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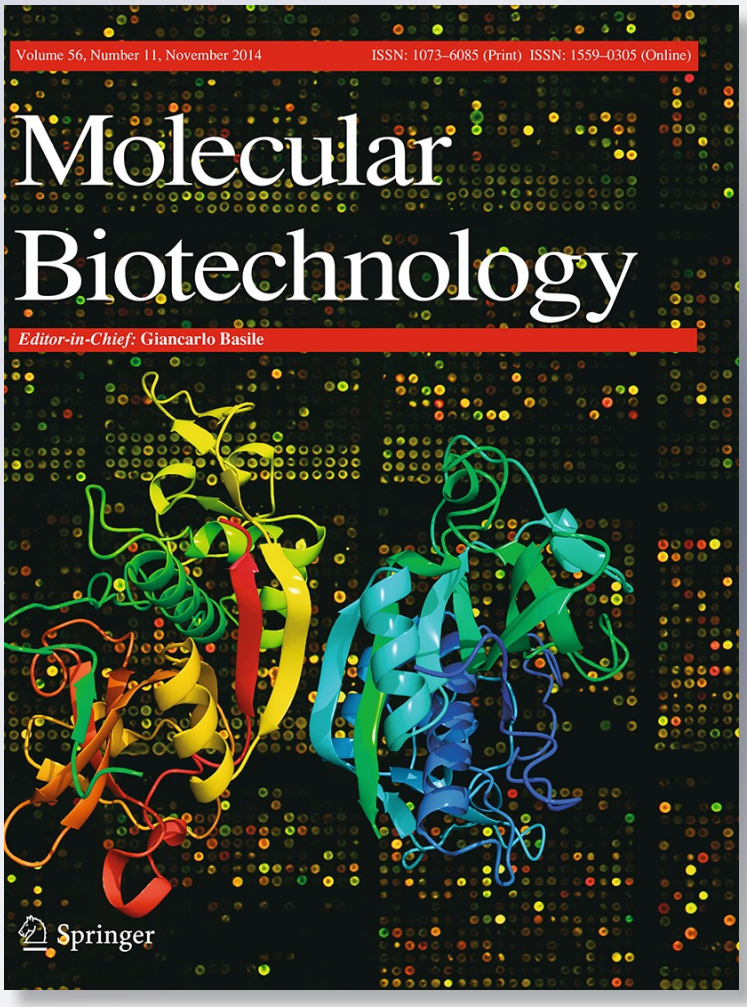

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RESEARCH

Comparison of Strategies for the Production of FMDV Empty Capsids Using the Baculovirus Vector System

V. Ruiz · A. C. Mignaqui · M. C. Nuñez · E. Reytor · J. M. Escribano · A. Wigdorovitz

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Abstract Recombinant FMDV empty capsids have been produced in insect cells and larvae using the baculovirus expression system, although protein yield and efficiency of capsid assembly have been highly variable. In this work, two strategies were compared for the expression of FMDV A/Arg/01 empty capsids: infection with a dual-promoter baculovirus vector coding for the capsid precursor (P12A) and the protease 3C under the control of the polyhedrin and p10 promoters, respectively (BacP12A-3C), or a single-promoter vector coding the P12A3C cassette (BacP12A3C). Expression levels and assembly into empty capsids were analyzed in insect cells and larvae. We observed that the use of the single-promoter vector allowed higher levels of expression both in insect cells and larvae. Recombinant capsid proteins produced by both vectors were recognized by monoclonal antibodies (mAbs) directed against conformational epitopes of FMDV A/Arg/01 and proved to self-assemble into empty capsids (75S) and pentamers (12S) when analyzed by sucrose gradient centrifugation.

Keywords Foot-and-mouth disease virus · Empty capsids · Baculovirus expression system · Sf9 cells · *T. ni* larva

Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals that causes severe economic losses. The etiologic agent, FMD virus (FMDV), is a member of the *Aphthovirus* genus of the *Picornaviridae* family and contains a single-strand positive-sense RNA genome of about 8,500 nucleotides that encodes a polyprotein. This polyprotein is processed by virus-encoded proteases to produce the structural and non-structural proteins required for virus assembly and replication. The capsid precursor P12A is cleaved by the 3C protease to produce the structural proteins VP0, VP3, and VP1. One copy of each of these proteins spontaneously forms the 5S protomer, which subsequently assembles into the 12S pentameric subunit.

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Twelve of these pentameric subunits associate to form the icosahedral empty capsid-like particle (75S) [1]. Encapsidation of viral RNA to produce mature virion (146S) is accompanied by the cleavage of VP0 to VP2 and VP4 [2].

At present, vaccination is the major means of FMD control in most endemic areas. Nowadays, FMD vaccine is produced by growing FMDV in BHK-21 cell cultures under biosecure conditions and inactivating it with binary ethyleneimine [3, 4]. Although this inactivated vaccine has been shown to be effective, it is associated with the risk of virus release during vaccine production and with the additional risk of improper inactivation of the virus, leading to vaccine-related outbreaks. Besides, there are other important shortcomings of current inactivated vaccines, including the problem of discriminating between vaccinated and field-virus infected animals, the need for adequate cold chain of formulated vaccines and difficulties of certain serotypes and subtypes to grow efficiently in cell cultures [5].

As an alternative to the current inactivated whole-virus vaccine, new vaccine production technologies have been investigated. Among these, empty capsids appear to provide an excellent subunit vaccine candidate since FMDV empty capsids are naturally produced in infected cells and are as immunogenic as virions [6, 7]. Additionally, animals inoculated with this type of vaccines could be easily distinguished from infected or convalescent animals. The use of FMDV empty capsids as immunogens has been reported by several groups using different expression systems such as transgenic plants [8], *E. coli* [9, 10], mammalian cells [11], and insect cells [12–19]. In this regard, the baculovirus expression vector system (BEVS) has been extensively explored using one of the following strategies: the co-expression of the capsid precursor P12A and the protease 3C from a dual-promoter baculovirus (encoding these genes under the control of two separate promoters) [13, 15], or the co-expression of these proteins from a single-promoter baculovirus encoding the complete cassette (P12A3C) as a single transcription unit [12, 14, 17–19]. Furthermore, several lepidopteran insect cell lines derived from *Spodoptera frugiperda* (Sf9 and Sf21), *Trichoplusia ni* (High Five, T.ni, T.nao38), and *Bombyx mori* (Bm-N) have been used to support baculovirus replication and produce FMDV empty capsids. In recent years, the use of silkworm larvae [14, 20] and pupae [21] as living bioreactors proved to be an innovative and more simple approach to produce FMDV empty capsids than insect cell lines. However, protein expression levels and the efficiency of capsid assembly have been highly variable in different reports using the same genetic design [14, 17, 19, 20].

The main purpose of the present work was to perform a comparative study of the different strategies that have been used for the production of FMDV empty capsids in the baculovirus expression system. Expression levels and assembly into empty capsids were analyzed in S9 cells and *T. ni* larvae, comparing the use of dual- and single-promoter baculoviruses encoding the capsid precursor (P12A) and the protease 3C or the P12A3C, respectively.

Table 1 Primer sequences used to amplify and clone FMDV capsid precursor and 3C protease fragments

Gene fragment	Primer sequence (5′ to 3′)	Amplicon size (pb)
P12A	F: *Bam*HIGGA TCC AAC **ATG** *GGA GCT GGG CAA TCC*	
	R1: *Xba*ITCT AGA **TTA** CCC AGG GTT GGA CTC	2,277/ 2,280
	R2: *Nsp*VTTC GAA *CCC AGG GTT GGA CTC*	
3C	F1: *Xho*ICTC GAG AAC **ATG** *AGT GGT GCC CCA CCG*	
	F2: *Nsp*VTTC GAA *AGT GGT GCC CCA CCG*	702/708
	R1: *Nhe*IGCT AGC **TTA** *CAT CAC GTG GAC GCG*	
	R2: *Xba*ITCT AGA **TTA** *CAT CAC GTG GAC GCG*	

Restriction enzymes recognition sequences are underline. ATG initiation and TTA termination codons are in bold. Sequences corresponding to viral genes are in italics. F: forward, R: reverse. P12A-R2 and 3C-F2 primers were used to link P12A and 3C fragments and generate the entire cassette P12A3C

Materials and Methods

Construction of Transfer Vectors and Generation of Recombinant Baculoviruses

DNA fragments encoding the viral capsid precursor P12A and the protease 3C were amplified by PCR from a recombinant plasmid containing the full-length cDNA of FMDV A/Arg/01 (kindly provided by Dr. García Nuñez, INTA, Argentina), cloned separately into pGEM-T Easy vector (Invitrogen) and bidirectionally sequenced. The primers used and the restriction sites added for cloning are summarized in Table 1. Two different transfer vectors were generated using the pFastBac Dual vector (Invitrogen), which contains two multiple cloning sites (MCS). To generate the dual-promoter vector, the P12A capsid precursor was cloned as a *Bam*HI-*Xba*I DNA fragment into the corresponding sites in the MCS I under the control of the polyhedrin (polh) promoter; and the 3C gene was cloned as a *Xho*I–*Nhe*I DNA fragment into MCS II under the control of the p10 promoter. The resultant vector was named pFB-P12A-3C. Similarly, target fragments P12A and 3C were digested with *Bam*HI/*Nsp*V and *Nsp*V/*Xba*I, respectively, and cloned together in frame into MCS I under the control of polh promoter. This

single-promoter vector was named pFB-P12A3C. Recombinant baculoviruses BacP12A-3C and BacP12A3C were subsequently generated using the Bac-to-Bac System (Invitrogen) and amplified by infecting Sf9 cells at a multiplicity of infection (MOI) of 0.01 pfu/cell. Infectious virus titers were calculated by end point dilution assay and converted to pfu/ml as described elsewhere [22].

Insect Cells and Larvae Infection

Sf9 cells were propagated in Sf-900 II SFM (Invitrogen) supplemented with 1 % heat-inactivated fetal bovine serum at 27 °C. For protein expression experiments, cells (8.4×10^6) were seeded into T-75 tissue culture flasks and infected with BacP12A-3C or BacP12A3C at a MOI of 5. Three days post-infection, when most of the cells showed cytopathic effect, cells were lysed with 2.5 ml of lysis buffer (0.1 % Triton X-100 in PBS, pH 7.4, with a protease inhibitor cocktail [Roche]) and analyzed for FMDV protein expression. *Trichoplusia ni* (*T. ni*, cabbage looper) larvae were reared under level 2 biosafety conditions following previously described methodology [23]. For larvae infection, fifth-instar larvae were sedated by incubation on ice for 15 min and then were injected with baculovirus preparations near the proleg (forward along the body cavity), using 5 µl of 5×10^3 pfu per larva. After 72 h at 28 °C, inoculated larvae were processed with an extraction buffer containing PBS, pH 7.2, 0.01 % Triton X-100, and a protease inhibitor cocktail (Roche). Extracts were centrifuged at 10,000×*g* for 10 min to eliminate larvae debris and supernatants filtered before they were analyzed for recombinant protein expression. Optimal conditions of infection and harvest time were previously established for both Sf9 cells and *T. ni* larvae (data not shown).

ELISAs

Quantification of recombinant proteins in cell lysates and soluble protein larvae extracts was carried out by a sandwich enzyme-linked immunosorbent assay (ELISA) as previously described [11]. To determine whether FMDV recombinant proteins could self-assemble to form subviral structures, cell lysates and larvae extracts (containing 300 ng/ml of specific protein) were analyzed by the same ELISA but incubating the captured antigens with four monoclonal antibodies (MAbs 1.5, 2.4, 3.2 and 3.3; kindly provided by Dra. Cristina Seki, Instituto Cesar Milstein, Argentina) directed against conformational epitopes of FMDV A/Arg/01 [24].

Sucrose Gradient

Protein extracts from cells and larvae infected with recombinant baculoviruses and mock-infected were loaded onto a 15–45 % sucrose gradient in NTE buffer (100 mM NaCl, 5 mM Tris HCl, 4 mM EDTA, pH 8) and centrifuged at 35,000×*g* for 16 h in a SW28.1 rotor at 4 °C. As a control, inactivated FMDV A/Arg/01 was run in the same conditions. Fractions were collected from the bottom of the gradient and tested by ELISA. The gradient fractions corresponding to empty capsids (fr. 15–17 from FMDV A/ArgA01; fr. 16–21 from larvae extracts) and fractions corresponding to pentamers (fr. 22–27 from Sf9 lysates) were pooled and ultracentrifuged at 107,000×*g* for 16 h in a SW28.1 rotor at 4 °C. The pellets were resuspended in NTE buffer and analyzed by Western blot.

Western Blot

Proteins from gradient fractions were separated by 12 % SDS-PAGE and transferred onto a nitrocellulose membrane for 1.5 h at 20 V. The membrane was blocked, and then incubated with anti-FMDV A/Arg/01 guinea pig serum and an anti-VP3 rabbit serum. After several washes, the membranes were incubated with the appropriate HRP-conjugates and the bound antibodies were detected by chemiluminescence (Western Lightning®Plus-ECL, Perkin Elmer).

Results and Discussion

DNA fragments encoding FMDV A/Arg/01 P12A and 3C were amplified and cloned to generate two transfer vectors, a dual-promoter vector coding for the capsid precursor and the protease 3C under the control of different promoters (pFB-P12A-3C), and a single-promoter vector coding for the entire cassette P12A3C under the control of the polh promoter (pFB-P12A3C). The recombinant baculoviruses named BacP12A-3C and BacP12A3C were generated after transfection of Sf9 cells with the corresponding recombinant bacmids (Fig. 1). These constructions were verified by sequence analysis and there were no mutations introduced in target genes. The titers of recombinant viruses were measured at 1.23×10^7 and 1.49×10^8 pfu/ml, for BacP12A-3C and BacP12A3C respectively, at the second passage. Sf9 cells and *T. ni* larvae were infected with both recombinant baculoviruses and harvested after three days. As shown in Fig. 2, the use of the single-promoter baculovirus BacP12A3C produced higher levels of FMDV recombinant proteins than BacP12A-3C, both in insect cells and larvae. Using the single-promoter baculovirus, recombinant protein yields were 0.58 µg/10^6 Sf9 cells and 31 µg/g of insect biomass (about three insect larvae), which corresponds to a ≈20-fold and threefold increase over the yield obtained with the dual-promoter baculovirus, respectively. Using the same strategy, Porta, et al. could not detect FMDV capsid protein expression in Sf9 cells unless 3C protease activity was reduced, in which case

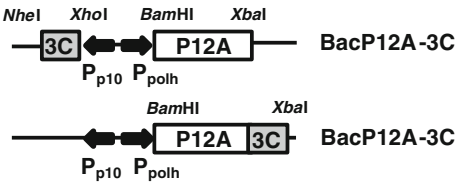

Fig. 1 Schematic illustration of recombinant baculovirus constructs. FMDV genomic fragments encoding P12A and 3C were cloned separately under the control of polyhedrin (P_{polh}) and p10 promoters (P_{p10}), respectively in the dual-promoter baculovirus (BacP12A-3C). The P12A and 3C fragments were cloned together under the control of polyhedrin promoter to generate the single-promoter baculovirus (BacP12A3C)

yields were similar to the ones reported in this work [17, 18]. FMDV empty capsids were also expressed in a hyperexpression variety of silkworm (JY1) by using expression vectors based on *B. mori* Nucleopolyhedrovirus (BmNPV) [14, 20]. In these reports, production yields were similar (30–45 μg/g of insect biomass) to the ones reached in this study.

The FMDV structural proteins produced in Sf9 cells and *T. ni* larvae were further characterized by ELISA by four specific MAbs directed against conformational epitopes of the virus. The four MAbs assayed reacted with protein extracts obtained from Sf9 cells and from larvae (Fig. 3), suggesting the presence of high order structures in these samples. Sedimentation in sucrose gradient was used to analyze the ability of recombinant capsid proteins to self-assemble into empty capsids. Larvae extracts obtained after inoculation with BacP12A3C or BacP12A-3C showed defined peaks of antigenic material that sedimented at the same rate (75S) as FMDV A/Arg/01 empty capsids (Fig. 4a). Other peaks with sedimentation coefficients of

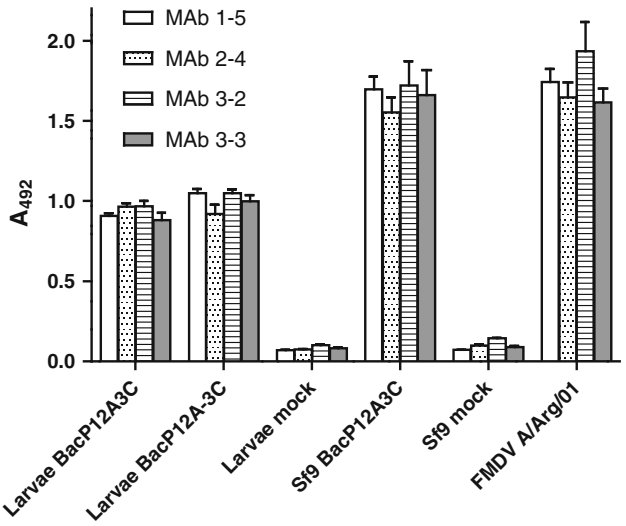

Fig. 3 Antigenic reactivity of recombinant structural proteins. Protein extracts of larvae inoculated with BacP12A3C and BacP12A-3C, lysates of Sf9 cells infected with BacP12A3C, their corresponding negative controls and FMDV A/Arg/01 were analyzed by ELISA with four MAbs (1.5, 2.4, 3.2 and 3.3) directed against conformational epitopes of the virus. Data represent the mean and SD of three independent experiments. Lysates of Sf9 cells infected with BacP12A-3C could not be analyzed because protein expression levels were very low

approximately 12S were also detected, suggesting the presence of pentamers as well (Fig. 4a). Recombinant proteins from the lysate of Sf9 cells infected with BacP12A3C assembled mostly into capsid intermediate structures sedimenting at 12S. However, a small peak of antigenic material was detectable at 75S, suggesting that further assembly of pentameric subunits into empty capsids occurred to a lesser extent under the conditions used in

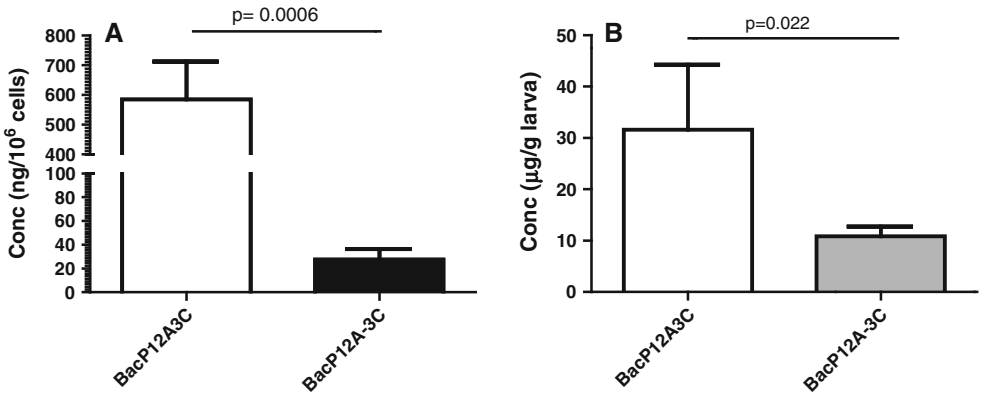

Fig. 2 Analysis of recombinant protein yield in Sf9 cell lysates (**a**) and larvae extracts (**b**) by ELISA. Sf9 cells and *T. ni* larvae were infected with BacP12A3C, BacP12A-3C, and mock-infected. After 3 days, cell lysates and larvae protein extracts were obtained, serially diluted and analyzed for FMDV capsid protein expression by ELISA. Quantification was performed with a standard curve generated with known amounts of inactivated FMDV A/Arg/01, after the negative-control (mock) absorbance was subtracted from each sample. Data represent the mean and SD of at least four independent experiments. *t* test with Welch's correction was performed using GraphPad Prism version 5.03 for Windows

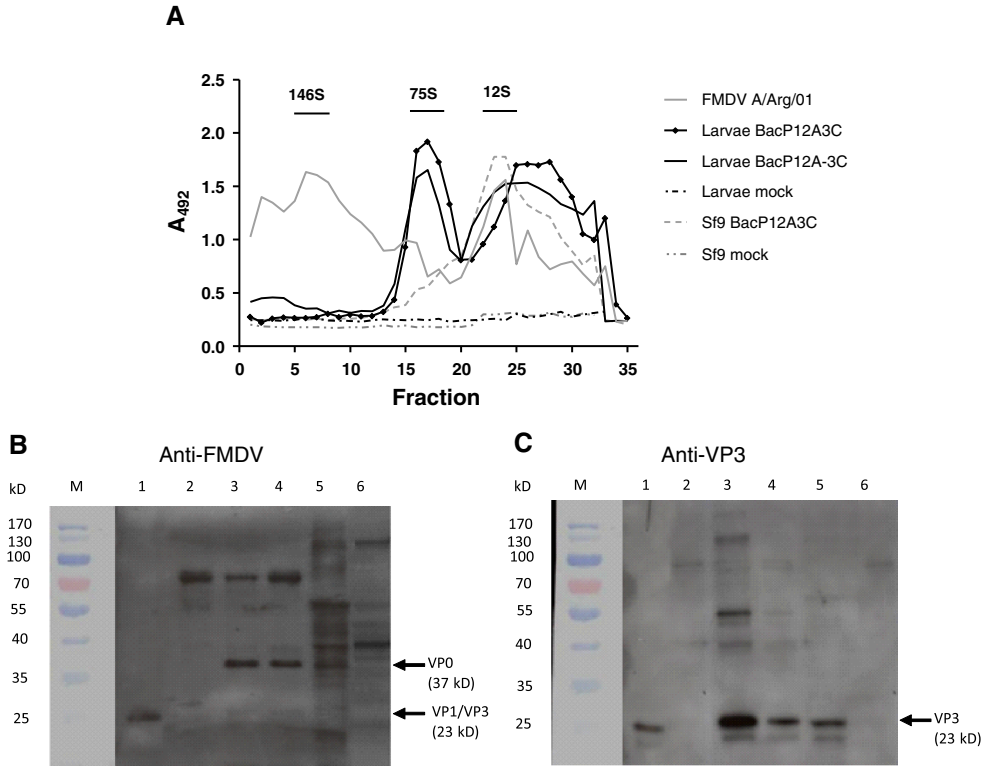

Fig. 4 Assembly of the recombinant structural proteins into subviral particles. Lysates of Sf9 cells and protein extracts of larvae infected with the different baculoviruses (or mock-infected) were loaded onto a 15–45 % sucrose gradient. Fractions were collected and analyzed by ELISA (**a**). The known positions of the FMDV A/Arg/01 virions (146S), empty capsids (75S), and pentamers (12S) are indicated. Fractions containing empty capsids (fr. 16–21 from larvae extracts; fr. 15–17 from FMDV A/Arg/01) and pentamers (fr. 22–27 from Sf9 cell lysates) were pooled and ultracentrifugated at 107,000×*g*. Aliquots of these fractions were analyzed by Western blot using an anti-FMDV A/Arg/01 guinea pig serum (**b**) and an anti-VP3 rabbit serum (**c**): *Lane* 1, fr. 15–17 from FMDV A/Arg/01; *lane* 2, fr. 16–21 from mock-inoculated larvae; *lanes* 3 and 4, fr. 16–21 from larvae inoculated with BacP12A3C and BacP12A-3C, respectively; *lanes* 5 and 6, fr. 22–27 from Sf9 cells infected with BacP12A3C and mock-infected, respectively. Molecular mass markers (kDa) are indicated on the left. Lysates of Sf9 cells infected with BacP12A-3C could not be analyzed because protein expression levels were very low

these experiments. This result could also suggests that the specific protein concentration in Sf9 cell lysates was below the critical threshold required before capsid formation can occur [25–27].

In order to confirm that the peaks observed in the sucrose gradient were in fact FMDV structures, fractions corresponding to empty capsids (75S) from FMDV A/Arg/01 and from soluble protein extracts of larvae, and fractions corresponding to pentamers (12S) from lysate of Sf9 cells, were pooled, ultracentrifuged, and analyzed by Western blot. In fractions corresponding to FMDV A/Arg/01, larvae extracts and Sf9 cell lysates, processed capsid proteins VP0 (37 kD), VP1 (23 kD) and VP3 (23 kD) were identified (Fig. 4b, c, lanes 1, 3–5), confirming that the structures isolated by density gradient centrifugation corresponded to FMDV empty capsids and pentamers. As expected, no FMDV proteins were detected by any of the sera in the fractions corresponding to the protein extract of the mock-infected controls (Fig. 4b, c, lanes 2 and 6).

Although the production of FMDV empty capsids has been reported in transgenic plants [8] and by SUMO-modified *E. coli* expression system [10], transient gene expression of mammalian cells and BEVS technology in insect cells and silkworm larvae are the more promising platforms in terms of expression levels and capsid assembly [11–21]. Regarding BEVS technology, both single- and dual-promoter baculoviruses have been used. In these reports, even when the same genetic design was used, expression levels were variable [14, 17, 19, 20]. In this study, both strategies were compared to determine the optimal genetic design for the production, processing, and assembly of FMDV capsid proteins in insect cells and larvae. The use of the single-promoter baculovirus BacP12A3C produced significantly higher levels of recombinant proteins than the use of the dual-promoter baculovirus, both in Sf9 cell lysates and larvae extracts. In the dual-promoter baculovirus BacP12A-3C, the 3C coding sequence is transcribed from the p10 promoter, which is

more active at an earlier time post infection than the polh promoter [28]. As it has been reported for mammalian cells [29, 30], Porta et al. recently demonstrated that 3C activity has a deleterious effect in protein synthesis in Sf9 cells and showed the efficient production of FMDV empty capsids following down regulation of 3C protease activity [17, 18]. Hence, the earlier expression of this enzyme could be responsible of the lower yield of FMDV capsid proteins observed with BacP12A-3C. The difference in protein yield was more significant in Sf9 cells than in insect larvae (Fig. 2). These results are in agreement with that observed by Chung [31], who reported that when the 3C coding sequence was transcribed using a weaker promoter, the enterovirus empty capsids production yields were improved in Sf9 cells but not in High Five cells (derived from *T. ni*). Since viral 3C protease cleaves not only the viral capsid precursor but also different host cell proteins such as translation initiation factors, histones, and cytoskeleton components [32–34], the differences observed in this study could be attributed to differences in the sequence or structure of these host cell targets between Sf9 cells and *T. ni* cells. The toxic effects of the viral protease could be more relevant in Sf9 cells than in many of the *T. ni* cell types where the baculovirus is producing the structural proteins and forming the empty capsids. Moreover, insect larvae as living biofactories have been shown to be especially useful for proteins difficult to express in other systems [35, 36].

T. ni larvae inoculated with BacP12A3C and BacP12A-3C produced FMDV empty capsids that were indistinguishable from authentic 75S empty capsids from FMDV A/Arg/01, in terms of their density and the presence of conformational epitopes, as revealed by sucrose gradient centrifugation and their reactivity against the four MAbs assayed, respectively.

This study is the first approach to shine some light on the relevance of genetic design for the production of FMDV empty capsids in Sf9 cells and *T. ni* larvae. We may conclude that both single- and dual-promoter baculoviruses are useful for the production of recombinant FMDV empty capsids. However, expression levels can be improved by the use of single-promoter vectors, especially in Sf9 cells which seem to be more susceptible to 3C toxic effects.

Baculovirus has been shown to induce host innate immune responses in various mammalian cells and in mice by inducing inflammatory cytokines (IL-6 and IL-12) and type I and II IFNs [37, 38]. Molinari et al. [39] have demonstrated that the inoculation of baculovirus in C57Bl/6 adult mice shortly before viral challenge with FMDV protects animals from infection. Although, this protective effect lasts only for 3 days, the co-inoculation of baculovirus together with a FMDV oil vaccine induced a very early and long-lasting immunity in the BALB/c murine model [40]. All these data suggest that the use of FMDV empty capsids produced by BEVS technology could be used as a subunit vaccine, whereby the presence of remaining baculovirus acts as natural adjuvant.

Using the single-promoter baculovirus, yields of FMDV recombinant proteins corresponded to 0.58 μg/10^6 Sf9 cells and 31 μg/g of insect biomass (about 3 *T. ni* larvae). A fivefold increase in expression levels in Sf9 cells may represent an amount of protein similar to that achieved in FMDV-infected BHK-21 culture during the current vaccine production process [20]. In the case of *T. ni* larvae, using the production method described here, 100 g of larval biomass (approximately 300 larvae) is comparable in productivity to 1 liter of BHK-21 cell culture. It is important to highlight that the use of insect larvae as living biofactories has advantages such as production costs of insect biomass (lower than 0.8€ for 1 g of insect biomass produced in a GMP pilot plant [41]), the easy scaling-up and the low associated biosafety risks. Experiments to improve production yields in both insect cells and larvae are in progress.

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