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Expression of the Severe Acute Respiratory Syndrome Coronavirus 3a Protein and the Assembly of Coronavirus-Like Particles in the Baculovirus Expression System

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Summary

The Bac-to-Bac Baculovirus expression system was used to generate a recombinant baculovirus capable of expressing the severe acute respiratory syndrome (SARS)-coronavirus (CoV) 3a protein. Using the same expression system, two structural proteins, membrane (M) and envelope (E), were co-expressed to form SARS-CoV virus-like particles (VLPs) within an insect cell. Expression of viral proteins was confirmed by Western blot analysis and the formation of VLPs was studied by transmission electron microscopy.

Key Words: Bac-to-Bac Baculovirus expression system; severe acute respiratory syndrome coronavirus; SARS-CoV; 3a protein; membrane protein (M); envelope protein (E); virus-like particles (VLPs); transmission electron microscopy; Western Blot.

1. Introduction

Baculovirus expression systems are widely used to allow for the expression of recombinant proteins ***(1)***. The Bac-to-Bac Baculovirus expression system ***(2)*** is often preferred as it is a rapid system where purified recombinant baculoviruses can be positively identified within 2 wk ***(3)***. The popularity of the system has further increased because the techniques used to isolate and purify the recombinant virus are relatively simple. A further advantage of insect cells is that they can fold, modify, traffic, and assemble newly synthesized polypeptides to form authentic, soluble end products ***(4–6)***. However, although the baculovirus-insect cell system has protein processing capabilities similar to

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those of higher eukaryotes, the insect protein processing pathways are not necessarily equivalent to those of higher eukaryotes ***(1)***. A good example of a similar but distinct processing pathway is the protein *N*-glycosylation pathway. Studies have shown that although insect cells could assemble *N*-glycans and transfer them to growing polypeptides, they have an unusual end-processing activity that trims an intermediate (common to both insect and mammalian pathways) to the insect-specific paucimannose end product ***(7)***. Nevertheless, baculovirus expression of viral proteins has been successfully used for the study of numerous viruses ***(1)***. This system has been particularly useful in the production of virus-like particles (VLPs) to study viral assembly processes and in several cases like the human papillomavirus and hepatitis C virus, such VLPs have been used in vaccine development ***(8,9)***. Another important application is the production of glycosylated viral antigens for immunization and protection against viral infection, for example, influenza A viral antigens expressed using baculovirus have been evaluated as potential vaccine candidates ***(10,11)*** **.**

The recent severe acute respiratory syndrome (SARS) epidemic, which affected more than 30 countries across five continents, has profoundly disturbed social and economic activities globally. A novel coronavirus, termed the SARS-coronavirus (CoV), was identified as the etiological agent of SARS ***(12)***. The SARS-CoV genome is nearly 30 kb in length and contains 14 potential open reading frames (ORFs) ***(13,14)***. Five of these ORFs encode for genes that are homologous to proteins found in all known coronaviruses, namely the replicase gene 1a/1b and the four structural proteins, nucleocapsid, spike, membrane (M), and envelope (E), whereas the remaining nine ORFs encodes for accessory proteins, varying in length from 39 to 274 amino acids, which are unique to SARS-CoV. The largest of these accessory proteins is termed 3a (also known as U274, X1, or ORF3). Antibodies specific for 3a have been found in convalescent patients ***(15,16)*** and 3a has also shown to be expressed in SARS-CoV-infected cells ***(17–19)***. 3a is a novel coronavirus structural protein as it is associated with virion purified from SARS-CoV-infected cells and it is incorporated into VLPs when co-expressed with M and E in the baculovirus system ***(20,21)***. 3a is predicted to have three transmembrane domains ***(13, 14)*** and when it is expressed on the cell surface, its N-terminus is facing the extracellular matrix whereas the C-terminus is facing the cytoplasm ***(19)***. It has also been reported that 3a is *O*-linked glycosylated, and this posttranslation modification may be important for its incorporation into virion ***(22,23)***. The formation of a recombinant baculovirus expressing the 3a protein fused with a myc-tag at the N-terminus will be used to illustrate the methods used to express this protein using the Bac-to-Bac Baculovirus expression system.

The formation of VLPs of SARS-CoV using recombinant baculovirus technology has been demonstrated ***(24,25)***. As has been observed for other

coronaviruses, the co-expression of two of the SARS-CoV structural proteins, M and E, is sufficient for the formation of VLPs. The M protein is a triple-spanning membrane glycoprotein that interacts with the nucleocapsid and spike protein during virion assembly ***(26)***. The small E protein has more recently been recognized as an essential structural component of the coronavirion. A large portion of this protein is embedded within the viral membrane; only its hydrophilic carboxy terminus protrudes inside the virion ***(27,28)***. Nal and co-workers recently showed that the SARS-CoV M protein is *N*-glycosylated, whereas the SARS-CoV E protein is not glycosylated ***(29)***. Recombinant viruses expressing M and E respectively will be used to form VLPs. The co-expression will be shown by Western blot analysis, and the formation of VLPs will be shown by transmission electron microscopy.

2. Materials

1. Bac-to-Bac Baculovirus expression system (Invitrogen, Life technologies).
2. pFastBac1 vector (Invitrogen).
3. pXJ40myc-3a.
4. *Escherichia coli* strains DH5α and DH10Bac.
7. Restriction enzymes and T4 DNA ligase.
8. Luria-Bertani (LB) agar plates and media.
9. QIAprep Miniprep kit and QIAgen Midiprep kit.
10. Agarose and DNA sequencing gel equipment.
11. Oligonucleotide primers.
12. Isopropyl-β-D-thio-galactopyranoside (IPTG) and X-gal.
13. Ampicillin, kanamycin, gentamicin, bacitracin and tetracycline.
14. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) equipment.
15. 5% SDS lysis buffer: 0.3 *M* Tris-Cl, pH 6.8; 5% SDS; 50% glycerol; 0.1 *M* dithiothreitol (DTT); 0.1% bromophenol blue.
16. Whatman Filter paper.
17. *Sf* 9 insect cells.
18. Sf-900 II SFM insect medium.
19. 1% penicillin/streptomycin solution.
20. Cellfectin reagent.
21. Unsupplemented Grace's insect medium.
22. 5% nonfat milk.
23. 3a antiserum, anti-myc monoclonal, M antiserum, E antiserum.
24. Phosphate buffered saline with 0.05% Tween 20.
25. Supersignal West Pico.
26. X-ray film.
27. TEN buffer: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, and 1 *M* NaCl with 1% Triton X-100.
28. Sucrose in TEN buffer.

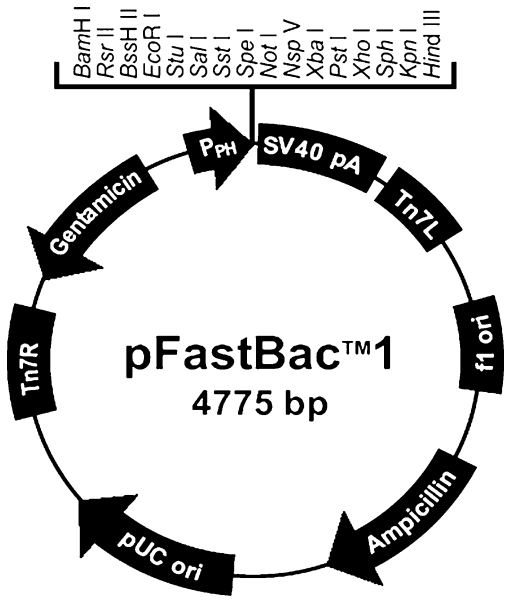

Fig. 1. Schematic drawing of pFastBac1 (Invitrogen).

29. Hybond-C Extra membrane.
30. Formvar coated copper grids and formvar coated nickel grids.
31. 2.5% gluteraldehyde.
32. Phosphotungsteic acid.
33. JEOL model: JEM1010 transmission electron microscope.
34. Incubation buffer: 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS).

3. Methods

The methods described below outline (1) the generation of the myc-3a recombinant virus, (2) the expression of the recombinant protein, (3) the co-expression of two structural proteins for VLPs formation, and (4) transmission electron microscopy staining and visualisation of the VLPs.

3.1. Generation of 3a Recombinant Virus

The vector utilised for this study, pFastBac1 **(Fig. 1.)**, contains an expression cassette, which includes the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin (PH) promoter ***(30)*** (which allows for high level of expression in insect cells), a gentamicin resistance gene (for selection), and an SV40 polyadenylation signal to form a mini Tn7. This expression cas-

sette is flanked by the right and left arms of Tn7. In the Bac-to-Bac Baculovirus expression system, recombinant bacmid DNA is formed using the site-specific transposition properties of the Tn7 transposon ***(32)***. Bacmid DNA (bMON14272) situated in *E. coli* DH10Bac cells, which allow for its propagation, contains the low-copy-number mini-F replicon, a kanamycin resistance marker, and *lac*Zα from pUC. The attachment site for the bacterial transposition Tn7 (mini-*att*Tn7) is inserted in the N-terminus of the *lac*Zα peptide. When these cells are grown in the presence of X-gal and the inducer IPTG, blue colonies are formed, because the *lac*Zα can complement a *lac*Z deletion present on the chromosome. The expression cassette present on the pFastBac1 (donor plasmid) is transposed to the mini-*att*Tn7 attachment site on the bacmid with Tn7 transposition functions provided in *trans* by a helper plasmid (pMON7124). The insertion of the mini-Tn7 of the pFastBac1 vector into the mini-*att*Tn7 attachment site of the bacmid disrupts the expression of the *lac*Zα peptide. Therefore, colonies containing recombinant bacmids will remain white in the presence of X-gal and IPTG because the *lac*Zα gene cannot express.

3.1.1. Cloning

1. The plasmid pXJ40myc-3a ***(19)*** was digested with restriction enzymes *Eco*RI and *Not*I as per the instructions of the manufacturer (New England Biolabs). This released a 0.822kb fragment, containing the 3a protein with the myc-epitope tag at the 5' end.
2. The fragment was ligated with pFastBac1 vector digested with the same combination of restriction enzymes. This was performed using T4 ligase enzymes according to the manufacturer's instruction and chemically transformed into *E. coli* DH5α cells by standard methods ***(31)***.
3. The *E. coli* DH5α cells were then plated onto LB agar plates containing ampicillin (100 μg/mL) and incubated overnight at 37°C.
4. Single colonies were selected and grown in LB broth with ampicillin. The plasmid was then isolated following the instructions of the QIAprep Miniprep Handbook and checked for the presence of the insert and for the correct orientation using restriction enzyme digestions. Alternatively, PCR can be used to screen for positive clones (*see* **Note 1**).

3.1.2. Generating the Recombinant Bacmid

1. One nanogram of the pFastBac1-myc-3a plasmid was chemically transformed into the DH10Bac cells using standard methods ***(31)***. The transformation mix was then incubated at 37°C at 225 rpm for 4 h.
2. After the 4-h incubation, 10-fold serial dilutions of the *E. coli* DH10Bac transformation mix were prepared to a dilution factor of 10^{-3}. One hundred microlitres of individual dilutions were plated onto LB agar plates containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100 μg/mL X-gal, and 40 μg/mL IPTG (*see* **Note 2**). Plates were then incubated for 48 h at 37°C.

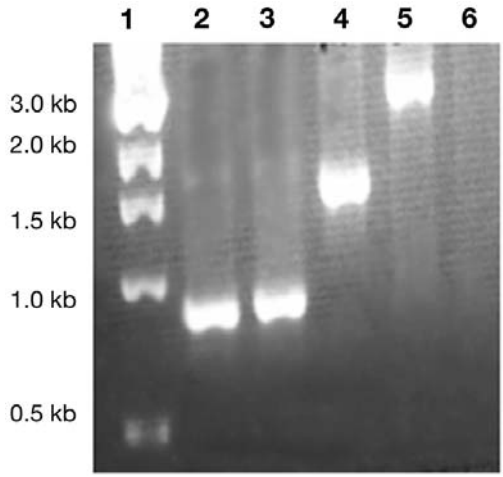

Fig. 2. PCR amplification of the recombinant bacmid DNA. Lanes 1, 1 kb marker (New England, Biolabs); lane 2, pFastBac1-myc-3a plasmid; lane 3, pXJ40myc-3a plasmid with gene specific primers (3a forward and 3a reverse); lanes 4 and 5, recombinant bacmid DNA with primer combinations of M13 Reverse and 3a forward, and M13 Forward (–40) and 3a reverse, respectively. Lane 6 was the water control.

3. To confirm the white phenotype, 10 white colonies were restreaked onto the same selection agar plates. These plates were incubated at 37°C for 16 h.
4. Once the phenotype was confirmed, a single colony was chosen and grown in LB media containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, and 10 μg/mL tetracycline.
5. The bacmid DNA was then isolated following the instructions of the QIAgen Midiprep Handbook.
6. Because of the large size of the bacmid DNA, restriction enzyme analysis is not recommended. The PCR using the M13 Forward (–40) and M13 Reverse primers is ideal to verify that the isolated bacmid DNA contain the gene of interest, because the bacmid contains these primer sites flanking the mini-*att*Tn7. PCR fragments were amplified using a combination of various primers: 3a reverse and 3a forward primers (**Fig. 2**, lanes 2 and 3), M13 Reverse and 3a forward primers (**Fig. 2**, lane 4), or 3a reverse and M13 Forward (–40) primers (**Fig. 2**, lane 5). The pFastBac1-myc-3a and the pXJ40myc-3a plasmids were used as positive controls for the PCR reaction and sterile distilled water was used as a negative control. For each primer set, a 50-μL standard PCR reaction was set up. The annealing temperature and extension time of a PCR cycle varied, depending on the primer combination. PCR fragments were visualized by agarose gel electrophoresis using standard procedures ***(31)*** (*see* **Note 3**).

3.1.3. Transfecton of Insect Cells With Bacmid to Produce Recombinant Virus

1. Two millilitres of 1 × 10^6/mL *Sf*9 insect cells, in Sf-900 II SFM medium (Invitrogen) containing a 1% penicillin/streptomycin solution (Sigma-Aldrich) (complete media), were seeded into six-well plates (*see* **Note 4**).
2. After the cells were allowed to attach for 1 h at 27°C, bacmid DNA which contained the inserted gene of interest was transfected into the *Sf*9 cells using Cellfectin reagent (Invitrogen). While the cells were attaching, 1 μg of Midiprep bacmid DNA was added to 100 μL of unsupplemented Grace's medium (Invitrogen) in a 1.5-mL microfuge tube (tube A). Six microliters of Cellfectin reagent was diluted in 100 μL of unsupplemented Grace's Medium in a separate 1.5-mL microfuge tube (tube B). The contents of tube A were added to the contents of tube B, and the solution was gently mixed, and incubated at room temperature for 30 min. Five minutes before the incubation time had expired, the Sf-900 II SFM medium was removed from the cells, and the cells were washed once with 2 mL of unsupplemented Grace's Medium after which the wash media was removed. A volume of 0.8 mL of unsupplemented Grace's Medium was added to the DNA–lipid complex, and this solution was added to the *Sf*9 cells.
3. The cells were incubated at 27°C incubator for 5 h, then the incubation media was removed and 2 mL of the complete growth media was added to the cells. The six-well plates were then incubated in a 27°C humidified incubator for 72 h (*see* **Note 5**).
4. The baculovirus infection cycle is characterized by a bi-phasic replication cycle during which two virion phenotypes are produced: (I) Occlusion derived virions (ODV) and (II) budded virus (BV) ***(33)***. Posttransfection, BV is usually released into the medium after 3 d. Depending on the transfection efficiency, a longer time period might be required to view the cytopathic effects (CPE). Some common CPE as time progresses, include enlarged nuclei, detachment, and finally cell lysis. Once CPE has been observed, the media from the wells were collected and transferred to a centrifuge tube.
5. The media was then centrifuged at 1500 rpm for 5 min at 4°C.
6. Aliquots of the supernatant, which constitute the P1 viral stock, was then transferred to sterile dark microfuge tubes, and stored at –80°C (*see* **Note 6**)
7. The P1 viral stock can be used to generate a viral stock of higher titer and volume. 1 × 10^7 *Sf*9 cells were added to 10 mL of Sf-900 II SFM medium and incubated for 1 h at room temperature to allow for cell attachment.
8. One milliliter of media was then removed and replaced with 1 mL of P1 viral stock, to allow for a final volume of 10 mL (*see* **Note 7**). The cells were then incubated for 48–72 h (until CPE was detected) in a 27°C humidified incubator.
9. After the incubation period, the media was collected and transferred to a centrifuge tube. The media was then centrifuged at 1500 rpm for 5 min at 4°C.
10. Aliquots of the supernatant were then transferred to sterile dark microfuge tubes, and stored at –80°C.
11. The P2 viral stock titer was determined by a plaque assay, as outlined **ref. *5***. Typically, the P2 virus stock has a 100-fold higher titer than P1.

3.2. Expression of the Recombinant Protein

The next step involved the confirmation of viral expression by Western blot analysis as outlined under **Subheadings 3.2.1–3.2.2**. This includes infection of cells with the recombinant P2 viral stock, *Sf*9 cell lysis, SDS-PAGE, and Western Blot analysis.

3.2.1. Infection of Sf9 Cells With the Recombinant P2 Viral Stock and Sf9 Cell Lysis

1. Viral stocks of the 3a recombinant virus were at a concentration of 1×10^8 plaque-forming units (pfu)/mL. 1×10^7 *Sf*9 cells were infected at an multiplicity of infection (MOI) of 1 and then incubated for 48–72 h (until CPE was detected) in a 27°C humidified incubator.
2. After the incubation period, cells were harvested by centrifugation at 1500 rpm for 5 min at 4°C.
3. The cells were then lysed with 5% SDS gel loading buffer (0.3 *M* Tris-Cl, pH 6.8; 5% SDS; 50% glycerol; 0.1 *M* DTT; 0.1% bromophenol blue). Because the 3a protein tends to form large aggregates when boiled, the lysate was incubated at 50°C for 15 min instead.
4. The lysate was then analyzed by a 15% SDS-PAGE.

3.2.2. Western Blot Analysis

1. Separated proteins were transferred onto Hybond-C Extra (Amersham Biosciences).
2. The membrane was blocked with 5% nonfat milk for 30 min and probed with primary antibodies, 3a rabbit antiserum (1:2000), or anti-myc monoclonal antibody (1:2000) (Santa Cruz Biotechnology, Santa Cruz, California) with rolling at 4°C overnight **(Fig. 3A,B)**. The antiserum used to probe the 3a protein was raised by immunizing rabbits with the amino acid fragment 134–274 of the 3a protein ***(19)***.
3. After three washes (15 min each) with PBS containing 0.05% Tween 20 (PBST) the blots were incubated in goat anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000, Pierce) at room temperature, with rolling for 1 h.
4. The blot was then washed with PBST three times for 15 min each, and visualised using Supersignal West Pico (Pierce) and developed on an X-ray film (Hyperfilm, Amersham Biosciences).

3.3. The Co-Expression of Two Structural Proteins for VLP Formation

The procedures outlined under **Subheadings 3.3.1.–3.3.3.** describe how the recombinant viruses expressing M and E, respectively, were co-expressed within insect cells to form VLPs. These procedures include the co-infection of insect cells with M and E recombinant viruses, the expression of viral proteins

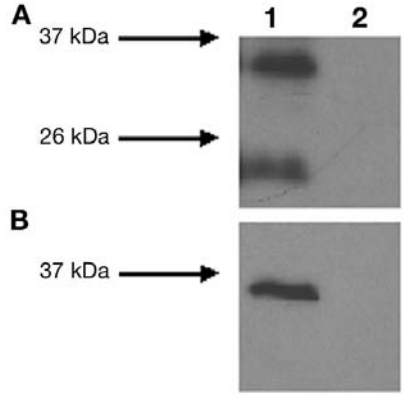

Fig. 3. Expression of severe acute respiratory syndrome (SARS)-coronavirus (CoV) 3a protein in insect cells. *Sf*9 cells were infected with a recombinant myc-3a baculovirus at a multiplicity of infection of 1 (lane 1). Cells were harvested at 72 h positinfection, lysed, and the cell lysate subjected to Western blot analysis using **(A)** anti-3a antibody and **(B)** anti-myc antibody. Two forms of myc-3a were detected by anti-3a antibody as previously reported ***(19–21)***. Mock infected *Sf*9 cells were used as a negative control (lane 2).

by Western blot, and the purification of VLPs. The methods used to purify the VLPs were similar to that outlined in **ref. *25***.

3.3.1. Infection of Insect Cells With M and E Recombinant Viruses

1. Triplicate 175-cm^2 tissue culture flasks with 2×10^7 cells of *Sf*9 insect cells were co-infected with the two recombinant viruses expressing M and E proteins at an MOI of 5:1. These recombinant viruses were kindly donated by Dr. Yu-Chan Chao ***(24)***.
2. Once CPE was observed (usually 72 h postinfection) cells were harvested. Three rounds of infection were completed for a total of nine flasks.
3. To confirm that the co-infected cells were expressing both M and E, infected *Sf*9 cells were lysed with 5% SDS gel loading buffer. Even though the E protein can withstand temperatures up to 100°C, previous studies have shown that the M protein forms insoluble aggregates when boiled ***(34)***. Thus, E:M lysate was heated at 50°C for 15 min.
4. The lysate was then subjected to Western blot analysis as described previously. The primary antibodies used were rabbit anti-M antibody (1:500) (anti-SARS virus PUPM C-term, purified Rabbit Pab, cat. no. AP6008b, ABGENT) and E polyclonal antibody (1:2000) at 4°C overnight, with rolling **(Fig. 4A,B)**. The antiserum used to probe the E protein was raised by immunising rabbits with amino acids 37–77 of the E protein (***35***).

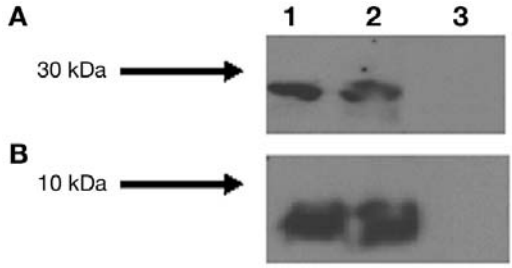

Fig. 4. Expression of severe acute respiratory syndrome (SARS)-coronavirus (CoV) M **(A)** and E **(B)** proteins in insect cells. *Sf*9 cells were infected with M only at a multiplicity of infection (MOI) of 10 (**A**, lane 1) and E only at an MOI of 2 (**B**, lane 1). The cells were also co-infected with the two recombinant baculoviruses, M and E, at an MOI of 5:1 respectively (**A,B**, lane 2). Cells were harvested at 72 h postinfection, lysed, and the cell lysate subjected to Western blot analysis using **(A)** anti-M and **(B)** anti-E antibody. Mock infected *Sf*9 cells were used as a negative control (lane 3).

3.3.2. Purification of VLPs

1. After the cells were sloughed in the growth media, the cells were separated from the media by centrifugation at 4000 rpm for 15 min.
2. The supernatant was then transferred to a sterile 50-mL centrifuge tube, while the cells were resuspended in 5 mL TEN buffer with 1% Triton X-100.
3. The cells were placed in liquid nitrogen (for quick freezing) and thawed at room temperature.
4. After the cells had thawed, they were sonicated at 2-min intervals for 10 min at 4°C. The lysed cells were then centrifuged at 3500 rpm for 30 min.
5. For each of the three rounds of amplification, the clarified supernatant of the triplicate flasks were pooled. The supernatant was then placed on a linear 30–45% (w/w) sucrose gradient in TEN buffer and centrifuged in a Beckman Ultracentrifuge at 27,000 rpm for 3 h. The opalescent band containing the particles was then collected at the interface (*see* **Note 8**).

3.4. Transmission Electron Staining and Visualization of the VLPs

Described as follows are the steps that can be used to stain and visualize the VLPs by transmission electron microscopy. These steps include negative staining and immunogold labelling. The immunogold procedure used was one modified from **ref. *37***.

3.4.1. Negative Staining of Grid

1. Aliquots of purified VLPs were placed on a block of parafilm and the formvar-coated copper grid was placed over the aliquot (rough surface in contact with the

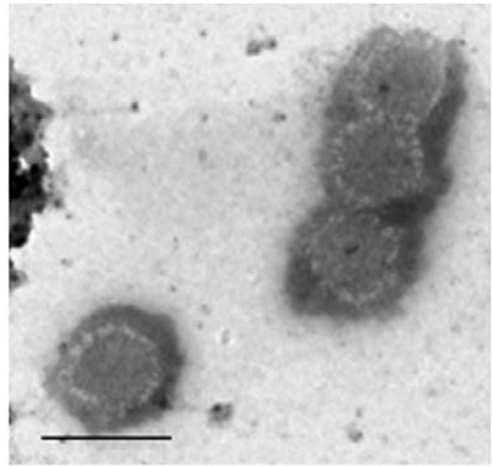

Fig. 5. Analysis of virus-like particles formed by co-infecting *Sf*9 cells with M and E and a multiplicity of infection of 5:1, respectively. Bar = 100 nm.

sample) for 1 min. The excess liquid was drained by touching the grid edge with a piece of filter paper.
2. To fix the sample onto the grid, the grid was placed onto a drop of 2.5% gluteraldehyde for 1 min. Once again, excess liquid was drained by touching the edge of the grid with a piece of filter paper.
3. To disseminate the sample evenly over the grid, the grid was placed on a drop of 30 μg/mL bacitracin for 1 min.
4. After the excess liquid was drained, the sample was negatively stained by placing the grid on a drop of phosphotungsteic acid (PTA), pH 6.0, for 1 min.
5. The excess liquid was finally drained, and the grid was allowed to dry thoroughly.
6. All samples were examined under a JEOL model: JEM1010 transmission microscope. As shown in **Fig. 5**, the VLPs of approx 100 nm can be detected and the size is slightly smaller than the spike-containing virions from SARS-CoV infected cells ***(36)***.

3.4.2. Immunogold Labeling of Absorbed VLPs

1. An aliquot of 500 μL purified VLPs were centrifuged at 8000 rpm for 5 min and the pellet was resuspended in distilled water.
2. Ten-microliter drops of VLPs were absorbed onto formvar-coated nickel 300 mesh electron microscopy grids for 15 min and washed with water.
3. Grids were then floated onto incubation buffer for 15 min and then floated for 30 min on a droplet of the appropriate primary antibody (the same M and E antibodies as used under **Subheading 3.3.2.**) with different dilutions for M (1:10

and 1:100 diluted in incubation buffer) or E (1:50 and 1:200 diluted in incubation buffer),
4. **Step 3** was followed by three washes in incubation buffer.
5. Grids were floated for 30 min on a droplet of gold particles (diameter, 10 nm) (1:20 diluted in incubation buffer) conjugated to protein A, followed by three washes in incubation buffer.
6. Grids were floated for 5 min on 2.5% gluteraldehyde (prepared in PBS) to fix the sample, followed by two washes in PBS, followed by four washes in distilled water and,
7. Grids were floated for 1 min in 2% uranyl acetate, followed by four washes in distilled water.
8. After the distilled water washes, the grids were placed onto filter paper and allowed to dry. After each stage, the grids were carefully blotted onto filter paper by holding the gird perpendicular to the paper.
9. All samples were examined under a JEOL model: JEM1010 transmission microscope. Examples of M or E-immunogold labelled VLPs can be found in **refs. *24*,*25***.

4. Notes

1. Colony PCR can be used as an alternative technique to screen for positive colonies. This could be done using a toothpick or sterile yellow tip to pick a colony and submerge the colony into the PCR mix. The fragment can then be amplified using a PCR program that is optimum for the primer combination, with the exception being an extended initial denaturation time. The primer combination used within the PCR can be specific for the gene of interest, or specific for priming sites on the pFastBac1 vector which flank the multiple cloning site (pFastBac_Fwd: 5'ACCATCTCGCAAATAAAG3' and pFastBac_Rev: 5'AACAACAATTGCATTCATTTT3').
2. When preparing the multiple antibiotic plates, the tetracycline concentration was increased from 10 μg/mL to 15 μg/mL. This was done to decrease the amount of satellites colonies obtained. After the 48-h incubation, plates were incubated at 4°C. This allowed for an enhancement of the blue colonies, which allowed for a more defined distinction between white and blue colonies.
3. The amplicon size between the two universal primers on the bacmid DNA is 2.3 kb when no gene is inserted. If the gene of interest together with the bacmid DNA is greater than 4 kb, it is recommended that a *Taq* polymerase such as the Expand High Fidelity PCR system (Roche) ***(38)*** is used, which will allow for amplification of larger fragments.
4. It is generally assumed that a confluent 25-cm^2 flask of *Sf*9 cells contains approx 1×10^7 cells. These cells, diluted in 10 mL of media, will then contain 1×10^6 cell/mL.
5. It is important to note that insect cells are not incubated in a CO_2 incubator but in a normal humidified incubator at 27°C. It is possible to obtain a slower growth at 19–22°C, but it is not advisable to exceed the temperature of 28°C, because the

insect cells do not grow well at higher temperatures. Also, it is extremely hard for the cells to recover once they have been placed under stress.

6. Repeated freeze–thaw cycles of virus samples are not recommended, as this can decrease titer. It is therefore recommended that a working stock of recombinant virus be stored at 4°C. Virus stocks can safely be stored this way, without loss of titer, for a least a year.
7. Assuming that the P1 viral stock has a titer of 1×10^6 pfu/mL, the number of cells to be infected is 1×10^7 cells/mL (1×10^6 cells in 10 mL of media) and the MOI required is 0.1 pfu/mL, it can be calculated that 1 mL of the P1 viral inoculum is required.
8. For a quick scan of VLPs, after the lysed cells have been separated from the cell debris, it is possible to place an aliquot of the clarified supernatant onto a formvar coated copper grid, stain the grid with PTA and view immediately by transmission electron microscopy.

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