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Identification of a *Lymantria dispar* Nucleopolyhedrovirus Isolate That Does Not Accumulate Few-Polyhedra Mutants during Extended Serial Passage in Cell Culture

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During *Lymantria dispar* multinucleocapsid nucleopolyhedrovirus (LdMNPV) replication in cell culture, few-polyhedra (FP) mutants arise at a high frequency and become predominant after only a few serial passages. The formation of LdMNPV FP mutants was an impediment to successful production of polyhedra in cell culture bioreactors. We have isolated and characterized a strain of LdMNPV, termed 122b, that did not accumulate FP mutants after extended serial passage in cell culture. Wild-type virus strains exhibited decreased polyhedra production and increased budded virus (BV) release, and FP mutants were the predominant virus type present after only a few serial passages in *L. dispar* 652Y cells. In contrast, isolate 122b exhibited stable polyhedra and BV production through 14 serial passages. Isolate 122b produced more BV than wild-type virus and essentially the same amount of BV as FP mutants. FP mutants derived from isolate 122b were isolated and partially characterized. These mutants produced approximately 10-fold fewer polyhedra than 122b and essentially the same amount of BV as 122b. These results suggest that FP mutants will not become predominant during serial propagation of 122b because 122b FP mutants do not release more BV than 122b. Isolate 122b was also found to be capable of generating productive infections at higher cell:virus infection ratios than wild-type virus.

Key Words: **baculovirus; *Lymantria dispar* nucleopolyhedrovirus; few-polyhedra mutant; cell culture; stable polyhedra production; *Lymantria dispar;* biological control.**

INTRODUCTION

Insect baculoviruses are a group of biological control agents that have received considerable attention in the past 20 years. These viruses have been isolated from over 300 insect species, exhibit relatively narrow or specific host ranges, can be aerially applied to agricultural crops and forests, and can be manipulated through genetic engineering to enhance viral efficacy (Bonning and Hammock, 1992; Winstanley and Rovesti, 1993; Miller, 1995). The *Lymantria dispar* multinucleocapsid nucleopolyhedrovirus (LdMNPV) product, Gypchek, is used as a biocontrol agent for the gypsy moth, *L. dispar* (L.) (Reardon and Podgwaite, 1994). The gypsy moth is a serious defoliating insect pest in the northeastern United States and is spreading into the south and midwest. Gypsy moth larvae feed on over 400 species of trees (Liebhold *et al.*, 1995).

The LdMNPV is a baculovirus within the family Baculoviridae, which is composed of invertebrate-specific viruses containing large double-stranded DNA genomes (Burgess, 1977; Schafer *et al.*, 1979). Nucleopolyhedroviruses (NPVs) have two distinct morphological forms (see Blissard and Rohrmann, 1990). The viral infection cycle begins with ingestion of a polyhedron, a crystalline protein matrix that contains virus particles. The polyhedron dissolves within the insect midgut, thereby releasing the occluded virus particles that infect the insect. Early after infection, a budded form of virus (BV) is produced that exits the infected cell and gives rise to a systemic infection within the insect host. Late in the infection cycle, virions are produced that become occluded into a polyhedron, which is the form of virus used for insect control. The LdMNPV has been completely sequenced and has a genome of 161,046 bp (Kuzio *et al.*, 1999), and the replication cycle of the LdMNPV has been characterized (Riegel and Slavicek, 1997).

The LdMNPV has the advantage of specificity for the gypsy moth in contrast to chemical insecticides and most other biological control agents (Lewis and Podgwaite, 1981). Consequently, LdMNPV is the agent of choice for gypsy moth control in environmentally sensitive areas and areas that contain threatened and endangered insect species. However, LdMNPV is not extensively used because of lack of commercial scale production. Currently, LdMNPV is produced on a limited basis by the U.S. Forest Service and the Animal

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and Plant Health Inspection Service in gypsy moth larvae. The Forest Service has registered the virus with the Environmental Protection Agency under the name Gypchek for use as a gypsy moth control agent. Production of Gypchek is expensive; it currently costs approximately $30.00 to generate sufficient virus to treat a hectare of forest. Production of virus in cell culture is an alternative to larval production and has the advantages of a controllable production system, a pure product, and a lower cost.

An impediment to successful production of LdMNPV in cell culture has been the formation of few-polyhedra (FP) mutants. During serial passage of NPVs in cell culture, FP mutants arise at a high frequency and become the predominant virus type due to production of more BV than wild-type virus. These mutants have the characteristics of altered plaque morphology, production of few polyhedra, synthesis of polyhedra that lack viral nucleocapsids, increased release and/or production of BV, defective envelopment of viral nucleocapsids within the nucleus, and, in the LdMNPV, a decrease in the percentage of infected cells that produce polyhedra in comparison to wild-type (many-polyhedra; MP) virus (Potter *et al.*, 1976; Fraser and Hink, 1982; Slavicek *et al.*, 1992, 1995; Harrison and Summers, 1995).

Specific mutations in FP mutants of *Autographa californica* NPV (Ac*M*NPV) and *Galleria mellonella* NPV (Gm*M*NPV) have been identified and characterized. The appearance of the FP phenotype during serial passage in cell culture often correlates with the presence of DNA insertions/deletions (Fraser *et al.*, 1983; Kumar and Miller, 1987; Cary *et al.*, 1989) and the absence of a specific 25-kDa protein (Fraser *et al.*, 1983). These insertions/deletions range from approximately 0.4 to 2.8 kb and occur predominately within a specific region (36–37 map units) on the Ac*M*NPV and Gm*M*NPV genomes. Analysis of this genomic region identified a gene coding for a 25-kDa protein that is necessary for the MP phenotype (Beames and Summers, 1989). Several different mutations in the *25K FP* gene generate all phenotypic characteristics of FP mutants in the Ac*M*NPV (Harrison and Summers, 1995). In contrast to other baculovirus FP mutants studied, most Ld*M*NPV FP mutants do not contain large insertions or deletions in the *25K FP* gene (Slavicek *et al.*, 1995). Of five Ld*M*NPV FP mutants characterized, only one contained a large DNA deletion. The others contained single nucleotide insertions or small deletions (Bischoff and Slavicek, 1997). Ld*M*NPV FP mutants arise very quickly during viral replication in cell culture and become predominant after only three to five serial passages (Slavicek *et al.*, 1995, 1996).

Production of baculovirus polyhedra in cell culture bioreactors involves the progressive scaleup into successively larger-volume bioreactors until the final stage is reached. The initial and intermediate bioreactor infections are used as the inoculum for the next-larger-volume bioreactor. These scaleup steps require serial passage of virus until the final infection. The very rapid formation of Ld*M*NPV FP mutants would prevent successful production of this virus in cell culture bioreactors unless a strain of virus that does not exhibit the FP mutant formation problem is developed. In this report, we describe the isolation and characterization of a strain of Ld*M*NPV that does not accumulate FP mutants during extended serial passage in cell culture.

MATERIALS AND METHODS

Cell Lines and Viral Isolates

The *L. dispar* 652Y (Ld652Y) ovarian cell line was used for serial passage studies (Goodwin *et al.*, 1978). The cells were propagated in complete medium [Goodwin's IPL-52B medium (JRH Biosciences, Lexena, KS), with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) and 6.25 mM glutamine (Gibco BRL, Rockville, MD)]. Plaque-purified Ld*M*NPV isolates A21, B21, 122, and 163 were used in this study as wild-type controls and were generated as previously described (Slavicek *et al.*, 1995). Isolate A21-MPV was plaque-purified from isolate A21 and exhibited greater polyhedra production stability than wild-type virus (Slavicek *et al.*, 1996). Ld*M*NPV isolates A21-2, B21-1, 122-2, 163-2, and 5-6 are FP mutants (Slavicek *et al.*, 1996). Ld*M*NPV isolates 122b, 122bFP1, and 122bFP2 were isolated as described below or under Results.

Identification of LdMNPV Isolate 122b

Ld652Y cells were plated at a density of 2×10^5/ml and infected with 0.1 tissue culture infectious dose ($TCID_{50}$) unit per cell of viral isolate A21, B21, 122, or 163. Seven days after infection, the cells were harvested and pelleted by centrifugation at 550*g* for 5 min. The supernatant containing the BV was removed and used to determine BV $TCID_{50}$ and for inoculum for a second passage (infections were performed at 0.1 $TCID_{50}$ unit per cell). This process was repeated twice more for a total of three passages. Budded virus from the third passage was plaque-purified, and plaques were used to infect Ld652Y cells (1×10^4 cells/well) in P96 well plates. Twenty viral isolates that exhibited a MP phenotype were then passaged 8 times in Ld652Y cells as described above. BV from the 8th passage was plaque-purified, and plaques were used to infect Ld652Y cells in P96 well plates. Forty-two viral isolates exhibiting a MP phenotype were serially passaged from 10 to 13 times as described above. Only one isolate, termed 122b, that retained a MP phenotype after all the serial passages was found.

$TCID_{50}$ Determination, Plaque Assay, Quantification of the Percentage of FP Mutants Present, and REN Analysis

Viral titers from cell culture medium were determined by the end-point dilution assay first described by Reed and Muench (1938) and adapted by Summers and Smith (1987). Ld652Y cells (1×10^4/well) were seeded in P96 well plates and allowed to attach for 1 h. The cells were infected with eight separate dilutions of virus at 10^{-1} to 10^{-11} and the plates were incubated at 27°C for 2 weeks. The wells of each separate dilution series were scored positive or negative for vial infection, and the results were used to calculate viral titer in number of infective doses per unit of inoculum according to the equation developed by Reed and Muench (1938). The viral titer was expressed as $TCID_{50}$ per milliliter of cell culture medium.

Viral isolates were plaque-purified after each selected passage as previously described (Slavicek *et al.*, 1995). After a 2-week incubation period at 27°C, the plaques that were clearly separated were transferred to P96 well plates seeded with Ld652Y cells. After 1 week at 27°C the infections were scored as exhibiting either a FP phenotype if the cells contained from approximately 1 to 10 polyhedra or a MP phenotype if the majority of cells appeared opaque due to the presence of many polyhedra per cell.

Viral isolate 122 and 122b genomic DNAs were analyzed for restriction fragment length polymorphisms. BV was pelleted from medium harvested from infected cells by centrifugation at 112,700*g* for 45 min at 4°C, and the pellet was resuspended in 0.1× TE (0.01 M Tris, pH 8.0, 1 mM EDTA, pH 8.0) overnight at 4°C. Viral DNA was isolated by the addition of 1 vol of extraction buffer (20 mM Tris, pH 7.5, 120 mM NaCl, 20 mM EDTA, pH 8.0, 1% SDS, and 0.04 mg/ml proteinase K) and incubation at 50°C for 1 h. The sample was then adjusted to 1% Sarkosyl (Sigma, St. Louis, MO) and incubated for an additional hour at 50°C. The solution was extracted with 1 vol of phenol saturated with 0.01 M Tris–0.001 M EDTA, pH 7.5, and 2 vol of chloroform:isoamyl alcohol (24:1), and the nucleic acid was precipitated with ethanol. Viral DNA was digested with the restriction enzymes *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Bam*HI, and *Pst*I, and the fragments were separated by agarose gel electrophoresis and visualized after staining with ethidium bromide.

Serial Passage of LdMNPV Isolates

The passage study was initiated by infection of Ld652Y cells, plated at a density of 2×10^5/ml, with viral isolates A21, B21, 122, 163, or 122b at 0.1 $TCID_{50}$ unit per cell. Passage 1 material was harvested on day 7 postinfection (p.i.). Polyhedra were pelleted by centrifugation at 550*g* for 5 min. The supernatant containing the BV was removed and used to determine BV $TCID_{50}$, for plaque assays to determine the percentage of virus that exhibited a MP or FP phenotype, and for inoculum for passage 2 (infections were performed at 0.1 $TCID_{50}$ unit per cell). The polyhedra pellet was resuspended in 5.0 ml water and sonicated for 30 s, and the number of polyhedra present was determined by the counting of three separate samples with a hemacytometer and expressed as polyhedra/ml. The number of polyhedra/ml was multiplied by 5 to calculate the number of polyhedra per flask. Serial passages 2 through 14 were performed as described above with BV from the previous passage as inoculum. Once the isolates had become predominately FP, as indicated by a 10-fold reduction in the number of polyhedra produced per flask and visual confirmation of the presence of few polyhedra/infected cell, serial passage was ended. Each viral strain was serially passaged three times and the results are expressed as the averages of three determinations. ANOVA and regression analysis of data were performed with the StatView program from Abacus Concepts (Berkeley, CA).

Transmission Electron Microscopy (TEM)

Polyhedra produced by the isolates 122b and A21-MPV were examined for the presence of viral nucleocapsids by transmission electron microscopy. The polyhedra were prepared and sectioned for electron microscopic analysis as previously described (Slavicek *et al.*, 1992). The relative size of polyhedra was determined by measurement of the diameter of polyhedral cross sections in TEM photographs.

Analysis of BV and Polyhedra Production and Bioassys

T25 flasks were seeded with 1×10^6 Ld652Y cells and the cells infected with LdMNPV isolates 122b, A21-MPV, A21-2, B21-1, 122-2, 163-2, 5-6, 122bFP1, or 122bFP2 at 0.1 $TCID_{50}$ unit per cell. All infections were performed in triplicate. BV was harvested 7 days p.i. and the amount present was determined by $TCID_{50}$ analysis. The number of polyhedra produced in infections by isolates 122b, 122bFP1, and 122bFP2 was determined as described above.

Viral isolates were bioassayed by the placement of 100 2nd instar *L. dispar* larvae on a disk of diet 10 cm in diameter which contained 1×10^5 surface-applied polyhedra of viral isolates 122bFP1, 122bFP2, or A21-MPV. After 24 h the larvae were placed on fresh diet and larval mortality was monitored daily and quantified.

Polyhedra Production as a Function of Increasing Virus:Cell Ratios

Production of baculovirus polyhedra in cell culture bioreactors requires several infections in progressively

greater volume bioreactors. A virus strain that produces more BV than wild-type virus should be more economical to produce since a smaller volume of inoculum would be required to achieve a successful infection, and potentially fewer intermediate stage bioreactors would be necessary. To determine the minimal amount of inoculum required to achieve successful polyhedra production, Ld652Y cells were infected with 122b or wild-type virus at increasing virus:cell ratios and polyhedra production was quantified.

T25 flasks were seeded with 1×10^6 Ld652Y cells and infected with either 122b or A21-15 (a wild-type plaque-purified isolate of genotypic variant A21). Appropriate amounts of virus inoculum were used to produce infections at virus:cell ratios of 1:1 (1×10^6 $TCID_{50}$ units:1×10^6 cells), 1:5 (2×10^5 $TCID_{50}$ units:1×10^6 cells), 1:10 (1×10^5 $TCID_{50}$ units:1×10^6 cells), 1:50 (2×10^4 $TCID_{50}$ units:1×10^6 cells), 1:100 (1×10^4 $TCID_{50}$ units:1×10^6 cells), 1:500 (2×10^3 $TCID_{50}$ units:1×10^6 cells), and 1:1000 (1×10^3 $TCID_{50}$ units:1×10^6 cells). The infections were harvested at 11 days p.i., and the number of polyhedra present was quantified. All infections were performed in quadruplicate. The amount of cells used in these infections was held constant since the number of cells that can be seeded into a T25 flask is limited due to contact inhibition of cell growth. The addition of 1×10^7 cells or greater would have stopped cell division due to contact inhibition, and the cells would have been refractory to viral infection.

RESULTS

Identification and Isolation of LdMNPV Isolate 122b

To identify LdMNPV isolates that did not or were less prone to accumulate FP mutants during serial passage in cell culture, a screen using serial passage coupled with plaque-purification was used. We hypothesized that a mutation that would impart a stable polyhedra production phenotype could occur in the virus during DNA replication. During serial passage under the conditions employed, the virus underwent several rounds of DNA replication within each passage, thereby providing many opportunities for mutations to occur. Plaque-purification of virus after serial passage and identification of virus exhibiting a MP phenotype provided a means of isolating the desired mutant.

LdMNPV isolates A21, B21, 122, and 163 were used in this study and are genotypic variants isolated from Gypchek (Forest Service formulation of a mixture of LdMNPV genotypes). These isolates were serially passaged in cell culture, and after the third passage virus was plaque-purified to isolate virus strains that exhibited a MP phenotype. A total of 20 viral plaques (6, 5, 5, and 4 of A21, B21, 122, and 163, respectively) that exhibited a MP phenotype after the first series of 3 serial passages were identified. These viral isolates were then serially passaged 8 times, virus from the 8th passage was plaque-purified, and a total of 42 isolates (1, 1, 17, and 23 of A21, B21, 122, and 163, respectively) that exhibited a MP phenotype were found. These viral isolates were then serially passaged 12 times. Most of the infections contained cells exhibiting a FP phenotype after only 3 to 6 serial passages, and with one exception, the rest did so by the 9th passage. Only one isolate, designated 122b, still exhibited a MP phenotype after the 12th passage and was selected for further studies.

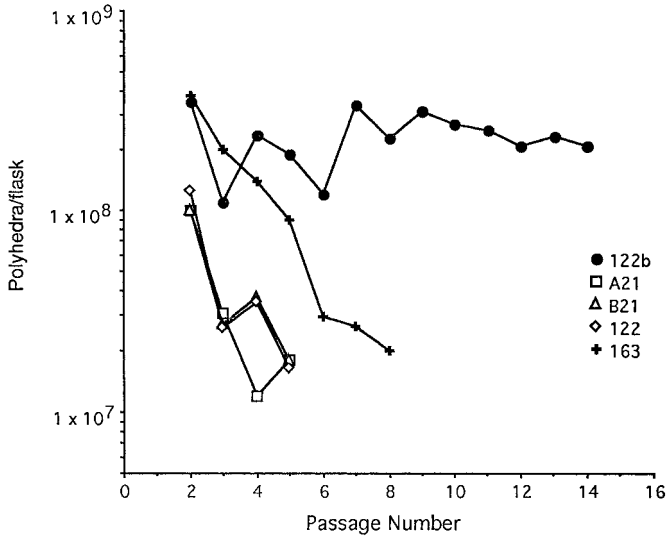

FIG. 1. Polyhedra production during serial passage in Ld652Y cells. The number of polyhedra produced after each serial passage was determined and expressed as the number of polyhedra/flask. All serial passages were initiated with plaque-purified viral isolates. The values shown are the averages of three determinations.

Serial Passage of 122b and LdMNPV Wild-Type Viral Lines

The stability of the MP phenotype of 122b during serial passage was compared to the stability of plaque-purified viral lines of wild-type LdMNPV isolates A21, B21, 122, and 163. Viral isolates 122b, A21, B21, 122, and 163 were serially passaged until the infected cells exhibited the FP phenotype or up to 14 times in Ld652Y cells. These infections and passages were carried out simultaneously under identical conditions with a pooled source of Ld652Y cells.

The amount of polyhedra produced per flask by wild-type isolates A21, B21, 122, and 163 decreased significantly (polynomial regression, A21, $R = 0.85$, $R^2 = 0.72$, $P = 0.003$, $df = 2, 11$; B21, $R = 0.88$, $R^2 = 0.77$, $P = 0.001$, $df = 2, 11$; 122, $R = 0.90$, $R^2 = 0.82$, $P = 0.001$, $df = 2, 11$; 163, $R = 0.82$, $R^2 = 0.67$, $P = 0.002$, $df = 2, 11$) through the first 5 serial passages (Fig. 1). After the 2nd passage the isolates

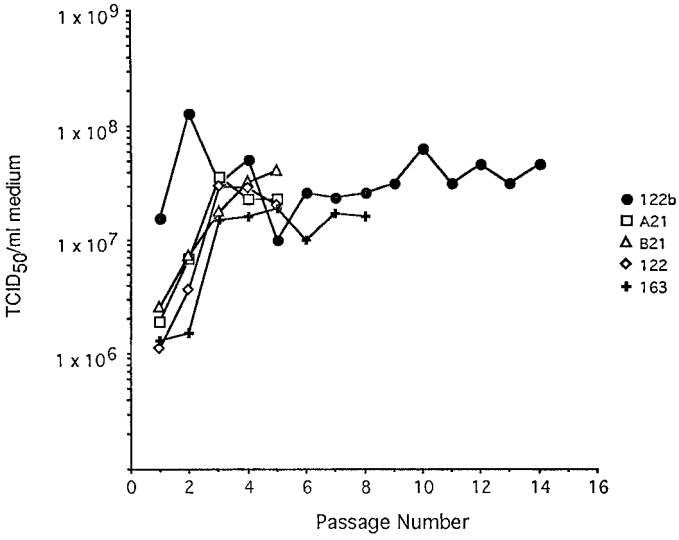

FIG. 2. Budded virus production during serial passage in Ld652Y cells. The $TCID_{50}$ values were determined after each serial passage and are expressed as the $TCID_{50}$/ml of cell culture medium. All serial passages were initiated with plaque-purified viral isolates. The values shown are the averages of three determinations.

produced approximately 1×10^8 polyhedra/flask (T25), and after the 5th passage, only $1–2 \times 10^7$ polyhedra/flask were generated. After the 5th passage, cells infected by these isolates contained very few polyhedra. Isolates A21, B21, 122, and 163 exhibited decreases in polyhedra production of approximately 10-fold during serial passage. In contrast, isolate 122b exhibited stable polyhedra production with no significant change (polynomial regression, $R = 0.17$, $R^2 = 0.03$, $P = 0.864$, $df = 2,38$) through 14 serial passages (Fig. 1).

The amount of BV produced, expressed as $TCID_{50}$ values, by isolates A21, B21, 122, and 163 increased significantly (polynomial regression, A21, $R = 0.78$, $R^2 = 0.61$, $P = 0.004$, $df = 2,14$; B21, $R = 0.84$, $R^2 = 0.71$, $P = 0.001$, $df = 2,14$; 122, $R = 0.82$, $R^2 = 0.67$, $P = 0.001$, $df = 2,11$; 163, $R = 0.75$, $R^2 = 0.57$, $P = 0.004$, $df = 2,15$) through the first 3 serial passages and then remained at approximately the same levels through passages 4 and 5 (Fig. 2). These isolates exhibited increases in $TCID_{50}$ values of approximately 10-fold from the 1st to the 3rd passage. In contrast, isolate 122b exhibited a $TCID_{50}$ value approximately 10-fold greater than that of the wild-type isolates at the 1st passage and then produced essentially the same amount of BV with no significant change (polynomial regression, $R = 0.294$, $R^2 = 0.09$, $P = 0.609$, $df = 2, 41$) through the 14th passage.

After the 14th serial passage of isolate 122b, FP mutants were not evident by visual inspection of infected cells. In contrast, after from 3 to 8 serial passages, infections by isolates A21, B21, 122, and 163 appeared to be mostly FP mutants. The percentage of FP mutants present in infections by these isolates after the 3rd (isolates A21, B21, and 122) or 8th (isolate 163) passage ranged from 71 to 92%.

Genotypic Analysis of Isolate 122b

The genomes of isolates 122b and 122 were analyzed for restriction fragment length polymorphisms to determine whether isolate 122b was derived from isolate 122 and whether genotypic differences between these isolates could be identified. Isolate 122b and 122 genomic DNA was restricted with B*gl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Bam*HI, and *Pst*I restriction endonucleases. The DNA fragments were separated by agarose gel electrophoresis and visualized following staining with ethidium bromide. There were no differences in the digestion profiles of isolates 122b and 122 generated with *Eco*RI, *Eco*RV, *Hin*dIII, *Bam*HI, and *Pst*I restriction endonucleases. Isolate 122b contained a *Bgl*II restriction endonuclease site at the genomic site of approximately 149.7 kb that was lacking in isolate 122. The presence of this site results in the formation of fragments of approximately 11.7 and 4.4 kb in isolate 122b, whereas isolate 122 contained a 16.1-kb fragment (data not shown).

Analysis of Isolate 122b Polyhedra

The size and morphology of isolate 122b polyhedra and virion occlusion were investigated through analysis of electron micrographs of polyhedra. Isolate 122b polyhedra exhibited the same morphology and approximately the same levels of virion occlusion as those of wild-type isolate A21 polyhedra (Figs. 3A and 3B, respectively). To determine whether the levels of virion occlusion were the same, the number of virions present in electron micrographs of polyhedra cross sections were counted and expressed as the number of virions/μm^2 of polyhedra cross-section area. The polyhedra were sectioned randomly with respect to the cutting plane, thereby generating representative cross sections from all areas of the polyhedra. Isolate 122b and A21 polyhedra contained 14.0 and 15.7 virions/μm^2 of polyhedra cross-section area, respectively, which were not statistically different ($F = 0.74$, $df = 1,49$, $P = 0.40$).

Visual comparison of 122b and A21 polyhedra micrographs suggested that 122b polyhedra were smaller than wild-type polyhedra. The relative diameters of 122b and wild-type polyhedra were determined by measurement of the diameter of polyhedra cross sections in electron micrographs. Since the polyhedra were sectioned randomly with respect to the cutting plane, measurement of cross section diameter provides an accurate relative determination of polyhedron diameter. The diameter of 122b polyhedra was found to be significantly ($F = 25.1$, $df = 1, 49$, $P = 0.0001$) less than that of wild-type polyhedra (Fig. 3C). The

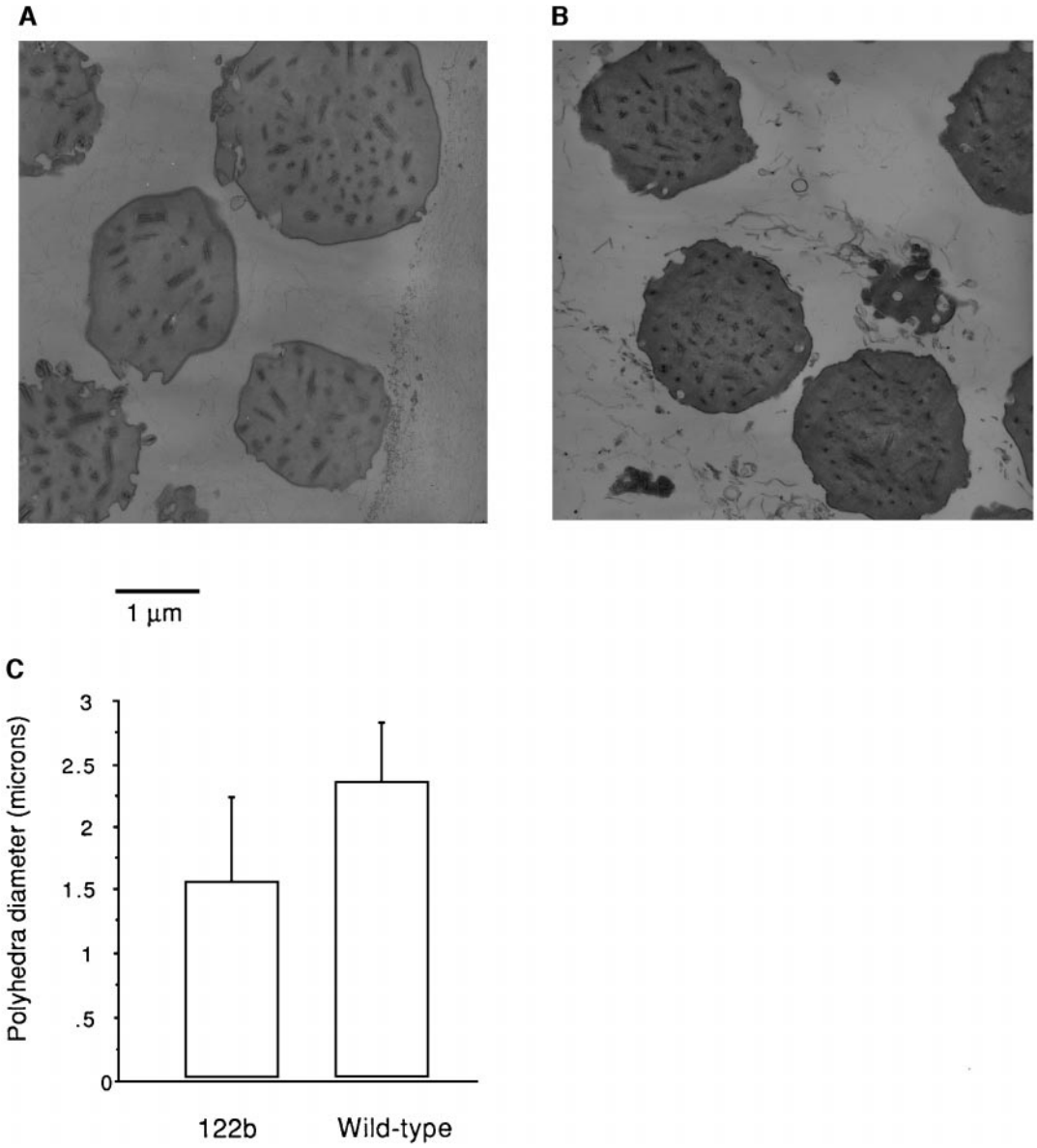

FIG. 3. Electron micrographs of polyhedra cross sections and determination of relative polyhedral diameter. Representative cross sections of polyhedra produced by isolate 122b (A) and A21-MPV (B). The relative diameters of isolate 122b and wild-type polyhedra were determined and expressed in microns (C). The values are the averages of 50 determinations for each isolate. One standard deviation is shown.

diameter of 122b polyhedra is approximately 38% less than that of wild-type polyhedra.

Comparison of 122b and FP Mutant BV Production

In agreement with previous studies (Slavicek *et al.*, 1995, 1996) BV production increased as FP mutants arose and became predominant during serial passage in Ld652Y cells (Fig. 2). In contrast, BV production by isolate 122b was stable during serial passage and was approximately 10-fold greater than that by wild-type virus. The finding that isolate 122b produced approximately the same amount of BV as isolates A21, B21, 122, and 163 after FP mutants became predominant suggested that 122b produces more BV than wild-type virus and an amount similar to that produced by FP mutants. The amount of BV produced by isolate 122b was compared to that by FP mutants to determine whether comparable levels of BV were produced (Fig. 4). FP mutants A21-2, B21-1, 122-2, 163-2, and 5-6 were used for this investigation. These mutants have deletions or single-nucleotide insertions in the *25K FP* gene (Bischoff and Slavicek, 1997). No significant difference was found in the amount of BV produced by isolate 122b compared to the amount produced by FP isolates A21-2, B21-1, 122-2, 163-2, and 5-6 (Fig. 4) (A21-2, $F = 0.03$, $df = 1, 5$, $P = 0.86$; B21-1, $F =$

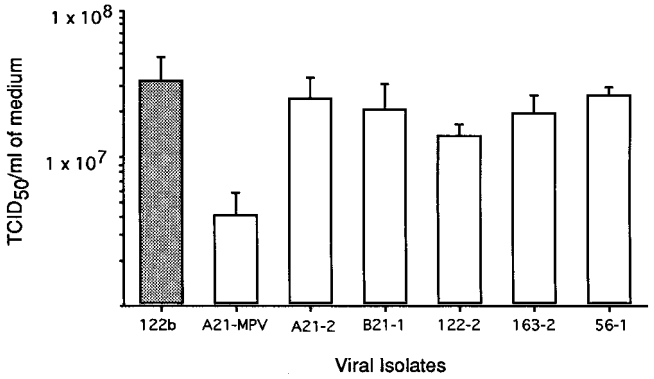

FIG. 4. Budded virus production by LdMNPV isolates. The amount of BV produced by isolates 122b and A21-MPV and the few polyhedra mutants A21-2, B21-1, 122-2, 163-2, and 5-6 was determined and expressed as the number of $TCID_{50}$ units/ml of medium. The values shown are the averages of three determinations + one standard deviation.

0.01, $df = 1, 5$, $P = 0.93$; 122-2, $F = 0.06$, $df = 1, 5$, $P = 0.82$; 163-2, $F = 0.08$, $df = 1, 5$, $P = 0.79$; 5-6, $F = 0.07$, $df = 1, 5$, $P = 0.80$). Isolate 122b did produce significantly more BV than isolate A21-MPV ($F = 33.1$, $df = 1, 5$, $P = 0.005$), as did the FP mutants (A21-2, $F = 17.9$, $df = 1, 5$, $P = 0.01$; B21-1, $F = 9.7$, $df = 1, 5$, $P = 0.04$; 122-2, $F = 24.2$, $df = 1, 5$, $P = 0.008$; 163-2, $F = 25.9$, $df = 1, 5$, $P = 0.007$; 5-6, $F = 23.1$, $df = 1, 5$, $P = 0.009$).

Analysis of FP Mutants Derived from Isolate 122b

The finding that isolate 122b and FP mutants produced equivalent amounts of BV suggests a possible basis for the lack of FP mutant accumulation during serial passage of isolate 122b. To address this possibility, 122b-derived viral isolates that exhibited characteristics of FP mutants were sought to determine whether these mutants produced amounts of BV equivalent those of 122b. Ninety-four plaques of isolate 122b were generated and then serially passaged 23 times in a P96 well plate. At the 20th and 22nd passages, two wells in which some infected cells produced fewer polyhedra than cells infected with 122b were found. Virus from these wells was plaqued, and two isolates that exhibited the FP mutant trait of reduced polyhedra synthesis were identified. The infectivity of these isolates was assessed through application of 1×10^5 polyhedra to a disk of diet, addition of 100 2nd instar *L. dispar* larvae, and quantification of larval mortality. Larvae feeding on diet containing A21-MPV polyhedra exhibited 100% mortality. In contrast, larvae feeding on diet containing the putative 122b FP mutant polyhedra exhibited 0% mortality. The amounts of BV and polyhedra produced by the putative 122b FP mutants, 122bFP1 and 122bFP2, and by isolate 122b were quantified and compared. The 122b FP mutants produced essentially the same amount of BV as isolate 122b (Fig. 5A). There was no significant difference in the amount of BV produced by these isolates compared to that by 122b (122bFP1, $F = 0.03$, $df = 1, 7$, $P = 0.86$; 122FP2, $F = 0.02$, $df = 1, 7$, $P = 0.89$). Isolates 122bFP1 and 122bFP2 produced significantly (122bFP1, $F = 288.3$, $df = 1, 7$, $P = 0.0001$; 122FP2, $F = 300.8$, $df = 1, 7$, $P = 0.0001$) fewer polyhedra, approximately 10-fold fewer, than isolate 122b (Fig. 5B). Fifty polyhedra cross sections of isolates 122bFP1 and 122bFP2 were visualized through electron microscopy to assess levels of virion occlusion. The majority (>90%) of isolate 122bFP1 and 122bFP2 cross sections contained no virions (Fig. 5C). The remaining polyhedra contained very few virions, an average of approximately three to five virions per cross section. The elevated BV titers, decreased polyhedra synthesis, and lack of virion occlusion exhibited by isolates 122bFP1 and 122bFP2 are traits consistent with traits exhibited by FP mutants (Slavicek *et al.*, 1995).

Analysis of Isolate 122b Polyhedra Production as a Function of Virus:Cell Infection Ratios

To determine the minimal amount of inoculum required to achieve successful polyhedra production, Ld652Y cells were infected with 122b or wild-type virus at increasing virus:cell ratios and polyhedra production was quantified. Isolate 122b produced greater than 1×10^8 polyhedra/flask at virus:cell ratios of 1:1 through 1:500 and almost 10^8 polyhedra at a ratio 1:1000 (Fig. 6). In contrast, wild-type virus exhibited a decrease in polyhedra production at virus:cell infection ratios of 1:5 through 1:1000. At a virus:cell ratio of 1:500, isolate 122b produced approximately 10-fold more polyhedra than wild-type virus. Polyhedra production by isolate 122b decreased at virus:cell infection ratios of 1:50 through 1:1000. However, at virus:cell ratios of 1:100 and 1:500 isolate 122b still produced more than 1×10^8 polyhedra per flask. In addition, isolate 122b produced significantly more polyhedra than wild-type virus at all virus:cell infections ratios assayed (1:1, $F = 89.2$, $df = 1, 7$, $P = 0.0001$; 1:5, $F = 41.4$, $df = 1, 7$, $P = 0.0007$, 1:10, $F = 83.2$, $df = 1, 7$, $P = 0.0001$; 1:50, $F = 190.9$, $df = 1, 7$, $P = 0.0001$; 1:100, $F = 109.7$, $df = 1, 7$, $P = 0.0001$; 1:500, $F = 37.5$, $df = 1, 7$, $P = 0.0009$; 1:1000, $F = 32.3$, $df = 1, 5$, $P = 0.005$).

DISCUSSION

We have isolated and characterized a LdMNPV isolate, 122b, that does not accumulate FP mutants after extensive serial passage in Ld652Y cells. In contrast, during serial passage of wild-type virus in Ld652Y cells, FP mutants arise and rapidly become the predominant virus type (Slavicek *et al.*, 1995, 1996; this investigation). Serial passage of baculoviruses in cell

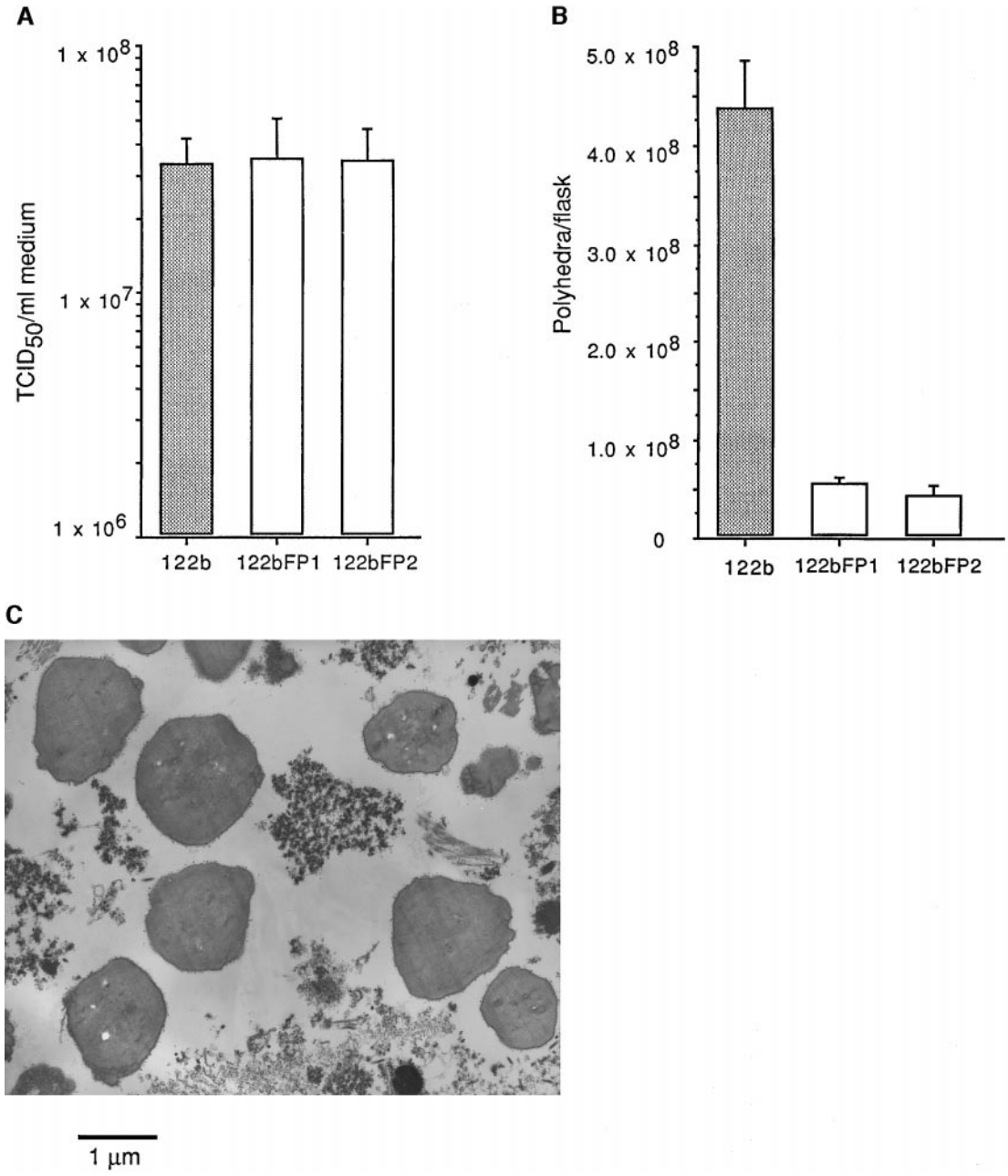

FIG. 5. Analysis of budded virus and polyhedra production and of polyhedra produced by few-polyhedra mutant isolates derived from isolate 122b. The amount of BV produced by isolates 122b, 122bFP1, and 122bFP2 was determined and expressed as the number of $TCID_{50}$ units/ml of medium (A). The number of polyhedra produced by isolates 122b, 122bFP1, and 122bFP2 was determined and expressed as the number of polyhedra/flask (B). The values shown in parts A and B are the averages of three determinations + one standard deviation. Cross sections of representative polyhedra produced by isolate 122bFP1 (C).

culture results in the formation of FP mutants at a high frequency (Hink and Strauss, 1976; MacKinnon *et al.*, 1974; Potter *et al.*, 1976; Fraser and Hink, 1982). LdMNPV FP mutants exhibit the traits of formation of few polyhedra per cell, a decrease in the percentage of infected cells that form polyhedra, increased release and/or production of BV, and synthesis of polyhedra with few viral occlusions compared to wild-type virus (Slavicek *et al.*, 1992, 1995). Under identical conditions in parallel serial passages, the release of BV increased and polyhedra production decreased during serial passage of wild-type LdMNPV strains, suggesting that FP mutants formed and became predominant. The presence of FP mutants was confirmed through analysis of viral plaques. These results are in agreement with our previous findings, in which wild-type viral strains ac-

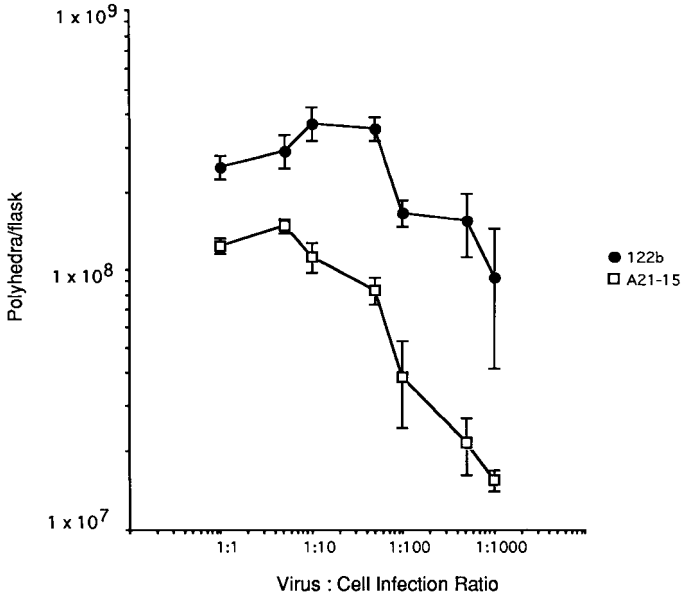

FIG. 6. Polyhedra production by isolate 122b and A21-15 (wild-type virus) at increasing virus:cell infection ratios. The number of polyhedra produced by isolate 122b and wild-type virus was determined and expressed as the number of polyhedra/flask. The values shown are the averages of four determinations ± one standard deviation.

quired mutations in the *25K FP* gene during serial passage in Ld652Y cells (Slavicek *et al.*, 1995, 1996). The enhanced stability of isolate 122b was confirmed by the finding that after 14 serial passages no FP mutants were evident. In contrast, after just a few serial passages, the wild-type virus strains were predominantly composed of FP mutants.

Analysis of 122b BV production revealed that this isolate produced approximately 10-fold more BV than wild-type virus and essentially the same amount as *25K FP* mutants. This finding provides a basis for the stable MP phenotype of 122b during serial passage. If isolate 122b produces the same amounts of BV as a FP mutant, the FP mutant could not become predominant during serial passage since it lacks the competitive advantage of increased BV production. For this to occur, however, a 122b FP mutant could not produce more BV than 122b. To investigate this theory we identified and isolated two 122b-derived viral strains that exhibited the traits of reduced polyhedra synthesis and lack of virion occlusion, both of which are traits of FP mutants. Marker rescue was performed with one of the putative 122b FP mutants with a clone containing the wild-type *25K FP* gene. The MP phenotype was restored, indicating that the mutant contained a mutation in the *25K FP* gene (data not shown). Analysis of BV production by the 122b FP mutants revealed that these mutants produced essentially the same amount of BV as isolate 122b. This finding supports the theory that the basis for the stable MP phenotype of isolate 122b during serial passage is due to elevated BV production.

Isolate 122b produced significantly more polyhedra than wild-type virus; however, the polyhedra were smaller than wild-type polyhedra. Isolate 122b produced approximately 3.5-fold more polyhedra than wild-type virus, which were about 35% smaller in diameter than wild-type polyhedra. Therefore, the volume of polyhedra produced by 122b was essentially the same as that produced by wild-type virus.

Genomic restriction endonuclease digestion profiles of isolates 122b and 122 were compared to determine whether isolate 122b was derived from 122 and to identify genotypic differences between the isolates. Analysis of digestion profiles with five different restriction endonucleases revealed no differences between the isolates, indicating that 122b is derived from isolate 122. Digestion with a sixth restriction endonuclease revealed that isolate 122b contained a *Bgl*II site lacking in isolate 122. This site has also been found in MP and FP strains of isolate 122 in a previous study (Slavicek *et al.*, 1995). The relevance, if any, of the additional *Bgl*II restriction endonuclease site to phenotypic traits of isolate 122b is under investigation.

Production of baculovirus polyhedra in cell culture bioreactors involves the progressive scaleup into successively larger-volume bioreactors until the final stage is reached. The initial and intermediate bioreactor infections are used as the inoculum for the next-larger-volume bioreactor. The fewer the number of intermediate bioreactors that are used to reach the final bioreactor, the more economical the production process will be. The number of intermediate bioreactor infections necessary will be dependent upon the amount of BV produced by the isolate used. With higher levels of BV production, a smaller volume of inoculum would be required to achieve a successful infection, and potentially fewer intermediate-stage bioreactors would be necessary. The minimal amount of inoculum required to achieve successful polyhedra production with isolate 122b was found to be significantly less than that with wild-type virus. Consequently, less inoculum of isolate 122b is necessary to generate a successful infection, which would decrease production costs compared to production of wild-type virus. In addition, fewer intermediate-stage bioreactors may be required when 122b is produced, which would also decrease production costs.

The stability of polyhedra production exhibited by isolate 122b in conjunction with the small amount of inoculum needed for productive infections suggest that isolate 122b would be more economical to produce than wild-type virus in cell culture bioreactors. In addition, FP mutant formation during production in cell culture bioreactors should not be a problem with isolate 122b due to its BV production levels. The molecular basis for increased BV release and/or production by isolate 122b

is currently under investigation. Isolate 122b has been patented and is available for licensing and research purposes (Slavicek and Hayes-Plazolles, 1999).

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