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# Functional Characterization of *Autographa californica* Multiple Nucleopolyhedrovirus ORF43 and Phenotypic Changes of ORF43-Knockout Mutant

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**ORF43 (*ac43*) of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is a highly conserved baculovirus gene of unknown function. To investigate the role of *ac43* in the baculovirus lifecycle, we constructed an *ac43*-deleted mutant AcMNPV, Ac43KO. After transfection into *Spodoptera frugiperda* cells, Ac43KO produced polyhedra much larger in size than those of wild-type AcMNPV. Interestingly, some of the nucleocapsids were singly enveloped in the polyhedrin matrix while the nucleocapsids of AcMNPV are known to be multiply enveloped. Furthermore, Ac43KO led to a defect in the transcription and expression of *polyhedrin*, which resulted in reduced occlusion body production. However, Ac43KO did not affect production of budded virus as there was no remarkable difference in budded virus titer. These results suggest that *ac43* plays an important role in the expression of *polyhedrin*, the morphogenesis of occlusion body, and the assembly of virions occluded in occlusion bodies.**

***Keywords*:** baculovirus, nucleopolyhedrovirus, *Autographa californica* NPV, *ac43*, virus morphology

## Introduction

Baculoviruses which are characterized by rod-shaped, enveloped nucleocapsids encompass a group of invertebrate-specific DNA viruses with circular, covalently closed, double-stranded DNA genomes of 80–180 kb (Ayres *et al.*, 1994; Herniou *et al.*, 2003; Jakubowska *et al.*, 2006). The family Baculoviridae is subdivided into four genera, alpha-, beta-, delta-, and gammabaculovirus, mainly based on their host insects and the morphology of their occlusion bodies (OBs) (Jehle *et al.*, 2006). *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the type species of the NPV genus. AcMNPV has a double-stranded DNA genome of approximately 134 kb which contains 154 predicted open reading frames (ORFs) of predicted polypeptides of 50 amino acids or more using the criterion that an ORF is a single, contiguous, non-overlapping coding region (Ayres *et al.*, 1994). The infection cycle of AcMNPV and many other baculoviruses includes two distinct viral phenotypes, occlusion-derived virus (ODV) and budded virus (BV). Both ODVs and BVs are identical in nucleocapsid structure and genetic information, but the composition of their envelopes is different to accommodate their respective functions in an infection cycle (Braunagel and Summers, 1994).

The ODVs, which are enveloped within the nucleus, initiate a primary infection of the insect by infecting epithelial cells in the midgut when the virions are released by the alkaline pH of the gut. The BVs are produced from the infected cells and can initiate a secondary infection (Federici, 1997). The ODVs are embedded within a paracrystalline matrix made of a protein called polyhedrin forming OBs, which are responsible for protecting the virions from UV radiation and desiccation, allowing infectious virions to survive for long periods in harsh environments before a susceptible host is infected. A baculovirus infection progresses in three phases, early, late, and very late, in cultured insect cells. A complex cascade of transcription and protein synthesis occurs within the first 6–8 h following viral infection (Friesen, 1997). The progression from early to late stages of infection coincides with the initiation of viral DNA replication between 6 and 12 h post-infection (p.i.) (Blissard, 1996). Newly replicated viral DNA is condensed and packaged into capsid structures within the nucleus to form nucleocapsids (Lu *et al.*, 1997). Nucleocapsids initially egress from the nucleus, migrate through the cytoplasm, and bud through a modified plasma membrane to acquire an envelope to form BVs (Monsma *et al.*, 1996). During the late phase of infection beginning at approximately 20 h p.i., there is a switchover from BV to ODV production by some unknown mechanism. Nucleocapsids then remain within the nucleus and develop an envelope *de novo* to form ODVs, which then become incorporated into the matrix of the OB (Williams and Faulkner, 1997).

The AcMNPV ORF43, *ac43*, is a highly conserved baculo-

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virus gene. Homologues of *ac43* are found in genomes of all group I and most of group II NPVs. Deletion of *Bm34*, a *Bombyx mori* NPV (BmNPV) homologue of *ac43*, resulted in reduced production of OBs and the slow killing of infected larvae. These effects were attributed to the reduced expression of *vlf-1* and *fp25k* (Katsuma and Shimada, 2009) which are required for efficient polyhedrin expression. In this study, an *ac43*-knockout virus, Ac43KO, was constructed via *in vitro* transposition of plasmid capture system donor-S (Choi *et al.*, 2005) into a novel AcMNPV bacmid, bAc-MK, to investigate the functional role of *ac43* in the AcMNPV life cycle. We identified the transcriptional phase of *ac43*, and the effects of *ac43* deletion on BV and OB production. In addition, the morphology of the OBs and ODVs in the Ac43KO-infected cells was also examined by electron microscopy. Our data indicated that *ac43* is involved in polyhedra morphogenesis by affecting the expression of the *polyhedrin* gene and ODV assembly.

## Materials and Methods

### Bacterial strains and bacmid DNA

*Escherichia coli* strains TOP10 and DH10B (Invitrogen, USA) were used throughout the experiments. All restriction endonucleases and modifying enzymes were from Roche Applied Science (Germany). All recombinant bacmids used in this study were propagated in *E. coli* strain DH10B.

### Insect cells, baculoviruses and transfection

The *Spodoptera frugiperda* cell line Sf9 was maintained at 27°C in TC-100 medium (WelGene, Korea) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (WelGene) and sub-cultured every 3–4 days. Wild-type AcMNPV C6 strain and all recombinant AcMNPVs used in this study were propagated in Sf9 cells maintained in TC-100 medium. Transfection was performed using the Cellfectin™ (Invitrogen) reagent according to the manufacturer's instructions.

### Tn7 *in vitro* transposition

The transposition procedure was carried out as described previously (Choi *et al*., 2005) with minor modification. For the transposition reaction, 12 ng of *Hin*dIII and *Sph*I-digested pPCS-S (donor-S) plasmid was combined with 200 ng of bAc-MK bacmid DNA. TnsABC* transposase (New England BioLabs, USA) was added to the transposition reaction, and the mixture was incubated at 37°C for 10 min. Next, 1 μl of start solution was added to the mixture, which was then incubated at 30°C for at least 2 h. Finally, the transposition reaction was stopped by heating it to 75°C for 10 min. The reacted DNA was transformed into competent *E. coli* DH10B cells (Invitrogen), and the transformed cells were subsequently plated onto nutrient agar plates containing kanamycin (50 μg/ml) and ampicillin (50 μg/ml). The plates were incubated at 37°C for 2 days. The colonies resistant to both kanamycin and ampicillin were selected, and successful transposition was verified by nucleotide sequence analysis.

### RNA and reverse transcription PCR (RT-PCR)

Sf9 cells were infected with viruses at a multiplicity of infection (MOI) of 10. Following gentle rocking for 1 h, the virus-containing culture medium was removed, and fresh medium was added after washing twice with unsupplemented TC-100 medium. Total RNA was isolated from infected Sf9 cells at 0, 0.5, 1, 2, 6, 12, 24, 48, 72, and 96 h p.i. using the TRIZOL Reagent (Invitrogen) according to manufacturer's instructions. After the RNA samples were treated with RNase-free DNase I (TaKaRa, Japan), RT-PCR was performed using the AccuPower® RT/PCR Premix (Bioneer, Korea) in a 20 ml volume according to manufacturer's instructions. To amplify the *ac43* gene, the oligonucleotides Ac43-F (5′-GGGGATCCCCAAGACAAAAAGATTGC-3′) and Ac43-R (5′-CCCCTCGAGCACCGTGAACACCTTGC-3′) were used. The *gp64* gene was amplified using the oligonucleotides AcGP64-F (5′-ATATGTGCTTTTGGCGG-3′) and AcGP64-R (5′-TTTGGCGCGTGGTGAAC-3′).

### Quantitative real-time PCR (qPCR)

Total RNA extracted from Sf9 cells infected with Ac-MK or Ac43KO at various times p.i. were treated with DNase I prior to cDNA synthesis. Single-strand cDNA was synthesized from the total RNA using the SuperScript™ III First-Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. The real-time PCR was conducted with 2× DyNAmo™ HS SYBR® Green qPCR Kit (FINNZYMES, Finland) and CFX96TM Real-Time System (Bio-Rad, USA). The cycling profile used for the qPCR was as follows: a preheating step for enzyme activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 15 sec, and 72°C for 30 sec. The relative transcription level was calculated using the 2-ΔCt method (Pfaffl, 2001). The 28S rRNA sequence was used as a reference gene (Xue *et al.,* 2010). Oligonucleotides specific to *ac43*, ORF43-RTF (5′-GCTATGTTTGCGACGAGTTGGTG-3′) and ORF43-RTR (5′-TGGGCACAGCACGATACCG-3′), and specific to *polyhedrin*, Polh-RTF (5′-ACCCAACCGTTGTTACAAATTCCT-3′) and Polh-RTR (5′-GCCGCCGCCCTTCTTAGC-3′), were used for qPCR.

### Titration of BV

Sf9 cells were infected with viruses at an MOI of 1.0. The collected media containing BVs were harvested at 24, 48, 72, and 96 h p.i., and cell debris were removed by centrifugation at 5,000×g for 5 min. The extracellular BV titers in the collected media were determined using the end-point dilution method in triplicate as previously reported (O'Reilly *et al.*, 1992).

### Quantification of OB

Sf9 cells were infected with viruses at an MOI of 10. Infected cells were harvested at 96 h p.i. by centrifugation at 5,000×g for 5 min. The resulting pellet was resuspended in lysis buffer (50 mM Tris-HCl; pH 8.0, 0.4% SDS, 10 mM EDTA, and 5% 2-mercaptoethanol), and OBs were pelleted by centrifugation at 5,000×g for 5 min and then resuspended in 0.5 M NaCl. To calculate the number of OBs, OB stocks were seri-

ally diluted and counted in triplicate using a hemocytometer.

### Electron microscopy

For transmission electron microscopy (TEM), Sf9 cells infected with viruses at an MOI of 10 were harvested at 72 h p.i. by centrifugation at 5,000×g for 5 min, and fixed for 4 h at 4°C with 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). After post-fixation by 1% $OsO_4$ in the same buffer, the samples were dehydrated in ethanol/propylene oxide series and embedded in an Epon-Araldite mixture. Ultra-thin sections were obtained with a RMC MT-X ultramicrotome and subsequently stained with a mixture of 2% uranyl acetate and Sato's lead. The transmission electron microscope JEM-1010 (JEOL, Japan) was used. For scanning electron microscopy (SEM), infected cells were fixed and dehydrated as described above for TEM, followed by drying in hexamethyldisilazane and mounting on metal stubs. Then the samples were gold coated and observed under the scanning electron microscope JSM-5410LV (JEOL).

## Results

### Generation of *ac43* knockout mutant virus

To determine whether *ac43* is essential for viral replication, we generated an infectious bacmid in which the *ac43* gene was knocked-out. For this, the baculovirus transfer vector pBacMKPol, which the *E. coli* origin of replication (Mini-F replicon) coupled with *polyhedrin* and kanamycin resistance gene (*Kan*) was cloned into a modified pBacPAK transfer vector (Clontech, USA), was inserted into the locus between *orf603* and *polh* of the AcMNPV sequence (Fig. 1A). The novel recombinant bacmid, bAc-MK, was generated via homologous recombination of pBacMKPol and AcMNPV genomic DNA in co-transfected Sf9 cells, and successful recombinants were selected in *E. coli* plated on nutrient agar plates containing kanamycin (50 μg/ml). The genomic structure of bAc-MK was verified by endonuclease digestion and nucleotide sequence analysis (Fig. 1B).

By means of Tn7-mediated transposition between the bAc-MK and the donor-S of the plasmid capture system, we generated the recombinant bacmid Ac43KO which the *ac43* gene was disrupted with a pUC origin (pUC ori) and an ampicillin resistance gene (*Amp*) (Fig. 1A). The genomic structure of the Ac43KO bacmid was verified by PCR using primers ORF43-RTF and ORF43-RTR specific to the *ac43* gene (Fig. 1C), and nucleotide sequence analysis result confirmed that *ac43* was disrupted at the amino acid position 41 in the middle of the ORF (data not shown).

RT-PCR analysis was performed to confirm the nullified *ac43* expression in Ac43KO-infected Sf9 cells (Fig. 1D). RT-PCR using the *gp64*-specific primer pair successfully amplified the corresponding gene from cDNAs of Sf9 cells infected with Ac-MK or Ac43KO, indicating that both of the viruses replicated in Sf9 cells. While a single RT-PCR product of the expected size was obtained from the Sf9 cells infected with Ac-MK, no product was amplified from Ac43KO-infected cells using the *ac43*-specific primer set. These results demonstrated that the *ac43* gene was successfully knocked-out from the bacmid Ac43KO.

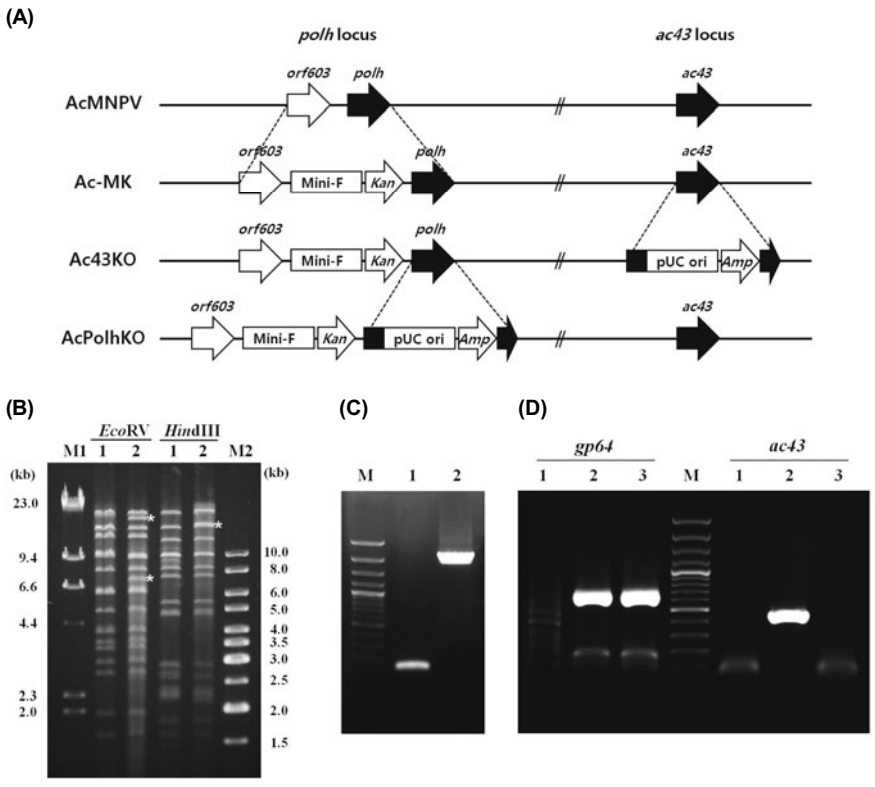


**Fig. 1. Construction of bAc-MK and Ac43KO bacmids.** (A) Schematic diagram of bAc-MK and Ac43KO bacmid. The pUC ori and *Amp* were inserted at amino acid 41 of *ac43*. (B) Restriction endonuclease digestion patterns of the bAc-MK. Lanes: M1, λ DNA digested with *Hin*dIII; 1, AcMNPV; 2, bAc-MK; M2, 1 kb Ladder. The insertion of Mini-F and *Kan* into the bacmid bAc-MK yielded *Eco*RI 21.1 kb and 8.4 kb, and *Hin*dIII 15.8 kb fragments (indicated with asterisks). (C) PCR analysis of *ac43* knockout virus. Lanes: M, 100 bp Ladder; 1, Ac-MK; 2, Ac43KO. The *ac43* PCR primers amplified 0.1 kb of *ac43* in Ac-MK, and 2.2 kb of pUC ori and *Amp* in Ac43KO at the *ac43* loci, respectively. (D) RT-PCR analysis of *ac43* transcription. Sf9 cells were infected at an MOI of 10, and total RNA was extracted at 72 h p.i. Lanes: M, 100 bp Ladder; 1, mock-infected Sf9 cells; 2, Ac-MK-infected Sf9 cells; 3, Ac43KO-infected Sf9 cells.

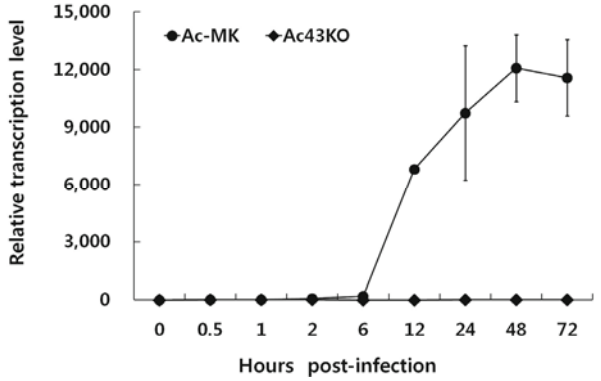


**Fig. 2. Transcription of *ac43* in Sf9 cells.** Total RNA was extracted from Sf9 cells infected with Ac-MK or Ac43KO at 0, 0.5, 1, 2, 6, 12, 24, 48, and 72 h p.i. and subjected to qPCR analysis using an *ac43*-specific primer set.

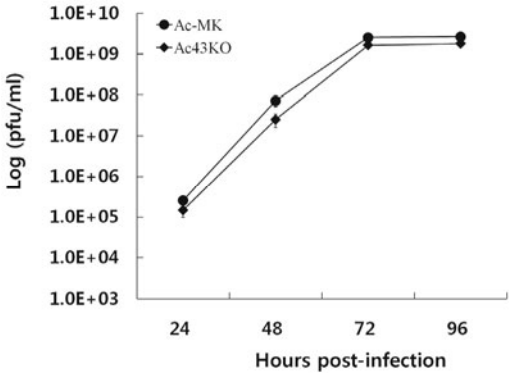


**Fig. 3. Effect of *ac43* on BV production in Sf9 cells.** $1\times10^6$ Sf9 cells were infected with Ac-MK or Ac43KO at an MOI of 1.0. The extracellular BV titers at 24, 48, 72, and 96 h p.i. were determined by end-point dilution assay. The error bars indicate one standard deviation (n=3).

### Transcriptional analysis of *ac43*

Temporal expression of the *ac43* gene during viral replication was investigated using qPCR (Fig. 2). In the Sf9 cells infected with Ac-MK, the transcription of *ac43* started at 6 h p.i, continued to increase until 48 h p.i. and then slightly declined by 72 h p.i. time point, indicating that *ac43* belongs to the late gene category. In contrast, no *ac43* transcription was detected at any time points in the Sf9 cells infected with Ac43KO as no functional copy of this gene was present on this bacmid.

### Effect of *ac43* deletion on BV and OB production

To examine the effects of *ac43* deletion on BV production, Sf9 cells were infected with Ac-MK or Ac43KO at an MOI of 1.0, and the resulting cell culture supernatant at selected time points were subjected to extra-cellular BV titration (Fig. 3). The result revealed that there were no significant differences in BV production between Ac-MK and Ac43KO, indicating that *ac43*-knockout virus is capable of infectious progeny virus production, and *ac43* does not play a role in viral replication in infected cells.

We also investigated the effect of *ac43* deletion on OB production in Sf9 cells and observed that OB production was markedly reduced in Ac43KO-infected Sf9 cells (Fig. 4A). Because polyhedrin is the major component of OBs, we characterized the effect of *ac43* deletion on *polyhedrin* gene expression. The qPCR analysis showed that the reduced production of OB was caused by the down-regulation of *polyhedrin* expression (Fig. 4B). While the expression of *polyhedrin* started at around 12 h p.i. and kept increasing until 48 h p.i. in both Ac-MK- and Ac43KO-infected Sf9 cells, the expression level of *polyhedrin* was reduced by approximately 50% in Ac43KO-infected Sf9 cells than that of Ac-MK-infected Sf9 cells at all time-points evaluated. These results suggest that the deletion of *ac43* causes a reduction in the level of *polyhedrin* expression and thereby also reduces OB production in Sf9 cells.

### Effect of *ac43* deletion on OB morphogenesis

The Sf9 cells infected with Ac-MK or Ac43KO were examined by microscopy to detect any effect of the *ac43* deletion on viral morphogenesis. Surprisingly, Sf9 cells infected with Ac43KO produced OBs were much larger in size (3.9±0.1 μm) than those from Sf9 cells infected with Ac-MK (2.7±0.1 μm) (Figs. 5A and B). There were no other differences in the external morphology between OBs from Sf9 cells infected with Ac-MK and Ac43KO. When $2.5\times10^5$ OBs were analyzed by SDS-PAGE, a much larger amount of polyhedrin protein from OBs of Ac43KO was present compared with OBs of

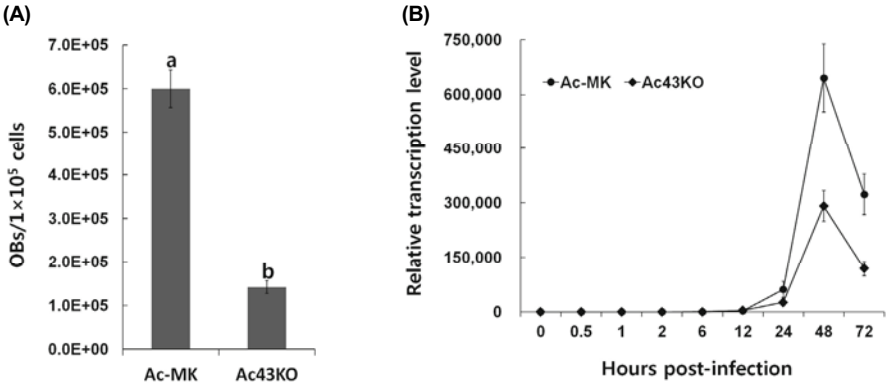


**Fig. 4. Effect of *ac43* on OB production and *polyhedrin* expression in Sf9 cells.** (A) OB production. $1\times10^5$ Sf9 cells were infected with the virus at an MOI of 10. Infected cells were harvested at 96 h p.i. and lysed, and three independent polyhedra counts were made using a hemocytometer. Different letters above the error bars (indicating standard deviation, n=3) indicate significant difference by Duncan's multiple range test ($P<0.05$). (B) *Polyhedrin* expression. Total RNA was extracted from Sf9 cells infected with Ac-MK or Ac43KO at 0, 0.5, 1, 2, 6, 12, 24, 48, and 72 h p.i., and subjected to qPCR analysis using a *polyhedrin*-specific primer set.

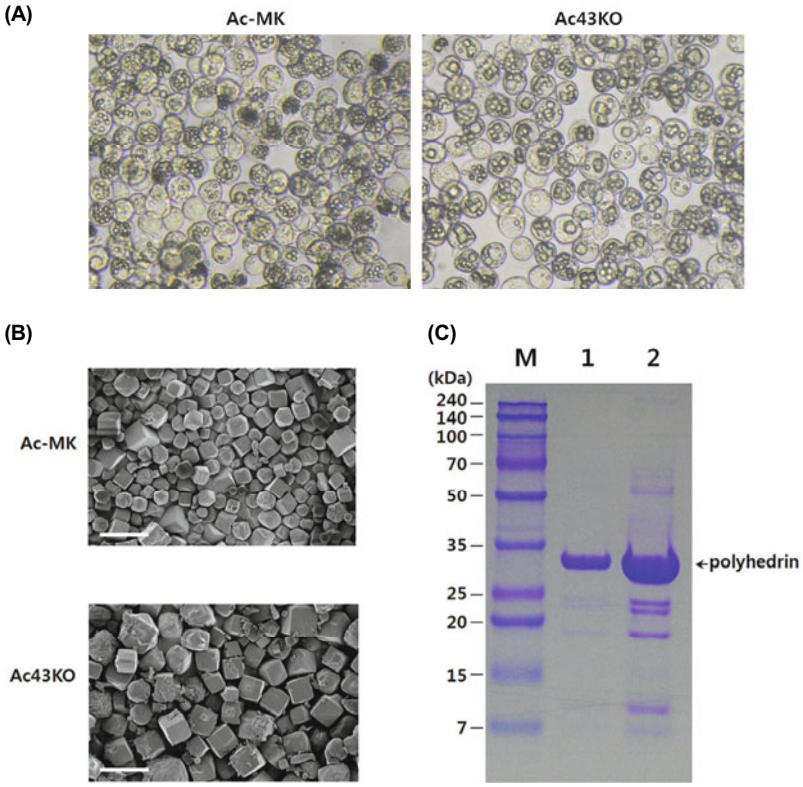


**Fig. 5. Effect of *ac43* on OB morphogenesis.** (A) Light microscopy of Sf9 cells infected with Ac-MK or Ac43KO. (B) Scanning electron microscopy of OBs produced from Sf9 cells infected with Ac-MK or Ac43KO (scalebar=10 μm). The average polyhedra sizes of Ac-MK and Ac43KO were 2.7±0.1 μm and 3.9±0.1 μm, respectively. (C) SDS-PAGE analysis of $2.5\times10^5$ OBs purified from Sf9 cells infected with Ac-MK or Ac43KO. Lanes: M, protein molecular weight marker; 1, Ac-MK-derived OBs; 2, Ac43KO-derived OBs.

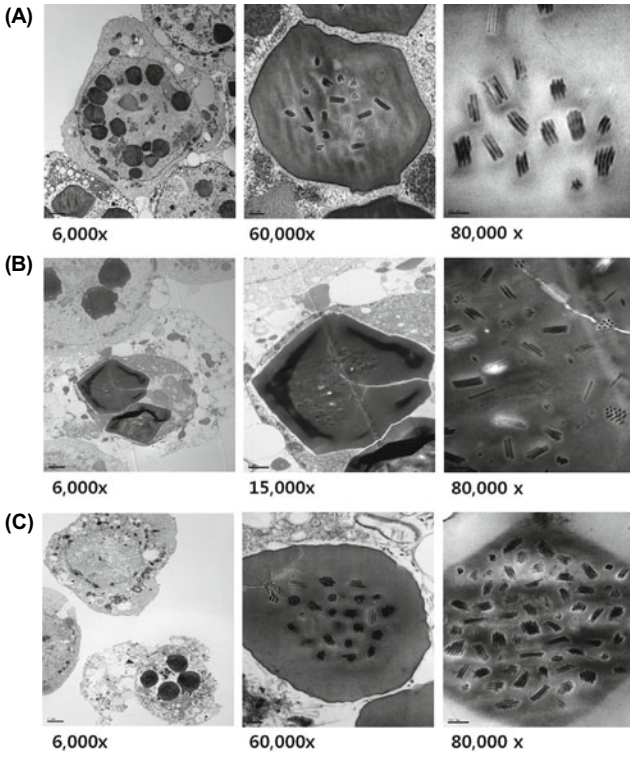


**Fig. 6. Effect of *ac43* on ODV assembly.** (A) Transmission electron microscopy of Sf9 cells infected with Ac-MK. (B) Transmission electron microscopy of Sf9 cells infected with Ac43KO. (C) Transmission electron microscopy of Sf9 cells co-infected with Ac43KO and AcPolhKO.

Ac-MK (Fig. 5C). These results indicated that the size of Ac43KO-derived OBs were significantly larger than Ac-MK-derived OBs.

To further study whether the deletion of *ac43* affects assembly and occlusion of ODVs into OBs, the internal structure of OBs produced from Sf9 cells infected with Ac-MK or Ac43KO was investigated using transmission electron microscopy. Whereas ODVs occluded in Ac-MK-derived OBs were of the normal multiple nucleocapsids phenotype, a comparatively larger number of ODVs with single nucleocapsid phenotype were occluded in the Ac43KO-derived OBs, although ODVs with multiple nucleocapsids were also occluded (Figs. 6A and 6B). When the deletion of *ac43* was complemented by co-infection of Ac43KO with AcPolhKO, which is a *polyhedrin*-knockout mutant generated by Tn7-mediated transposition of pUC ori-*Amp* donor cassette into *polyhedrin* locus, only ODVs with multiple nucleocapsids were occluded in OBs (Fig. 6C). These results suggest that *ac43* is involved in multiple nucleocapsid packaging in ODV assembly.

## Discussion

The infection of Sf9 cells with Ac43KO showed that the deletion of *ac43* did not affect BV production, but led to a defect in OB production. In addition, deletion of *ac43* resulted in markedly down-regulated transcription and expression of the *polyhedrin* gene. These results were consistent with a previous report that *Bm34*, the *ac43* homolog of BmNPV, was required for efficient OB production and *polyhedrin*

expression. Furthermore, *Bm34* played an important role in the transcription of *vlf-1* and *fp25K* (Katsuma and Shimada, 2009) which indirectly control the expression of late and very late genes. In this study, reduced expression of *polyhedrin* in Ac43KO-infected cells might also result from the down-regulation of *vlf-1* and *fp25K* expression.

It has been reported that the *Bm34* gene product is mainly located in the nucleus and is not a component of either BV or ODV. Interestingly, whereas OBs from BmN cells infected with *Bm34*-knockout virus showed normal shapes and sizes (Katsuma and Shimada, 2009), OBs from Sf9 cells infected with Ac43KO had significantly larger sizes compared with those from AcMNPV-infected cells. Furthermore, these OBs contained a much larger amount of ODVs of the single nucleocapsid phenotype. The processes of virion occlusion in the OBs and the genes that regulate this occlusion process have not been well identified yet. Previous analysis had suggested that OB formation depends on amino acid sequence and the secondary structure of polyhedrin (Carstens *et al.*, 1992). Four spontaneous mutant viruses with altered OB morphology have been isolated and characterized in AcMNPV. In each case, a single amino acid substitution in the polyhedrin sequence was responsible for the abnormal phenotype of the OBs. One of those mutants, bearing a substitution at position 118, produced large amounts of small particles instead of crystalline OBs (Ribeiro *et al.*, 2009). The other reported mutants, which had a substitution at residue 58 (Carstens *et al.*, 1986), 25 (Lin *et al.*, 2000) or 130 (Lopez *et al.*, 2011), produced a single large cubic OB in the nuclei of the infected cells, and only a small portion of these OBs contained nucleocapsids in them. These OB-deficient mutants have been classified as class I and II based on their different characteristics (Ji *et al.*, 2010).

In contrast, several studies have demonstrated that OB formation is determined by the interactions between polyhedrin and other proteins present on the viral envelope (Woo *et al.*, 1998; Wang *et al.*, 2009). The co-infection of AcMNPV and BmNPV generated a recombinant virus which produced OBs varying in morphology when infecting different host cell types. The *polyhedrin* gene of this recombinant virus showed 100% identity with the *polyhedrin* gene of the BmNPV, suggesting that the size and morphology of the OBs might be affected by host cell factors as well as viral genes or regulatory sequences other than *polyhedrin* (Woo *et al.*, 1998). Other results also indicated that some viral or host factors may play a role in the process of virion occlusion (Hu *et al.*, 1999). No virions were detected in the OBs of an AcMNPV mutant, in which the *p26*, *p10*, and *p74* genes were deleted, demonstrating that *p26*, *p10*, and *p74* are all required for proper virion occlusion into the OBs of AcMNPV (Wang *et al.*, 2009). In this study, deletion of *ac43* not only altered the size of OBs but also affected the virion occlusion in the polyhedra.

For AcMNPV, the development of two viral morphotypes (i.e., BV and ODV) and the multiple nucleocapsid packaging of ODV are major evolutionary developments that contribute to its success as a pathogen of lepidopteran larvae (Washburn *et al.*, 2003). In this study, deletion of *ac43* resulted in the production of ODVs with singly packaged nucleocapsids. The evolutionary history and host ranges of the NPVs suggest that the MNPV has evolved from the SNPV and, therefore, may offer selective advantages (Washburn *et al.*, 1999). At least two hypotheses have been proposed to explain the multiple nucleocapsid packaging strategy of the MNPVs. First, it is well documented that viral occlusions rapidly lose viability when exposed to sunlight, presumably because UV radiation damages viral DNA. By infecting primary target cells with multiple copies of the viral genome, it is possible that gene complementation compensates for the damaged DNA and facilitates productive infections (Washburn *et al.*, 1999). The second hypothesis postulates that infection of primary target cells with multiple copies of the viral genome increases infection efficiency *in vivo* by accelerating the onset of secondary infection (Blissard, 1996; Volkman, 1997).

Although the biological basis for multiple nucleocapsid envelopment has not been determined, *ac142* was recently shown to be essential for ODV nucleocapsid envelopment (McCarthy *et al.*, 2008). In addition, deletion of *sf29* from *Spodoptera frugiperda* MNPV (SfMNPV) reduced the number of ODVs occluded in OBs but had no apparent effect on ODV nucleocapsid content (Simon *et al.*, 2008). Another gene, *ac23*, homologous to the F-proteins of group II NPVs, is also involved in ODV formation. Like the *ac43*-knockout virus in this study, a greater percentage of ODVs with singly-enveloped nucleocapsids are occluded in OBs of the *ac23*-null mutant virus compared with that of wild-type virus, suggesting that Ac23 somehow facilitates multiple nucleocapsid envelopment (Yu *et al.*, 2009). However, OBs of *ac23*-null mutant and wild-type virus do not differ significantly in size or the number of ODVs occluded (Yu *et al.*, 2009). However, OBs produced by the *ac92*-knockout mutant lacked ODVs with multiply-enveloped nucleocapsids, but singly-enveloped nucleocapsids were detected (Wu and Passarelli, 2010). The mutant virus, in which the Ac92 C155XXC158 amino acids (important for sulfhydryl oxidase activity) were mutated to A155XXA158, exhibited a similar phenotype to the *ac92*-knockout virus, suggesting that the C-X-X-C motif was responsible for the altered ODV phenotype (Wu and Passarelli, 2010).

In the present study, we showed that *ac43* is a novel late gene required for efficient expression of the *polyhedrin* gene and production of OBs. Additionally, *ac43* appears to play an important role in the process of OB formation and multiple nucleocapsid packaging of ODVs. Further studies to address whether *ac43* interacts with other viral sequences, viral proteins, or even other factors from the host cells would provide greater insight into the processes of OB morphogenesis and nucleocapsid packaging in the AcMNPV life-cycle.

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