Insect viruses capable of killing at least one target insect pest quicker than previously described viruses and DNA sequence conferring that phenotype of faster killing are provided. Further improvement in the speed of killing is obtained when the virus of this invention also contains a non-functional egt gene to reduce feeding by the infected larvae, inhibit growth and further mediate the earlier death of the infected insect. A specifically exemplified faster-killing insect virus is the V-8 strain of AcMNPV. The faster killing phenotype is carried on a M13 fragment from 1.93 to 3.27 map units within the AcMNPV genome, and its sequence is provided herein as SEQ ID NO:.... V8eGTDDEL is the egt-inactivated derivative of AcMNPV V-8; the combination of the increased virulence of the V-8 genotype, for example, and the inactivation of the gene encoding ecysteoyl glycosyl transferase provides further improvement (as further decrease in time after infection until insect death). Additionally, such an Egt-deficient baculovirus may be still further modified to express a protein which affects ecdysis. Methods for producing the faster-killing insect virus, improved insecticidal compositions and improved methods of controlling insects are also included within the scope of this invention.
FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| AT | Austria |
| AU | Australia |
| BB | Barbados |
| BE | Belgium |
| BF | Burkina Faso |
| BG | Bulgaria |
| BJ | Benin |
| BR | Brazil |
| BY | Belarus |
| CA | Canada |
| CF | Central African Republic |
| CG | Congo |
| CH | Switzerland |
| CI | Côte d'Ivoire |
| CM | Cameroon |
| CN | China |
| CS | Czechoslovakia |
| CZ | Czech Republic |
| DE | Germany |
| DK | Denmark |
| ES | Spain |
| FI | Finland |
| FR | France |
| GA | Gabon |
| GB | United Kingdom |
| GE | Georgia |
| GN | Guinea |
| GR | Greece |
| HU | Hungary |
| IE | Ireland |
| IT | Italy |
| JP | Japan |
| KE | Kenya |
| KG | Kyrgyzstan |
| KP | Democratic People's Republic of Korea |
| KR | Republic of Korea |
| KZ | Kazakhstan |
| LI | Liechtenstein |
| LK | Sri Lanka |
| LU | Luxembourg |
| LV | Latvia |
| MC | Monaco |
| MD | Republic of Moldova |
| MG | Madagascar |
| ML | Mali |
| MN | Mongolia |
| MB | Mauritania |
| MW | Malawi |
| NE | Niger |
| NL | Netherlands |
| NO | Norway |
| NZ | New Zealand |
| PL | Poland |
| PT | Portugal |
| RO | Romania |
| RU | Russian Federation |
| SD | Sudan |
| SE | Sweden |
| SI | Slovenia |
| SK | Slovakia |
| SN | Senegal |
| TD | Chad |
| TG | Togo |
| TJ | Tajikistan |
| TT | Trinidad and Tobago |
| UA | Ukraine |
| US | United States of America |
| UZ | Uzbekistan |
| VN | Viet Nam |
INSECT VIRUSES, SEQUENCES, INSECTICIDAL COMPOSITIONS AND METHODS OF USE

Technical Field

The present invention relates to methods and compositions using baculoviruses and sequences therefrom for improved biological control of insect pests. More particularly, the present invention relates to a recombinant baculovirus which has improved properties in insect control and the segment therefrom conferring improved properties, i.e., more rapid death for at least one target insect. The present invention also relates to genetically modified baculoviruses with further improved killing properties and methods of use.

Background of the Invention

Interest in the biological control of insect pests has arisen as a result of disadvantages of conventional chemical pesticides. Chemical pesticides generally affect beneficial as well as nonbeneficial species. Insect pests tend to acquire resistance to such chemicals so that new insect pest populations can rapidly develop that are resistant to these pesticides. Furthermore, chemical residues pose environmental hazards and possible health concerns. Biological control presents an alternative means of pest control which can reduce dependence on chemical pesticides.

The primary strategies for biological control include the deployment of naturally-occurring organisms which are pathogenic to insects (entomopathogens) and the development of crops that are more resistant to insect pests. Approaches include the
identification and characterization of insect genes or gene products which may serve as suitable targets for insect control agents, the identification and exploitation of previously unused microorganisms (including the modification of naturally-occurring nonpathogenic microorganisms to render them pathogenic to insects), the modification and refinement of currently used entomopathogens, and the development of genetically engineered crops which display greater resistance to insect pests.

Viruses that cause natural epizootic diseases within insect populations are among the entomopathogens which have been developed as biological pesticides. Baculoviruses are a large group of viruses which infect only arthropods (Miller, L.K. (1981) in Genetic Engineering in the Plant Sciences, N. Panopoulous, (ed.), Praeger Publ., New York, pp. 203-224; Carstens, (1980) Trends in Biochemical Science 52:107-110; Harrap and Payne (1979) in Advances in Virus Research, Vol. 25, Lawfer et al. (eds.), Academic Press, New York, pp. 273-355; The Biology of Baculoviruses, Vol. I and II, Granados and Federici (eds.), CRC Press, Boca Raton, Florida, 1986.). Many baculoviruses infect insects which are pests of commercially important agricultural and forestry crops. Such baculoviruses are potentially valuable as biological control agents. Four different baculoviruses have been registered for use as insecticides by the U.S. Environmental Protection Agency. Among the advantages of baculoviruses as biological pesticides is their host specificity. Not only do baculoviruses as a group infect only arthropods, but also individual baculovirus strains usually only infect one or a few species of insects. Thus, they pose no risk to man or the environment, and can be used without adversely affecting beneficial insect species.

Baculoviruses, including AcMNPV, have been found in approximately 400 different species across several different insect orders; the vast majority of these viruses occur in the order Lepidoptera. Baculoviruses are known to infect insects in
both natural ecosystems (e.g., forests and prairies) and monocultural agroecosystems (e.g., cotton fields).

_Autographa californica_ nuclear polyhedrosis virus, (AcMNPV) is the most extensively characterized baculovirus known. AcMNPV belongs to the family Baculoviridae, subfamily Eubaculovirinae, genus Nuclear Polyhedrosis Virus, and the subgenus Multiple Nucleocapsid Virus, which are characterized by the formation of viral occlusion bodies (or polyhedra) in the nuclei of infected host cells. The virus was first isolated more than 20 years ago from an alfalfa looper, _Autographa californica_ during a naturally occurring epizootic infection in California. Since then, the virus has been characterized extensively using biochemical and molecular techniques, and extensive DNA sequence within the 128 kbp genome is known. AcMNPV has been designated as the type species for the subgenus Multiple Nucleocapsid Virus.

Baculovirus subgroups include nuclear polyhedrosis viruses (NPV), granulosis viruses (GV), and non-occluded baculoviruses. In the occluded forms of baculoviruses (GV and NPV), the virions (enveloped nucleocapsids) are embedded in a crystalline protein matrix. This structure, referred to as an inclusion or occlusion body, is the form found extraorganismally in nature and is responsible for spreading the infection between organisms. The characteristic feature of the NPVs is that many virions are embedded in each occlusion body. The NPV occlusion bodies are relatively large (up to 5 micrometers). Occlusion bodies of the GV viruses are smaller and contain a single virion each. The crystalline protein matrix of the occlusion bodies of both forms is primarily composed of a single 25,000 to 33,000 dalton polypeptide which is known as polyhedrin or granulin. Baculoviruses of the non-occluded subgroup do not produce a polyhedrin or granulin protein, and do not form occlusion bodies.

In nature, infection is initiated when an insect ingests food contaminated with baculovirus particles, typically in the form of occlusion bodies for an NPV such as AcMNPV. The
occlusion bodies dissociate under the alkaline conditions of the insect midgut, releasing individual virus particles which then invade epithelial cells lining the gut. Within a host cell, the baculovirus migrates to the nucleus where replication takes place. Initially, certain specific viral proteins are produced within the infected cell via the transcription and translation of so-called "early genes." Among other functions, these proteins are required to allow replication of the viral DNA, which begins 4 to 6 hours after the virus enters the cell. Extensive viral DNA replication proceeds up to about 24 hours post-infection (pi). From about 8 to 20 hours pi, the infected cell produces large amounts of "late viral gene products." These include components of the nucleocapsid which surrounds the viral DNA during the formation of progeny virus particles. Production of the progeny virus particles begins around 12 hours pi. Initially, progeny virus migrate to the cell membrane where they acquire an envelope as they bud out from the surface of the cell. This non-occluded virus can then infect other cells within the insect. Polyhedrin synthesis begins about 18 hours after infection and increases to very high levels by 24 hours pi. At that time, there is a decrease in the number of budded virus particles, and progeny virus are then embedded in occlusion bodies. Occlusion body formation continues until the cell dies or lyses. Some baculoviruses infect virtually every tissue in the host insect so that at the end of the infection process, the entire insect is liquified, releasing extremely large numbers of occlusion bodies which can then spread the infection to other insects. (Reviewed in The Biology of Baculoviruses, Vol. I and II, Granados and Federici (eds.), CRC Press, Boca Raton, Florida, 1986.)

Larvae become increasingly resistant to viral infection as they grow and mature. Both adult tissues and pupal tissues appear to be refractory to viral infection. The primary means of infection by AcMNPV appears to be via horizontal transmission. Insects typically acquire AcMNPV by consuming contaminated food.
The occlusions dissolve in the insect mid-gut and release virions which establish a primary site of infection in mid-gut cells. The ability of AcMNPV to persist and spread in the environment is governed by many interrelated factors (reviewed by Evans, H. (1986) *The Biology of Baculoviruses, Ecology and Epizology of Baculoviruses*, Granados, R.R. and Federici, B.A. (eds.) pp.89-132). Factors such as the relative sensitivity of the insect host to virus, as well as developmentally determined sensitivity to AcMNPV, are important. Host density also appears to play an important role in determining persistence and spread of baculoviruses. There are important implications concerning the role of biotic and abiotic forces that determine AcMNPV environmental transmission and persistence. For example, predators compete with virus for available insect hosts and tend to reduce potential virus productivity by removal of these virus-susceptible hosts from the environment. On the other hand, predators can also indirectly increase the survival capacity and spread of MNPVs by increasing virus dispersal and by making more efficient use of available host populations. This predator-aided transmission is generally by passage of infectious MNPVs through the gut of predatory insects, birds, and mammals. Likewise, abiotic factors (such as ultraviolet (UV) light, rainfall, temperature, and pH) have a major influence on virus survival and spread in the environment. For example, baculoviruses appear to be particularly sensitive to UV irradiation and to alkaline pH. Persistence of field applied virus without UV protection can be as little as 1-2 days in the field. Soil appears to be a particularly important reservoir for persistence of baculoviruses. The decline of viruses in the soil is slow and wide range of times for persistence and viability have been reported. The ubiquitous and harmless association between baculoviruses and humans and other species due to dietary exposure underscores their safety and value as insecticides.

One potential disadvantage to using baculoviruses as pesticides is the length of time between virus ingestion and

There is a need for biological pesticides, specifically insect viruses, which reduce feeding by the insect before death and/or which result in a shorter time between infection and death when compared to prior art insect viruses. A biological pesticide is preferred because it creates less of an environmental hazard than a chemical pesticide. The exploitation of a recombinant insect which results in more rapid insect killing than available viruses will allow improved biological control of insect pests.
Summary of the Invention

This invention specifically provides a purified and isolated recombinant baculovirus (called AcMNPV V-8 herein) which has improved killing properties as compared with prior art Autographa californica Nuclear Polyhedrosis Virus strains, against at least one insect pest including, but not limited to, Spodoptera frugiperda. The object baculovirus has been deposited with the American Type Culture Collection as ATCC VR-____ (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD). Improved killing properties against at least one insect pest means that when at least one species of insect pest is infected, the time between infection and insect death is shorter than with a comparison AcMNPV, e.g., AcMNPV E2 (ATCC VR-1344).

A further specific object of the present invention is an AcMNPV V-8 derivative which has been genetically engineered to inactivate the gene encoding ecdysteroid glucosyltransferase (egt). A preferred embodiment is V8vEGTDEL, which is the AcMNPV V8 derivative in which a portion of egt is deleted, with the result that a functional ecdysteroid glucosyltransferase is not produced during the viral infection process.

An additional object of the present invention is a segment of insect virus DNA which confers the improved (i.e., faster) killing phenotype when incorporated in the genome of a heterologous insect virus. As specifically exemplified such a DNA fragment is the MluI to EspI fragment from the region of the AcMNPV V-8 genome of 1.93 to 3.27 map units (or from the V1000 nuclear polyhedrosis virus, which appears to be closely related to or identical to Rachiplusia ou Nuclear Polyhedrosis Virus); the DNA sequence of the exemplified V-8 fragment is given in Fig. 4 (SEQ ID NO:...). This DNA segment carries sequences encoding a late expression factor (lef-2), and polypeptide of 603 amino acids (function unknown) and sequences upstream of the polyhedrin gene. Within this MluI to EspI fragment of the V-8 is a smaller region which is capable of conferring the improved killing
phenotype when incorporated into the genome of a (recombinant) AcMNPV; this smaller region is between nucleotide 3026 and 4231 (V-8 sequence) of Fig. 4. Also within the scope of the invention are other recombinant insect viruses, including baculoviruses, in which such a DNA fragment conferring the improved killing phenotype has been incorporated into the genome with the result that at least one insect pest is killed quicker after infection than the corresponding baculovirus which has not been so genetically engineered or which otherwise does not contain a related heterologous DNA segment which confers increased virulence.

The present invention also provides methods for improving the killing properties of a baculovirus by introducing a segment of DNA conferring a phenotype of improved killing, e.g., faster insect death of at least one species of insect pest after infection, e.g., and by confirming the improved killing property by determining that the LT₉₀ (time required for killing 50% of test larvae at a standard virus dose, using a dose killing 90% of test larvae by set time post infection) is shorter for the genetically engineered strain than for the parental baculovirus. The genetically engineered strain may be produced by molecular biological techniques using an insect virus selected from the group consisting of nuclear polyhedrosis viruses including, but not limited to, Lymantria dispar NPV, Autographa california NPV, Synographa falcifera NPV, Spodoptera lilturalis NPV, Spodoptera exigua NPV, Spodoptera frugiperda NPV, Heliothis armigera NPV, Mamestra brassicae NPV, Choristoneura fumiferana NPV, Trichoplusia ni NPV, Helicoverpa zea NPV and Manduca sexta NPV; granulosis viruses including, but not limited to, Cvdia pomonella GV, Pieris brassicae GV and Trichoplusia ni GV. Non-occluded viruses from the Baculovirinae may also be genetically modified to improve their killing properties for particular target insects; examples of such non-occluded baculovirinae include, but are not limited to, those of Orctyes rhinoceros and Heliothis zea non-occluded baculovirinae. Functionally equivalent sequences conferring improved killing properties may be isolated from
viruses other than as specifically exemplified herein, and incorporated into viral genomes which do not naturally contain them to produce insect viruses with improved insect control properties.

Alternatively it may be produced by co-infection of a first baculovirus with a second baculovirus whose genome comprises said DNA fragment, wherein said first and second baculoviruses are sufficiently related (i.e., have sufficient DNA sequence identity at least over limited distances on regions flanking the sequences conferring the improved killing phenotype) so that recombination occurs to result in a recombinant baculovirus being produced which incorporates the DNA conferring the improved killing phenotype.

Further objects of the present invention are insecticidal compositions comprising the baculoviruses with improved killing properties against at least one insect pest. Preferred viruses include V8vEGTDEL, AcMNPV V-8, vEcoRlHyb1, vEcoRlHybIFS and nuclear polyhedrosis viruses and granulosis viruses and non-occluded baculovirinae viruses in a non-limiting fashion as set forth herein above into which a segment of DNA conferring the improved killing phenotype has been genetically engineered or incorporated by recombination. Preferred the insecticidal compositions of the present invention are formulated as wettable powders. The composition of a preferred wettable powder insecticidal composition is as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Nominal Percent (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V8vEGTDEL polyhedrin inclusion bodies</td>
<td>10.0%</td>
</tr>
<tr>
<td>Morawet D425</td>
<td>30.0%</td>
</tr>
<tr>
<td>Morawet EFW</td>
<td>20.0%</td>
</tr>
<tr>
<td>Kaolin Clay</td>
<td>16.0%</td>
</tr>
<tr>
<td>Microcel E</td>
<td>16.0%</td>
</tr>
<tr>
<td>UV-9 oxybenzone or charcoal</td>
<td>5.0%</td>
</tr>
<tr>
<td>Eudragit S100</td>
<td>2.0%</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>0.9%</td>
</tr>
<tr>
<td>polyethylene glycol MW400</td>
<td>0.1%</td>
</tr>
</tbody>
</table>
Optionally, a stilbene brightener can be added to the formulation to enhance infectivity or potentiate the insecticidal effects of the insect virus.

The preferred composition described above is formulated as follows:

a) preparing an aqueous suspension of Eudragit S100 (1% w/v);

b) dissolving the Eudragit S100 by adding the pH of the suspension of step (a) to 9.0 to 9.5;

c) adding viral PIBs and UV-9 oxybenzone or charcoal to the solution of step (b), and blending to produce an even suspension;

d) air drying the even suspension of step (c);

e) milling the dried material of step (d) to produce milled material; and

f) dry blending the milled material of step (e) with Morwet D425, Morewet EFW wetting agent, Kaolin Clay as a bulking agent, Microcell E as a flow agent, citric acid and polyethylene glycol MW400 to provide flexibility to the milled material.

30 **Brief Description of the Drawings**

Fig. 1 is a schematic representation of the AcMNPV genome showing the location of the eot and genes. The AcMNPV genome is presented in map units and as EcoRI and HindIII restriction maps.

Fig. 2A is a schematic representation of the structures of the eot gene regions of AcMNPV; Figs. 2B and 2C illustrate the
structures of the corresponding regions of the recombinant viruses vEGTZ and vEGTDEL, respectively, and the pEGTZ and pEGTDEL plasmids used in the construction of those recombinant viruses. The hatched box in Fig. 2B represents the lacZ gene.

Fig. 3A-3D represents partial restriction maps of the 327 ORF, lef-2, the 603 ORF, and the polyhedrin gene (polh) region of AcMNVPV strains. Fig. 3A is the map for the AcMNVPV L-1 wild type (thin line represents L-1 DNA). Fig. 3B is the map of AcMNVPV V-8 (thick line represents V-8 DNA). The extra HindIII site in lef-2 is one distinguishing (physical) characteristic of V-8. V-8 is missing both the MluI site within the 603 ORF and the EcoRV site between the 603 ORF and polh. The V-8 603 ORF has a premature stop codon generated by an insertion and is predicted to produce an incomplete, non-functional polypeptide product (note "X" through the 603 ORF). Fig. 3C is the map of vEcoRIHybI recombinant virus containing the portion of the V-8 genome indicated by the thick bar. Although the transfer plasmid used to construct this hybrid contained V-8 sequence to the MluI site at 1.93 m.u., allelic replacement limited V-8 sequences to the portion of lef-2 indicated. Fig. 3D is the map of vEcoRIHybIFS recombinant virus containing the entire V-8 MluI (1.93 m.u.) to EspI (3.27 m.u.) fragment. The NaeI site in what was the 603 ORF has been destroyed via a four base pair deletion (asterisk denotes missing NaeI site).

Fig. 4 presents the DNA sequence of AcMNVPV L-1 (SEQ ID NO:1) from the 327 ORF MluI site (nucleotide 2470) to the polh EspI site (nucleotide 4186) aligned with the corresponding sequence from the AcMNVPV V-8 variant (SEQ ID NO:2). The V-8 sequence has a multitude of point mutations and four insertions as compared to the L-1 sequence. Identities are indicated by a vertical line. Sequence differences and insertions are in bold type. Startpoints of lef-2, the 603 ORF and polh are marked with asterisks (*). The natural termination codons of the L-1 lef-2 and 603 ORF are marked with pound signs. Crossover in vEcoRIHybI occurred in the dashed region between the two dollar signs at
nucleotides 3003 and 3027. The premature stop codon generated by the insertion in the V-8 603 ORF is indicated by three consecutive carets. Sequence numbering in parentheses corresponds to that in O'Reilly et al. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*, W.H. Freeman & Co., NY.

Fig. 5 presents the predicted amino acid sequences of AcMNPV L-1 and the V-8 *lef-2* gene products (SEQ ID NO: ___ and SEQ ID NO: ___, respectively). Identities are designated by vertical lines. Differences are designated by bold type and a period between the differing amino acid residues. DNA sequence differences between the L-1 and the V-8 are responsible for six amino acid differences between the two *lef-2* gene products. Amino acid differences occur at residues 14, 89, 101, 114, 153 and 190. The recombinant virus vEcoRIHybIFS is expected to contain all these *lef-2* amino acid differences, but vEcoRIHybI is predicted to contain only the mutations at residues 153 and 190. The crossover event in vEcoRIHybI occurred in the DNA region that encodes the residues marked by a line of asterisks.

Fig. 6 presents the nucleotide sequence of AcMNPV DNA in the region of the *egt* gene. The codons for translation initiation (atg) and termination (taa) for the *egt* open reading frame are indicated over the sequence. The 1094 bp fragment deleted in the EGTDEL virus is underlined. The positions from which the oligonucleotide primers (EGTDEL1 and EGTDEL2) used for PCR amplification are shown.

Fig. 7 presents DNA sequences for AcMNPV E2 (SEQ ID NO: ...), AcMNPV V-8 (SEQ ID NO: ...) and V1000 (SEQ ID NO: ...) virus strains beginning at the Esp31 site upstream of the polyhedrin gene and extending into the polyhedrin coding region. The sequences were from one strand using primer PV1 Reverse, and nucleotides indicated as N were not identified.
Detailed Description of the Disclosed Embodiments

Because faster acting insect viruses are desirable as insect control agents, a study was undertaken to search for baculovirus strains with such improved killing properties. Toward this end, a minimally passaged (in insect larvae) AcMNPV virus stock was amplified, plated in culture to obtain clonal isolates, and these isolates were examined for restriction site polymorphisms and for increased virulence in insect larvae. A minimally passaged stock was used as the starting material for this survey, in part because serial passage in cell culture was known to lead to mutations and perhaps reductions in virulence in AcMNPV (see, e.g., Kumar and Miller (1987) *Virus Research* 7:335-349). Genotypic variants of AcMNPV were known (Lee and Miller (1978) *J. Virol.* 27:754-767). AcMNPV was the baculovirus for which a more virulent (i.e., faster killing) variant was sought because it is known to infect a relatively large number of insect pests of particular economic importance in agriculture.

The AcMNPV V-8 isolate was one of ten viral clones plaque purified on SF-21 cell monolayers inoculated with diluted hemolymph from *Heliothis virescens* larvae that had been orally infected with a minimal passage stock of the original Vail AcMNPV isolate (Vail et al., (1971) *Proc. IV Int. Collog. Insect Pathology*, College Park, MD pp.297-304). All ten viral isolates, V-1 through V-10, were initially characterized by restriction endonuclease analysis with *Bam*H1, *Bgl*II, *Eco*RI, *Hind*III, *Pst*I, and *Xho*I and compared to AcMNPV L-1. The pattern of V-10 is identical to that of L-1. The profiles of V-1, V-2, V-3, V-6, V-7, V-8 and V-9 all lack the approximately 8.5 kb of *Hind*III-F fragment and instead contain two novel fragments of approximately 7.4 kb and 1.1 kb. Two isolates, V-4 and V-5, have a restriction pattern intermediate between the first two viral types (containing submolar quantities of the *Hind*III-F fragment and both the novel fragments of 7.4 kb and 1.1 kb). The presence of submolar fragments suggests that V-4 and V-5 are incompletely purified viral stocks, as all samples were plaque purified only
once to preserve the virulence of the isolates. No differences in restriction profiles were detected between any of these ten clones and L-1 using BamHI, BglII, EcoRI, PstI, and XhoI digestion.

The V-8 isolate of AcMNPV was selected as representative of the predominant genotype of the ten isolates and was further characterized using Spodoptera frugiperda neonate bioassays. Data from representative bioassays evaluating oral infectivity (LC₃₀) and virulence (LTₕ₀) are presented in Table 1. LC₃₀ is the amount of virus at which 50% of infected larvae are dead within ten days after infection. LTₕ₀ is the time after infection when 50% of the infected larvae are dead when exposed to virus at LC₃₀ unless otherwise indicated hereinbelow. In S. frugiperda and Trichoplusia ni neonates, the LC₃₀s of the L-1 and V-8 AcMNPV strains are very similar, but the LTₕ₀s are significantly different in S. frugiperda neonates. For AcMNPV strains E-2 and L-1, death from infection normally occurs at about the same time after infection while V-8 causes death more quickly post infection than the L-1 and E2 strains. There is variability in the actual time until death from experiment to experiment, but the results are consistent from experiment to experiment for comparisons of the percent difference in time until death in the V-8 versus L-1 or E-2 comparisons. The average LTₕ₀ at LC₃₀ of the V-8 isolate in S. frugiperda neonates is consistently about 12% shorter than the average LTₕ₀ of L-1.

Initial restriction analysis of AcMNPV V-8 versus AcMNPV L-1 with a battery of different restriction endonucleases (BamHI, BglII, EcoRI, HindIII, PstI, and XhoI) showed only the HindIII restriction polymorphism discussed above. The six restriction endonucleases used to characterize AcMNPV V-8 recognize a total of 95 sites in the AcMNPV genome, counting each EcoRI hr region (six short regions with highly repetitive DNA sequences and multiple EcoRI sites) as one site. Since each recognition site is a hexanucleotide, a total of 570 bp have been screened for mutations by restriction endonuclease analysis. The only
difference found in this screen was a *HindIII* restriction polymorphism in *lef-2* (a 0.18% mutation rate (1/570). The V-8 strain was subsequently shown to lack the *EcoRV* site which is located at about 90 bp upstream of the polyhedrin translation start site (see, e.g., Figure 7).

Sequence analysis of 1.72 kb in the region surrounding the *HindIII* polymorphism revealed numerous nucleotide differences between L-1 and V-8 sequences in and around *lef-2*, the 603 ORF, and the polyhedrin gene (*polh*) (1.93 map units (m.u.) to 3.27 m.u.) (Fig. 2). There are 73 nucleotide changes in the 1.72 kb sequenced region. The *HindIII* restriction polymorphism in V-8 is due to a C to T mutation at nucleotide 3243. Both the *MluI* site (3389) in the 603 ORF and the *EcoRV* site (4001) between the polyhedrin gene and the 603 ORF were destroyed by single nucleotide changes (Figures 4 and 5). Several nucleotide substitutions in this region result in amino acid sequence changes in the predicted polypeptide products of *lef-2*, while insertions and substitutions substantially alter the 603 ORF. The six predicted amino acid changes in *lef-2* are shown in Fig. 5. A 26 bp insert in the 603 ORF creates a stop codon within the open reading frame of the 603 ORF and is predicted to cause premature termination during 603 ORF translation. No sequence differences were discovered in the 327 ORF as far upstream as the *MluI* site. Only three DNA sequence differences between V-8 and E-1 were discovered in *polh*. These are third base pair changes which do not change the encoded amino acids. The region about 90 bp upstream of the polyhedrin translation start site of *polh* was generally unchanged, although the *EcoRV* site present in L-1 is absent in V-8.

Therefore, based on sequence and restriction analysis, this region of V-8 contains an unusually high density of mutations using L-1 as a wild-type comparison. Without wishing to be bound by any particular theory, it is postulated that AcMNPV V-8 arose by recombination between AcMNPV and a virus relatively distantly related to AcMNPV. Furthermore, considering the mutation density
of V-8 in this region, the differences at nucleotides 2703 and 4194 of V-8 (Fig. 4) may be the limits of the recombination, as no sequence differences were found as far downstream of the BamHI site beginning at nucleotide 4264 in polh as shown in Fig. 4 (i.e., nucleotide ___ of SEQ ID NO:___) and as far upstream as the 327 ORF MluI site (beginning at ___ in SEQ ID NO:___) at nucleotide 2470 (Fig. 2). Most of the mutations are concentrated in the 603 ORF and, to a lesser extent, in lef-2. Furthermore, complete V-8 vs. L-1 sequence analysis of the relatively distant 504 ORF (a phosphatase gene located between 0.0 m.u. and 0.4 m.u.) revealed no differences between L-1 and V-8.

The *H. virescens* colony at the American Cyanamid Agricultural Research Center, Princeton, NJ, was derived from a field isolate (Stoneville, MS) in 1966, and has been maintained since 1966. Air, water and diet are not thoroughly sterilized before coming in contact with *H. virescens*. It has been discovered that there are sporadic viral outbreaks in this colony. Virus, termed V1000, has been isolated from this colony, partial genomic DNA sequence has been determined and various properties of the virus have been characterized. The V1000 Nuclear Polyhedrosis Virus appears to be most closely related to *Rachiplusia ou* Nuclear Polyhedrosis Virus (RoNPV) based on restriction endonuclease analysis.

Based on a sequence comparison of V-8 and V1000 polyhedrin regions, but without wishing to be bound by any particular theory, it is postulated that AcMNPV strain V-8 is a recombinant between AcMNPV and the V1000 virus. A comparison of sequence between AcMNPV E-2 (ATCC VR-1344, American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD), V1000 and AcMNPV V-8 is presented in Fig. 7. The last sequence difference between the AcMNPV V-8 and E-2 strains occurs at the twentieth codon of the polyhedrin coding sequence.

Two recombinant viruses, vEcoRIhybI and vEcoRIHybIFS, were constructed by allelic replacement (see Example 3 and Fig. 2) to
determine if the reduced LT<sub>50</sub> of AcMNPV V-8 was correlated with the sequence differences observed in the lef-2 region. Sequence analysis established which V-8 characteristic sequence differences these recombinants possessed following allelic recombination. Both recombinants had recombined downstream of the KpnI site in polh as evidenced by their occlusion positive phenotype. The parent virus vSynVlgal lacks polh sequences upstream of the KpnI site. The virus vEcoRIHybIFS contained the entire 1.72 kb MluI to EspI (1.93-3.27 m.u.) fragment with a four bp deletion at the NaeI site in what was the 603 ORF. The deletion in the V-8 603 ORF was intended to destroy the function of the product of the 603 ORF, but subsequent sequence analysis revealed that the V-8 603 ORF was already disrupted. Thus, this deletion is expected to have no additional effect on viral infectivity and virulence. Crossover during the allelic replacement event generating VEcoRIHybI occurred at some point between nucleotides 3003 and 3027 of AcMNPV sequence in Fig. 4 (between nucleotides ___ and ___ of SEQ ID NO: ___ Figures 4 and 6) and before the KpnI site within the polyhedrin gene. Thus, the lef-2 gene product of VEcoRIHybI is predicted to be a hybrid containing L-1-like amino acid residues upstream of the crossover and V-8-like residues downstream of the crossover (Figures 4 and 6).

Bioassays to determine infectivity and virulence of the viruses L-1, V-8, VEcoRIHybI, and VEcoRIHybIFS were performed on Spodoptera frugiperda neonates. Both LC<sub>50</sub>s and LT<sub>50</sub>s were computed using probit analysis (Daum (1970) Bulletin of the Entomological Society of America 16:10-15) for each virus (Table 1). The LC<sub>50</sub>s of all four viruses were statistically equivalent. As previously noted, V-8 has a 12.4% shorter LT<sub>50</sub> at LC<sub>50</sub> than L-1, reflecting increased virulence. The differences between the LT<sub>50</sub>s at LC<sub>50</sub> of V-8, vEcoRIHybI and vEcoRIHybIFS are not statistically significant. However, the differences between the LT<sub>50</sub>s at LC<sub>50</sub> of L-1 and each of the three viruses containing V-8 DNA are statistically significant; V-8 and the two hybrid viruses each had a significantly shorter LT<sub>50</sub> than L-1. Hybrid virus
vEcoRIHybI contains only a small region of the V-8 sequences from the middle of lef-2 to the 5' end of polh but possesses the increased virulence characteristic of V-8. The fact that the lef-2 gene product of vEcoRIHybI has an L-1-like amino-terminus and a V-8 carboxy-terminus but still retains the V-8 virulence phenotype indicates that the increased virulence (decreased LT₉₀) of V-8 is due either to one or both of the lef-2 carboxy-terminal amino acid differences or to the absence of a functional 603 ORF gene product, or some combination thereof. Alternatively, the V-8 faster killing (increased virulence) phenotype may be due to cis-acting sequence effects.

Whether the two carboxy-terminal lef-2 amino acid differences, the non-functional 603 ORF or a combination of these differences is responsible for the increased virulence of V-8 remains to be determined. Gearing and Possee (1990) J. Gen. Virol. 71:251-262 determined that the 603 ORF is not essential for production of budded virus in cell culture, production of polyhedra, or the infectivity (LC₅₀) of AcMNPV. However, Gearing and Possee presented no data relevant to the virulence as measured by LT₉₀ of their 603 ORF deletion mutant, so it is not known what effects the destruction of the 603 ORF had on the virulence of their mutant. Passarelli and Miller (1993) J. Virol. 67:2149-2158 reported that lef-2 and its 630 amino acid expression product is required for late and very late gene expression in transient expression assays. Without wishing to be bound by any particular theory, it is postulated that one or both of the V-8 lef-2 and 603 ORF alleles affect the rate of virus replication and therefore the virulence of the virus. It is possible that the faster killing by the V-8 strain is due to a cis or trans effect. The phenotype is believed to be carried within the region between nucleotides 3027 and 4231 in the V-8 sequence shown in Fig. 4.
Table 1A. Bioassays of the infectivity of AcMNPV variants in S. frugiperda neonates.

<table>
<thead>
<tr>
<th>Virus</th>
<th>LC₉₀</th>
<th>Upper</th>
<th>Lower</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-1</td>
<td>4.6x10³</td>
<td>6.8x10³</td>
<td>3.0x10³</td>
<td>0.81</td>
</tr>
<tr>
<td>V-8</td>
<td>3.0x10³</td>
<td>4.3x10³</td>
<td>2.0x10³</td>
<td>0.97</td>
</tr>
<tr>
<td>vEcoRIHybI</td>
<td>5.5x10³</td>
<td>1.3x10⁴</td>
<td>1.9x10³</td>
<td>0.96</td>
</tr>
<tr>
<td>vEcoRIHybIFS</td>
<td>2.1x10³</td>
<td>2.9x10³</td>
<td>1.4x10³</td>
<td>1.06</td>
</tr>
</tbody>
</table>

LC₉₀s (#PIBs/ml diet; polyherin inclusion bodies/ml) for L-1, V-8, vEcoRIHybI, and vEcoRIHybIFS were statistically equivalent.

Table 1B. Bioassays of the Virulence of AcMNPV Variants in S. frugiperda neonates

<table>
<thead>
<tr>
<th>Virus</th>
<th>Fiducial Limits</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LT₉₀</td>
<td>Upper</td>
<td>Lower</td>
<td>Slope</td>
</tr>
<tr>
<td>L-1</td>
<td>129.4</td>
<td>134.1</td>
<td>125.7</td>
<td>12.35</td>
</tr>
<tr>
<td>V-8</td>
<td>113.3</td>
<td>116.1</td>
<td>110.8</td>
<td>14.39</td>
</tr>
<tr>
<td>vEcoRIHybI</td>
<td>116.9</td>
<td>120.7</td>
<td>113.7</td>
<td>10.64</td>
</tr>
<tr>
<td>vEcoRIHybIFS</td>
<td>115.0</td>
<td>117.9</td>
<td>112.3</td>
<td>13.50</td>
</tr>
</tbody>
</table>

(B) The LT₉₀s (in hours) at LC₉₀ of V-8, vEcoRIHybI, and vEcoRIHybIFS were 10-12% faster than the LT₉₀ of L-1 at LC₉₀; this difference is statistically significant, as evidenced by the upper and lower fiducial limits.

The AcMNPV V-8 was genetically modified to inactivate the egt gene (egt encodes ecysteoid glycosyl transferase) following substantially the same procedure as described in U.S. Patent No.
5, 180,581. Then the LT$_{50}$ values were determined using *S. frugiperda* neonates for AcMNPV L-1, the egt-deficient derivative of L-1 (VEGTDEL), AcMNPV V-8 and the V-8 derivative in which the egt gene was inactivated (V8VEGTDEL). The results are shown in Tables 2 and 3. Clearly, AcMNPV V-8 killed faster than L-1, and V8VEGTDEL killed even faster than the AcMNPV V-8.

### Table 2  *S. frugiperda* neonate bioassays

**A. Dose: 2 x 10$^7$ PIB/ml (100% mortality)**

<table>
<thead>
<tr>
<th>Virus</th>
<th>V8</th>
<th>V8VEGTDEL isolate 1</th>
<th>L1</th>
<th>VEGTDEL isolate 1</th>
<th>V8VEGTDEL isolate 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper limit</td>
<td>90</td>
<td>75.7</td>
<td>106</td>
<td>86.8</td>
<td>77</td>
</tr>
<tr>
<td>LT50</td>
<td>84</td>
<td>70.6</td>
<td>103.6</td>
<td>81.5</td>
<td>72</td>
</tr>
<tr>
<td>Lower limit</td>
<td>84</td>
<td>66</td>
<td>101</td>
<td>76.5</td>
<td>68</td>
</tr>
</tbody>
</table>

**B. Dose: 5 x 10$^4$ PIB/ml (92% to 100% mortality)**

<table>
<thead>
<tr>
<th>Virus</th>
<th>V8</th>
<th>V8VEGTDEL isolate 1</th>
<th>L1</th>
<th>VEGTDEL isolate 1</th>
<th>V8VEGTDEL isolate 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper limit</td>
<td>91.8</td>
<td>83</td>
<td>109</td>
<td>101</td>
<td>85.5</td>
</tr>
<tr>
<td>LT50</td>
<td>86.4</td>
<td>76.5</td>
<td>105</td>
<td>93</td>
<td>79</td>
</tr>
<tr>
<td>Lower limit</td>
<td>81</td>
<td>70.6</td>
<td>102</td>
<td>85.6</td>
<td>73</td>
</tr>
</tbody>
</table>
TABLE 3  Bioassays of LT₅₀ S V8, L-1 and egt deletions in S. frugiperda neonates

A.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Upper Limit</th>
<th>LT₅₀</th>
<th>Lower Limit</th>
<th>50% Kill</th>
</tr>
</thead>
<tbody>
<tr>
<td>V8VEGTDEL</td>
<td>81.6</td>
<td>75.4</td>
<td>69.8</td>
<td>96</td>
</tr>
<tr>
<td>V8</td>
<td>99.6</td>
<td>95</td>
<td>91</td>
<td>90</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Upper Limit</th>
<th>LT₅₀</th>
<th>Lower Limit</th>
<th>50% Kill</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-1</td>
<td>106</td>
<td>102</td>
<td>98</td>
<td>90</td>
</tr>
<tr>
<td>VEGTDEL</td>
<td>89.7</td>
<td>84.2</td>
<td>78.9</td>
<td>96</td>
</tr>
</tbody>
</table>

In diet overlay bioassays using second instar H. virescens larvae, LT₅₀ values for V8VEGTDEL were lower than for the corresponding V-8 virus, and V-8 had lower LT₅₀ values than the L-1 strain. The VEGTDEL and V-8 had similar LT₅₀ values, and V8VEGTDEL had lower LT₅₀ than VEGTDEL (L-1). Using diet overlay tests with second instar Helicoverpa zea larvae, V8VEGTDEL exhibited significantly faster killing than AcMNPV E2. In insect larval tests, V8VEGTDEL appeared to kill infected insects faster than the AcMNPV L-1 egt-deletion strain (VEGTDEL). LC₅₀ values calculated on PBs/16 cm² arena in diet overlay bioassays for AcMNPV wild-type strains (as E2 or L-1) were 1 x 10⁵ to 1 x 10⁶ for S. frugiperda and H. zea; 1 x 10⁵ to 1 x 10⁴ for S. eridania; and 1 x 10⁴ to 1 x 10² for T. ni, S. exigua and H. virescens.

Thus, preferred viruses for insect control carry both a genetic region conferring the increased virulence phenotype and a genetic modification inactivating the gene encoding ecdysteroid glycosyl transferase. Preferred regions carrying the increased
virulence phenotype are those from region of the \textit{lef-2} and ORF 603 region of the RoNPV genome. Functional equivalents of this regions from other baculoviruses can be readily identified, isolated and manipulated using the teachings of the present disclosure and technology well known to the art.

Lepidopteran insects undergo a well characterized sequence of events during development from egg to adult insect (see \textit{Comprehensive Insect Physiology, Biochemistry and Pharmacology,} Vols. 7 and 8, Kerkut and Gilbert (eds.), Pergamon Press, Oxford, 1984 for detailed reviews). After hatching, the insect larva enters a period of extensive feeding during which time it will molt several times to allow continued growth. Stages between successive molts are known as instars. At the end of the larval growth period, the larva pupates and finally emerges as an adult insect. The processes of molting and pupation (collectively termed ecdysis) are regulated by the concerted actions of several different groups of hormones. The initial stimulus is the release of prothoracicotropic hormone (PTTH) by certain brain cells. This stimulates the prothoracic glands to produce and secrete ecdysteroids, often referred to as insect molting hormones. In the presence of juvenile hormone, a larval molt will ensue, while in the absence of juvenile hormone, the larvae will pupate. Eclosion hormone is also important in mediating several behavioral changes associated with ecdysis.

AcmNPV, which is used as a model system for much baculovirus research, interferes with the process of insect development. Insect larvae infected with AcmNPV are no longer able to molt or pupate because AcmNPV directs the synthesis of an enzyme, known as ecdysteroid UDP-glucosyl transferase (EGT), which specifically inactivates the insect ecdysteroids by conjugating them to galactose \textit{in vivo} (O’Reilly et al. (1991) Insect Biochem. Molec. Biol. 22:313-320) or glucose \textit{in vitro} (O’Reilly et al. Science 245:1110-1112). Other baculoviruses carry \textit{egt} genes as well.
The AcMNPV gene encoding EGT extends from 8.4 to 9.6 map units on the AcMNPV genome (Figs. 1 and 2). Figure 2 shows the restriction map of the egt region of the genome. The nucleotide sequence of the AcMNPV (strain L1) egt gene and the deduced amino acid sequence of 506 amino acids are shown in SEQ ID NO:1 and 2, respectively. The coding sequence of egt extends from nucleotide 149 to about nucleotide 1670.

In a preferred embodiment of the present invention, the egt gene of the AcMNPV V-8 strain is inactivated by replacing a portion of the egt gene with a bacterial sequence encoding β-galactosidase. This recombinant baculovirus is designated V8vEGTDEL herein. In a second preferred embodiment, part of the egt gene of the V8 strain AcMNPV is deleted without replacement, for example, by deleting an EcoRI/XbaI segment from within the egt coding sequence (See Fig. 6; U.S. Patent No. 5,180,581; Example 7 hereinbelow). An alternate mechanism for the inactivation of the insect virus egt gene is the insertion of a gene encoding an insect hormone affecting ecysis, an enzyme which inactivates an insect hormone affecting ecysis, which gene is expressible in an insect cell infected with said insect virus or an insect-specific toxin gene.

Using the AcMNPV egt gene as a probe, an egt gene has been identified in the baculovirus Orgyia pseudotsugata nuclear polyhedrosis virus (OpMNPV). It will be recognized by those skilled in the art with the benefit of this disclosure that the egt gene of any baculovirus can be characterized and isolated in a similar manner as AcMNPV (see, e.g., U.S. Patent No. 5,180,581, incorporated by reference herein in its entirety). egt genes with at least 70% nucleotide sequence homology to the egt coding sequence in Fig. 6 from nucleotides 149 to 1666 (and in SEQ ID NO:1, from nucleotide 149 to 1666) are considered equivalent to said sequence, provided those homologous genes encode an enzyme which is an ecdyalosterone UDP-glycosyl transferase, and their identification, isolation and manipulation will be readily achieved by the skilled worker using the sequences and assay
information provided, taken together with what is well known in
the art.

Functional equivalents of the eot gene are those which also
catalyze the inactivation of ecdysteroids such as ecdysone by
transferring a glucose or galactose moiety from UDP-glucose to
the ecdysteroid(s). Those functional equivalents of eot may be
identified using the assay methods described herein.

By inhibiting molting and pupation via the effects of
ecdysteroid glycosyl transferase, wt AcMNPV infection can
actually prolong larval feeding time. Baculoviruses which lack
a functional eot gene do not prolong larval feeding time. Larvae
infected with eot-deficient baculoviruses may pupate, and they
succumb to the viral infection even more rapidly than larvae
infected with the corresponding wt virus. The more rapid killing
by a baculovirus lacking a functional eot gene is most
dramatically seen when newly hatched first instar larvae are
infected with wt AcMNPV and with vEGTZ. Larvae infected with
vEGTZ succumb to the viral infection 3 to 4 days sooner than
larvae infected with wt AcMNPV. Therefore, baculoviruses lacking
a functional eot gene are considerably more effective as insect
control agents than wild-type baculoviruses. It will be apparent
to those skilled in the art with the benefit of this disclosure
that the eot gene can be rendered nonfunctional in any
baculovirus by any means known to the art.

Although the length of time progeny virus can accumulate in
larvae infected with baculoviruses lacking a functional eot gene
is somewhat truncated and the infected insect displays reduced
growth, there is substantial production of progeny virus. The
amount of virus obtained per larva following vEGTZ infection of
late instar larvae is about 15 to 50% that obtained with wt
virus. This is sufficient to allow cost-effective preparation
of large quantities of virus particles.
In another embodiment of the present invention, an insect virus lacking a functional egt gene is modified by genetic engineering techniques so that its effectiveness as a biological control agent is further enhanced by incorporating a second gene whose product affects insect development.

The gene encoding PTTH (a peptide hormone) can be inserted into the viral genome with the egt gene inactivated and PTTH can be expressed at levels sufficiently high to affect ecdysis. Insect larvae infected with such a virus experience extreme disruption in the hormonal control of development. These insects become sick rapidly resulting in severely compromised growth and development, reduced feeding, and earlier death. PTTH sequences are described in Kawakami et al. (1990) Science 247:1333).

Eclosion hormone is also a small peptide hormone whose gene can be inserted into the viral genome with a nonfunctional egt gene, using techniques known to the art. Because eclosion hormone governs many behavioral changes associated with ecdysis, insects infected by an Egt- virus producing sufficiently high levels of this hormone will display behavioral and/or developmental abnormalities, such as reduced feeding. Eclosion hormone sequences are described in Horodyski et al. (1989) Proc. Natl. Acad. Sci. USA 86:8123-8127).

A prime regulator of juvenile hormone titers in the insect is the enzyme juvenile hormone esterase, which inactivates juvenile hormone. A recombinant virus lacking a functional egt gene and expressing sufficiently high levels of juvenile hormone esterase can adversely affect insect behavior and/or development. Juvenile hormone esterase sequences are described in Hanzlik et al. (1989) J. Biol. Chem. 264:12419) and have been enhanced through genetic engineering (Hammock et al. (1990) Nature 344:458-461; Hammock et al. (1993) Arch. Biochem. Biophys. 307:231-241).
It is important to note that, while all of the above genes could be added to wild-type virus genome using disclosure provided herein and/or in U.S. Patent 5,180,581 and techniques well known to the art, they would not be expected to significantly affect insect behavior in the wild-type virus because expression of the egt gene by wild-type virus inactivates the ecdysteroid molting hormones and ecdysis is prevented, regardless of the production of other hormones. Thus, successful strategies involving the generation of viruses designed to interfere with insect ecdysis depend upon prior inactivation of the egt gene.

It will be understood by those skilled in the art that mutant organisms lacking an intact egt gene or incapable of expressing a functional egt product and those which are further genetically modified so as to express another hormone-modifying enzyme or a peptide developmental hormone are included as insect control agents of the present invention.

An isolated and purified insect virus is one which has been cloned through plague purification in tissue culture, for example, or otherwise prepared from a single viral genotype. A recombinant insect virus, as used herein, is one which has at least one portion of its genotype derived from a heterologous insect virus, i.e., an insect virus of different taxonomic viral species. A recombinant insect virus may be generated by coinfection of one insect cell or insect with one than one viral species, or it may be the result of introducing insect virus genomic DNA and a heterologous insect DNA virus segment into the same insect or insect cell, with the result that a portion of the heterologous DNA becomes incorporated in the insect virus genome by recombination process. It is understood in the art that such a recombinant virus can be recognized via restriction endonuclease analysis, DNA sequencing at least a portion of the putative recombinant genome or via a change in phenotype. As specifically exemplified herein, recombinant insect viruses are recognized by their increased virulence phenotype (lower LT50) in
at least one target insect as compared with the parental insect virus. A recombinant insect virus phenotype with the faster killing phenotype can be further genetically modified and further improved as an insect control agent by inactivating an ecdysteroid modifying enzyme, for example.

As used herein, an insecticidal composition has at least one active ingredient which has an adverse affect on insect pests, preferably which kills said pests. The present invention is the use of a recombinant insect virus which has been isolated or which has been genetically engineered to kill at least one insect pest faster than the corresponding wild-type comparison using a segment of heterologous insect virus DNA. A heterologous insect virus DNA segment is one which is not normally associated with the genome of the parent of the recombinant virus. As specifically exemplified herein, a portion of a baculovirus genome has been isolated and identified and shown to confer a phenotype of faster insect killing (as measured using the LT₉₀ assay) when inserted into the AcMNPV L-1 genome, with Spodoptera frugiperda as the target insect. When an Egt-deficient derivative of that recombinant insect virus is used, feeding by insects is reduced in response to the insect egt-deficient recombinant virus, normal insect ecdysis is disrupted and death of the insect is further accelerated relative to the isogenic wild-type strain (i.e., with functional egt). A recombinant virus of this invention can also be an insect virus genetically engineered to inactivate a gene encoding an ecdysteroid modifying enzyme or one which is further engineered to express a heterologous gene encoding a protein which affects insect development, so as to minimize the time of insect feeding or to cause more rapid killing after virus infection.

It will be understood by those skilled in the art that the insect pests can be exposed to the viruses of the present invention by conventional methods including ingestion, inhalation or direct contact of the insect control agent.
A primary use of the recombinant and/or genetically engineered baculoviruses of the present invention will be as active ingredients of agricultural compositions for applying to plants to effect the biological control of insect pests of plants. Many variations of preparing such agriculturally suitable compositions for insect control are known in the art. The insecticidal compositions of this invention are typically administered at dosages in the range of $2.4 \times 10^4$ to $2.4 \times 10^{12}$ PIBs/hectare of recombinant insect virus.

Insecticidal compositions suitable for applications to plants to control insect pests comprise an agriculturally suitable carrier and an insect control agent, i.e., an insect virus, preferably a baculovirus. Conventional formulation technology known to persons skilled in the art is used to prepare the compositions of this invention. The compositions can be in the form of wettable powders, dispersible granular formulations, granules, suspensions, emulsions, solutions for aerosols, baits and other conventional insecticide preparations. Wetting agents, coating agents, agents to promote physical flexibility, UV protectants, dispersants and sticking agents are desirable additives in at least some formulations. The compositions will frequently include an inactive carrier, which can be a liquid such as water, alcohol, hydrocarbons or other organic solvents, or a mineral, animal or vegetable oil, or a powder such as talc, clay, silicate or kieselguhr. A nutrient such as sugar may be added to increase feeding behavior and/or to attract insects. Flow agents, for example, clay-based flow agents, may be added to minimize caking of the wettable powders or other dry preparations during storage. Application of an insecticidal composition of this invention can protect plants from insect pests by reducing feeding by and killing of susceptible insects.

The skilled artisan knows how to choose an insect virus which is suitable for the control of a particular insect pest. The concentration of the insect control agent that will be required to produce insecticidally effective agricultural
compositions for plant protection will depend on the type of crop, target insect, virus genotype used and the formulation of the composition. Insecticidal compositions may be formulated, for example, as wettable powders, with about 10% (w/w) polyhedrin inclusion bodies. The insectically effective concentration of the insect control agent within the composition can readily be determined experimentally by a person of ordinary skill in the art.

Agricultural compositions must be suitable for agricultural use and dispersal in fields. Generally, components of the composition must be non-phytotoxic and not detrimental to the integrity of the occluded virus. Foliar applications must not damage or injure plant leaves. In addition to appropriate solid or, more preferably, liquid carriers, agricultural compositions may include sticking and adhesive agents, emulsifying and wetting agents, but no components which deter insect feeding or any viral functions. It is desirable to add components which protect the insect control agent from UV inactivation. Agricultural compositions for insect pest control may also include agents which stimulate insect feeding.


Field trials in which AcMNPV E-2, V8VEGTDEL and a commercial Bacillus thuringiensis subsp. kurstaki insecticide (DIFEL 2X, Abbott Laboratories, Chicago, IL) were carried out during the fall growing season in Arizona. Although the pest infestation
was relatively light, results from this study indicated that 
V8vEGTDEL was efficacious against T. ni in young lettuce (Table 
4). Following the fourth application of treatments (on ca. 5-day 
intervals), V8vEGTDEL at \(1 \times 10^{11}\) and \(1 \times 10^{12}\) PIBs/A provided 
better control of T. ni than similar doses of AcMNPV-E2 "wild-
type". Additionally, V8vEGTDEL at \(1 \times 10^{11}\) and \(1 \times 10^{12}\) PIBs/A 
provided control of the T. ni infestation at levels equal to that 
provided by DIPEL 2X at 1 lb/A. Based on data collected after 
only three applications, however, DIPEL 2X provided better pest 
control than either baculovirus.

After completion of data collection, the test site (as well 
as 10-ft wide perimeter) was sprayed with an aqueous dilution of 
1% (v/v) bleach. The treated crop, as well as a 10-ft wide 
perimeter, was then destroyed by using tractor-mounted tillage 
equipment. About 3 weeks later, soil samples were collected from 
several sites located within 100 ft of the test site. No 
V8vEGTDEL virus were detected in soil surrounding the test site, 
and no additional action was taken.
TABLE 4. Efficacy of selected baculovirus treatments against *Trichoplusia ni* in lettuce

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose/A²</th>
<th>Mean # larvae/10 plants at 3DA3T</th>
<th>Mean # larvae/10 plants at 5DA4T</th>
</tr>
</thead>
<tbody>
<tr>
<td>V8vEGTDEL</td>
<td>1x10⁶ PIBs</td>
<td>15 a b²</td>
<td>20 a</td>
</tr>
<tr>
<td></td>
<td>1x10⁷ PIBs</td>
<td>20 a</td>
<td>2 c</td>
</tr>
<tr>
<td></td>
<td>1x10⁸ PIBs</td>
<td>12 b</td>
<td>7 b c</td>
</tr>
<tr>
<td>AChNPV E2</td>
<td>1x10⁶ PIBs</td>
<td>10 b</td>
<td>18 a</td>
</tr>
<tr>
<td></td>
<td>1x10⁷ PIBs</td>
<td>18 a</td>
<td>20 a</td>
</tr>
<tr>
<td></td>
<td>1x10⁸ PIBs</td>
<td>12 b</td>
<td>10 b</td>
</tr>
<tr>
<td>DIPEL 2X</td>
<td>1 lb form</td>
<td>0 c</td>
<td>7 b c</td>
</tr>
<tr>
<td>Untreated</td>
<td>—</td>
<td>20 a</td>
<td>18 a</td>
</tr>
</tbody>
</table>

1 Baculovirus compositions were formulated as water-soluble wettable powders (1 x 10⁶ PIBs/10 gm).

2 Baculovirus compositions were applied at 1x10⁶, 1x10⁷, and 1x10⁸ PIBs/A on day 15, however, due to poor mixing and spray characteristics of the 1 x 10⁶ dose, both baculovirus were applied at 1 x 10⁶, 1 x 10⁷ and 1 x 10⁸ PIBs/A in all subsequent applications at days 5, 10 and 15. DIPEL 2X was also applied on days 1, 5, 10 and 15.

3 Means within columns followed by the same letter are not significantly different (DMRT, P=0.05).

In a second fall field trial, the efficacy of V8vEGTDEL, AChNPV E2 and a commercial *B. thuringiensis* subsp. kurstaki insecticide (DIPEL 2X, Abbott Laboratories, Chicago, IL) against the cabbage looper in New Jersey. Viral insecticidal compositions were formulated as wettable powders.

Due to the light pest infestation in this study, differences among treatments in control of *T. ni* larvae were very slight (and generally not statistically significant). However all treatments had significantly fewer live larvae and less plant defoliation than untreated cabbage (Table 5). At 7 days after last application of treatments, untreated plots averaged 18% defoliation whereas cabbage treated with V8vEGTDEL or AChNPV-E2
"wild type" (rates of $1 \times 10^6$, $1 \times 10^7$, and $1 \times 10^8$ PIBs/A) averaged 8-10% defoliation and DIPEL-treated (1 lb/A) cabbage averaged 4% defoliation. At 12 days after last application, untreated plots had a mean of 6.5 live larvae/10 plants whereas baculovirus- (1$ \times 10^7$ and 1$ \times 10^8$ PIBs/A) and DIPEL-treated plots averaged < 2 larvae/10 plants.

After data collection was complete, the test site (as well as 10-ft wide perimeter) was sprayed with an aqueous dilution of 1% (v/v) bleach. The treated crop, as well as a 10-ft wide perimeter, was then destroyed by using tractor-mounted cultivation equipment. About five months after the bleach treatment, soil samples were again collected from several sites located within 100 ft of the test site. Also on this date, the test site was treated with AcMNPV-E2 "wild-type" at a rate of $1 \times 10^9$ PIBs/A. No V8VEGTDEL was detected in these later soil samples.
TABLE 5. Efficacy of selected baculovirus treatments against Trichoplusia ni in cabbage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose/A$^2$</th>
<th>Mean # larvae/10 plants at 7DAJT</th>
<th>Mean # larvae/10 plants at 12DAJT</th>
</tr>
</thead>
<tbody>
<tr>
<td>V8VEGTDLEL</td>
<td>1 x 10$^5$ PIBs</td>
<td>10 b$^3$</td>
<td>2.0 b</td>
</tr>
<tr>
<td></td>
<td>1 x 10$^6$ PIBs</td>
<td>11 b</td>
<td>1.2 b c</td>
</tr>
<tr>
<td></td>
<td>1 x 10$^7$ PIBs</td>
<td>7 b c</td>
<td>1.7 b c</td>
</tr>
<tr>
<td>AcMNPV E2</td>
<td>1 x 10$^5$ PIBs</td>
<td>8 b c</td>
<td>2.0 b</td>
</tr>
<tr>
<td></td>
<td>1 x 10$^6$ PIBs</td>
<td>8 b c</td>
<td>0.8 b c</td>
</tr>
<tr>
<td></td>
<td>1 x 10$^7$ PIBs</td>
<td>11 b</td>
<td>0.8 b c</td>
</tr>
<tr>
<td>DIPEL 2X</td>
<td>1 lb form.</td>
<td>4 c</td>
<td>0.2 c</td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td>8 a</td>
<td>6.5 a</td>
</tr>
</tbody>
</table>

1 Both types of baculoviruses were formulated as water-soluble wettable powders (1E11 PIBs/10 gm of WP).

2 "EGT-deleted" and "Wild-type" (at 1 x 10$^6$ and 1 x 10$^7$ PIBs/A) and DIPEL 2X were applied three times. Due to severe clogging of nozzles, the planned baculovirus doses of 1 x 10$^6$ PIBs/A, so no baculovirus "high dose" was applied at the first application and V8VEGTDLEL and AcMNPV E2 at 1 x 10$^7$ PIB/A were applied and were subsequently applied only twice (5 and 10 days later).

3 Means within columns followed by the same letter are not significantly different (DMRT, P=0.05).

5 A third field trial for efficacy of V8VEGTDLEL, AcMNPV E2 and a commercially available B. thuringiensis subsp. awaizai insecticide (Xentari, Abbott Laboratories, Chicago, IL) for control of T. ni in lettuce was carried out in spring in Florida.

The data are summarized in Table 5. V8VEGTDLEL provided significantly faster control of T. ni than AcMNPV V8. Five days after treatment with V8VEGTDLEL (1 x 10$^{12}$ PIBs/A) caused 100% larval mortality whereas V8 at the same dose caused only 29% larval mortality (up to 97% mortality by day 7). Also, V8VEGTDLEL (1 x 10$^{11}$ PIBs/A) exhibited larval control at a rate equal to that of V8 at 1 x 10$^{12}$ PIBs/A.
The commercial Bt product Xentari (1 lb form./A), provided 76% larval control by day 4 vs. only 40% larval control from V8vEGTDEL (1 x 10^{12} PIBs/A). However, by day 5, V8vEGTDEL (1 x 10^{12} PIBs/A) and Xentari (1 lb/A) exhibited 100% and 89% larval mortality, respectively.

**TABLE 6. Efficacy of field applications of V8vEGTDEL and AcMNPV V8 ("wild-type") against *Trichoplusia ni* in lettuce**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose per acre</th>
<th>Day 4</th>
<th>Mean % larval mortality</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>V8vEGTDEL</td>
<td>1 x 10^{11} PIBs</td>
<td>0</td>
<td>35</td>
<td>88</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>V8vEGTDEL</td>
<td>1 x 10^{12} PIBs</td>
<td>40</td>
<td>100</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>AcMNPV V-8</td>
<td>1 x 10^{12} PIBs</td>
<td>4</td>
<td>29</td>
<td>68</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>Xentari</td>
<td>1 lb</td>
<td>76</td>
<td>89</td>
<td>92</td>
<td>95</td>
<td></td>
</tr>
</tbody>
</table>

1. Baculovirus compositions were formulated as wettable powder.

2. Treatments were applied to six true-leaf lettuce (4 plots/treatment, RCT design). About 3 hrs. after application, leaves were harvested from field-plots, individually placed into petri dishes containing water-moistened filter paper, and then infested with three-day-old *T. ni* larvae (ca. 10 larvae/leaf). Two days later, larvae were placed in CD-International trays containing untreated Stoneville artificial diet (1 larva/diet-well), and percent mortality was rated on each of several days post-treatment.

The examples provided herein use many techniques well known and accessible to those skilled in the arts of molecular biology, in the manipulation of recombinant DNA in plant tissue and in the culture and regeneration of transgenic plants. Enzymes are obtained from commercial sources and are used according to the vendors' recommendations or other variations known to the art. Reagents, buffers and culture conditions are also known to the art. References providing standard molecular biological procedures include Sambrook et al. (1989) *Molecular Cloning*, second edition, Cold Spring Harbor Laboratory, Plainview, NY; R.

Abbreviations and nomenclature, where employed, are deemed standard in the field and are commonly used in professional journal such as those cited herein. All references cited in the present application are expressly incorporated by reference herein.

This invention is illustrated by the following examples, which are not to be construed in any way as imposing limitations on the scope thereof. It is understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

THE EXAMPLES

Example 1. Isolation of AcMNPV V-8

A minimally passaged AcMNPV stock from the original AcMNPV isolated by Pat Vail (Vail et al. (1971) Proc. IV Int. Colloq. Insect Pathology, College Park, MD pp. 297-304) was amplified in Heliothis virescens larvae from the H. virescens colony at American Cyanamid, Princeton, NJ. The H. virescens are reared on a soybean-wheat germ agar-ased diet at 28 C under constant fluorescent light. Virus was then further amplified in H. virescens larvae. Ten viral clones were plaque-purified from diluted hemolymph from the latter infected H. virescens larvae. Methods for plaque assay, plaque purification, virus amplification and viral DNA preparation are described in O'Reilly et al. (1992) Baculovirus Expression Vectors; A Laboratory Manual, W.H. Freeman & Co., New York, NY. Unless otherwise indicated, viruses were propagated in the IPLB-SF-21 cell line.
(SF-21) (Vaughn et al., 1977) In Vitro 12:213-217) using TC100 medium (Gibco BRL, Gaithersburg, MD) supplemented with 0.26% tryptose broth and 10% fetal bovine serum (Intergen, Purchase, NY). SF-21 cells are commercially available (e.g., Invitrogen Corporation, San Diego, CA). DNA was prepared from each isolate and characterized by restriction endonuclease analysis in parallel with DNA prepared from the L-1 strain of AcMNPV, which is described in Lee and Miller (1978) J. Virol. 27:754.

Example 2. Analysis of the AcMNPV lef-2 Region

Molecular biology techniques were used as previously described (Maniatis et al., 1989). Plasmid pRI-I contains the 7.33 kb AcMNPV L-1 EcoRI-I fragment cloned in the EcoRI site of pBR322. Plasmid pECori-IV8 contains the V-8 EcoRI-I fragment in the EcoRI site of Bluescript KS+ (Stratagene, La Jolla, CA). Plasmid pECoriHybI was constructed by replacing the 1.72 kb MluI to EspI fragment (1.93-3.27 m.u.) in the L-1 EcoRI-I fragment with the corresponding fragment from V-8. The hybrid EcoRI-I fragment was then recloned into a pUC19 vector, producing pUC19HybI, a plasmid with a unique NaeI site in the 603 ORF. A plasmid with a frameshift mutation at this NaeI site, pUC19HybIFS, was produced by digesting pUC19HybI with NgOAIV (an isoschizomer of NaeI which produces cohesive ends), blunt-ending the overhanging ends with mung bean nuclease, and relegating the blunt ends to produce a four base pair deletion that destroys the NaeI site and disrupts the 603 ORF reading frame. This frameshift, which was confirmed by dideoxynucleotide sequencing (United States Biochemical Corp. Sequenase kit, Cleveland, OH), was predicted, on the basis of the published L-1 DNA sequence of AcMNPV [Possee et al., (1991) Virology 185:229-241], to cause premature termination of 603 ORF translation at a site fourteen amino acids downstream of the deletion.

Example 3. Virus Bioassays

Polyhedral inclusion bodies (PIBs) of L-1, V-8, vEcoRIHybI, and vEcoRIHybIFS were prepared simultaneously from infected Trichoplusia ni larvae as previously described (O'Reilly et al.,
1992). LC$_{50}$ data (the concentration of virus [PIBs/ml of diet] required for one half of the larvae to die by ten days post infection) and LT$_{50}$ data (the time taken, at a specific viral concentration, for one half of the larvae to die) were collected from neonate bioassays performed on Spodoptera frugiperda larvae. Neonates were allowed to feed for 24 hours on diet containing various concentrations of PIBs from the viruses being assayed and then transferred to individual cups containing diet without virus. The seven doses of each virus assayed were 5x10$^4$, 2x10$^5$, 5x10$^5$, 1x10$^6$, 2x10$^6$, 5x10$^6$, and 2x10$^7$ PIBs/ml. Sixty larvae were assayed per dose. Larval mortality was recorded at 48, 72, 84, 90, 96, 102, 108, 120, 132, and 144 hours post infection (p.i.). A final mortality count was performed at ten days post infection. LT$_{50}$ and LC$_{50}$ values were determined using probit analysis (Daum (1970) Bulletin of the Entomological Soc. of America 16:10-15).

Alternate virulence testing was done as follows: Trays were purchased from C-D International, Inc., and contained 32 separate arenas per tray. Each 4x4 cm (16 cm$^2$) arena contained 5 ml of appropriate artificial diet. Clear vented adhesive tops from C-D International, Inc., enclosed the insect in the arena following treatment and infestation. These clear tops allowed for easy scoring. The surface of the Stoneville (soybean/wheat germ diet) or pinto bean (Bio-Serv, Inc., Frenchtown, NJ Diet #9393) diet was contaminated with 0.4 ml of aqueous viral solution. The dilutions ranged from 1 x 10$^{-4}$ to 1 x 10$^{-1}$ PIBs/ml, in 10-fold dilutions, depending upon the insect species tested. The applications were evenly distributed by rotating the tray and solutions were allowed to dry in a laminar flow hood. Bioassay trays were held at 28°C in continuous fluorescent light throughout the study period. Readings were taken twice a day to observe early onset time of infection. LC$_{50}$ values were calculated from the BASIC log/probit statistics package and based on mortality versus dose at 8 days post-treatment. The T$_{50}$ (time at 0 hours) was based on initial average time when the larva was exposed to the treated diet. The LT$_{50}$ value was calculated from the BASIC log/probit statistics package based on mortality versus
hours. The LT₉₀ data calculated were derived from the LD₉₀ value (based on a dose that was preferably less than 2 logs greater than the LC₉₀ value).

Example 4. Recombinant Virus Construction
Recombinant viruses are prepared essentially as described in O'Reilly et al. (1992) supra. The recombinant viruses vEcoRIHybI and vEcoRIHybIFS were constructed by cotransfecting SF-21 cells with vSynVI'gal DNA (Wang et al. (1991) Gene 102:131-137) and either pUC19HybI plasmid DNA (for vEcoRIHybI) or pUC19HybIFS plasmid DNA (for vEcoRIHybIFS) (See Example 2). The virus vSynVI'gal expresses the E. coli lacZ gene instead of the polyhedrin gene and forms occlusion negative (OCC), blue plaques in the presence of the chromogenic β-galactosidase indicator X-gal. Both pUC19HybI and pUC19HybIFS contain a polyhedrin gene; thus recombination between plasmid DNA derived from the polyhedrin region and viral DNA produced white occlusion positive (OCC⁺) viral plaques. Viruses forming white OCC⁺ plaques have lost the lacZ gene and acquired a functional polyhedrin gene through allelic replacement.

Example 5. Field Testing of Variant Baculovirus
The field trial program evaluates the efficacy of V8vEGTDEL relative to AcMNPV wild-type against important lepidopteran pests which attack vegetables. Pest organisms targeted in these field trials include cabbage looper, Trichoplusia ni; beet armyworm, Spodoptera exigua; fall armyworm, Spodoptera frugiperda; southern armyworm, Spodoptera eridania; tobacco budworm, Heliothis virescens; corn earworm, Helicoverpa zea, diamondback moth, Plutella xylostella; cabbageworm, Pieris rapae. Each test is conducted on land currently used for growth/production of row crops (i.e., commercial or research farms). The crop used in each test is a leafy vegetable (e.g., lettuce) or a crucifer (e.g., cabbage). Each field trial consists of the following eight treatments: V8vEGTDEL (see Examples 6 and 7 hereinbelow) at 1 x 10⁴, 10⁶, and 10⁹ PIBs/acre; AcMNPV at 1 x 10⁴, 10⁶, and 10⁹ PIBs/acre, and untreated control. Within a given test, each
treatment will be applied to the crop no more than six (6) times; treatments will be applied on an "as needed" basis (i.e., as pest populations warrant, probably 5- to 14-day intervals).

Within each test, there is a maximum of six applications of each treatment. Treatments are applied to plots in each test by using ground equipment, either small tractor sprayers or CO$_2$-driven backpack sprayers. Treatments are diluted in water and applied through standard agricultural hydraulic spray booms and nozzles. The maximum size of a treatment plot (i.e., replicate) in each test is 0.018 acres (i.e., 4 rows wide x 60 ft. long, row spacing of 40 in.). The maximum number of plots (i.e., replicates) per treatment in each test is four. Each test is monitored on at least a weekly basis for the duration of the study. Each of these trials will be conducted on secured private farm land or research farms (no trespassing by unauthorized individuals). At the conclusion of each test, the test area and a 10 ft.-wide untreated test perimeter undergo "crop destruction" (i.e., rather than being harvested for commercial use, the treated and adjacent crop is shredded and plowed underground).

Soil is perhaps the most important reservoir for persistence of virus in the environment. The monitoring program consists of the collection of 4 soil samples (each 7.6 cm in depth) totaling 500 g from within the test site and from an area 100 ft outside the treatment zone. Samples are taken approximately midway through the test. A second set of samples are collected at the end of the test after all disinfection procedures (as described below) have been completed.

Monitoring for viable, infectious virus is important because immunodetection and PCR methods make no distinction between infectious occlusion bodies and non-viable remnants of viral particles. The only reliable method for determining if viable, infectious viral particles are present in the soil samples is to perform bioassays of the samples on a highly susceptible insect host such as Heliothis virescens. From each 500 g sample of
soil, 25 g is used in the bioassay. A standard method for isolation of viral occlusion bodies from soil is used. This method efficiently recovers approximately 46% of polyhedra from soil. The LC₅₀ for AcMNPV in our standard diet overlay assay is 300-1000 polyhedra/arena for H. virescens. Therefore, if each larvae to be bioassayed is fed the isolate from 1g of soil this assay reliably detects 600-2000 viral occlusion bodies per gram of assayed soil. Larvae which exhibit typical symptoms of viral infection in the bioassay are examined for the presence of occlusion bodies using light microscopy. If polyhedra are observed, they are isolated from the cadaver for DNA isolation from the occlusion bodies and a standard PCT assay (routinely performed in the lab) is done using primers flanking the vEGTDEL deletion (See Fig. 6, e.g.). The efficiency of DNA recovery and the PCR assay approaches 100%. If the virus present is vEGTDEL, then a DNA fragment of a characteristic size is observed, allowing unambiguous identification of the virus as vEGTDEL. Other viruses generate DNA fragments of differing sizes.

AcMNPV variants having deletions in the egt gene can arise spontaneously in nature, and such viruses are subject to a severe replicative disadvantage that will not allow them to compete effectively with indigenous viruses in the environment. Furthermore, since egt-inactivated virus produce 30%-50% fewer polyhedra following a successful infection, environmental persistence is further compromised. Contaminated plants within the test site and 10 ft.-wide buffer, tools, and farm implements are topically sterilized with a 1% bleach wash to prevent unnecessary dispersal of the viral insecticide.

The V8vEGTDEL formulation for the field trial program is in the form of a wettable powder. On a weight:weight basis, ingredients of this formulation are as follows:
Eudragit S100 (Rohm Pharma Co.) comprises methyl methacrylic and methyl methacrylate. It is a pH dependent coating agent which holds UV9 on the PIBs, and it slightly prolongs photostability of the formulation. UV-9 oxybenzone (Cytech Ind.) also provides slight photostability to the formulation. Polyethylene glycol MW400 (Aldrich Chemical Co.) provides flexibility to the UV-protectant coatings. MiraSperse (Staley Co.) is a starch-based "sticker", and provides rainfastness to the formulation after it is applied to the crop. REAX ATN (West Waco Co.) is a lignin sulfonate, and it is used as a dispersant and keeps the particles separate in the liquid phase (i.e., in the water diluent). Sugar is used as an insect feeding stimulant and/or attractant. Morewet EFW (Witco Co.) is a wetting agent, so that the formulation can more effectively spread across the surface of a treated leaf. Microcel E (Manville Co.) is a clay-based flow agent that prevents the wettable powder from caking during storage.

For use in the test formulations, the PIBs (polyhedrin inclusion bodies) are air-milled to under 10μm in size, and coated with an organic solution containing Eudragit S100, UV-9, and MW400. The other aforementioned inerts are blended and Fitz-milled to make a pre-blend. The coated PIBs and the pre-blend are blended together and Fitz-milled, and then the formulation is packaged. No extraneous microorganisms will be present in the formulation since production in tissue culture requires the use
of sterile procedures. In each 10 g of wettable(382,82),(630,94)(383,97),(634,109) powder formulation, there is 1 g (2 × 10¹¹ PIBs) of V8vEGTDEL.

A preferred wettable powder insecticidal composition is as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Nominal Percent (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V8vEGTDEL polyhedrin inclusion bodies</td>
<td>10.0%</td>
</tr>
<tr>
<td>Morwet D425</td>
<td>30.0%</td>
</tr>
<tr>
<td>Morwet EFW</td>
<td>20.0%</td>
</tr>
<tr>
<td>Kaolin Clay</td>
<td>16.0%</td>
</tr>
<tr>
<td>Microcel E</td>
<td>16.0%</td>
</tr>
<tr>
<td>UV-9 oxybenzone or charcoal</td>
<td>5.0%</td>
</tr>
<tr>
<td>Eudragit S100</td>
<td>2.0%</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>0.9%</td>
</tr>
<tr>
<td>polyethylene glycol MW400</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

The active ingredient is V8vEGTDEL. Morwet D425 is used as a dispersant and keeps the particles separate in the liquid phase (i.e., in the water diluent). Morwet EFW (Witco Co.) is a wetting agent, so that the formulation can more effectively spread across the surface of a treated leaf. Kaolin Clay is a bulking agent. Microcel E (Manville Co.) is a flow agent that prevents the wettable powder from caking. UV-9 (or charcoal) provides slight photostability to the formulation. Eudragit S100 (Rohm Pharma Co.) also slightly prolongs photostability of the formulation. Citric acid is used for pH adjustment. MW400 (Aldrich Chemical Co.) provides flexibility to the UV-protectant coatings.

A stilbene brightener is optionally added (at approximately 5% w/w) to PIBs in alternative preferred wettable powder formulations, and the percentages of other inert ingredients are then adjusted accordingly. Stilbenes provide some protection against UV inactivation and can also serve to enhance or potentiate virus infectivity, particularly in insects which are less susceptible to the insect virus, see, e.g., U.S. Patent No.
5,246,936 (issued September 21, 1993, Treacy et al.), which is incorporated by reference herein.

In this formulation the PIBs are first coated using an aqueous coating procedure. A 1% (w/v) suspension of Eudragit S100 is prepared in water. The Eudragit is dissolved by adjusting the pH to between 9.0 and 9.5. Viral PIBs, Blankophor BBH (stilbene brightner, Miles Inc; if used), and UV-9 oxybenzone or charcoal in the proper proportions are added. The mixture is blended to create an even suspension and then air-dried. The dried coated PIBs are then air-milled to achieve a small particle size. This material is then dry blended with the prescribed amounts of Morvet 425, Morewet EFW, Kaolin Clay, Microcell E, Citric acid and polyethylene glycol MW400 and then packaged as the final formulation. The preferred particle size of the blended material is less than 20 μm.

**Example 6.**

The position of the egt gene on the AcMNPV genome is illustrated in Fig. 2B. A scale in map units is presented above the map of the AcMNPV genome in Fig. 1A. The nucleotide sequence of the AcMNPV L-1 egt gene and flanking regions has been determined (Fig. 6, SEQ ID NO: ____). Fig. 1A shows a linear map of the AcMNPV L-1 genome after cleavage with restriction endonucleases EcoRI and HindIII. Fig. 1B is an enlargement of the AcMNPV genome from 7.6 to 11.1 map units showing the location of the egt gene. The AcMNPV strain L-1 has been described (Lee and Miller (1978) J. Virol. 27:754). Cloned L-1 DNA fragments and the names of the resultant plasmids are shown in Fig. 1C. Fragment 1, which extends from the PstI site at 7.6 μu to a BamHI site at 11.1 μu, is cloned into the plasmid vector pUC19; fragments 2 and 3 (from PstI (7.6 μu) to EcoRI (8.65 μu) and from EcoRI (8.65 μu) to SalI (10.5 μu), respectively) are both cloned into the vectors Bluescript M13+ and Bluescript M13− (Stratagene, San Diego, California). Fragment 5 (BstEII (8.35 μu) to BstEII (8.7 μu) is cloned into Bluescript M13+.
The nucleotide sequence of the egt gene and the predicted amino acid sequence of the egt gene product, are presented in SEQ ID NO:___ and SEQ ID NO:___, respectively.

Example 7.

To construct AcMNPV recombinant viruses (e.g., V-8) incapable of expressing a functional egt gene, further manipulation of the plasmid clones described in Example I is required. Plasmid pUCBCPsB is cleaved with restriction endonucleases EcoRI and XbaI (see Fig. 3 for sites within the egt gene) and the small fragment is discarded. The Escherichia coli lacZ gene, excised from pSKSI04 (Casadaban et al. (1983) Methods Enzymol. 100:293-303) with EcoRI and AhaIII, is then inserted between the EcoRI and XbaI sites after the XbaI overhanging ends are filled in using T4 DNA polymerase. The resultant plasmid is designated pEGTZ. In this plasmid, the inserted lacZ gene is in frame with the preceding egt coding sequences. Alternatively, the plasmid pEGTDEL is constructed by simply ligating the EcoRI and XbaI sites together (after both sites have been blunt-ended) without inserting any sequences between them.

Plasmid pEGTZ is then cotransfected with AcMNPV V-8 DNA into SF cells as described in Miller et al. (1986) supra. This procedure allows for homologous recombination to take place between sequences in the viral and plasmid DNAs, resulting in replacement of the viral egt gene with the egt-lacZ gene fusion from the plasmid. Because the remaining egt coding sequence is in frame with the lacZ sequences, such a recombinant virus will produce a fusion protein comprising the first 84 amino acids of egt joined to β-galactosidase. The recombinant virus, termed VEGTZ, can be identified because β-galactosidase expression gives rise to blue viral plaques in the presence of a chromogenic indicator such as 5-bromo-4-chloro-3-indoly1-β-D-galactopyranoside (X-gal).
Recombinant virus V8vEGTDEL is obtained by cotransfecting the plasmid pEGTDEL and DNA from the virus vEGTZ into SF cells. Homologous recombination results in the replacement of the egt-lacZ fusion gene in V8vEGTZ with the deleted egt gene from pEGTDEL. The recombinant virus VEGTDEL is identified by its failure to form blue plaques in the presence of X-gal.

In a specific embodiment of an AcMNPV V-8 virus in which the egt is inactivated, a DNA fragment from 7.6-11.1 map units on the physical map of AcMNPV was cloned into a plasmid vector as described in U.S. Patent No. 5,180,581, incorporated by reference herein.

This AcMNPV fragment contains the egt gene and flanking viral DNA. An internal deletion was made in the egt gene and the E. coli lacZ gene was fused in frame. The initial egt-deleted virus, designated vEGTZ, was constructed using this fusion plasmid to replace the egt gene in AcMNPV by allelic recombination mediated by cellular recombination mechanisms. Presence of a functional lacZ gene facilitated the identification of the recombinant virus by its blue color in plaque assays in the presence of an appropriate chromogenic indicator. An additional egt-deleted virus, vEGTDEL, was constructed by deleting an internal portion of the egt gene from the plasmid vector containing the 7.6-11.1 map unit region of the AcMNPV genome using PCR mediated mutagenesis. The sequence of the egt coding region and flanking sequences are shown in Figure 6, along with the locations of the PCR primers. Deletion at the precise sites indicated in Fig. 3 results in the formation of two novel and easily characterized restriction enzyme sites (EcoRI and XbaI) at the deletion junction. This deletion plasmid was then used to replace the egt-deleted lacZ gene.

Example 8.

Egt enzymatic activity protein can be determined as follows: SF cells are infected with egt-deleted as described hereinbefore.
Twelve hours post infection the cells and extracellular media are collected and processed separately. Uninfected cells are treated in parallel. Cell lysates or extracellular media are incubated in the presence of 1 mM UDP-glucose, UDP-galactose and 0.25 μCi[3H]ecdysone as described in O’Reilly and Miller (1989) Science **245**:1110-1112. Ecdysteriod UDP-glucosyl transferase activity in the cell lysates or media catalyze the transfer of glucose from the UDP-glucose to ecdysone to form an ecdysone-glucose conjugate. Ecdysone and the ecdysone-sugar conjugate are separated from one another by silica gel thin layer chromatography (Bansal and Gessner (1988) Anal. Biochem. **169**:321) and visualized by autoradiography. Ecdysone-glucose conjugates (G) are only formed when wt AcMNPV-infected cell lysate or extracellular medium is assayed. No conjugates are observed when uninfected or egt-inactivated virus infected cell lysates or media are used, showing that the activity is due to egt expression. Most of the activity is located in the extracellular medium.

It should be understood that the foregoing relates only to preferred specific embodiments of the present invention and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the invention as set forth in the appended claims.
Docket No ACY 32430

Microorganism: Deposit Information

The ATCC deposit accession number for the deposit described at page 7 is VR 2465.
WE CLAIM:

1. An isolated DNA molecule comprising a nucleotide sequence as given in Fig. 4 from nucleotide 3002 to 4231, V-8 sequence, wherein said nucleotide sequence confers improved killing properties for at least one insect pest when said nucleotide sequence is incorporated in an insect virus genome.

2. The isolated DNA molecule of claim 1 comprising the nucleotide sequence as given in Fig. 4 from nucleotide 2469 to 4231.

3. An insect virus having improved killing properties for at least one insect pest, said insect virus being genetically engineered to contain the nucleotide sequence of claim 1 or 2.

4. The insect virus of claim 3 which is a baculovirus.

5. The insect virus of claim 4 wherein said baculovirus is Autograph californica Nuclear Polyhedrosis Virus.

6. The insect virus of claim 5 wherein said genetically engineered baculovirus is one of vEcoRIHybI and vEcoRIHybIFS.

7. The insect virus of claim 3 which has been further genetically engineered to inactivate a gene encoding ecdysteroid glucosyltransferase.

8. The baculovirus of claim 7 in which said ecdysteroid glucosyltransferase gene has been inactivated by deleting at least a portion thereof.
9. An isolated and purified recombinant baculovirus having incorporated within its genome a segment of heterologous insect virus DNA conferring improved killing properties has been incorporated, which recombinant baculovirus effects faster killing for at least one insect pest as compared with an isogeneic parental baculovirus lacking said segment of DNA.

10. The isolated and purified recombinant baculovirus of claim 9 which is an *Autographa californica* Nuclear Polyhedrosis Virus.

11. The recombinant baculovirus of claim 10 wherein said segment of DNA is derived form *Rachiplusia ou* Nuclear Polyhedrosis Virus or form Nuclear Polyhedrosis Virus V1000.

12. The recombinant baculovirus of claim 11 wherein said segment of DNA comprises a nucleotide sequence as given in Fig. 4 from nucleotide 3002 to nucleotide 4231.

13. The recombinant baculovirus of claim 11 wherein said segment of DNA comprises a nucleotide sequence as given in Fig. 4 from nucleotide 2469 to 4231.

14. The baculovirus of claim 13 which is AcMNPV V-8.

15. The baculovirus of claim 9 which has been further improved as an insect control agent by inactivating a gene encoding ecdysteroid glucosyltransferase.

16. The baculovirus of claim 15 wherein the baculovirus in which the gene encoding ecdysteroid glucosyltransferase has been inactivated is AcMNPV V-8.
17. An insecticidal composition comprising an effective amount of the baculovirus of claim 9 and a suitable carrier.

18. The insecticidal composition of claim 17 wherein said improved killing properties of the baculovirus are determined by a shorter time between infection of neonate larvae of said insect pest and the time when half of the infected larvae are dead than is observed for the comparison Autographa californica Nuclear Polyhedrosis Virus.

19. The insecticidal composition of claim 18 wherein said baculovirus is at least one of AcMNPV V-8 and V8vEGTDEL.

20. The insecticidal composition of claim 18 wherein said baculovirus is AcMNPV V-8 which has been genetically engineered to inactivate a gene encoding ecdysteroid glucosyltransferase.

21. A wettable powder insecticidal composition comprising V8vEGTDEL polyhedrin inclusion bodies, 10% weight; methacrylic-methyl methacrylate pH dependent coating agent, 0.45% (w/w); UV-9 oxybezone, 2.5% (w/w); polyethylene glycol, molecular weight 400, 0.10% (w/w); starch-based sticking agent, 39.1% (w/w); lignin sulfonate, 4.90% (w/w); sugar, 19.45% (w/w); wetting agent, 19.60% (w/w); and clay-based flow agent, 3.90% (w/w) by weight.

22. A method of preparing the insecticidal composition of claim 21, said method comprising the steps of:

a) air-milling V8vEGTDEL polyhedrin inclusion bodies to under 10 μm in size;
b) coating the air-milled polyhedrin inclusion bodies of step (a) with an organic solution comprising a pH-dependent coating agent, oxybenzone and polyethylene glycol molecular weight 400 to produce coated particles;

c) blending the starch-based sticking agent, the lignin sulfonate, sugar, the wetting agent and the clay-based flow agent to make a pre-blend;

d) blending together the coated particles of step (b) and the pre-blend of step (c), whereby a wettable powder insecticidal composition is produced.

23. A wettable powder insecticidal composition comprising V8EGTDEL polyhedrin inclusion bodies, 10% weight; methacrylic-methyl methacrylate pH dependent coating agent, 2.0% (w); UV-9 oxybenzone or charcoal, 5.0% (w); polyethylene glycol, molecular weight 400, 0.10% (w/w); wetting agent, 20% (w/w); and kaolin clay bulking agent, 16.0% (w/w); citric acid, 0.9%; dispersant, 30.0% (w/w), and clay-based flow agent, 16.0% (w/w).
FIG. 4B
SUBSTITUTE SHEET (RULE 26)
FIG. 4D
FIG. 5
TCATTTGAG TACCTAGAC TGACACACA ATCTGGCAA TGAAAATTT
GCTTTGTCG GTTCTGCAG GTCTCAGCA GTGTCAACG CCAGTACGT GTTTGGCAAA GCACGCCTCC
ATGTGACAA AGATCGACAG GCACACCGA GTCTGCTGAC GCCGCCGTTT GCTCCGCGG TCATAGTTTA
TGAAAATAGT CGCCGCTTCG AGTTTATAAA TCAGAGAGAC ATGCTAGTCG GAAATTTTTT AATAATAAA
CATTTGCAA GTGAAATCTCA AAGTCTATTT AACGCTATT AATTGAAAG GTGAGACAGA GCCAAATTCG
GGATGCAGCA TTACCATAT GCCATGTATT TAAATGATA CGAGATCTG TTTAAAATGT AGATGCCTTT
CTCCCATTGC CAAATTCGAA GTATTCAATTA TTATCAAGAA CTGATATCTC GCCATATACG AGCGCATT
TGGAGCATT ACCGATAGTG TAAACATTT TAAACACTT AACGAAAAGC ATGTAACGT TGGCCGTTT
GTAAGGGCGT ACGATGAAGG TACAACATG CCTATGTCA TGGACCATT ATATCTGTGC GAC

FIG. 6B
FIG. 7