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Engineering of Biosafe Baculoviruses with Improved Insecticidal Properties: Developments and Prospects

Hu Zhihong Just M. Vlak*

(*Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, 430071 P. R. China*)

*(*Department of Virology, Wageningen Agricultural University, Wageningen, The Netherlands*)

Dedicated to professor Xie Tian-en

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具有生物安全性的杆状病毒杀虫剂基因工程技术的发展与前景*

胡志红 Just M. Vlak*

(中国科学院武汉病毒研究所,武汉 430071)

*(*Department of Virology, Wageningen Agricultural University, Wageningen, The Netherlands*)

提要 杆状病毒作为杀虫剂在世界各地已被广范应用,但与化学农药相比,杆状病毒具有杀虫速度慢,对高龄害虫需用量大,杀虫谱窄等缺点。随着基因工程技术的发展,从80年代末期起,科学家开始尝试对杆状病毒的遗传性状进行各种分子生物学改造,以获得更优良的病毒杀虫剂。近年来这方面的研究已取得了可喜进展,同时重组病毒的安全性也引起了世界范围的广泛关注。因此,研制既有优良杀虫性能,又有生物安全性的重组病毒,已成为当今病毒杀虫剂的发展方向。本文及时地总结了重组杆状病毒杀虫剂研究的历史,从提高病毒杀虫速度、增强病毒杀虫毒性、以及病毒宿主特异性等三个方面进行了系统归纳,并对重组病毒的安全性进行科学地分析。重点阐述了新时期研制具有生物安全性的重组病毒的各项基因工程策略,如对重组病毒进行混合包装、前包装;或生产缺陷型的重组病毒(*p10*基因,*p74*基因,*egt*基因,*pp34*基因的缺失)等。这些措施将把重组病毒对环境可能造成的危害控制在最小范围。理想的重组病毒杀虫剂应具有杀虫快、杀虫谱广、不危害其它生物、在环境中滞留时间短等特点。

关键词 杆状病毒,杀虫剂,基因工程,生物安全性

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通讯作者:胡志红

INTRODUCTION

Baculoviruses are viral pathogens that cause fatal disease in insects, mainly in members of the families Lepidoptera, Diptera, Hymentoptera and Coleoptera, and in crustaceans. More than 800 baculovirus isolates (Family **Baculoviridae**) have been described and categorized in two genera: **Nucleopolyhedrovirus** and **Granulovirus** (formerly classified together as subfamily Eubaculovirinae). These two genera comprise the nucleopolyhedroviruses (NPV) and the granuloviruses (GV). The non-occluded baculoviruses (formerly the subfamily **Nudibaculovirinae**) and occurring in insects and crustaceans, e.g. shrimp, are now no longer classified awaiting more detailed characterization[1]. Depending on whether the nucleocapsids are packaged singly (S) or in multiples (M) the viruses are designated SNPV of MNPV. Nineteen baculoviruses have received species status including *Autographa californica* MNPV, *Spodoptera exigua* MNPV, *Lymantria dispar* MNPV, *Heliothis zea* SNPV and *Trichoplusia ni* GV. The other viruses are tentative species and their names are derived from the first two letters of the Linnaean insect genus and species names, e.g. Busu NPV for *Buzura suppressaria* NPV[1]. An updated list of baculovirus isolates will become available as part of the Ecological Database of the Worlds Insect Pathogens (Internet: http://www.inhs.uiuc.edu).

Baculoviruses are highly specific for insects and can cause epizootics in nature which reduce the size of insect populations. These viruses are therefore recognized as attractive biological control agents of insect pests in agriculture and forestry as alternatives to chemical insecticides. Baculoviruses have been successfully used in the control of a variety of pest insects, including velvetbean caterpillar, tea moth, codling moth, pine beauty moth, cotton bollworm, Douglas fir tussock moth, beet army worm, fall army worm and many other pest insect species, on all continents. As of 1995 eight viruses have been registered in the USA. A few viral products have been commercialized, such as the MNPV for treatment of S. *exigua* (Spodex^R, BioSys, USA) in the USA (on cotton), Thailand (on shallot, garden pea, grape and Chinese kale) and the Netherlands (on flowers and ornamentals)[2,3]. Others are produced through government sponsoring, such as Gypcheck^R against *L. dispar* in the USA. In China about 100 000 hectares of cotton and hot pepper are treated annually with the SNPV from *H. armigera*[4,5]. In Brasil 1 000 000 hectares have been treated annually against the velvetbean caterpillar, *Anticarsia gemmatalis*[6]. Baculoviruses are also well suited for use in Integrated Pest Management Programs as they are compatible with other (chemical or biological) control agents, they have minimal effects on non-target insects such as honey bees, they do not present safety and health problems to humans as encountered with chemical insecticides and lastly these viruses do not encounter resistance in insects[7].

The major limitation to a more wide-spread use of baculoviruses as insect control agents is their relatively slow speed of action, in particular in crops with low damage thresholds such as flowers. Upon infection insects stop feeding only after a few days, whereas immediate insecticidal

effect is often sought. In this respect the entomopathogenic bacterium *Bacillus thuringiensis* has a clear advantage over viruses as biological insecticide at the moment. Their toxins are quick acting, but resistance against these toxins has already become apparent[8]. By genetic engineering it has now become possible to generate baculoviruses with improved insecticidal properties, including increased speed of action (see[9-13], for review). A further drawback in the use of baculoviruses is the relatively low virulence for older instars (Table 2). Fourth and fifth instars cause the majority of damage in a crop. Recombinants with enhanced virulence have been engineered[14,15] and may prove efficacious in the field. Finally, baculoviruses are often too specific (Table 3) to meet the commercial requirements of broad spectrum activity, but recombinants with extended host range have been made[16]. From the application and commercial point of view the host range extension of baculoviruses in order to be able to combat various insect pests with the same virus is highly desirable. From a safety perspective, however, the limited host specificity of baculoviruses is an asset which is needs to be maintained in recombinants.

Table 1 Hallmarks in the engineering of baculovirus insecticides

1980	Polyhedrin is a virus-coded protein
1981	Polyhedrin and p10 are hyperexpressed genes with strong promoters
1983	Nucleotide sequence of polyhedrin determined
	First engineered baculovirus (polyhedron-negative)
1986	First release of a genetically modified (marked) baculovirus in the field
1987/88	P10 gene is dispensable for virus replication
1989/90	Failed attempts to engineer an improved baculovirus insecticide (toxins)
1989	Engineering of a baculovirus insecticide with enhanced virulence
1990/91	Successful engineering of baculovirus with improved insecticidal properties (speed of action)
1993/94	Engineering of a baculovirus insecticide with extended host range
1994	First environmental release of a baculovirus with improved insecticidal properties (toxin)
1995/96	Field releases of genetically improved baculovirus insecticides by commercial companies

Table 2 Biological activity of *Mamestra brassicae* MNPV against *Mamestra brassicae* larval instars[a]

		95% fiducial limits	
Instar	LD_{50}^{b}	upper	lower
L1	7	5	11
L2	947	796	1127
L3	5420	4334	4777
L4	59225	47629	73645
L5	238370	184408	308123
L5(mud)	$>5\times10^{7}$	-	-

[a] from Evans[17]

[b] polyhedra per instar of *Mamestra brassicae*

Table 3 Host range of selected baculoviruses in insects

Virus Species	Insect family	Insect species
Autographa californica MNPV	13	73
Mamestra brassicae MNPV	4	36
Helicoverpa zea SNPV	1	7
Gilpinia hercyniae NPV	1	7
Cydia pomonella GV	1	4
Spodoptera litura	1	1
Spodoptera exigua MNPV	1	1
Euproctis chrysorrhoea NPV	1	1

Detailed knowledge about baculovirus gene structure, function and regulation has allowed the manipulation of the viral genome and the development of the baculovirus expression vector system[18-20]. This technology is now being exploited and tailored for the construction of baculoviruses with novel insecticidal properties to combat insect pests more effectively (Table 4). The purpose of this communication is to give an overview of the progress that has been made over the years in the engineering of baculoviruses for improved insecticidal properties. AcMNPV has been the model virus for most of the data obtained, but other viruses will follow suit quite quickly. Also, an assessment of the potential risks associated with release of genetically modified baculoviruses in the environment as well as potential strategies to improve their biosafety will be disussed.

Table 4 Insecticidal proteins that have been inserted into baculovirus recombinants to improve their speed of action

Recombinant expressing	General effect on target insects	Effect on infected insect
Buthus eupeus toxin	paralysis	none
Bacillus thuringiensis toxin	gut destruction	none
Androctonus australis toxin (AaIT)	paralysis	redeced LT_{50}(20%)/feeding
Pyemotes tritici toxin PxP-1	paralysis	redeced LT_{50}(30%)/feeding
Agelenopsis aperta toxin μ-Aga-1V	paralysis	redeced LT_{50}
Dugueta canites toxin DTX9.2	paralysis	redeced LT_{50}
Tegenaria agrestis toxin TaITX-1	paralysis	redeced LT_{50}
Leiurus quinquestriatus toxin	paralysis	redeced LT_{50}(30%) and feeding
Maize mitochondrial protein URF13	male sterility in plants	toxic to insects
Manduca sexta diuretic hormone	diuresis	reduction LT_{50}(20%)
Manduca sexta eclosion hormone	eclosion	none
Bombyx mori prothoracicotropic hormone	accelerate molt	none
Heliothis virescens juvenile hormone esterase	regulates molt	reduced LT_{50}(10%) decrease weight gain

IMPROVEMENT OF INSECTICIDAL PROPERTIES

Strategies to improve the speed of action involved the introduction into baculoviruses of insect

– specific toxins, hormones and enzymes (Table 4). The results of these strategies will be sequentially reviewed as well as those leading to viruses with enhanced virulence and with extended host range.

Increased speed of action

Toxins derived from the bacterium *B. thuringiensis*[21~25] and the scorpin *Buthus eupeus*[26] did not enhance the speed of action of recombinant AcMNPV. The introduction of neurotoxin genes of the scorpions *Androctonus australis*, AaIT[27,28] and *Leiurus quinquestriatus hebraeus*, LqhIT2[29] and the mite *Pyemotes tritici*, PxP – 1[30] resulted in a considerable reduction in time (days) required to cause cessation of feeding and/or to incapacitate the host insect due to paralysis (LT_{50}). AaIT has been the toxin of choice since it is a highly insect – specific toxin and a considerable amount of toxicological data has been available[28]. Further improvements can possibly be made by optimizing codon usage for these toxins, by expressing mutated toxins with stronger potency, by expressing the toxins earlier after infection using different baculovirus promoters (IE – 1, p6.9, DA26) or by testing novel toxins.

Another strategy to improve the speed of action of baculoviruses includes the over – expression of peptide hormones regulating diuresis (diuretic hormone[31]), or insect metamorphosis such as eclosion hormone[32] and prothoracicotropic hormone[33]. The products of these genes, when expressed at high level, could in principle interfere with the insect metabolism resulting in, among others, faster cessation of feeding. However, up to this point these strategies, although more attractive from a safety point of view, have been relatively unsuccessful so far. Other protein factors regulating the molt, such as receptors, transcription factors, etc. may be future targets for improvement of baculoviruses using insect – derived genes.

The introduction of the juvenile hormone esterase (JHE) gene from *H. virescens*[34] in recombinant baculoviruses under the control of a strong baculovirus promoter (polyhedrin) resulted in a slight increase in virulence to young larval instars as compared to control viruses. Site – specific mutations in the JHE gene resulting in resistance of the JHE against degradation enhanced the stability of the recombinant enzyme in the insect and, hence, further increased the speed of kill[35].

Inadvertently, some other proteins cause an enhancement of the insecticidal effect, such as the overexpression of methyltransferase[36], an enzyme which adds methyl groups to nucleic acids, or the maize mitochondrial protein (URF13)[37], which cause male sterility in plants. The mode of action in these cases is entirely unknown.

Another significant finding has been the identification in baculoviruses of a gene, producing ecdysteroid UDP – glucosyltransferase (*egt*)[38,39]. Upon glycosylation this viral enzyme inactivates ecdysteroids and, hence, causes a delay in the molting of the insect. This is much to the benefit of the virus as the larvae can grow larger and produce more virus. Deletion of this gene from AcMNPV resulted in the abortion of this delay and in normal larval development and, hence, in a lower yield of virus. Surprisingly, this deletion also caused a quicker death of the

insect[40], which could be attributed to malfunctioning of the Malpighian tubules[41]. Recently, the deletion of the *egt* genes from *L. dispar* MNPV[42] and *Choristoneura fumiferana* MNPV[43] have been achieved but the effect of these recombinants on the respective insects needs to be determined. Baculoviruses with deletions of the *egt* gene are likely to be first generation of recombinant viral insecticides that are aceptable for commercial use. Very much depends on the agronomic behaviour of these viruses when tested in the field. These experiments are being carried out in the USA and elsewhere.

Another agronomic parameter that needs to be taken into consideration is the FT_{50}, the time it takes for insects to cause 50% of the final feeding damage[44]. It could well be that a recombinant virus kills faster, but that the insect does not stop feeding much earlier than when infected with the wild type virus, or *vice versa*. Therefore, this parameter needs to be determined as well in addition to the LD_{50} and LT_{50} to evaluate the agronomic potential of the recombinant.

Virulence

Usually, first, second and third instar larvae are very susceptible to baculovirus infection, but fourth and fifth instars require much more virus to cause morbidity and mortality. The values (LD_{50}) can range between 10 polyhedra for a first instar to over 200 000 for a fifth instar. It would therefore be desirable to engineer the virus in such a way that less virus is required to obtain the same level of mortality. One successful engineering strategy has been the removal of the polyhedral envelope by inactivating the gene encoding the polyhedra envelope protein[14]. As a consequence the occlusion body - derived virus particles are more efficiently released from the polyhedra and less polyhedra are necessary to cause the same biological activity (LC_{50})[15]. However, the agronomic performance of this recombinant in the field remains to be tested.

Host range

A host - range mutant has been engineered by swapping parts of the DNA helicase of *Bombyx mori* NPV into AcMNPV[16,45]. The DNA helicase is involved in baculovirus DNA replication[46] and may determine, in part, host specificity. This type of engineering extended the host range of AcMNPV to *B. mori*, an insect that would otherwise not be infected with AcMNPV. In principle, host - ranges of baculoviruses may now be expanded and restricted at will by genetic engineering, but it also poses an additional risk if other yet undetermined host range factors are involved.

BIOSAFETY CONSIDERATIONS

The combination of a highly specific insecticidal protein with a highly selective group of insect viruses presents an attractive concept for the safe use of baculovirus recombinants. If there are risks, then these are partly similar to those associated with the deliberate release of wild - type baculoviruses, such as the effects on non - target hosts. Although baculoviruses have been used for over five decades in the field and have a perfect safety record, very little has been learned

about the ecology of virus infections in the field. The additional risks associated with the use of genetically modified baculoviruses can not easily be identified and quantified. These risks may involve alteration of the host range and sensitivity, the spread of the engineered virus from the field site to other ecosystems, the physical instability of the viral genome, the activation of latent viruses and mobile elements (transposons), the possible exchange of genetic information, in particular the insecticidal gene, with other organisms, and toxicity of the insecticidal protein for non-target insects.

Some of these perceived risks can be experimentally tested, but others are most difficult. Genetically engineered baculoviruses can aid in these studies by performing contained releases in the environment[47,48]. Host range and virulence of wild-type and AaIT toxin-containing baculoviruses were very similar[49] suggesting that their use in the environment would not constitute a significant risk to indigenous insect pests. Toxins produced by these recombinants did not have an effect on predators, parasites or honey bees[50~52]. Field release of genetically modified AcMNPV, either with neurotoxins or with *egt* deletions, are being carried out in various parts of the world. The first report of such a field study with AaIT toxin-containing AcMNPV indicated more effective control than wild type virus and, as expected, lower yield of progeny virus[48]. The presence of transposons in baculoviruses is also a concern, as these elements can in principle transfer insecticidal genes to other baculoviruses or even cross insect species borders[53].

If risk is defined as exposure times hazard and when hazards are difficult to be quantified, the biosafety of genetically engineered baculoviruses is enhanced by adding genetic traits to the virus limiting its exposure to insects. In many instances described above this prerequisite is already met, since the enhanced insecticidal activity and low virus yield render these recombinants reduced recycling potential. Additional 'biological containment' can be achieved by affecting the spread and survival of the recombinants even further. Various molecular strategies are being evaluated.

ENGINEERING STRATEGIES FOR BIOSAFETY

The ecological and environmental fitness of such genetically modified baculovirus may be reduced by various strategies. The principle is to make these viruses less fit as compared to wild type viruses or even suicidal. The latter strategy has some commercial advantage as the product can be sold more often.

By co-occlusion of polyhedrin-negative recombinant viruses with wild-type viruses the insecticidal gene in the recombinant is in the polyhedrin locus and will only recombine with the polyhedrin gene in the wild type virus. It is a prerequisite to produce this recombinant in cell culture in order to regulate the degree of co-occlusion. Since the recombinant will never make polyhedra, it will be eventually inactivated in the field, whereas the wild-type virus will prevail in the end[54,55]. Field experiments indicated that this was indeed the case[56].

By pre-occlusion of the polyhedrin-negative recombinant the virus particles waiting to be

occluded (pre－occluded) is formulated and used[57]. This recombinant will have limited survival as polyhedra are absent and thus unable to protect the pre－occluded virus from decay once it is in the environment (H. A. Wood, personal communication).

The most promising strategy encompasses deletion mutagenesis. Several baculovirus genes have now been described, which are not essential for viral replication, but whose inactivation reduces the persistence and spread of the virus in the environment. The genes for p10[58], chitinase[59] and cathepsin[60] are involved in insect lysis and thus enhance polyhedra release from the insect. The pp34 gene is associated with the formation of the polyhedral envelope giving protection to polyhedral decay[14]. The *egt* gene inhibits molting of the infected host and affect virus yield[40]. Single and multiple deletion mutants are now being made and will be tested for their capacity of reduced spread and survival. In case of a p10－minus recombinant it could already be demonstrated that its spread was indeed impaired[61]. Field releases in the USA with an *egt* deletion mutant of AcMNPV are carried out since 1993 by American Cyanamid. Deletion mutagenesis and replacement of the deleted gene by toxins is a relative safe strategy as recombination with its wild type relative will take place over the same locus (see also co－occlusion strategy above).

Another attractive strategy involves the deletion of the p74 gene from the virus. This gene plays an essential role in the entry of the virus into the midgut epithelial cells of insect larvae[62]. P74 is not required for infection of other larval or tissue culture cells. Absence of this gene product on the outside of occluded virions aborts the infection of larvae. When polyhedra are produced in transgenic cell lines producing p74 the occluded viruses can be complemented with this protein, but are then only able to infect insects only once; progeny virus will lack the p74 gene and is thus unable to infect other insects[63]. This strategy is thus dependent on the availability of specifically transformed cell lines.

The construction of baculovirus recombinants which cause quick cessation of feeding has been achieved. In addition, by deletion mutagenesis these recombinants can be made less persistent in the environment. The recombinants can be produced in insects, when the insecticidal action does not cause immediate mortality. Otherwise, cell culture is the logical alternative when inexpensive media and large－scale bioreactors become available[64]. Development of additional strategies for improvement of baculoviruses requires a more detailed understanding of insect biochemistry and physiology. There is a strong quest for additional insecticidal genes for a variety of insect species in order to tailor baculoviruses further.

With the pioneering work on AcMNPV, it now becomes possible to engineer baculoviruses of other, economically important pest insects, such as *S. exigua*, *H. zea* and *H. armigera*. So far, efforts have had limited success, mainly due to recalcitrant cell culture systems. This makes engineering of these baculoviruses more difficult, but technical advances will be quickly made here as well.

When infected with recombinant with increased speed of kill, insect larvae may die before

prior to polyhedra production. To produce these recombinants in equal amounts as wild type viruses in insect larvae, it is essential to have large scale cell culture systems available (up to 10 000 L scale). Major advances have yet to be made in this area, in particular the development of cheap media and the productivity of the cell culture systems (number of polyhedra produced per cell per unit volume). This may also require the development of new cell lines producing more polyhedra per cell[65].

CONCLUSION

The ideal improved baculovirus insecticide should have a broad but defined host - range, cause cessation of feeding upon infection and should be noncycling. Only baculovirus insecticides with these specifications are commercially attractive to develop in the future. Recently, the AcMNPV recombinant expressing the AaIT toxin has been tailored for optimal expression and now reduces the time to render insects ineffective in feeding in about 1.5 day[66]. Field tests of AcMNPV carrying toxins have been carried out in the United Kingdom in 1993 and 1994[48] and in the U.S.A in 1996 (American Cyanamid and Dupont) and proved to be positive. The present properties make baculoviruses competitive with *B. thuringiensis* preparatimns. It remains to be seen, however, whether resistance against the neurotoxins is generated in insects as is the case against *B. thuringiensis* toxins.

The availability of genetically engineered baculovirus insecticides with genes coding for insect - specific toxins, hormones or metabolic enzymes are not likely to impose additional environmental risks. These proteins are specific for certain insect species and they are all natural elements of the insect biosphere. Recombinant baculovirus insecticides containing genes of this nature may therefore be considered as natural, insect - specific biocontrol agents producing biorational compounds. In addition, safety or suicide strategies as outlined above can render these viruses noncycling working more as a proteinaceous insecticide and may be employed if hazards are anticipated. Reduced persistence and host specificity are important features to keep as they reduce the risks associated with the deliberate release of genetically modified viral insecticides in the environment. However, studies on the behaviour of these baculoviruses in microcosm, greenhouse and field, will provide information not only on the agronomic properties on different crops but also if the perceived risks are hypothetical or real.

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