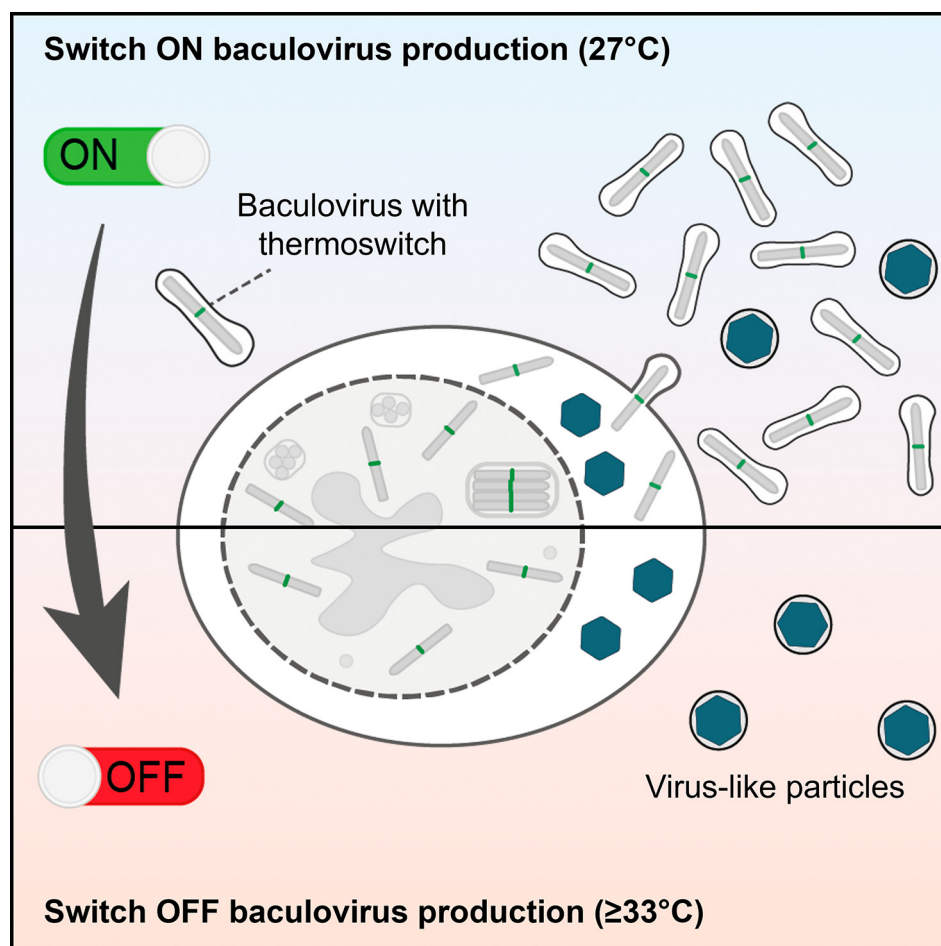


Research Article

Engineered thermoswitch in the baculovirus expression vector system for production of virus-like particle vaccines with minimized baculovirus contaminants



This work presents an innovative baculovirus expression system for virus-like-particle (VLP) vaccine production. A genetic thermoswitch was engineered in the baculovirus expression vector (BacFree^{ts}) to regulate baculovirus particle production. Temperature elevation from 27°C to 33°C results in a multilog reduction of co-produced baculovirus contaminants in the upstream VLP production phase.

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Highlights

The removal of baculovirus particle contaminants is a major challenge in downstream processing (DSP) of virus-like particles (VLPs) produced in insect cells.

A genetic ON/OFF thermoswitch was engineered in the baculovirus vector by a mutation in the essential *AcMNPV gp41* gene (BacFree^{ts}).

BacFree^{ts} produces high baculovirus titers at 27°C, while contaminating baculovirus production switches off at the elevated temperature of 33°C.

BacFree^{ts} is used for efficient and scalable VLP production of chikungunya virus (CHIKV), West Nile virus (WNV), coxsackievirus A6, and foot-and-mouth disease virus (FMDV), concomitant with baculovirus particle reduction up to 99.97%.

The novel BacFree^{ts} system is compatible with commonly used insect cells and with both high and low multiplicity of infection strategies in bioreactors.

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Research Article

Engineered thermoswitch in the baculovirus expression vector system for production of virus-like particle vaccines with minimized baculovirus contaminants

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The baculovirus expression vector system (BEVS) is commercially used for producing recombinant (glyco)proteins, gene therapy vectors, and virus-like particles (VLPs) in insect cells. A major challenge in VLP downstream processing (DSP) is the removal of similarly sized baculovirus particle contaminants. Herein we engineered a genetic ON/OFF thermoswitch in the baculovirus vector by mutating the essential *gp41* gene. This temperature-sensitive (ts) baculovirus expression system (BacFree^{ts}) produces high baculovirus titers at 27°C, while contaminating baculovirus production switches off at elevated temperatures. We demonstrate scalable VLP production for chikungunya virus (CHIKV), West Nile virus (WNV), coxsackievirus A6, and foot-and-mouth disease virus (FMDV) at 33°C, concomitant with baculovirus particle reduction up to 99.97%. However, elevated temperature moderately affected the yield of two out of four VLP designs. BacFree^{ts} is compatible with commonly used insect cells and scalable infection strategies. BacFree^{ts} effectively reduces baculovirus contaminants in the upstream VLP production phase, which will facilitate DSP of human and veterinary VLP-based vaccines.

Introduction

VLPs (see [Glossary](#)) are excellent immunogens used in a number of commercial vaccines, because they provide the safety benefits of protein subunits combined with the efficacy of inactivated virus vaccines [1,2]. For example, human papillomavirus (HPV) VLPs are very efficacious cervical cancer vaccines and provide complete and durable protection after single-dose immunization [3] and recently a CHIKV enveloped (e)VLP vaccine has received a positive recommendation by the European Medicines Agency (EMA) on market authorization (<https://www.ema.europa.eu/en/news/new-chikungunya-vaccine-adolescents-12-adults>). **BEVS** is an insect cell-based production platform with excellent scalability, safety, and cost-effectiveness. With over 40 years of experience, including successful commercial human vaccine manufacturing [HPV vaccine Cervarix, GlaxoSmithKline; Influenza virus vaccine FluBlok, Sanofi; coronavirus disease 2019 (COVID-19) vaccine NVX-CoV2373, Novavax] the BEVS is often the system of choice for the production of recombinant proteins, viral gene therapy vectors, and VLPs in insect cells [4–6].

Technology readiness

The baculovirus expression system is commercially developed and widely used for production of recombinant proteins and virus-like particles, for veterinary as well as human applications. The proposed ts BacFree^{ts} system is compatible with all existing baculovirus expression systems (transfer vectors, cell lines, etc.), because only a mutation in the viral backbone is required, combined with elevated incubator temperature. The system is robust in laboratory settings for production of various virus-like particles and the maturity of the technology can be considered as a Technology Readiness Level 4 (TRL4).

A crucial next step is the technology transfer of BacFree^{ts} to commercial scale, where precise temperature control of 33°C will be critical. Lower temperatures (≤32°C) will induce production of contaminating baculovirus particles, while higher temperatures (≥34°C) reduce production yields. Moreover, as impurities in the VLP preparations still remain, advances and optimization of downstream processes and analytical methods, tailored to each VLP, are required to obtain high-quality, clinical-grade vaccine material.

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However, the purification of VLPs is problematic in the BEVS because of the physical similarities between the VLPs/eVLPs and the co-produced baculovirus particles. Here, we developed a solution by preventing the co-production of baculovirus particles during the VLP production phase by engineering an ON/OFF thermoswitch in the baculovirus vector. An intrinsic characteristic of the BEVS is the production of **budded viruses (BVs)** that are actively secreted into the culture fluid during the late phase of infection. BVs are needed to spread the infection in the culture to enable recombinant protein or VLP production (Figure 1A). While occlusion bodies (also known as polyhedra) are not formed due to the absence of the polyhedrin (*pol/h*) gene, occlusion-derived viruses (ODVs) are still being assembled in the nucleus and are released upon cell lysis. These baculovirus virions (BV and ODV) are co-produced with the recombinant proteins or VLPs and have to be inactivated (veterinary applications) or removed (human applications) during **DSP**.

Baculoviruses are considered relatively safe, as they cannot replicate in mammalian cells [7–10]. However, BVs are able to enter mammalian cells and can deliver their DNA inside these cells. This characteristic forms a risk for unwanted antiviral immune responses [11] and possibly host genome integration [12], although this latter concern is mainly theoretical. Immune responses activated in mammals (mice) by baculoviruses include the production of type-I interferons and

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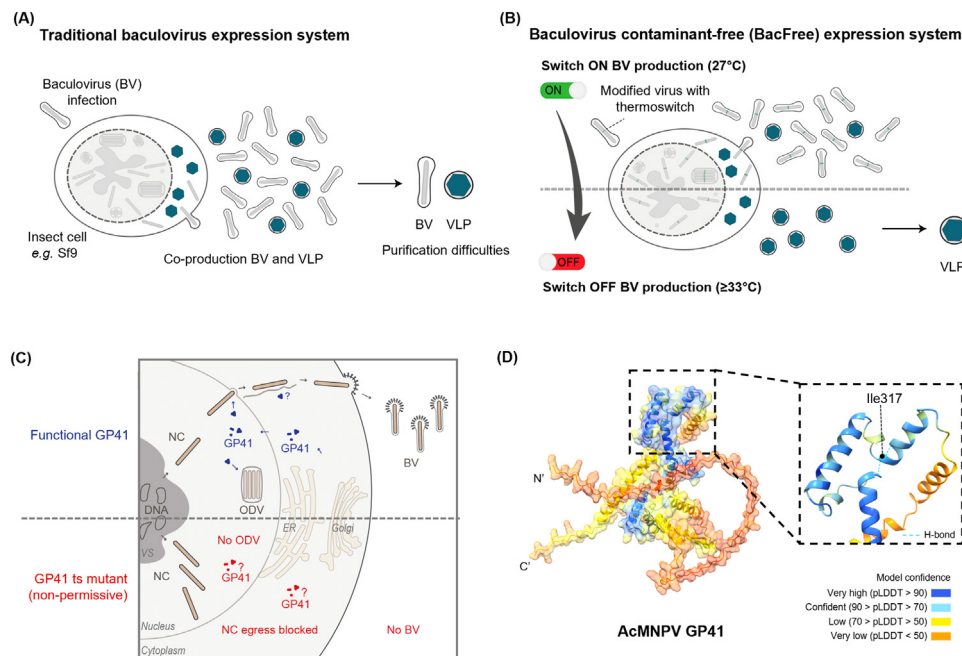


Figure 1. Design of a temperature-sensitive (ts) baculovirus expression system (BacFree^{ts}) for controlled baculovirus virion production in insect cells. (A) In the traditional virus-like particle (VLP) production process using the baculovirus expression vector system (BEVS), insect cells are infected with recombinant budded virus (BV). The baculovirus replicates and co-produces new BV and heterologous VLPs. A mixture of BVs and VLPs results in subsequent purification difficulties in downstream processing. (B) In the novel BacFree^{ts}, the baculovirus is modified to contain an ON/OFF switch. BV production is maintained in the ON state, but when switched to the OFF state, the co-production of BVs is blocked while VLP production continues. (C) The role of GP41 during viral infection. GP41 is found in multiple oligomeric states in both cytoplasm and nucleus, where it is essential for occlusion-derived virus (ODV) assembly and nuclear egress of nucleocapsids (NC). In the GP41 ts mutant at non-permissive temperature, ODVs are not formed and NC egress is blocked, but the exact effect on the GP41 protein is unknown. (D) Predicted protein structure of AcMNPV GP41 constructed with AlphaFold3. The residues are colored according to the per-residue confidence score (pLDDT) of AlphaFold3. The box shows a close-up of the tip of the protein, where Ile317 is located.

activation of genes associated with Toll-like receptors, complement activation, and cytokine signaling [9,11,13,14]. As such, BVs are regarded as **vaccine contaminants** and must be removed from the active vaccine components during the purification process of human vaccines [15].

VLPs have a large size (nanometer scale) and complexity compared with subunit proteins, and similarities in size and physical/biochemical properties between the VLPs and the co-produced BVs complicate DSP [16,17]. The commercial manufacturing process of HPV VLPs involves the purification of monomeric L1 subunit proteins from baculovirus-infected cells and these monomers are subsequently re-assembled into VLPs [18]. Specifically for the HPV VLP production process, BV contaminants are not a major problem as monomers can rather easily be separated from baculovirus particles. However, for most other VLPs, especially the more complex eVLPs and viral vector particles, such a disassembly–reassembly step is not feasible and a complicated downstream process is required with concomitant product yield loss [19]. The regulations for veterinary vaccines are comparatively less strict and allow for residual baculoviruses in the final vaccine formulation after a viral inactivation step. However, inactivation, often with chemical treatments like formalin, β -propiolactone, or binary ethylenimine, can alter the **antigenicity** of recombinant proteins [20,21], potentially requiring higher vaccine doses to compensate for the loss of **immunogenic epitopes** [22].

The extensive DSP issues caused by these BV ‘contaminants’ were identified as a key hurdle for commercialization of BEVS VLP production [23–26]. For example, BEVS could provide a more scalable expression system for production of CHIKV eVLPs [27] with higher yields compared with mammalian cell expression systems [28,29]. Consequently, there is growing demand for the development of **baculovirus-contaminant free (BacFree)** expression systems for simplified manufacturing of VLPs and eVLPs [16,17,30–32]. The BacFree idea is that by blocking BV secretion in the upstream production phase, the downstream process of VLP production can be simplified.

First-generation BacFree systems were based on baculovirus deletion mutants defective in BV production [33] in combination with engineered cell lines expressing the deleted gene *in trans* to rescue baculovirus amplification during BV seed stock production. This approach was initially implemented by disrupting the essential nucleocapsid protein VP80 [34] and later by deleting the gene for the envelope glycoprotein - GP64 [35] or the late essential factor - LEF-5 [36]. While these deletion mutants are indeed highly effective in blocking BV production, the **trans-complementing** cell line cannot readily produce high titer BV seed stocks [34]. Moreover, the constitutive expression of the trans-complementing gene might have cytotoxic effects on the cells and is associated with cell line instability [34]. Placing the trans-complementing gene under an inducible promoter resulted in the rapid accumulation of revertants – baculovirus escape mutants that re-gained the previously deleted gene by recombination [36].

Because baculovirus deletion mutants cannot spread in cell culture, it is essential to use a high **multiplicity of infection (MOI)** in the production process. This is not preferred in large-scale production processes as it requires very large BV seed stock volumes/bioreactors and results in more dilution of the production bioreactor with spent medium. The possibility of using low-MOI infections for VLP production would eliminate an extra BV amplification step beforehand, which will also reduce adverse passage effects [37,38] and is compatible with commercial large-scale production [23,39]. Thus, even though baculovirus deletion mutants effectively block BV production, the first-generation BacFree systems have significant limitations for implementation on large scale.

Glossary

Antigenicity: the ability to activate an immune response.

Baculovirus-contaminant free (BacFree): a modified expression system designed to minimize the production of baculovirus particle contaminants.

Baculovirus expression vector system (BEVS): a widely used platform for production of recombinant proteins in insect cells, driven by baculovirus infection.

Budded viruses (BV): extracellular baculovirus virions that mediate cell-to-cell spread of the virus.

Downstream processing (DSP): the purification steps required after production to obtain the final product.

Immunogenic epitopes: a small peptide or region that is recognized by the immune system and triggers an immune response.

Multiplicity of infection (MOI): the number of infectious virus particles added per host cell in a viral infection experiment.

Temperature-sensitive (ts): a mutant that displays an alternative phenotype when the incubation temperature is changed.

Trans-complementing: restoring the defective (viral) function by providing the missing gene in a separate genetic element, for example, on a separate plasmid or integrated into the host genome.

Vaccine contaminants: impurities in vaccine preparations, such as host cells proteins and residual viral particles.

Virus-like particles (VLPs): non-infectious, self-assembling nano-scale structures that mimic the shape and surface morphology of native viruses.

Herein we present a novel and innovative BacFree expression system for VLP vaccine production by engineering a genetic thermoswitch in the baculovirus expression vector (BacFree^{ts}). With BacFree^{ts}, temperature elevation from the standard production temperature of 27°C to 33°C results in a multilog reduction of co-produced baculovirus contaminants in the upstream VLP production phase. BacFree^{ts} is compatible with both high and low MOI strategies and makes the BEVS more competitive for high-quality production of large biomolecules, VLPs, and viral vector particles in insect cell bioreactors.

Results

Design of a ts baculovirus expression system for controlled baculovirus virion production in insect cells

We aimed to develop a novel BacFree system based on a BV-production shut-off switch (Figure 1B). Our approach employs a **ts** baculovirus with an ON/OFF switch regulated by temperature. Such a ts BacFree (BacFree^{ts}) expression system would offer numerous advantages over the previously developed BacFree approaches. A simple switch to a higher incubation temperature would block BV formation and solve the problem of producing contaminating particles. Moreover, standard insect cells can be used for both baculovirus amplification and VLP production. Finally, it also allows for the use of a low MOI in the production phase, where the correct timing of the temperature switch will likely become important.

A literature search was performed to identify a suitable candidate among previously characterized baculovirus ts mutants [40–44]. An improved BacFree system has several requisites. First, efficient baculovirus amplification is needed at permissive temperatures to produce sufficient BV seed stocks. Second, the BVs should still be able to enter the cell line and the virus should then go through all infection stages including DNA replication, the late phase, and hyperexpression of the gene(s) of interest in the very late phase. Only the production, assembly, or intercellular transport of new BV particles should be inhibited [34]. Of the >35 described ts mutants, one suitable candidate was identified [45]: the ts AcMNPV mutant ts-B1074 [42]. This virus was generated by mutagenesis with 5-bromodeoxyuridine (BUdR), followed by screening at 32.5°C for temperature sensitivity. ts-B1074 produced similar numbers of BVs as the wild-type virus at 23.5°C [$\sim 10^8$ plaque forming unit (pfu)/ml], but shows a single-cell infection phenotype at 32.5°C, where the infection did not visibly spread to neighboring cells. Moreover, a high MOI (10 pfu/cell) resulted in 100% of the cells producing polyhedral occlusion bodies with equal onset of occlusion body formation as the wild-type virus [42]. The ts-B1074 mutant contained an isoleucine-to-threonine point mutation at residue 317 (I317T) in the viral gene *ac80*, encoding the ODV tegument protein GP41 [46], which was later shown to also be an essential protein in the secretion pathway of BV [47] (Figure 1C).

GP41 is encoded by a baculovirus core gene, which is expressed in the late phase of infection as a 45.4 kDa glycoprotein [48]. The protein localizes to both the cytoplasm and cell nucleus where it is essential for ODV assembly and BV secretion [47]. GP41 is found in several oligomeric states, where the GP41 trimers are selectively incorporated into virions [47], but the exact role of GP41 in BV secretion remains enigmatic. There are two leucine zippers and several important cysteine residues identified in GP41 [47], but any other secondary or tertiary structural features of GP41 have not been resolved. The protein structure of GP41 was therefore predicted using the deep learning protein folding software AlphaFold3 [49] (Figure 1D), showing that GP41 has an elongated tertiary structure, which noticeably appears disorganized. Both the N and C terminus are located at the base of the protein and the N terminus contains a long disordered region. The AcMNPV ts-B1074 mutant contains an isoleucine-to-threonine mutation at residue 317 (I317T) [46]. Residue Ile317 is situated at the tip of GP41, N-terminal of a short helix within a helix–

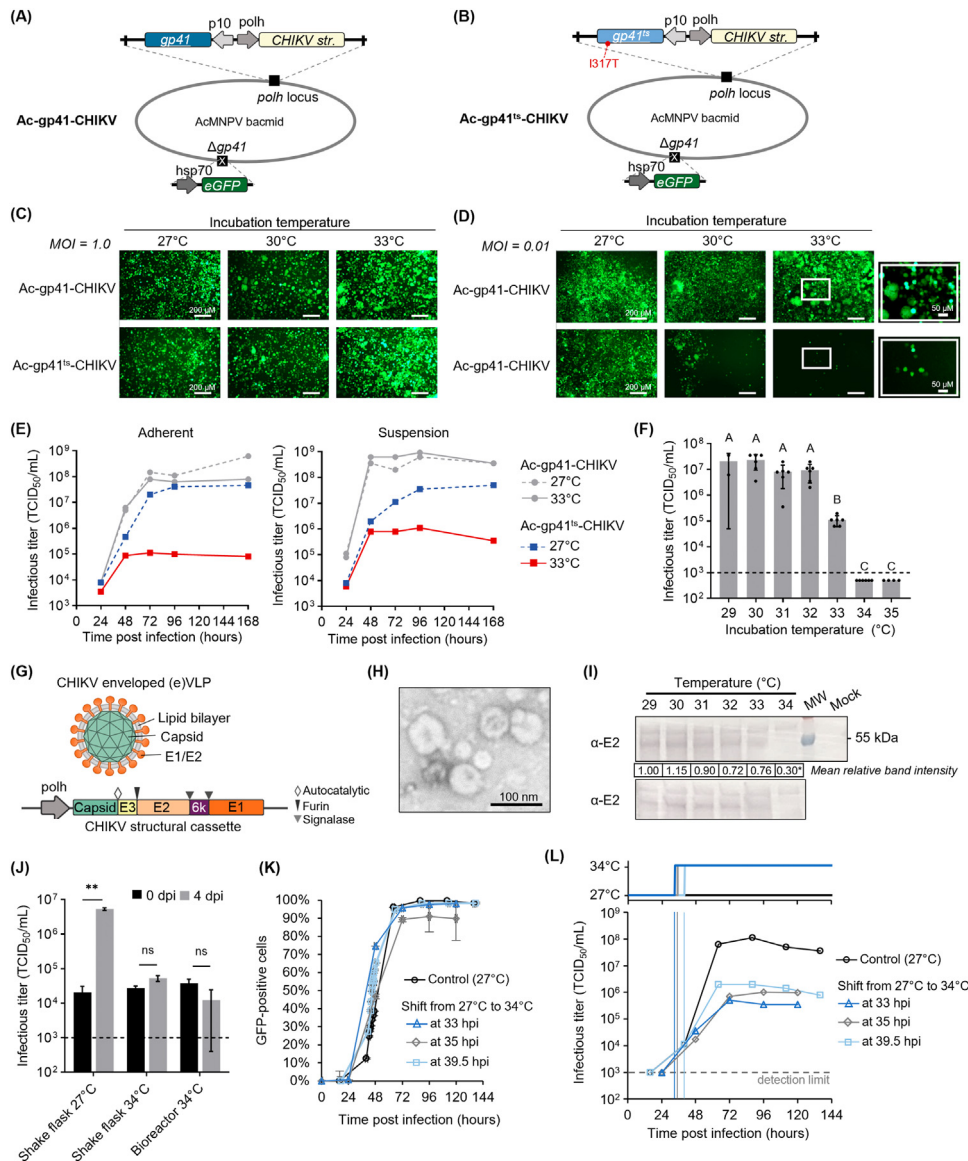
loop-helix-loop-helix motif (Figure 1D). The Ile317 residue and the structural motif are conserved among baculovirus GP41 orthologs, as demonstrated by multiple sequence alignments (Figure S1A in the supplemental information online) and AlphaFold3 predictions (Figure S1B). The ts-B1074 mutant has not been characterized in depth. It is unclear how its viral replication is affected by temperature, nor whether it might be applicable in the BEVS, let alone how this ts mutant would function in a larger-scale production process for VLPs.

Construction and characterization of a ts baculovirus expressing chikungunya VLPs

The I317T mutation in baculovirus GP41 creates a ts virus, enabling BV production at 23°C while significantly restricting it at 33°C, but the exact switching temperature was not studied [42,46]. We first verified this previous research by constructing a proof-of-concept ts baculovirus, accurately identifying its permissive and restrictive temperatures, and assessed the production of secreted VLPs. An AcMNPV bacmid was engineered to replace the native *gp41* gene (including the codon for residue 317) with a green fluorescent protein (GFP) marker gene under the control of the hsp70 promoter (AcΔgp41 [47]). The *gp41* expression was rescued under the very late p10 promoter and the VLP cassette from CHIKV was expressed under the very late polh promoter at the polh locus (Ac-gp41-CHIKV, Figure 2A). A similar strategy was used to create the ts mutant by introducing a single point mutation in *gp41* that resulted in an I317T mutation (Ac-gp41^{ts}-CHIKV, Figure 2B). Green fluorescence, by eGFP expression (hsp70 promoter) was observed in nearly all cells at 27°C, 30°C, and 33°C after an infection with MOI of 1 50% tissue culture infectious dose (TCID₅₀/cell (Figure 2C). A restricted viral spread was observed at 30°C and 33°C after infection with Ac-gp41^{ts}-CHIKV at low MOI (0.01 TCID₅₀/cell), indicating temperature sensitivity, although a complete single-cell infection phenotype at 33°C could not be confirmed (Figure 2D). This was substantiated by viral growth curves at 27°C and 33°C in both adherent and suspension Sf9 cell cultures. Infectious BV secretion in the culture fluid was reduced with several log-scales at 33°C but was not completely inhibited (Figure 2E).

Microbioreactor screening with ExpiSf9 suspension cultures was performed to identify the optimal permissive and non-permissive temperatures. Initial screening with low MOI infections (0.01 TCID₅₀/cell) showed that Ac-gp41-CHIKV retained efficient BV production from 22°C up to 34°C, and for Ac-gp41^{ts}-CHIKV the optimal BV production (>10⁸ TCID₅₀/ml) was found from 26°C to 30°C (Figure S2 in the supplemental information online). Temperature sensitivity of Ac-gp41^{ts}-CHIKV was observed from 33°C to 35°C, where the BV concentration did not reach the detection limit of 1.0 × 10³ TCID₅₀/ml (Figure S2). Infection with a slightly higher MOI (0.3 TCID₅₀/cell) demonstrated severe attenuation at 33°C and full inhibition of BV production at 34–35°C (<10³ TCID₅₀/ml) (Figure 2F); this was 1°C higher than previously reported [46]. At this non-permissive temperature, a single-cell infection phenotype could be observed after a low MOI infection (Figure S3A in the supplemental information online), with no measurable infectious BV present (Figure S3B). Moreover, transmission electron microscopy (TEM) of infected cell nuclei showed production of nucleocapsids that accumulated at the nuclear membrane but failed to egress at these high temperatures and ODVs were not observed (Figure S4 in the supplemental information online), in line with previous reports of the ts phenotype [46]. Expression of the CHIKV VLP cassette (Figure 2G) resulted in secretion of eVLPs (Figure 2H). CHIKV structural proteins could be detected in the culture fluid at all temperatures (Figure 2I). However, the elevated incubation temperatures of 34°C resulted in significantly lower E2 concentrations, presumably due to attenuated viral spread and/or low productivity, and was therefore a suboptimal temperature for producing these VLPs.

The system was also scalable to shake flasks and stirred-tank bioreactors. Under control conditions at 27°C, BV titers significantly increased between 0 and 96 hours post-infection (hpi). By



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Figure 2. Construction and characterization of a temperature-sensitive (ts) baculovirus expressing chikungunya virus (CHIKV)-like particles. (A,B) Baculoviruses were constructed expressing either the wild-type *gp41* (A, Ac-gp41-CHIKV) or *gp41* containing the I317T ts mutation (B, Ac-gp41^{ts}-CHIKV) under control of the p10 promoter, and CHIKV S27 structural cassette [27] under the polyhedrin (polh) promoter. The virus backbone contains a deletion of the native *gp41* gene (AcΔgp41), expressing eGFP (hsp70 promoter). (C,D) The infection phenotype of Ac-gp41-CHIKV and Ac-gp41^{ts}-CHIKV at elevated temperatures. Sf9 cells were infected at multiplicity of infection (MOI) 1 50% tissue culture infectious dose (TCID₅₀/cell) (C) or 0.01 TCID₅₀/cell (D) and incubated at 27°C, 30°C, or 33°C. Viral spread was observed by fluorescence microscopy at 4 days post-infection (dpi). (E) Growth curves of Ac-gp41-CHIKV and Ac-gp41^{ts}-CHIKV at 27°C and 33°C. Sf9 cells were either grown as adherent cells in a six-well plate or as suspension cells in 250 ml shake flasks and were infected with MOI 1 TCID₅₀/cell, followed by incubation at either 27°C or 33°C. The baculovirus titer was measured by end-point dilution assay at 27°C. (F) Microbioreactor characterization of Ac-gp41^{ts}-CHIKV. Suspension ExpiSf9 cells were infected with Ac-gp41^{ts}-CHIKV at MOI = 0.3 TCID₅₀/cell and incubated at the indicated temperatures. At 96 hours post-infection (hpi) the infectious budded virus (BV) titer was determined [Mean ± standard deviation (STDEV)]. The broken line indicates the lower limit of detection. Significance was calculated by one-way ANOVA on log10- (Figure legend continued at the bottom of the next page.)

contrast, no new BVs were produced in the infections at 34°C in either shake flasks or 500 ml bioreactor (Figure 2J). Since the Ac-gp41^{ts}-CHIKV virus did not spread at 34°C, the temperature was kept at 27°C for low MOI processes until all cells became infected. Only then was the temperature increased to 34°C to inhibit baculovirus contaminant release. To assess the impact of timing on low MOI processes, several low MOI bioreactor infections (scaled-up to 3 l reactors) with temperature shifts at different times post-infection were performed. Previous experiments with low MOI infections (0.01 TCID₅₀/cell) observed complete baculovirus infection around 40 hpi [50]. Therefore, the temperature shift was conducted between 33 and 40 hpi, when an increase in average cell diameter was detected (Figure S5 in the supplemental information online). Complete infection was achieved for all conditions (Figure 2K) but the final contaminating BV titers were approximately 100-fold lower in the bioreactors that included a temperature shift from 27°C to 34°C, compared with the control run at 27°C (Figure 2L).

We observed a significant reduction in CHIKV VLP productivity at 34°C. However, this was not caused by the ts mutation, as the same reduction in transgene expression was observed with a standard Ac-eGFP virus (eGFP under the control of the polh promoter) (Figure S6 in the supplemental information online). Similarly, occlusion bodies and P10 protein were hardly observed in cells infected with a wild-type AcMNPV isolate at 34°C (Figure S6). The reduction was caused by lower mRNA transcript levels from both p10 and polh promoter, as opposed to viral DNA copies, measured by qPCR on Ac-eGFP infected cells (Figure S6). The reduction in transgene expression at 34°C was seen in several insect cell lines. This includes a *Trichoplusia ni* High Five cell line adapted through adaptive laboratory evolution to grow at high temperatures (Figure S6) [51].

Implementation of the gp41^{ts} thermoswitch in the Bac-to-Bac expression system (BacFree^{ts})

The gp41^{ts} expression was controlled by the p10 promoter at the polh locus and this bacmid was not readily compatible with standard Bac-to-Bac transfer vectors (the pFastBAC series, ThermoFisher). Since gp41^{ts} was not at its native locus and was under the control of a strong heterologous promoter, its overexpression in the very-late phase of infection might affect the infection kinetics. The I317T mutation was therefore incorporated in gp41 at its original locus in the bacmid by scar-less recombineering [52]. To visualize and monitor viral spread, eGFP was expressed under the control of the polh promoter at the attTn7 transposition site present at the odv-e56 locus of this bacmid (Figure 3A) [53]. The Ac-eGFP virus (used in the standard BEVS)

transformed titers ($P < 0.0001$). (G) The Ac-gp41-CHIKV and Ac-gp41^{ts}-CHIKV baculoviruses encode the CHIKV structural cassette (polh promoter), which is post-translationally processed into the individual CHIKV capsid and envelope (E) proteins by autocatalytic cleavage and processing by host furin and signalases. The structural proteins self-assemble into secreted CHIKV enveloped virus-like particles (eVLPs). (H) Transmission electron micrograph of CHIKV eVLPs. CHIKV VLPs were purified from the culture fluid on a 40–70% (w/v) sucrose gradient and imaged by negative staining transmission electron microscopy. (I) Western blot immunodetection of CHIKV eVLPs in the culture fluid. Samples were collected at 96 hpi from the microbioreactor run at 29–34°C after infection with Ac-gp41^{ts}-CHIKV at MOI = 0.3 TCID₅₀/cell. The western blot membrane was stained with polyclonal antibody against CHIKV E2. Relative CHIKV VLP expression was calculated by densitometry analyses with ImageJ. Band intensities were normalized to the sample at 29°C and averaged for two biological replicates. Significance was calculated by one-way ANOVA ($^*P = 0.0293$). (J) Scale-up to a 300 ml bioreactor. The bioreactor and shake flasks were infected with Ac-gp41^{ts}-CHIKV at a high MOI (10 TCID₅₀/cell). The temperature remained at 27°C, or was increased to 34°C at 3 h post-infection. The infectious BV titer in the culture fluid was determined at 0 and 96 hpi. Error bars represent the STDEV of duplicate measurement values. $^{**}P = 0.0032$. (K,L) Temperature shift timing in low MOI infections with Ac-gp41^{ts}-CHIKV in 3 l bioreactors. ExpiSf9 cells were infected with MOI 0.01 TCID₅₀/cell at 27°C and the incubation temperature in the bioreactor was elevated to 34°C at 39.5 hpi (light blue), 35 hpi (grey), or 33 hpi (dark blue), or remained at 27°C (black). (K) The percentage of GFP-positive cells, measured by flow cytometry. Error bars represent duplicate measurement values. (L) Infectious BV titers, measured by end-point dilution assay at 27°C. The broken grey line indicates the measurement threshold of the TCID₅₀ assay. The moment of the temperature shift (at 40, 35, or 33 hpi) is indicated in vertical lines and in the top graph. Abbreviation: MW, molecular weight marker; ns, not significant.

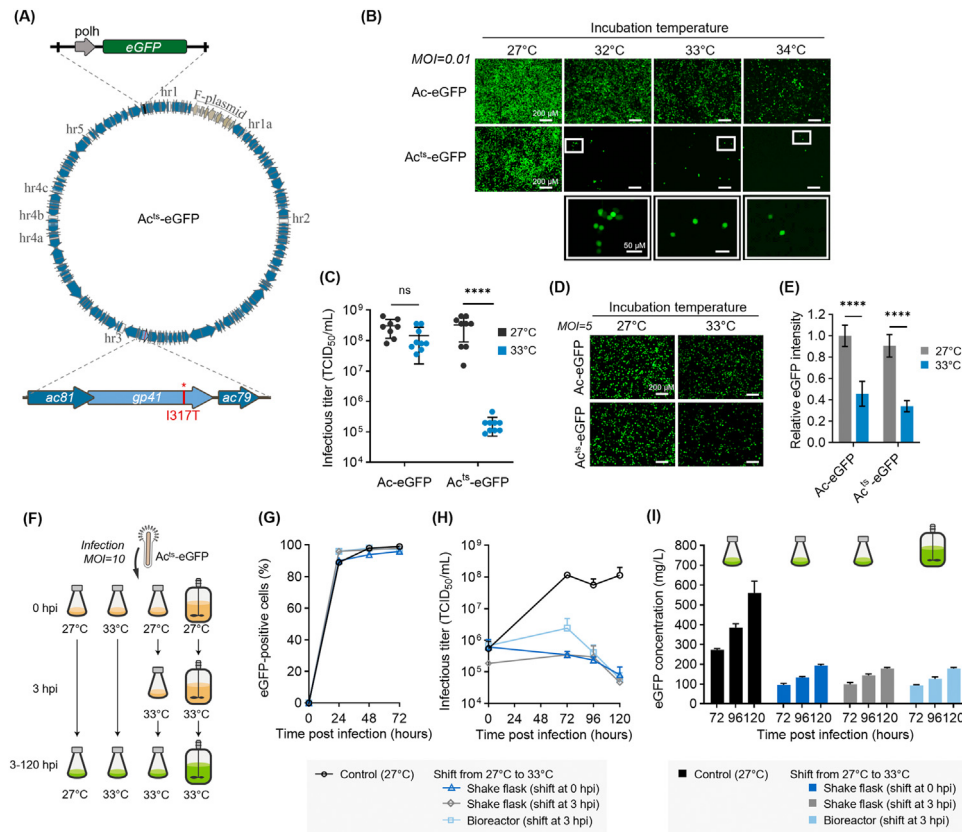


Figure 3. Implementation of the *gp41^{ts}* thermoswitch in the Bac-to-Bac expression system (BacFree^{TS}). (A) The *Ac^{TS}* bacmid comprises the AcMNPV genome with mini-F plasmid at the polyhedrin locus and an attTn7 transposon site at the *odv-e56* locus (AcBACE56 [53]). The I317T mutation was introduced in *gp41* at the *gp41* locus by recombinering. The eGFP gene (*polh* promoter) was inserted into the *odv-e56* locus by Bac-to-Bac transposition, resulting in *Ac^{TS}-eGFP*. (B) The non-permissive temperature (temperature at which a single-cell infection phenotype was observed) was determined by low multiplicity of infection (MOI) infections (MOI = 0.01 TCID₅₀/cell) of the wild-type *Ac-eGFP* virus or the temperature-sensitive (ts) mutant *Ac^{TS}-eGFP* in six-well plates. Cells were incubated at 27°C, 32°C, 33°C, or 34°C and were monitored for viral spread by eGFP production at 4 days post-infection (dpi). The white box zooms in on a group of cells. (C–E) Comparison between the standard baculovirus expression vector system (BEVS) and the BacFree^{TS} BEVS. Sf9 cells were infected at MOI = 5 50% tissue culture infectious dose (TCID₅₀/cell) with *Ac-eGFP* or *Ac^{TS}-eGFP*. At 3 hours post-infection (hpi) the inoculum was washed and cells were incubated at 27°C or the lowest non-permissive temperature of 33°C. (C) The budded virus (BV) titers were determined at 4 dpi. The experiment was performed with triplicate infections and the experiment was repeated a total of three times. The mean $\pm$ standard deviation (STDEV) is shown, significance was calculated by one-way ANOVA on log₁₀-transformed titers ($P < 0.0001$). (D) Fluorescence microscopy pictures at 4 dpi. Pictures are representative of the entire well and replicate infections. (E) eGFP signal in the fluorescence pictures was quantified with ImageJ and were normalized to the average intensity of *Ac-eGFP* at 27°C. The mean $\pm$ STDEV was calculated with fluorescence pictures of all replicate infections. Significance was calculated with one-way ANOVA ($P < 0.0001$). (F) Experimental design of scale-up of *Ac^{TS}-eGFP* infections to shake flasks and table-top bioreactor. ExpiSf9 cells were cultured in 125 ml shake flasks and 300 ml bioreactor and were infected with *Ac^{TS}-eGFP* at MOI = 10 TCID₅₀/cell and cell concentration at time of infection (CCI) 2×10^6 viable cells/ml. The temperature in the bioreactor and parallel shake flask was increased from 27°C to 33°C at 3 hpi. A control flask was either kept at 27°C or was incubated at 33°C directly after infection (0 hpi). (G) The fraction of eGFP-positive cells was determined by flow cytometry in technical triplicates. (H) Infectious BV titers were measured at 0 hpi (5–10 min after addition of the virus), 72 hpi, 96 hpi, and 120 hpi by end-point dilution assay on ExpiSf9 cells. The error bars indicate the variation between duplicate titration results. (I) Total eGFP productivity (intra- and extracellular) over time, measured by fluorescence plate reader in technical triplicate. **** indicates $P < 0.0001$. Abbreviation: ns, not significant.

could spread throughout the cell culture at all tested temperatures, as expected. By contrast, single-cell infections were observed for the Ac^{ts}-eGFP mutant at 33°C and 34°C (Figure 3B). At 32°C, the virus was largely attenuated and only local spread of the virus infection was observed. This non-permissive temperature (33°C) is consistent with previous reports on ts-B1074 [42,46] and is a more favorable temperature for VLP expression. The point-mutation and ts phenotype were stable for at least eight viral passages and expression of the eGFP cassette was also retained, while overall BV titers were reduced (Figure S7 in the supplemental information online), which was expected in high MOI passaging of baculovirus stocks [37,54]. We conclude that the Ac^{ts} baculovirus is suitable for testing large-scale pharmaceutical production, which requires multiple viral passages for generation of seed stocks.

A side-by-side comparison of the Ac^{ts}-eGFP BacFree^{ts} virus and the standard Ac-eGFP virus was conducted in terms of BV production at 27°C and 33°C (Figure 3C). At the permissive temperature (27°C), Ac^{ts}-eGFP produced a high BV titer ($>10^8$ TCID₅₀/ml) similar to Ac-eGFP. By contrast, titers of Ac^{ts}-eGFP at the non-permissive temperature (33°C) only reached 10^5 TCID₅₀/ml at 4 days post-infection (dpi). This is a 99.94% ($-3.2 \log_{10}$) reduction of BV contaminants compared with 27°C, corresponding to a release of ~ 0.2 TCID₅₀ per cell. Viral DNA replication was not affected by elevated temperatures as measured with qPCR quantification of intracellular viral DNA (Figure S8 in the supplemental information online). The eGFP intensity was still visibly lower at 33°C compared with 27°C (Figure 3D) and was reduced with almost threefold in both viruses (Figure 3E).

We assessed whether a similar BV reduction could be achieved in scalable cultivation systems. The high MOI infection process (MOI 10 TCID₅₀/cell) with Ac^{ts}-eGFP was scaled-up to ExpiSf9 suspension cultures in 125 ml shake flasks and a table-top bioreactor containing 300 ml culture medium (Figure 3F). The infection was monitored for 72 hpi, until $>95\%$ GFP-expressing cells was achieved for all conditions (Figure 3G). As expected, BV titers of Ac^{ts} were several log-scales higher under control conditions (27°C) compared with the bioreactor and shake flasks cultured at, or shifted to 33°C (Figure 3H). A similar reduction in BV titers was measured in bioreactors and shake flasks, so the temperature-switch strategy is suitable for scale-up. eGFP was produced to 100–200 mg/l at 33°C and was similar for the shake flask and bioreactor cultures, but approximately threefold lower compared with production at 27°C (Figure 3I).

Production of stabilized West Nile VLPs with BacFree^{ts}

We next investigated the application of the BacFree^{ts} system for higher-value products. Here, we produced secreted eVLPs of WNV (family *Flaviviridae*). WNV subviral particles, which are eVLPs lacking the WNV capsid protein, are formed and secreted when the WNV pre-membrane (prM) and envelope (E) proteins are coexpressed (Figure 4A). The E protein was stabilized by introduction of an A261C mutation at the twofold axis (Figure S9A in the supplemental information online), an approach similar to stabilization of Zika virus and dengue virus eVLPs [55,56]. The introduction of the A261C mutation led to a substantial proportion of cysteine-linked E-multimers on non-reducing SDS-PAGE, but alternative cysteine insertions did not further increase the fraction of stable E multimers (Figure S9). Production of assembled WNV subviral particles was confirmed by TEM imaging (Figure 4B). Expression of stabilized WNV eVLPs with Ac^{ts} resulted in a 99.70% ($-2.5 \log_{10}$) reduction of infectious BV contaminants at 33°C compared with 27°C (Figure 4C and Table 1). WNV E protein monomers and dimers were detected in the cell pellet and concentrated culture fluid by western blot analyses, with equal volumes loaded between samples (Figure 4D). Moreover, production of WNV VLPs at 33°C was accompanied by reduced baculovirus VP39 capsid contaminants (Figure 4E). WNV eVLPs were produced in similar amount at 27°C and 33°C (Figure 4F).

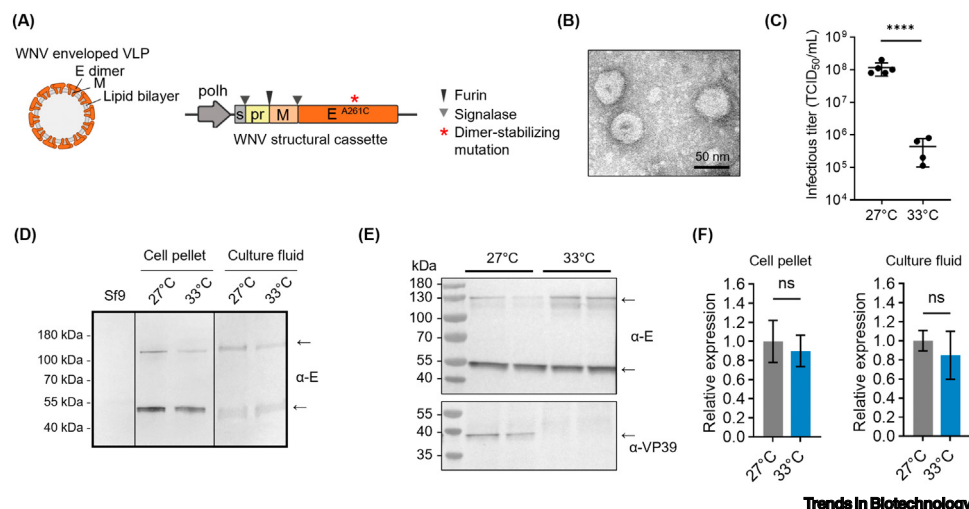


Figure 4. Production of stabilized West Nile virus (WNV)-like particles with BacFree™. (A) Schematic overview of the WNV structural cassette, encoding a signal sequence (s, end of capsid), precursor membrane (prM), and envelope (E) protein, and resulting in production of secreted enveloped virus-like particles (eVLPs). The E protein contains an A261C dimer-stabilizing cysteine mutation. (B) Transmission electron micrograph of WNV A261C eVLPs produced at standard culturing conditions, or at elevated temperature (33°C). VLPs were purified from the culture fluid by sucrose density centrifugation and visualized with negative staining. (C–F) Expression of WNV eVLPs in the BacFree™ system. Sf9 cells were infected with Ac™-WNV prME A261C at high multiplicity of infection (MOI) and incubated at 27°C or 33°C. (C) Infectious budded virus (BV) titer in the culture fluids was measured at 3 days post-infection (dpi), showing the average +/- standard deviation of five replicate infections. Significance was determined by an unpaired *t*-test on log10-transformed titers ($P < 0.0001$). (D) Western blot immunodetection of stabilized WNV eVLPs in the cell pellet and concentrated culture fluid at 3 dpi. (E) Presence of contaminating baculovirus capsid protein (anti-VP39), and WNV VLPs (anti-E, 4G2) in the culture fluid detected by western blot immunodetection. Samples of the culture fluid of duplicate infections after incubation at 27°C or 33°C [MOI = 10 50% tissue culture infectious dose (TCID₅₀/cell, 3 dpi)]. (F) Relative expression of WNV VLPs in the culture fluid. The E protein concentration was determined by densitometry analyses of western blot bands (ImageJ). Band areas were normalized against the average at 27°C (five replicates among three independent experiments). (*t*-test, $P = 0.2687$). Abbreviation ns, not significant.

Production of coxsackievirus A6 non-enveloped VLPs with BacFree™

Apart from eVLPs, we also expressed non-enveloped capsid-based VLPs from coxsackievirus A6 (CVA6; family *Picornaviridae*). CVA6 has become the major causative agent of hand, foot, and mouth disease in young children and adolescents [57,58]. The virus is difficult to propagate

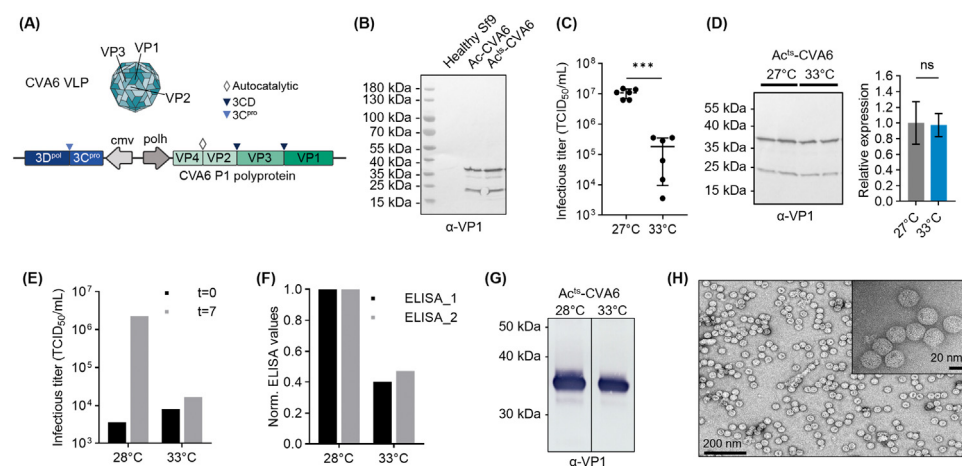
Table 1. Summary of the reduction in contaminating baculovirus particles compared with productivity in the BacFree™ system^a

Product	Contaminating BV			Productivity	
	Log10 reduction	Percentage reduction	Corresponding figure in main text	Relative product yield	Corresponding figure in main text
CHIKV eVLPs	>4.0	>99.99%	Figure 2F	0.30	Figure 2I
eGFP (monolayer)	3.2	99.93%	Figure 3C	0.34	Figure 3E
eGFP (bioreactor)	3.2	99.94%	Figure 3G	0.32	Figure 3H
WNV eVLPs	2.5	99.70%	Figure 4C	0.85 (ns)	Figure 4F
CVA6 VLPs (Sf9 cells)	1.8	98.32%	Figure 5C	0.97 (ns)	Figure 5D
CVA6 VLPs (Tni High five cells)	2.1	99.26%	Figure 5E	0.44	Figure 5F
FMDV VLPs	3.5	99.97%	Figure 6C	1.74	Figure 6F

^aThe table depicts the average reduction in contaminating BV titers (log-converted and percentages) and the relative VLP productivity at 33°C compared with 27°C. For WNV eVLPs and CVA6 VLPs (from Sf9 cells) the productivity was not significantly different (ns).

in cell culture, hindering the development of traditional inactivated vaccines [58]. VLPs are produced by coexpressing the P1 polyprotein (polh promoter) and the viral 3CD protease (CMV promoter) [59,60] (Figure 5A). We expressed the P1/3CD cassette in the BacFree^{ts} expression system using Ac-CVA6 (wild-type) and Ac^{ts}-CVA6, where in both cases the expression and efficient P1 polyprotein processing was seen in the culture fluid (Figure 5B). Infection with Ac^{ts}-CVA6 at MOI 10 TCID₅₀/cell resulted in a reduction of 98.32% (−1.8 log10) of contaminating BV titers in the culture fluid at 33°C compared with 27°C (Figure 5C and Table 1). VLPs were produced and secreted at similar levels at 33°C compared with 27°C (Figure 5D).

The production of CVA6 VLPs with the BacFree^{ts} system was scaled-up to 150 ml suspension cultures in Tni High Five cells (MOI 5 TCID₅₀/cell). Baculovirus production was observed in the ts virus at 28°C between $t = 0$ and $t = 7$ dpi (Figure 5E). By contrast, hardly any BVs were produced at 33°C for Ac^{ts}-CVA6, resulting in a 99.26% reduction (−2.1 log10) in contaminating BV titers compared with 28°C. This confirms that the BacFree^{ts} expression system can also be used in combination with the Tni High Five cell line in suspension cultures. Correctly folded VLPs were quantified by ELISA using two different monoclonal antibodies that bind conformational epitopes on assembled CVA6 A-particles. The yield of correctly assembled VLPs at 33°C was 56% lower compared with 28°C in the Ac^{ts}-CVA6 virus (Figure 5F). Western blot immunodetection confirmed the ELISA results, detecting CVA6 VP1 in the culture fluid at both



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Figure 5. Production of coxsackievirus A6 non-enveloped virus-like particles with BacFree^{ts}. (A) For the production of CVA6 virus-like particles (VLPs), the P1 polyprotein (polh promoter) and the 3CD^{pro} protease (CMV promoter) were coexpressed. The P1 polyprotein is post-translationally processed into VP1-VP4, which self-assemble into icosahedral VLPs. (B) Expression and polyprotein cleavage of CVA6 P1 in a standard baculovirus (Ac-CVA6) and in Ac^{ts} (Ac^{ts}-CVA6) at 27°C. The culture fluid was analyzed by western blot immunodetection against CVA6 VP1. (C,D) High multiplicity of infection (MOI) infection of adherent Sf9 cells with Ac^{ts}-CVA6. Infected cells were incubated for 4 days at 27°C or 33°C. (C) Infectious budded virus (BV) titer in the culture fluid was measured by end-point dilution assay. The average $\pm$ standard deviation of six replicate infections are shown and significance was calculated via an unpaired t -test on log10-transformed titers (***, $P = 0.0001$). (D) The clarified culture fluid was analyzed by western blot immunodetection, stained against CVA6 VP1. Band intensity was quantified and normalized against the intensity at 27°C for five replicates. (E–H) Production of CVA6 VLPs in suspension Tni High Five cells with Ac^{ts}-CVA6. Suspension cells (150 ml) were infected with Ac^{ts}-CVA6 [MOI = 5 50% tissue culture infectious dose (TCID₅₀)/cell] and were incubated at 28°C or 33°C for 7 days. (E) The flasks were sampled at 0 days post-infection (dpi) and 7 dpi and the infectious BV titer in the culture fluid was determined by end-point dilution assay. (F) Assembled CVA6 A-particles were quantified in the culture fluid at 7 dpi with two different antibody ELISAs. (G) The culture fluid was also analyzed by western blot immunodetection, stained with an antibody detecting VP1. (H) Electron micrograph of CVA6 VLPs produced with Ac^{ts}-CVA6 at 33°C. The VLPs in the culture fluid were purified by ultracentrifugation before negative staining and imaging with transmission electron microscopy. Abbreviation ns, not significant.

28°C and 33°C (Figure 5G). The VLPs in the culture fluid of Ac^{ts}-infected cells at 33°C were purified and a homogeneous population of assembled VLPs was observed by TEM (Figure 5H) with the expected size and morphology [61,62]. In short, BV contaminants were reduced by over 2 logs, while compromising on VLP yield by 56% (Table 1).

Production of FMDV VLPs with minimized baculovirus contamination

Foot-and-mouth disease is an economically important veterinary disease caused by the FMDV (family *Picornaviridae*). Coexpression of the FMDV P1/2A polyprotein and 3C protease (3C^{pro}) in insect cells [63] (Figure 6A), resulted in production of secreted VLPs (Figure 6B). The FMDV VLPs were produced by high-MOI infections with Ac^{ts}-FMDV P1/3C in shake flask cultures. A reduction in infectious BV titer of 99.97% (−3.5 log₁₀) was measured at 33°C compared with 27°C (Figure 6C). qPCR quantification of secreted viral genomes (Figure 6D) and western blot analyses of the culture fluid (Figure 6E, top) confirmed contaminant reduction by orthogonal methods. Thus, the decrease in infectious BV titer at 33°C represented a reduction in both viral DNA contaminants and baculovirus capsid protein contaminants. The quantity of FMDV VLPs in equal volumes of culture fluid samples, measured as the amount of FMDV VP2 detected on western blots, was comparable in infections incubated at 27°C and 33°C (Figure 6E, bottom). The concentration of FMDV VLPs in these samples, measured by ELISA, ranged from 1 to 2 mg/l, with a higher VLP concentration in the 33°C samples (Figure 6F). This suggests that the BacFree^{ts} production of FMDV VLPs results in >99% reduction in contaminating baculovirus particles without yield loss. Overall, BacFree^{ts} reduces contaminating BV titers by >98% with a moderate impact on VLP productivity (Table 1).

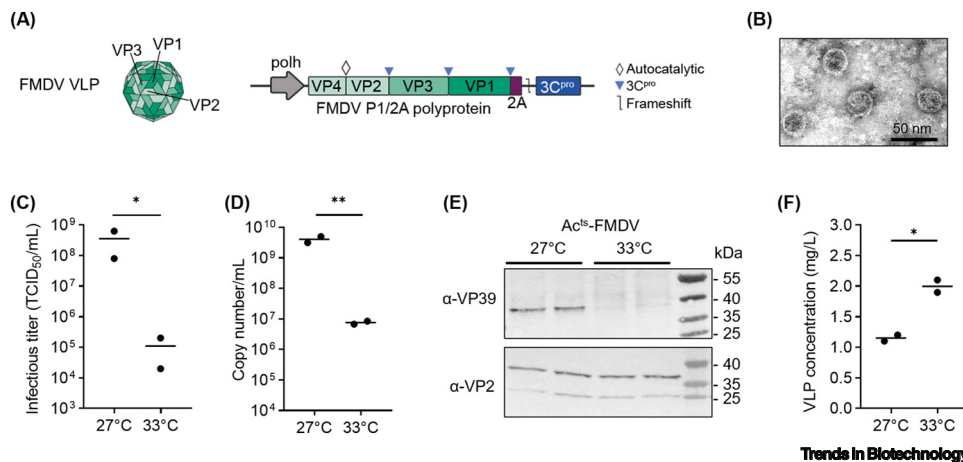


Figure 6. Production of foot-and-mouth disease virus (FMDV) virus-like particles (VLPs) with minimized baculovirus contamination. (A) The P1/2A polyprotein and 3C^{pro} (protease) of FMDV were cloned behind the polh promoter. 3C^{pro} is preceded by a NotI sequence and is translated after a ribosomal frameshift [63]. Processing of the polyprotein into VP1-VP4 results in production of icosahedral VLPs. (B) FMDV VLPs were purified from the culture fluid by sucrose density centrifugation and visualized with negative staining transmission electron microscopy. (C–F) Production of FMDV VLPs with reduced baculovirus contaminants. Suspension Sf9 cells were infected with Ac^{ts}-FMDV at high multiplicity of infection (MOI) [10 = 50% tissue culture infectious dose (TCID₅₀)/cell] and were incubated at 27°C or 33°C for 4 days. (C) Infectious budded virus (BV) titer in the culture fluid, quantified by end-point dilution assay on Sf9-ET cells. Significance was calculated by unpaired *t*-test on log₁₀-transformed titers (* *P* = 0.0341). (D) Baculovirus DNA copies in the culture fluid, quantified by qPCR with primers targeting *vp80*. The copy number was calculated via a ¹⁰log standard curve of pJET-vp80 plasmid with known concentration. Significance was calculated by unpaired *t*-test on log₁₀-transformed values (** *P* = 0.0016). (E) The clarified culture fluid was analyzed by western blot immunodetection, stained for AcMNPV VP39 (top) and FMDV VP2 (bottom). (F) The FMDV VLPs in the culture fluid were quantified by ELISA. Significance was calculated by unpaired *t*-test (* *P* = 0.0169). Abbreviation ns, not significant.

Discussion

This study presents Bacfree^{ts}, which is a significant advancement in baculovirus contaminant-free expression systems for the production of VLPs in insect cells. Bacfree^{ts} is based on a recombinant baculovirus expression vector carrying an ON/OFF thermoswitch to prevent contaminating BV during VLP vaccine production. Baculovirus ts mutants have been valuable tools for investigating the function of essential viral genes [42,46,64–66], but herein we utilized a GP41^{ts} mutant in a completely innovative way to improve the BEVS. With Bacfree^{ts}, a multilog reduction of BV titers during VLP production was achieved by increasing the production temperature from 27°C to 33°C. The novel BacFree^{ts} system is based on a mutant bacmid with a single amino acid mutation in GP41 and thereby offers compatibility with the state-of-the-art baculovirus expression methods, preserving the inherent flexibility and speed that defines the BEVS. Using the same cell line and baculovirus constructs throughout the entire process simplifies production from start to finish. As GP41 is conserved in members of the family *Baculoviridae*, the Bacfree^{ts} system is potentially broadly applicable across other baculovirus expression systems such as silkworm expression using *Bombyx mori* nuclear polyhedrosis virus (NPV) [67].

The BacFree^{ts} system also has several limitations. Product yield is a key parameter for process optimization, especially for veterinary vaccines where low cost per dose are generally required. A reduction in yield was found in the BacFree^{ts} system at elevated temperatures when expressing eGFP under control of the p10 and polh promoters, which displayed lower transcriptional activity. Still, the total eGFP concentration at 33°C reached 200 mg/l, which is at least ten times higher than the generally reported yields for eVLPs in the standard BEVS at 27°C [28,29]. A small reporter protein like eGFP is expressed in high quantities and an impaired promoter activity directly impacts protein yield. By contrast, the limiting factors for (e)VLP production are likely protein synthesis rate and/or maturation speed (i.e., post-translational modifications and processing, and the associated intracellular transport) rather than mRNA transcript levels [68,69]. Indeed, we did not measure a reduction in yield when expressing FMDV VLPs and WNV eVLPs with BacFree^{ts} (Table 1). Nonetheless, in case a significant reduction in yield is measured when producing at 33°C (e.g., for CVA6 VLPs), further optimization of the baculovirus vector, cell line, and/or process parameters are required.

The BEVS is intrinsically associated with baculovirus and host cell impurities. While the BacFree^{ts} system results in a significant reduction of BV contaminants up to 99.97% and produces no ODVs, efficient purification methods are still required to obtain high-quality, clinical-grade vaccine material. VLPs are typically purified by a combination of filtration steps, ultracentrifugation, and/or affinity chromatography [26]. Ultracentrifugation is non-scalable and an alternative DSP pipeline (e.g., chromatography-based) must be developed for a reliable industry-scale process [17]. Product characterization by sophisticated biophysical methods are required to confirm that the produced VLPs have the expected integrity and functionality. Thus, the actual impact on vaccine DSP and formulation in a real industrial process remains to be investigated, but is outside the scope of the current proof-of-concept research. Still, our results with four veterinary and medically relevant VLP products give confidence that reducing BV contaminants by several logarithmic factors improves the product-to-contaminant ratio and will no doubt streamline the DSP process.

A key feature of the BEVS is its scalability. We show effective scalability of BacFree^{ts} from tissue culture flasks to shake flasks and 3 l laboratory-scale bioreactors, yet implementation to commercial-scale production (e.g., in 1000 l bioreactors) is an important next step. With respect to shear, gas transfer, and mixing, the BEVS has proven to be well scalable to large-scale bioreactors [70]. However, the BacFree^{ts} puts additional demands on the scale-up related to the

required temperature control. At larger scales temperature gradients and/or fluctuations may occur in the bioreactor. This is not well studied in general, yet it may determine the required accuracy of temperature control during the production process and the requirement for high-quality hardware.

BacFree^{ts} can also be used in low MOI strategies, which are often preferred in large-scale processes because smaller BV seed stock volumes are required. The infection must be monitored closely to accurately determine the timing of the temperature shift. In this case, online monitoring of the infection (e.g., by measuring cell diameter) [50,71,72] could be an effective tool for determining the optimal timing. In general, stirred-tank bioreactors (STRs) have efficient heat transfer, and increasing the temperature by several degrees (to ≥33°C) during the VLP production phase is feasible at commercial scale.

The first-generation BacFree systems were based on the deletion of a baculovirus gene required for BV production. A trans-complementing cell line rescues baculovirus amplification for seed stock production [34–36]. Even though this approach is highly effective in blocking BV production, the limitation lies in the scalability of the system because of the high MOI requirement. Recently, a next-generation BacFree system was presented that uses CRISPR-Cas9 to create conditional baculovirus gene knockouts. This system either requires two cell lines, one expressing the Cas9 gene, and a recombinant baculovirus expressing the single guide (sg)RNA [73], or requires co-infection by two baculoviruses carrying either Cas9 or sgRNA [74]. In the first case, a reduction in BV titers up to 99% was achieved, but flexibility regarding cell line choice and MOI was lost [73]. The two-baculovirus approach could achieve a reduction in BV titers of >90% by targeting *gp64* or *vp80*, but also negatively impacted transgene production under control of the *polh* promoter [74], suggesting that further research and development is needed before such systems can be successfully utilized by the industry. BacFree^{ts} does not have these limitations and may be easier to implement.

Concluding remarks

Challenges in VLP purification from BV contaminants are common and solutions typically coincide with high cost or substantial product loss [17,30–32]. BacFree^{ts} can be seen as a less-complicated, more elegant strategy that retains all the advantages of the current BEVS and combines that with the option to reduce BV contaminants prior to DSP. The fewer polishing steps required with BacFree^{ts} will lead to cost savings which makes it an attractive proposition for implementation in industrial production of VLPs and other multisubunit proteins in insect cells (see Outstanding questions).

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
 - Prediction of GP41 structure
 - Construction of Ac-gp41-CHIKV and Ac-gp41^{ts}-CHIKV
 - Growth curves of Ac-gp41-CHIKV and Ac-gp41^{ts}-CHIKV at 27°C and 33°C
 - Micro-bioreactor runs
 - Viral infection experiments with Ac-gp41-CHIKV and Ac-gp41^{ts}-CHIKV in stirred-tank bioreactors
 - Construction of Ac^{ts}-eGFP and phenotypic characterization
 - qPCR of intracellular baculovirus DNA and RNA
 - Viral infection experiments with Ac^{ts}-eGFP at elevated temperatures in stirred-tank bioreactors
 - Production of stabilized West Nile virus-like particles with BacFree^{ts}

Outstanding questions

Does the VLP vaccine material produced at 33°C have the same biophysical characteristics and immunogenicity compared with VLP vaccine material that is produced at 27°C?

What strategies can be developed to account for the potential yield loss at 33°C, for example, using alternative baculovirus promoters to drive VLP expression or lowering the switch-temperature by engineering GP41?

How can the residual baculovirus inoculum be effectively removed or inactivated during DSP?

What is needed for effective technology transfer of the BacFree^{ts} system to commercial-scale biomanufacturing of VLPs?

- Purification and transmission electron microscopy of enveloped VLPs
- Production of coxsackievirus A6 non-enveloped virus-like particles with BacFree^{ts}
- Purification and transmission electron microscopy of capsid VLPs
- Production of foot-and-mouth disease virus VLPs with BacFree^{ts}
- QUANTIFICATION AND STATISTICAL ANALYSES

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Gorben Pijlman (gorben.pijlman@wur.nl).

Materials availability

Bacmids generated in this study will be made available on request, but we may require a payment and/or a completed materials transfer agreement if there is potential for commercial application.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Author contributions

Conceptualization, L.v.O., J.J.A., G.N., D.E.M., M.M.v.O., and G.P.P.; investigation, methodology, visualization, and validation, L.v.O., J.J.A., G.N., D.K., T.N., V.I.P.T., J.v.d.H., S.M., W.W., C.S.H., L.K., L.v.d.P., A.R., R.C., and E.v.d.B.; writing – original draft, L.v.O., J.J.A., D.K., C.S.H., L.K., D.E.M., M.M.v.O., and G.P.P.; writing – review and editing, L.v.O., J.J.A., D.E.M., M.M.v.O., and G.P.P.; funding acquisition, R.H.W., D.E.M., M.M.v.O., and G.P.P.

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Declaration of interests

A patent application has been filed entitled ‘Temperature-sensitive baculovirus expression vector system for the production of baculovirus virion-free biopharmaceuticals (PCT/NL2024/050220 – Bacfree)’. Inventors: Linda van Oosten, Gorben P. Pijlman, Jort J. Altenburg, Dirk E. Martens, Monique M. van Oers.

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STAR★METHODS

KEY RESOURCES TABLE

Reagent or resource	Source	Identifier
Antibodies		
Chikungunya virus E2dTm antibody, rabbit serum	Metz <i>et al.</i> [94]	N/A
Pan-flavivirus monoclonal antibody 4G2	McCown <i>et al.</i> [91]	N/A
Coxsackievirus A6 VP1 polyclonal antibody	Thermo Fisher	Cat# PA5-112001
Coxsackievirus A6 A-particle monoclonal antibody 1D5	Creative Biolabs	Cat# PABC-483
Foot-and-mouth disease virus VP2 antibody	Provided by MSD Animal Health	N/A
AcMNPV VP39 monoclonal antibody	Kind gift from Loy Volkman	N/A
Goat-anti-mouse IgG-HPR	Southern Biotech	Cat# 1030-05
Chemicals, peptides, and recombinant proteins		
Gateway LR clonase II	Thermo Fisher Scientific	Cat# 11791020
Recombinant purified EGFP	ChromoTek	Cat# EGFP
TRIzol reagent	Thermo Fisher Scientific	Cat# 15596026
RQ1 RNase-free DNase	Promega	Cat# M6101
SuperScript II Reverse Transcriptase	Thermo Fisher Scientific	Cat# 18064014
Critical commercial assays		
SYBR Select Master Mix	Applied Biosystems	Cat# 4472908
DNeasy Blood and Tissue Kit	QIAGEN	Cat# 69504
BaculoQUANT virus extraction and titration kit	Oxford Expression Technologies	Cat# 100602
Experimental models: cell lines		
<i>Spodoptera frugiperda</i> Sf9 cells	Gibco	Cat# 11496015
<i>Spodoptera frugiperda</i> Sf9 easy titration (Sf9-ET) cells	Hopkins and Esposito [75]	N/A
<i>Spodoptera frugiperda</i> ExpiSf9 cells	Gibco	Cat# A35243
<i>Trichoplusia ni</i> High Five cells	Gibco	Cat# B85502
Oligonucleotides		
Primers for <i>Spodoptera frugiperda ecd</i> , F: GCCGTATCCAAAGCATGACA; R: TGGTGACGGCCAAAGGAA	Salem <i>et al.</i> [85]	N/A
Primers AcMNPV <i>vp80</i> , F: GAAACGGCTGTACGAATAC; R: GATCGAGATTGTAC AGGTTTAG	This paper	N/A
Primers AcMNPV <i>gp64</i> , F: CGGCGTGAGTATGATTCTCAAA; R: ATGAGCAGACA CGCAGCTTTT	This paper	N/A
Primers for AcMNPV <i>p6.9</i> , F: GTACCACATATGGTTCGACAC; R: GTTCTGTAAC TCGGCGAC	This paper	N/A
Primers for AcMNPV <i>odv-e18</i> , F: AAAGGATTTACAAAACCCCCC; R: ATGTTC TTGACCATCTTGCG	This paper	N/A
Primers for AcMNPV <i>odv-e56</i> , F: CGCGGCTTAGCATTTTAAC; R: TCACTGAAA CCAACGACAC	This paper	N/A
Primers for <i>eGFP</i> , F: GGCACAAGCTGGAGTACAAC; R: AGTTCACCTTGATGCC GTTC	This paper	N/A

(continued)

Reagent or resource	Source	Identifier
Recombinant DNA		
pFastBac Dual expression vector	Gibco	Cat# 10712024
Chikungunya S27 structural cassette	Metz <i>et al.</i> [27]	N/A
AcMNPV <i>gp41</i> with I317T mutation (codon 317 mutated to ACA), synthetic DNA	This paper	N/A
AcMNPV AcΔgp41 bacmid	Li <i>et al.</i> [47]	N/A
AcMNPV BACe56 bacmid	Pijlman <i>et al.</i> [53]	N/A
AcMNPV BACe56 bacmid with I317T mutation in <i>gp41</i>	De Jong <i>et al.</i> [52]	N/A
West Nile virus prME structural cassette, synthetic DNA	This paper	N/A
Gateway pDEST8 vector	Thermo Fisher Scientific	Cat# 11804010
Coxsackievirus A6 P1 and 3CD, synthetic DNA	This paper	N/A
pFastBac1 vector without polh promoter (pFB1ΔAcPpol)	Pijlman <i>et al.</i> [93]	N/A
Foot-and-mouth disease virus P1-2A and 3C coding sequences, synthetic DNA	This paper	N/A
Software and algorithms		
AlphaFold 3	Abramson <i>et al.</i> [49]	https://alphafoldserver.com
UCSF ChimeraX version 1.8	Pettersen <i>et al.</i> [76]	https://www.cgl.ucsf.edu/chimerax/
SMART	Letunic <i>et al.</i> [79] Letunic <i>et al.</i> [80]	http://smart.embl-heidelberg.de/
DISOPRED3	Jones and Cozzetto [77]	http://bioinf.cs.ucl.ac.uk/disopred
fIDPnn	Hu <i>et al.</i> [78]	https://biomine.cs.vcu.edu/servers/fIDPnn/
FlowJo software v10	BD Biosciences	https://www.flowjo.com/
ImageJ version 1.54f	Schindelin <i>et al.</i> [84]	https://imagej.net/software/fiji/
JalView	Waterhouse <i>et al.</i> [82]	https://www.jalview.org/
Disulfide by Design 2.0	Craig and Dombkowski [90]	http://cptweb.cpt.wayne.edu/DbD2/
GraphPad Prism 9 and 10	GraphPad	https://www.graphpad.com/
Other		
Micro-bioreactor microMatrix cultivation system	Wiegmann <i>et al.</i> [83]	https://www.getinge.com/
Applikon miniBio reactor vessels with Applikon my-Control	Getinge	https://www.getinge.com/nl-be/products/applikon-minibio/
iLineF Double Differential Digital Holographic Microscopy (D3HM)	Ovizio	https://ovizio.com/
BD Accuri C6 Plus Flow Cytometer	BD Biosciences	Cat# 660517
JEM-1400 plus transmission electron microscope	JEOL	www.jeol.com

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Spodoptera frugiperda (Sf9 and ExpiSf9) insect cells were maintained in 250 ml suspension flasks shaking at 110 rpm at 27°C. Sf9 and Tni High Five cells (Gibco) were cultured in Sf900II serum-free medium (Gibco) supplemented with 50 µg/ml gentamicin. ExpiSf9 cells (ThermoFisher Scientific) were grown in ExpiSf chemically defined (CD) medium (Gibco), also supplemented with 50 µg/ml gentamicin. Monolayers of Sf9-Easy Titer cells (Sf9-ET) were grown in Sf900II medium (Gibco) supplemented with 100 µg/ml geneticin (G418, Gibco) and 5% fetal bovine serum (FBS; Gibco) [75].

METHOD DETAILS

Prediction of GP41 structure

The folding structure of the full AcMNPV GP41 protein was constructed by AlphaFold3 [49] with default settings. Structure visualization was performed with UCSF ChimeraX [76] and was colored according to the per-residue confidence score (pLDDT) of

AlphaFold3, or a consensus hydrophobicity scale of ChimeraX [76]. The presence of intrinsically disordered regions at the N-terminus and C-terminus were also confirmed by machine-learning-based predictors DISOPRED3 [77] and flDPnn [78]. A multiple sequence alignment of AcMNPV GP41 with homologs, identified by SMART search [79,80], was constructed with MUSCLE [81] and was visualized in JalView [82].

Construction of Ac-gp41-CHIKV and Ac-gp41^{ts}-CHIKV

pFastBacDual (pFBD, Gibco™) plasmids were constructed with the wild type *gp41* or *gp41^{ts}* (with I317T mutation) sequence downstream the p10 promoter as XhoI/NheI fragment with N-terminal HA tag (MYPYDVPDYA). The chikungunya virus structural cassette (CHIKV) [27] was inserted downstream of the polyhedrin (ph) promoter as BamHI/XbaI fragment. The AcMNPV bacmid with deletion of *gp41* (AcΔ*gp41*) was kindly received from Li and co-authors (2018) [47]. In this bacmid, a large part of the *gp41* open reading frame (including residue 317) was replaced with a chloramphenicol acetyltransferase (*cat*) and enhanced green fluorescent protein (eGFP) gene downstream the heat-shock protein 70 (*hsp70*) promoter. The pFBD plasmids were inserted into the AcΔ*gp41* bacmid by transposition in *Escherichia coli* (Bac-to-Bac baculovirus expression system, ThermoFisher Scientific). Recombinant bacmids were purified by alkaline lysis followed by isopropanol precipitation. Baculovirus stocks were amplified at 27°C in Sf9 cells and viral titers were determined on Sf9-ET cells by end-point dilution assay [75].

Growth curves of Ac-gp41-CHIKV and Ac-gp41^{ts}-CHIKV at 27°C and 33°C

Growth curves were constructed of the wild type and *ts* virus at low and elevated temperatures in both monolayer cultures and suspension cultures. Sf9 cells were diluted in Sf900II supplemented with 5% FBS. The monolayers were seeded in 24-well plates containing 0.25×10^6 cells per well, whereas suspension flasks contained 15 mL of 1.0×10^6 cells/mL. Cells were infected with MOI=1 TCID₅₀/cell and either kept at 27°C or shifted to 33°C at 4 hpi. Wells and flasks were sampled daily and BV titers in the clarified culture fluid were determined by end-point dilution assay.

Micro-bioreactor runs

The infection kinetics at a range of temperatures was screened in a high-throughput micro-bioreactor cultivation system (microMatrix, Getinge) [83]. ExpiSf9 cells were diluted to 1.0×10^6 cells/mL in ExpiSf CDM with 50 µg/mL gentamycin and infected with Ac-gp41-CHIKV or Ac-gp41^{ts}-CHIKV virus at MOI=0.01 or MOI=0.3 TCID₅₀/cell. Cells were incubated at 27°C for 30–45 minutes before aliquoting 5 mL per well of the 24-well micro-bioreactor cassette (Getinge). The reactor was run for 96 hours shaking at 250 rpm, and the pH and DO were not controlled. The incubation temperature was set for each column of wells with a temperature difference between adjacent wells of 1°C and an accuracy of ±0.1°C. Runs were performed with a temperature range of 22–27°C, 29–34°C, or 30–35°C. The CHIKV eVLP production was detected in the clarified culture fluid by western blot immunodetection by staining for the E2 protein (E2dTm, Rabbit serum).

Viral infection experiments with Ac-gp41-CHIKV and Ac-gp41^{ts}-CHIKV in stirred-tank bioreactors

ExpiSf9 cells were grown in 0.5 L miniBio or 3.0 L glass reactor vessels (Getinge) controlled by myControl bioreactor controllers (Getinge). The working volume of each reactor vessel was 0.3–2.0 L. Reactors were inoculated at target starting densities of 0.5 – 1.0×10^6 viable cells/mL. The pH in the culture fluid was not controlled but was maintained by the buffering capacity of the medium and fluctuated between pH 5.7–6.1. The dissolved oxygen concentration was controlled at 30% of air saturation using Lumisens optical dissolved oxygen sensors (Getinge) and sparging pure oxygen through open pipe spargers. A constant headspace aeration rate of 0.01 vvm air was also applied. Agitation with marine impellers was set to 266–600 rpm by keeping the tip speed constant among the different reactor sizes. Online average cell diameter was measured every 0.5 h by a D3HM digital microscope (OVIZIO [50]). The fraction of GFP-positive cells was measured in three technical replicates using a BD Accuri C6 Plus Flow Cytometer (BD Biosciences) at an emission of 533/30 nm. Data was analyzed by FlowJo Software v10 (BD Biosciences). Healthy, non-infected ExpiSf9 cells were measured for gating single cells and autofluorescence.

Construction of Ac^{ts}-eGFP and phenotypic characterization

The I317T mutation (codon ATA to ACA) [46] was introduced in the *gp41* gene of the AcMNPV BACe56 bacmid [53] by scarless, two-step homologous recombineering in *E. coli* as described elsewhere [52]. The integrity of the modified Ac^{ts} bacmid and the presence of the I317T mutation were confirmed by Illumina sequencing (Macrogen). Bac-to-bac transposition was performed with pDEST8-eGFP to introduce the eGFP gene in the *odv-e56* locus (Ac^{ts}-eGFP). The ‘wild type’ control virus Ac-eGFP was constructed

similarly by Bac-to-Bac transposition into the non-mutated BACe56 bacmid. Virus stocks were amplified and titrated on Sf9 cells at 27°C. The non-permissive temperature of Ac^{ts}-eGFP was determined by low MOI infections (0.01 TCID₅₀/cell). The plates were incubated at 27°C for 3 hours, then the inoculum was removed and replaced with fresh medium and the temperature was switched to 32°C±0.1°C, 33°C±0.1°C, or 34°C±0.1°C. A control plate was kept at 27°C. The viral spread was visualized at 4 days post-infection (dpi) by fluorescence microscopy. The non-permissive temperature was identified as the lowest temperature at which a single-cell infection phenotype could be observed. The experiment was repeated to confirm the observed phenotype. Production of eGFP was visualized by high MOI infections (10 TCID₅₀/cell) at 27°C. At 3 hpi, the inoculum was removed and the cell monolayer was washed three times with pre-warmed medium. The cells were then further incubated at 27°C or 33°C±0.1°C. At 4 dpi, culture fluid samples were collected, and the infectious BV titer was determined by end-point dilution assay at 27°C. eGFP fluorescence intensity was quantified with ImageJ software [84] and was normalized by dividing to the average intensity of Ac-GFP at 27°C. The stability of the baculovirus and I317T mutation was assessed by serial passaging [37,53,54]. The initial infection was carried out with Ac^{ts}-eGFP P1 virus stock at MOI of 10 TCID₅₀/cell. The inoculum was replaced by fresh culture medium at three hours post-infection (passage 1). At 3 dpi, 1 ml culture fluid was passaged to a new flask of cells (passage 2). This was repeated for a total of 8 passages at 27°C and was performed in three parallel experiments (replicates A, B, C).

qPCR of intracellular baculovirus DNA and RNA

Viral genome copies inside the infected cells was determined by quantitative PCR (qPCR). Sf9 cells were infected with an MOI=10 TCID₅₀/cell. After a 3-hour incubation at 27°C, the inoculum was removed, and 1 mL of fresh cell culture medium was added to the cells. The cells were further incubated at 27°C or 34°C. At 24 hours post-infection (hpi), the cells were harvested and DNA was purified from the cells with the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's instructions, including an RNase treatment. Quantitative Real-Time PCR was performed using the SYBRTM Select Master Mix (Thermo Fisher Scientific) with primers targeting AcMNPV *gp64* or *vp80*. Melt curve analyses were conducted to confirm specific amplification. The average Cq values of triplicate qPCR wells were determined, and the experiments were conducted as three biological replicates. Primers targeting the Sf9 ecdysoneless (*ECD*) gene were used for data normalization (ΔCq) [85] and data analysis was further carried out using the $2^{-\Delta\Delta Cq}$ method. Similarly, relative transcript levels of various baculovirus genes were determined by reverse transcriptase followed by qPCR (RT-qPCR). Infected cells were harvested at 48 hpi and total RNA was extracted with TRIzol reagent (Invitrogen). Residual DNA contamination was removed by RQ1 RNase-free DNase (Promega). Subsequent cDNA synthesis was performed using SuperScript II Reverse Transcriptase (Invitrogen) utilizing 1 µg of DNA-free total RNA template and random hexamers (Invitrogen) according to the manufacturer's instructions. A control reaction without SuperScript II was included as non-RT control to assess residual DNA contamination in qPCR. qPCR was performed as described above, with 1 µl cDNA as template, with primers targeting eGFP, or AcMNPV *p6.9*, *gp64*, *odv-e18*, or *odv-ec27*.

Viral infection experiments with Ac^{ts}-eGFP at elevated temperatures in stirred-tank bioreactors

The high MOI infection was scaled-up to shake flasks and a stirred-tank bioreactor. ExpiSf9 cells were grown in 0.5 l miniBio reactor vessels (Gethinge) with a 300 ml working volume, controlled by myControl bioreactor controllers (Gethinge). The reactor was inoculated at a starting density of 1.0x10⁶ viable cells/ml and was infected at cell concentration at time of infection (CCI) of 2.0x10⁶ cells/ml with Ac^{ts}-eGFP at an MOI of 10 TCID₅₀/cell. The pH in the culture fluid was not controlled but was maintained by the buffering capacity of the medium. The dissolved oxygen concentration was controlled at 30% of air saturation using Lumisens optical dissolved oxygen sensors (Gethinge) and sparging of pure oxygen through open pipe spargers. A constant headspace aeration rate of 0.01 vvm air was also applied. Agitation with marine impellers was set to 300 rpm. At 3 hpi, the temperature was elevated from 27°C to 33°C. The temperature of the working volume was maintained at setpoint by a heating block (miniBIO). In parallel, shake flasks were inoculated and infected at the same CCI and MOI and were either incubated at 27°C, at 33°C, or followed the same temperature switch regime as the bioreactor (from 27°C to 33°C at 3 hpi). Samples were collected at 0 dpi (5-10 minutes after addition of the virus), and at 1-5 dpi. The concentration of eGFP in the samples was determined by fluorescence measurements relative to that of an eGFP standard. For this procedure, cell suspensions were incubated for 10 minutes on ice in 4X lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 0.1% Triton X-100, pH8.0, supplemented with 1 mM PMSF protease inhibitors) and centrifuged for 10 minutes at 13,000 x g. The cell lysates were further diluted 10 to 100 times with lysis buffer according to their expected concentration, to ensure an appropriate fit within the calibration curve. The standard curve was prepared from recombinant eGFP (Chromotek) diluted to 0-20 ng/µL. Fluorescence was measured in technical triplicate using an FLx800 micro-plate reader (BioTek) with a 485/528 excitation/emission filter.

Production of stabilized West Nile virus-like particles with BacFree^{ts}

West Nile virus (WNV) subviral particles, eVLPs lacking the capsid, were designed to contain a stable E-dimer (see below). The structural cassette of WNV prME was based on the Dutch isolate (MW228499) and comprised the prM and E genes [86]. A peptide consisting of the final 18 residues of the WNV capsid (GGTAGFTILLGLIACAGA) preceded by the residues 'MAA' was N-terminally fused to prME and served as a signal sequence to direct the polyprotein to the ER membrane [87,88]. The lobster tropomyosin (L21) cDNA leader sequence was placed at the 5' end for efficient translation initiation [89]. This prME cassette was flanked by Gateway attB sites to enable Gateway cloning (Invitrogen) and was ordered as a synthetic DNA construct (Integrated DNA Technologies). An envelope-stabilizing cysteine mutation A261C was introduced in the E gene by site-directed mutagenesis PCR, predicted using sequence alignment to dengue virus (DENV) and Zika virus (ZIKV) envelope sequences [56] (see Figure S9A in the supplemental information online). Additional stabilizing mutations were predicted by Disulfide by Design 2 webserver [90]. The mutated prME cassette was subsequently cloned into pDest8 (Invitrogen) by Gateway LR clonase (Invitrogen) and recombinant baculoviruses were constructed by Bac-to-Bac transposition into Ac^{ts} bacmid. Infection experiments at high MOI were performed described above for Ac^{ts}-eGFP. The WNV eVLPs were detected in equal volumes of cell pellets (5 µl per well) and equal volumes of acetone-precipitated culture fluid (10 µl per well, ten times concentrated) by western blot immunodetection with pan-flavivirus anti-E monoclonal antibody 4G2 (E domain II fusion loop, 1:100, from mouse) [91]. Densitometry analyses was performed in ImageJ, and the band intensity (area) was normalized against the average area at 27°C.

Purification and transmission electron microscopy of enveloped VLPs

CHIKV and WNV VLPs were purified from the culture fluid by ultracentrifugation [27,92]. Secreted proteins were precipitated with 7% (w/v) polyethylene glycol (PEG)-6000 and 0.5 M NaCl for 2 hours at room temperature. Pellets were resuspended in GTNE buffer (200 mM glycine, 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.3). VLPs were purified on 40-70% (w/v) sucrose gradient (CHIKV VLPs) or a 30-80% (w/v) sucrose gradient (WNV VLPs) as described previously [27,92]. Samples were blotted on an electron microscopy grid (Electron Microscopy Sciences, carbon-supported, Cu-200), negatively stained in 2% uranyl acetate, and visualized using the JEM-1400 plus transmission electron microscope (JEOL).

Production of coxsackievirus A6 non-enveloped virus-like particles with BacFree^{ts}

Production of human enterovirus VLPs was achieved through the co-expression of coxsackievirus A6 (CVA6) P1 (polh promoter) and 3CD (CMV early promoter plus enhancer) [59]. This construct, P1-C3CD, was cloned as XbaI/NotI fragment into the multiple cloning site of a derivative of pFastBac1 (Thermo Fisher) that lacked the polh promoter (pFB1ΔAcPpol, deletion from Bst1107I to StuI) [93]. Recombinant Ac^{ts} baculoviruses were constructed as described above. *Trichoplusia ni* High Five cells (CCI=0.5×10⁶ cells/ml, 150 ml per flask) were infected with Ac^{ts}-CVA6 at MOI=5 TCID₅₀/cell in 1 L Erlenmeyer culture flasks at 28°C. At 2.5 hpi, one flask was shifted to 33°C while the control flask remained at 28°C. The flasks were sampled at t=0 dpi (2.5 hpi) and at t=7 dpi. The CVA6 VLPs were detected in the clarified culture fluid by western blot immunodetection and quantified by ELISA. SDS-PAGE was performed with 20 µl culture fluid per well. The western blot membrane was stained with polyclonal anti-VP1 antibody (Thermo Fisher PA5-112001, 1:1000, from rabbit). ELISA was performed for relative quantification of the assembled CVA6 particles in the culture fluid samples. High-binding ELISA plates (Greiner Bio-One) were coated with 100 µl sample in duplicate wells overnight. ELISA was performed with either primary antibody 1D5 / PABC-483 (Creative Biolabs, 1:1000), targeting the CVA6 A-particle. The secondary antibody goat-anti-mouse IgG-HPR (Southern Biotech, 1030-05) was diluted 1:5000 and detection was performed with Ultra TMB-ELISA substrate (Thermo Fisher Scientific). The relative expression levels were calculated by normalization of the ELISA values.

Purification and transmission electron microscopy of capsid VLPs

CVA6 and FMDV VLPs were purified from the culture fluid by ultracentrifugation before imaging by transmission electron microscopy [61,62]. The clarified culture fluid was supplemented with 0.1% Triton-X and first filtered through 0.45 µm pore size and then through a 0.22 µm filter (Millex). The filtered culture fluid was then pelleted through a 30% (w/w) sucrose cushion in 1X PBS (Gibco) for 5 hours at 141,000 × g. The pellet was resuspended in 1 mL 1X PBS and was centrifuged for 10 min at 10,000 × g. The resulting supernatant was loaded onto a discontinuous 15-45% (w/w) sucrose gradient that was ultracentrifuged for 3 hours at 141,000 × g. The gradient was collected in eight fractions. The fraction containing the VLPs was blotted on an electron microscopy grid (Quantifoil, carbon-supported, Cu-400), negatively stained in 2% uranyl acetate, and visualized using the JEM-1400 plus transmission electron microscope (JEOL).

Production of foot-and-mouth disease virus VLPs with BacFree^{ts}

The foot-and-mouth disease virus strain O1/Manisa/TUR/69 P1-2A and 3C coding sequence, flanked by Gateway attB sites, was ordered as a synthetic gene cloned into pUC57 (Genscript). The construct was designed based on research by Porta and co-authors (2013) [63] where an HIV-1 ribosomal frameshift site was introduced between the P1-2A and 3C sequence to downregulate 3C levels for improved P1 processing [63]. The attB sites allowed for re-cloning of the cassette from pUC57 into pDONR207 and subsequently into pDest8 by Gateway clonases (Invitrogen), placing the cassette behind the polh promoter. Recombinant Ac^{ts}-FMDV baculovirus stocks were constructed as described for Ac^{ts}-eGFP. Infections with Ac^{ts}-FMDV at elevated temperatures were performed in Sf9 suspension cells at CCI=1.0x10⁶ cells/ml and MOI=10 TCID₅₀/cell in shake flasks. Infected cells were incubated at either 27°C or 33°C±0.1°C and were sampled at 4 dpi. Baculovirus DNA copies in the culture fluid were quantified by qPCR according to the BaculoQUANT virus extraction and titration kit (Oxford Expression Technologies) with in-house primers targeting *vp80*. A ¹⁰log dilution series of a plasmid containing the viral *vp80* ORF (pJET-*vp80*) served as a calibration curve for calculating viral genome copy numbers. Equal volumes of culture fluid (15 µl) were analyzed by SDS-PAGE and immunodetection with antibody staining FMDV VP2 (1:400, from mouse) and AcMNPV VP39 (1:1500, from mouse). The FMDV VLPs were also quantified by ELISA with the antibody targeting VP2.

QUANTIFICATION AND STATISTICAL ANALYSES

Standard data processing and statistical analyses were performed with GraphPad Prism 9 or GraphPad Prism 10 software. Data is presented as mean ± Standard Deviation (SD), unless indicated otherwise in the figure legend. Various statistical tests were employed to calculate P values, this is specified in the STAR methods and figure legends. Significance was defined as follows: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.