

Supplementary data - Production of LLOV Antigen for the Immunoblot assay

Recombinant transfer vectors

The C-terminal domain of the LLOV GP (GP₂, GenBank AN: JF828358) was recovered from bat LLOV samples and cloned into the pCRII vector by traditional TA cloning procedures. LLOV GP₂ DNA was re-amplified by PCR from the pCRII templates using the following forward and reverse primers, F-LL (5' GCG CGA ATT CCG ATG CCA TTG GGA GGG TCT TCG 3') and R-LL (5' CGC GGT CGA CTC ATC GTG TTA TTC TGC ACA GAC AAA TCA AG 3'). EcoRI and SalI restriction sites are underlined, respectively. Primers were designed based on the nucleotide sequence of LLOV. Standard amplification conditions were applied: one cycle at 94 °C for 2 min, followed by 40 cycles of 94 °C for 1 min; 50°C for 2 min 30 s; 72°C for 1 min and a final extension of 72°C for 10 min.

The 963 bp amplified GP₂ fragment was directionally subcloned into the EcoRI and SalI sites of pfastBac HT B donor plasmid (Invitrogen, cat n°10359-016) to construct a recombinant vector pfastbac-HIS-LLOV-GP₂ with a hexahistidine (6xHis) tag sequence. We used pfastbac-HT-CAT (chloramphenicol acetyltransferase gene, Invitrogen, cat n°10359-016) as control. pfastbac-HIS-LLOV-GP₂ was sequenced to rule out any mutation. Both pfastBac-HIS-LLOV-GP₂ and pfastBac-HT-CAT were transformed into DH10bac *E. coli* respectively, for transposition into the bacmid (Invitrogen, cat n°10359-016). The recombinant bacmid DNA (Bacmid-6xHis-LLOV-GP₂), containing the entire LLOV GP₂ gene, and the control bacmid containing a CAT gene (Bacmid-6xHis-CAT), were isolated using anion exchange resin columns (Purelink HiPure Plasmid DNA Miniprep Kit, Invitrogen), screened by PCR and confirmed by sequencing.

Producing recombinant baculoviruses stock

Serial dilutions (10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷) of the purified recombinant Bacmid-6xHis-LLOV-GP₂ and Bacmid-6xHis-CAT viral stocks were used to infect a *Spodoptera frugiperda* 21 (Sf21) insect cell line (5x10⁵ cells/ml) to determine the viral titer. Single plaques were isolated to check for quality. Characterization was performed by PCR with the PUC/M13 Forward primer (5' CCC AGT CAC GAC GTT GTA AAA CG 3') and PUC/M13 Reverse primer (5' AGC GGA TAA CAA TTT CAC ACA GG 3') and standard conditions (Bac to Bac Expression System, Invitrogen Kit, cat n ° 10359-016). Once clones were qualified, cells were infected as follows: 2x10⁶ cells Sf21 cells were diluted in a supplemented Grace's Insect Medium, seeded in a 6-well plate, and incubated 1 hour at 27 °C. Then, 100 µl of each viral stock was added to each well for 1 h adsorption at room temperature. Cells were incubated 4 days at 28 °C or until demonstration of typical signs of late infection, when the cells and the supernatant was collected and transferred to sterile tubes. Tubes were centrifuged at 1000 xg for 5 min to remove cells and large debris. The clarified supernatants constituted the P1 viral stock. The titers were determined as 1.3x10⁸ pfu/ml for bacmid GP₂ and 1.4x10⁹ pfu/ml for bacmid CAT. Finally, the P1 stocks were amplified to obtain the working P2 viral stock by infecting 100 ml of Sf21 cells in culture (0.5x10⁶ cells/ml) at a low multiplicity of infection (MOI, 0.5) with the corresponding baculovirus, as recommended (viral plate assay, Invitrogen, cat n° 10359-016). The final titers were 1.5x10⁸ pfu/ml and 1.75x10⁸ pfu/ml for the recombinant bacmids GP₂ and CAT, respectively.

Production of LLOV GP₂ antigen

Sf21 insect cell suspensions (1.0x10⁸ cells) were infected at a MOI of 5 with the recombinant Bacmid-6xHis-LLOV-GP₂ (1.5x10⁸ pfu/ml) and with the control Bacmid-6xHis-CAT (1.75x10⁸ pfu/ml), respectively. Both infections were incubated at 28°C for four days. Then, cultures were harvested and centrifuged at 12,000 rpm at 4°C for 10 min. Supernatant fractions were discarded and cell pellets lysed by addition of 1 ml of lysis buffer (cat. N° 554778, BD) and 1µl of protease inhibitor cocktail per each 100x10⁶ cells (cat. 554779,

BD). Lysates were held on ice for 45 min and further sonicated (Bandelin Sonopuls, program: pulse on 20 seconds, 3 times). The supernatant (soluble fraction) and the pellet (insoluble fraction) were separated by centrifugation at 10.000 rpm for 10 min. The expression of recombinant 6xHis-LLOV-GP₂ and 6xHis-CAT proteins (40 and 28 KDa respectively) was analyzed in both (supernatant and pellet fractions) by Immunoblot using the monoclonal anti-His antibody (His Tag Mouse mAb HRP conjugate; cat. 9991s, Cell Signaling Technology, dilution 1:2500) as primary antibody, and the polyclonal anti-mouse IgG-HRP-link as a secondary antibody (cat. 7076; Cell Signaling Technology, dilution 1:2000). Protein visualization was performed using a peroxidase solution and DAB (3,3'-diaminobenzidine tetrahydrochloride) as substrate (CN/DAB substrate kit, thermo scientific, cat. 34000). The supernatant and pellet fractions were positive for both proteins (**Figure 1a**).

The recombinant 6xHis-LLOV-GP₂ protein (40 KDa) to be used as antigen in Immunoblot assays was obtained from a crude extract of the pellet fraction after its treatment to break up the inclusion bodies: the insoluble pellet of infected cells was resuspended in 500 µl of Inclusion Body Solubilization reagent (IBS, Thermo Fisher scientific). Recombinant 6xHis-CAT protein was obtained in the same conditions in order to have a negative control.