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Baculovirus: A Powerful Tool for Various Biotechnological Applications

Quentin Sandro, Rachid Benchaouir*

Coraliotech SARL, MonacoTech SAM and Centre Scientifique de Monaco, Monaco

***Corresponding author:** Rachid Benchaouir, Coraliotech Sarl, c/o MonacoTech SAM, 6 avenue Albert II, 98000 Monaco. Tel: + 33-607934764; Email: rbenchaouir@coraliotech.mc

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Abstract

Since its introduction in the early 1980s, the protein production system based on the Baculovirus/Insect cell pair has continuously evolved and has been used in a growing number of applications. A better knowledge of the baculovirus infectious cycle and the strong expression of two of its non-essential proteins led to the brilliant idea of using its great capacity to propagate in insect cells to produce large quantities of recombinant proteins. Many other features of this interesting system, particularly its ability to be scaled for industrial applications, have allowed it to distinguish itself from other historical methods such as those using bacteria or yeasts. Here, through a short historical point of view and a general description of its process, we describe the applications that have been progressively performed by this system, from the production of a single recombinant protein to that of vaccine manufacturing or even viral vectors for gene therapy approaches. The baculoviral system thus continues to evolve by opening up new technological, scientific and industrial perspectives that undoubtedly make it a key player for many forthcoming innovative biotechnology applications.

Keywords: Baculovirus; Insect Cells; Recombinant Proteins; Production Process; Viral Vectors; Gene Therapy; Marine Biotechnologies

Abbreviations

ICTV: International Committee on Taxonomy of Viruses; AcMNVP : Autographa Californica Multiple NucleoPolyhedroVirus; BEVS : Baculovirus Expression Vector System; cDNA : complementary Desoxyribonucleic Acid; Polh : Polyhedrin; TIPS : Titerless Infected-Cells Preservation and Scale-Up; BIICs : Baculovirus-Infected Insect Cells; GMPs : Good Manufacturing Practices; VLPs: Virus-Like Particles; AAVs : Adeno-Associated Viruses.

Introduction

A Brief Historical Point of View

Baculoviridae family contains invertebrate-restricted viruses that are currently divided in four distinctive genera as edited by the International Committee on Taxonomy of Viruses (ICTV). These large enveloped and rod-shaped viruses have a circular double-stranded DNA genome of 80 to 180 kbp in size. In-depth knowledge of the detailed characteristics of the baculoviruses has required decades as it took more than 20 years of fundamental and technological investigations to propose a system of protein production strictly based on these viruses. To summarize, we could divide the early developmental stages into three key steps. Firstly, the establishment in the 1960s of conditions allowing the culturing of insect cells *in vitro* was an essential prerequisite. These conditions have been performed through the co-development of optimal insect cell lines and their specific culture media [1-3]. Secondly, in the 1970s, the discovery of a specific species of Baculovirus, The Autographa Californica Multiple NucleoPolyhedroVirus (AcMNPV), followed by the development of methods dedicated on its isolation and *in vitro* propagation, was decisive [4-6]. Finally, based on the practical knowledge accumulated on the AcMNPV, the early 1980s were marked by the emergence of maps and precise genetic sequences of baculoviral genome [7,8]. These achievements led in 1983 to the publication of the first production of one recombinant protein, the human interferon-bêta by the current so-called Baculovirus Expression Vector System or BEVS [9]. From this technological feat, the number of publications mentioning the use of BEVS to produce recombinant proteins increased exponentially to reach a peak in the late 1990s, and since then their number were stabilized at a high level [10].

BEVS: Competitive Characteristics

Compared with other conventional recombinant protein production systems (Bacteria, yeasts, mammalian cells), BEVS offers an advantageous alternative in many respects [11]. The first one is the capacity of the insect cells to be grown in suspension, giving them the opportunity to be easily scaled up to reach industrial levels of production. In addition, the strength of the two baculoviral promoters upstream of which the genes of interest are cloned (polh and p10, see later) makes BEVS a system guaranteeing one of the highest protein yield. The other property concerns the ability of the insect cell environment to promote the most commonly post-translational modifications found in mammalian cells, particularly glycosylations and phosphorylations, which are known to be of great importance for retaining the functionality of many classes of recombinant proteins. Another interesting property of BEVS is related to the cell culture conditions that do not require neither carbon dioxide nor serum supplementation for their growth. The serum-free advantage facilitates the purification steps by drastically reducing the viscosity and clogging constraints which can occur during the clarification and/or concentration of the bulk. Furthermore, serum avoidance reduces the risk of contamination of the final product by molecules of animal origin that can cause inflammatory reactions or anaphylactic shock, and which is in any case particularly restricted by the regulatory authorities.

Methods: Baculoviral Process Overview

Genetic Constructions

The production of recombinant proteins by BEVS requires many steps and some preliminary genetic constructions. Nowadays, the raw material is commercially available and it can be purchased from various suppliers, such as the BaculoGold™ baculovirus expression system (BD Biosciences), the Bac-to-Bac™ expression system (Life Technologies), or the flashBAC™ system (Oxford Expression Technologies). The most important content of these commercial kits is undoubtedly the baculovirus genome itself. Depending on the kit used, this genome may be in a "Free" state or already transformed within specific competent bacteria. In the first case, the baculovirus genome is linearized before being co-transfected into an insect cell line in combination with a plasmid vector carrying the complementary DNA (cDNA) corresponding to the gene to be expressed. The integration of the cDNA into the baculovirus genome is achieved in insect cells by homologous recombination. In the second case, the competent bacteria containing the baculovirus genome are first transformed by a plasmid vector containing the cDNA of interest. In this case, the integration of the cDNA into the baculovirus genome is achieved by a complex transposition reaction. A final step of extraction and purification of the recombinant baculovirus genome is necessary before its transfection into an insect cell line. It is important to note that the cDNAs are inserted downstream of one of the two strong baculovirus promoters: polyhedrin (polh) or p10. These two promoters control the expression of the so-called very late genes, expressed well after viral particles synthesis, a particularity of the baculovirus infectious cycle. These genes have been shown to be non-essential for the production of viruses, leading to the idea of replacing them with the cDNA sequences of interest.

Production of the Recombinant Baculoviruses

The introduction of a recombinant baculovirus genome into insect cells (Sf9, Sf21 or High Five cell lines are among the most used) leads to its replication and the production of the viral particles as previously described [12]. Indeed, despite the introduction of a transgene downstream the strong polh or p10 promoters, the recombinant baculovirus genome carries all its other endogenous genes allowing the initiation and the spreading of the infectious cycle through the insect cell culture. Nevertheless, the post-transfection derived baculoviruses may exhibit genomic heterogeneity due to viral genome instability, in particular through the accumulation of the so-called Defective Interfering (DI) particles [13]. In this context, the establishment of a plaque assay in 1977 was a decisive step to select baculovirus clones and is still used today to isolate the most optimal variants [6]. In addition to its ability to isolate baculovirus clones, the plaque assay provides one other important information: the titer of the baculoviral supernatant (expressed in plaque forming units per milliliter or pfu/ml). The selected variants are then amplified through successive cell passages to reach the volumes required for the production of the recombinant proteins. The volumes of supernatant to be used for the upstream processes are dependant of both the quantified baculoviral titers and the required volume of production. The passages involve the infection of the insect cell line with the previous viral supernatant following by the monitoring of the proliferation rate (or cell viability) and the average cell size, two physical parameters that control the effectiveness of the infection.

Preservation of the Baculovirus Stocks

To produce the recombinant protein of interest the supernatants are generally used extemporaneously because of the difficulty of baculoviruses to be preserved for long term in their conditioned medium. The cryopreservation methods do not give optimal results despite the many experimental tests carried out so far in this context [14]. A little used alternative was proposed through the conservation of newly synthesized baculoviruses inside the pre-infected insect cells [15]. More specifically, the method, called The Titer Less Infected-Cells Preservation and Scale-Up (TIPS), consists in infecting the insect cells and early stopping the infectious cycle before the start of cell lysis, i.e. at a time where recombinant baculovirus production is barely started. These Baculovirus-Infected Insect Cells (BIICs) are directly cryo-

preserved as classically done for a large majority of cell lines. This approach has the merit of protecting the virus particles inside cells, reaching a high cell concentration storage (10e7 cells/ml). The other advantage is the use of a very limited volume of BIIC to initiate the production step [16]: a 10e3 to 10e4 ratio (v/v) between insect cell culture and BIIC respectively is enough to initiate the upstream process, and even to reach industrial production volumes.

Upstream and Downstream Processes

The steps for producing the recombinant proteins are initiated through the infection of a growing insect cell culture by recombinant baculoviruses or by co-cultivation with BIICs according to previously described conditions [15,16]. It should be reminding that insect cell cultures do not require neither serum supplementation nor carbon dioxide for their growth. However, optimizations can be made by increasing the percentage of dissolved oxygen inside the cell culture. In the context of R&D processes, the best results are obtained by culturing cells in Erlenmeyer-type flasks with ventilated plugs and subjected to constant and moderate agitation for better gas diffusion (with a sufficient air / liquid interface to effectively promote these exchanges). For pilot and industrial processes, the best production solution inevitably involves the implementation of bioreactor systems. The automation of the system by the real time control of the physicochemical parameters of the cell culture, as well as the ease of diffusion and gas exchange, give to the bioreactor process a real added value compared to the R&D processes.

The purification processes of the products generated by the BEVS are obviously dependent on their specific nature. Concerning the recombinant proteins, it is usually necessary to implement a variety of steps and techniques to achieve high levels of purification. These are generally divided into four key stages: clarification, concentration, purification and polishing. The clarification step essentially uses centrifugation and / or filtration approaches to get rid of cells, cell debris and other macroscopic structures. For pilot or industrial processes, in which the volumes generated can be very important, a step of concentration is necessary. The method is generally performed by tangential flow filtration approaches. The purification steps of the recombinant proteins are mainly based on chromatographic techniques which will not be more detailed here. The so-called polishing phase generally uses gentle methods of separation such as exclusion chromatography, dialysis or lyophilization methods. Beyond their capacity to increase the purity of the final product, they also make it possible to reformulate it in a buffer solution adapted for its long-term preservation.

BEVS Applications

Recombinant Proteins

As previously discussed, BEVS was first used for the production of recombinant proteins [17]. Its advantages in terms of yields, post-translational modifications, or scaling up give it a place of choice among other existing production systems. The proteins produced by BEVS are for the most part used for enzymatic analysis, diagnostics or for functional and structural research studies. They can be produced and accumulated in the intracellular compartment of the insect cell lines or secreted into the extracellular medium. In the latter case, it should be noted that a large number of insect signal peptides have been identified and can be fused to the mature protein sequence for its final excretion. In another hand, many other original signal peptides are also effectively recognized by insect cell specific proteases, once again demonstrating the great flexibility of BEVS to accommodate and express heterologous proteins from different origins. Moreover, the development and implementation of processes based on Good Manufacturing Practices (GMPs) have gradually led regulatory authorities to allow the use of certain BEVS products for human clinical and veterinary applications. Much of the recombinant proteins produced by BEVS are of human or mammal's origin. We will discuss below the emerging possibilities of using BEVS for the production of proteins from other lineages. A chronological list of some examples of recombinant proteins produced by BEVS is given in Table 1.

Product Name	Laboratory / Company	Year	References
BEVS for Recombinant Proteins			
Human Interferon-bêta	Texas A&M University, USA	1983	Smith, et al, 1983
E. coli bêta-Galactosidase	University of Idaho, USA	1984	Pennock, et al, 1984
Human Insulin Holoreceptor (HIR)	University of Chicago, USA	1990	Steiner, et al, 1990
Human interleukin-7	McGill University, Canada	2008	Mirzaei, et al, 2008

Human Glycogen Synthase-1 (hGYS1)	Indiana University School of Medicine, USA	2013	Khanna, et al, 2013
Human Thyroid Peroxydase	East China University of Science and Technology, China	2018	Lou, et al, 2018
BEVS for Vaccine Applications			
Human papillomavirus L1 protein (Cervarix®)	Dartmouth Medical School, USA	2008	Harper, 2008
Porcine circovirus ORF2 (CircoFLEX®)	Boehringer Ingelheim Vetmedica Inc., USA	2009	Desrosiers, et al, 2009
Influenza HA (Flubock®)	Protein Sciences, USA	2009	Cox, et al, 2009
Sipuleucel-T (Provenge®)	Dana-Farber Cancer Institute, Harvard Medical, School, USA	2010	Kantoff, et al, 2010
BEVS for rAAV Production			
Production of rAAV vector serotype 2	Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, USA	2002	Urabe, et al, 2002
rAAV vector with lipoprotein lipase transgene (Glybera®)	Uniqure, The Netherlands	2013	Haddley, 2013
OneBac Platform for rAAV serotype 1-12	Charité Universitätsmedizin Berlin, Germany	2014	Heilbronn, et al, 2014
Rapid, scalable, and low-cost purification of rAAV	SQY Therapeutics Inc., France	2016	Buclez, et al, 2016
Clinical grade rAAV production by BEVS	University of Massachusetts Medical School, USA	2017	Cecchini, et al, 2011
Baculovirus as Gene Transfer Vector			
Gene-transfer into human hepatocytes	Max Delbrück Center for Molecular Medicine, Germany	1995	Hofmann, et al, 1995
Transduction of mammalian cells with rBac-VSVG	Biogen Inc., USA	1997	Barsoum, et al, 1997

Table 1: Non-exhaustive list of the four main BEVS applications.

Vaccines Applications

The use of baculovirus for human and veterinary vaccination approaches was among the first commercial successes of BEVS [18]. Applications cover areas as important as anti-viral or anti-tumor vaccination. For instance, vaccines for cervical and prostate cancers are some of the success stories of BEVS [19,20]. The vaccines against the porcine circovirus or the seasonal human influenza are also part of the most consistent advances of the baculoviral system [21,22]. Among these applications, some of them use the expression of soluble protein antigens, others are based on the production of Virus-Like Particles (VLPs), self-assemblies of genome-free viral proteins mimicking the structure of the native virus without any infectious capacity [23]. In the same context, the insertion of antigenic epitopes on the baculovirus surface has been used for the development of vaccines [24]. The approach consists in fusing the antigen of interest to the baculovirus envelope glycoprotein GP64. These fused epitopes are then displayed on the extracellular membrane of the infected cells and could be used for functional studies, drug screening or development of vaccines.

Production of Gene Transfer Vectors

The ability of BEVS to produce both correctly folded recombinant proteins and VLPs led Robert M. Kotin's team to develop, in the early 2000s, the production of viral vectors for gene transfer and gene therapy [25]. This challenge was carried out for the production of vectors belonging to the *Parvoviridae* family: the Adeno-Associated Viruses (AAVs). AAVs have quickly emerged as ideal candidates for *in vivo* gene transfer and rapidly as potentially powerful tools for gene therapy. These viruses were initially produced by so-called tri-transfection methods in mammalian cells [26,27]. By this approach, the best viral titers are obtained during productions made in adherent cells. The prospects for using AAVs in clinics have raised the question of scaling production levels. The previously mentioned beneficial features of BEVS production have led researchers to use this system to circumvent the technology gap they were facing [25]. One of the

methods that we previously described for the AAV production by BEVS consists of cloning the structural and functional AAV genes (called *cap* and *rep* genes respectively) within a first baculovirus genome under the control of the polh and p10 promoters [28]. Another baculovirus genome is used to clone the expression cassette carrying the cDNA of interest. In this configuration, production of AAV vectors is initiated by co-infection of insect cell lines with the two recombinant baculoviruses [28]. More than ten years after the development of this production system, the first marketing authorization for an AAV gene therapy vector was approved by the competent authorities [29].

Baculovirus as Gene Transfer Vector

The virus-derived gene transfer vectors remain the most powerful molecular tools for transporting a transgene in cell nuclei and allowing its expression. Viral families derived from lentiviruses, adenoviruses, parvoviruses or even herpesviruses are among the most used. In recent years, developments around baculovirus processes and biology have led several teams to focus on their use as direct gene vectors [30]. Among the most interesting advantages, we can note their effectiveness in transducing a large number of mammalian cells, their inability to replicate outside insect cells, their large packaging capacity and their ability to be easily pseudotyped by other envelope glycoproteins allowing them to potentially adopt various specific cell tropisms. Moreover, their disadvantage of being neutralized *in vivo* by the complement system has been recently circumvented by approaches exploiting their capacity to express some specific complement inhibitory factors on the viral envelope [31]. The use of baculovirus vectors in gene transfer has applications in the screening of gene functions, drug testing or more recently in the gene therapy of genetic diseases, cancer and other type of pathologies.

Discussion

Our personal experience in the use of BEVS has led us to use its biological characteristics to investigate the production of proteins from more exotic organisms, especially some from the marine world. The challenge is exciting as there are still few proteomic libraries available for a large part of these organisms. Numerous scientific programs and expeditions are currently taking place over the world to enrich the genomic databases of organisms from these specific and still little investigated environments. In this context, BEVS could serve as a springboard to extend these efforts towards the establishment of new proteomic banks. The resulting structural and functional studies would enrich our knowledge while enhancing the discovery of innovative products for human or animal health or well-being.

From the technological point of view, future optimizations will certainly be performed to improve the production yields of BEVS. The construction of baculovirus genomes deleted from a number of non-essential genes, especially those encoding viral proteases (particularly cathepsin and chitinase) have already yielded positive results [32]. In another context, the impressive efforts made in recent years to improve both the tissue tropism and the immune escape of baculovirus vectors used for gene transfer augur exciting prospects in the field of human or animal therapeutics.

Conclusion

We have summarized the major applications of BEVS to point to the growing attraction of this biological system in the areas of R&D, diagnosis and for the human and veterinary clinic. It is clear to us that the coming years will see the emergence of increasingly potent systems based on the use of baculovirus, both in aspects of recombinant protein production and the use of these viruses in gene transfer. Finally, we hope that this review may trigger the curiosity and attractiveness of groups with regard to these very promising biotechnological approaches.

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Conflict of Interest

The authors declare no economic or conflict of interest.

References

1. Gaw ZY, Liu NT, Zia TU (1959) Tissue culture methods for cultivation of virus grasserie. Acta Virol: 55-60.
2. Grace TDC (1962) Establishment of four strains of cells from insect tissues grown *in vitro*. Nature 195: 788-789.
3. Hink WF (1970) Established insect cell line from the cabbage looper, Trichoplusia ni. Nature 226: 466-467.
4. Vaughn JL, Faulkner P (1963) Susceptibility of an Insect Tissue Culture to Infection by Virus Preparations of the Nuclear Polyhedrosis of the Silkworm (Bombyx Mori L.). Virology 20: 484-489.
5. Henderson JF, Faulkner P, MacKinnon EA (1974) Some biophysical properties of virus present in tissue cultures infected with the nuclear polyhedrosis virus of Trichoplusia ni. J Gen Virol 22: 143-146.
6. Brown M, Faulkner P (1978) Plaque assay of nuclear polyhedrosis viruses in cell culture. Appl Environ Microbiol 36: 31-35.
7. Adang MJ, Miller LK (1982) Molecular cloning of DNA complementary to mRNA of the baculovirus Autographa californica nuclear polyhedrosis virus: location and gene products of RNA transcripts found late in infection. J Virol 44: 782-793.
8. Vlak JM, Smith GE (1982) Orientation of the Genome of Autographa californica Nuclear Polyhedrosis Virus: a Proposal. J Virol 41: 1118-1121.

9. Smith GE, Summers MD, Fraser MJ (1983) Production of human beta interferon in insect cells infected with a baculovirus expression vector. Mol Cell Biol 3: 2156-2165.

10. van Oers MM, Pijlman GP, Vlak JM (2015) Thirty years of baculovirus-insect cell protein expression: from dark horse to mainstream technology. J Gen Virol 96: 6-23.

11. Chambers AC, Aksular M, Graves LP, Irons SL, Possee RD, et al. (2018) Overview of the Baculovirus Expression System. Curr Protoc Protein Sci 91: 1-5.

12. Burand JP, Summers MD, Smith GE (1980) Transfection with baculovirus DNA. Virology 101: 286-290.

13. Pijlman GP, van den Born E, Martens DE, Vlak JM (2001) Autographa californica baculoviruses with large genomic deletions are rapidly generated in infected insect cells. Virology 283: 132-138.

14. Jorio H, Tran R, Kamen A (2006) Stability of serum-free and purified baculovirus stocks under various storage conditions. Biotechnol Prog 22: 319-325.

15. Wasilko DJ, Lee SE, Stutzman-Engwall KJ, Reitz BA, Emmons TL, et al. (2009) The titerless infected-cells preservation and scale-up (TIPS) method for large-scale production of NO-sensitive human soluble guanylate cyclase (sGC) from insect cells infected with recombinant baculovirus. Protein Expr Purif 65: 122-132.

16. Cecchini S, Virag T, Kotin RM (2011) Reproducible high yields of recombinant adeno-associated virus produced using invertebrate cells in 0.02- to 200-liter cultures. Hum Gene Ther 22: 1021-1030.

17. Martinez-Solis M, Herrero S, Targovnik AM (2019) Engineering of the baculovirus expression system for optimized protein production. Appl Microbiol Biotechnol 103: 113-123.

18. Felberbaum RS (2015) The baculovirus expression vector system: A commercial manufacturing platform for viral vaccines and gene therapy vectors. Biotechnol J 10: 702-714.

19. Harper DM (2008) Impact of vaccination with Cervarix (trade mark) on subsequent HPV-16/18 infection and cervical disease in women 15-25 years of age. Gynecol Oncol 110: S11-S17.

20. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, et al. (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 363: 411-422.

21. Cox MM, Hollister JR (2009) FluBlok, a next generation influenza vaccine manufactured in insect cells. Biologicals 37: 182-189.

22. Treanor JJ, El Sahly H, King J, Graham I, Izikson R, et al. (2011) Protective efficacy of a trivalent recombinant hemagglutinin protein vaccine (FluBlok(R)) against influenza in healthy adults: a randomized, placebo-controlled trial. Vaccine 29: 7733-7739.

23. Liu F, Wu X, Li L, Liu Z, Wang Z (2013) Use of baculovirus expression system for generation of virus-like particles: successes and challenges. Protein Expr Purif 90: 104-116.

24. Grabherr R, Ernst W (2010) Baculovirus for eukaryotic protein display. Curr Gene Ther 10: 195-200.

25. Urabe M, Ding C, Kotin RM (2002) Insect cells as a factory to produce adeno-associated virus type 2 vectors. Hum Gene Ther 13: 1935-1943.

26. Xiao X, Li J, Samulski RJ (1998) Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. J Virol 72: 2224-2232.

27. Dias Florencio G, Precigout G, Beley C, Buclez PO, Garcia L, et al. (2015) Simple downstream process based on detergent treatment improves yield and *in vivo* transduction efficacy of adeno-associated virus vectors. Mol Ther Methods Clin Dev 2: 15024.

28. Buclez PO, Dias Florencio G, Relizani K, Beley C, Garcia L, et al. (2016) Rapid, scalable, and low-cost purification of recombinant adeno-associated virus produced by baculovirus expression vector system. Mol Ther Methods Clin Dev 3: 16035.

29. Gaudet D, Methot J, Dery S, Brisson D, Essiembre C, et al. (2013). Efficacy and long-term safety of alipogene tiparvovec (AAV1-LPLS447X) gene therapy for lipoprotein lipase deficiency: an open-label trial. Gene Ther 20: 361-369.

30. Ono C, Okamoto T, Abe T, Matsuura Y (2018) Baculovirus as a Tool for Gene Delivery and Gene Therapy. Viruses 10.

31. Kaikkonen MU, Yla-Herttuala S, Airenne KJ (2011) How to avoid complement attack in baculovirus-mediated gene delivery. J Invertebr Pathol 107: S71-S79.

32. Galibert L, Merten OW (2011) Latest developments in the large-scale production of adeno-associated virus vectors in insect cells toward the treatment of neuromuscular diseases. J Invertebr Pathol 107: S80-S93.