Int. J. Indust. Entomol. Vol. 2, No. 2, 2001, pp. 155~160

International Journal of
Industrial Entomology

Generation of Baculovirus Expression Vector Using Defective *Autographa californica* Nuclear Polyhedrosis Virus Genome Maintained in *Escherichia coli* for Occ^+ Virus Production

Yeon Ho Je*, Jin Hee Chang, Jong Yul Roh and Byung Rae Jin[1]
School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea.
[1]College of Natural Resources and Life Science, Dong-A University, Pusan 604-714, Korea.

(Received 11 April 2001; Accepted 10 May 2001)

We have generated a novel baculovirus genome which can be maintained in *Escherichia coli* that facilitates the rapid and efficient generation of recombinant baculovirus expression vectors. To make Occ^+ recombinant expression vectors, polyhedrin gene under the control of p10 promoter was inserted to bAcGOZA and this genome was designated bApGOZA. As in bAcGOZA, bApGOZA lacks a portion of the essential ORF1629 gene, but includes a mini-F replicon and selectable kanamycin-resistance marker. This occusion-producing activity of bApGOZA can be used very conveniently for its oral infectivity to insect larvae in mass production of foreign protein and insecticides.

Key words : AcNPV, Bacmid, Baculovirus, Expression Vector, Mini-F replicon, Occlusion-positive recombinant

Introduction

The baculovirus expression vector system is a helper-independent system that has found extensive use for the expression of heterologous genes. Its popularity stems from a combination of high level expression with the ability to carry out most eukaryotic post-translational modifications authentically (Jarvis, 1997; O'Reilly *et al.*, 1992 for review).

Autographa californica nucleopolyhedrovirus (AcMNPV) is the prototype baculovirus strain and the virus most commonly used in expression vector systems. The target gene is cloned into an appropriate transfer vector such that it is flanked by viral DNA sequences to allow recombination. Insect cells are then cotransfected with viral DNA and the modified transfer vector. Double recombination between these DNAs results in the transfer of the target gene to the viral genome. Most baculovirus vector systems use a strong viral promoter, which is activated very late in the infection process, to drive gene expression. Two viral promoters are commonly used in baculovirus vector design: P_{ph}, the promoter of the polyhedrin gene and P_{p10}, the promoter of the p10 gene. Both polyhedrin and p10 are abundantly expressed but are nonessential for virus replication in cell culture so that heterologous genes can be inserted in their places (Wang *et al.*, 1991). One of the most laborious steps in the construction of a baculovirus expression vectors is the identification of a virus that has incorporated foreign DNA by recombining with a transfer vector (Kitts *et al.*, 1990).

To remove this disadvantage, several alternatives including the linearization of viral DNA using restriction endonuclease (Kitts *et al.*, 1990) and the generation of a "bacmid", a recombinant baculovirus genome carrying a mini-F replicon and a Tn7 transposition site (Luckow *et al.*, 1993) were originated and have been used. In the previous report about bAcGOZA (Je *et al.*, 2001), we described a system for the generation of baculovirus expression vectors that has advantages over both of the above approaches. It employs a modified baculovirus genome that lacks part of ORF1629 but includes a mini-F replicon, and so can be maintained in *E. coli*. The target gene is transferred into the viral genome by recombination in insect cells. No selection steps are required and only recombinant virus is obtained because the parental DNA cannot form viable virus.

In addition to bAcGOZA, in this study, we describe bApGOZA system for the generation of occlusion-posi-

*To whom correspondence should be addressed.
School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea. Tel: 82-31-290-2492; Fax: 82-31-296-0926; E-mail: kskipl@plaza.snu.ac.kr

tive vector by insertion of polyhedrin gene under the p10 promoter is constructed and can be used very conveniently for its oral infectivity to insect larvae in mass production of foreign protein and insecticides.

Materials and Methods

Cells and Viruses

Sf9 cells (Summers and Smith, 1987) were maintained in TC-100 medium (GIBCO BRL, USA) supplemented with 10% fetal bovine serum (GIBCO BRL, USA). The wild-type AcNPV-C6 strain was used throughout these experiments. Virus stocks were generated, propagated and titered on Sf9 cells as described (O'Reilly *et al.*, 1992).

Construction of the transfer vector pTen21-Pol

The pAcGFPPOL (Je, 1998) was digested with *Xho*I and *Sna*BI and subcloned into pTen21 (QBIOgene, USA) digested with *Pst*I (blunt ended) and *Bam*HI to give the plasmid pTen21-Pol.

Generation of baculovirus genomes maintained in *E. coli*

The generation of the bApGOZA genome maintained in *E. coli* is summarised in Fig. 1. The transfer vector

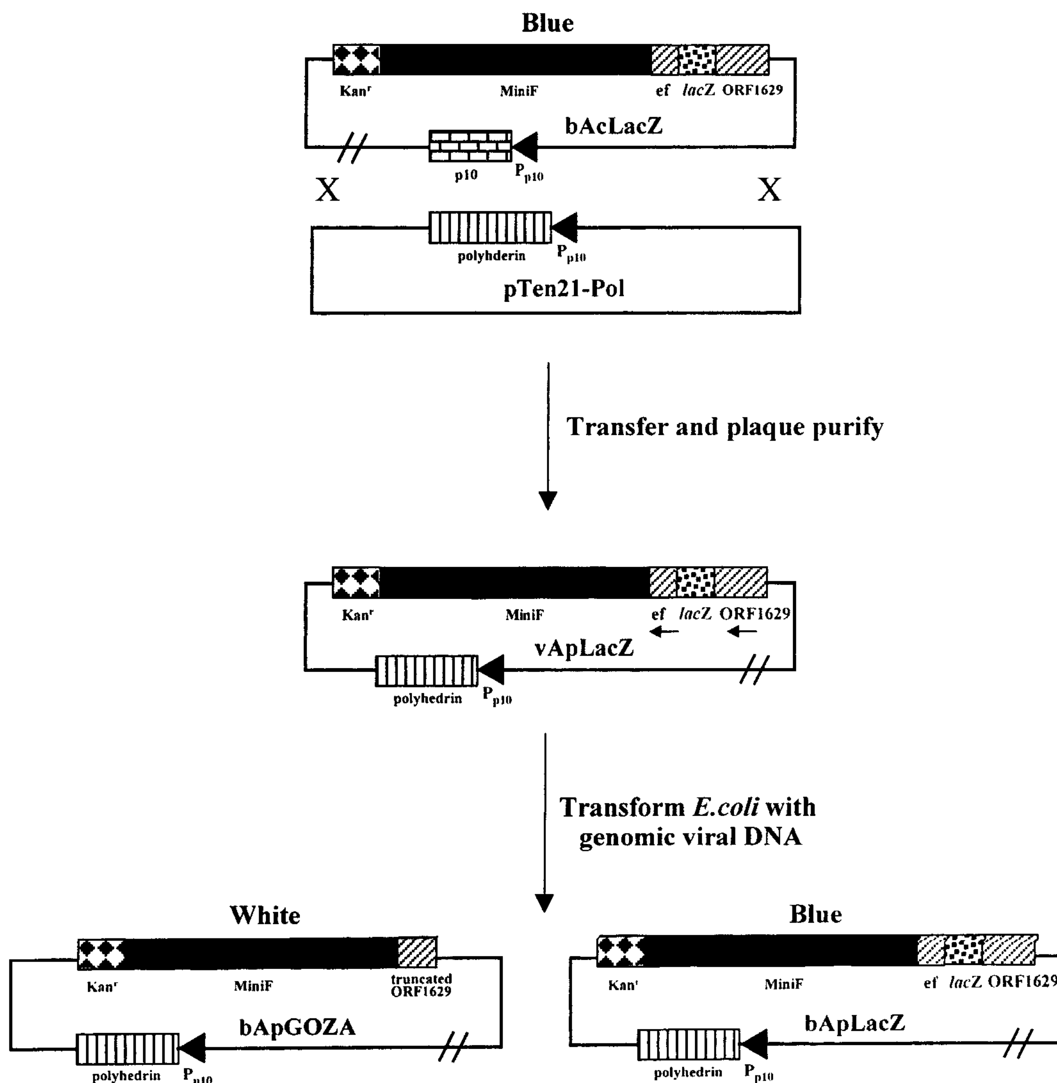

Fig. 1. Schematic outline of the generation of bacmids maintained in *E. coli.* Hatched sections represent ORF1629 sequences. ef indicates the central *Hin*dIII-*Cla*I section of ORF 1629. The solid section represents the mini-F element, whereas the heavy and light stippled sections denote *kan*ʳ and *lacZα* elements, respectively. The vertical striped and brick type region indicate the polyhedrin and p10 locus, respectively. The solid triangle represents promoter and the direction of ORF1629 segments is indicated by arrows.

pTen21-Pol was cotransfected into Sf9 cells with bacmid bAcGOZA using Lipofectin™ (GIBCO BRL, USA) and the recombinant virus vApLacZ isolated by screening for occlusion positive plaques. When vApLacZ was propagated in Sf9 cells, recombination can take place between the partial and complete copies of ORF1629. Thus, vAp LacZ infected cells retain both vApLacZ and vAp GOZA.

Crude genomic viral DNA isolated from the nuclei of cells infected with recombinant virus vApLacZ was transformed into *E. coli* DH10B. Transformants were distinguishable as light blue or white colonies on agar plates containing kanamycin, X-Gal, and IPTG. Recombinants (white colonies) were identified based on the loss of the *lacZα* element and were designated bApGOZA. (The prefix b indicates a bacterially-derived baculovirus genome). The C-terminus of ORF1629 is absent from bApGOZA. The non-recombinant genome was designated bApLacZ. To confirm the structures of these genomes, DNA was isolated from 250 ml cultures grown in 2× YT medium by alkaline lysis, and purified over Qiagen-tip 500 columns (Qiagen, Germany) according to the manufacturers instructions. Purified DNA was analysed by restriction digestion, agarose gel electrophoresis and southern blotting using standard procedures. Probe for southern blotting was labelled with dioxigenin using a DIG DNA labelling kit (Boehringer Mannheim, Germany).

Co-transfection of bacmid and transfer vector DNAs

Thirty-five-millimiter dishes were seeded with 1-1.5× 10^6 Sf9 cells and incubated at 28°C for 2 to 24 hrs to allow the cells to attach. One hundred nanograms of bApGOZA DNA, 500 ng of transfer vector DNA in TE and sterile water to make a total volume of 50 μl were mixed in a polystrene tube. To determine the viability of bApGOZA, bApGOZA DNA without transfer vector was prepared alone in the same amount. Fifty microliters of 100 μg/ml Lipofectin™ (GIBCO BRL, USA) were gently mixed with the DNA solution, and the mixture was incubated at room temperature for 15 - 30 min. Meanwhile, the medium was removed from the cell monolayers, and the cells were washed twice with 2 ml serum-free TC-100 insect medium (GIBCO BRL, USA) containing 50 units of penicillin and 50 μg streptomycin per ml. Serum-free TC-100 (1.5 ml) containing antibiotics was added to each dish. The Lipofectin-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After incubating at 28°C for 5 hrs, 1.5 ml TC-100/10% FBS/antibiotics were added to each dish and the incubation at 28°C continued. Five days after adding the Lipofectin-DNA complexes to the cells, the medium containing viruses released by the transfected cells was transferred to a sterile container and stored at 4°C.

Recombination assay

A standard plaque assay procedure (OReilly *et al.*, 1992) was used to obtain viral plaques from dilutions of the media harvested from the co-transfections. Individual plaques were examined under a microscope and scored for green fluorescence and polyhedra production.

Microscopy

Microscopy of Sf9 cells infected with wild-type AcNPV and recombinant virus were performed using light and fluorescent microscope (Axiophot Universal Microscope, Germany).

Results

Construction of a novel baculovirus genome that can be maintained in *E. coli*.

The transfer vector pTen21-Pol comprised polyhedrin gene under the control of p10 promoter. This pTen21-Pol plasmid was co-transfected into Sf9 cells with bacmid bAcLacZ DNA and recombinants selected based on their occlusion-positive phenotype. Thus the recombinant virus designated vApLacZ, contained the entire *kan*^{r} - *lacZα* cassette and polyhedrin gene inserted in place of the polyhedrin gene and the p10 gene, respectively. When vApLac Z DNA was amplified in Sf9 cells, recombination can occur between the incomplete and complete segments of ORF1629. This resulted in deletion of the *lacZα* element and consequent loss of β-galactosidase expression in transformed *E. coli* DH10B cells. β-galactosidase-positive and negative clones were isolated and designated bApLacZ and bApGOZA, respectively.

To confirm the structure of these genomes, bacmid bAcLacZ, bApLacZ and bApGOZA DNAs were purified and characterized by restriction mapping and southern blotting (Fig. 2). As seen in Fig. 2A, all recombinant bacmids were cut from 4 to 5 times, but one more cutting site of *Sse*8387I in bAcLacZ and bApLacZ than bApGOZA existed in *lacZα* fragment and these differences reflected the loss of the *lacZα* element and part of ORF1629 from bApGOZA (Fig. 2C). Hybridization of S*se*8387I digested DNAs with a probe comprising wild-type AcNPV polyhedrin element revealed bands just in bApLacZ and bApGOZA, confirming that only bAp-series genomes contain polyhedrin and can be occlusion-positive phenotype (Fig. 2B).

Generation of recombinant baculovirus vector using bApGOZA

To assess the utility of bApGOZA as parental viral genome for the generation of recombinant baculovirus

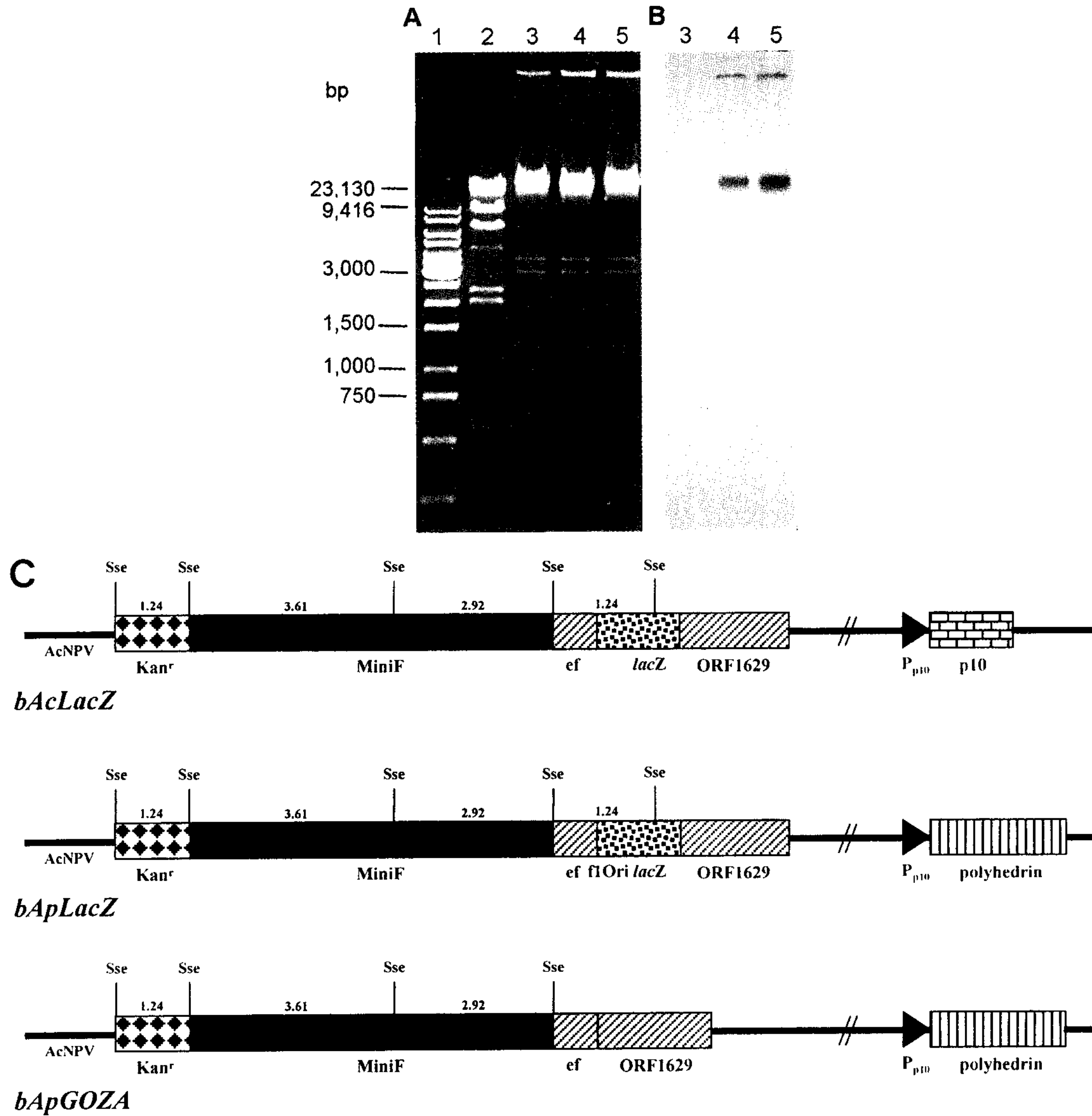

Fig. 2. Restriction analysis and southern blotting of bacmids maintained in *E. coli*. (A) Ethidium bromide-stained agarose gel. Lane 1, 1 kbp DNA ladder; lane 2, *Hin*dIII-digested lambda DNA; lane 3, bAcLacZ; lane 4, bApLacZ; lane 5, bApGOZA. Samples in lanes 3 - 5 were digested with *Sse8387I*. (B) Southern blots of the gel probed with the polyhedrin fragment of wild-type AcNPV. (C) Schematic representations of the structures of bAcLacZ, bApLacZ and bApGOZA. *Sse*8387I site is indicated by Sse. Sizes are indicated in kbp. The different genetic elements are shaded as in Fig. 1.

expression vectors, bApGOZA DNA purified from bacteria was co-transfected into Sf9 cells with the polyhedrin based transfer vector in which the GFP (green fluorescent protein) gene was coupled to the polyhedrin promoter (pAcGFP; Je, 1998). To determine the viability of bApGOZA, bApGOZA DNA without transfer vector was prepared and transfected alone in the same amount. Titration of the progeny from co-transfection with bApGOZA and pAcGFP gave rise to mean titers of 2.76×10^5 pfu/ml and 1.73×10^6 pfu/ml for recombinant virus at 4 days and 5 days after co-transfection, respectively (Table 1). As expected, transfection of bApGOZA alone gave rise to no viable virus. Plaque purification and characterization of the virus formed in both co-transfections with bApGOZA

Table 1. Recombination of bApGOZA with or without transfer vector pAcGFP

Viral DNA	Transfer vector	No[a]	Titer (pfu/ml)	
			4 days after transfection	5 days after transfection
bApGOZA	pAcGFP	3	2.76×10^5	1.73×10^6
bApGOZA	None	3	0	0

[a] Number of transfection

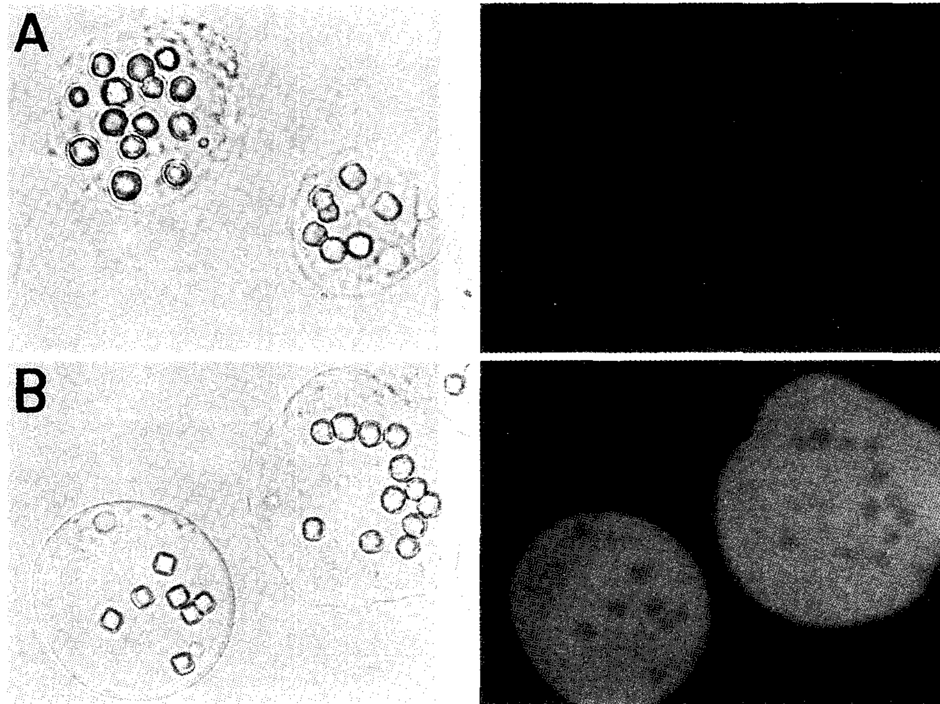

Fig. 3. Microscopy of Sf9 cells infected with recombinant virus. Sf9 cells infected by wild-type AcNPV (A) and bApGOZA with pAcGFP (B) were observed by light (left panels) and fluorescent (right panels) microscope (× 1,000).

and pAcGFP confirmed that only the expected recombinant virus was formed.

As shown in Fig. 3, the fluorescence of GFP in Sf9 cells infected by bApGOZA with pAcGFP was easily detected by fluorescent microscope. The recombinant virus resulted from co-transfection with bApGOZA and pAcGFP produced occlusion-positive plaques similar to those of wild-type AcNPV and bright glow of GFP clearly appeared in the whole cells.

Discussion

In this study, bApGOZA system, AcNPV based recombinant baculovirus was constructed and verified their ability for baculovirus expression vector systems. As in our previously report in bAcGOZA system (Je *et al.*, 2001), a novel method for the generation of recombinant baculoviruse using bApGOZA combines rapid and easy generation of the parental viral DNA with 100% recombination efficiency. The method combines recombination with a deleted viral genome lacking part of the essential ORF1629 (Kitts & Possee, 1993) and recombination with a viral genome maintained in *E. coli* (Luckow *et al.*, 1993) and facilitates the rapid and efficient generation of recombinant virus. Since the frequency of the recombination events within wild-type viral DNA is typically 0.1 to 1% (Kitts *et al.*, 1990), the identification of a recombinant virus against a background of parental virus is often a very tedious step. Considering this problem, the increasing the percentage of recombinants among the progeny viruses and no background of parental viruses can remove the time-killing steps. Our finding using defective baculovirus genome can make these screening steps very easy and rapid for its no background of non-recombinant viruses. Although the protein production in cell culture system has advantages of its easy purification and uniformity of protein quality, for scale-up of foreign gene production, it is too expensive for its high cost of media and specialized equipment. The alternative for mass production of protein is the *in vivo* expression system using insect larvae. This larval production can generate the higher yields and efficiency of the certain type of post-translational modification than cell culture and avoid the high costs of cell culture technology (OReilly *et al.*, 1992). In mass production with insect larvae, however, injecting larvae with budded virus is also can be used but especially in a large amount of production, it is too laborious and time-killing step. In both production systems, bAcGOZA and bApGOZA can be used separately and efficiently by its applying system. In cell culture system, bAcGOZA system can be used and the isolated recombinant virus can be amplified in insect cells easily by its occlusion-negative phenotype. And the bApGOZA system results in occlusion-

positive phenotype recombinants and these progeny viruses can be used to orally infect insect larvae for mass scale protein production or for pesticide evaluation or use (Wang *et al.*, 1991).

Furthermore, it is particularly easy to generate large quantities of pure DNA using standard methods and kits for the purification of DNA from *E. coli* and the DNA can be used directly for transfection without digestion.

References

Jarvis, D. L. (1997) Baculovirus Expression Vectors; in *The baculoviruses,* Miller L. K. (ed.), pp. 389-431, Plenum Press, New York.

Je, Y. H. (1998) Construction of a novel baculovirus insecticide producing recombinant polyhedra with *Bacillus thuringiensis* Cry1Ac crystal protein and green fluorescent protein. Ph.D. thesis, pp. 1-164, Seoul Nat'l Univ.

Je, Y. H., J. H. Chang, J. Y. Choi, J. Y. Roh, B. R. Jin, D. R. O'Reilly and S. K. Kang. (2001) A Defective Viral Genome Maintained in *Escherichia coli* for the Generation of Baculovirus Expression Vectors. *Biotechnol. Lett.* **23**, 575-582.

Kitts, P. A., M. D. Ayres and R. D. Possee (1990) Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. *Nucleic Acids Res.* **18**, 5667-5672.

Kitts, P. A. and R. D. Possee (1993) A method for producing recombinant baculovirus expression vectors at high frequency. *BioTechniques* **14**, 810-817.

Luckow, V. A., S. C. Lee, G. F. Barry and P. O. Olins (1993) Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli. J. Virol.* **67**, 4566-4579.

O'Reilly, D. R., L. K. Miller and V. A. Luckow (1992) *Baculovirus Expression Vectors - A Laboratory Manual.* W. H. Freeman and Company, New York.

Summers, M. D. and G. E. Smith (1987) A manual of methods for baculovirus vectors and insect cell culture procedures. *Texas Agricultural Experiment Station Bulletin. 1555.*

Wang, X., B. G. Ooi and L. K. Miller (1991) Baculovirus vectors for multiple gene expression and for occluded virus production. *Gene* **100**, 131-137.