

# Deletion of *v-chiA* from a Baculovirus Reduces Horizontal Transmission in the Field

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**Nucleopolyhedroviruses (NPVs) can initiate devastating disease outbreaks in populations of defoliating Lepidoptera, a fact that has been exploited for the purposes of biological control of some pest insects. A key part of the horizontal transmission process of NPVs is the degradation of the larval integument by virus-coded proteins called chitinases, such as V-CHIA produced by the *v-chiA* genes. We used recombinant and naturally occurring strains of the *Lymantria dispar* NPV (LdMNPV) to test horizontal transmission in the field, release of virus from dead larvae under laboratory conditions, and cell lysis and virus release in cell culture. In the field, strains of LdMNPV lacking functional *v-chiA* genes showed reduced horizontal transmission compared to wild-type or repaired strains. These findings were mirrored by a marked reduction in released virus in laboratory tests and cell culture when the same strains were used to infect larvae or cells. Thus, this study tests the pivotal role of liquefaction and the *v-chiA* gene in field transmission for the first time and uses complementary laboratory data to provide a likely explanation for our findings.**

Nucleopolyhedroviruses (NPVs) are viruses belonging to the *Baculoviridae*, members of which infect insects and other arthropods (1). NPVs possess large circular and double-stranded DNA genomes and have a unique infection cycle. These viruses produce two infectious morphological forms: a budded virus form (BV) and an occlusion-derived virus (ODV), a form that is occluded into a protein structure termed a polyhedron (2). Lepidopteran larvae, which represent a large proportion of the taxa affected by NPVs, are infected after they ingest polyhedra, which dissolve in the alkaline environment of the midgut and release ODV. BV is then produced within larvae early during infection, budding from the plasma membrane and infecting other cell types to cause a systemic infection. During the late phase of infection, ODV are produced and become embedded into polyhedra, and the infection process terminates upon larval death (1). Many NPVs code for proteins that degrade the larval integument, aiding in the release of polyhedra contained within the larvae (2). These released ODV are protected within the polyhedron from environmental elements until polyhedron-contaminated leaf material is ingested and the cycle is repeated (3). Microbial insecticides used for biological control of agricultural and forest insect pests utilize occlusion bodies or granulins (reviewed in references 4 and 5).

The *Lymantria dispar* (gypsy moth) multinucleocapsid NPV (LdMNPV) product, Gypchek, is an example of an ODV-derived biopesticide currently in use (6). Gypchek is an Environmental Protection Agency-registered gypsy moth control agent containing the ODV form of LdMNPV as its active ingredient (7). The gypsy moth is a serious defoliating lepidopteran pest in the north-eastern United States that is spreading into the south and midwest (8, 9). LdMNPV has the advantage of specificity for the gypsy moth, in contrast to chemical insecticides and most other biological control agents, including *Bacillus thuringiensis* (10), which kills many species of Lepidoptera in the larval form. Consequently, LdMNPV is the agent of choice for gypsy moth control in environmentally sensitive areas and areas that contain threatened and endangered insect species (6). Although effective, Gypchek is

also more expensive on a per-hectare basis than other sprayed controls. Currently LdMNPV is produced on a limited basis *in vivo* by the USDA Forest Service and Animal and Plant Health Inspection Service.

LdMNPV is also a major naturally occurring cause of mortality in high-density gypsy moth populations in the United States and within its native range. In field populations of gypsy moth, horizontal transmission of LdMNPV is initiated by first-instar larvae (11). These larvae ingest LdMNPV-contaminated eggs during eclosion, become infected, die, and disintegrate on the surface of leaves (12, 13). In this context, the disintegration of virus-killed larvae is termed liquefaction. In the field, the process of liquefaction contaminates leaves, and LdMNPV is then later ingested by other larvae, producing several waves of mortality during the season (14, 15). It is generally assumed that larval liquefaction facilitates dissemination of virus in the field and, thus, horizontal transmission (16), although until now no studies have directly addressed this question.

If larval liquefaction is assumed to be integral to virus transmission, key to the process of liquefaction is the production of V-CHIA, a virally encoded chitinase, and V-CATH, a virally encoded cysteine protease. Deletion strains of the *Autographa californica* MNPV (AcMNPV) and the *Bombyx mori* NPV (BmNPV), lacking functional *v-chiA* or *v-cath*, do not cause host liquefaction (17–20). The *v-chiA* genes of NPVs encode proteins that are members of family 18 of hydrolytic enzymes (21). Among most V-CHIAs, including the LdMNPV *v-chiA* (22), a C-terminal KDEL endoplasmic reticulum retention signal is present that is necessary

Received 16 January 2013 Accepted 20 April 2013

Published ahead of print 26 April 2013

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doi:10.1128/AEM.00152-13

for the protein to be targeted to the endoplasmic reticulum of infected cells (23, 24). AcMNPV V-CATH is expressed as 35.5- and 32-kDa precursor forms that are processed to a 27.5-kDa mature form (20). In AcMNPV and BmNPV, V-CHIA is necessary for the proper processing of V-CATH to its mature form (25, 26). The functions of these liquefaction-related genes and their products may have serious ramifications for the efficacy and persistence of control agents derived from them. However, because most studies of microbial pesticides focus on the initial response of pests to a sprayed product, the effect of genotype on subsequent horizontal transmission remains largely unexplored, with the exception of some engineered viruses (1, 27).

Strains of LdMNPV from various sources are screened in our laboratory as part of our efforts to develop improved viral strains and formulations to control gypsy moth infestations. Some of the most promising of these strains have advanced to field trials, and logically this has been done primarily in the context of efficacy (28–30). In circumstances where increased potency has been coupled with atypical infections or poorly understood genetic variation, however, we have initiated additional studies. Observations of a decrease in larval liquefaction in bioassays led us to investigate the role of *v-chiA* in horizontal transmission of LdMNPV in the field. In this study, we found that deletion of *v-chiA* diminished horizontal viral transmission in the field, and we have quantified the assumed role of *v-chiA* for the first time.

## MATERIALS AND METHODS

**Cells and virus strains.** *L. dispar* 652Y (Ld652Y) cells were grown as previously described (31). LdMNPV strain 203 was isolated from Gypchek. Isolate 203 was initially semipurified by the *in vivo* method of Smith and Crook (32) in fourth-instar larvae and then plaque purified 3 times in the Ld652Y cell line using standard techniques (33). During bioassays of several 203 plaque lines, one was found that did not liquefy virus-killed larvae and was designated 203-NL. A line that did liquefy virus-killed larvae was designated 203-WT. Viral strain 203-*chiA*-del is a recombinant virus lacking the chitinase gene and was generated as described below.

**Cloning, sequence analysis, and generation of recombinant viruses.** DNA from LdMNPV isolates 203-WT and 203-NL was purified from polyhedra using a modification of the method from O'Reilly et al. (33). DNA was digested with SpeI (New England BioLabs). The resulting digest showed an 8.3-kbp band in 203-NL instead of the 12.1-kbp band found in the 203-WT isolate. This area of the genome contains the chitinase gene (bp 64801 to 66477) based on the LdMNPV 5-6 sequence (22). The 12.1-kbp band from isolate 203-WT and the 8.3-kbp band from 203-NL were excised from a Tris-borate-EDTA (TBE) agarose gel, purified from the agarose using the GeneClean spin kit (Bio101), and ligated into SpeI-digested pBluescriptSK+ vector after dephosphorylation using calf intestinal alkaline phosphatase (New England BioLabs). The ligation mix was used to transform competent DH5 $\alpha$  cells using standard techniques. DNA from the correct clones was purified using the Concert high-purity Maxi plasmid purification kit (Life Technologies). The 8.3- and 12.1-kbp SpeI clones were digested with several restriction endonucleases to delineate the basis for the size difference in these clones. This analysis identified a region of about 3.8 kbp that was absent from the 203-NL 8.3-kbp SpeI clone. The missing region of this clone was identified through sequencing using 11 sequencing primers (Invitrogen) that covered the region from bp 63045 to 68408 of the 203-WT 12.1-kbp clone. Purified plasmid DNA of the SpeI clones of both 203-WT and 203-NL were sequenced at the Plant-Microbe Genomics Facility of the Ohio State University, Columbus, Ohio.

To generate a recombinant virus lacking the chitinase gene, the 12.1-kbp SpeI clone from 203-WT was digested with SpeI/HpaI, and the 5.5-kbp band containing the chitinase gene was excised, gel purified, and

ligated into pBluescriptSK+ cut with SpeI/HincII. To delete most of the chitinase gene, the 5.5-kbp clone was digested with ClaI/RsrII and end filled using T4 polymerase (BRL), which removed all but 108 bp from the C terminus of the gene and 49 bp downstream of the N terminus. The 6.8-kbp band, which included pBluescriptSK+ vector, was gel purified. The *lacZ* gene was excised from plasmid p216.1 by digestion with XbaI/BamHI. The 3.8-kbp band was isolated and gel purified. This band was end filled using T4 polymerase and cloned into the 6.8-kbp ClaI/RsrII fragment using the quick ligation kit. Several clones were analyzed for the presence of the *LacZ* gene and the absence of the chitinase gene, and clone pLdchidellacZ-1 was cotransfected with 203-WT genomic DNA using Lipofectin reagent (BRL). The resulting transfection was plaque purified by a standard plaque assay technique, with the addition of an 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) solution (100 mg/ml in dimethyl sulfoxide [DMSO]) to the agarose overlay (0.8% SeaPlaque [FMC], ExCell 420 medium [JRH], and 5% Serum Plus medium supplement [JRH]). The final concentration of X-Gal was 0.1 mg/ml in the overlay. After 2 weeks, blue plaques were identified in the highest dilution plates. These blue plaques were transferred to a p96 cell culture plate (Corning) seeded with  $1.0 \times 10^4$  cells/well and 200  $\mu$ l of media/well. After 1 week, 50  $\mu$ l of the medium from the infected wells was removed to a 0.6-ml microcentrifuge tube, and 2  $\mu$ l of X-Gal stock solution was added. The media from the p96 wells that had the darkest blue color development in the least amount of time were transferred to T25 cell culture flasks (Corning) seeded with  $1 \times 10^6$  cells. The resulting budded virus media from the flasks that showed only minimal cell lysis were harvested 1 week postinfection and were tested for the presence of *lacZ* using X-Gal solution. The one flask that showed the lowest degree of cell lysis and the greatest reaction with X-Gal was further plaque purified using a standard plaque assay technique and the addition of X-Gal to the overlay. After 2 weeks, blue plaques were identified in the highest dilution and were transferred to a p96 cell culture plate as described before. As before, 50  $\mu$ l of viral medium was tested in the presence of X-Gal solution. The sample that tested the darkest blue and had no cell lysis, plaque 4F, was grown up for budded virus production and DNA isolation. The DNA restriction profile of 203-chitinasedel-4F digested with both EcoRI and SpeI showed the presence of the *lacZ* gene and absence of the chitinase gene and was named 203-*chiA*-del. 203-NL-rep was generated by rescue of 203-NL with cosmid clone C-77 that contains the genomic region from approximately 48.1 to 82.4 kbp (34). 203-NL and C-77 DNA were cotransfected into Ld652Y cells as described above. BV from this transfection was plaque purified as described above, and 200 well-separated plaques were picked and used to infect Ld652Y cells. Virus from two plaques that caused cell lysis of Ld652Y cells was plaque purified again. Both isolates were found to cause larval liquefaction, and one was used in this study and designated 203-NL-rep.

**Quantification of larval liquefaction and polyhedron release from Ld652Y cells.** To quantify larval liquefaction, newly molted 4th-instar *L. dispar* organisms were placed on a diet that contained  $1 \times 10^6$ /cm<sup>2</sup> surface-applied polyhedra of isolates 203-WT, 203-NL, 203-*chiA*-del, or 203-NL-rep. The larvae were removed and placed on new diet 24 h later and monitored for virus-caused mortality. Once larvae began to die from viral infection, larvae that were on the verge of death were removed, weighed, placed into individual 15-ml plastic tubes, and reexamined 4 h later to confirm larval death. Larvae that had died were retained and those alive were discarded, so that 22 to 33 virus-killed larvae were able to be used for the following test. Twenty-four hours after confirmation of larval death, 20  $\mu$ l/mg larval weight of phosphate-buffered saline (PBS) was added, the contents of the tube were mixed on a Fisher Vortex Genie 2 (Fisher Scientific) at a setting of 6 for 5 s, large debris was allowed to settle for 5 s, and a 2-ml aliquot was removed. The 2-ml sample was remixed and an aliquot removed, and the number of polyhedra present was determined by counting using a hemacytometer. The number of polyhedra present was expressed as the number of polyhedra per mg of larval weight, and the results were compared by a one-way analysis of variance (ANOVA). Differences

between means were determined by a Tukey honestly significant difference (HSD) test.

To quantify polyhedra release from infected Ld652Y cells, T-25 flasks were seeded with  $1 \times 10^6$  Ld652Y cells, the cells were allowed to attach for 1 h, and then they were infected with 203-WT, 203-NL, 203-*chiA*-del, or 203-NL-rep budded virus at a dose of 5 50% tissue culture infectious dose (TCID<sub>50</sub>) units per cell. After a 1-h infection period, the viral inoculum was removed from the flask and replaced with medium. All infections were performed in quadruplicate. At 7 days postinfection, the medium was removed from each flask and the number of polyhedra present was quantified. Five ml of PBS was added to the flasks, the unlysed cells were removed, the solution was sonicated, and the number of polyhedra released from the cells was quantified. The average number of polyhedra generated by each viral strain was compared by a one-way analysis of variance, and differences between means were determined by a Tukey HSD test.

**Laboratory bioassays.** To assess the relative efficacy of the different strains of LdMNPV, we first performed laboratory bioassays under controlled conditions. In 2004, 203-NL and 203-WT were assayed; in 2007, we assayed 203-NL, 203-WT, and 203-*chiA*-del. These were the same strains that were used later each year in the field. Five groups of 10 newly molted 2nd-instar gypsy moths (Standard New Jersey-colonized strain) were challenged with each dose of viral occlusion bodies (OBs) incorporated into artificial diet. Doses were  $10^2$ ,  $10^3$ ,  $10^4$ ,  $10^5$ , and  $10^6$  OBs per ml of diet. Larvae fed *ad libitum* on the virus-laced diet for 2 days and then fed on uncontaminated diet until day 14, at which time mortality was assessed. Bioassay conditions were, with minor modifications, the same as those described by Slavicek et al. (35). The droplet feeding bioassay method was also used as previously described (31). Probit analysis (Polo-Plus 2.0; LeOra Software, Berkeley CA) was used to determine and compare lethal concentration (LC) values from the larval mortality data.

**Field experiments.** Our field experiments were performed with a technique described in D'Amico et al. (36). This technique entails confining each treatment/dose combination of larvae in mesh bags on single branches of live, naturally growing trees in the field. These experiments maximize realistic field effects on transmission while preventing escape of larvae. The mesh bags allow free passage of air and rain, have insignificant effects on temperature, and do not impact foliage for the confinement period (37). The methodology was common to all of the experiments and is described here briefly.

(i) Experiments were performed on pin oaks (*Quercus palustris*) in the Cedar Swamp State Wildlife Management Area east of Smyrna, DE. (ii) Small branch tips with 40 ( $\pm 3$ ) leaves were used for the experiments to reduce variability in the final density of virus on foliage. (iii) Virus was applied to foliage of branches in the form of living first instars infected with 203-WT, 203-NL, 203-*chiA*-del, or 203-NL-rep. Larvae were fed for 2 days in a 6-oz cup on LdMNPV-contaminated diet; the different strains of LdMNPV were used to produce these infected larvae (203-NL and 203-WT in the first field trial and 203-NL, 203-WT, and 203-*chiA*-del in the second field trial). Regardless of strain, diet contained LdMNPV incorporated at  $10^6$  occlusion bodies per 100 ml of diet. This dose caused 99 to 100% mortality in first instars (data not shown). These infected larvae were placed on branches in various numbers, which determined the dose: 2, 5, 10, 25, or 50 larvae per branch in 2004 and 2, 5, 10, 20, or 40 in 2007. Larvae remained free to move among leaves on a branch but were confined on each treatment branch within a mesh bag until all were dead (10 days). Once dead, these larvae liquefied to various degrees according to the strain of virus used and served as the source of virus for horizontal transmission to second-instar test larvae (step 4). (iv) Twenty-five healthy second-instar larvae were placed, within bags, on the same branches among the LdMNPV-killed larvae for 7 days. They were then removed from the branches and reared individually in diet cups for 3 weeks in an outdoor insectary. Mortality and cause of death were determined and recorded. The first field trial started on 10 May 2004, when infected first-instar larvae were placed on branches. In the second trial, the experiments

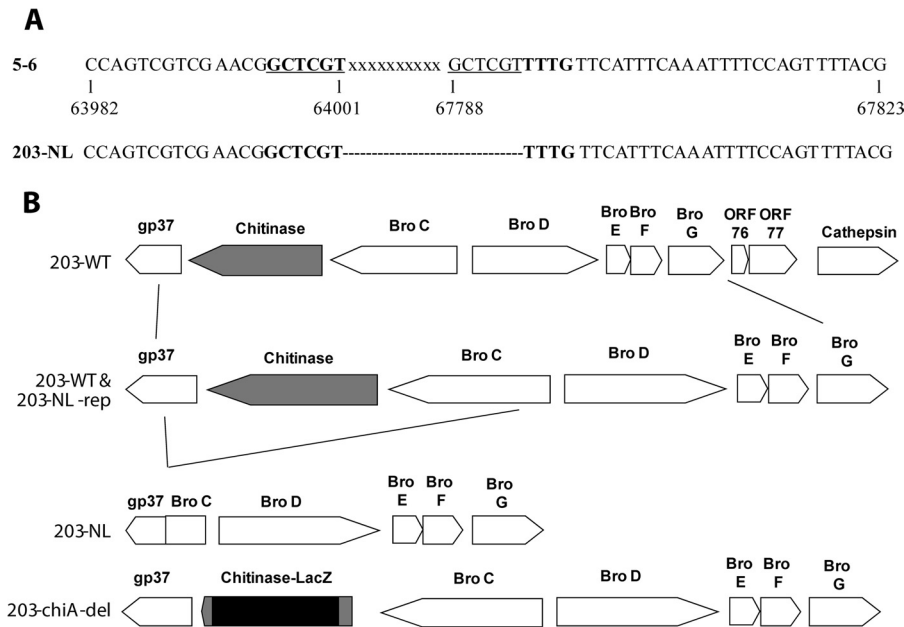
were begun on 5 June 2007. Dose in this case is represented as the number of larvae used on each 40-leaf branch. Under ideal conditions, a single first-instar larva produces approximately  $4 \times 10^8$  polyhedra (38), although factors in the field, such as UV radiation and precipitation, likely reduce actual availability of virus as the inoculum for horizontal transmission. Data were analyzed using PASW Statistics 18 (release version 1800; SPSS, Inc., Chicago, IL). For data from both field tests, the effect of number of infected first-instar larvae (dose) versus different virus strains was analyzed with two-way analysis of variance. A one-way analysis of variance was then performed to compare the effects of virus strains at each dose tested. In all cases, means were separated using the Tukey HSD test (39).

## RESULTS

### Identification and characterization of LdMNPV isolates 203-WT and 203-NL

Several genotypes of LdMNPV were isolated from Gypchek, and their biological activities were investigated through bioassays to determine if significant differences in biological activity existed between genotypic variants. One of these isolates, strain 203, exhibited a potency that was about 2- to 4-fold greater than that of Gypchek (for strain 203, 50% lethal dose [LD<sub>50</sub>] of 2,335 OBs/ml and 95% confidence limits of 921 to 6,647 OBs/ml; for Gypchek, LD<sub>50</sub> of 7,151 OBs/ml and 95% confidence limits of 3,642 to 14,086). During plaque purification of strain 203, a variant was observed that did not cause lysis of infected Ld652Y cells, and it was also found not to cause liquefaction of infected larvae, in contrast to all other plaque-purified isolates of strain 203. This strain was designated 203-NL, for nonliquefaction. Analysis of genomic restriction endonuclease digestion profiles of isolates 203-WT and 203-NL revealed the presence of an *SpeI*-generated 12.1-kbp fragment only in digests of 203-WT and an 8.3-kbp *SpeI*-generated fragment specific to 203-NL (data not shown). The 12.1-kbp *SpeI* fragment contains the sequences from bp 56731 to 68850 within the sequenced genome of LdMNPV isolate 5-6 (22). Restriction endonuclease digestion of the 12.1- and 8.3-kbp *SpeI* fragment clones using *HpaI*, *MfeI*, *NotI*, *SpeI*, *BamHI*, *SspI*, *ScaI*, and *ClaI* revealed a potential deletion of about 3.8 kbp in length within the genomic region from bp 63426 to about 67500 (data not shown). Sequencing of the area from bp 63045 to 68408 revealed a deletion of 3,782 bp from bp 64002 to 67793 (Fig. 1A). The sequences in isolate 5-6 from bp 63982 to 64001 and 67794 to 67823 matched the corresponding sequences in isolate 203-NL (Fig. 1A). It is interesting that the GCTCGT sequence in isolate 5-6 is repeated at the beginning and end of the sequence that was deleted in 203-NL. Isolate 203-NL contains this sequence only at one end of the remaining sequence that matches 5-6. The deletion in 203-NL causes the loss of 131 of 269 amino acids of *gp37* from the amino-terminal end, all of open reading frame 69 and *v-chiA*, and 394 of 528 amino acids of *bro-c* from the carboxyl-terminal end (Fig. 1B).

**Characterization of larvae and Ld652Y cells infected with isolates 203-WT, 203-NL, 203-NL-rep, and 203-*chiA*-del.** Larvae infected with viral isolate 203-WT exhibited larval liquefaction (Fig. 2). In contrast, larvae infected with isolate 203-NL did not liquefy (Fig. 2). To determine whether the lack of liquefaction by 203-NL was due to the loss of viral genes caused by the deletion in this isolate, the deleted genes were replaced to generate 203-NL-rep (Fig. 1B). Restriction endonuclease digestion of genomic 203-NL-rep DNA with *SpeI* revealed a 12.1-kbp *SpeI* fragment in place of the 8.1-kbp *SpeI* fragment present in the genomic DNA of 230-NL (data not shown). Larvae infected with this isolate lique-



**FIG 1** (A) Nucleotide sequence demarking the region in 203-NL that is deleted compared to the sequence of isolate 5-6. The sequences in 203-NL at the ends of the deletion and the corresponding sequences in 5-6 are shown in boldface. The sequences that are duplicated in 5-6 that border the deletion in 203-NL are underlined. (B) Analysis of 203-NL and schematic representation of viruses used in this study. Arrows show the location and direction of ORFs in the genomic region containing the *v-chiA* gene. The *v-chiA* gene is shaded gray, and the *lacZ* gene is shaded black.

fied (Fig. 2). To determine if the lack of liquefaction by 203-NL was due to the loss of the *v-chiA* gene, a recombinant virus was generated in which the *v-chiA* gene was deleted and replaced with the *lacZ* gene. This viral construct, 203-*chiA*-del, retains 157 bp of the 1,676 bp that encodes V-CHIA (Fig. 1B). Larvae infected with this isolate did not liquefy (Fig. 2).

A novel approach was used to provide a quantifiable method for liquefaction of larvae through assessment of larval cuticle strength. Briefly, after virus-caused larval death, the larvae were weighed and placed in test tubes, a proportional amount of fluid to larval weight was added, the tube was vortexed, and the number of polyhedra was quantified and expressed as the number of poly-



**FIG 2** Photographs of larvae infected with 203-WT, 203-NL, 203-*chiA*-del, or 203-NL-rep. Fourth-instar larvae were infected with the indicated virus strains and photographed shortly after death.

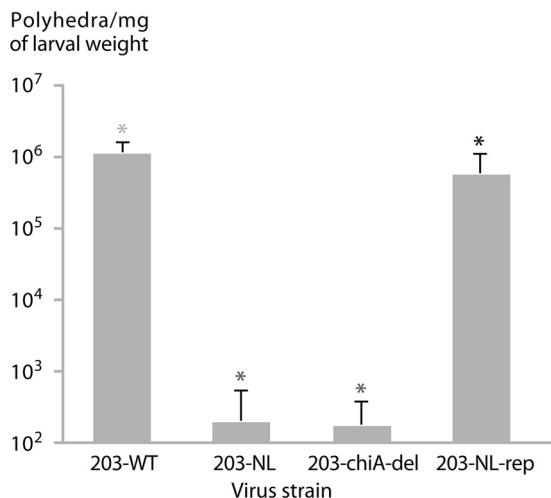


FIG 3 Average number of polyhedra released from larvae infected with the indicated viral isolates  $\pm$  standard errors. The average values are derived from between 22 and 33 larvae. Where differences were seen between strains, identical asterisk shading indicates that they are not significantly different from each other ( $P < 0.05$  by Tukey-Kramer test).

hedra released per mg of larval weight. Since V-CHIA is necessary for host cuticle degradation during the process of host liquefaction, it was anticipated that the cuticle of larvae treated as described above would readily break and release polyhedra into the fluid. If the integrity of the cuticle remained intact, breakage would occur to a far lesser extent. Larvae infected with 203-WT released an average of  $1.1 \times 10^6$  polyhedra/mg of larval weight after treatment (Fig. 3), which was significantly more polyhedra released from larvae infected with 203-NL (210/mg larval weight;  $P < 0.0001$ ) and 203-chiA-del (177/mg larval weight;  $P < 0.0001$ ). The number of polyhedra released from larvae infected with 203-

NL-rep,  $5.6 \times 10^6$ , was significantly greater than the average number of polyhedra released from larvae infected with 203-NL and 203-chiA-del ( $P < 0.0001$  for each). There was no significant difference in the number of polyhedra released after treatment of larvae infected with 203-NL and 203-chiA-del. One-way ANOVA was conducted to analyze the effect of LdMNPV strains on the number of polyhedra produced per mg of larval weight. There was a significant effect of LdMNPV strain at the  $P < 0.001$  level on the number of released polyhedra [ $F(3, 109) = 68.3$ ;  $P < 0.0001$ ].

Infection of Ld652Y cells with isolate 203-WT caused cell lysis (Fig. 4). In contrast, cells infected with isolate 203-NL or 203-chiA-del did not result in lysed cells. Infection of cells with 203-NL-rep did cause cell lysis (Fig. 4). These results were quantified by analysis of polyhedra release from infected cells. Cells infected with 203-NL or 203-chiA-del released significantly fewer ( $P < 0.001$  for each) polyhedra than cells infected with 203-WT (Fig. 5). Cells infected with isolate 203-NL-rep released significantly more polyhedra than cells infected with 203-NL or 203-chiA-del ( $P < 0.001$  for each). There was no significant difference in the number of polyhedra released from cells infected with 203-NL or 203-chiA-del. Cells infected with 203-NL-rep released significantly more polyhedra than cells infected with 203-WT (Fig. 5). One-way ANOVA was conducted to analyze the effect of LdMNPV strain on the number of polyhedra released from infected cells. There was a significant effect of LdMNPV strain at the  $P < 0.05$  level on the number of released polyhedra [ $F(3, 12) = 124.9$ ;  $P < 0.001$ ]. The total numbers (released from lysed cells plus present in intact cells) of polyhedra produced by isolates 203-WT, 203NL, 203-chiA-del, and 203-NL-Re were not significantly different (ANOVA).

**Laboratory bioassays.** The 50% lethal concentration ( $LC_{50}$ ) of 203-WT polyhedra used for the first field transmission experiments was about 4-fold lower than that of 203-NL polyhedra (203-WT, 3,349 polyhedra/ml diet and 95% fiducial limits of

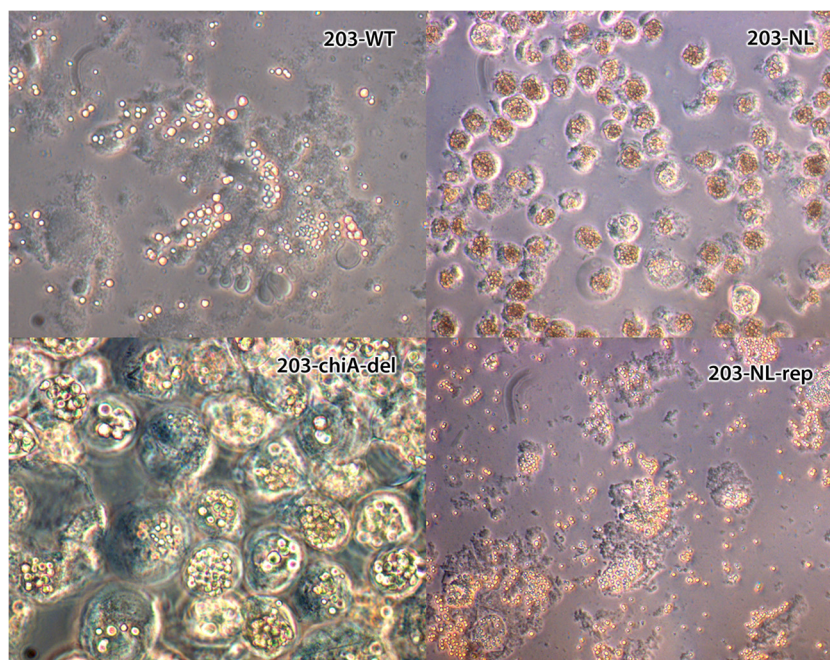


FIG 4 Photographs of Ld652Y cells infected with the indicated viral strains 7 days postinfection.

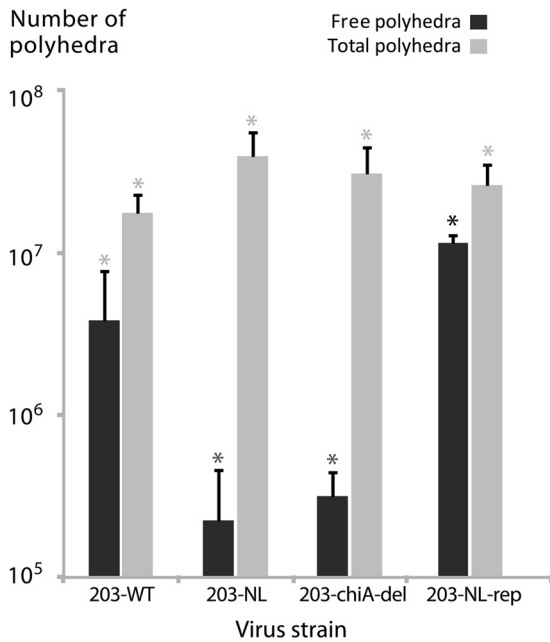


FIG 5 Average number of polyhedra released from infected Ld652Y cells 7 days after infection with the indicated viral isolates. The averages from 4 separate infections  $\pm$  standard errors are shown. Where differences were seen between strains, identical asterisk shading indicates that they are not significantly different from each other ( $P < 0.05$  by Tukey-Kramer test).

2,342 to 5,086; 203-NL, 745 polyhedra/ml diet and 95% fiducial limits of 269 to 2,000). The  $LC_{50}$  for 203-WT polyhedra was approximately 3-fold lower than that of 203-NL and 203-chiA-del polyhedra, which were used in the second field transmission study (203-WT, 46,519 polyhedra/ml diet and 95% fiducial limits of 30,634 to 71,087; 203-NL, 15,651 polyhedra/ml diet and 95% fiducial limits of 9,953 to 24,682; 203-chiA-del, 14,071 polyhedra/ml diet and 95% fiducial limits of 6,108 to 33,588). In both laboratory bioassays,  $LD_{50}$  values for 203-WT were higher than those of 203-NL and 203-chiA-del, in opposition to results of field trials.

**Field tests.** Our first field transmission experiment compared mortality in test larvae feeding on foliage contaminated by the cadavers of larvae killed by 203-WT or 203-NL. In every case, the greatest mortality in the field was seen in insects feeding on branches contaminated with the cadavers of larvae killed by 203-WT (Fig. 6A). Doses of 5, 10, 25, and 50 larvae infected with 203-WT resulted in significantly more death in test larvae than did test larvae placed on foliage that was dosed with larvae infected with 203-NL (Fig. 6A). The largest difference in larval mortality, 40% dosed with 203-WT versus 12% dosed with 203-NL, occurred at a dose of 25 infected larvae. At the highest dose of 50 larvae, significantly more larvae died on foliage dosed with 203-WT (38%) than on foliage dosed with 203-NL (22%).

The field transmission studies were repeated with the addition of viral strain 203-chiA-del. Similar to the results of the first field trial, there was greater larval mortality in larvae that fed on foliage dosed with 203-WT-infected larvae than in larvae that fed on foliage dosed with 203-NL (Fig. 6B). At doses of 5, 10, and 40 larvae, the differences were significant ( $P < 0.05$  for each). At these same doses, there was greater larval mortality of larvae that

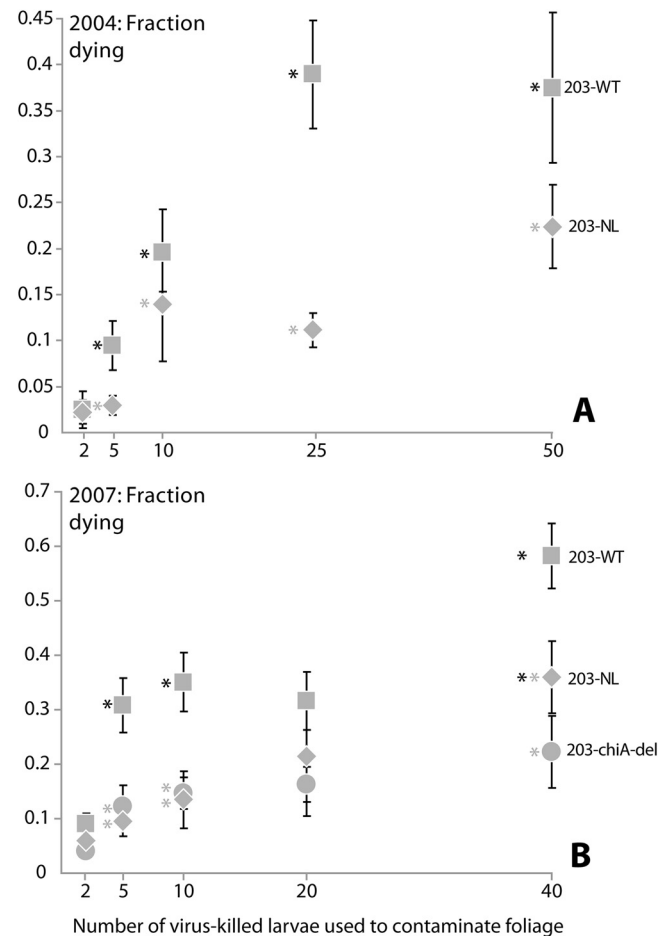


FIG 6 Mean mortality  $\pm$  SE in test of horizontal transmission of LdMNPV strains in confined field experiments. (A) In 2004, two strains were tested using 2 to 50 infected first-instar larvae as the dosing inoculum on red oak branches in bags (10 bags per dose,  $n = 10$ ). Twenty-five healthy second-instar larvae were put into each bag as test insects. (B) In 2007, three strains were tested, with 2 to 40 infected larvae at 8 bags per dose ( $n = 8$ ). Where differences were seen between strains, identical asterisk shading indicates that they are not significantly different from each other ( $P < 0.05$  by Tukey-Kramer test).

fed on foliage dosed with 203-WT-infected larvae than on foliage dosed with 203-chiA-del-infected larvae. At a dose of 40 larvae, significantly more larvae died on foliage dosed with larvae infected with 203-NL larvae than with larvae that fed on foliage dosed with larvae infected with 203-chiA-del. At all doses, there were no significant differences in mortalities of larvae feeding on foliage dosed with 203-NL or 203-chiA-del (Fig. 6B).

## DISCUSSION

Liquefaction of larvae infected with LdMNPV 203-WT indicated that *v-chiA* generates an active protein, and the presence of *v-chiA* was previously confirmed by sequencing of the LdMNPV genome (22). This study confirms that LdMNPV *v-chiA* is necessary for larval liquefaction. The occurrence of larval liquefaction has been presumed to enhance dissemination of polyhedra in the field and consequently to facilitate horizontal virus transmission. The results of this study showed significantly greater mortality of larvae feeding on foliage dosed with larvae lethally infected with 203-WT than on foliage dosed with 203-NL and 203-chiA-del. These re-

sults offer the first experimental evidence that larval liquefaction enhances viral dissemination and horizontal transmission in the field. This result was consistently found over the course of two separate field tests using 203-NL, as well as with one field test that included 203-*chiA*-del for comparison. The differences found were not due to 203-WT having a greater potency than 203-NL or 203-*chiA*-del. These isolates exhibited 3- to 4-fold greater potencies in laboratory bioassays than 203-WT.

To find a means of quantifying the liquefaction differences between larvae infected with 203-WT and 203-NL other than sight of melting, we developed a method that assesses cuticle integrity through release of polyhedra from viral cadavers. The integrity of the cuticle of virus-killed larvae infected with 203-WT and 203-NL-rep was easily ruptured, in contrast to that of larvae infected with 203-NL and 203-*chiA*-del. Cuticle rupture caused the release of polyhedra liberated from lysed cells within larvae, in contrast to larvae infected with 203-NL and 203-*chiA*-del. This method provides a means of quantification of liquefaction and a means of assessing the impact of viral genes on the liquefaction process. Our results also show that the loss of *v-chiA* prevented lysis of Ld652Y cells in culture, thereby preventing polyhedron release from infected cells. A recent study by Katsuma et al. (40) showed that loss of V-CATH n-linked glycans in recombinant BmNPV strains with altered *v-cath* N-linked glycosylation sites prevented polyhedron release from virus-infected BmN cells in culture.

Viral isolate 203-NL also has mutations in the *gp37* and *bro-c* genes, where approximately 49 and 75%, respectively, of these genes were deleted. The *gp37* gene in baculoviruses generates a protein that forms the matrix of a structure termed a spindle body (41, 42). It has been suggested (43) that the *gp37* gene expressed in LdMNPV functions to disrupt the peritrophic matrix lining the host midgut. Analysis of the *Spodoptera litura* MNPV GP37 indicated that it can bind to the chitin component of the peritrophic membrane (44). While the function of GP37 in MNPVs remains unclear, the results of this study indicate that deletion of most of the *gp37* gene from 203-NL did not have a negative impact on viral replication, polyhedron formation, or viral potency. Earlier studies by Cheng et al. (45) indicated that the AcMNPV *gp37* gene is not essential for viral replication. The function of baculovirus *bro* genes is also unknown. These genes constitute a multigene family that is typically present in multiple copies within double-stranded DNA viruses (46). In the BmNPV, Kang et al. (47) were unable to purify mutant strains of virus that lacked *bro-d* or strains that lacked both *bro-a* and *bro-c*, suggesting that these genes are necessary for viral replication. In addition, Zemskov et al. (48) proposed that BmNPV BRO-A and BRO-C function as DNA binding proteins. The deletion of most of the LdMNPV *bro-c* gene from 203-NL had no obvious impact on viral replication, polyhedron formation, or potency.

Horizontal transmission of LdMNPV in the field has been explored by a number of researchers, often in the context of explaining observed disease dynamics (14, 15, 36, 37, 49–51). In the majority of these studies, the exact strain of virus infecting larvae was unknown and considered peripherally important, because the primary purpose of the research was to replicate or observe epizootics in the field, which likely involve hundreds of strains of LdMNPV. Only one such field study has involved the use of a patently mutated or engineered strain of LdMNPV (27). In that study, the insertion of the *lacZ* reporter gene into the baculovirus genome had no observed effect on larval liquefaction; indeed, liquefaction

of dead larvae was a necessary part of the sampling protocol. Therefore, the discovery of a nonliquefying variant of LdMNPV allowed the importance of this aspect of infection to be tested.

An obvious way that liquefaction, or a lack thereof, could impact transmission is via the mechanism by which infection occurs: ingestion of OBs on the surface of leaves. In the case of an insect not liquefied by a virus strain lacking functional *v-chiA* or *v-cath*, the larval cadaver could remain intact until weather conditions dislodge it from leaves or UV radiation inactivates the ODV inside (52–56). Such a viral genotype would reduce the opportunity for horizontal or vertical transmission to occur, and this reduced fitness would quickly remove it from the population. In contrast, when early-instar gypsy moths are killed by wild-type LdMNPV, they create a silk pad on the surface of leaves, attach to it, and remain in place until dead and liquefied (V. D'Amico, personal observation). Under these circumstances, rainfall has been shown to move LdMNPV from the site of a cadaver to other parts of leaves and branches, facilitating transmission (57). The effects of changes in the viral *v-chiA* and *v-cath* on other behaviors of infected larvae are not known. Larvae infected with a recombinant strain of LdMNPV that does not produce the enzyme ecdysteroid UDP-glucosyltransferase (EGT) exhibited changes in climbing behavior (58), and LdMNPV infection in general inhibits molting and, presumably, the behaviors associated with that process (59). The behavior of virus-infected larvae has been shown to have important effects in the LdMNPV system (60). Larvae that moved off leaves or failed to secure themselves may, in our experiments, have reduced overall mortality in the test larvae. Such an effect could be even more pronounced in field populations, when larvae are not confined in any way.

It is somewhat uncommon for researchers to explore both the genetic structure of baculoviruses and the effects of differing genotypes on field transmission. This may not be surprising, considering how few baculoviruses are used for microbial control, but such studies can be fruitful in cases where the virus also plays a large role in the natural population dynamics of a pest insect and may persist through dissemination and transmission (27, 61–65). In the case of LdMNPV, field transmission experiments can help to predict the total impact of a biopesticide over the field season. Key to the usefulness of such experiments, however, is the extent to which they mirror actual field conditions. This entails balancing two opposing requirements: allowing insects as much freedom of movement and exposure to natural environmental conditions as possible while retaining the test insects in numbers sufficient to show differences in mortality caused by different virus strains. We believe that the field method used in this study is a reasonable approximation of environmental conditions during gypsy moth outbreaks (36, 37), giving us confidence that the amount of secondary transmission seen in the treatments is representative of what would actually occur following a virus application in the field. The importance of using these techniques is seen in the contrast observed between the results of our laboratory bioassays and the transmission experiments we performed in the field. These effects often cannot be observed or accurately predicted using laboratory assays alone.

## ACKNOWLEDGMENTS

We thank Phil Taylor for his untiring help in the field and Nancy Hayes-Plazolles for her untiring help in the laboratory. Many thanks to our

esteemed colleague Kelli Hoover of Pennsylvania State University for her presubmission review of our manuscript.

This work was funded by the U.S. Forest Service.

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