

Construction of occluded recombinant baculoviruses containing the full-length *cry1Ab* and *cry1Ac* genes from *Bacillus thuringiensis*

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Abstract

The administration of baculoviruses to insects for bioassay purposes is carried out, in most cases, by contamination of food surfaces with a known amount of occlusion bodies (OBs). Since *per os* infection is the natural route of infection, occluded recombinant viruses containing crystal protein genes (*cry1Ab* and *cry1Ac*) from *Bacillus thuringiensis* were constructed for comparison with the baculovirus prototype *Autographa californica* nucleopolyhedrovirus (AcNPV). The transfer vector pAcUW2B was used for construction of occluded recombinant viruses. The transfer vector containing the crystal protein genes was cotransfected with linearized DNA from a non-occluded recombinant virus. The isolation of recombinant viruses was greatly facilitated by the reduction of background "wild type" virus and the increased proportion of recombinant viruses. Since the recombinant viruses containing full-length and truncated forms of the crystal protein genes did not seem to improve the pathogenicity of the recombinant viruses when compared with the wild type AcNPV, and in order to compare expression levels of the full-length crystal proteins produced by non-occluded and occluded recombinant viruses the full-length *cry1Ab* and *cry1Ac* genes were chosen for construction of occluded recombinant viruses. The recombinant viruses containing full-length and truncated forms of the crystal protein genes did not seem to improve its pathogenicity but the size of the larvae infected with the recombinant viruses was significantly smaller than that of larvae infected with the wild type virus.

Key words

- Baculovirus
- AcNPV
- *Bacillus thuringiensis*
cry 1A
- *Heliothis virescens*
- *Pieris brassicae*

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Introduction

Baculoviruses are a potential source of biopesticides for the control of insect pests. Recombinant baculoviruses which express toxins have been constructed and shown to have an improved insecticidal activity (1-5).

Bacillus thuringiensis delta endotoxins or crystal proteins have also been introduced into the genome of baculoviruses and, although highly expressed and immunologically similar to their native counterparts, they did not improve the insecticidal activity of the recombinant baculoviruses (6-10). Cry-

tal proteins are potent insecticides and exhibit a very high degree of insect specificity. They are produced in large amounts during sporulation of *B. thuringiensis* and appear as large crystals. When they are ingested by a susceptible insect the crystal proteins are dissolved and proteolytically cleaved to a smaller active polypeptide in the alkaline environment of the insect midgut. The activated toxin has a cytolytic effect on the midgut cells. The insect stops feeding and eventually dies. In a previous study, we introduced the full-length and truncated forms of the *cryIAb* gene and the full-length *cryIAc* gene of *Bacillus thuringiensis* into the genome of the baculovirus *Autographa californica* nucleopolyhedrovirus (AcNPV) under the control of the polyhedrin promoter (6). These recombinant viruses expressed the toxins and were non-occluded. In order to bioassay the virus we had to inject the budded form of the virus into the hemocele of the insect. In the present paper we report the introduction of the full-length *cryIAb* and *cryIAc* genes from *B. thuringiensis* into the genome of AcNPV under the control of the p10 promoter. The recombinant viruses constructed had the polyhedrin gene and consequently produced the occluded form of the virus. Since *per os* infection is the natural route of infection for baculoviruses, the occluded recombinant viruses containing the crystal protein genes were used for comparison with the baculovirus prototype AcNPV. The expression of crystal proteins in insect cells and insect larvae, and the pathogenicity of recombinant viruses towards a susceptible insect were analyzed.

Material and Methods

Restriction enzymes and reagents

All restriction enzymes used in the present study were obtained from Gibco-BRL (Gaithersburg, MD). The reagents were obtained from Gibco-BRL and Sigma Chem-

ical Company (St. Louis, MO).

Virus, cells and insects

AcNPV strain C6 (11) and AcRP5 β gal virus (Ribeiro BM, unpublished results) were used to infect *Spodoptera frugiperda* (IPLB-SF-21) cells grown in TC100 medium supplemented with 5% fetal calf serum. Virus was titrated by the plaque assay (12).

Heliothis virescens and *Pieris brassicae* were reared under hygienic conditions using artificial diets (13,14). *H. virescens* was used for the bioassays with polyhedra since this insect is susceptible to the AcNPV virus. *P. brassicae* was used to test the Cry protein activity since this insect is not susceptible to AcNPV but is highly sensitive to the CryIA toxins.

Construction of a transfer vector containing the full-length genes *cryIAb* and *cryIAc*

The strategy for cloning the full-length genes *cryIAb* and *cryIAc* is shown in Figure 1. Ten micrograms of the plasmids pUCBtm and pUCBt73 (6) was digested with *Bam*HI and separated on 1% agarose gel. The bands containing the genes were recovered from the gel (15) and suspended in a small volume of TE buffer. The transfer vector pAcUW2B was digested with *Bgl*III, dephosphorylated and ligated to the *cryIAb* and *cryIAc* genes. The ligation mixture was transformed into *E. coli* DH5 α cells, and colonies were screened for the presence of the crystal protein genes. The recombinant transfer vectors pAcOBtm and pAcOBt73 respectively containing the *cryIAb* and *cryIAc* genes were then isolated from a large-scale plasmid DNA preparation and stored at -20°C.

Co-transfection of *Spodoptera frugiperda* cells and isolation of recombinant viruses

DNA of the recombinant transfer vectors pAcOBtm and pAcOBt73 was co-transfected

with linearized DNA from the non-occluded AcRP5 β gal virus. The non-occluded AcRP5 β gal virus was constructed by introducing the *Escherichia coli* β -galactosidase gene into the AcNPV genome in place of the polyhedrin gene (Ribeiro BM, unpublished results). In this study, DNA from AcRP5 β gal was digested with *Bsu36I* which hydrolyzes the viral DNA at only one location (5'-end of the β -galactosidase gene). Linear viral DNA (500 ng) was co-transfected with 1-2 μ g of transfer vector DNA into *S. frugiperda* cells using the liposome-mediated transfection method (16). After 48 h the whole medium from transfected cells was mixed with 1×10^6 *S. frugiperda* cells and seeded onto a 96-well plate. The plate was incubated at 27°C for 5 days and the wells were screened for the presence of polyhedra by light microscopy and by dot blot hybridization (data not shown). Medium from strongly positive wells was mixed and passed twice in 96-well plates. Medium from single positive wells from the later passage was then used to infect *H. virescens* larvae. After insect death, polyhedra were purified and the DNA isolated. The purity of the recombinant viruses was checked by restriction endonuclease digestion of viral DNA (Figure 2).

Expression of crystal proteins in insect larvae by the occluded recombinant viruses

Insect cells were infected with AcOBtm and AcOBt73 (10 pfu/cell) and 3rd-instar insect larvae were infected by spreading 100 to 1000 occlusion bodies on small cabbage leaf discs. After the liquid dried, the leaf discs were given to individual larvae and incubated at 27°C overnight. Insects which consumed all the leaf disc were transferred to replica dishes containing a plug of artificial diet and incubated at 27°C until they succumbed to the virus. The expression of the crystal proteins in insect larvae was analyzed by SDS-PAGE (Figure 3) and immunoblotting (data not shown).

Bioassay of occluded recombinant viruses

The droplet feeding method (17) was used for the bioassay of the occluded recombinant viruses. The amount of OBs ingested by the larvae of *H. virescens* was calculated indirectly by the determination of the ingested volume by fluorescence spectroscopy (18). Mean lethal concentrations (LC₅₀) were estimated by probit analysis. Homogenates from insects (*H. virescens*) infected with AcOBtm and AcOBt73 were serially diluted and incorporated into the artificial diet. Twenty newly hatched *P. brassicae* larvae were incubated with the diet for 5 days and two parameters of insect mortality, mean

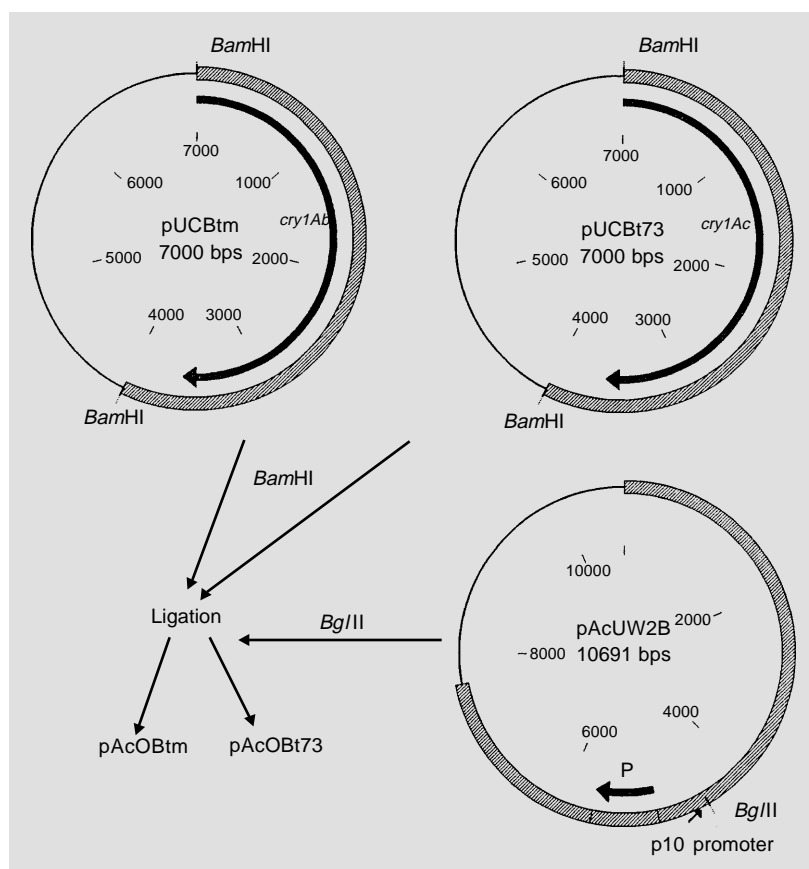


Figure 1 - Construction of transfer vectors pAcOBtm and pAcOBt73. Plasmids pUCBtm and pUCBt73, containing the *cry1Ab* and *cry1Ac* genes, respectively, were digested with *Bam*HI and the fragments containing the genes were cloned into plasmid pAcUW2B previously digested with *Bg*III. The large arrows in pUCBtm, pUCBt73 and pAcUW2B represent the open reading frames of the *cry1Ab*, *cry1Ac* and polyhedrin (P) genes, respectively. The small arrow in pAcUW2B represents the p10 promoter.

lethal dose (LD_{50}) and lethal half-time (LT_{50}), were estimated by probit analysis (Table 1). For the measurement of LD_{50} , serially diluted OBs from the recombinant and wild type virus were given to the insects (25-50 newly hatched larvae). Mortality was recorded 10 days post-infection. For the measurement of LT_{50} , 300 OBs/nl were used, mortality was recorded at 8-h intervals from day 4 to day 10, and the bioassays repeated 4 times. Calculated mean values and their stan-

dard error of the mean were analyzed by ANOVA.

Results

Construction of a transfer vector containing the full-length genes *cry1Ab* and *cry1Ac*

Figure 1 shows the strategy for the cloning of the full-length *cry1Ab* and *cry1Ac* genes. The *cry1Ab* and *cry1Ac* genes from *B. thuringiensis* were removed from the plasmids pUCBtm and pUCBt73 (6) and cloned into the transfer vector pAcUW2B. The resulting plasmids pAcOBtm and pAcOBt73 were used for the construction of the recombinant viruses.

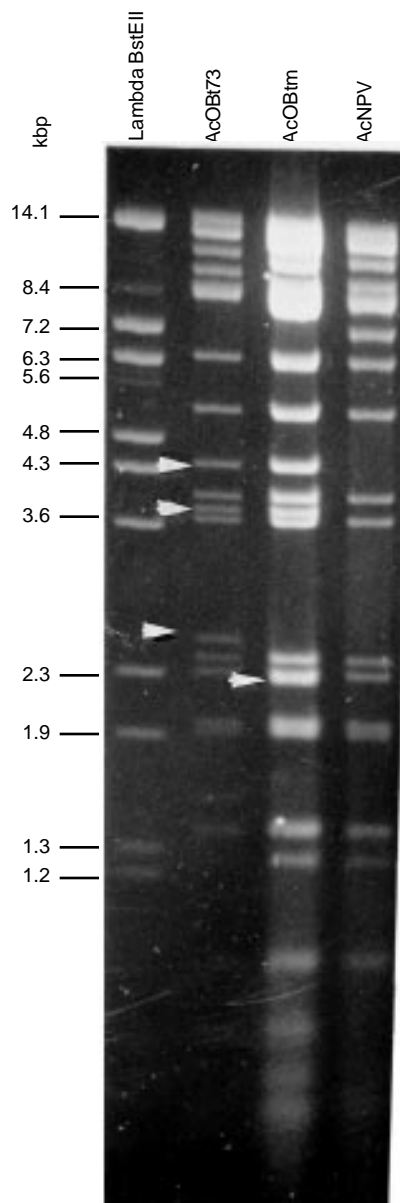
Co-transfection of *Spodoptera frugiperda* cells and isolation of recombinant viruses

The plasmids pAcOBtm and pAcOBt73 were used together with linearized DNA from the non-occluded AcRP5 β gal virus for the co-transfection of IPLB-SF-21 cells. After 48 h, the cell supernatant was collected and used for the isolation of the recombinant viruses by a combination of the end-point dilution method and Southern-blot (16). The purified recombinant virus was amplified in IPLB-SF-21 cells and the DNA extracted (16). The DNA was subjected to restriction enzyme analysis and compared to the DNA of the wild type virus (Figure 2). The purity of the recombinant virus was confirmed by the appearance of new bands of the expected sizes for each gene (Figure 2, arrowheads), and by Southern-blot (data not shown).

Expression of crystal proteins in insect larvae by the occluded recombinant viruses

The full-length crystal proteins produced by the occluded recombinant viruses AcOBtm and AcOBt73 were similar in size (as determined by SDS-PAGE) to the authentic crystal proteins expressed by the *B.*

Figure 2 - Analysis of the DNA from the recombinant viruses. Electrophoresis on 1% agarose gel of an *EcoRI* digest of DNA from the occluded recombinant viruses AcOBtm and AcOBt73. Arrowheads indicate the extra DNA fragment bands corresponding to the insertion of the toxin gene constructs into the AcNPV genome.



thuringiensis (Figure 3). They also proved to be immunologically similar since they reacted with antiserum raised against authentic Cry1Ac crystal protein (data not shown).

The levels of expression of the *cry1Ab* and *cry1Ac* proteins in insect cells infected with the occluded viruses AcOBtm and AcOBt73 were shown to be at least 1/5 of the expression of the same proteins in insects infected with the non-occluded viruses AcBtm and AcBt73 (see Tables 1 and 2 in Ribeiro and Crook (6)).

Biological activity of the crystal proteins expressed by the occluded recombinant viruses

Crystal protein genes expressed in insect larvae were shown to be biologically active against *P. brassicae*. Insect suspensions from insects infected with AcOBtm and AcOBt73 were serially diluted and incorporated into the artificial diet. Twenty newly hatched *P. brassicae* larvae were incubated with the diet for 5 days and insect mortality was analyzed by probit analysis (Table 1). The proteins expressed by the two recombinant viruses showed the same level of toxicity towards *P. brassicae* larvae.

Bioassay of occluded recombinat viruses

The mean volume ingested by larvae of *H. virescens* by the droplet feeding method was 14.48 nl, with a standard error of 6.88. The results of the LD₅₀ bioassays using the susceptible *H. virescens* (Table 2) showed that the LD₅₀ of AcOBtm and of AcOBt73 were at least 2 times greater than that of the wild type virus. The LT₅₀ bioassays (Tables 3 and 4) showed that the values for AcOBtm and AcOBt73 were significantly higher than the values for AcNPV.

Discussion

Occluded recombinant viruses contain-

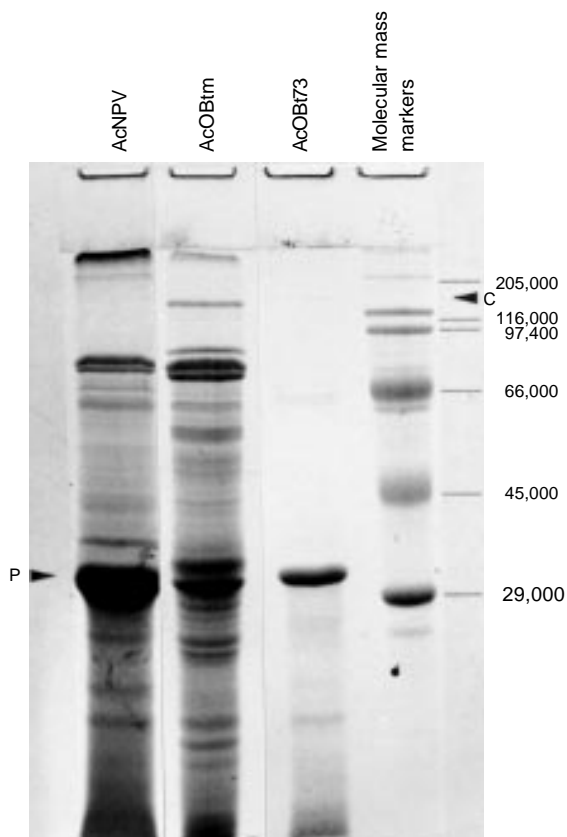


Figure 3 - Expression of crystal proteins in insect larvae. Polyacrylamide gel electrophoresis (PAGE) of extracts prepared from insects infected with the occluded recombinant viruses containing the *cry1Ab* and *cry1Ac* genes. The arrowheads show the position of the crystal proteins (C) and polyhedrin (P).

Table 1 - LC₅₀ values for the crystal proteins produced by the occluded viruses containing the full-length *cry1Ab* and *cry1Ac* genes.

C.L. = Confidence limits, CHI-SQ = residual chi-square, d.f. = degrees of freedom, SEM = standard error of the mean. The residual chi-squares are not significant.

Virus	CHI-SQ	d.f.	slope	SEM (slope)	LC ₅₀ (µg insect/g diet suspension)	C.L.
AcOBtm	2.12	2	1.2	0.2	0.08	0.04 + 0.13
AcOBt73	4.19	2	1.6	0.3	0.05	0.03 + 0.08

Table 2 - LD₅₀ values for the occluded recombinant viruses AcOBtm and AcOBt73 using *H. virescens*.

C.L. = Confidence limits, CHI-SQ = residual chi-square, d.f. = degrees of freedom, SEM = standard error of the mean. The residual chi-squares are not significant.

Virus	CHI-SQ	d.f.	slope	SEM (slope)	LD ₅₀ (OBs)	C.L.
AcNPV	0.81	2	1.8	0.3	168	114 + 253
AcOBt73	1.76	2	1.8	0.4	453	305 + 820
AcOBtm	10.0	2	0.7	0.14	380	155 + 1258

Table 3 - LT₅₀ values for the occluded recombinant viruses AcOBtm and AcOBt73 in 4 separate assays (letters A-D).

SEM = Standard error of the mean. LT₅₀ is in hours.

Virus (300 OBs)	Bioassay A LT ₅₀	SEM	Bioassay B LT ₅₀	SEM	Bioassay C LT ₅₀	SEM	Bioassay D LT ₅₀	SEM
AcNPV	112	10	115	17	110	12	115	20
AcOBtm	138	25	159	25	154	31	149	35
AcOBt73	131	35	169	27	177	40	153	38

Table 4 - LT₅₀ analysis of the 4 bioassays for the occluded AcOBtm and AcOBt73 recombinant viruses.

SEM = Standard error of the mean.

Virus	LT ₅₀	SEM
AcNPV	112	2
AcOBtm	148	5
AcOBt73	158	6

ing the full-length genes *cryIAb* and *cryIAc* from *B. thuringiensis* were constructed. The crystal proteins produced in insect larvae by the recombinant occluded viruses were shown to be immunologically similar to the authentic crystal proteins produced in *B. thuringiensis*. Furthermore, they proved to be biologically active against *P. brassicae*. We have shown previously (6) that the expression of full-length and truncated forms of the *cryIAb* gene and the full-length *cryIAc* gene in insect cells and insects by recombinant non-occluded baculoviruses produced proteins biologically and immunologically similar to their native counterparts. Martens et al. (8,9) also reported the expression of different forms of the Cry1Ab crystal protein from *B. thuringiensis* in insect cells, showing that the expressed proteins were biologically active. Full-length (72 kDa) and truncated (61 kDa) Cry11Aa proteins from *B. thuringiensis* were also expressed in insect cells by recombinant baculoviruses. These proteins formed cuboidal crystals and only the full-length protein was shown to be

toxic to insects (10). The crystal protein from *B. thuringiensis* subsp. *kurstaki* HD-73 was expressed in insect cells by a recombinant baculovirus and also showed biological activity (7).

Despite the fact that the crystal proteins were expressed at high levels in insects, the pathogenicity of the recombinant occluded viruses was not improved by the presence of either crystal protein. The LD₅₀ values for the occluded recombinant viruses AcOBtm and AcOBt73 were about 2 to 3 times higher than for the wild type virus. This result is in accordance with the data obtained by Merryweather et al. (7). They also obtained an LD₅₀ about 2 times higher for an occluded recombinant baculovirus expressing the HD-73 crystal protein. The levels of expression of the Cry1Ab and Cry1Ac proteins in insect cells infected with the occluded viruses AcOBtm and AcOBt73 were shown to be at least 1/5 of the expression of the same proteins in insects infected with the non-occluded viruses AcBtm and AcBt73 ((6), Tables 1 and 2). This lower expression could be due to the simultaneous expression of the polyhedrin gene, since they might compete for the same resources inside the cell. The failure to improve the insecticidal activity is not due to the production of the protein inside the cell. Martens et al. (9) showed that the production and or secretion of the Cry1Ab protoxin or the mature toxin by the recombinant baculoviruses did not increase insecticidal activity. They postulated that the

protoxins produced are inactive and not likely to be activated *in vivo*, secretion of the protoxins is poor and production of the mature toxins results in cytotoxicity. Although the expression of crystal proteins from *B. thuringiensis* in insect cells by recombinant baculovirus does not increase pathogenicity, as measured by the bioassay results, we noted that the size of the larvae infected with the recombinant viruses was significantly smaller than that of larvae infected with the wild type virus. This is probably due to contamination of the OBs with crystal proteins. The expression of *B. thuringiensis* crystal pro-

teins in insect cells could be useful for the study on the mode of action of such proteins since they assemble in crystals similar to those found in bacteria and have been shown to be biologically active (6,10).

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References

- Lu A, Seshagiri S & Miller LK (1996). Signal sequence and promoter effects on the efficacy of toxin-expressing baculoviruses as biopesticides. *Biological Control*, 3: 320-332.
- Stewart LMD, Hirst M, Ferber ML, Merryweather AT, Cayley PJ & Possee RD (1991). Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. *Nature*, 352: 85-88.
- McCutchen BF, Choudary PV, Crenshaw R, Maddox D, Kamita SG, Palekar N, Volrath S, Fowler E, Hammock BD & Maeda S (1996). Development of a recombinant baculovirus expressing an insect-selective neurotoxin: potential for pest control. *Bio/Technology*, 9: 848-852.
- Tomalski MD & Miller LK (1991). Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. *Nature*, 352: 82-85.
- Tomalski MD & Miller LK (1992). Expression of a paralytic neurotoxin gene to improve insect baculoviruses as biopesticides. *Bio/Technology*, 10: 545-549.
- Ribeiro BM & Crook NE (1993). Expression of full-length and truncated forms of crystal protein genes from *Bacillus thuringiensis* subsp. *kurstaki* in a baculovirus and pathogenicity of the recombinant viruses. *Journal of Invertebrate Pathology*, 62: 121-130.
- Merryweather AT, Weyer U, Harris MP, Hirst M, Booth T & Possee RD (1990). Construction of genetically engineered baculovirus insecticides containing the *Bacillus thuringiensis* subsp. *kurstaki* HD-73 delta endotoxin. *Journal of General Virology*, 71: 1535-1544.
- Martens JWM, Honee G, Zuidema D, Van Lent JWM, Visser B & Vlak JM (1990). Insecticidal activity of a bacterial crystal protein expressed by a recombinant baculovirus in insect cells. *Applied and Environmental Microbiology*, 56: 2764-2770.
- Martens JW, Knoester M, Weijts F, Groffen SJ, Hu Z, Bosh D & Vlak JM (1995). Characterization of baculovirus insecticides expressing tailored *Bacillus thuringiensis* CryIA(b) crystal proteins. *Journal of Invertebrate Pathology*, 66: 249-257.
- Pang Y, Frutos R & Federici BA (1992). Synthesis and toxicity of full-length and truncated bacterial Cry11Aa mosquito-cidal proteins expressed in lepidopteran cells using a baculovirus vector. *Journal of General Virology*, 73: 89-101.
- Possee RD, Sun T-P, Howard SC, Ayres MD, Hill-Perkins M & Gearing K (1991). Nucleotide sequence of the *Autographa californica* nuclear polyhedrosis virus 9.4 kbp *EcoRI*-I and -R (polyhedrin gene) region. *Virology*, 185: 229-241.
- Brown M & Faulkner P (1977). A plaque assay for nuclear polyhedrosis viruses using a solid overlay. *Journal of General Virology*, 36: 361-364.
- David WAL & Gardiner BOC (1965). Rearing *Pieris brassicae* L. larvae on a semi-synthetic diet. *Nature*, 207: 882-883.
- Guennelon G, Audemard H, Fremond JC & El Idrissi Ammari MA (1981). Progrès réalisés dans l'élevage permanent du carposcapse (*Laspeyresia pomonella*) sur milieu artificiel. *Agronomie*, 1: 59-64.
- Sambrook J, Fritsch EF & Maniatis T (1989). *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, New York.
- O'Reilly DR, Miller LK & Luckow VA (1992). *Baculovirus Expression Vectors. A Laboratory Manual*. W.H. Freeman and Company, New York.
- Hughes PR & Wood HA (1981). A synchronous peroral technique for the bioassay of insect viruses. *Journal of Invertebrate Pathology*, 37: 154-159.
- van Beek NAM & Hughes PR (1986). Determination by fluorescence spectroscopy of the volume ingested by neonate lepidopterous larvae. *Journal of Invertebrate Pathology*, 48: 249-251.