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Characterization of a multiple-nucleocapsid nucleopolyhedrovirus isolated from *Perina nuda* (Fabricius) (Lepidoptera: Lymantriidae) larvae

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Abstract

A multiple-nucleocapsid nucleopolyhedrovirus (MNPV), named *Perina nuda* MNPV (or PenuNPV), isolated from *P. nuda* (Fabricius) (Lepidoptera: Lymantriidae)—a major banyan pest—was studied in terms of its main morphological, biochemical, and biological properties. The pleiomorphic occlusion bodies were 1.7–5.4 μm in diameter with a mean size of 2.2±0.52 (μm±SE), and their most prominent protein had an apparent molecular mass of 32 kDa. The DNA genome size was estimated to be 120.38±0.38 (kbp±SE) based on restriction endonuclease fragment sizes. The median lethal dose response (LD_{50}) for this virus in 2nd to 5th instar *P. nuda* larvae were estimated at 154, 1,431, 14,458, and 169,137 occluded bodies per larva, respectively. In host range tests, other nine lepidopteran species from five families were not susceptible to PenuNPV. The high pathogenicity and specificity of this newly described MNPV indicate that it is indeed a good candidate for the biological control of this moth.

Key words: *Perina nuda*; PenuNPV; restriction profiles; histopathology; biological activity

INTRODUCTION

Members of the family Baculoviridae are arthropod-specific pathogens and have been isolated primarily from lepidopteran (moth and butterfly) species. The family is taxonomically characterized by their large and covalently closed genome of double-stranded DNA (range from 80 to 180 kbp), which is packaged singly or multiply in an enveloped, rod-shaped virion (Blissard et al., 1999; Friesen and Miller, 2001). In addition, baculoviruses are also distinguished by their two morphologically distinct forms of infectious particles: occlusion derived virions (ODVs), comprising enveloped virions embedded within a crystalline matrix of protein (polyhedrin or granulin), and budded virions (BVs), comprising a single virion enveloped by a plasma membrane. Baculoviruses involve two genera: the nucleopolyhedroviruses (NPVs) and granuloviruses (GVs) (Blissard et al., 1999). The NPVs are characterized by the presence of large occlusion bodies (OBs) in the nucleus of the infected cells. Due to their economic importance especially in pest control and in foreign gene expression, the NPVs have attracted the greatest attention and are the best characterized baculoviruses. Currently, more than 500 different insect species infected by baculoviruses have been reported in the literature (Friesen and Miller, 2001).

The transparent wing moth, *Perina nuda* (Fabricius), is a serious pest of banyan (*Ficus* spp.) and is a major defoliator of forest and shade trees in Southeastern Asia (Su et al., 1983). To date, utilization of broad-spectrum chemical insecticides is the only practical method of controlling this caterpillar, but this method may cause safety issues in the city, as well as it may cause the natural enemies to delay or suppress field colonization. A safe alternative pest control method using their pathogens was considered. Among pathogens, a nucleopolyhedrovirus (PenuNPV) and an insect picorna-like virus (PnPV) were detected as the main mortality factors (Su et al., 1983; Wang et al., 1998, 1999). Nevertheless, PenuNPV could be more suitable than PnPV for inclusion in banyan pest biological control because of its high virulence to the *P. nuda* larvae (Lo et al., 1990; Wang and Tsai, 1995; Wang et al., 1998). So far, both the success of *in vitro* propagation of this NPV in its homogeneous cell line, NTU-PN-HH (Wang et al., 1996) and the analysis of the nucleotide sequence of polyhedrin

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and *p10* genes (Chou et al., 1996, 1997) facilitate further molecular biological studies of this virus. However, former investigations were either directed towards molecular level studies of this virus or toward evaluating its virulence, and none of the previous reports addresses its identification or characterization.

In this paper we attempt to get a better overview of this virus, including its main morphological, biochemical, and biological properties. The data presented here give further support to the argument that PenuNPV is a distinct baculovirus species and is a good candidate for the biological control of *P. nuda* moth outbreaks in the future.

MATERIALS AND METHODS

Virus isolation and multiplication. A colony of *Perina nuda* (Lepidoptera: Lymantriidae) caterpillars were obtained and reared in the laboratory with leaves of Banyan, *Ficus* spp. as in the previous paper (Wang and Tsai, 1995). The PenuNPV isolate used in this study originated from a single infected larva of *P. nuda*, collected on the campus of Fu Jen Catholic University (Taipei, Taiwan). The genetic homogeneity of the isolate was further verified following plaque purification in *P. nuda* culture cells (NTU-PN-HH; Wang et al., 1996). Viral amplification was carried out, allowing 3rd-instar larvae to feed on fresh Banyan leaves, superficially contaminated with occlusion bodies (about 2.5×10^3 OBs per larva). OBs were purified from homogenized larvae by centrifugation on continuous 40 to 65% (wt/wt) sucrose gradients at $100{,}000\times g$ for 30 min as previously described (Chou et al., 1996). The purified OBs were examined with an optic microscope (400×) and fifty OBs were measured to determine their mean size. ODVs were released by hydrolyzing OBs in diluted alkaline solution (100 mM Na_2CO_3, 170 mM NaCl, 10 mM EDTA, pH 10.5) at 37°C for 30 min. Following the dissolution of the OBs, the suspension was centrifuged on the same sucrose gradient at $100{,}000\times g$ for 1 h. About five viral bands were transferred to a new centrifuge tube, diluted with 3×volume of 1×TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), and then precipitated at $100{,}000\times g$ for 30 min. The purified ODVs were resuspended in 1×TE buffer and stored at −20°C. BVs of PenuNPV were collected from *in vitro* propagation medium after a 3-day infection and purified by sucrose gradient centrifugation as previously described (Wang et al., 1996). The purified BVs were then prepared for negative staining.

Transmission electron microscopy. The infected and control larvae at 5-day postinoculation were dissected to small pieces, fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h, postfixed with 1% osmium tetroxide for 2 h, dehydrated through an alcoholic gradient series, and then embedded in Epon-Araldite resin (Luft, 1961). Thick and ultrathin sections were cut on a Reichert OMU3 Ultramicrotome. Thick sections were stained with 0.05% toluidine blue (prepared in 1% borax solution), while ultrathin sections were stained in 2% uranyl acetate and lead citrate. The micrographs were taken with a HITACHI H7100 electron microscope operated at an accelerating voltage of 75 kV. For negative staining, a drop of either purified ODVs or BVs were placed on a carbon coated grid for 3 min and excess virus suspension was removed with filter paper. An additional drop of 2% aqueous solution of phosphotungstic acid was placed on the grid, excess stain being removed from the grid with filter paper. The preparation was allowed to dry before examination.

Protein profiles of occlusion body. The major protein profiles of the OBs were carried out following the method of Reinganum (1984) with slight modifications. Briefly, the purified OBs were pretreated with 1% SDS and 0.5% 2-mercaptoethanol (2-ME) for 30 min at pH 7.2 and 70°C, then washed to remove soluble material. The washed OBs were then centrifuged at $7{,}650\times g$ for 10 min. The pellets were dissociated with 1× SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% 2-ME, 0.1% bromophenol blue, and 2% SDS) at 100°C for 10 min. The soluble proteins were electrophoresed in 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels using the Laemmli (1970) buffer system. Proteins were visualized after staining with silver nitrate, and sizes were estimated using molecular weight markers (Amersham). The analysis was also performed on *Autographa californica* NPV (AcMNPV), kindly supplied by Dr. M. J. Fraser of the University of Notre Dame. The other two viruses, *Bombyx mori* NPV Taiwan isolate (BmNPV-TWN) (unpublished) and *Spodoptera litura* NPV Taiwan strain (SpltNPV-TWN) (Shih et al., 1995a), were isolated

in our laboratory from NPV-infected larvae, *B. mori* and *S. litura*, respectively.

Genome. ODV suspensions were adjusted to a final concentration of 0.5% SDS and incubated with proteinase K (0.25 mg/ml at 37°C for 3 h), and the DNA was isolated by phenol extraction and ethanol precipitation (Chou et al., 1996; Wang et al., 1996). The DNA fragments obtained after digestion with *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, and *Sma*I endonucleases were separated by electrophoresis on 1% (wt/vol) agarose gels, stained with ethidium bromide, and photographed under UV light. Alternatively, DNA digests separated by electrophoresis were then blotted onto Hybond-N nylon papers (Amersham Biosciences) using a vacuum transfer unit (Hoefer TE80). A DIG-labeled probe prepared by a PCR DIG Probe Synthesis Kit (Roche) with primer set (35/36, designed for amplification of a 680 bp product within the polyhedrin (*polh*) ORF, Chou et al., 1996; Wang et al., 2000) and PenuNPV DNA were used for localization of the polyhedrin gene. Briefly, 1 μl of the PenuNPV DNA used in a 50 μl PCR reaction containing: 0.2 mM DIG-dNTPs (Roche), 2.5 units of *Taq* DNA polymerase (Promega), 0.5 μM of each primer, and 1×PCR Reaction Buffer (Promega) containing 1.5 mM $MgCl_2$. The thermal profile consisted of a single 2 min denaturation step at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, the reaction was completed with a single cycle of 72°C for 5 min using a PCR machine (AG-9600 Thermal Station, AcuGen Systems). The Southern hybridization was preformed according to the standard procedure of Sambrook et al. (1989) and the manufacturer's recommended condition (5×SSC, 1% blocking reagent, 0.1% *N*-laurosarcosine, and 0.02% SDS) for 16 h at 65°C. After hybridization, the nylon paper were washed twice in 2×SSC+0.1% SDS for 10 min at room temperature followed by two washes at 65°C for 15 min each in 0.5×SSC+0.1% SDS. Standard chemiluminescent detection was performed according to the manufacturer's instructions (Roche), and the blot was exposed to X-ray film (Kodak XAR-5).

Biological activity. In order to estimate the virulence of the PenuNPV isolate, bioassays were conducted using the modified droplet feeding technique (Hughes and Wood, 1981). The OBs were suspended in a solution of 1% sucrose–0.1% Coomassie brilliant blue (Begon et al., 1993). The mean ingested volume per larvae was measured by feeding newly molted and 8-h (for 2nd- and 3rd-instar) and 16-h (for 4th- and 5th-instar) starved larvae the standard PBS solution with 1% sucrose, mixed with a known concentration of fluoresbrite carbosylate particles (FCP, Polysciences, Inc., about 0.5 μm in diameter). The FCP suspensions were offered in small droplets, applied in a circle on a layer of parafilm placed on the bottom of a petri dish. Experiments were performed with 20 larvae per instar. After 10 min-ingestion, larvae were placed individually in 1.5 ml eppendroff tubes and then homogenized in PBS solution. The amount of FCP uptake was estimated using a hemocytometer under a fluorescence microscope (FITC filter, a little bright light was needed for visualization of the grid lines of the hemocytometer). FCP counts from individual larvae were converted to volumes of ingested solution. On the basis of these measurements, dilutions were adjusted to obtain the six doses (4.4×10, 8.8×10, 8.8×10^2, 1.76×10^2, 8.8×10^3, and 4.4×10^4 OBs per larva) for 2nd- and 3rd-instar larvae, and six doses (8.8×10^3, 1.76×10^4, 8.8×10^4, 1.76×10^5, 4.4×10^5, and 8.8×10^5 OBs per larva) for 4th- and 5th-instar larvae in the 50% lethal dose (LD_{50}; dose that produces a lethal infection in 50% of the larvae in 7 days) assays. Three replicate assays of 32 larvae each were carried out for each dose. An additional 32 larvae fed on the colored sucrose solution were used as controls. Those larvae which had ingested the virus suspension were transferred individually to multiwell dishes containing fresh leaves of Banyan, *Ficus* spp. and held at 20±1°C and 80±5% RH and for a 12-h photoperiod. Mortality was recorded daily and NPV infections were confirmed using the Giemsa staining (Wigley, 1980). Dosage-mortality response data were analyzed using Probit analysis (Finney, 1972), with the computer program, POLO-PC (LeOra Software, Berkeley, CA). Larvae which died of unexplained causes (e.g. <10% of 2nd-instar larvae at the highest dose tested that died quickly within a day after inoculation, however, no viral OBs were observed under microscopy) were not included in the analysis. Host-specificity examination was performed on the three noctuid pests, *S. litura*, *S. exigua*, and *Helicoverpa armigera*, and they were reared as previously described (Shih et al., 1995a, b). Six other

lepidopteran species—*Trichoplusia ni*, *Plutella xylostella*, *Pieris rapae*, *Homona coffearia*, and *Ocinara varians*, collected in the field or on campus, and the silkworm *B. mori*, kindly provided by the Taiwan Apicultural and Sericultural Experiment Station—were also included in the assays. The fifteen 3rd-instar larvae of each species were tested by diet surface contamination with the same six doses (4.4×10, 8.8×10, 8.8×10^2, 1.76×10^2, 8.8×10^3, and 4.4×10^4 OBs per larva) described above. Fifteen additional larvae of each species were treated only with 1% sucrose solution as controls. Following virus inoculation, larvae were individually reared on artificial diet or on natural foliage (Table 2) without viral OBs and maintained at 25±1°C and 60±5% RH and for a 12-h photoperiod. Mortality was recorded daily until all larvae had either pupated or died. Each deceased larva was checked microscopically to confirm NPV infection.

RESULTS AND DISCUSSION

Virus morphology

The OBs of PenuNPV were pleiomorphic and 1.7–5.4 μm in diameter with a mean size of 2.2±0.52 (μm±SE). On the surface of mature OBs, an intact, additional thin envelope, known as the polyhedron calyx was observed under transmission electron microscopy (Fig. 1). Within the OBs, multiple virions were embedded in a crystalline matrix of polyhedrin. Each occluded virion contained up to six nucleocapsids surrounded by a membrane envelope. The ODVs were 268±14.4 (nm±SE) long and had various diameters (38–172 nm) depending on the number of enveloped nucleocapsids (Figs. 1 and 2a). The ultrastructural observations revealed that PenuNPV is a multiple-nucleocapsid type NPV (MNPV). In our observation of BVs, the estimated size was 272±6.9 nm in length and 36±4.1 nm in diameter. In addition, BVs exhibited a distinctive hemispherical cap (approximately 56 nm×40 nm), with the attached peplomers at their anterior ends (Fig. 2b and 2c). The outermost layer was a very thin leaflet and the inner layer of cap decreased gradually in thickness from the tip down to both sides. The peplomers were rooted to the inner layer and passed through the outermost layer, about 11 nm in length (Fig. 2c).

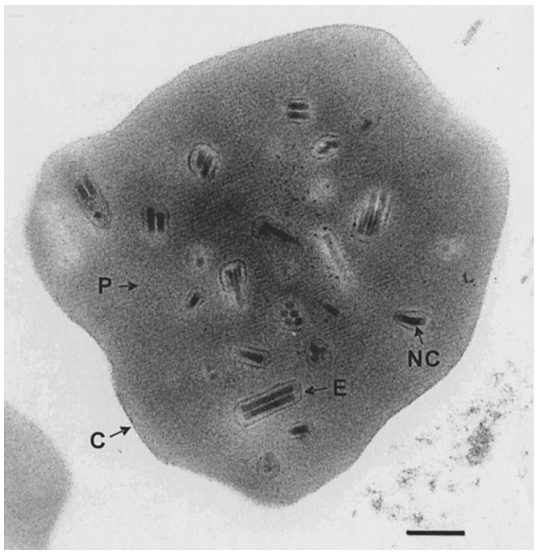

Fig. 1. Transmission electron micrograph of the mature PenuNPV occlusion body isolated from infected larva showing that nucleocapsids (NC) embedded in the crystalline matrix of polyhedrin protein (P) are multiply encapsulated within the virion envelope (E) with many virions occluded in the occlusion body. On the surface, an additional thin envelope, known as the polyhedron calyx (C) was also observed. Bar: 0.2 μm.

Protein profiles of occlusion body

Twelve major occlusion body protein bands of PenuNPV—39.8, 32.0, 31.2, 26.8, 25.8, 23.1, 20.1, 18.5, 17.8, 16.5, 15.0, and 14.2 kDa—with molecular weights from around 43.0 to 14.4 kDa were observed. This protein profile diverges from those of AcMNPV, BmNPV-TWN, and SpltNPV-TWN (Fig. 3). The most abundant occlusion body proteins of the four NPVs had almost the same mobility and fell within the 25–33 kDa range, characteristic for polyhedrins (Blissard et al., 1999). The molecular weight of PenuNPV polyhedrin was estimated to be 32 kDa.

Genome

The DNA fragments obtained after cleavage by *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, and *Sma*I endonucleases were separated by electrophoresis on 1% agarose gel (Fig. 4), and the restriction patterns of PenuNPV DNA were distinct from those published for other NPVs, such as AcMNPV (Smith and Summers, 1979), SeMNPV (Brown et al., 1984), AfMNPV (Chen et al., 1996), SlMNPV-B (Croizier et al., 1989), LdMNPV (McClintock and Dougherty, 1988; Smith et al., 1988), and OpMNPV (Chen et al., 1988). To minimize the inaccu-

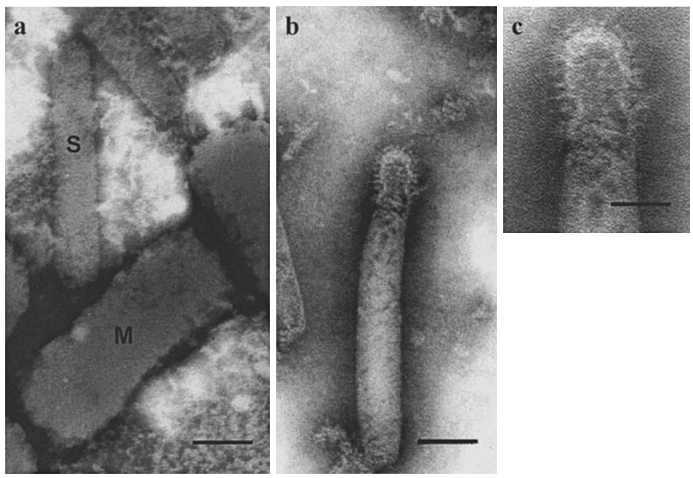

Fig. 2. Negative electron micrographs of purified virions stained with 2% phosphotungstic acid showing: a, the rod-shaped blunt-ended occluded virions with single nucleocapsid (S) and multiple nucleocapsid (M), Bar: 50 nm; b, the budded virion with the cap structure at the anterior end, Bar: 50 nm; c, the high magnification of the entire cap of the budded virion, emphasizing the distribution of peplomers over the cap. It is worth noting that the outermost layer (arrow) was a very thin leaflet and the inner layer (double arrow) of the cap decreased gradually in thickness from tip down to both sides. The peplomers rooted to the inner layer and passed through the outermost layer. Bar: 25 nm.

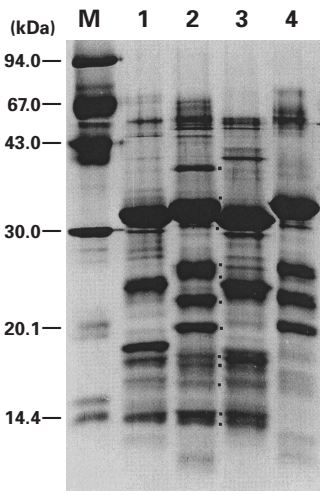

Fig. 3. SDS-polyacrylamide gel electrophoresis of PenuNPV and three other NPV occlusion body proteins. Protein molecular weight estimates for the occlusion bodies of SpltNPV-TWN (lane 1), PenuNPV (lane 2), BmNPV-TWN (lane 3), and AcMNPV (lane 4) were derived by comparison with the electrophoretic mobilities of protein molecular weight standards (M: LMW-SDS Marker Kit, Amersham Biosciences). The major bands of PenuNPV occlusion body proteins in gel with molecular weight around 43.0 to 14.4 kDa are indicated with dots.

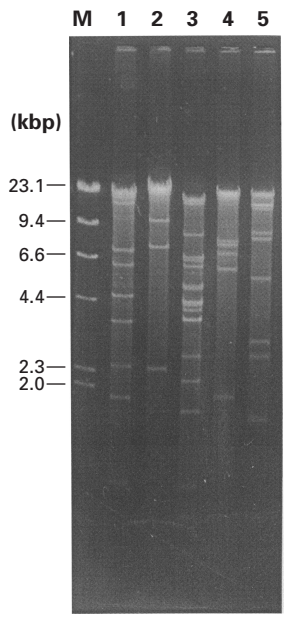

Fig. 4. Restriction endonuclease (REN) profiles of PenuNPV DNA cleaved with *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, and *Sma*I (lanes 1–5, respectively) and separated on 1% agarose gel. Size markers represented as *Hin*dIII restricted lambda phage DNA fragments (lane M).

racy associated with the determination of the sizes of very large restriction fragments, double digests using combinations of restriction endonucleases were also analyzed (data not shown). A mean size of 120.38±0.38 (kbp±SE) for the PenuNPV genome was estimated from the sum of the fragments (Table 1). This result is consistent with the known genomes of different NPVs, which range from 80 to 180 kbp (Blissard et al., 1999). Detailed

Table 1. Estimated sizes (in kbp) of PenuNPV DNA fragments following restriction with five endonuclease enzymes and the location of their polyhedrin gene (*)

Fragment	Restriction enzyme				
	*Eco*RI	*Eco*RV	*Hin*dIII	*Kpn*I	*Sma*I
A	21.9	22.3*	18.6	23.4	21.9*
B	20.1	20.1	17.9	22.6	21.9
C	19.4	19.9	16.6	21.9	16.0
D	17.1	19.1	9.0	20.1	16.0
E	16.0	17.8	7.0	8.3	14.4
F	7.5	11.5	6.6*	7.9*	9.0
G	6.3*	7.7	6.0	6.9	8.8
H	4.6	2.3	5.2	5.7	5.4
I	3.5		5.0	1.9	2.8
J	2.4		4.4	0.6	2.6
K	1.9		4.3		1.6
L	0.8		4.0		0.6
M			3.8		
N			3.8		
O			2.5		
P			2.1		
Q			1.6		
R			0.9		
S			0.6		
Total	121.5	120.7	119.9	119.3	121.0

genome analysis is under way to complete the physical restriction map and sequencing of PenuNPV, which would confirm the PenuNPV species' taxonomic status. Since no submolar fragments were apparent in the restriction endonuclease analyses, the PenuNPV isolate studied in our laboratory seems to be homogeneous. However, further studies on the viral genome and the comparison of DNA digests from other geographical and temporal isolates of PenuNPV will allow us to determine if variants of this virus exist, as previously described for other NPV isolates (Gettig and McCarthy, 1982; Smith and Crook, 1988; Richards et al., 1999).

A polyhedrin gene fragment of about 680 bp was synthesized by PCR with degenerate primer set (35/36) and used as a probe to localize this gene by Southern blotting (data not shown). The results were summarized in Table 1, the PenuNPV polyhedrin gene was located in *Eco*RI-G (6.3 kbp), *Eco*RV-A (22.3 kbp), *Hin*dIII-F (6.6 kbp), *Kpn*I-F (7.9 kbp), and *Sma*I-A (21.9 kbp) fragments, respectively. Previous papers report that both the polyhedrin and P10 protein of PenuNPV has a high amino acid sequence identity to their counterparts in OpMNPV, 99% and 96% respectively (Chou et al., 1996, 1997). The following clues allow us to distinguish PenuNPV from OpMNPV: The restriction enzyme profiles of PenuNPV (Fig. 4) and those of OpMNPV (Chen et al., 1988) are different. In addition, the polyhedrin gene of PenuNPV is located in the *Eco*RI-G and *Hin*dIII-F fragments while the polyhedrin gene of OpMNPV is located in the *Eco*RI-B (28 kbp) and *Hin*dIII-A (21 kbp) fragments (Leisy et al., 1984; Rohrmann, 1986; Chen et al., 1988). Nevertheless, the overall homology of their genomic DNA awaits further studies.

Histopathology

Our results, together with the previous studies (Wang et al., 1998), showed that PenuNPV infection was polyorganotropic and spread throughout most tissues, especially adipose tissue, tracheal matrix, midgut, and epidermis at 5-day postinfection. The infected cells or tissues were characterized by presence of OBs and virogenic stroma in the hypertrophic nuclei (Fig. 5). The proliferation cells, muscle cells, and hemocytes showed heavy infection, and their nuclei were filled with OBs (Fig. 5a). In the hind-gut, several infected cells ruptured and released the OBs to the hemocoelum. Besides, some free OBs were also found in the coelum of the hind-gut (Fig. 5b). The adipose tissue showed heavy infection during the late stages of pathogenesis. The viral nucleocapsids, multiple embedded virions, virogenic stroma, and developing polyhedra were observed in the hypertrophic nuclei of the adipocytes. Both in the cytoplasm and nuclei, the expansive fibrillar structures associated with a coiled coil-domain protein (P10, reviewed in O'Reilly, 1997) were also observed. Except for the mitochondria and the endoplasmic reticulum, no obvious organelles were found (Fig. 5c). Therefore, we suggest that infected adipocytes are the primary location of occluded virus production in *P. nuda* larvae.

Biological activity

The larvae infected by PenuNPV showed symptoms typical of an NPV infection at a late stage of the disease, i.e., loss of appetite, decrease in mobility, and change of color due to the accumulation of occlusion bodies in the infected tissues. Shortly

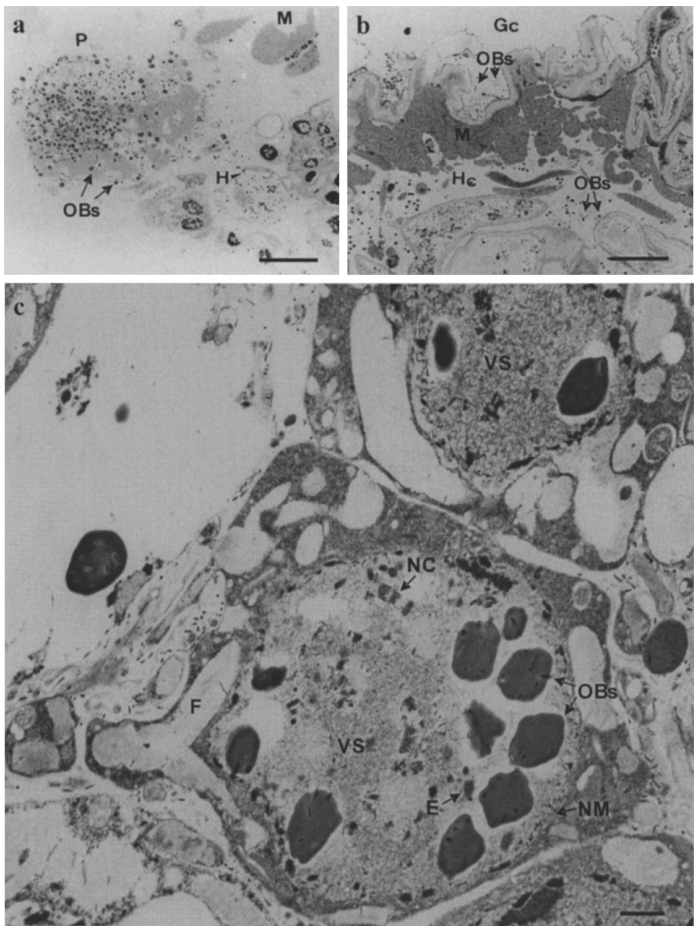

Fig. 5. Transmission electron micrographs of PenuNPV-infected larvae at 5-day postinfection. a, The thick section showing heavy infection caused the hypertrophic nuclei of the proliferation cells (P), muscle cells (M), and hemocytes (H) filled with occlusion bodies (OBs). Bar: 50 μm; b, The thick section through the hind-gut showing free OBs in the gut coelum (Gc) and hemocoelum (Hc). M, muscle. Bar: 50 μm; c, Electron micrograph of PenuNPV-infected fat body tissue during the late phase of replication. The nucleus, surrounded by the nuclear membrane (NM), contains the virogenic stroma (VS) with associated nucleocapsids (NC). Newly assembled nucleocapsids associate with intranuclear membrane envelopes (E), then become envelopes, and are embedded within OBs. A large amount of fibrillar materials (F) often collect in the cytoplasm and nucleus. Bar: 1 μm.

after death, the larvae became swollen and turned brownish, and the integument was easily disrupted. The mean volumes ingested by 2nd- to 5th-instar larvae were estimated as 0.643±0.062, 1.986±0.148, 7.465±0.437, 13.943±2.185 (μl±SE), respectively. The dosage-mortality response data estimated by probit analysis of *P. nuda* larvae inoculated with PenuNPV were shown in Table 3. As expected, the susceptibility was decreased rapidly with larval age. The LD_{50} value of the 5th-instar larvae was as much as 1,100 fold greater than that of the 2nd-instar larvae. In host range studies, a total of nine lepidopteran species from five families (Noctuidae, Plutellidae, Pieridae, Tortricidae, and Bombycidae) were tested and none of these species succumbed to infection when challenged *per os* with PenuNPV (Table 2) even though the dose of inoculation was up to 30×the LD_{50} for *P. nuda* 3rd-instar larvae. In all cases, larvae fed on PenuNPV contaminated diet pupated simultaneously with the controls. These results indicate PenuNPV has a narrow host range, while the unavailability of any lymantriid species prevented a more detailed assessment.

PenuNPV is a potential control agent for *P. nuda*

PenuNPV was considered a tentative species in the genus *nucleopolyhedrovirus* (Baculoviridae) based on electron microscopy evidence and biochemical data that confirmed its generic identity in

Table 2. Results of virus specificity tests for 9 species of Lepidoptera representing 5 families

Test species[a]	Common name	Family	Food type	Permissive (Yes/No)
Spodoptera litura (Fabricius)	Cotton leafworm	Noctuidae	Artificial diet[c]	No
Spodoptera exigua (Huebner)	Beet armyworm	Noctuidae	Artificial diet	No
Helicoverpa armigera (Huebner)	Tobacco budworm	Noctuidae	Artificial diet	No
Trichoplusia ni (Huebner)	Cabbage looper	Noctuidae	Artificial diet	No
Plutella xylostella (Linnaeus)	Diamondback moth	Plutellidae	Cabbage	No
Pieris rapae (Linnaeus)	Cabbage white	Pieridae	Cabbage	No
Homona coffearia (Nietner)	Tea tortrix	Tortricidae	Tea leaves	No
Ocinara varians Walker	—[b]	Bombycidae	Banyan	No
Bombyx mori (Linnaeus)	Silkworm	Bombycidae	Morus spp.	No

[a] Third-instar larvae of each species received a *per os* dose up to 44,000 OBs (equal to 30 LD_{50} for *Perina nuda*) of PenuNPV.
[b] No verified common name.
[c] Composition of the artificial diet was according to Shih et al. (1995a, b) reports.

Table 3. Dosage-mortality responses for 2nd- to 5th-instar larvae of *P. nuda* infected with PenuNPV

Instar	LD_{50}[a] (95% confidence limits) (OBs/larvae)	Dosage-mortality regression line ($Y=bX+a$)[b]	SE of b
2nd	154 (101–202)	$Y=1.03X+4.88$	0.11
3rd	1,431 (1,230–1,798)	$Y=0.86X+4.01$	0.08
4th	14,458 (12,021–18,794)	$Y=0.62X+3.80$	0.14
5th	169,137 (133,816–212,319)	$Y=0.79X+4.03$	0.16

[a] Recorded at the 7th day after inoculation.
[b] Y=mortality in probits, X=log dose (OBs).

this study. The biological properties and DNA restriction patterns are consistent with an NPV different from any previously described in the literature. On the basis of host range studies and the estimates for LD_{50} obtained from 2nd- to 5th-instars larvae, it can be concluded that PenuNPV is highly specific and virulent to *P. nuda* larvae and, indeed, is a good candidate for the microbial control of this pest. However, in order to develop a viral insecticide for *P. nuda*, further studies on PenuNPV efficacy under field conditions are necessary. A significant decrease of susceptibility with increasing larval age was apparent, a phenomenon observed elsewhere in several other baculoviruses (Bourcias et al., 1980; Smits and Vlak, 1988; Engelhard and Volkman, 1995; Kirkpatrick et al., 1998). In this context, it is also important to establish complementary methods to monitor the population dynamics of *P. nuda*, in order to determine the application times that would ensure control during the early instars of the larvae, when they are more exposed and susceptible to the virus. In general, last-instar larvae were less susceptible to NPV, only causing the infected pupae to fail to emergen at most, and few OBs are yielded from NPV-infected pupae (Wang and Tsai, 1995). However, many black-dead pupae filled with OBs can be found in fields (Wang and Tsai, 1995). This phenomenon leads to a conclusion that pupae of *P. nuda* are also susceptible to PenuNPV, although more studies, especially the time of infection, are necessary to elucidate the virus replication during pupation. Furthermore, the moribund male pupae and female pupae after injection with PenuNPV suspension (10 μl) could yield 4.72×10^8 and 9.07×10^8 OBs per gram, respectively (Wang and Tsai, 1995). Therefore, it is a good idea to harvest the OBs from moribund pupae rather than from larvae due to the need to avoid food contamination and losing most of the OBs from the fragile body (e.g. infected pupae are less fragile than infected larvae). In summary, providing a description of PenuNPV has clear value, not only as a prerequisite to more detailed evaluations of its potential as a biocontrol

agent (Su et al., 1983) but also as a foundation for future investigations of mass-production.

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