, a biological containment strategy is inherent to the BES. Most of the baculovirus vectors are constructed by replacing the polyhedrin or the *p10* coding regions by the gene of interest (Kidd and Emery, 1993). The deletion of polyhedrin or *p10* reduces the environmental fitness of the recombinant viruses and, thus, serves as containment measure (Vlak and Hu, 1997).

However, these deletion mutants retain their oral infectivity to their natural hosts (Wood et al., 1993; Bianchi et al., 2000) and the possibility of recombinant baculoviruses being carried out from the laboratory by invertebrates has been addressed (O'Reilly et al., 1992). In such scenario, gene transfer may take place in the environment. Homologous baculoviruses are highly recombinant in vitro (Croizier and Ribeiro, 1992; Martin and Weber, 1997; Hajos et al., 2000) and recombinant baculoviruses can recombine with wild relatives in vivo (Merryweather-Clarke et al., 1994). Host transposable elements have been identified in baculovirus genomes and can mediate genetic transfer between baculoviruses (reviewed by Friesen, 1993).

Furthermore, certain modifications of baculovirus vectors have involved the insertion of genetic infor-

mation, which might give a selective advantage to the recombinant or alter its host range. For example, hybrid baculoviruses containing human-virus genomes or baculovirus with improved infection efficiencies have been created (Yap et al., 1997; Delaney and Isom, 1998; Fipaldini et al., 1999; Tani et al., 2003). Although the utilisation of recombinant baculoviruses seems safe, recombinants with modifications as the ones mentioned above may require further confinement measures to avoid their possible replication in the environment in case of accidental release.

The deletion of baculovirus genes required for host infectivity is a confinement measure that would avoid virus replication in nature (Vlak and Hu, 1997). Candidate genes for deletion should be essential for host infection but unnecessary for replication and protein production in cell culture. Such genes are present in the baculovirus genome as a result of the unusual life cycle of these viruses.

During the baculovirus cycle, two types of virions are produced. The first type, the budded virion (BV), is responsible for the cell-to-cell spread of infection whereas the second type is responsible for the transmission between hosts. The second type of virion is embedded in the occlusion body, a protective proteinaceous matrix. This type of virion is named pre-occluded virion (POV) before their embedding in the occlusion body (Wood et al., 1993) and occlusion derived virion (ODV) when it is liberated from the occlusion body by digestion.

Both BVs and ODVs/POVs are produced in all infected cells, in a timely regulated pattern. Budded virions are produced earlier in the infection. They bud out from the infected cell into the insect hemolymph or into the culture medium, spreading the infection to other cells. Later in the infection, virions of the second type are produced in the nucleus where they accumulate and the occlusions bodies are formed (Granados and Lawler, 1981). Baculovirus infection eventually leads to cell lyses and host death. The occlusion bodies are then liberated in the environment. New hosts become infected while feeding on food contaminated with occlusion bodies. Once in the host midgut, the occlusion bodies are dissolved, liberating the ODVs. These virions infect the midgut epithelial cells by attaching and fusing the viral envelope with the cell membrane (Horton and Burand, 1993). Infection of the midgut cells yields BVs and

the infection spreads all through the host (Federici, 1997).

The BV is practically non-infectious to insects when orally administered (Volkman, 1997) but it is highly infectious to cells in culture and essential for the virus cycle in vitro. On the contrary, the second type of virion hardly infects cell culture or cells other than the midgut cells (Volkman, 1997). Most baculovirus vectors used in biotechnological applications do not produce occlusion bodies because they lack the gene coding for the main protein of the occlusion body. In such cases, the second type of virion is not occluded and only POVs are produced. POVs are less persistent in the environment due to the lack of a protective structure but they are fully infectious (Wood et al., 1993). Therefore, ODVs or POVs have little or no utility during the virus cycle in vitro but they could launch infection if ingested by a host. In principle, the genes exclusively involved in the infectivity of ODVs/POVs could be deleted to block oral infectivity without affecting virus replication and recombinant protein production in cell culture.

Several authors have suggested the use of vectors lacking such genes to increase the biosafety of the BES (Vlak and Hu, 1997; Pijlman et al., 2003). However, to our knowledge, no such vector has been examined. One possible candidate gene is *pif* (per os infectivity factor). The *pif* gene has been characterised in the *Spodoptera littoralis* NPV (SpliNPV). SpliNPV *pif* codes for an ODV protein essential for oral infection but not required for BV infectivity in *S. littoralis* larvae (Kikhno et al., 2002). The presence of genes homologous to SpliNPV *pif* in all the sequenced baculoviruses suggests that its role is conserved among this family of viruses. Therefore, the deletion of this gene seems an attractive confinement measure.

Our aim in this study was to improve the biosafety of the baculovirus-based technologies by constructing a vector lacking oral infectivity. Since AcMNPV is the baculovirus most commonly used as expression vector, we have constructed an AcMNPV vector which lacks the *pif* gene.

In this report, we first show that the deletion of AcMNPV *pif* blocked oral infection as previously observed with SpliNPV *pif*. Secondly, we verified that the deletion of *pif* did not hamper the production of the reporter protein chloramphenicol acetyltransferase (CAT) in cell culture. Surprisingly, *pif* deletion sig-

nificantly promoted CAT yield in early times in the infection.

## 2. Materials and methods

### 2.1. Cell line, larvae and viruses

*Spodoptera frugiperda* insect cells (Sf9) (Vaughn et al., 1977) were maintained at 28 °C in TC100 medium supplemented with 5% fetal calf serum. *S. frugiperda* and *S. littoralis* larvae, two AcMNPV hosts widely distributed, were obtained from our laboratory colony and reared as previously described (Kikhno et al., 2002). Infected larvae were reared individually. Wild type AcMNPV clone 1.2 (AcMNPV-1.2) (Croizier et al., 1988) and recombinant baculoviruses were propagated in Sf9 cells. Occlusion bodies of AcMNPV-1.2 and Ac $\Delta$ *pif* were isolated from virus-killed *S. frugiperda* larvae as described (Croizier and Ribeiro, 1992).

### 2.2. Plasmid transfer vectors

#### 2.2.1. p269

This vector was used to obtain an AcMNPV mutant with a deletion of the *pif* coding region. p269 contains the *pif* region with a deletion of the open reading frame *pif* (ORF119). A *Bam*HI site has been inserted at the deletion site. To obtain p269, a plasmid vector containing the *pif* region was created. The ORF *pif* was then deleted using a PCR approach. First, the *Eco*RI restriction fragment from AcMNPV-1.2 containing from ORF114 to ORF127 was ligated into plasmid pSP70 (Promega). DNA fragments corresponding to the sequences upstream and downstream from *pif* were generated using PCR. The PCR product encompassing 301-bp upstream from the ORF *pif* was obtained with FOR119A (5'-CCAAGAATCCCGTACAACGTTAAATGTTGATACGCATTATTAC) and BAC119A (5'-GGACAAGCTTCAGTATAATAGCAAAAATGGATCCTACT) primers. The PCR product containing 213-bp downstream from the *pif* stop codon was obtained with the FOR119B (5'-CTTGAACTTACCCCAACTACTCTGGATCCCAACATGAG) and BAC119B (5'-GGACAAGCTTCTCTGTAGT T TCTTGTTTAACATGACTGCCAC) primers. Both PCR products were ligated and the resulting fragment cloned in the previously modified pSP70 vector to yield p269. The

construction was verified by sequencing (Genome Express).

### 2.2.2. p274

This plasmid was used to create a *pif* deletion AcMNPV producing blue plaques and, thus, easily isolable. p274 originates from p269. The polyhedrin promoter and the *Escherichia coli LacZ* coding sequence were inserted in the *Bam*HI site of p269.

### 2.2.3. p299

This transfer vector was used to obtain recombinant viruses expressing CAT. The *cat* gene was inserted in the polyhedrin locus and under the control of the polyhedrin promoter. The *cat* coding sequence was obtained by digestion of the plasmid pBLCAT2 (Luckow and Schutz, 1989) with *Bgl*III and *Kpn*I. This fragment was ligated into the polyhedrin transfer vector pGmAc1 16T (Poul et al., 1995), yielding p299.

## 2.3. Generation of recombinant baculoviruses

Sf9 cells were cotransfected with viral DNA and a transfer vector using the lipofection reagent DOTAP (Roche). All recombinant viruses were isolated from the transfection supernatant through three rounds of plaque purification. Virus stocks were generated by propagating viruses in Sf9 cells (75 cm<sup>2</sup> flasks) and titrated using end-point dilution assays (King and Possee, 1992). For all the recombinants, the presence of the different inserts at the right locus was verified using restriction enzyme digestion and Southern blot analysis (King and Possee, 1992) (data not shown).

The construction of Ac $\Delta$ *pif*, an AcMNPV mutant with a deletion of the *pif* coding region, was done in two steps. First, a recombinant virus expressing *LacZ*, Ac $\Delta$ *pif*-*LacZ*, was obtained after cotransfection of Sf9 cells with AcMNPV-1.2 DNA and p274. Ac $\Delta$ *pif*-*LacZ* was plaque purified by selecting plaques staining blue in the presence of X-Gal (King and Possee, 1992). Then, Ac $\Delta$ *pif*-*LacZ* DNA and p269 were cotransfected in Sf9 cells to obtain Ac $\Delta$ *pif*. Ac $\Delta$ *pif* was purified from the parental virus by selecting plaques, which did not turn blue after X-Gal staining. Sequencing confirmed that the cassette containing the polyhedrin promoter and the *lacZ* coding region had been deleted from the *pif* locus of Ac $\Delta$ *pif*.

Ac $\Delta$ *pif*-CAT and AcCAT, two recombinants expressing *cat* under the control of the polyhedrin promoter, were constructed by cotransfecting Sf9 cells with the p299 vector and Ac $\Delta$ *pif* DNA or AcMNPV-1.2 DNA, respectively. These recombinants were purified by selection of the occlusion body-negative plaques.

## 2.4. Oral infectivity of Ac $\Delta$ *pif* to lepidopteran larvae

The oral infectivity of occlusion bodies of Ac $\Delta$ *pif* and AcMNPV-1.2 to *S. frugiperda* and *S. littoralis* larvae was determined using the droplet feeding technique (Hughes and Wood, 1981). Cohorts of 24 neonates of each species were fed with four doses of virus suspensions (10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup> and 10<sup>8</sup> occlusion bodies/ml). Larva mortality was recorded until all larvae had either died or pupated. Death larvae were checked for virus infection by microscope observation. All experiments were repeated at least three times.

The presence of viral DNA in orally-inoculated *S. frugiperda* and *S. littoralis* larvae was determined in a time-course experiment. Newly-moulted second-instar larvae were fed with a virus suspension containing 10<sup>8</sup> occlusion bodies per ml of Ac $\Delta$ *pif* or AcMNPV-1.2. Although neonates are more susceptible to baculovirus infection, second-instar larvae were chosen because their bigger size facilitated subsequent experiments. Fifty larvae per virus and host species were processed for DNA isolation at 24 and 48 h post inoculation (p.i.). Before 24 h p.i., the colorant diluted in the virus suspension was excreted with faecal pellets. We assumed that the virus inoculum followed a similar pattern. Larvae of the same species and infected with the same virus were separated in cohorts of ten. Each cohort was then grinded separately in 500  $\mu$ l 1  $\times$  TE. The resulting suspension was treated with 200  $\mu$ l SDS 10% and 100  $\mu$ l proteinase K (10  $\mu$ g/ $\mu$ l). After digestion, debris was removed by centrifugation and DNA purified by sequential phenol, phenol-chloroform, and chloroform extractions. Then, DNA was ethanol precipitated and quantified by spectrometry. The presence of viral DNA in each cohort was examined using PCR. PCR amplifications were performed with the Expand High Fidelity System (Roche) and the primers Acpif150up (5'-ACTGACAGATCTATGGAGATAAT-TAAAATGATAACC) and Acpif150down (5'-CTAG-CAGGATCCATTTATAGTTTTTTTATTAC). These

primers were located 150-bp upstream and 150-bp downstream from the *pif* coding region, respectively. When using these primers, a PCR product of 300 bp or 1.9 kb is obtained when using Ac $\Delta$ *pif* or AcMNPV-1.2 DNA as a template, respectively.

## 2.5. Complementation assays

Mixtures of occlusion bodies of Ac $\Delta$ *pif* and AcMNPV-1.2 were fed to *S. frugiperda* larvae so as to determine whether Ac $\Delta$ *pif* occlusion bodies were orally infectious when co-inoculated with wild-type AcMNPV occlusion bodies. Occlusion bodies from Ac $\Delta$ *pif* and AcMNPV-1.2 were mixed in equal proportions to obtain a suspension of  $2 \times 10^7$  occlusion bodies/ml. This suspension was fed to newly-moulted second-instar larvae using the droplet-feeding method (Hughes and Wood, 1981). One hundred virus-dead larvae were separated in groups of five. The virus progeny of each group was isolated and the viral DNA was extracted as described by (Hunter-Fujita et al., 1998). The presence of Ac $\Delta$ *pif* and AcMNPV-1.2 DNA in the DNA of the virus progeny was analysed using PCR. The primers Acpif150up and Acpif150down (see above) were used to check for the presence of Ac $\Delta$ *pif* DNA. Preliminary PCR experiments with these primers showed that very low proportions of Ac $\Delta$ *pif* DNA (1%) could be detected in template mixtures of Ac $\Delta$ *pif* and AcMNPV-1.2 DNA. In fact, PCR only yielded the Ac $\Delta$ *pif* DNA derived amplification unless AcMNPV-1.2 DNA proportion in the template was over 60% (data not shown). As a result, we used the primers Acpifright (5'-TTTTGTGCAACTGCTTACGG) and Acpifleft (5'-CCACATGACGCAACGATAAC) to specifically detect AcMNPV-1.2 DNA. These primers were internal to the *pif* coding sequence and amplified a 561-bp PCR product.

## 2.6. BV injections

To assess susceptibility to systemic infection, *S. frugiperda* larvae were injected with a BV suspension (cell-culture supernatant). Twenty plaque forming units (p.f.u.) of Ac $\Delta$ *pif*, AcMNPV or a mixture of each virus in equal proportions were injected into the haemocoel of third instar larvae (12 larvae per virus) with a sharp-needle. Larva mortality was recorded until all larvae

had either died or pupated. Virus offspring and DNA were isolated from each dead larva and analysed using PCR as described above.

## 2.7. CAT expression assays

Sf9 cells ( $4 \times 10^6$  per 25 cm<sup>2</sup> flask) were inoculated with a BV suspension (5 p.f.u./cell) of Ac $\Delta$ *pif*-CAT, AcCAT or AcMNPV-1.2. After 1 h of adsorption at room temperature, the viral inoculum was removed and fresh culture medium was added. Cells were further incubated at 28 °C. Samples ( $4 \times 10^6$  cells per virus and time point) were collected at 2 days and 4 days p.i. The amount of CAT enzyme present within the cells was determined with the CAT ELISA kit (Roche) following manufacturer's instructions. Infections and CAT quantifications were done in triplicate. A one-way analysis of variance was used to test for CAT-level differences between Ac $\Delta$ *pif*-CAT- and AcCAT-infected cells at each time point (SPSS software, SPSS Inc.).

## 3. Results

### 3.1. *pif* deletion effects on AcMNPV oral infectivity

We compared the oral infectivity of occlusion bodies of Ac $\Delta$ *pif* and AcMNPV-1.2 in *S. frugiperda* and *S. littoralis* neonates. None of the larvae inoculated with Ac $\Delta$ *pif* died from virus infection at any of the doses (Table 1). On the contrary, AcMNPV-1.2-induced mortality levels ranged approximately from 20 to 100%. Similar results were obtained with POVs of the CAT re-

Table 1  
Average virus-induced mortalities observed after oral inoculation of *Spodoptera frugiperda* and *Spodoptera littoralis* neonates with different doses of Ac $\Delta$ *pif* and AcMNPV-1.2 occlusion bodies (OB)

OB (ml)	AcMNPV-1.2		Ac $\Delta$ <i>pif</i>	
	<i>frugiperda</i> (%)	<i>littoralis</i> (%)	<i>frugiperda</i> (%)	<i>littoralis</i> (%)
10 <sup>8</sup>	100	89	0	0
10 <sup>7</sup>	96	60	0	0
10 <sup>6</sup>	67	28	0	0
10 <sup>5</sup>	25	19	0	0

Twenty-four larvae of each species were infected per experiment and each experiment was repeated three times.

combinants. *AcΔpif*-CAT POVs did not kill any larva whereas larvae which had fed on AcCAT POV suspensions became infected and died (data not shown). Next, we compared the infectivity of AcMNPV-1.2 and *AcΔpif* using BV injections in *S. frugiperda* larvae. We found that larvae were sensitive to both viruses at roughly the same levels (92% mortality for *AcΔpif*, 85% for AcMNPV-1.2). In addition, we did not observe any substantial difference between the replication of AcMNPV-1.2 and *AcΔpif* in Sf9 cell culture. These data show that the deletion of the *pif* gene prevented AcMNPV oral infection in two host species. Moreover, this deletion does not seem to have effect on BV infectivity. These results are consistent with previous observations on the role of *pif* during SpliNPV infection of *S. littoralis* larvae (Kikhno et al., 2002).

We examined the progression of *AcΔpif* and AcMNPV infections in *S. frugiperda* and *S. littoralis* larvae after oral inoculation using PCR. Table 2 summarizes the results of the PCR analysis. We did not detect *AcΔpif* DNA in any cohort of larvae. At 24 h p.i., AcMNPV DNA was detected in one cohort out of five for each host species whereas all the cohorts were AcMNPV DNA positive at 48 h p.i. These results indicate that the *pif* deletion probably blocks oral infection at a very early stage.

### 3.2. *pif* deletion mutants are not complemented by wildtype virus during oral inoculation

To investigate whether AcMNPV-1.2 occlusion bodies can complement *AcΔpif* occlusion bodies during oral inoculation, we fed *S. frugiperda* larvae with a mixture of occlusion bodies from both viruses. We collected the virus progeny from the virus-killed lar-

Table 2  
Detection of viral DNA in orally-inoculated larvae at 24 and 48 h post inoculation (p.i.) using PCR

Viral DNA	24 h p.i.		48 h p.i.	
	<i>frugiperda</i>	<i>littoralis</i>	<i>frugiperda</i>	<i>littoralis</i>
AcMNPV-1.2	1	1	5	5
<i>AcΔpif</i>	0	0	0	0

Each number corresponds to the number of cohorts of larvae which were found to contain viral DNA. Five cohorts were tested for AcMNPV-1.2 or *AcΔpif* DNA for each time point and host species. Each cohort consisted of ten *Spodoptera frugiperda* or *Spodoptera littoralis* larvae.

vae and we examined this progeny for the presence of DNA of each virus using PCR. A sample of the results is presented in Fig. 1A. We did not detect *AcΔpif* DNA in none of the 20 samples (each sample = viral DNA from five larvae) whereas AcMNPV-1.2 DNA was detected in all the samples. We injected larvae with a mixture of BV from AcMNPV-1.2 and *AcΔpif* to determine if these viruses could replicate together in vivo. The virus progeny obtained from the dead larvae was isolated. PCR analysis of the virus offspring revealed the presence of DNA of both viruses (Fig. 1B). Hence, both viruses could replicate together when injected but AcMNPV-1.2 could not complement *AcΔpif* in oral infections.

### 3.3. *pif* deletion effects on recombinant protein production

We have examined the effect of *pif* deletion on the production of a recombinant protein with the BES. Results are shown in Fig. 2. CAT was readily detected in cells infected with the *pif* deletion recombinant, *AcΔpif*-CAT, at both sampling times. Surprisingly, CAT yield was significantly higher 2 days after infection in *AcΔpif*-CAT-infected cells than in AcCAT-infected cells ( $p=0.02$ ). *AcΔpif*-CAT-infected cells produced over twice the CAT amount compared to AcCAT-infected cells at this time point. The difference in CAT amounts was not significant at 4 days after infection ( $p=0.08$ ). Nevertheless, *AcΔpif*-CAT-infected cells produced around 15% more CAT than AcCAT-infected cells. CAT yield increased substantially with time for both viruses. This is consistent with previous observations on CAT expression with the BES (Carbonell et al., 1985; King et al., 1991). This increase in CAT yield was less marked for *AcΔpif*-CAT-infected cells than for AcCAT-infected cells (double and almost four times, respectively). Our results show that the deletion of *pif* did not interfere with the expression of a recombinant protein. Moreover, this deletion significantly increased protein yield early in the infection.

## 4. Discussion

The main purpose of this study was to improve the biosecurity of technologies that use baculoviruses. We aimed to do so by constructing an expression vector in-

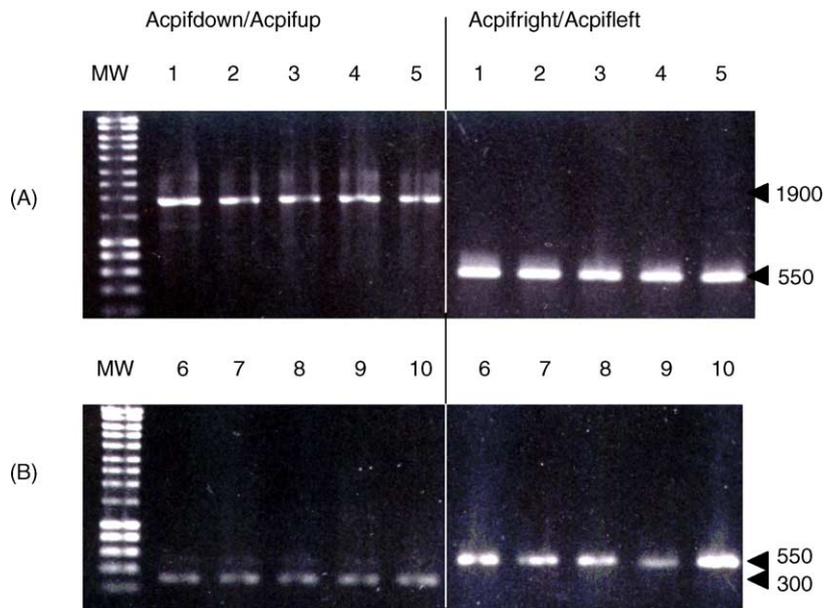


Fig. 1. PCR analysis of the virus offspring isolated from *Spodoptera frugiperda* larvae inoculated with a mixture of AcMNPV-1.2 and Ac $\Delta$ pif. Two different PCRs were performed with each DNA sample: PCR with the primers Acpifdown and Acpifup (on the left of the central line) or PCR with the primers Acpifright and Acpifleft (on the right of the central line). PCR products sizes (in base pairs) appear on the left. (A) PCR products of viral DNA isolated from orally-infected larvae. Each DNA sample (lanes 1–5) was obtained from five virus-killed larvae. (B) PCR products of viral DNA isolated from larvae infected by injection. Each lane corresponds to a PCR performed with the viral DNA obtained from one larvae (lanes 6–10). MW: molecular weight marker Smartladder (Eurogentec).

capable of oral infection, the common way of infection in natural conditions. This vector could not replicate and spread in nature, reducing potential environmental risks.

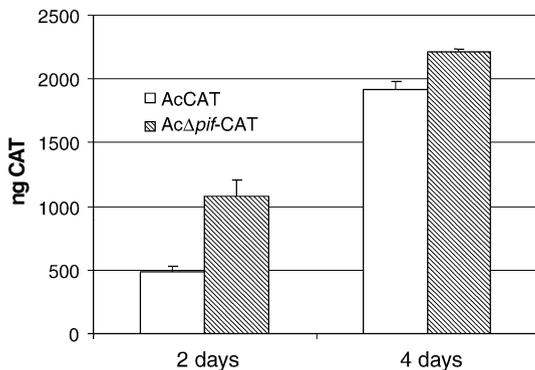


Fig. 2. CAT yields obtained with two baculovirus vectors in  $4 \times 10^6$  Sf9 cells (multiplicity of infection = five plaque forming units per cell). CAT quantities were measured at 2 and 4 days after infection. Each point represents the mean and the standard error of the mean of three repetitions.

Our findings show that the deletion of the *pif* gene of AcMNPV, the most used baculovirus expression vector, prevents oral infection of two host species. When orally administered, the *pif* deletion mutant failed to yield productive infections. Moreover, this mutant did not replicate within two different hosts or replicated at levels undetectable by PCR. The blockage caused by the deletion could not be overcome by co-inoculation with a wild-type AcMNPV. Nevertheless, the *pif* deletion mutant was fully infectious when administered by injection or in cell culture. Similar results have been obtained with an AcMNPV *pif* deletion mutant in *Trichoplusia ni* larvae (R. Possee, personal communication). Thus, the role of AcMNPV *pif* seems restricted to oral infection as previously determined for SpliNPV *pif* (Kikhno et al., 2002). The deletion of AcMNPV *pif* reduces dramatically the chances of replication and transmission in natural conditions but it does not seem to affect virus performance in cell culture.

SpliNPV PIF has been only detected in the ODV envelope (Kikhno et al., 2002). This location suggests that

PIF plays a role during ODV attachment and/or fusion with midgut cell membranes. AcMNPV PIF location is not known yet but the conservation of a transmembrane domain in the different PIF proteins (Kikhno et al., 2002) suggests that AcMNPV PIF might have the same location as SpliNPV PIF. Our results indicate that AcMNPV *pif* deletion blocked infection soon after inoculation, which agrees with a role of PIF in ODV infection of midgut cells. A blockage at this stage would imply that the viral DNA is not liberated from the nucleocapsid, and that the virus does not replicate in any host cell. Such blockage would be desirable in biosafety strategies because the probability of gene transfer becomes extremely low.

The common role of *pif* in two baculovirus species and the presence of *pif* homologues in all the genomes of the sequenced baculovirus suggest a conservation of the *pif* role among baculoviruses. Thus, the deletion of *pif* may block oral infection in other hosts and other baculoviruses used as expression vectors.

Once the *pif* deletion vector was shown to be non infectious per os, we examined the effect of the deletion on protein production. Not only the deletion had no negative effect on CAT yield but CAT yield was significantly higher at early times in the infection (a two-fold increase). We have not studied the mechanisms involved in these surprising findings. A possible explanation may come from previous work on SpliNPV *pif* expression. Although SpliNPV *pif* is essential for the virus in nature, it is expressed at very low levels (Gutiérrez et al., 2004). A hypothesis for this low expression is that high PIF levels may have adverse effects on the virus cycle. In that case, PIF might somehow interfere with protein production; above all at early times in infection when PIF is synthesized in the cytoplasm before being transported to the nucleus for ODV formation.

Recombinant protein processing can be inefficient late in the baculovirus infection (Jarvis, 1997). Higher levels of expression at earlier times of the infection are, thus, desirable to limit inefficient post-translational modification. The expression pattern obtained with the *pif* deletion vector followed the desired trend. Hence, the use of the *pif* deletion vector might yield higher quality protein although we have not verified this hypothesis.

This study proves that the deletion of the *pif* gene is an efficient confinement measure that minimizes the

possibility of spread of the recombinant AcMNPVs used in biotechnology and of the genetic information they carry. Furthermore, this measure is easily feasible and cheap. We recommend, thus, the use of this measure in the biotechnological applications of the baculoviruses. Although the role of *pif* is likely to be conserved, it may be possible that the oral infection of certain AcMNPV hosts does not require this gene. The *pif* deletion vector should be tested with other hosts in order to check the validity of our approach. In addition, more work on the specific function of AcMNPV PIF is required to confirm the blocking step suggested by the presented results. Besides the advantages from a biosafety point of view, the deletion of *pif* seems to increase recombinant protein production early in infection. Such production pattern may yield higher quality protein. This result should be further studied by examining the expression of other proteins with the *pif* deletion vector.

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