INTRODUCTION

Baculoviruses are a family of enveloped dsDNA viruses that infect insects of the orders Lepidoptera, Hymenoptera and Diptera (Rohrmann, 2013). Based on their whole-genome sequences, the family Baculoviridae can be divided into four genera: Alphabaculovirus [lepidopteran nucleopolyhedroviruses (NPVs)], Betabaculovirus [lepidopteran granuloviruses (GVs)], Gammabaculovirus (hymenopteran NPVs) and Deltabaculovirus (dipteran NPVs). The members of the genus Alphabaculovirus [type species Autographa californica multiple nucleopolyhedrovirus (AcMNPV)] can be further subdivided into Group I and Group II (Jehle et al., 2006). During the baculovirus infection cycle, two phenotypically distinct but genotypically identical progeny virions, budded virions (BVs) and occlusion-derived virions (ODVs), are produced. BVs are responsible for transmitting infection between susceptible insect tissues, whereas ODVs mediate horizontal virus transmission between insects.

Baculoviruses replicate in the nuclei of infected cells; therefore, their genomes must enter the host nucleus. Based on transmission electron microscopy observations, previous studies inferred that betabaculoviruses eject viral DNA through the nuclear pore complex (NPC), leaving an intact empty capsid on the cytoplasmic side of the NPC (Summers, 1971). In contrast, alphabaculoviruses translocate intact DNA-containing nucleocapsids into the nucleus through the NPC, after which the nucleocapsid is uncoated and viral DNA is released (Au & Pant, 2012; Ohkawa et al., 2010). Although nuclear import is a vital event in the alphabaculovirus life cycle, the viral and cellular proteins involved in this process remain largely unknown.

AcMNPV possesses a genome of approximately 134 kbp that encodes 154 putative ORFs (Ayres et al., 1994; Maghodia et al., 2014). The genomic sequence of the ac132 gene predicts a gene product of 219 aa with a putative molecular mass of 25.1 kDa (Ayres et al., 1994). Orthologs of Ac132 are found in all sequenced Group I alphabaculovirus genomes (Rohrmann, 2013). Proteomic studies have shown that Ac132 is a component of both BV and ODV of AcMNPV (Braunagel et al., 2003; Wang et al., 2010). A more recent study showed that Ac132 is a nucleocapsid-associated protein and that the deletion of ac132 seriously impacts the production of infectious BVs (Yang et al., 2014). Similarly, the deletion of Bm109, the ac132 ortholog in Bombyx mori NPV (BmNPV), also led to a defect in the production of infectious BVs (Ono et al., 2012). However, the exact role of Ac132 in viral replication remains obscure.

The *Autographa californica* multiple nucleopolyhedrovirus Ac132 plays a role in nuclear entry

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A recent study found that the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) Ac132 is a nucleocapsid-associated protein and required for budded virion (BV) production. We therefore initiated experiments aimed at understanding how Ac132 is involved in AcMNPV infection. An 80 bp region of ac132 was replaced with a chloramphenicol resistance gene to construct vAc132KO. Transfection of vAc132KO into Sf9 cells resulted in a single-cell infection phenotype, consistent with findings reported in a previous study. Interestingly, BVs were observable in the supernatants, and the BV production in the supernatant was comparable with that present in supernatants from a WT control. These results suggest that the ac132 deletion does not affect the egress of nucleocapsids from the transfected cells to form BVs, but the BVs are non-infectious. Transfection with DNA extracted from vAc132KO BVs could establish infection in Sf9 cells, indicating that the deletion does not affect the integrity of the viral genomic DNA in non-infectious progeny BVs. To monitor the traffic of nucleocapsids without Ac132, two mCherry proteins were fused with the major capsid protein VP39 to construct vAc132KO : 2mC. Using confocal microscopy, we observed that the nucleocapsids of vAc132KO : 2mC could not enter the nucleus and instead remained docked at the nuclear membrane. This study provides a new understanding of the nuclear entry of baculoviruses.
In this study, an ac132-knockout virus (vAc132KO) was generated to investigate the role of ac132 in the AcMNPV life cycle. As expected, the deletion of ac132 results in a defect in cell-to-cell virus infection, which is consistent with findings reported in previous studies. Subsequently, we found that the major nucleocapsid protein VP39 was detected in supernatants from vAc132KO-transfected cells, and the amount of this protein present in the cell supernatants was similar to the amount present in supernatants from WT controls. Negative staining and electron microscopy confirmed the presence of BVs. Finally, we demonstrated that the nucleocapsids of vAc132KO:2mC are unable to enter the cell nucleus.

RESULTS

ac132 deletion leads to non-infectivity of AcMNPV BVs

The ac132 ORF is 660 bp in length. To generate the ac132-knockout bacmid bAc132KO, an 80 bp region of ac132 was replaced with a 1038 bp chloramphenicol resistance gene (Cm) via ET homologous recombination as previously described (Wu et al., 2006). To avoid affecting neighbouring genes, 120 bp from the 5’ end and 460 bp from the 3’ end of ac132 were retained in the present study (Fig. 1). The knockout region of ac132 was different from that reported by Yang et al. (2014) in which a 310 bp N-terminal fragment (Ac132 nt 1–310) was deleted from ac132. To determine whether the deletion of ac132 had any effect on occlusion body (OB) morphogenesis and to facilitate the examination of viral infection, the polyhedron gene (polh) of AcMNPV and the enhanced green fluorescence protein gene (egfp, referred to as gfp in this study) were inserted into the polh locus of bAc132KO via Tn7-mediated transposition as described previously (Wu et al., 2006). Thus, an ac132-knockout AcMNPV, which we designated vAc132KO, was constructed. To confirm the phenotype resulting from the deletion of ac132, a rescue virus was generated by inserting a haemagglutinin (HA) epitope-tagged ac132 ORF under the control of its native promoter together with the polh and gfp genes into the polh locus of bAc132KO, and the rescue virus was designated vAc132:HA. To generate a WT control virus, the polh and gfp genes were inserted into the polh locus of bMON14272 (Luckow et al., 1993); the resulting virus was designated vAcWT (Fig. 1).

Sf9 cells were transfected with the recombinant viral DNAs and were monitored by fluorescence microscopy. At 24 h post-transfection (p.t.), no obvious differences in the number of fluorescent cells were observed, indicating that the transfection efficiencies of the three viruses were comparable (Fig. 2a). At 48 h p.t., a significant increase in the number of fluorescent cells was detected in the vAcWT- and vAc132:HA-transfected cells. In contrast, the number of fluorescent cells in the vAc132KO-transfected cell population did not increase (Fig. 2a). At 72 h p.t., fluorescence was observed in almost all of the cells transfected with vAcWT or vAc132:HA; however, the fluorescence intensity of the cells transfected with vAc132KO remained unchanged (Fig. 2a). These results suggest that the ac132 deletion affects the generation of infectious BVs (Fig. 2a). Light microscopy showed that OBs were present in the transfected cells at 96 h p.t. (Fig. 2a), indicating that the ac132 deletion does not affect the progression of infection to the very late phase. Titre assays showed that the kinetics of

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Fig. 1. Schematic diagram of the recombinant viruses used in this study. The 80 bp fragment of the ac132 ORF was replaced with a 1038 bp Cm cassette via ET homologous recombination to generate the ac132-knockout bacmid bAc132KO. The ac132-knockout virus (vAc132KO) was generated by inserting the polh and gfp genes into the polh locus of bAc132KO via Tn7-mediated transposition. Similarly, the ac132-repaired virus (vAc132:HA) was generated by inserting the gene ac132 with HA, polh and gfp genes into the polh locus of bAc132KO; the WT control virus (vAcWT) was generated by inserting the polh and gfp genes into the polh locus of bMON14272.
the infectious BV production of vAc132 : HA and vAcWT were similar; however, no titre of vAc132KO BV could be detected at any time point up to 96 h p.t. (Fig. 2b). Taken together, these results suggest that the ac132 deletion affects the generation of infectious BVs, which is consistent with those of a recent study (Yang et al., 2014).

To investigate if there were any BV particles produced by vAc132KO, Western blotting was subsequently conducted to detect the presence of the major nucleocapsid protein VP39 and the BV envelope-specific protein GP64 in culture supernatants from vAc132KO- or vAcWT-transfected Sf9 cells. As shown in Fig. 2(c), VP39 and GP64 were clearly detected in the culture supernatants of cells transfected with either of these viruses at 24 h p.t., and the protein levels were comparable. Using negative staining, we found that BVs were produced in the vAc132KO-transfected cells, and there were no discernible differences in BV morphology between vAc132KO and vAcWT (Fig. 2d). Thus, although the deletion of ac132 does not affect BV production, the BVs produced by vAc132KO are non-infectious.

To further investigate whether ac132 deletion affects the integrity of the viral genomic DNA, viral genomic DNA
extracted from vAc132KO BVs was transfected into Sf9 cells. As shown in Fig. 2(e), fluorescence was detected in the transfected cells at 24 h.p.t. (Fig. 2e), indicating that the deletion of ac132 does not affect the integrity of the viral genomic DNA in non-infectious progeny BVs. However, the number of fluorescent cells did not increase at 72 h.p.t. (Fig. 2e), indicating that vAc132KO failed to establish a secondary infection. By contrast, infection by the WT control virus proceeded in a typical fashion.

**Ac132 is a component of the nucleocapsid and the ODV envelope**

BVs and ODVs were purified from vAc132:HA-infected *Trichoplusia ni* larvae. The presence of Ac132 in virions was detected by Western blotting with a monoclonal anti-HA antibody. As shown in Fig. 3(a), Ac132 was detected in both BVs and ODVs, consistent with the results of previous proteomic studies (Braunagel et al., 2003; Wang et al., 2010). The virions were further fractionated into envelope and nucleocapsid fractions, and Ac132 was found to be associated with the nucleocapsids of both BVs and ODVs, and with the ODV envelope but not with the BV envelope (Fig. 3a). The effectiveness of the fractionation was confirmed by the detection of the major nucleocapsid protein VP39, the BV envelope-specific protein GP64 and the BV/ODV envelope-associated protein ODV-E25 (Fig. 3a).

Our finding that Ac132 is present in the envelope fraction of ODVs is inconsistent with results reported by Yang et al. (2014) in which Ac132 was not detected in the ODV envelope fractions. The sensitivity of the two antibodies may account for the difference. To further confirm our results, immunoelectron microscopy was performed with thin sections generated from vAc132:HA-infected cells. As shown in Fig. 3(b), immunogold-labelled Ac132 was specifically localized to the nucleocapsids within the electron-dense edges of the virogenic stroma (VS) (Fig. 3b, i), the virus-induced intranuclear microvesicles (Fig. 3b, ii), and the nucleocapsid and the envelope of ODV in the ring zone (Fig. 3b, iii). Immunogold-labelled Ac132 was not detected in the nuclear membrane. Very few gold particles were detected in control vAcWT-infected cells (data not shown). Taken together, the results from the Western blotting and the immunoelectron microscopy presented above suggest that Ac132 is a component of the nucleocapsid and the ODV envelope.

**ac132 is required for nucleocapsid entry into the nucleus**

To investigate the effects of the deletion of ac132 on AcMNPV infection, two recombinant viruses, vAc132KO:2mC and vAcWT:2mC, were generated. In these recombinants, two mCherry proteins were fused with the major capsid protein VP39 to make it possible to monitor the traffic of nucleocapsids without or with Ac132, respectively, into the nucleus. BVs were harvested from Sf9 cells transfected with vAc132KO:2mC or vAcWT:2mC and used to inoculate fresh Sf9 cells. The infected cells were fixed and observed by confocal microscopy. Forty vAc132KO:2mC-infected cells and 27 vAcWT:2mC-infected cells were examined. In the cells infected with vAc132KO:2mC, red signals, indicative of nucleocapsids, were detected on the plasma membrane, in the cytoplasm and on the edges of nuclei but not within the nuclei (Fig. 4a). In contrast, in the cells infected with vAcWT:2mC, nucleocapsids were observed in the cytoplasm and in the nucleus, as expected (Fig. 4a), and nucleocapsids were observable in the nucleus in 57.14±12.37 % of the 27 cells examined (Fig. 4b). These results suggest that the deletion of ac132 does not affect the entry of BVs into Sf9 cells but does impair the capacity of nucleocapsids to pass through the nuclear envelope.

**Mutation of the SDXXYK motif affects infectious BV production**

A SMART search showed that the 103–134 aa region of Ac132 is homologous to an NEBU domain that is known to bind actin (Pfuhl et al., 1996). The NEBU domain is also present...
Ac132 was carried out. The four conserved amino acids in the motif \(124\text{SDXXYK}\) of Ac132 were altered to alanine by using site-directed, ligase-independent mutagenesis. By using the same strategy for the construction of vAc132 : HA, a mutant, named vAc132 : \(124\text{SDXXYK}\), was generated. At 24 h.p.t., fluorescence was detected in vAcWT- and vAc132 : \(124\text{XXA}\) -transfected Sf9 cells, and equal numbers of cells were fluorescent after transfection by either virus, indicating relatively similar efficiencies of transfection (Fig. 5b). At 72 h.p.t., the number of fluorescent cells increased slightly in the vAc132 : \(124\text{XXA}\) -transfected cells, whereas fluorescence spread throughout most of the vAcWT-transfected cells (Fig. 5b), and the titre of vAc132 : \(124\text{XXA}\) was approximately 1000-fold lower than that of vAcWT (Fig. 5c). These results indicate that the mutation of the SDXXYK motif significantly affects the ability of AcMNPV to infect cells.

**DISCUSSION**

*ac132* is one of the 12 genes that are conserved in and unique to all Group I alphabaculovirus genomes sequenced to date. The conservation of the *ac132* gene suggests that it may play a crucial role in virus replication. Specifically, *ac132* was recently shown to be an essential gene, and its product was characterized as a component of the viral nucleocapsid (Yang et al., 2014). However, what role *ac132* plays in viral replication remains unknown. In the present study, a recombinant AcMNPV, vAc132KO, was constructed, in which a small region of *ac132* was replaced with a Cm gene. Consistent with a previous report by Yang et al. (2014), the *ac132* deletion led to a defect in the transmission of viral infection (Fig. 2a) but the deletion had no effect on viral morphogenesis (data not shown). Whether the phenotypic differences between the current study and the previous report by Yang et al. (2014) were due to the slight difference of Ac132 knockout region remains to be determined. Further investigation by our group showed that BVs were present in the culture supernatant of vAc132KO-infected cells; however, the BV progeny of the mutant virus were non-infectious (Fig. 2c, d).

Previous studies showed that Ac132 is associated with both BVs and ODVs (Braunagel et al., 2003; Wang et al., 2010; Yang et al., 2014). In the present study, Ac132 was further confirmed to be a component of the nucleocapsid by Western blotting (Fig. 3a). Mutations of viral structural proteins generally lead to impairment in the packaging, assembly or structural integrity of the nucleocapsid; to alterations in the transport or egress of nucleocapsids within the cell; or to defects in intranuclear microvesicle formation, ODV envelopment or the subsequent embedding of virions into OBs. Interestingly, in contrast to the deletions of the other nucleocapsid proteins, deletion of *ac132* did not affect viral morphogenesis. This finding implies that Ac132 is a nucleocapsid protein that is essential to viral infectivity rather than to virion assembly. Western blotting results showed that Ac132 is a
component not only of the nucleocapsid but also of the ODV envelope. Localization of Ac132 to the nucleocapsid and the ODV envelope was further demonstrated using immunoelectron microscopy. In AcMNPV, VP80 is in a complex along with actin that is involved in the polar transport of nucleocapsids out of their assembly sites to the periphery of the nucleus (Marek et al., 2011). Like Ac132, VP80 was also found to be located in the envelope fraction of ODV (Hou et al., 2013). The biological significance of such proteins that can be a nucleocapsid component and an ODV envelope component remains unknown.

BVs in the supernatant of vAc132KO-transfected cells were non-infectious but had a normal appearance. It would be of interest to determine why the vAc132KO virus is non-infectious. The presence of GP64 in the vAc132KO BVs strongly suggests that the deletion of ac132 did not affect the binding of vAc132KO BVs to host cells or the release of nucleocapsids into the cytoplasm. Previous findings have shown that AcMNPV nucleocapsids enter the nucleus through nuclear pores (Ohkawa et al., 2010) and that actin cables are crucial for the efficient translocation of the nucleocapsids to the nucleus (Lanier & Volkman, 1998). In the present study, virions could be observed in the cytoplasm and on the edge of the nucleus, but not within the nucleus, after ac132 deletion (Fig. 4). Thus, Ac132 may be required for the transport of the nucleocapsid into the nucleus through nuclear pores. Previous work has shown that ac109-knockout viruses show a phenotype similar to that of the ac132 deletion mutant used in this work (Alfonso et al., 2012; Fang et al., 2009; Lehiy et al., 2013). It would therefore be interesting to determine whether Ac132 interacts with Ac109 and to elucidate the precise role of each of these viral proteins in the transport of nucleocapsids through the nuclear pores.

Nebulin is a 600–800 kDa protein found in the thin filaments of vertebrate striated muscle; up to 97 % of the nebulin sequence consists of repeats of a 35 aa motif referred to as the NEBU domain. The tandem arrays produced by this motif are presumed to play a role in binding and stabilizing F-actin (Pfuhl et al., 1996). Actin-based motility plays a role in the transit of baculovirus nucleocapsids to the nucleus (Ohkawa et al., 2010). While visualizing movements in the vicinity of the nucleus, Ohkawa et al. (2010) noticed that nucleocapsids frequently collided with and stuck to the nuclear periphery. Strikingly, these viruses continued to polymerize actin, which radiated from the nucleus and underwent corkscrew-like motions (Ohkawa et al., 2010). In the present study, a potential NEBU domain was found in Ac132 in which the mutation of the hallmark motif SDXXYK resulted in a significant reduction of BV production. It is therefore intriguing to speculate that Ac132 may play a role in binding and stabilizing F-actin in the infected cell. After nucleocapsids are released into the cytoplasm during infection, the naked nucleocapsids are quickly transported to the nuclear periphery and dock at the cytoplasmic filaments of nuclear pore complexes prior to entering the nucleus through the nuclear pores. We speculate that the NEBU domain of Ac132 may stabilize F-actin, which might attach to nucleocapsids and then push the nucleocapsids into the nucleus.

In summary, the results of this study show that Ac132 plays a role in the entry of viral nucleocapsids into the nucleus. This finding broadens our understanding of the baculovirus infection pathway and provides a better understanding of the transport of nucleocapsids through the nuclear pore complex. In the biomedical field, baculoviruses are well known as potent protein expression vectors and gene therapy vectors (Cox, 2012; Rivera-Gonzalez et al., 2011). Understanding the mechanism of the nuclear import of...
baculoviruses will facilitate the use of baculoviruses as efficient vectors.

**METHODS**

**Cell lines and viruses.** The Sf9 cell strain, which was derived from *Spodoptera frugiperda* (Vaughn et al., 1977), was cultured at 27°C in TNM-FH medium (Invitrogen) supplemented with 10% FBS, 100 µg penicillin ml⁻¹ and 30 µg streptomycin ml⁻¹. The bacmid bMON14272 (Invitrogen), containing an AcMNPV genome, was propagated in the *Escherichia coli* strain DH10B as previously described (Lluckow et al., 1993). BV titres were determined with a 50 % TCID₅₀ endpoint dilution assay in Sf9 cells (O’Reilly et al., 1992).

**Construction of an ac132 knockout bacmid.** An ac132 knockout AcMNPV bacmid was generated via ET homologous recombination as previously described (Wu et al., 2006). Briefly, a transfer vector in which an ac132 locus region was replaced with a Cm gene for antibiotic selection in *Escherichia coli* was constructed as follows. Using primers ac132SU1 [5'-GGAGCTCAAGCCATCCGGTAAACATTTCATTCG-3' (the SacI site is underlined)] and ac132SU2 [5'-GGATCCAGGCGATGGC-3' (the BamHI site is underlined)], a 508 bp fragment homologous to the 5' region of the ac132 ORF (AcMNPV nt 117 368–117 468) was PCR-amplified from bMON14272. Similarly, a 490 bp fragment homologous to the 3' region of the ac132 ORF (AcMNPV nt 117 549–118 038) was PCR-amplified using primers ac132DS1 [5'-CTCGAGATAGATGGAAATGAGCAGTTGTA-3' (the PstI site is underlined)] and ac132DS2 [5'-AAGCTTCATGAGTTCGTTAGAGAATGCAGTTGTAGTA-3' (the HindIII site is underlined)]

**Construction of an ac132 knockout virus.** To facilitate the examination of viral infection and to determine whether the mutant virus exhibited any structural abnormalities, the BVs of vAc132KO were purified and negatively stained as previously described (Wang et al., 2011). Western blotting was performed using one of the following primary antibodies: mouse monoclonal anti-AcMNPV ODV-E25 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2011). Western blotting was performed using one of the following primary antibodies: mouse monoclonal anti-AcMNPV ODV-E25 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2011). Western blotting was performed using one of the following primary antibodies: mouse monoclonal anti-AcMNPV ODV-E25 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2011). Western blotting was performed using one of the following primary antibodies: mouse monoclonal anti-AcMNPV ODV-E25 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2011). Western blotting was performed using one of the following primary antibodies: mouse monoclonal anti-AcMNPV ODV-E25 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2011). Western blotting was performed using one of the following primary antibodies: mouse monoclonal anti-AcMNPV ODV-E25 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2011). Western blotting was performed using one of the following primary antibodies: mouse monoclonal anti-AcMNPV ODV-E25 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2011). Western blotting was performed using one of the following primary antibodies: mouse monoclonal anti-AcMNPV ODV-E25 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyclonal anti-AcMNPV VP39 antibody (1:2000) (Wang et al., 2010) or polyc Check for any errors or omissions in the transcription process.
is underlined]). The three DNA fragments were digested with the corresponding enzymes and successively cloned into pFastBac1 (Invitrogen) to generate the donor plasmid pFB1-VP39-2mCherry. pFB1-VP39-2mCherry was site-specifically transposed into bAc132KO and hM014272 as described above; the resulting viruses were designated vAc132KO:2mC and vAcWT:2mC, respectively.

**S9** cells (3.0×10⁶) were chilled at 4 °C for 30 min and then infected with 1 ml of culture supernatant containing vAc132KO:2mC BVs or vAcWT:2mC at an m.o.i. of 0.1 TCID₅₀ per cell. After incubation of the cells for 90 min at 4 °C, the inoculum was removed and the cells were washed twice with chilled TNM-FH medium and shifted to 27 °C in TNM-FH medium supplemented with FBS, penicillin and streptomycin. After incubation at 27 °C for 90 min, the cells were fixed with 4% paraformaldehyde and examined with a Zeiss 7 DUO NLO confocal microscope using a ×63 oil immersion objective.

**Construction of the Ac132 S¹²⁴D¹²⁵XXXY¹²⁸K¹²⁹ to A¹²⁴/¹²⁵XXXA¹²⁸/¹²⁹ point mutation virus.** To identify Ac132 homologues, the Ac132 amino acid sequence was searched against non-redundant protein sequences in the National Center for Biotechnology Information (NCBI) database using the position-specific iterated (PSI) BLAST algorithm. Multiple sequence alignments were performed using CLUSTAL X (Larkin et al., 2007) and edited using GeneDoc (Nicholas et al., 1997). The presence of an NEBU domain was predicted by a SMART search (Letunic et al., 2009).

To investigate the role of the NEBU domain in Ac132, the conserved S¹²⁴D¹²⁵XXXK¹²⁹ motif was altered to A¹²⁴/¹²⁵XXXA¹²⁸/¹²⁹ by using site-directed, ligase-independent mutagenesis as described previously (Chiu et al., 2004). The primers ac132-SDXXY-Ft (5'-TGCTGCAAG TATGCTGCTGATTCGACATTCTAGT-3'), ac132-SDXXY-Es (5'-TCTGATTCTGGCACATTCTAGT-3'), ac132-SDXXY-Rt (5'-GCACATATTGGCAGCCAACTTGATC-3') and ac132-SDXXY-Rs (5'-AACAAAGACGAATCGCATAT-3') were used, and the plasmid pUC18-Ac132; HA-SV40 was used as the template. The PCR products were digested with DpnI (NEB) and hybridized to generate the plasmid pUC18-Ac132; A¹²⁴/¹²⁵XXXA¹²⁸/¹²⁹-SV40. pUC18-Ac132; A¹²⁴/¹²⁵XXXA¹²⁸/¹²⁹-SV40 was digested with SacI and XhoI, and the resulting fragments were inserted into pFB1-PH-GFP to generate the donor plasmid pFB1-Ac132; A¹²⁴/¹²⁵XXXA¹²⁸/¹²⁹-PG. Then, vAc132; A¹²⁴/¹²⁵XXXA¹²⁸/¹²⁹ was generated by site-specific transposition as described above.

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**REFERENCES**


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