Effects of a protease-expressing recombinant baculovirus insecticide on the parasitoid Cotesia marginiventris (Cresson)

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Effects of a protease-expressing recombinant baculovirus insecticide on the parasitoid *Cotesia marginiventris* (Cresson)

by

Tyasning Nusawardani

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This is to certify that the master's thesis of

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has met the thesis requirements of Iowa State University

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ABSTRACT

Baculoviruses have been used for management of lepidopteran pests in a number of niche markets. Advantages of use of baculovirus insecticides include that they have a narrow host range and are relatively safe for nontarget organisms. However, the commercial use of baculovirus insecticides has been limited because of the relatively slow speed of kill compared to chemical insecticides. Recombinant baculoviruses have been constructed for improved insecticidal efficacy. AcMLF9.ScathL is a recombinant baculovirus that expresses a basement membrane-degrading protease. AcMLF9.ScathL reduces the survival time and feeding damage of AcMLF9.ScathL-infected Heliothis virescens larvae when compared to those of wild type virus, AcMNPVC6-infected larvae. Before field trials can be conducted with AcMLF9.ScathL, risk assessment studies are required. One of the categories of tests required by the United States Environmental Protection Agency concerns the risk posed by use of AcMLF9.ScathL to nontarget organisms, including those that play a beneficial role in agriculture, such as pollinators, insect predators, and parasitoids.

Laboratory studies were conducted to identify any deleterious effects of AcMLF9.ScathL on the life history traits of the parasitoid Cotesia marginiventris. Choice tests showed that infection of H. virescens with AcMLF9.ScathL did not affect host preference of the parasitoid. AcMLF9.ScathL reduced the larval and adult survival of C. marginiventris compared to the wild type virus when the hosts were infected with a high dose of virus (>LC99) at 72 hour post parasitism, but not at a low dose (LC50). The survival of the parasitoid was not significantly different between the wild type virus and recombinant virus treatments at 96- and 120 hour post parasitism. There were no significant differences between AcMLF9.ScathL and wild type virus treatments for the parasitoid larval emergence time, adult emergence time, sex ratio, size, and fecundity. One-third of the parasitoids that developed within diseased hosts tested positive for AcMLF9.ScathL and were able to transmit virus to
healthy larvae. These results indicate that the use of AcMLF9.ScathL may pose a slightly higher risk to parasitoids when compared to use of wild type virus if infection occurs at 72 hours post parasitism.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Insect pests cause major economic losses on a variety of agricultural and horticultural crops. Because commonly used chemical insecticides are environmentally damaging, and targeted species readily develop resistance, alternative means of pest control are needed (Hunter-Fujita et al., 1998). The incidence of insecticide resistant pest populations against the main groups of chemical insecticides is increasing (Hunter-Fujita et al., 1998). The development of resistance in many pest species and the environmental pollution has increased the pressure to reduce the use of synthetic organic chemical insecticides for insect pest control (Inceoglu et al., 2001). The environmental impact, safety, and production costs must however be considered for any alternative control agents used for pest management (Hunter-Fujita et al., 1998).

The use of natural enemies of insect pests provides an alternative approach for pest management (Wood, 1997). Some pathogens of insects or microbial insecticides can be applied for pest management by using the same formulation and application methods as synthetic chemical insecticides (Inceoglu et al., 2001). Unlike the synthetic chemical insecticides, the microbial insecticides are safe for human health and the environment (Wood, 1997). In addition, microbial insecticides lack mammalian toxicity and do not produce toxic residues (Federici, 1993). Microbial insecticides commonly replicate and persist in the environment thereby exerting sustained pressure on the insect population (Inceoglu et al., 2001).

The most successful of the microbial insecticides is *Bacillus thuringiensis* (Bt). Bt is a gram-positive bacterium and effective for control of both agricultural and household pests as well as vectors of animal and human disease (Harrison & Bonning, 2000). Approximately 95% of the biopesticide market consists of Bt products (Harrison & Bonning, 2000). Two limitations of Bt are the relatively poor
persistence under field conditions and that many lepidopteran pests, such as *Spodoptera*, *Heliothis*, and *Helicoverpa* spp. are not very susceptible to Bt (Federici, 1993). There are many other pests for which no effective Bt toxins are known. Viral pathogens have potential for development as control agents against many of the species that cannot be effectively controlled by Bt (Federici, 1993).

Insect viruses, such as baculoviruses, are suited for use in integrated pest management (IPM), because they are compatible and often synergistic with other control agents (Murhammer, 1996). Baculoviruses have been used successfully for control of specific lepidopteran pests, and also for control of a hymenopteran, the European pine sawfly *Neodiprion sertifer* (Geoffroy) (Moscardi, 1999). One of the first successful applications of a baculovirus was in Germany in 1892 for control of populations of *Lymantria monacha* in pine forest (Moscardi, 1995). In North America, the use of baculoviruses started as early as 1930 with *Diprion hercyniae* nucleopolyhedrovirus (NPV) for protection of pine trees (Bird and Burk, 1961).

The first registration of a baculovirus insecticide in the United States was in the 1970s for Elcar™ for application against the cotton bollworm, *Helicoverpa zea* (Black et al, 1997). Elcar™ consists of the HzNPV, a baculovirus which infects all of the major *Helicoverpa/Heliothis* species, and is used in cotton, soybean, sorghum, maize, tomato, chickpea, and navy beans (Moscardi, 1999). Elcar™ failed commercially in the market because of its low speed of action compared to synthetic pyrethroid insecticides (Inceoglu et al., 2001).

Another baculovirus, *Spodoptera frugiperda* NPV (SfNPV), has been used for insect control on maize. SfNPV was applied on 5,000-10,000 hectares annually until 1995 and 20,000 hectares annually since 1995 (Moscardi et al., 1999). Moscardi (1999) demonstrated successful use of *Anticarsia gemmatalis* MNPV for control of the velvetbean caterpillar on soybean in 1982/1983 in Brazil and this virus has been used effectively for more than 20 years. Applications of virus were conducted with approximately $1.5 \times 10^{11}$ occlusion bodies (OB)/hectare before larvae reached the
fourth instar and with fewer than 20 larvae/m of row. The area treated with AgMNPV increased from 2000 hectares in 1982/1983 to 1.2 million hectares in 1997/1998. Production of AgMNPV is now coordinated by private industry. Virus production involves collecting and storing dead larvae before processing. The cost of the formulated product is about US $0.70/ha, and it is sold to farmers at a mean cost of US $1.20-$1.50/ha, which is cheaper than the cost of chemical insecticides. The success of AgMNPV in Brazil is due to its high virulence at a relatively low dose and efficient transmission by natural enemies and abiotic factors. Unsuccessful applications of AgMNPV can result from inappropriate timing of application, or use in regions with low mean temperatures (20 °C) and in years with extended drought periods (Moscardi, 1999).

One of the limitations of baculovirus insecticides is the slow speed of kill of the infected host insect (Murhammer, 1996). To overcome this limitation of use of wild type baculoviruses, recombinant baculoviruses that produce insect-specific toxins or physiological effectors have been constructed (Inceoglu et al., 2001). A recombinant baculovirus expressing a cathepsin L-like protease, AcMLF9.ScathL, has been constructed to increase the speed of kill of the wild type baculovirus Autographa californica multiple NPV (AcMNPV) clone C6 (Harrison and Bonning, 2001). The United States Environmental Protection Agency (EPA) requires testing before field trials can be conducted with the recombinant baculovirus. One of the tests concerns the risk posed by use of the recombinant baculovirus to nontarget organisms, including predators and parasitoids. The issue addressed in this thesis is whether the recombinant baculovirus AcMLF9.ScathL deleteriously affects the insect parasitoid Cotesia marginiventris (Cresson) (Hymenoptera: Braconidae) which parasitizes the targeted pest, Heliothis virescens. These data are needed for risk assessment of AcMLF9.ScathL before its release into the field.
Literature Review

Baculovirus biology

Baculoviruses (family Baculoviridae) are occluded viruses with the infectious virions embedded within a proteinaceous occlusion body (OB) (Hunter-Fujita et al., 1998). Occlusion bodies protect the virus from dessication allowing for persistence in the environment (Hunter-Fujita et al., 1998). Baculoviruses have a large circular double-stranded DNA genome contained within a rod-shaped, enveloped nucleocapsid (Hunter-Fujita et al., 1998). Nucleocapsids are packaged together or singly within occlusion derived viruses (ODV). ODV in turn are enclosed within OB.

The family Baculoviridae is composed of two genera: Nucleopolyhedrovirus (NPV) and Granulovirus (GV) (Volkman, 1995). The genera are characterized by the size of the occlusion bodies. The NPVs produce large polyhedron-shaped structures called polyhedra, which contain many virions (Funk et al., 1997). The GVs have smaller occlusion bodies called granules that contain a single virion (Funk et al., 1997). The occlusion bodies of GVs are 0.3-0.5 µm in diameter and the occlusion bodies of NPVs are 1-5 µm in diameter (Hunter-Fujita et al., 1998).

Baculovirus viral particles exist in two morphologically distinct forms: Virus particles within the occlusion bodies are called occlusion-derived viruses (ODV) and are responsible for infection of the epithelial cells of the midgut (Hunter-Fujita et al., 1998). The second phenotype is budded virus (BV). Budded viruses are responsible for dissemination of virus throughout the insect body (Hunter-Fujita et al., 1998). The envelopes of occlusion-derived virus are synthesized within the nucleus during occlusion body morphogenesis (Hunter-Fujita et al., 1998). The envelopes of BV consist of virally-modified cell membrane acquired when the nucleocapsids bud through the host cell membrane (Hunter-Fujita et al., 1998). Species within the genus *Nucleopolyhedrovirus* are divided into two groups, based on whether
occlusion-derived viruses contain multiple (M) or single (S) nucleocapsids (Washburn et al., 2003).

Baculoviruses have been isolated from more than 400 insect species from 7 insect orders: Hymenoptera, Diptera, Coleoptera, Neuroptera, Thysanura, Trichoptera, and Lepidoptera (Volkman et al., 1995). GVs have only been reported from members of the order Lepidoptera. Approximately 100 lepidopteran species are reported susceptible to GVs (Volkman et al., 1995). The infectivity of most NPVs is restricted to members of the genus or family of the original host (Groner, 1990). A few exceptions to this restricted host range are known. For example Autographa californica MNPV (AcMNPV) is known to infect approximately 43 species of Lepidoptera within 11 families (Groner, 1990).

**Nucleopolyhedrovirus Life Cycle**

The baculovirus life cycle starts when the virus enters the insect host via ingestion of contaminated foliage (Hunter-Fujita et al., 1998). The virus can also enter transovarially (passage within the egg) and transovum (contamination of the egg surface), through spiracles into the tracheal system, or via parasitism (Cory et al., 1997). On ingestion of polyhedra by a host insect, the polyhedrin matrix dissolves in the alkaline midgut solution (Bonning et al., 2002). The ODV released from the polyhedra pass through the peritrophic membrane (Federici, 1997). The midgut epithelia and specifically the columnar cells, are the sites for virus attachment and entry (Granados, 1980).

The ODV infect the midgut cells and virus replication takes place in the nucleus (Bonning et al., 2002). Budded viruses that are produced penetrate the basement membrane at sites of penetration by the tracheal system and enter the hemocoel (Engelhard, 1994). Budded viruses circulate in the hemolymph and initiate sites of secondary infection throughout the host body (Federici, 1997). The midgut, hemocytes, tracheal matrix, and fat body cells are the most readily infected in
susceptible hosts (Federici, 1997). Infection is also observed in nerve, muscle, pericardial cells, reproductive tissues, and glandular tissues (Federici, 1997). Hemocytes are capable of clearing infection through encapsulation in less susceptible hosts (Trudeau et al., 2001).

Occlusion-derived viruses and polyhedra are produced in the late phase of infection (Federici, 1997). The larvae appear swollen and pale at late stages of infection, because of the presence of polyhedra accumulating in epidermal and fat body nuclei (Federici, 1997). Cell lysis releases polyhedra into the hemolymph at advanced stages of disease (Federici, 1997). Death of infected larvae can occur as soon as 5 days after infection (Inceoglu et al., 2001). Prior to death the larvae of many lepidopteran species crawl to the top of the vegetation. The larvae lose their turgor after death and the cuticle ruptures easily thereby releasing polyhedra into the environment (Federici, 1997).

**Properties of baculovirus insecticides**

One of the advantages of the use of baculoviruses for pest control is that they do not infect vertebrates or plants (Groner, 1990). Thus, baculoviruses are harmless for nontarget organisms. Baculoviruses can be applied alone, or in combination with chemical insecticides, or other biological control agents (Hawtin & Possee, 1993).

Baculoviruses can persist in the environment for considerable periods of time. Fuxa et al. (2001) detected viable occlusion bodies in the soil 17 months after virus application. Both biotic and abiotic factors influence baculovirus persistence. Baculoviruses are inactivated by ultraviolet light (Murhammer, 1996). Rain and crop residues make virus less accessible to insects, although rain can also splash virus on to plants (Hunter-Fujita et al., 1998). Temperature, humidity and pH may also affect baculovirus persistence (Hunter-Fujita et al., 1998). Dissemination of virus is facilitated by predators, parasitoids, auto-dissemination, casual dissemination
(unintentional movement by humans), and the availability of alternative host species (Hunter-Fujita et al., 1998).

If used repeatedly, such as use of AgMNPV in Brazil, pests will develop resistance to baculovirus infection. Moscardi (1999) demonstrated the velvetbean caterpillar, *A. gemmatalis*, in Brazilian colonies is more resistant to infection by AgMNPV than colonies in the United States. The resistance of *A. gemmatalis* from Brazil was lost after backcrossing the highly resistant population with the respective nonselected population (Moscardi, 1999). One way to prevent the development of resistance is by allowing migration of adults from areas not exposed to AgMNPV to areas where AgMNPV has been extensively applied (Moscardi, 1999).

Baculoviruses can be produced by *in vivo* and *in vitro* methods. *In vivo* production of baculoviruses can be conducted in the laboratory, in field cages, or in farmers’ fields (Moscardi, 1999). The cost of *in vivo* production in the laboratory is relatively high in terms of labor, equipment, and diet (Moscardi, 1999). The cost of *in vivo* production is relatively low compared to *in vitro* production in insect cell culture however. The disadvantages of *in vivo* production are inclusion of large amounts of insect parts in the product, the amount of labor, and vulnerability of workers to microbial contamination (Black et al., 1997). One method of *in vitro* production for baculoviruses is use of conventional stir-tank bioreactors (Murhammer et al., 1996). This method can produce large quantities of virus, although the amounts vary according to the virus strain, cell line, and media used (Black et al., 1997). Both the *in vitro* and *in vivo* processes can be fully automated (Hunter-Fujita et al., 1998).

**Genetic modification to improve insecticidal activity**

Baculoviruses have been extensively used for control of insect pests, although they are not currently in widespread use (Moscardi, 1999). The use of baculoviruses has been restricted because of several factors, including problems
with long term storage, persistence in the field, and the relatively slow speed of kill of the targeted pest (Inceoglu, 2001).

Baculoviruses have a slow speed of action compared to the classical synthetic chemical insecticides (Inceoglu et al. 2001). Infected larvae continue to feed for several days until their death and may cause significant damage to crops (Hawtin and Possee, 1993). Crops such as pine forests are able to tolerate the damage that occurs during this period (Hawtin and Possee, 1993). The cost of such damage to orchard and field crops is too high, and therefore limits the commercial use of baculovirus insecticides (Hawtin and Possee, 1993). Genetically modified baculoviruses have been constructed to augment the action of wild type baculoviruses (Bonning & Hammock, 1996; Possee et al., 1997; van Beek & Hughes, 1998; Inceoglu, 2001, Bonning et al., 2002).

The purpose of genetic enhancement of baculoviruses for use as insecticides is to combine the pathogenicity of the virus with the insecticidal action of a toxin, hormone, or enzyme (Bonning et al., 2002). The foreign protein is expressed during infection of the insect larva. The insect dies rapidly from the effect of the toxin rather than from the virus infection itself. The goal of genetic enhancement is to reduce the time from infection with the recombinant virus to death of insect (Bonning et al., 2002). The goal is to reduce the feeding damage caused by the insect to below the economic threshold. Other goals of genetic enhancement of baculoviruses are for example to improve the production of virus, modify host range and enhance the utility of various insect viruses as biopesticides (Bonning & Hammock, 1996). About 20 recombinant baculovirus insecticides have been constructed to express insect-specific toxins derived from venomous animals such as scorpions and spiders, or hormones or enzymes for disruption of insect development (Bonning & Hammock, 1996; Harrison & Bonning, 2000; Inceoglu et al., 2001; Bonning et al., 2002).

Fewer polyhedra are produced by recombinant baculoviruses because of the enhanced speed of kill (Black et al., 1997). This can be viewed as a disadvantage
because it limits the persistence of recombinant baculoviruses in an agroecosystem. On the other hand, this can be viewed as an advantage in that it restricts spread of the genetically modified virus. Lee et al. (2001) showed that the recombinant baculovirus expressing a scorpion toxin (AcNPV.AaIT) and the recombinant baculovirus expressing a mutated juvenile hormone esterase (AcJHE.SG) were both rapidly outcompeted by the wild type baculovirus (AcNPV.WT).

A new recombinant baculovirus with potential for insect pest control expresses a basement membrane-degrading protease. Harrison & Bonning (2001) demonstrated that the recombinant baculovirus (AcMLF9.ScathL) expressing a cathepsin L-like protease significantly reduced the median survival time of infected larvae by 51% and the feeding damage by 80% compared to larvae infected with the wild type virus, AcMNPV C6.

**Mode of action of the protease-expressing recombinant baculovirus, AcMLF9.ScathL**

Basement membranes are extracellular sheets of protein that provide structural support, a filtration function and a surface for cell attachment, migration, and differentiation (Yurchenco and O'Rear, 1993). The basement membrane of insects may act as a potential barrier for secondary infection of the virus within the host body (Harrison & Bonning, 2000).

The *Sarcophaga peregrina* cathepsin L (ScathL), is a protease derived from the flesh fly *S. peregrina* that is secreted from cells in response to 20-OH ecdysone. ScathL degrades the basement membrane of the imaginal discs (Homma & Natori, 1996; Fujii-Taira et al., 2000). For the recombinant baculovirus that expresses ScathL, it was hypothesized that degradation of the basement membrane would facilitate movement of the virus within the infected host insect (Harrison & Bonning, 2001).
The rapid death of larvae infected with AcMLF9.ScathL does not appear to be caused by acceleration of systemic infection of the host. If the protease-expressing virus damages the basement membranes and facilitates secondary infection, a dual infection will result in a rapid widespread infection of other tissues by a second virus. If the second virus encodes a toxin, larger quantities of toxin will be expressed and a further reduction of survival time may be expected (Harrison & Bonning, 2001). Harrison & Bonning (2001) did not find any synergistic or additive effect on host survival time arising from dual infections with the protease-expressing virus and a toxin expressing virus. The fact that even fewer polyhedra are produced following infection with AcMLF9.ScathL suggests that larvae are dying as a result of protease activity, rather than from enhanced virus dissemination.

Another proposed hypothesis is that the insecticidal action of the protease may be due to activation of prophenoloxidase which can be toxic for the insect (Harrison & Bonning, 2001). Prophenoloxidase is a key enzyme required for production of melanin (Nappi et al., 1995). The production of quinones during the process of melanization may cause cytotoxic effects, via production of reactive oxygen groups, such as superoxide anions and hydroxyl radicals (Nappi et al., 1995). These cytotoxic effects may be important in the killing of parasites and pathogens and may contribute to the insecticidal effect of ScathL. Popham et al. (2004) found an antiviral activity in the plasma of susceptible larvae which is active against baculovirus in vitro. The virucidal activity is also coincident with phenoloxidase activity (Popham et al., 2004). It is possible that increased phenoloxidase levels resulting from ScathL activity may inhibit AcMLF9.ScathL. Research is still needed to determine the relationship between virucidal activity and the generation of cytotoxic free radicals in the hemolymph of susceptible insects (Popham et al., 2004).

Late instar larvae infected with AcMLF9.ScathL melanize prior to death (Harrison & Bonning, 2001). The melanization observed is assumed to be caused by
ScathL protease-induced damage to the basement membrane. Melanization, or dark pigmentation occurs when an insect is wounded (Gillespie et al., 1997). Melanization is important in insect defense by protecting the wound and in sequestering pathogens (Gillespie et al., 1997). Wilson et al. (2001) found a significant positive correlation between phenoloxidase activity in hemolymph, midgut, and cuticle with cuticular melanization. There was also a positive correlation between dark coloration of larvae, the number of melanized ectoparasitoid eggs and decreased fungus-induced mortality (Wilson et al., 2001). The authors suggested that the structure of the melanized cuticle provided a secure physical barrier that prevents penetration of pathogens and parasites. Research is still needed to examine the relationship between host melanization and virucidal activity.

Research is underway in the Bonning laboratory to explore the relationship between phenoloxidase activity, melanization, and possible virucidal activity of cytotoxic free radicals in the hemolymph of AcMLF9.ScathL infected-insects. This research will provide a better understanding of the mode of insecticidal action of AcMLF9.ScathL.

**EPA requirements for registering a microbial control agent**

The use of baculovirus insecticides results in fewer detrimental effects to nontarget organisms than the use of synthetic chemical insecticides. Guidelines have been established by EPA to assess potential risks associated with a new microbial control agent. The EPA guidelines use a tier testing scheme that is designed to provide the minimum data for scientific regulatory decisions. Tier I testing consists of maximum dose, single species hazard tests on nontarget organisms. If adverse effects are detected in Tier I tests, ecological exposure tests are performed in Tier II testing. Tier II testing is performed to quantify the levels of the control agent to which the susceptible nontarget organism may be exposed. If Tier II tests indicate that there may be significant exposure to the control agent, Tier
III studies are adopted to determine a dose response effect and whether other factors would decrease adverse effects in the environment. Pathogenic effects occurring at Tier III or beyond would reduce the likelihood of registration of the control agent. Tier IV tests are designed to address problems that are not resolved by lower tier testing (Bonning et al., 2002).

Tier I toxicology and nontarget organism toxicity tests are conducted for registration of new microbial pest control products. If data generated from Tier I testing indicate no adverse effects, no further testing would be required. The purpose of nontarget organism testing is to assess the potential hazard of the control agent to terrestrial wildlife, aquatic animals, plants, and beneficial insects. For testing of nontarget insects, Tier I testing is used to assess the toxicity and pathogenicity of the agent to the honey bee, and three species of predaceous, and parasitic insects. At least two of the following groups should be selected: parasitic Diptera, parasitic Hymenoptera, predaceous Hemiptera, Coleoptera, Neuroptera, and predaceous mites. The species tested should have relevance to the ecosystem which will be exposed to the control agent (Bonning et al., 2002).

A previous study was conducted by Boughton et al. (2003) to examine the effects of the recombinant baculovirus, AcMLF9.ScathL, on the life history traits of predators. Additional research on the effects of AcMLF9.ScathL9 on a parasitoid is required for the EPA requirements for Tier I toxicity testing of nontarget arthropods and to examine more subtle effects of AcMLF9.ScathL on the parasitoid.

Risk assessment of recombinant baculoviruses to nontarget organisms

Richards et al. (1998) proposed a conceptual model for the impact evaluation of recombinant baculovirus insecticides. The model has three main components: impact identification, exposure identification, and impact evaluation. The impact identification may identify species and populations which are susceptible to direct or indirect impacts of the recombinant baculovirus. The exposure identification pathway
may identify the dispersal and transmission of the recombinant baculovirus in the environment. As a part of risk assessment the impact evaluation is then used in the design of risk management strategies. The risk of using recombinant baculoviruses is that they may affect nontarget organisms in the environment. There are nontarget organisms which play a role as beneficial insects in the agricultural systems, for example: pollinators, predators, and parasitoids. They may come into contact with the foreign protein expressed from baculovirus-infected larvae and suffer adverse effects (Richards et al., 1998).

The pollinators may come into contact with plants contaminated by baculovirus. However, studies showed no ill effects on the honey bee, *Apis mellifera* L., when fed with sucrose solution mixed with wild type baculoviruses (Cantwell et al., 1966; Knox, 1970). Morton et al. (1975) also showed that *AcMNPV* had minimum or no effect on honey bee longevity, egg laying by the queen, egg hatch, care of larvae by workers, growth of larvae, pupation, or development and emergence of adult workers. In addition, polyhedra were not detected in the gut or fat body of honey bees. Heinz et al. (1995) found no adverse effects of the recombinant baculovirus expressing a scorpion toxin (*AcAalT*) on the survivorship of adult honey bees.

Predators may be exposed to baculoviruses through ingestion of infected larvae. Li et al. (1999) found that the recombinant viruses did not detrimentally affect the life history traits of three predators: the red imported fire ant *Solenopsis invicta* Buren, the big-eyed bug *Geocoris punctipes* Say, and the convergent lady beetle *Hippodamia convergens* Guérin-Méneville. The experiment used five recombinant baculoviruses (four *AcMNPVs* and one *HzSNPV*) and two wild type (*AcMNPV* and *HzSNPV*) baculoviruses. The life history traits observed were the rates of food consumption, travel speed, fecundity, and survival. The recombinant baculoviruses expressed the scorpion-derived insect specific toxins LqIT or AalT. PCR results
showed that only 2% of *S. invicta* workers, 13% of *G. punctipes*, and 12% of *Hippodamia convergens* harbored virus.

Boughton et al. (2003) found that there were no detrimental risks posed by the protease-expressing recombinant baculovirus, AcMLF9.ScathL, on predators. There were no significant differences in developmental rates between the lacewing *Chrysoperla carnea* (Stephen) fed on recombinant virus-infected prey and wild type virus-infected prey. The results showed the survival of *C. carnea* reared on *Sitotroga* eggs or recombinant virus-infected larvae were significantly higher than for *C. carnea* reared on mock-inoculated or wild type virus infected larvae. It is hypothesized that ScathL degradation of host tissues facilitates the process of extra-oral digestion used by lacewing larvae. The recombinant virus had no effect on time to onset of oviposition and the daily egg production of *C. carnea*. Suspensions of adult lacewing meconial pellets fed on virus-infected prey caused virus-induced mortality of 100% of the inoculated test larvae. This experiment showed the potential of *C. carnea* as carriers of virus in the environment. The results also showed the recombinant virus posed no adverse effect on the survival of the ladybird beetle *Coleomegilla maculata* (DeGeer). Neither predator distinguished between prey that were uninfected or infected with wild type or recombinant virus.

Viruses can be transported in the environment by predators or scavengers by voiding of viable NPV in feces. Lee and Fuxa (2000a) showed that there were no differences in the preference of the predator spined soldier bug *Podisus maculiventris* (Say), the scavenging fly *Sarcophaga bullata* (Parker), and the scavenger house cricket *Acheta domesticus* (L.) for larvae killed with a wild type virus (AcNPV.WT) or a recombinant virus expressing a scorpion toxin (AcNPV.AalT) or a recombinant virus expressing a mutated juvenile hormone esterase (AcJHE.SG). The feces of the insects contained viable virus in a large amounts. However, the mortality of the insects was higher in the predator and scavengers fed with any of the viruses than the uninfected larvae. The virus infected larvae may not
be as good a source of food as healthy larvae. Lee and Fuxa (2000b) showed that predators and scavengers transported the wild type and the recombinant NPVs. Their data showed that *P. maculiventris*, and *S. bullata*, transported the recombinant and the wild type viruses at a rate of up to 62.5 cm/day, and *A. domesticus* (L.) at 125 cm/day.

Host-parasitoid-virus interactions

Parasitoids are organisms that live and feed on or inside the body of another arthropod during the larval stage and eventually kill the host. The parasitoid needs only one host to complete its development (Godfray, 1994). Baculoviruses do not replicate in parasitoids, and thus do not have any direct effects on the parasitoid. Indirect effects on parasitoids may arise, because the virus and parasitoid compete for the same host resources (Bonning et al., 2002). Deleterious effects of pathogens on parasitoids may result from premature death of the host before the parasitoid has completed its development, production of substances that are toxic to parasitoids within the host, and alterations in the host that affects the preference of parasitoids in oviposition behavior (Brooks, 1993).

Virus infected hosts may become unattractive to parasitoids, and hence oviposition behavior may be affected. The ovipositional preference of parasitoids between virus infected hosts and uninfected hosts is highly variable (Brooks, 1993). In some studies, some parasitoids did not show any preference when exposed to uninfected hosts and virus-infected hosts (Beegle & Oatman, 1975; Levin et al., 1983). However, some parasitoids did show an ovipositional preference for uninfected hosts over virus infected hosts. Kyei-Poku and Kunimi (1997) showed that the parasitoid *Cotesia kariyai* drummed the host with its antennae and then adopted the oviposition posture on entomopoxvirus-infected larvae or uninfected larvae. There was no signficicant difference in the numbers of infected or uninfected hosts parasitized. The oviposition time was significantly different however between
uninfected hosts and entomopoxvirus-infected hosts when the hosts were exposed to parasitism nine days after virus infection. The parasitoids rejected later instar hosts. The number of rejections also increased in virus infected hosts as the period between infection and parasitism increased. Infection with the virus results in differences in appearance and color of the host insects. From observing parasitoid behavior, parasitoids could not recognize the presence of virus before they made contact with the hosts. The ovipositor or probing behavior may provide information about host suitability for parasitoids. The decision of *C. kariyai* to lay more eggs in uninfected larvae than in the virus infected larvae may result from information from the sensor on the ovipositor. Hegazi and Abd-Allah (2004) also found that the parasitoid *Microplitis croceipes* parasitized more uninfected hosts than granulovirus infected hosts.

Premature death of the infected host may result in the death of a slower developing parasitoid which is not able to complete its development (Beegle and Oatman, 1975). Irabagon and Brooks (1974) showed that survival of the parasitoid *Campoletis sonorensis* occurred only when exposure to NPV followed parasitism by more than 48 hours. The research also showed that when the hosts were infected prior to parasitism, parasitoid development rarely proceeded beyond the first instar.

Parasitoids that survive in virus infected hosts may have a faster or slower development time than those from uninfected hosts. Escribano et al. (2000) found that *C. sonorensis* developed faster in many of the hosts infected with *Spodoptera frugiperda* MNPV, especially if parasitism occurred 4-6 days following viral infection. Another study showed that parasitoids took longer to develop in virus-infected hosts than in uninfected hosts. Nakai and Kunimi (1997) found that the parasitoid *Ascogaster reticulatus* developing in granulovirus-infected hosts grew more slowly than parasitoids developing in noninfected hosts. Virus infection has been shown to adversely affect the development and the size of parasitoid larvae. Parasitoid larvae
from entomopoxvirus-infected hosts showed little or no development and died between 7 and 8 days after parasitism (Kyei-Poku & Kunimi, 1998).

Kaya and Tanada (1972) showed that a granulovirus-produced protein was toxic to the parasitoid Apanteles militaris in armyworm larvae. The toxin affected the parasitoid in the egg stage. More parasitoids were able to survive the toxic effect at later stages or if parasitism occurred before toxin injection. The authors suggested that the eggs or later stages of development might have some protection from exposure to the toxin.

Parasitism by parasitoids may cause the host to become more susceptible to pathogens by blocking the host immune system. Following infection with AcMNPV, Washburn et al. (2000) found that parasitism or polydnavirus injection caused Manduca sexta (which is semipermissive to AcMNPV) to become permissive to AcMNPV.

Parasitoids also play a role in dispersal of baculoviruses in the environment (Hunter-Fujita et al., 1998). Injection of polyhedra into a lepidopteran larva is unlikely to cause infection of the larva, because infectious virions would not be released from the polyhedrin matrix at neutral pH. In addition to that, polyhedra in the midgut of parasitoids that have developed in virus-infected hosts are voided with the meconium during pupation (Stolz et al., 1988). However, contamination of the parasitoid with budded virus could result in successful infection (Irabagon and Brooks, 1974; Volkman and Summers, 1977). Brown et al. (1989) found that the parasitoid Microplitis croceipes transmitted NPV to healthy Heliothis virescens larvae. Mortality of hosts exposed to contaminated parasitoids was significantly higher than in the control treatments.

The effects of recombinant baculoviruses on endoparasitoids

Unlike pollinators or predators, endoparasitoids live and feed inside their hosts throughout the parasitoid’s larval stage. Beagle and Oatman (1975) showed
that polyhedra and budded viruses were found in the gut lumen of parasitoids, but not in the gut epithelium or in parasitoid tissues. Endoparasitoids may be bathed in and ingest the toxin or enzyme expressed by recombinant baculoviruses (McCutchen et al., 1996).

Kaya & Tanada (1972) showed that a toxin produced by wild type granulovirus could affect the survival of parasitoids in the early stages, because the parasitoid eggs were more likely to be exposed to the toxin than the late stage larvae. Thus, the toxin would kill the parasitoids developing inside the host before the host died from viral infection. Kyei-Poku & Kunimi (1998) showed that *C. kariyai* did not complete their development when virion-free plasma was injected into the hosts suggesting that entomopoxvirus produces a toxin which adversely affects development of *C. kariyai*.

Infection of the host with wild type baculovirus can affect the survival of parasitoids, when the virus kills the host before the parasitoid completes its development. Few parasitoids complete development to the cocoon stage when host larvae are infected with wild type virus at 0 and 48 h post parasitism (Beegle & Oatman, 1975; Teakle et al., 1985; Brown et al., 1989). A period of at least 72 hours is required between parasitism and infection to allow the parasitoid to complete development (Smith et al., 2000). Recombinant baculoviruses kill the hosts of parasitoids faster than the wild type virus, and may therefore have a detrimental effect on development and survival of the parasitoid.

McCutchen et al. (1996) showed that there was no significant difference in survival of the parasitoid *M. croceipes* at either the larval or adult stage between the recombinant virus and wild type virus treatments. The survival of parasitoids was not affected even though the recombinant baculoviruses expressing scorpion toxin (AcAalT) and juvenile hormone esterase (AcJHE.KK) kill the host 30-40% faster than the wild type NPV. In field studies, Smith et al. (2000) showed that there was no difference in emergence of the parasitoid *M. croceipes* from host larvae infected with
a recombinant virus expressing a scorpion toxin (LqIT2), wild type virus, and untreated control treatments.

McCutchen et al. (1996) suggested that parasitoids might develop faster or prematurely emerge in response to the deteriorating condition of the host. Parasitoid larvae exposed to AcAalT infected hosts emerged earlier than those emerging from the wild type virus infected hosts. There was no difference in the larval emergence time between the AcJHE.KK and the wild type virus treatments. There were no differences between treatments in emergence times of adult parasitods (McCutchen et al., 1996). The accelerated growth and development of parasitoids may result in smaller adult parasitoids. McCutchen et al. (1996) found that parasitoids that emerged significantly earlier from the recombinant virus-infected larvae were smaller than those that emerged from the uninfected control group. There was no difference in fecundity or virus transmission between parasitoids emerging from recombinant virus-infected hosts and from wild type virus infected hosts. No differences were detected in either the laboratory or the field study in sex ratio of the parasitoids between the recombinant virus, wild type virus, and uninfected control treatments (McCutchen et al., 1996; Smith et al., 2000).

Parasitoids emerging from virus infected larvae could mechanically transmit the recombinant virus and wild type virus to nontarget hosts including rare lepidopterous species (McCutchen et al., 1996). The female parasitoids can pass the virus to healthy hosts. Beegle and Oatman (1975) suggested that the virus was carried on the ovipositor. Cabellero et al. (1991) showed that three parasitoids Apanteles telengai, Aleiodes gasteratus, and Campoletis annulata transmitted virus from GV infected larvae to healthy larvae. McCutchen et al. (1996) showed that parasitoids from wild type NPV, AcAalT, and AcJHE.KK treatments produced 4.0, 10.6, and 8.3 % NPV infection rates on parasitism of new uninfected hosts. Approximately 40 % of adult parasitoids that developed in AcAalT infected larvae tested positive for AcAalT by PCR in a laboratory trial (McCutchen et al., 1996),
although none of the parasitoids tested positive for viral DNA in a field experiment (Smith et al., 2000). Vail (1981) reported that the parasitoids emerging from NPV-infected larvae carried the virus in their digestive tracts until the gut contents (including the meconium and virus particles) were voided soon after emergence.

The recombinant virus, AcMLF9.ScathL killed *H. virescens* larvae 30% faster than AcMLF9.LqIT2, which expresses a scorpion toxin (Harrison & Bonning, 2001). AcMLF9.ScathL may adversely affect survival of parasitoids, because the virus may kill the host before the parasitoid is able to complete development. The melanization of larvae that is associated with the insecticidal action of ScathL may alter parasitoid host choice, or affect emergence of larval parasitoids from the host insect. In this thesis, we present the results of a laboratory study to determine the effects of the recombinant baculovirus AcMLF9.ScathL on the life history traits of the parasitoid *C. marginiventris*.

**The biology of the parasitoid Cotesia marginiventris**

*Cotesia marginiventris* is a solitary larval endoparasitoid of many lepidopteran insects. Boling and Pitre (1970) described the life cycle of *C. marginiventris*. *C. marginiventris* oviposits within several minutes of emerging from the cocoon. The female finds a host by using her antennae, inserts an egg, and moves away from the host. *C. marginiventris* resides in the hemocoel of the host until it reaches the third instar. The third instar larva then chews its way out of the host, emerges, and spins a cocoon. The adults live for up to 5 days and oviposition events continue for several days. In *H. virescens*, the parasitoids emerge 6 days after oviposition at 30° C (Boling and Pitre, 1970).

*C. marginiventris* attacks many pests of agricultural crops, most of which are in the family Noctuidae and one in the family Pyralidae (Tillman, 2001). In 1992, the predominant pest that emerged from late season cotton in north-central Florida was the fall armyworm *Spodoptera frugiperda* (Tingle et al. 1994). Parasitoids may play a
role in suppressing other pests, such as the tobacco budworm *H. virescens* and corn earworm *H. zea* (Tingle et al., 1994). Indeed, 50 to 100% of larvae collected from the field in August 1992 were parasitized, the most common parasitoid being *C. marginiventris* (Tingle et al., 1994).

**Research Problem**

A recombinant clone of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) expresses a protease, cathepsin L (ScathL) that degrades the basement membrane of infected-lepidopteran larvae (Harrison & Bonning, 2001). The recombinant baculovirus, AcMLF9.ScathL, has potential for commercial use in pest management, because it kills the host faster than wild type baculovirus and reduces feeding damage caused by the infected host. Before field trials can be conducted with AcMLF9.ScathL, research is required to determine the potential deleterious effects of AcMLF9.ScathL on a parasitoid of the targeted pest. This study will contribute toward EPA requirements for toxicity testing of nontarget organisms, and will also provide extensive data on more subtle effects on behavior, life history traits, and potential transmission of the virus by the parasitoid. These data will have implications for the environmental impact of the transgene and potential for dissemination of the recombinant baculovirus away from the site of application.

*C. marginiventris* plays an important role as a beneficial insect in agricultural crops and particularly in cotton. Thus, in this study *C. marginiventris* represents a model nontarget organism that may be exposed to the recombinant baculovirus under field conditions. The first objective of the research is to identify any deleterious impact of the new recombinant baculovirus on the behavior of the parasitoid *C. marginiventris*. The second objective is to determine whether AcMLF9.ScathL has any impact on the survival and development of parasitoids relative to wild type virus. The third objective is to assess whether AcMLF9.ScathL alters the fecundity of parasitoids emerging from baculovirus-infected larvae, relative to the wild type virus. The fourth objective is to determine what role the parasitoids may play in baculovirus
dissemination. The central hypothesis for the research is that baculovirus-expressed ScathL will affect survival and emergence of parasitoids from the host. The rationale for this project is that potential hazards to non target organisms and the degree of exposure of susceptible species must be considered before release of the recombinant baculovirus.

**Thesis Organization**

The thesis contains a General Introduction (Chapter 1). Chapter 2 is a manuscript entitled "Effect of a protease-expressing recombinant baculovirus insecticide on the parasitoid Cotesia marginiventris (Cresson)". This manuscript will be submitted to *Biological Control* and is co-authored by Drs. John R. Ruberson, John J. Obrycki, and Bryony C. Bonning. Dr. John R. Ruberson provided the parasitoid *C. marginiventris*. Drs. John J. Obrycki and Bryony C. Bonning helped with the experimental design. Dr. Bonning has been involved in direction of the project, the interpretation of the results and editorial comments during preparation of the manuscript. The thesis also contains a General Discussion (Chapter 3). References are cited at the end of each chapter.

**References**


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CHAPTER 2. EFFECTS OF A PROTEASE-EXPRESSING RECOMBINANT BACULOVIRUS INSECTICIDE ON THE PARASITOID 
*COTESIA MARGINIVENTRIS* (CRESSON)

A paper to be submitted to *Biological Control*

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Abstract

A recombinant baculovirus insecticide, AcMLF9.ScathL, has been constructed that expresses a cathepsin L-like protease. This protease degrades the basement membrane of infected-lepidopteran larvae. AcMLF9.ScathL kills larvae significantly faster than the wild type baculovirus. Before field trials can be conducted with this virus, risk assessment studies are required to examine the potential impact of the virus on nontarget organisms. We examined the impact of AcMLF9.ScathL on the survival, development, and behavior of the parasitoid *Cotesia marginiventris* (Cresson) that parasitizes infected larvae. Larvae of *Heliothis virescens* were exposed to the parasitoid and infected with the wild type or recombinant virus at >LC99 at 72, 96, or 120 hours after parasitism. Control larvae were mock infected. Choice tests showed that infection with AcMLF9.ScathL did not affect the host preference of the parasitoid. At 72 h post parasitism, the survival of parasitoids emerging from hosts infected with AcMLF9.ScathL was lower than for parasitoids emerging from hosts infected with wild type virus or uninfected controls. There were no significant differences between AcMLF9.ScathL and wild type virus
treatments for the larval emergence time, adult emergence time, sex ratio, size, and fecundity of *C. marginiventris*. These results indicate that use of AcMLF9.ScathL in insect pest management poses a slightly greater risk to the parasitoid at early stages of parasitoid development than use of the wild type virus at >LC99 dose. This effect was not seen with infection at LC50.

**Introduction**

Wild type baculoviruses have been used for management of lepidopteran pests in cotton, vegetable crops, forest, and ornamental plants (Moscardi, 1999; Inceoglu et al., 2001). The commercial use of baculovirus insecticides has been limited however in part because of the relatively slow speed of kill compared to chemical insecticides (Inceoglu et al., 2001). Recombinant baculoviruses that express insect-specific toxins, hormones or enzymes have been constructed for improved insecticidal efficacy (Bonning & Hammock 1996; Bonning et al., 2002; Inceoglu et al., 2001). The infected insect dies from the effects of the expressed protein rather than from the virus infection itself.

Before a recombinant baculovirus can be tested under field conditions, risk assessment studies are required to evaluate the potential risks posed by the recombinant baculovirus to nontarget organisms (Black et al., 1997). Beneficial insects, such as pollinators, predators, and parasitoids, may come into contact with the recombinant baculovirus under field conditions (Richards et al., 1998). Risk assessment studies on potential nontarget effects of a recombinant baculovirus expressing the scorpion venom-derived toxins AalT or LqhIT2 showed that the recombinant baculoviruses posed no greater risk to honey bees or predatory arthropods than the wild type virus (Heinz et al., 1995; Li et al., 1999).

Parasitoids and viruses compete for host resources. Baculoviruses do not replicate in parasitoids so any impact of virus infection of the host insect on parasitoids is indirect (Bonning et al., 2002). McCutchen et al. (1996) found no
adverse effects of recombinant baculoviruses expressing AalT or JHE on the parasitoid Microplitis croceipes. The parasitoid _M. croceipes_ transmitted the recombinant baculoviruses to uninfected larvae in this laboratory study (McCutchen et al. 1996), although virus was not detected in parasitoids in field experiments (Smith et al., 2000).

A recombinant baculovirus AcMLF9.ScathL that expresses a basement membrane-degrading protease has been constructed. The ST50 and feeding damage of AcMLF9.ScathL-infected larvae were 49% and 20% lower respectively than those of wild type virus infected larvae (Harrison & Bonning, 2001). Boughton et al. (2003) reported that there are no greater risks to predatory arthropods associated with use of AcMLF9.ScathL than with use of wild type virus. However, given that a parasitoid would be bathed in the protease, AcMLF9.ScathL may represent a greater risk to parasitoids than to predators. In addition, AcMLF9.ScathL-infected larvae melanize, which may alter parasitoid host choice, or affect emergence of parasitoid larvae from the infected host. The purpose of this study was to evaluate the effect of AcMLF9.ScathL on the behavior, survival, development, and fecundity of a model parasitoid, _C. marginiventris_ (Cresson). We also evaluated the potential of the parasitoid _C. marginiventris_ to transmit the recombinant baculovirus to other hosts.

**Materials and methods**

**Viruses.** Construction of the recombinant baculovirus, AcMLF9.ScathL has been described previously (Harrison and Bonning, 2001). The parental virus, AcMNPV clone C6 (Possee, 1986), was used as the wild type virus control.

**Virus amplification and purification.** The recombinant and wild type viruses were amplified and purified as described previously (O’Reilly et al., 1992).

**Insects.** The parasitoids _C. marginiventris_ (Cresson) (Hymenoptera: Braconidae) were maintained on larvae of the beet armyworm, _Spodoptera exigua_ (Hübner) (Lepidoptera: Noctuidae). Tobacco budworms, _H. virescens_ were reared
from eggs purchased from AgriPest (Zebulon, North Carolina) at 27°C, 70% RH on *H. virescens* diet obtained from Southland Products (Lake Village, AR). All insects were maintained with a photoperiod of 14 h light, 10 h dark.

**Choice tests.** Two types of choice tests were conducted to determine whether infection with AcMLF9.ScathL affected parasitoid host preference. The first choice test was conducted to examine whether feeding *H. virescens* larvae on different food coloring dyes affected the preference of parasitoids for the “colored” hosts. The dyes were used in the second choice test to indicate which *H. virescens* larvae received which treatment (virus or control). We examined whether the virus treatment affected parasitoid host preference in the second choice test. Both choice tests were performed in the laboratory. Lighting was provided by fluorescent ceiling lights and the temperature was 23-26°C. The experimental arena consisted of 35 x 10 mm tissue culture dishes filled one third full with *H. virescens* diet. The dishes were then divided into three equal sized 120° sections. Prior to use in the first choice test *H. virescens* larvae were reared on different colored diets for 48 hours. Red or blue food color dyes (2%; McCormick, Hunt Valley, MD) were added to the *H. virescens* diet during diet preparation. A single *H. virescens* larva reared on red diet was placed at the center of the first section, a larva reared on blue diet was placed at the center of the second section, and a larva reared on plain diet was placed at the center of the third section. A female parasitoid was then introduced into the center of each arena. The female was exposed to fresh larvae for oviposition prior to being transferred to the choice test arena. Experimental arenas containing host larvae and one female parasitoid were observed until the first attack. The choice of larva for the first attack was recorded. The first color-preference choice test was repeated three times, with 20 observations per replicate.

The second choice test was conducted to evaluate the effect of virus infection on parasitoid host preference. The droplet feeding method of Hughes and Wood (1981) was used for infection of *H. virescens* larvae. Prior to being used in the
choice tests *H. virescens* larvae were reared on different colored diets for 24 hours and then starved for another 24 hours before virus infection. *H. virescens* larvae reared on red diet were infected with wild type virus, larvae reared on blue diet were infected with AcMLF9.ScathL, and larvae on plain diet were mock infected. One *H. virescens* larva reared on red diet was placed at the center of the first section, a larva reared on blue diet placed at the center of the second section, and a larva reared on plain diet was placed at the center of the third section. A single female parasitoid was then introduced into the center of each arena. Experimental arenas containing host larvae and a single parasitoid were set up at two minute intervals and were run in batches of 5. Three replicates were performed for a total of 20 observations per replicate. The dose used for each virus was a 100 x LC50. The concentration used for the wild type virus, AcMNPV C6, was $4.34 \times 10^6$ occlusion bodies (OB)/ml and for the recombinant virus, AcMLF9.ScathL was $3.44 \times 10^6$ OBs/ml. The LC50 dose of each virus was determined from the mean value of two replicate lethal concentration bioassays conducted in prior experiments (data not shown). Data were recorded for the first attack only.

**Survival and Development Tests.** Experiments to examine the impact of AcMLF9.ScathL on survival and development of the parasitoid were based on methods described by McCutchen et al. (1996). Mated experienced female *C. marginiventris* were placed individually into petri dishes (35 mm x 10 mm) with one second instar for each female. *H. virescens* larvae were infected with AcMNPV C6 or AcMLF9.ScathL at 72, 96, or 120 h after parasitism (5-10 larvae per treatment per time point) by using the diet cube inoculation technique as described by Boughton et al. (2003). The larvae were infected with > LC99 ($5 \times 10^4$ OB) dose of NPV (McCutchen et al., 1996). Any larvae that died before parasitoid emergence were dissected to determine whether a larval parasitoid was present. Survival and development experiments were replicated four times. Preliminary studies showed that viral treatments at 0 and 48 hour post parasitism resulted in only a few
parasitoids completing development to the cocoon stage in wild type virus infected larvae. Uninfected parasitized larvae served as controls. Emergence of parasitoid larvae from the host and emergence of parasitoid adults from cocoons were monitored at 12 h intervals. Survival of larvae and adult parasitoids from all treatments was monitored. The head widths of adults surviving from each treatment group used in the survival and development tests were recorded by using an ocular micrometer. The sex ratio of F1 adults from the survival tests was also determined.

An additional experiment was conducted to examine survival of parasitoids developing in larvae infected at an LC50 dose of virus (50 OB) using the same procedure. Two replicates were conducted with 5-15 parasitoids per replicate.

**Fecundity tests.** In fecundity tests, parasitoid adults (F1) surviving from *H. virescens* larvae infected with virus (> LC99) at 96 h post parasitism or from the control treatment were allowed to mate and each female was provided with five healthy second instar larvae of *H. virescens* for oviposition each day until death. *H. virescens* larvae were then housed individually and the number of progeny parasitoids (F2) recorded. Successful oviposition was quantified as emergence of the larval parasitoid from the host (MCCutchen et al., 1996). Fecundity experiments were replicated twice.

**Virus transmission.** The host larvae from fecundity tests were monitored for evidence of virus infection by examination for virus symptoms (pale coloration or melanization). In addition, the F1 generation *C. marginiventris* adults from each treatment of the survival and development tests were stored at -20 °C and analyzed for the presence of virus by using PCR. The DNA extraction method used was as described previously with slight modification (Noda et al., 2002). Individual parasitoids were homogenized in 10 µl STE buffer (100 mM NaCl, 1 mM ethylenediamine-tetraacetic acid [pH 8.0], 10 mM Tris-HCl [pH 8.0]) and digested with 0.05% proteinase K. Samples were boiled for 2 minutes and used as template for polymerase chain reaction (PCR). PCR for the detection of recombinant virus
was performed by using two primers for AcMLF9.ScathL. The two primers were OScL335 5’ CCA CCT ACA TTC CTC CAG CAC 3’ and OScL822R 5’ ATT GTA TAC ACC TTC GCT GTA C 3’. These primers were used at an annealing temperature of 55°C for 45 cycles with an extension temperature of 72 °C for 1 minute. Viral DNA samples purified from insect cell culture medium (from approximately 5 plaque-forming units of virus) were used as AcMLF9.ScathL positive controls in PCR reactions.

**Statistical analyses.** The $\chi^2$ test (SAS-Institute, 2002-2003) was used to identify differences in parasitoid host choice. ANOVA and Tukey adjustment’s means separation test (SAS-Institute, 2002-2003) were used to analyze differences in parasitoid survival, emergence times, and proportions of parasitized hosts. The survival, sex ratio, and fecundity data were transformed by arcsin square root before analysis. Means plus standard deviations were reported after they were back-transformed to their original form.

**Results**

**Choice tests.** The parasitoids observed in the color choice tests and virus treatment choice tests did not show any preference for a particular color or treatment (Figure 1). The parasitoid behaviors observed were walking around the dish, approaching the hosts, drumming the hosts with antennae, and attacking the host by inserting the ovipositor into the host larva. Most of the parasitoids parasitized as soon as they came into contact with the hosts, and most attacked more than one host. Data for the three replicates of each choice test were pooled with 61 parasitoid females observed for the color treatment choice test and 60 for the virus treatment choice test. The choice tests did not show any differences in parasitoid preference between replicates or for pooled data for the hosts reared on blue, red or plain diet ($X^2 = 1.01$, df=2, $P = 0.6016$; Figure 1 A). No preference was detected for uninfected
H. virescens larvae or for larvae infected with the wild type virus or recombinant virus ($X^2 = 0.7$, df=2, $P = 0.7047$; Figure 1 B).

**Survival and development tests.** Larvae of H. virescens were parasitized and then infected with virus (lethal concentration > LC99) at 72-, 96-, 120-h post parasitism. The emergence of parasitoids from the hosts was monitored. There was a significant difference in mean survival percentage of parasitoid larvae ($F=74.84$; df=2,24; $P=0.0001$) and adults ($F=93.88$; df=2,24; $P=0.0001$) among the treatments following virus infection at 72 h post parasitism (Figure 2). At 72 h post parasitism, the number of survivors was significantly lower for parasitoids emerging from recombinant virus-infected hosts than from wild type virus infected hosts and from uninfected controls. At 96 h post parasitism, the mean percentage of larval and adult survival showed no significant difference between the wild type and the recombinant virus treatment, but was significantly lower than for control treatments. The mean percentage parasitoid survival showed no significant difference between the virus treatments and the control for virus infection at 120 h post parasitism. For infection at an LC50 dose of virus, larval and adult parasitoid survival was not significantly different between the wild type virus and AcMLF9.ScathL treatments ($F=11.52$; df=2,8; $P=0.0044$) in contrast to infection at a high virus dose (>LC99). Data were collected from a total of 10-17 parasitoids per treatment at the LC50 dose. The timing of infection of the parasitized host did not affect parasitoid survival at the LC50 dose.

Our experiments showed no significant differences for mean time of larval ($F=1.35$; df=2,190; $P=0.2626$) and adult emergence ($F=1.15$; df=2,185; $P=0.3186$), or pupal period ($F=0.45$; df=2,185; $P=0.6398$) among the three treatments for infection at >LC99 (Table 1). Parasitoids that emerged from the recombinant virus-infected hosts were not significantly smaller as determined by head width than those from the wild type infected-hosts (Figure 4). Parasitoids emerging from the virus-treated hosts were significantly smaller than those emerging from uninfected larvae.
Fecundity. No significant difference was detected in fecundity between the parasitoids that emerged from the wild type virus infected hosts and recombinant virus-infected hosts (Table 2; F=5.0; df=2,29; P=0.0180).

Virus transmitted by parasitoids. Five and 3 % respectively of hosts exposed to parasitoids that had emerged from wild type and recombinant virus infected hosts died from virus infection rather than from parasitization (Table 2). The 488 bp-PCR product was detected in 33.33 % of the parasitoids that emerged from recombinant virus infected hosts (Figure 5). 25 and 29 parasitoids were analyzed for uninfected and AcMLF9.ScathL-infected treatments respectively. During the course of these experiments, we observed that parasitized larvae infected with AcMLF9.ScathL did not melanize (Figure 6).

Discussion

Choice tests. Parasitoids use cues to assess the quality of potential hosts (Godfray, 1994). They attack the hosts by inserting their ovipositor to obtain additional information about host suitability, after externally examining the host by stroking or drumming with their antennae (Godfray, 1994). Some studies suggest that virus infection may affect parasitoid ovipositional behavior. Kyei-Poku and Kunimi (1997) suggested that the parasitoid Cotesia kariyai might use sensory structures on the ovipositor to detect cues inside the host before making a decision to oviposit. In some cases, female parasitoids prefer to oviposit in uninfected hosts rather than virus infected hosts (Versoi & Yendol, 1982; Cabellero, 1991; Kyei-Poku & Kunimi, 1997; Hegazi & Abd Allah, 2004). However, in other cases no parasitoid preference was observed (Beegle & Oatman, 1975; Levin et al., 1983).

Harrison and Bonning (2001) noted that infection with AcMLF9.ScathL resulted in melanization of late instar larvae which might affect parasitoid ovipositional behavior. However, Boughton et al. (2003) observed that first and second instar larvae infected with AcMLF9.ScathL did not melanize completely prior to death, although they showed some dark mottling, indicating a low level of
melanization. Our data show no effect of AcMLF9.ScathL infection on parasitoid preference or ovipositional behavior. The parasitoids may not detect the low level of melanization or physiological alterations caused by AcMLF9.ScathL infection. The implication is that *C. marginiventris* will readily oviposit in hosts infected with AcMLF9.ScathL.

**Survival and development tests.** Host larvae infected by baculoviruses may die from virus infection before the parasitoids complete their development. Thus, the parasitoids may not survive (Brooks, 1993). Recombinant baculoviruses have been constructed to hasten the speed of kill relative to the wild type virus. McCutchen et al. (1996) showed that survival of parasitoids was not affected by recombinant baculoviruses expressing a scorpion-derived insect specific toxin (AcAalT) or JHE (AcJHE.KK), even though the recombinant baculoviruses killed larvae 30-40% faster than the wild type virus. Their results also showed that parasitoid larvae emerged earlier from AcAalT-infected hosts than from wild type virus infected hosts. These investigators suggested that the parasitoids might develop faster in response to declining nutrients in the host insect.

The survival time of AcMLF9.ScathL-infected larvae was reduced by 51% when compared to that of wild type virus infected larvae (Harrison & Bonning, 2001). In addition, AcMLF9.ScathL killed *H. virescens* approximately 30% faster than recombinant baculovirus expressing the scorpion toxin, LqhIT2 (Harrison & Bonning, 2001). The rapid death of hosts infected with recombinant baculoviruses may impact parasitoid survival. The results of our study showed that the larval and adult survival of the parasitoids was deleteriously impacted by the recombinant virus relative to the wild type virus when the hosts were infected 72 h post parasitism. Parasitoids did not survive if hosts were infected at less than 72 h post parasitism. In contrast to the results of McCutchen et al with AcAalT, there was no significant difference between the recombinant- and wild type virus treatment in time to larval or adult emergence. The low survival rate of *C. marginiventris* when hosts were infected 72 h post
parasitism may be caused by the inability of the parasitoids to complete their development within the AcMLF9.ScathL-infected hosts before the hosts died.

Kyei-Poku & Kunimi (1998) observed retarded growth of the parasitoid C. kariyai in entomopoxvirus infected larvae. They also found that the parasitoid died inside the virus infected host before host death. Dissection of the dead H. virescens infected with AcMLF9.ScathL at 72 h post parasitism indicated that the parasitoid larvae were still alive and had developed to second or third instar. These observations suggest that the protease does not harm the parasitoid directly, because the parasitoid larvae were still able to develop to the later instars. Our results indicate that AcMLF9.ScathL infection at 72 h post parasitism results in indirect adverse effects on parasitoid survival. However, when host larvae were infected at a low virus dose (LC50), we found no significant difference in larval and adult parasitoid survival between wild type virus and recombinant virus treatments. This result shows that AcMLF9.ScathL only poses increased risk to parasitoid survival relative to the wild type virus at high viral doses when infection occurred at 72 h post parasitism.

On oviposition, hymenopteran endoparasitoids inject polydnaviruses into the host insect (Shelby & Webb, 1999). Polydnaviruses inhibit the immune response of the host thereby allowing the parasitoid to develop within the host without detection and inactivation (Shelby & Webb, 1999). Host inactivation of invading organisms results from encapsulation with hemocytes and melanization (Lavine & Strand, 2002). Given that the protease ScathL induces melanization of AcMLF9.ScathL-infected larvae, it will be interesting to determine whether the presence of the polydnavirus alters the physiological effects induced by ScathL. If the presence of the polydnavirus reduces the impact of ScathL on the host insect, this may explain why no significant difference was detected in emergence times between wild type and recombinant virus infected hosts. Indeed parasitized larvae infected with AcMLF9.ScathL exhibited no melanization in contrast to unparasitized larvae. The
sex ratio and the size of F1 parasitoids may be affected by a recombinant baculovirus. Kunnalaca & Muller (1979) reported that C. marginiventris female development takes slightly longer at 25 °C, although the development times for both sexes were the same at 30 °C. Thus, the sex ratio of parasitoids may change if the virus kills the hosts before most females complete their development. However, we found no difference in the sex ratio of F1 parasitoids between treatments. McCutchen et al. (1996) also did not find significant differences in the sex ratio or in oviposition for parasitoids emerging from recombinant virus or wild type virus treatments at 27 °C. McCutchen et al. (1996) found that parasitoids that emerged from AcAalT- and AcJHE.KK-infected hosts were significantly smaller than those from the uninfected hosts, and that parasitoids developing in AcAalT-infected hosts were significantly smaller than those from wild type virus infected hosts. In contrast, the parasitoids that emerged from AcMLF9.ScathL-infected larvae were not significantly smaller than those from the wild type virus infected larvae. As the protease ScathL disrupts the basement membrane, it may increase the availability of nutrients for the developing parasitoid in the hemolymph. Hence, reduced resources available to the parasitoid as a result of virus infection may be countered in part by the activity of the protease. Indeed, larvae of the green lacewing, Chrysoperla carnea appeared to benefit from the increased availability of nutrients from the proteolytic action of ScathL in AcMLF9.ScathL-infected prey (Boughton et al., 2003).

**Fecundity tests.** The size of the adult female parasitoid may influence fitness and reproductive success by affecting searching efficiency, longevity, or egg production (Godfray, 1994). McCutchen et al. (1996) found that parasitoids that emerged significantly earlier from the recombinant virus infected larvae were smaller than for the uninfected control group. However, there was no difference in fecundity or percentage parasitized hosts among parasitoids emerging from recombinant virus infected hosts, from wild type virus infected hosts, and from uninfected hosts (McCutchen et al., 1996). Our results showed that there was no significant difference
in fecundity of F1 parasitoids emerging from wild type virus- and AcMLF9.ScathL-infected hosts. The protease ScathL expressed by AcMLF9.ScathL does not affect the reproductive success of female parasitoids that develop in infected hosts. Fewer F2 progeny were produced by parasitoids that emerged from virus infected larvae compared to the uninfected control larvae however. This may result from competition between the parasitoid and virus for resources, such that parasitoids from virus-infected larvae have fewer resources for production of progeny.

**Virus transmission.** Female parasitoids can carry baculoviruses on the ovipositor (Beegle & Oatman, 1975). Eller et al. (1988) reported that the parasitoid *M. croceipes* did not transmit HzSNPV from infected larvae to uninfected larvae via oviposition. They suggested that the inability of the parasitoids to transmit the virus was caused by the brief duration of oviposition and the short ovipositor. However, by using PCR McCutchen et al. (1996) recorded virus transmission by *M. croceipes* from wild type NPV, AcAalT, and AcJHE.KK treatments with 4.0, 10.6, and 8.3 % infection of the uninfected larvae. They also reported that approximately 40% of parasitoids that developed in AcAalT-infected hosts carried the virus (McCutchen, 1996). However, in a field experiment none of the parasitoids tested positive for viral DNA (Smith et al., 2000).

Our study showed that *C. marginiventris* transmitted virus to uninfected larvae. Virus was detected by PCR analysis in approximately 33.33 % of parasitoids that developed in AcMLF9.ScathL infected hosts. Five and 3 % of *H. virescens* larvae exposed to female *C. marginiventris* that developed from wild type and AcMLF9.ScathL treatments respectively died from virus infection rather than parasitism. In other cases, the virus dose transmitted by the parasitoids is assumed to have been sufficiently low that the parasitoid was able to outcompete the virus. The overall rate of virus transmission was not determined. These results confirm that parasitoids may carry and transmit recombinant baculoviruses to other host larvae.
Taken as a whole, our results indicate that use of AcMLF9.ScathL in insect pest management may pose a slightly greater risk to parasitoids at early stages of development than use of the wild type virus if host larvae are heavily infected. However, the degree of exposure of parasitoids under field conditions may not be as high as in this worst-case scenario study, such that the actual risk is likely to be negligible.

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**References**


Table 1. Development and sex ratio of *C. marginiventris* exposed to recombinant and wild type nucleopolyhedroviruses

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean time to larval emergence ± SD in hours (n)</th>
<th>Mean time to adult emergence ± SD in hours (n)</th>
<th>Mean pupal period ± SD in hours (n)</th>
<th>Sex Ratio (% Males) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (uninfected)</td>
<td>199 ± 3a (81)</td>
<td>308 ± 4a (81)</td>
<td>109 ± 2a (81)</td>
<td>35 a (81)</td>
</tr>
<tr>
<td>Recombinant AcMLF9.ScathL</td>
<td>195 ± 3a (52)</td>
<td>304 ± 4a (50)</td>
<td>108 ± 2a (50)</td>
<td>43 a (50)</td>
</tr>
<tr>
<td>Wild type AcMNPV C6</td>
<td>200 ± 3a (63)</td>
<td>310 ± 4a (60)</td>
<td>110 ± 2a (60)</td>
<td>36 a (60)</td>
</tr>
</tbody>
</table>

Numbers with the same letter within a column are not significantly different at the 5% significance level as determined by among treatment comparisons by using 1-way ANOVA with Tukey adjustment’s means separation. n, number of parasitoids
Table 2. Oviposition and virus transmission by F1 parasitoids that emerged from virus infected or uninfected hosts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of parasitoid females</th>
<th>% of exposed hosts from which F2 parasitoids emerged (n)</th>
<th>% of exposed hosts in which virus outcompeted parasitoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (uninfected)</td>
<td>13 (uninfected)</td>
<td>83 A (324)</td>
<td>0</td>
</tr>
<tr>
<td>Recombinant AcMLF9.ScathL</td>
<td>11 (347)</td>
<td>66 B (347)</td>
<td>3</td>
</tr>
<tr>
<td>Wild type AcMNPV C6</td>
<td>11 (293)</td>
<td>68 B (293)</td>
<td>5</td>
</tr>
</tbody>
</table>

Numbers with the same letter are not significantly different at the 5% significance level as determined by among treatment comparisons using 1-way ANOVA with Tukey adjustment's means separation.
**Figure Legends**

**Fig. 1.** Frequency of first attack in color (A) and color/treatment (B) choice tests for *C. marginiventris*. Data are based on 60 (A) and 61 (B) *C. marginiventris* attacks, pooled from three replicates. Columns with the same letter are not significantly different at the 5% significance level as determined by the chi-square test.

**Fig. 2.** Mean percentage survival of the larval (A) and adult (B) stage of *C. marginiventris* exposed to healthy hosts (control) or hosts infected at a high dose (>LC99) of wild type virus or AcMLF9.ScathL, at 72, 96, or 120 hours post parasitism. Columns with the same letter are not significantly different at the 5% significance level as determined by 1-way ANOVA and the Tukey means separation test. Data were collected from 4 replicates with a total of 26-32 parasitoids per treatment.

**Fig. 3.** Mean percentage survival of larval (A) and adult (B) stage of *C. marginiventris* emerged from healthy hosts (control) or hosts infected at a low dose (LC50) of wild type virus or AcMLF9.ScathL, at 72, 96, or 120 hours post parasitism. Columns with the same letter are not significantly different at the 5% significance level as determined by 1-way ANOVA and Tukey means separation test. Data were collected from 2 replicates with a total of 10-17 parasitoids per treatment.

**Fig. 4.** Mean head width (mm) of F1 adults of *C. marginiventris* surviving from hosts infected with wild type or recombinant virus. F1 adults from recombinant virus-infected hosts were significantly smaller than for the control group (p=0.05). Columns with the same letter are not significantly different at the 5% significance level as determined by among treatment comparisons using 1-way ANOVA with Tukey’s means separation test. Data were pooled from 4 replicates with a total of 43-76 parasitoids per treatment.

**Fig 5.** Analysis of F1 adults of *C. marginiventris* for the presence of AcMLF9.ScathL by PCR. Lane 1, 1 kb ladder. Lane 2, positive control. Lanes 3-5, 3
individual parasitoids from uninfected larvae. Lanes 6-8, 3 individual parasitoids from larvae infected with AcMLF9.ScathL. The 488 bp PCR product (arrow) was detected in 33.33% of parasitoids that developed in AcMLF9.ScathL-infected hosts.

**Fig 6.** Effect of parasitism on cuticular melanization of AcMLF9.ScathL-infected larvae. Nonparasitized (A-C) and parasitized (D-F) larvae of *H. virescens*: uninfected (A, D), infected with wild type AcMNPV C6 (B, E), or AcMLF9.ScathL (C, F). Note the absence of cuticular melanization in F relative to C. Larvae were infected at fourth instar and photographed 4 days post infection. Bars: 3.5 mm.
Fig. 1
Fig. 2

A

![Bar chart showing mean larval survival (percentage) over infection time (hours post parasitism).](image)

B

![Bar chart showing mean adult survival (percentage) over infection time (hours post parasitism).](image)
**Fig 3.**

A. Mean larval survival (control, AcMNPV C6, AcMLF9.ScathL) over infection time (hours post parasitism).

B. Mean adult survival (control, AcMNPV C6, AcMLF9.ScathL) over infection time (hours post parasitism).
Fig. 4
Fig. 5
CHAPTER 3. GENERAL CONCLUSIONS

Summary

The recombinant baculovirus AcMLF9.ScathL reduced the larval and adult survival of the parasitoid *Cotesia marginiventris* compared to the wild type virus when the hosts were infected at >LC99 at 72 hour post parasitism. The survival of the parasitoid was not significantly different between the wild type virus and recombinant virus treatments at 96- and 120 h post parasitism. At any infection time there were no significant differences between the wild type virus treatment and recombinant virus treatment in parasitoid development time, sex ratio, size, or fecundity. Parasitoids did not discriminate between uninfected hosts, wild type virus-infected hosts, and AcMLF9.ScathL-infected hosts. Some parasitoids that developed within diseased hosts tested positive for virus and transmitted virus to healthy larvae. This study shows that there is slightly increased risk with use of AcMLF9.ScathL at >LC99 over use of wild type virus, with significantly lower survival of parasitoids infected at 72 h post parasitism. Our studies confirm the potential of parasitoids to transmit recombinant baculoviruses to other host larvae.

Further Research

Our laboratory study to evaluate the risks posed by a recombinant baculovirus expressing ScathL may not reflect events in the field. Indeed, larvae were infected with a high baculovirus dose (>LC99) for assessment of potential risks in the worst-case scenario. Under field conditions, various factors would result in a much lower infection rate of host larvae. For example, phytochemicals produced by plants may inhibit the infectivity of baculoviruses against the tobacco budworm, *Heliothis virescens* (Hoover et al., 1998; Ali et al., 1999). Further research could be conducted to determine whether a high dose of AcMLF9.ScathL would pose the same risk to the parasitoid at 72 h post parasitism under field condition. Our results
with infection at a low dose (LC50) at 72 h post parasitism indicated that AcMLF9.ScathL will not pose a greater risk than wild type virus to parasitoid survival. Parasitoids developing in hosts infected with a low dose (LC50) of AcMLF9.ScathL had a higher survival rate when infected at 72 h post parasitism than treatment with a high dose (>LC99) of virus. The higher the virus dose, the faster the speed of kill of the host larva. By using different virus concentrations, it may be possible to determine a virus dose that is effective for the control of pests but that poses minimal risk to parasitoids.

The mode of insecticidal action of AcMLF9.ScathL is under investigation. The protease ScathL activates prophenoloxidase either directly, or indirectly, causing melanization of the host larva. The phenoloxidase cascade is an important component of the insect immune system. Relating to the study of the mode of action of ScathL and to the parasitoid research described here, polydnaviruses, a group of mutualistic viruses in some ichneumonid and braconid wasps, disrupt the immune system of the host insect (Trudeau & Strand, 1998; Shelby & Webb, 1999). Campoletis sonorensis polydnavirus reduces the plasma melanization of the host (Shelby & Webb, 1999). Does the presence of the Cotesia marginiventris polydnavirus alter the insecticidal action of ScathL? Our observations indicate that parasitized, AcMLF9.ScathL-infected larvae exhibited less melanization than non-parasitized AcMLF9.ScathL-infected larvae. Further research will be done using specific polydnavirus genes to examine the interaction between the protease ScathL, melanization, and the immune system. The results of this research may provide a better understanding of the potential impact of the protease ScathL on the host immune system and on parasitoid survival.

Given that the protease ScathL disrupts the basement membrane, it may increase the availability of nutrients to the parasitoid, which is bathed in the hemolymph. We did not observe any decrease in development time, or increase in head width which might occur with increased nutrition. However, increased nutrition
may have been offset by competition with the virus for resources, which may explain the difference between our results and those of McCutchen et al. (1996) for AcAalT. Infection with AcAalT resulted in shorter development time and smaller head width of parasitoids that developed in AcAalT infected hosts compared to those from wild type virus infected hosts (McCutchen et al., 1996).

The total rate of virus transmission was not determined in our study. The use of PCR for detection of virus in host larvae would provide a sensitive method for determining the overall rate of mechanical transmission of virus.

Field testing is needed to determine the potential use of AcMLF9.ScathL as an insecticide. Before a permit for field testing is provided, the US EPA requires information regarding: identity of the microorganism, description of the natural habitat of the parental strain, information on the host range with an assessment of infectivity and pathogenicity to nontarget organisms, information on the survival and the ability of the microbial pesticide to replicate in the environment, identity of possible transmission vectors, data on the relative environmental competitiveness compared to the parental strain, description of genetic modification methods, data on potential for genetic exchange and on genetic stability of the inserted sequences, a description of the proposed field program, and a statement of composition for the formulation to be tested (Black et al., 1997). Thus, further research would be needed to examine the competitiveness of AcMLF9.ScathL versus the wild type virus, the environmental persistence of AcMLF9.ScathL and protease ScathL, and the possibility of genetic exchange with other viruses.

**General Discussion**

Before field trials can be conducted, the US EPA requires testing to evaluate the potential risks posed by recombinant baculoviruses to nontarget organisms in the environment. Evaluation of the potential risks associated with recombinant organisms includes risk to other organisms and the fate of the recombinant material
(Inceoglu et al., 2001). At the organism level, several studies concluded that baculoviruses are safe for use as pest control agents (Bonning et al., 2002). Baculoviruses do not replicate in mammals or plants and are not toxic or allergenic to mammals, birds, or fish (Groner, 1990). The insertion of a foreign gene expressing an insect-selective toxin, hormone, or enzyme will not change the safety of the virus to nontarget vertebrates (Bonning et al., 2002). Genetic engineering is not expected to alter the host range of the parent baculovirus. Genetic modification of AcMNPV C6 to produce AcMLF9.ScathL did not disrupt any of the baculovirus genes that play a role in determining the host range of the virus. In addition, the use of p6.9 promoter to drive expression of ScathL ensures that the protease will not be expressed in non-permissive insects (Miller and Lu, 1997).

The environmental fate of wild type and recombinant baculoviruses has been discussed in several reviews (Cory & Entwistle, 1990; Moscardi, 1999; Inceoglu et al, 2001; Cory & Myers, 2003). Baculoviruses can be transmitted horizontally through ingestion of polyhedra by susceptible hosts and vertically from adults to young (Cory & Myers, 2003). Biotic factors such as parasitoids, scavengers and predators, may also disseminate baculoviruses in the environment (Hunter-Fujita, 1998; Lee and Fuxa, 2000). In laboratory studies, predators and parasitoids transmitted AcMLF9.ScathL to healthy larvae, and are likely to disseminate the recombinant virus in the environment.

The behavior of baculovirus infected larvae can enhance the spread of wild type baculoviruses in the environment. Before death, wild type virus-infected larvae climb to the top of vegetation and the cuticle readily ruptures to release the polyhedra into the environment (Federici, 1997). Larvae infected with fast-acting recombinant baculoviruses do not exhibit this climbing behavior and do not rupture easily. In addition, unlike wild type virus-infected larvae, which may melanize after death, larvae infected with AcMLF9.ScathL melanize prior to death. Dissemination of
the virus in the environment may be further inhibited by the melanized cuticle of AcMLF9.ScathL-infected larvae.

Occlusion bodies of baculoviruses can persist for many years in the soil after an epizootic outbreak (Black et al., 1999). A question that remains to be answered relates to the ability of the recombinant baculovirus AcMLF9.ScathL to persist in the environment. The reduction in ST50 of recombinant virus infected larvae results in fewer cycles of baculovirus replication and a reduced number of virus progeny such that they are readily outcompeted by wild type virus (Black et al., 1999). AcMLF9.ScathL produces fewer polyhedra per larva than any other recombinant baculovirus developed to date and hence would be rapidly outcompeted by the wild type virus in the environment.

Two issues must be considered when evaluating the potential risk of movement of a transgene from a recombinant baculovirus into another organism: the likelihood of a genetic exchange between the recombinant baculovirus and another organism and the consequences of such an event (Black et al., 1997). The genetic exchange requires a shared location of replication between the donor and the recipient (Black et al., 1997). The most likely recipient for the exchange of genetic material is another virus that replicates within the nucleus of the host insect cell (Black et al., 1997). Even if two viruses share the host and the same replication compartment, differences in the mode of replication, the composition of the viral genome, and the degree of homology between the two viruses can limit the exchange of genetic information (Black et al., 1997). These barriers limit the likelihood of genetic exchange. As a consequence of possible genetic exchange, new genetic traits become fixed in the population only if they provide a selective advantage to the species (Black et al. 1997). Insertion of the ScathL gene increases the speed of kill of the baculovirus, thereby decreasing the fitness of the recombinant baculovirus. The decreased environmental fitness of AcMLF9.ScathL would be a strong selective disadvantage for its persistence in the environment.
The genetic engineering of AcMNPV C6 to produce AcMLF9.ScathL is not expected to change the host range of the virus or the safety to mammals and plants. The slightly increased risk to parasitoids relative to the wild type virus is not likely to be significant under field conditions. Field trials have been conducted recently in China to test the potential of recombinant *Helicoverpa armigera* NPV that expresses ScathL for control of pests on cotton. Hence, baculovirus-expressed ScathL may be applied for pest management in other countries. The ScathL gene may also be used in transgenic plants when combined with an appropriate system to deliver the protease to its target, the basement membrane. In conclusion, ScathL delivered by a virus or a transgenic plant, may be employed as an environmentally benign alternative to synthetic chemical insecticides for insect pest management.

References


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