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# Baculovirus-Derived Vectors for Immunization and Therapeutic Applications

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*M. Laura Fabre, Paula N. Arrías, Tomás Masson, Matías L. Pidre and Víctor Romanowski*

Laboratory of Molecular Virology, Institute of Biotechnology and Molecular Biology (IBBM), University of La Plata-CONICET, La Plata, Argentina

## ABBREVIATION

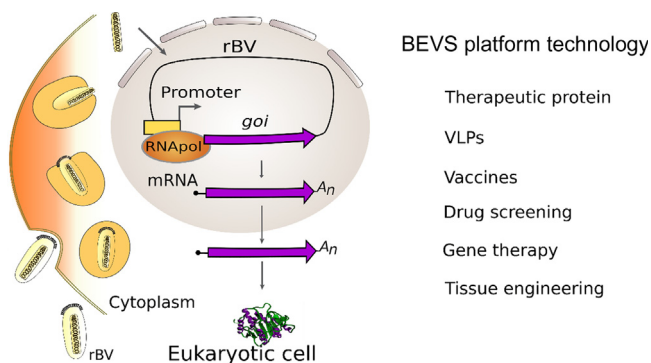
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<b>AAV</b>	adeno-associated viruses
<b>AcMNPV</b>	<i>Autographa californica</i> nucleopolyhedrovirus
<b>BEVS</b>	Baculovirus Expression Vector System
<b>BV</b>	budded virus
<b>CMV</b>	cytomegalovirus
<b>DNA</b>	deoxyribonucleic acid
<b>HA</b>	hemagglutinin
<b>ITR</b>	inverted terminal repeats
<b>NC</b>	nucleocapsid
<b>OB</b>	occlusion body
<b>ODV</b>	occlusion derived virus
<b>ORF</b>	open reading frame
<b>PCV</b>	porcine circovirus
<b>POLH</b>	polyhedrin
<b>rAAV</b>	recombinant adeno-associated virus
<b>RNA</b>	ribonucleic acid
<b>VLP</b>	virus-like particle
<b>VSV</b>	vesicular stomatitis virus

## BIOLOGY OF BACULOVIRUSES

Baculoviruses are insect-specific pathogens that have been isolated from about 600 species belonging to the orders Lepidoptera, Hymenoptera, Diptera, Orthoptera, Coleoptera, Neuroptera, Thysanura, and Trichoptera. Taxonomy of *Baculoviridae* family includes four genera: *Alphabaculovirus* (nucleopolyhedroviruses specific of lepidopterans), *Betabaculovirus* (granuloviruses specific of lepidopterans), *Gammabaculovirus* (nucleopolyhedroviruses specific of hymenopterans), and *Deltabaculovirus* (granuloviruses specific of dipterans) (Jehle et al., 2006; Herniou et al., 2011). These viruses infect arthropod larvae and possess circular double-stranded deoxyribonucleic acid (DNA) genomes that range in size from 80 to 180 kbp. The baculoviral genome is packed inside a distinct rod-shaped nucleocapsid (NC). The life cycle of baculoviruses is biphasic, and each phase is characterized by a different viral phenotype. In the environment, baculoviruses are found as occlusion bodies (OBs). A protein matrix encloses virions and provides protection against harsh environmental conditions such as temperature, dehydration, and ultraviolet light until a susceptible larva ingests the OBs. Briefly, the baculovirus infection cycle of insect hosts is as it follows: OBs are ingested together with foliage by larvae and dissolved in the midgut-releasing occlusion-derived viruses (ODVs), and the damaged peritrophic membrane allows the receptor-mediated endocytosis of the ODV by epithelial cells (Adams and McClintock., 1991). ODVs may contain one or multiple rod-shaped NCs depending on the baculovirus (or less frequently on growth conditions). The NCs enter the cytoplasm and translocate to the nucleus where the viral DNA is transcribed and replicated. Newly synthesized genomic DNA assembles with specific viral proteins to form the NCs in the nucleus. The NCs travel to the plasma membrane where budding takes place to yield the budded viruses (BVs), the second viral phenotype, responsible for the systemic infection of the larvae.

Gene expression is temporally regulated; early genes are transcribed directly by host ribonucleic acid (RNA) polymerase II, whereas late and very-late gene transcription is dependent on viral RNA polymerase. Late genes code mostly for structural proteins characteristic of the beginning of systemic infection while very-late genes code for most ODV structural proteins including the major OB matrix protein (Horton and Burand, 1993; Haas-Stapleton et al., 2004; Au et al., 2013). BVs can infect both in vivo tissues and insect cell cultures (Laakkonen et al., 2008; Long et al., 2006a,b). The possibility to propagate these viruses in cell culture has made baculoviruses an accessible tool for genetic engineering (Goodwin et al., 1970), both for the study of their biology and for the development of a recombinant protein expression system (*BEVS: baculovirus expression vector system*) (Smith et al., 1983). This system is

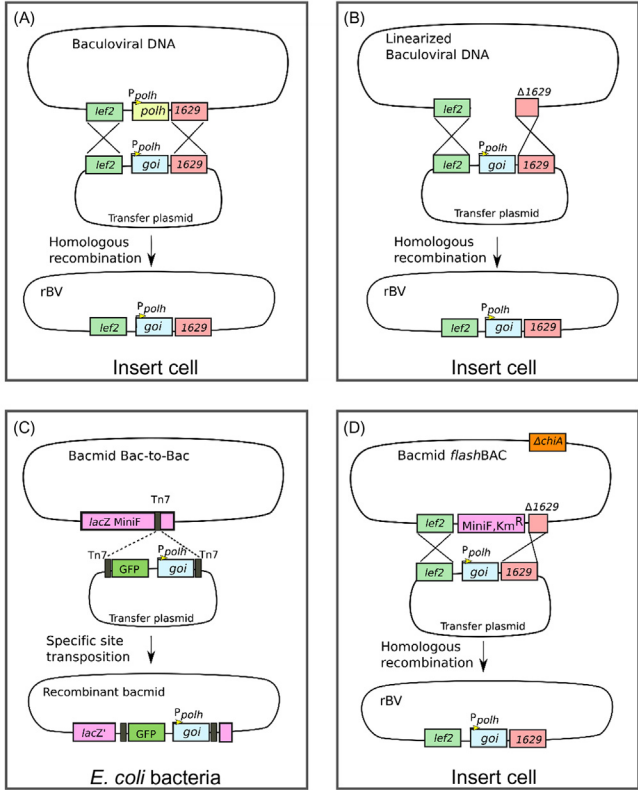


**FIGURE 11.1 Baculovirus expression vector system (BEVS).** The recombinant baculovirus (rBV) enters the cell via endocytosis. Following this event, the nucleocapsid (NC) is released into the cytoplasm and translocated into the nucleus, where viral DNA will be expressed. A simplified scheme of the viral DNA is presented, highlighting the promoter and the coding sequence of the gene of interest (*goi*). Alternative promoters: Immediate early protein *ie1*, *polh*, *p10*, *gp64*, *p6.9*, *vp39* promoters from baculovirus genomes are used for protein expression in insect cells and promoters such as Simian Virus SV40, immediate early protein *ie1* cytomegalovirus (CMV), elongation factor (EF)-1 $\alpha$ , hybrid CAG promoter, human ubiquitin C, U6, Pol III H1, and *Drosophila melanogaster* *hsp70*, among others, are commonly used for expression in mammalian cells. On the left of the schematic: different applications of the baculovirus expression system.

considered a useful, efficient, and robust tool in many areas, including but not limited to biotechnology, clinical biochemistry, and molecular biology (Fig. 11.1).

## GENETIC ENGINEERING OF BACULOVIRUSES

Even though in the last three decades, different strategies have been developed for the generation of recombinant baculoviruses, two remain the most widely used. The more traditional approach consists of cotransfecting insect cells with both a baculoviral genome and a transfer plasmid. The transfer plasmid carries the genes of interest flanked by two regions containing sequences for homologous recombination; meanwhile, the viral genome provides the genetic information of a wild-type virus. Isolation of the recombinant event was made by selecting the OB-phenotype (nonoccluded viruses) observed by microscopy. However, due to a low recombination frequency ranging from 0.1% to 1%, this original technique yielded a high proportion of nonrecombinant wild-type virus (Fig. 11.2A) (O'Reilly and Miller, 1991). This contamination with parental virus makes isolation and purification of recombinant viruses laborious since it involves multiple plaque



**FIGURE 11.2 Methods for the generation of recombinant baculovirus (rBV).** (A) Classic methodology. rBV are generated by homologous recombination between a baculoviral wild-type genome and a transfer plasmid containing the gene of interest (*goi*) in insect cells; (B) Linearized viral genome approach. The viral genome is linearized and the essential gene *orf1629* is truncated. This approach facilitates the recombinant virus' selection steps. Homologous recombination reconstitutes *orf1629*; (C) Bac-to-Bac transposition-based technology. The *goi* is inserted by site-specific transposition into a bacmid inside *Escherichia coli* cells; (D) *FlashBAC* system. Homologous recombination occurs between a bacmid and a transfer plasmid in insect cells. Bacterial sequences are eliminated after the recombination event.

selection steps that are time consuming. In order to reduce the parental virus progeny, the baculoviral genome was linearized in a unique restriction site (*Bsu36I*) in the accessory gene *polh*, which codes for the major protein of the nucleopolyhedroviruses' OB, polyhedrin (POLH) and has a strong baculoviral promoter for high expression in insect cells. Since linear genome cannot replicate (noninfective), only the genomes that become circular after homologous recombination with the transfer vector are able to produce progeny. This modification to the system provides a recombination frequency near 30%, but the

possibility of isolating wild-type virus still exists due to genetic repair events or incomplete digestion (Fig. 11.2B) (Possee et al., 1991). Further modifications have been made to this system, by inserting additional unique restriction sites, one of which is inserted in the essential gene *orf1629*, which will be disrupted during linearization of the viral DNA by restriction enzyme digestion. This open reading frame (ORF) codes for a phosphoprotein that is a key component of the NC and its deletion renders undesired recircularization events nonviable (Kitts and Possee, 1993).

The second and most widely used methodology of site-specific transposition of Tn7 transposon into a bacmid containing the baculoviral genome (Fig. 11.2C). This system is commercially available as Bac-to-Bac Expression System (Invitrogen, Inc.). Shortly, this technique for generating recombinant baculoviruses consists in the site-specific transposition of a transfer vector (pFastBac) that carries the *goi* under a strong baculoviral promoter, and a bacmid. The bacmid contains Tn7 sites and a bacterial origin of replication (*mini-F*) and is propagated in DH10Bac *Escherichia coli*. This *E. coli* strain contains a helper plasmid that codes for Tn7 transposase. When both bacmid and transfer plasmid are transformed into bacteria containing the helper plasmid, the site-specific transposition takes place generating a recombinant baculoviral genome. The selection of clones is made by white and blue colony screening. The bacmid can be purified from bacterial cultures and transfected into Sf9 cells inside of which it can replicate, thus generating recombinant baculoviruses. The downside to this system when developing therapeutic products for both veterinary and human use is the presence of bacterial genomic sequences and selection markers such as antibiotic resistance.

Oxford Expression Technologies has made a safer approach with its *flashBAC* platform, which combines the two most widely used methodologies for the generation of recombinant baculoviruses (Fig. 11.2D). This platform is based on a bacmid where an essential gene such as *orf1629* has been partially deleted (other accessory genes such as *p74*, *p10*, *p26* have been also knocked out) and homologous recombination that occurs in insect cells with transfer plasmid containing part of *orf1629* restores the complete sequence of this essential gene, while eliminating bacterial sequences (Je et al., 2003; Hitchman et al., 2010). The *flashBAC* platform also offers variants to increase yields for specific target proteins (GOLD, ULTRA, and PRIME). More recently, a new platform known as MultiBac was developed (Geneva Biotech) for the expression of multiple polypeptides, especially suitable for the production of eukaryotic multiprotein complexes (Berger et al., 2013). This system allows the insertion of foreign genes into two different *loci* of an engineered baculoviral genome: *polyhedrin* locus and *chiA/γ-cath* locus.

The advantages of MultiBac are the tandem recombining that permits expression of proteins with various subunits and the reduction of proteolytic activity in the cell.

## EXPRESSION OF HETEROLOGOUS PROTEINS FOR DIAGNOSIS AND IMMUNIZATION

Expression systems for the production of therapeutic proteins have been established using recombinant DNA technology in mammalian, plant, insect, fungal, and bacterial cells, as well as transgenic multicellular organisms (Wu et al., 2002; Steinbach et al., 2002; Chen et al., 2003; Katagiri et al., 2003; Hammonds et al., 2007).

The BEVS can be divided into two stages: the first is the generation and isolation of a recombinant baculovirus carrying the *goi* and the second is the infection of a susceptible host (cultured cells or larvae) with this recombinant virus (O'Reilly and Miller, 1991). The very first recombinant protein that was produced using BEVS was human interferon beta (Smith et al., 1983), but to date thousands of proteins have been expressed using this system. By far the most widely used virus is *Autographa californica* nucleopolyhedrovirus (AcMNPV); however, *Bombyx mori* nucleopolyhedrovirus is also used due to the higher protein expression using silkworm larvae or its pupae (Motohashi et al., 2005). The most commonly used promoters for the expression of heterologous proteins in BEVS are the POLH promoter (*polh*) and p10 promoter (*p10*). These promoters have a high expression capacity and they become active following the late stages of infection.

In terms of cell lines, those derived from *Spodoptera frugiperda* ovary (Sf9, Sf21, and ExpressSF+) and *Trichoplusia ni* (HighFive, BTI-TN-5B1-4, Invitrogen, Carlsbad, CA, United States) are the ones most frequently chosen by the users of BEVS. All these lines are able to grow both adherent or in suspension, in serum-free media and without requirements of CO<sub>2</sub> (Grace, 1962; Granados, 1994; Vaughn et al., 1997; Lynn, 2001; Ikonomou et al., 2003; Agathos, 2010). It has been reported that HighFive cells have the highest heterologous protein expression yield (Wang et al., 2011, 1992; Yamaji et al., 2006). Larvae belonging to the order Lepidoptera, such as the silkworm, can also be used for the expression, with similar protein yields, the higher rate of production and, lower costs compared to those of cell culture. However, the requirements of Good Manufacturing Practice and quality control using larvae are more difficult to achieve, which makes the use of these insects not as common. Although insect cell lines are more secure than mammalian ones they have to be screened for adventitious agents that

pose a risk to human and veterinary health, such as *Alphanodavirus* and *Rhabdovirus* that have been isolated from *Helicoverpa armigera* and *S. frugiperda* cells, respectively (Bai et al, 2011; Ma et al., 2014). A disadvantage associated with the use of both cell culture and larvae is that after the first round of infection, cells lyse, and the entire culture has to be replaced, which does not allow a continuous expression of the *goi*. However, the use of low-cost serum-free media has somewhat helped overcome this disadvantage.

The recombinant protein production has also been improved by the deletion of baculoviral accessory genes such as chitinase and cathepsin that had a positive effect on the integrity of both the intracellular and secreted recombinant protein (Kaba et al., 2004; Hitchman et al., 2010).

There are many advantages of BEVS over other expression systems. It provides a eukaryotic platform for adequate folding, disulfide bridge establishment, oligomerization, and other posttranslational modifications needed for biological activity of proteins. Moreover, high protein yields can be obtained using strong viral promoters, and the system allows the simultaneous expression of multiple genes due to the baculoviral genome's capacity to insert DNA fragments up to 34 kbp (O'Reilly et al, 1994). It is important to mention that the production of recombinant proteins using this system has great scale-up potential and low-associated cost; low biosecurity requirements are needed since baculoviruses have a very limited host range and are nonpathogenic to humans.

In terms of posttranslational modifications of recombinant proteins, such as the eukaryotic glycosylation, BEVS presents simple protein glycosylation patterns. However, the *N*-glycosylation of proteins in insect cells does not resemble fully that of mammalian cell culture, while insect *N*-glycans have terminal mannose residues, and mammalian *N*-glycans often have terminal sialic acid residues. This difference may result in a lower or even total loss of biological function of the protein of interest (Harrison and Jarvis, 2006, 2016). In order to overcome this problem, humanized insect cells have been developed that express the mammalian glycosylating enzymes (Harrison and Jarvis, 2006; Jarvis et al., 1998; Mabashi-Asazuma et al., 2014; Okada et al., 2010). Another strategy consists in modifying the baculoviral genome in order to express the *goi* along with these glycosylating enzymes (SweetBac) (Palmberger et al., 2012).

Product contamination with baculoviruses might be a problem when using BEVS for development of human and veterinary vaccines. Marek et al. have developed a system called Bacfree. This methodology uses *vp80* deficient baculovirus that can only be



propagated in cells that provides VP80 in *trans*. Deletion of this essential gene prevents the assembly of the viral phenotypes BV and ODV (Marek et al., 2011).

The rapid, scalable, and low-cost production using this technology is particularly important in terms of confronting sanitary emergencies such as the influenza pandemic of 2009. Fedson and Dunnill (2007) reported that if just 25% of global bioreactor capacity was used in the generation of anti-flu vaccines with the BEVS, 425 million vials of 10 µg/dose could be produced in only 1 month, as opposed to the classic platform used for this purpose, which involves embryonated chicken eggs and takes about 6 months (Wong and Webby, 2013). This means that nowadays, the production of influenza vaccines begins months before the exact prevalent strain of influenza is known for certain. If BEVS platform was used instead, the time required for the production would be just 45 days (Cox, 2012).

Over the last 30 years, the scientific community has used this platform for the production of recombinant proteins at a laboratory scale. However, in the last decade, the BEVS has started to gain priority in the biotechnological industry. To date, five veterinary vaccines and four products for human use exist on the market including, vaccines, immunotherapy, and gene therapy vectors (Table 11.1).

FluBlok is the first recombinant vaccine to be licensed by the US Food and Drug Administration for the prevention of seasonal influenza and is manufactured using BEVS in expresSF + insect cells. It is a trivalent recombinant hemagglutinin (HA) vaccine, produced by Protein Sciences Corporation, that contains HA antigen derived from three influenza virus strains, selected for inclusion in the annual influenza vaccine by the World Health Organization and updated on an annual basis (Holtz et al., 2003; Wang et al., 2006). Since in this system, there cannot be replication, subsequent inactivation steps of the live influenza virus that are crucial in the licensed egg-based vaccines are not needed using this alternative.

Diamyd is a therapeutic vaccine produced using BEVS in Sf9 cells aimed to treat type 1 diabetes. Diamyd is a recombinant 65 kDa glutamate decarboxylase corresponding to human glutamate decarboxylase 65 that is the major autoantigen in type 1 diabetes. Diamyd is developed by Diamyd Medical and produced by Protein Sciences Corporation (Hinke, 2008).

In summary, the BEVS is a valuable platform for the manufacture of a great variety of biotechnological products since with just one insect cell line and a single method for the construction of recombinant baculoviruses it is possible to express a large variety of products.

**TABLE 11.1** Commercial Vaccines and Therapies Based on Baculovirus Expression Technology for Veterinary or Human Use

Pathogen of disease	Expressed product	Vaccine type	Commercial name	Company	Year	References
<b>VETERINARY USE</b>						
Classical swine fever	E2 glycoprotein	Subunit/ marker	Porcilis Pesti	Merck	1998	<a href="#">Kirnbauer et al. (1992)</a>
Classical swine fever	E2 glycoprotein	Subunit/ marker	Bayovac CSF E21	Bayer AG	2001	<a href="#">Small et al. (2000)</a>
Porcine circovirus type 2	PCV ORF2	VLP	Circumvent PCV	MSD Animal Health	2005	<a href="#">Blanchard et al. (2003)</a>
Porcine circovirus type 2	PCV ORF2	VLP	Ingelvac CircoFLEX	Boehringer Ingelheim	2008	<a href="#">Fachinger et al. (2008)</a>
Porcine circovirus type 2	PCV ORF2	VLP	Porcilis PCV	Merck	2009	<a href="#">van Aarle (2003)</a>
<b>HUMAN USE</b>						
HPV	HPV L1 protein (strain 16 and 18)	VLP	Cervarix	GlaxoSmithKline	2007	<a href="#">Muñoz et al. (2003)</a>
Prostate cancer	PAP–GM–CSF <sup>a</sup> AAV	Immunotherapy	Provenge	Dendreon	2010	<a href="#">Cheever and Higano (2011)</a>
Familial lipoprotein lipase deficiency	AAV vector with lipoprotein lipase transgene <sup>b</sup>	Gene therapy	Glybera	uniQure	2012	<a href="#">Ferreira et al. (2014)</a>
Avian influenza virus	HA	Subunit	Flublok	Protein Sciences Corporation	2013	<a href="#">Holtz et al. (2003)</a>

<sup>a</sup>Prostatic acid phosphatase coupled to granulocyte macrophage colony-stimulating factor.

<sup>b</sup>Discontinued.

AAV, Adeno-associated viruses; HA, hemagglutinin; HPV, human papillomavirus; VLPs, virus-like particles.

## THE BACULOVIRUS EXPRESSION VECTOR SYSTEM FOR THE GENERATION OF VIRUS-LIKE PARTICLES

The baculovirus/insect cell expression system has been widely used for the generation of antigens and the development of viral vaccines both for human and veterinary use (Cox, 2012; Mena and Kamen, 2011; van Oers, 2006; Yamaji, 2014). Classic viral vaccines (first-generation vaccines), such as those for measles, rubella, rabies, and yellow fever, use attenuated viral strains or inactivated viruses. Attenuation corresponds with limited viral replication after vaccination, and although the immune response induced by this kind of vaccines is similar to natural infection adverse, reactions cannot be dismissed. Conversely, inactivated strains cannot replicate since the genetic material has been destroyed, making them more secure than the attenuated ones. However, the immune response generated by these formulations is not as efficient, and three to five doses are generally required to attain optimal antibody titers; in addition, risks of incomplete inactivation can lead to events such as the Cutter incident, in which about 40,000 polio cases were caused by a defective polio vaccine in the United States (Fitzpatrick, 2006).

An alternative that is set to outclass this type of vaccines consists of subunit or recombinant protein formulations, which include virus-like particles (VLPs). These recombinant vaccines carry a limited number of viral proteins, either complete or truncated, in a nonpathogenic viral vector. VLPs are self-assembling, complex protein structures that resemble wild-type viral capsids or enveloped virions. The difference with the latter is that VLPs do not contain pathogenic (replicative) genomes. Vaccines formulated with these structures combine the advantages of attenuated vaccines with those of subunit vaccines since the epitopes can be recognized by host's immune system and efficiently stimulate the adaptive immune response while avoiding the possible reversion to virulent phenotypes.

VLPs are classified in two types based on structure: enveloped VLPs, that include a host-derived lipid membrane and naked or nonenveloped (simply referred to as VLPs). Likewise, they are also classified as simple or multiple VLPs depending on the number of different recombinant proteins included in the structure (Noad and Roy, 2003; Roldão et al., 2011). Some difficulties may be associated with VLP production: for example, protein stoichiometry can be a problem since the incorporation of adequate proportions of each protein may not occur (Latham and Galarza, 2001). However, even if the VLPs do not fully resemble the wild-type virus, they can stimulate the host immune system to an extent high enough to meet the requirements of a vaccine candidate.

VLPs represent a significant advance in terms of developing preventive tools against viruses that cannot be propagated in cell lines or *ex vivo* cultures, such as the hepatitis B virus and enteric adenoviruses types 40 and 41.

VLP production using BEVS can be accomplished by one of two alternatives, or by the combination of both. The first consists of the coinfection of insect cells with as many recombinant viruses as proteins the manufacturer wants to assemble in the VLP structure (monocistronic baculoviruses) or with a single polycistronic virus that codes for all the proteins. Complex multiprotein VLPs present difficulties for their production since the infection with multiple recombinant baculoviruses are not homogeneous. Vieira *et al.* compared both strategies for the production of rotavirus VLPs containing VP2, VP6, and VP7. They observed that the coexpression strategy was superior to the coinfection one; meanwhile Roldão *et al.* for the same VLPs found the opposite (Vieira *et al.*, 2005; Roldão *et al.*, 2006). To this day, a vast array of taxonomically and structurally different VLPs has been produced using BEVS. Many of these already are in preclinical and clinical trial stages, and seven VLP-based vaccines have already been approved and are currently being commercialized (Table 11.2). One of the most promising products already on the market is Cervarix, a VLP-based vaccine against human papillomaviruses 16 and 18, which are responsible for 70% of cervical cancer cases, produced by GlaxoSmithKline (Muñoz *et al.*, 2003; Smith *et al.*, 2007).

## BACULOVIRUS SURFACE DISPLAY

As mentioned earlier, the BEVS can be used to produce substantial amounts of heterologous proteins for their use as immunogens. This strategy, although key to subunit vaccines development, can be replaced by peptide display on virion surfaces. Baculovirus surface display refers to the display of heterologous peptides or proteins on the surface of BVs by different strategies all of which involve a translational fusion of a peptide to GP64 or a portion of it (Kost *et al.*, 2005; Xu *et al.*, 2011). GP64 is the major surface protein and a class III integral membrane glycoprotein of budded virions that is able to mediate membrane fusion under acidic pH conditions. AcMNPV GP64 is a 512-amino acid long phosphoglycoprotein with 15 cysteine residues that participate in disulfide bridge formation required for stability and biological activity (Hefferon *et al.*, 1999; Garry and Garry, 2008).

The surface display of the heterologous polypeptide can be achieved by constructing a chimera with complete wild-type GP64 or only the transmembrane, multimerization, and carboxy-terminal (cytoplasmic tail) domains to the target protein, or by expression of a recombinant

**TABLE 11.2** Generation of Virus-Like Particles (VLPs) Using Baculovirus Expression Vector System

<b>Virus</b>	<b>Type of VLP<sup>a</sup></b>	<b>Cells</b>	<b>Recombinant proteins</b>	<b>Expression strategies</b>	<b>References</b>
Feline leukemia virus	eVLP	<i>Sf9</i>	Gp85 and Gag	Coinfection	Thomsen et al. (1992)
Norwalk virus	VLP	<i>Sf9</i>	Capsid protein	Single infection	Jiang et al. (1992)
Porcine parvovirus	VLP	<i>Sf9</i>	VP2	Single infection	Martinez et al. (1992)
Bluetongue virus	VLP	<i>Sf21</i>	VP2, VP6, VP7, and NS1	Coexpression	Belyaev and Roy (1993)
Poliovirus	VLP	<i>Sf21</i>	VP0, VP3, and VP1	Coinfection	Bräutigam et al. (1993)
HPV	eVLP	<i>Sf9</i>	L1 and L2	Coexpression	Kirnbauer et al. (1992), Boxus et al. (2016), Huber et al. (2017)
Herpes simplex virus	VLP	<i>Sf21</i>	VP23, VP5, VP22, <sup>a</sup> VP21–VP24, VP26–VP19c	Coinfection	Tatman et al. (1994)
Rotavirus	VLP	<i>Sf9</i>	VP2, VP6, VP7, and VP4	Coinfection	Crawford et al. (1994)
Porcine parvovirus (LCMV) <sup>b</sup>	VLP	<i>Sf9</i>	VP2 containing LCMV epitope	Single infection	Sedlik et al. (1997)
African horse sickness virus	VLP	<i>Sf9</i>	VP3 and VP4	Coinfection	Maree et al. (1998)
Human immunodeficiency virus	eVLP	<i>Sf9</i>	Gag and Gp120	Coexpression	Buonaguro et al. (2001)
Human severe acute respiratory syndrome coronavirus	eVLP	<i>Sf21</i>	Spike, membrane and envelope proteins	Coinfection	Ho et al. (2004)
Human astrovirus	VLP	<i>Sf9</i>	ORF2	Single infection	Caballero et al. (2004)
Enterovirus-71	VLP	<i>Sf9</i>	P1 and 3CD	Coexpression and coinfection	Chung et al. (2006)

(Continued)

TABLE 11.2 (Continued)

Virus	Type of VLP <sup>a</sup>	Cells	Recombinant proteins	Expression strategies	References
Feline calicivirus	VLP	<i>Sf9</i>	Capsid	Single infection	Di Martino et al. (2007)
Simian virus 40	VLP	<i>Sf9</i>	VP1, VP2, and VP3	Single infection and coinfection	Kosukegawa et al. (1996)
Rift Valley fever virus	eVLP	<i>Sf9</i>	Gn, Gc, and N	Coexpression	Liu et al. (2008b)
Porcine circovirus	VLP	<i>Tn</i>	VP2	Single infection	Liu et al. (2008a)
Avian influenza virus	eVLP	<i>Sf9</i>	HA, NA, and others	Coexpression	Tao et al. (2009), Pushko et al. (2016)
Encephalomyocarditis virus	VLP	<i>Sf9</i>	P1-2A-3C	Single infection	Jeoung et al. (2011)
Rous sarcoma virus	eVLP	<i>Tn</i> and <i>Sf9</i>	Gag and Gp120	Single infection	Deo et al. (2011)
Japanese encephalitis virus	eVLP	<i>Sf9</i>	prM and gE	Coinfection	Yamaji et al. (2006)
Coxsackievirus A16	VLP	<i>Sf9</i>	P1 and 3CD	Coexpression	Liu et al. (2012)
Foot-and-mouth disease virus	VLP	<i>Sf9</i>	P1-2A-3C	Single infection	Mohana Subramanian et al. (2012)
RHDV	VLP	<i>SL</i>	Capsid protein	Single infection	Zheng et al. (2016)
PRRS	eVLP	<i>Sf9</i>	Gp2, Gp3, Gp4, Gp5, E, and M	Single infection and coinfection	García Durán et al. (2016), Nam et al. (2013)
Chikungunya virus	eVLP	<i>Sf21</i>	Structural polyprotein	Single infection	Metz et al. (2013)
Ebola virus	eVLP	<i>Tn</i> and <i>Sf9</i>	GP, NP, and VP40	Coexpression and coinfection	Sun et al. (2009), Ye et al. (2006), Warfield et al. (2007, 2015)
Marburg virus	eVLP	<i>Tn</i> and <i>Sf9</i>	GP, NP, and VP40	Coexpression and coinfection	Warfield et al. (2007)

(Continued)

TABLE 11.2 (Continued)

Virus	Type of VLP <sup>a</sup>	Cells	Recombinant proteins	Expression strategies	References
Sudan virus	eVLP	Tn and Sf9	GP, NP, and VP40	Coexpression and coinfection	Warfield et al. (2015)
Duck hepatitis A virus	VLP	Sf9	P1 and 3CD	Coexpression	Wang et al. (2018)

<sup>a</sup>Classification of VLP: VLP (nonenveloped VLP), eVLP (enveloped VLP).

<sup>b</sup>LCMV, lymphocytic choriomeningitis virus; Sf, *Spodoptera frugiperda*; Tn, *Trichoplusia ni*; SL, *Silkworm larvae*. HPV, Human papillomavirus; PRRS, porcine reproductive and respiratory syndrome; RHDV, rabbit hemorrhagic disease virus.

This table has been modified and expanded on the basis of 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.

GP64 fused to a short or long heterologous peptide (Grabherr and Ernst, 2010).

Baculovirus display of heterologous proteins by fusion to GP64 was demonstrated to be very effective immunogens, successfully able to induce antibody response to a variety of displayed proteins (Kost et al., 2005). In addition, baculoviruses by themselves are able to trigger a robust innate immune response by activation of professional antigen-presenting cells.

Baculoviruses displaying heterologous envelope proteins, such as vesicular stomatitis virus (VSV)-G protein, have also been constructed. Several studies have shown that VSV-G is capable of improving transduction efficiency of baculovirus in vertebrate cells (Barsoum et al., 1997). Different strategies have been developed to increase the effectiveness of virus display (Chapple and Jones 2002). The polypeptides displayed by VSV-G exhibited higher densities of recombinant proteins than GP64 fusions because this protein is homogenous distributed in the BV compared with GP64 that is mostly apical.

In contrast to surface display, heterologous protein can be displayed on the NC of the virion. The NC core is composed of a circular, covalently closed dsDNA genome and the major basic core protein P6.9, and the NC sheath is formed by stacked ring-like subunits consisting of one major polypeptide, VP39, and several minor proteins. The NC display strategy involves the translational fusion of a peptide to VP39 (Kukkonen et al., 2003; Molinari et al., 2011; Pidre et al., 2013).

Depending on the host immune response that is targeted, one strategy might be chosen over the other. Antigen displayed on the NC should be able to reach the cytosol and preferentially trigger major

histocompatibility complex class I presentation pathway and mount a strong CD8 + T-cell response (Molinari et al., 2011). In turn, the BV displaying antigens on the surface elicit a humoral response (Gronowski et al., 1999; Abe et al., 2005).

Several studies have shown that baculovirus display can generate high titers of specific antibodies that protect against different pathogens such as Japanese encephalitis virus, avian reovirus, human enterovirus, influenza, and malaria (Xu et al., 2011; Lin et al., 2014; Marek et al., 2011; Jin et al., 2008; Madhan et al., 2010).

## BACULOVIRUS EXPRESSION VECTOR SYSTEM PLATFORM FOR THE PRODUCTION OF ADENO-ASSOCIATED VIRUSES

Adeno-associated viruses (AAV) have become relevant for in vivo gene-therapy (Nayerossadat et al., 2012; Flotte, 2013; Wang and Gao, 2014). This therapy involves the incorporation of genes into cells. AAV used as viral a vector is the tool that allows the safe, efficient, and specific incorporation of the gene inside the organism. The first group of hereditary diseases for which gene-therapy was investigated included hemophilia A and B, Leber's macular degeneration, and Duchenne's muscular dystrophy, but nowadays there are many studies focused on central nervous system diseases such as Parkinson's, different types of cancers, cardiovascular afflictions, and bone regeneration (Flotte et al., 1996; Kay et al., 2000; Kaplitt et al. 2007; Jiang et al., 2011). An estimate of 70%–80% of human population has been exposed to at least one event of infection with AAV, and no adverse consequences have been associated to the infection and latency of these viruses (Boutin et al., 2010; Erles et al., 1999; Tobiasch et al., 1998). AAVs have a single-stranded DNA genome and are nonenveloped viruses of 18–25 nm of diameter. They belong to the Parvovirus family and are classified as members of the *Dependovirus* genus. Twenty human and nonhuman primate strains have been isolated, and all serotypes except for serotype 5 share a very similar capsid structure, genome size, and organization. AAV genome has a size of approximately 4.7 kb and contains two ORFs that code for four regulatory proteins. These ORFs are flanked by two inverted terminal repeats (ITRs) that contain *cis*-regulatory sequences needed for viral infection. Major proteins Rep78 and Rep68 are involved in genome excision, rescue, replication, and integration. Integration into the host genome is site-specific and takes place in chromosome 19, more specifically into *locus* AAVS1 of the human genome, without adverse consequences. Minor proteins Rep52 and Rep40 are responsible for the



accumulation of DNA and packaging. VP1, VP2, and VP3 are structural proteins encoded in the Cap ORF.

Recombinant AAVs are constructed by replacement of *rep* and *cap* genes with the *goi* flanked by ITR regions. This viral vector is able to transduce both active division and quiescent cells. It is very important to highlight that, in contrast to wt AAV, the recombinant AAV has no site-specific integration capacity since *rep* genes are absent; thus the integration occurs at random. However, the estimated integration frequency of these recombinant adeno-associated viruses (rAAVs) is 0.1%–0.5% in mammalian cell lines (McCarty et al., 2004). In view of this, to establish stable and persistent expression in patients, an alternative to genome integration has been developed consisting in double-stranded DNA that persists as an episome inside the cells (Yang et al., 1999). To date, adeno-associated vectors have been modified to be tissue-specific, the preferred organs being those that are immune-privileged such as the eye or the brain.

The classic technique for the production of AAVs involves HEK293, A549, or HeLa cells cotransfected with two plasmids, one containing the recombinant DNA (recombinant adeno-associated virus plasmid) and the other the *rep* and *cap* genes (pHelper). The cells are then infected with a helper virus such as an adenovirus or a herpes simplex virus to allow the replication of the recombinant AAV (Samulski et al., 1987). The downside to this methodology is the possibility of undesired recombination events between plasmids and that purification of the helper virus is time consuming, and the scale-up process is difficult to achieve. A good alternative has been the use of BEVS platform [revised in Galibert and Merten (2011)], which involves the use of three recombinant baculoviruses and Sf9 cells. Each of the three recombinant baculoviruses contains one of the following genes: *rep*, *cap*, and the *construct of interest* flanked by the ITRs. Alternatives involving a polycistronic baculovirus containing *rep* and *cap* genes are also used (Smith et al., 2009). The OneBac system involves transgenic insect cell lines that express the genes *rep* and *cap* of the AAV and only one recombinant baculovirus. The efficiency of this technique and the scale-up feasibility are higher than the previous alternatives, and it provides a more flexible strategy since it allows the production of other AAV serotypes by simply changing cell lines (Mietzsch, 2014). Such as other products derived from BEVS, the contamination of rAAVs with baculovirus particles has to be reduced. There is already one rAAV produced by BEVS on the market commercialized as Glybera (alipogene tiparvovec) developed and marketed by uniQure that compensates for lipoprotein lipase deficiency, which received regulatory approval by the European Medicines Agency in 2012, although now it has been discontinued due to lack of demand.

## BACULOVIRUSES AS MAMMALIAN TRANSDUCTION VECTORS

It was demonstrated in the 1990s that the baculoviral genome was capable of transducing mammalian cells and that if heterologous genes were under control of adequate mammalian promoters, the ORFs carried by this baculoviral genome were transiently expressed in these cells. The viral promoters such as Simian Viruses SV40, *immediate early protein* ie1 CMV, elongation factor-1 $\alpha$ , hybrid CAG promoter, human ubiquitin C, U6, Pol III H1, and *Drosophila melanogaster* hsp70 among others are commonly used for expression in mammalian cells. Following the first days, the baculoviral genome is degraded and unable of insertion in the mammalian genome in the absence of selection pressure (Volkman and Goldsmith, 1983; Carbonell et al., 1985; Boyce and Bucher, 1996). At present, the baculoviral transduction ability has been documented both in vitro and in vivo for a variety of cell types including embryonic stem cells and pluripotent stem cells (Kost et al., 2005; Ho et al., 2005; Airene et al., 2010; Chen et al., 2011). It is suggested that the mechanism by which the baculoviruses ingress mammalian cells is clathrin and dynamin-dependent endocytosis and macropinocytosis. Makkonen showed that this process also requires the host cell proteins heparan sulfate proteoglycans and syndecan-1 that serves as a receptor (Long et al., 2006a,b; Kataoka et al., 2012; Makkonen et al., 2013, 2014; Nasimuzzaman, 2014). Once inside the cell, the baculovirus virions are transported into the endosome, where acidic conditions promote membrane fusion mediated by GP64, and the genetic material is thus liberated into the cytoplasm. Actin cytoskeleton reorganization promotes translocation of the baculoviral genome into the nucleus where transcription occurs by host machinery.

One of the advantages of using baculovirus as vectors for mammalian cell transduction is that in contrast to other viruses used to the same end, they are not human pathogens, they do not replicate in mammalian cells, no integration in the genome has been documented, and there is no preexisting immunity to baculoviruses (Strauss et al., 2007; Jin et al., 2008). A human study was made in which volunteers consumed OBs in their diet; the results were promising since no inflammatory response, allergies, or adverse effects were observed in a 5-day follow-up (Heimpel et al., 1973). These studies have been extended to animal models to evaluate possible side-effects such as skin irritation and cytotoxicity, all of which have been negative (Kost and Condrey, 2002; Jin et al., 2008).

Even though a downside to the use of baculovirus as viral vectors for gene delivery is the lack of long-term expression of the transgene,

alternatives to overcome this have been developed. For example, Lo et al. engineered a baculovirus vector system with Cre/loxP sites. In their approach, the construct is excised and remains as an episome in the cells that prolong expression (Lo et al., 2017).

All this evidence, together with the fact that baculoviruses infect no other organisms besides those of the phylum *Arthropoda*, shows they are safe vectors for developing DNA vaccines, immunotherapy, and gene therapy. A reflection of this is the increasing number of peer-reviewed publications that use recombinant baculoviruses over other viral vectors. For example, Swift et al. (2013) used a baculovirus with a prodrug approach to mediate cell-death of prostate cancer cells with success, even observing the good capacity of cell-layer penetration compared to other viral vectors. In the last few years, gene silencing by RNA interference has become more relevant for gene regulation. Baculoviral vectors have been engineered to deliver microribonucleic acid and short hairpin ribonucleic acid into insect and mammalian cells successfully. Gottardo and Pidre showed that baculovirus-mediated gene silencing of human in increased survival and reduced the progression of pituitary tumor growth in vivo (Gottardo et al., 2018). This type of strategy can be also applied to interfere with viral pathogens infecting humans and other animals (Makkonen et al., 2015).

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## DISCUSSION

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For over 30 years, baculoviruses have been exploited in molecular biology laboratories, and the trend has never stopped increasing. Since they are a highly versatile tool, with low-associated costs and scalable production, pharmaceutical giants such as Merck are taking advantage of them. Baculoviruses are not mere biological control agents anymore but are also key in the state of the art of gene-therapy and immunization departments. One of the most relevant advantages of BEVS is the rapid response under an epidemiological emergency such as an influenza pandemic. Meanwhile, the classical first-generation, egg-based approach for developing flu vaccines can be unsuitable for allergic users and takes about six months to develop; the BEVS recombinant technology approach can give a fully functional vaccine within the first 45 days after the outbreak. Using the BEVS is not only inexpensive and direct, but it also provides unique plasticity. Together with the fact that almost no special biosafety measures have to be taken into account for its application, it makes an excellent platform when compared to other systems.

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