The black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae), is a serious localized pest of vegetable and field crops. We have characterized a newly discovered baculovirus, the *Agrotis ipsilon* multicapsid nucleopolyhedrovirus (AgipMNPV), that was isolated from *A. ipsilon* in Illinois. Restriction enzyme fragment profiles of AgipMNPV DNA were distinct from those of previously described nucleopolyhedroviruses. Electron microscopy of AgipMNPV-infected tissues indicated that nucleocapsids of this virus are multiply enveloped. *A. ipsilon* was highly susceptible to infection by AgipMNPV and significantly more susceptible to infection by AgipMNPV than by *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV). Host range studies showed that *Heliothis virescens* and *Helicoverpa zea* were moderately susceptible to infection; *Pseudaletia unipuncta* and *Spodoptera frugiperda* were only partially susceptible, and *Anticarsia gemmatalis,* *Spodoptera exigua,* *Trichoplusia ni,* and *Ostrinia nubilalis* were not susceptible to infection by AgipMNPV. Because of its high virulence, AgipMNPV has potential as an alternative to chemical insecticides for control of *A. ipsilon.*

Key Words: *Agrotis ipsilon*; *Agrotis ipsilon* multicapsid nucleopolyhedrovirus; Baculovirus; virus characterization and host range studies.

**INTRODUCTION**

The Baculoviridae is a large family of entomopathogenic viruses. Within the family, two genera of viruses are recognized, the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs) (Volkman et al., 1995). Baculoviruses have a narrow host range, are highly pathogenic, and have occlusion bodies which make them more environmentally stable than some other families of entomopathogenic virus (Entwistle and Evans, 1985). These characteristics enable baculoviruses to be used as microbial control agents against insect pests, and there are many examples of the successful use of baculoviruses in this role (Entwistle, 1998; Moscardi, 1999).

We have isolated a new NPV from the black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae). *A. ipsilon* is a worldwide pest of over 30 important crops including tobacco, cotton, tomato, potato, cabbage, barley, and oats (Rings et al., 1975). In the U.S. corn belt, *A. ipsilon* can be a serious localized pest of field corn (Clement and McCartney, 1982; Engelken et al., 1990). All instars of *A. ipsilon* feed on the leaves of corn seedlings, but the most serious damage results from leaf and stem cutting by the late instars (Clement and McCartney, 1982). Present management of *A. ipsilon* is based on population monitoring and rescue applications of chemical insecticide against damaging larval populations (Stockdale, 1977). Several Microsporidia, bacteria, fungi, and viruses infect *A. ipsilon,* and among these pathogens, baculoviruses appear to have the most potential as alternatives to chemical insecticides (Ignoffo and Garcia, 1979).

In this paper we describe characterization of a new NPV, *A. ipsilon* multicapsid nucleopolyhedrovirus (AgipMNPV). We present restriction enzyme profiles, ultrastructural morphology, and lethal concentration data for nine species of agriculturally important Lepidoptera.

**MATERIALS AND METHODS**

**Insects**

Nine species of Lepidoptera were used in the study: eight from the family Noctuidae and one species, *Ostrinia nubilalis* (Hübner), from the family Crambidae. *A. ipsilon,* *Pseudaletia unipuncta* (Haworth), and *O. nubilalis* were obtained from USDA-ARS Corn Insects and Crop Genetics Research Unit (Ames, IA). *Helicoverpa zea* (Boddie) was obtained from a colony maintained by Dr. T. C. Baker (Entomology Department, Iowa State University, Ames, IA). *Spodoptera frugiperda* (J. E. Smith) was obtained from a colony.
maintained by Dr. F. Davis (USDA-ARS, Crop Research Laboratory, Mississippi State University, Starkville, MS). *Heliotis virescens* (Fabricius), *Spodoptera exigua* (Hübner), and *Anticarsia gemmatalis* Hübner, were obtained from Southern Insect Management Laboratory (Stoneville, MS). *Trichoplusia ni* (Hübner) was obtained from a colony maintained by Dr. T. Coudron, USDA-ARS (Biocontrol of Insects Laboratory, Columbia, MO). All insects were reared from eggs at 27°C with a photoperiod of 12 h light, 12 h dark. *A. ipsilon*, *P. unipuncta*, *H. zea*, and *H. virescens* were reared on pinto bean diet (Hendrix *et al.*, 1991), and *O. nubilalis* was reared on wheat germ diet (Guthrie, 1987). *S. frugiperda*, *S. exigua*, and *A. gemmatalis* were reared on general purpose soy flour diet (Southland Products, Lake Village, AR). *T. ni* was reared on Insecta-diet (Bio-Serv, Frenchtown, NJ).

**Viruses**

The original isolate of AgipMNPV was obtained from infected *A. ipsilon* larvae collected in Illinois by Dr. J. Maddox (Illinois Natural History Survey, Champaign, IL). AcMNPV clone C6 (Possee, 1986) and *Rachiplusia ou* multicapsid nucleopolyhedrovirus (RoMNPV) clone R1 (Smith and Summers, 1980) were used for comparison in the restriction enzyme studies.

**Virus Amplification and Purification**

Amplification of AgipMNPV was performed in fifth instar *A. ipsilon*, and virus concentrations were quantified with a phase contrast microscope and a Neubauer bright-line hemocytometer (Fisher Scientific, Pittsburgh, PA). Larvae were placed in individual 17-ml jelly cups (Fill-Rite Inc., Newark, NJ) and starved overnight. One 3-mm cube of diet inoculated with 800,000 polyhedral occlusion bodies (POBs) of AgipMNPV was added to each cup. Larvae that consumed the inoculated diet were reared from eggs at 27°C under phase contrast microscopy. Subsequent amplification of virus was accomplished by injection of hemolymph, when viewed under phase contrast microscopy. Subsequent amplification of virus was accomplished by injection of hemolymph (5 µl) from infected larvae, into uninfected sixth instar *A. ipsilon*, which were maintained on diet until death. For purification of viral POBs, cadavers were homogenized using a 30-ml dounce homogenizer (Wheaton Scientific Products Inc., Millville, NJ) for 10 min in 0.1% SDS (1 ml per cadaver) and filtered through five layers of cheesecloth. POBs were pelleted by centrifugation at 3600g for 10 min at room temperature in 42-ml glass centrifuge tubes. The pellet was resuspended in 0.5% SDS, and centrifugation and resuspension repeated with 0.5 M NaCl before final resuspension of POBs in distilled water (O’Reilly *et al.*, 1992). POBs were mixed with 0.01 M phosphate-buffered saline (PBS), pH 7.4, loaded (2 ml per tube) onto 34-ml linear sucrose gradients (40–65%) in 0.01 M PBS, and centrifuged at 93,000g for 3 h at room temperature in SW28 ultracentrifuge tubes (Beckman Inc., Palo Alto, CA). The bands of viral occlusions were recovered with a tissue culture pipet and diluted fivefold in distilled water. The resulting mixture was centrifuged at 900g for 10 min at room temperature in 42-ml glass centrifuge tubes, and the pelleted POBs resuspended in a small volume of distilled water. AgipMNPV used in all subsequent experiments came from this stock.

**Extraction of Viral DNA**

Virus purified by sucrose gradient centrifugation was diluted to 1 × 10⁶ POBs/µl, and sodium carbonate added to a final concentration of 0.1 M. This mixture was incubated at room temperature for 30 min, and then Tris–HCl was added to a final concentration of 0.1 M (O’Reilly *et al.*, 1992). The resulting suspension was centrifuged at 3600g for 5 min at room temperature in 50-ml tissue culture tubes (Fisher Scientific, Pittsburgh, PA). The supernatant, containing the released virions, was then layered onto 3-ml cushions of 25% sucrose in 5 mM NaCl and 10 mM EDTA and made up to a final volume of 35 ml with 0.01 M PBS and then centrifuged at 76,000g for 75 min at 4°C in SW28 ultracentrifuge tubes. The supernatant and underlying sucrose cushion were decanted, and the virion pellets resuspended in 0.5 ml of TE (10 mM Tris, 1 mM EDTA, pH 8). Virions and nucleocapsids were digested by incubation with 5% SDS and 0.1 M NaCl and 100 units/ml of proteinase K (200–400 units/ml) for 1 h at 56°C. DNA was extracted by a series of organic extractions using phenol, 25:24:1 phenol:chloroform:isoamyl alcohol, and 24:1 chloroform:isoamyl alcohol. DNA isolated from *E. coli* was precipitated with 0.3 M sodium acetate and 95% ethanol and, following overnight incubation at 4°C, was pelleted by centrifugation at 16,000g for 15 min. The resulting DNA pellets were washed in 70% ethanol, dried, and then resuspended in a small volume of TE.

**Restriction Enzyme Digests**

Viral DNA (3 µg) from AgipMNPV, AcMNPV C6, and RoMNPV R1 was digested with 25 units of *HindIII* or *EcoRI* for 3 h at 37°C. Digested DNA, together with 1-kb ladder DNA (0.5 µg) and Lambda-*HindIII* marker DNA (0.5 µg), was loaded onto a 20 × 25-cm 0.8% agarose gel and run at 75 V for 14 h. The gel was stained for 30 min in ethidium bromide solution (0.5 µg/ml) and then photographed under ultraviolet light. AgipMNPV DNA was also digested with 11 other restriction enzymes and run out on an agarose gel under similar reaction conditions. Restriction enzyme (RE) fragment patterns from digests of AgipMNPV DNA were then compared with published RE digest profiles of DNA from 28 other NPVs.
**Ultrastructural Studies**

Sixth instar *A. ipsilon* were fed 3-mm cubes of diet inoculated with $6 \times 10^6$ POBs of AgipMNPV and subsequently maintained at 27°C. Hemolymph was collected 6 days postinfection from anesthetized larvae (chilled to 4°C) by making a small incision at the base of one of the abdominal prolegs, and collecting hemolymph in an Eppendorf tube on ice. Dithiothreitol was immediately added to the hemolymph to a final concentration of 1 mM to inhibit the phenol oxidase cascade (O’Reilly et al., 1992). Virus infections were confirmed by examining larval hemolymph for the presence of POBs within hemocytes, using phase contrast microscopy. Hemolymph was centrifuged at 400g for 4 min at 4°C to pellet hemocytes. Fat body tissue was dissected from infected larvae bathed in 1% electron microscopy (EM) grade glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA). Hemocyte pellets and fat body tissue were fixed overnight at 4°C in 1% EM grade glutaraldehyde. Following fixation, samples were washed three times in 0.01 M PBS and postfixed in 1% osmium tetroxide in PBS for 1 h at room temperature. Samples were dehydrated through a standard ethanol series (25–100% ethanol, 15 min per step) and cleared with three washes in acetone. Tissues were infiltrated with EMBED 812 epoxy resin (Electron Microscopy Sciences) in a stepwise fashion with ratios of acetone to EMBED of 3:1, 1:1, and 1:3 and finally several changes of pure resin. Resin blocks were dried in an oven at 60°C for 48 h. Thin silver interference sections (50–80 nm thick) were cut with a Reichert Ultracut S ultramicrotome using a diatome diamond knife and collected on 200-mesh copper grids. Thin sections were counterstained with 10% methanolic uranyl acetate for 15 min, followed by Sato-S lead acetate stain for 10 min. Sections were examined with a Jeol 1200EX scanning transmission electron microscope at 80 KV.

**Bioassays**

Lethal concentration bioassays were carried out with first instar *A. ipsilon*, using a droplet feeding technique (Hughes and Wood, 1981). First instars were allowed to drink from suspensions of distilled water containing 1/25 vol blue food coloring dye and one of six concentrations of virus (0 [control], 200, 1000, 5000, 25,000, 125,000 POBs/µl) in 60-mm petri dishes. After 15 min, larvae that had ingested these suspensions were moved into 24-well tissue culture plates (Fisher Scientific) containing diet using paint brushes. Treatments were checked after 24 h for deaths resulting from handling and were scored for mortality at 10 days postinfection, and subsequently until no further mortality occurred. *A. ipsilon, H. virescens, H. zeas, S. frugiperda, P. unipuncta, A. gemmatalis, S. exigua, T. ni, and O. nubilalis* were tested for susceptibility to infection by AgipMNPV. Thirty-five larvae were used per dose, and bioassays in each species were replicated at least twice. For comparative purposes, bioassays in *A. ipsilon* were also performed using AcMNPV C6 and RoMNPV R1. Data were subjected to probit analysis (Russell et al., 1977) and the assumptions of the models verified (Robertson and Preisler, 1992).

**RESULTS**

**Restriction Enzyme Digests**

Restriction enzyme digest profiles of AgipMNPV DNA lacked submolar bands. AgipMNPV DNA digested with HindIII and EcoRI yielded fragment patterns distinct from those produced by digestion of AcMNPV C6 and RoMNPV R1 DNA (Fig. 1). Digests of AgipMNPV DNA carried out with 11 additional restriction enzymes (data not shown) yielded restriction enzyme profiles distinct from those of 28 NPVs in the literature.

**Ultrastructural Studies**

Electron microscopy of tissues from insects infected with AgipMNPV revealed multiple POBs within the nuclei of infected cells (Fig. 2a). Regions of virogenic stroma were visible in the nuclei of infected host cells. POBs were seen to contain many virions, and multiple nucleocapsids were encapsulated within the membrane of each virion (Fig. 2b). Within the nuclei of some cells, nucleocapsids were stacked along sheets of membrane. Fibrillar bodies were also apparent in the nuclei of infected cells (Fig. 2c).

**Bioassays**

Preliminary data indicated that susceptibility to infection by AgipMNPV varied greatly among the lepidopteran species used in the host range studies (Table 1). First instar *A. ipsilon* were the most susceptible, with an LC$_{50}$ of 269 POBs/µl. *H. virescens and H. zeas* were moderately susceptible to infection, while *P. unipuncta* and *S. frugiperda* were only semipermissive to infection. *A. gemmatalis, S. exigua, T. ni,* and *O. nubilalis* were not susceptible to infection by AgipMNPV at the concentrations used in this study. *A. ipsilon* was susceptible to AcMNPV C6 and RoMNPV R1, with LC$_{50}$ values of 1478 and 27,626 POBs/µl, respectively.

**DISCUSSION**

Ultrastructural studies of infected insect tissue showed AgipMNPV to have a typical baculovirus morphology. POBs were present in the nuclei of infected cells, confirming that this new virus is an NPV, and multiple nucleocapsids were seen within each virion, indicating that this virus is a multicapsid NPV (Volkman et al., 1995). Restriction digest fragment patterns
of AgipMNPV DNA were distinct from published fragment patterns from 28 NPVs. It was confirmed that restriction fragment profiles of AgipMNPV were different from the fragment profiles of *S. frugiperda* MNPV (Shapiro *et al.*, 1991) and *A. gemmatalis* MNPV (Johnson and Maruniak, 1989), which are both common throughout the geographic range of *A. ipsilon*. The restriction fragment profiles of AgipMNPV were also determined to be distinct from the fragment profiles of *Agrotis segetum* MNPV (AsMNPV) (Allaway and Payne, 1983). The 4.2-kb *HindIII* K fragment of the Agip-MNPV genome was sequenced and yielded a nucleotide sequence unlike that of any previously published baculovirus nucleotide sequence (data not shown). Although no submolar bands were detected in the restriction enzyme digests, the possibility of a small amount of contaminating virus in the stock suspension cannot be excluded.

Preliminary bioassay data indicated that *A. ipsilon* was the most susceptible species tested, although *H. virescens* and *H. zea* were also susceptible to Agip-MNPV. Other species were not susceptible, or were only semipermissive to infection by AgipMNPV even at the highest infection concentration of 125,000 POBs/µl. In addition to differences in susceptibility between different host species, the slopes of the dose mortality curves differed markedly. Steep slopes indicate that mortality levels change significantly in response to relatively small changes in virus dose. The volumes of fluid ingested by first instars of several noctuid species during droplet feeding bioassays have been quantified (Hughes *et al.*, 1986; van Beek and Hughes, 1986; Kunimi and Fuxa, 1996). In all cases, larvae ingested 6–11 nl of fluid. Assuming that first instar *A. ipsilon* ingest an average of 11 nl of virus suspension during droplet feeding bioassays, a conservative estimate for

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**FIG. 1.** Comparison of restriction enzyme digest profiles of AgipMNPV DNA (*Ai*₁ and *Ai*₂) with those of AcMNPV C6 DNA (Ac) and RoMNPV R1 DNA (Ro). Purified DNA was digested with *HindIII* or *EcoRI* and electrophoresed on 0.8% agarose gel for 14 h at 75 V. 1-kb DNA ladder (1 kb) and λDNA *HindIII* (λHIII) size standards are shown.
the LD<sub>50</sub> of AgipMNPV, is 3 POBs per larva. This LD<sub>50</sub> is similar to that of AcMNPV against a susceptible host, such as <i>T. ni</i> (Hughes et al., 1986).

The high susceptibility of <i>A. ipsilon</i> to AgipMNPV suggests that this virus will be more effective for <i>A. ipsilon</i> control than other viruses that have been tested, such as <i>A. segetum</i> granulovirus, AcMNPV C6, and RoMNPV R1 (Johnson and Lewis, 1982; Zethner and Ogaard, 1982; Allaway and Payne, 1984).

In summary, AgipMNPV is a new baculovirus isolated from <i>A. ipsilon</i>. AgipMNPV has high virulence against <i>A. ipsilon</i> and appears to have greater potential as a microbial control agent for use against this insect than other baculoviruses investigated to date. Green-
TABLE 1
Preliminary Lethal Concentration Data for AgipMNPV against First Instar Lepidoptera*

<table>
<thead>
<tr>
<th>Host species</th>
<th>LC50a (POBs/µl)</th>
<th>95% Confidence interval</th>
<th>Heterogeneity (χ²/DF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrotis ipsilon</td>
<td>269 (145–407)</td>
<td>1.77 0.26</td>
<td></td>
</tr>
<tr>
<td>Heliocloodes tiarensens</td>
<td>797 (324–1533)</td>
<td>0.78 0.25</td>
<td></td>
</tr>
<tr>
<td>Helicoverpa sea</td>
<td>7,083 (3359–1578)</td>
<td>0.86 0.34</td>
<td></td>
</tr>
<tr>
<td>Spodoptera frugiperda</td>
<td>108,260 (44,647–550,820)</td>
<td>0.69 0.96</td>
<td></td>
</tr>
<tr>
<td>Pseudaletia unipuncta</td>
<td>125,370 (36,639–694,510)</td>
<td>1.03 1.88</td>
<td></td>
</tr>
<tr>
<td>Anticarsia gemmatalis</td>
<td>NS</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Spodoptera exigua</td>
<td>NS</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Trichoplusia ni</td>
<td>NS</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ostrinia nubilalis</td>
<td>NS</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Note. NS, not susceptible to AgipMNPV at the doses used in this study.

*Thirty-five larvae were exposed per virus concentration. Bioassays were replicated twice for each species, and data reported are not significantly different from the second replicate for that species.

b Data analyzed by probit analysis (Russell et al., 1977). Probit-dose relationship linear at α = 0.05 confidence level. Data fit probit model by χ² test at α = 0.05.

house and field trials are underway to assess the potential of AgipMNPV for control of A. ipsilon.

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