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Recent Advances in Our Knowledge of Baculovirus Molecular Biology and Its Relevance for the Registration of Baculovirus-Based Products for Insect Pest Population Control

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1. Introduction

Public demand for safer, environmentally-benign alternatives to synthetic chemical pesticides and more stringent barriers put in place by regulatory agencies worldwide has led to increased interest in microbial pest control agents (MPCA) based on viruses, bacteria, fungi, protozoa and nematodes as the active ingredient. The MPCA market has recently experienced an increase of 47% between 2004/2007 with sales worth $396 million in 2007/2008 (CPL Consultants, 2010). Despite this increase, the microbial biopesticide market still only represents about 1% of the sales of chemical pesticides (CPL Consultants, 2010; Marrone, 2007). Factors impeding the establishment of strong MPCA markets are complex (Chandler et al., 2011; Marrone, 2007; Ravensberg, 2011) but include the burdensome costs associated with the registration of commercial products that are aimed at relatively small niche markets (Chandler et al., 2011; Ehlers, 2011). The main priority of regulatory agencies is to protect human health and safety and the environment from potential risks associated with the use of pest control products. A common feature of MPCA registration processes around the world is that they grew out of registration processes designed for chemical pesticides with adjustments allowing for the reduced risks of MCPAs (Chandler et al., 2011; Ehlers, 2011). Even though public attitudes to the use of biological control agents has been favourable (63%) a large proportion of the public (46%) has expressed concerns about the consumption of food treated with microbial pesticides (Cuddleford, 2006).

Baculoviruses only infect insects, are ubiquitous in the environment and are known to be important in the regulation of many insect populations. Baculoviruses are host specific, infecting only one or a few closely-related species, helping to make them good candidates for management of crop and forest insect pests with minimal off-target impacts (Cory, 2003; England et al., 2004; Hewson et al., 2011; Raymond et al., 2005). In fact, baculoviruses have been recognized as being amongst the safest of pesticides (Black et al., 1997; Gröner, 1986)

and have been included on lists of "low risk" biocontrol agents by the European Union (Leuschner, 2010; Regulation of Biological Control Agents in Europe; http://www.rebeca-net.de). Since the start of their commercial use, baculoviruses have been tested extensively to assess their safety in order to meet registration requirements (reviewed in Burges et al., 1980a, 1980b; Gröner, 1986; Ignoffo, 1973). As of 2010, over 24 baculovirus species have been reported to be registered for use in insect pest management throughout the world (Kabaluk et al., 2010; Quinlan & Gill, 2006). Market share of baculoviruses is 6% of all microbial pesticides (Marrone, 2007; Quinlan & Gill, 2006) and millions of hectares have been treated with registered baculovirus products over the years (Kabaluk et al., 2010; Moscardi, 2011; Szewczyk et al., 2009). Despite many years of use and testing against nontarget organisms, no adverse effects have ever been attributed to baculoviruses (McWilliam, 2007; OECD, 2002). In this review of baculoviruses, we discuss how baculovirus evolution, host range determination and pathogenesis have contributed to their inherent safety for nontarget organisms including humans.

2. Classification and origins of baculoviruses

The virus family *Baculoviridae* is divided into four genera that are restricted to three insect orders: *Alphabaculovirus* (nucleopolyhedrovirus or NPV) in Lepidoptera, *Betabaculovirus* (granulovirus or GV) also in Lepidoptera, *Gammabaculovirus* (NPV) in Hymenoptera, and *Deltabaculovirus* (NPV) in Diptera (Jehle et al., 2006). The large, covalently-closed, double-stranded DNA genome is packaged into an enveloped, rod-shaped capsid. The virions of NPVs are enveloped either singly (SNPV) or in groups of multiple virions (MNPV) which are then occluded in a protein called polyhedrin to form the occlusion body (OB). Virions of GVs are enveloped and occluded singly in an ovicylindrical granule (also an OB) formed from the protein granulin. Baculoviruses are normally named for the initial host from which they were first isolated. The International Committee on Taxonomy of Viruses (ICTV) lists the designated members of the Baculoviridae (http://www.ictvdb.org/Ictv/index.htm).

To date, 58 baculovirus genomes have been sequenced; 41 alphabaculoviruses, 13 betabaculoviruses, three gammabaculoviruses and one deltabaculovirus. Baculovirus genome sizes range from the smallest gammabaculovirus, *Neodiprion lecontei* nucleopolyhedrovirus (NeleNPV), at 81,755 bp (Lauzon et al., 2004) to the largest betabaculovirus, *Xestia c-nigrum* granulovirus (XcGV) at 178,733 bp (Hayakawa et al., 1999). No matter the genus or genome size, all baculoviruses share 31 core genes in common (Miele et al., 2011). These are essential genes involved in oral infection (*pif-0 (p74), pif-1*, *pif-2*, *pif-3*, *pif-4/19kd/odv-e28*, *pif-5/ odv-e56*), cell cycle arrest (*odv-ec27*, *ac81*), replication (*dnapol*, *helicase*, *lef-1*, *lef-2*), late gene transcription (*lef-4*, *lef-5*, *lef-8*, *lef-9*, *p47*) and virus assembly, packaging and release (*38k/ac98*, *alk-exo*, *desmoplakin*, *gp41*, *odv-e18*, *odv-nc42*, *odv-ec43*, *p6.9*, *p33/ac92*, *p49*, *vlf-1*, *vp39*, *vp91*, *vp1054*) (Miele *et al*. 2011). Twenty of these core genes (*p47*, *lef-4*, *lef-5*, *lef-8*, *lef-9*, *vlf-1*, *pif-0*, *pif-1*, *pif-2*, *pif-3*, *pif-4*, *pif-5*, *vp91*, *vp39*, *38k*, *ac68*, *ac81*, *p33*, *dnapol*, *helicase*) are also found in insect dsDNA viruses belonging to the genus *Nudivirus* (Wang et al., 2011) and a number (e.g., *lef-4*, *lef-5*, *lef-8*, *p47*, *38k/ac98*, *vp91*, *pif-0*, *pif-1*, *pif-2*, *pif-3*) are also found in bracoviruses (polydnaviruses) associated with parasitic wasps belonging to the family Braconidae (Bézier et al., 2009). It has been suggested that bracoviruses arose from the insertion of a nudivirus ancestor into braconid wasps about 100 million years ago (mya) (Bézier et al., 2009). Nudiviruses and baculoviruses, however, are

thought to have shared a common ancestral virus (Wang & Jehle, 2009). Deltabaculoviruses and gammabaculoviruses are thought to be more primitive than the alphabaculoviruses and betabaculoviruses because of their smaller genomes and tissue tropism which is limited to midgut epithelial cells (Lauzon et al., 2004) and, in the case of deltabaculoviruses, cells of the posterior midgut and gastic caeca (Moser et al., 2001). Gammabaculoviruses, however, are thought to be more closely (although still distantly) related to the alphabaculoviruses and betabaculoviruses than are the deltabaculoviruses (Herniou et al., 2004). The virions of *Culex nigripalpus* deltabaculovirus (CuniNPV) are occluded in a 90-kDa protein that bears no similarity to polyhedrin/granulin proteins or any other protein in available sequence databases (Perera et al., 2006).

The occlusion derived virions (ODVs) that emerge from OBs are the universal virion phenotype for all baculoviruses as they are responsible for the initial oral (*per os*) infection of host insect gut cells. In lepidopteran hosts, the initial, primary infection of midgut cells by ODVs is followed by secondary infection of tissues within the insect hemocoel that is effected by the budded virion (BV) phenotype. The genome content of ODVs and BVs is identical but differences in virion morphology, structural proteins, envelopes, antigenicity, and cellular site of maturation are the basis for their respective patterns of infectivity. In mosquito hosts, following the primary infection of the gastric caeca and posterior midgut by ODVs, deltabaculovirus BVs also spread the infection further from cell to cell but only to these same tissues (Moser et al., 2001). Sawfly gammabaculoviruses do not appear to have a BV phenotype (Duffy et al., 2006; Garcia-Maruniak et al., 2004; Lauzon et al., 2004) and OBs are only produced in the nuclei of midgut epithelial cells (Federici, 1997).

In Lepidoptera, NPVs have been reported from 28 families and GVs from 19 families (Martignoni & Iwai, 1981). In the Diptera, NPVs have been reported from the Calliphoridae, Chironomidae, Culicidae, Sciaridae, Tachinidae and Tipulidae (Martignoni & Iwai, 1981). Fewer families of sawflies (Argidae, Diprionidae, Pamphiliidae and Tenthredinidae) are reported to be infected by NPVs (Martignoni & Iwai, 1981). However, due to a general lack of viral isolates, sequence data and other information, most of the baculoviruses listed by Martignoni & Iwai (1981) are not yet considered as valid species by ICTV (http://www.ictvdb.org/Ictv/index.htm). For example, the NPV of the pamphiliidid sawfly, *Acantholyda erythrocephala,* has been reported to occur in the fat body (Jahn, 1967), something which is not characteristic of gammabaculoviruses.

Recent phylogenetic analyses have indicated that the Hymenoptera, not the Coleoptera, are basal to the holometabolous insects that also include the Lepidoptera and Diptera (Savard et al., 2006). When and how the four genera of baculoviruses came to infect their different insect hosts is not known but selection pressure and co-evolution with their respective hosts appears to have constrained each baculovirus genus to a single insect order (Herniou et al., 2004). Historically, the Hymenoptera have been subdivided into the more advanced Apocrita, including ants, bees and wasps, and the basal Symphyta that includes the sawflies. It now appears, however, that the evolution from the ancestral hymenopteran to the Euhymenoptera (Apocrita and Orussoidea) was monophylletic and that the different superfamilies of sawflies constitute separate branches off the lower end of this lineage (Farris & Schulmeister, 2011). In this light, gammabaculoviruses have only been confirmed in the Diprionidae and, considering the paraphyletic origins of the different groups of

sawflies, it may be the case that gammabaculoviruses are restricted to the Diprionidae or closely related families within the superfamily, Tenthredinoidea.

3. Baculovirus pathogenesis and potential blocks to infection

As is the case for all viral pathogens, baculovirus replication is dependent upon the availability of permissive host cells. The accessiblity and susceptibility of host cells to viral invasion and replication is classified into three categories; permissive, semi-permissive and non-permissive. A permissive infection results in successful viral replication and subsequent production of infectious virions that can transmit the infection to other permissive cells and individuals. Semi-permissive infections result in limited viral progeny resulting from defects in some replication events, such as gene expression or viral DNA replication. In non-permissive infections, cells do not support viral replication and the process does not yield infectious progeny. Determining what factors influence the level of permissiveness of an insect cell to a particular baculovirus has proven to be challenging because baculovirus host range is affected not only by the interactions between the baculovirus and the host cell at the molecular level (reviewed in Miller & Lu, 1997; Thiem & Cheng, 2009) but also by aspects of insect behaviour and physiology (reviewed in Cory & Hoover, 2006).

As hosts for viruses, insects can present challenges because of their sporadic and/or episodic availability and their relatively short life spans. Long periodicity of population fluctuations, for example the spruce budworm (*Choristoneura fumiferana*) which can span over 30 years (Royama, 1992), indicates that baculoviruses must be able to persist in the environment for long periods while waiting for permissive hosts to become available. The OB and its surrounding polyhedral envelope (PE) (a protein/carbohydrate matrix) (Gross & Rohrmann, 1993; Gross et al., 1994; Russell & Rohrmann, 1990) help protect the ODVs from degradation by such environmental factors as dessication and ultraviolet radiation (UV) (reviewed in Slack & Arif, 2007). OBs and ODVs can be further protected from UV radiation by establishing natural reservoirs in sheltered environments such as those in and on plants and in soil (Raymond et al., 2005; Witt, 1984).

3.1 Midgut lumen and pH

Baculoviruses are predominantly diseases of the larval stages of insects. When a larval host consumes foliage or water that is contaminated with OBs, the alkaline pH (8-11) of the larval midgut (Fig. 1) dissolve the PE and OB matrix within minutes (Adams & McClintock, 1991) releasing ODVs into the midgut lumen. The gut environment, into which OBs enter, is a first deciding specificity factor as OBs will only dissolve in an alkaline environment. The dissolution of OBs is further facilitated by OB-associated alkaline proteases. While the PE lattice is sufficiently narrow to restrict access by large digestive enzymes of vertebrates, it does allow infiltration by anions from the alkaline midgut of insects. Midgut pH of Lepidoptera averages 10.5, while within the Diptera, only in the Culicidae does the gut pH reach 10 (Terra et al., 1996). Within the Hymenoptera, only sawflies are known to harbor baculoviruses and their midgut pH, although lower than those of lepidopteran and culicid mosquitoes, is between 6.7 and 8.7 (Heimpel, 1955), which is more alkaline than that of bees and wasps (Apidae) at pH 5.7 (Terra et al., 1996). When compared to the midgut of other coleopteran families, such as the Coccinelidae, Chrysomelidae and Cerambycidae (midgut

pH 5.4 to 6.9), the midgut pH of Scarabeidae is 10.4 (Terra et al., 1996). Although not a baculovirus *per se*, the nudivirus of the scarab *Oryctes rhinoceros* (OrN) shares homologies with baculoviruses *per os* factors and other core genes (Wang et al., 2011). Although the nudiviruses have established more complex tissue tropisms and transmission routes than baculoviruses, their primary route of infection is also *per os* (Wang & Jehle, 2009).

In addition to protecting the ODVs against environmental factors, the stability of the crystalline structure of the OBs has been shown to assist in the dispersal of the virus by vertebrates. The acidic pH of the stomachs (from pH 1 to 7) of vertebrates (Fig. 1) helps to preserve the integrity of the OBs. Excreted OBs, recovered from the digestive tracts of non-host invertebrate and vertebrate animals (Lautenschlager et al., 1980; Vasconcelos et al., 1996) were found to remain infectious to their insect larval hosts, leading to the suggestion that the consumption of baculovirus-infected larvae by various non-target animals plays a role in the dissemination of OBs (Entwistle et al., 1977; Lautenschlager et al., 1980).

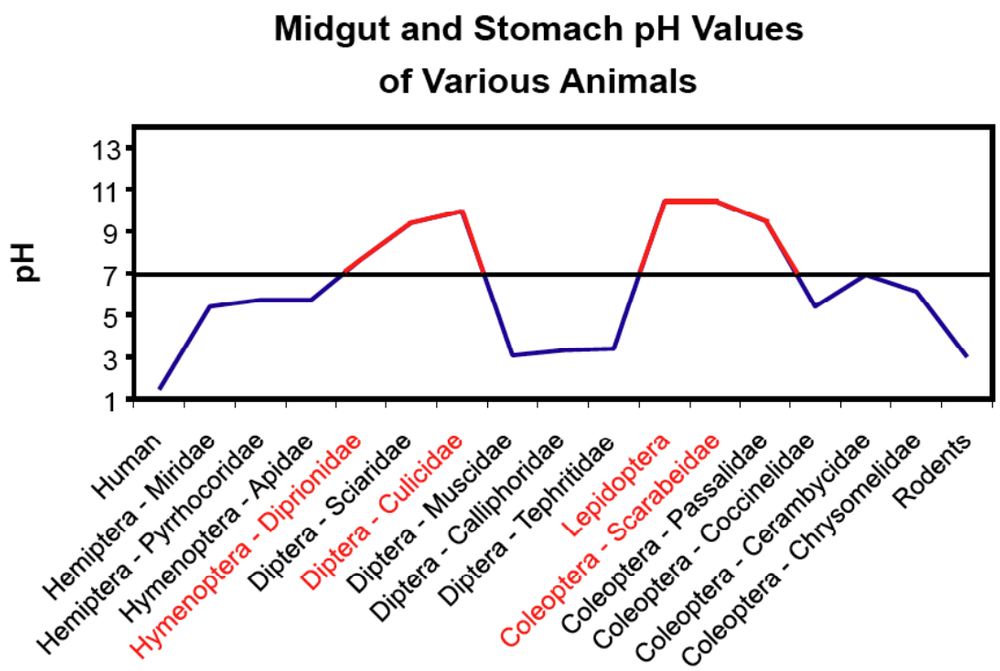

Fig. 1. Midgut and stomach pH values of various organisms. Red line shows alkaline values and blue line, acidic values. Insect orders and families that have been identified as baculovirus or nudivirus hosts are labeled in red. pH value of midguts of Hemiptera, Hymenoptera (Apidae), Diptera, Lepidoptera and Coleoptera (Terra et al., 1996), pH value of Hymenoptera (Diprionidae) (Heimpel, 1955) and pH values for stomach of human (Guyton & Hall, 2006) and rodents (McConnell et al., 2008).

3.2 Peritrophic membrane

The peritrophic membrane (PM) of insects is an acellular sleeve-like structure that lines and protects the gut. In insects, the PM consists of chitin sheets, proteoglycans and chitin-binding proteins such as peritrophins and intestinal mucins, that cross-link to form a thick, three-dimensional mesh (Barbehenn & Martin, 1995; Lehane, 1997; Peters, 1992; Tellam et al.,

1999). The chitin sheets provide the PM with strength and flexibility and the hydrating capacity of the proteoglycans is implicated in determining the permeability of the PM (Lehane, 1997). The pores, ranging from 21 to 36 nm (Barbehenn & Martin, 1995), allow bidirectional trafficking of small molecules such as digestive enzymes and the regulation of the flow of hydrolytic products (water and nutrients) between endoperitrophic and ectoperitrophic spaces (reviewed in Lehane, 1997). Insect intestinal mucins (IIM) consist of potentially-glycosylated, mucin-like domains and cysteine-rich chitin-binding domains (CBD) (Sarauer et al., 2003; Wang & Granados, 1997a), which are thought to have a similar lubricating and protective role as vertebrate mucins (Sarauer et al., 2003; Tellam et al., 1999; Wang & Granados, 1997a).

The small pore size of the PM limits the passage of larger materials such as pathogens, toxins and noxious phytochemicals (e.g. tannins) that are also ingested into the endoperitrophic space during feeding (Barbehenn, 2001). The size of baculovirus nucleocapsids, being between 40–70 nm in diameter and 250–400 nm in length (Boucias & Pendland, 1998), precludes them from crossing the PM. Disruptions in the links between PM chitin sheets and proteins can result in a decrease in the ability of the PM to act as a barrier to pathogens (Plymale et al., 2008; Wang & Granados, 1997b; 2000). ODVs passively cross the PM through physical breaches caused by mechanical abrasions, chemical means (Wang & Granados, 2000) or possibly by natural degradation of the PM. Diet has also been shown to affect the PM thickness. When fed cotton foliage rather than artificial diet, tobacco budworm larvae (*Heliothis virescens*) were shown to form a thicker PM that, by decreasing the number of primary infection foci on the midgut epithelium (Plymale et al., 2008), led to lower susceptibility to *Autographa californica* MNPV (AcMNPV).

An active mechanism of disruption of the PM has evolved in many GVs and a few lepidopteran group II NPVs whose genomes encode a metalloprotease called enhancin. Enhancins are occluded in the OB matrix either inside or on the surface of ODVs (Hashimoto et al., 1991; Lepore et al., 1996; Slavicek & Popham, 2005; Wang et al., 1994). In *Trichoplusia ni*, TnGV ODV enhancin results in disruption of the PM through the degradation of IIM structures (Derksen & Granados, 1988; Hashimoto et al., 1991; Wang & Granados, 1997b). LdMNPV enhancins are located on the ODV surface and were found to be essential for *per os* infectivity of *Lymantria dispar* larvae even when the PM was absent suggesting that enhancins may play a role in addition to PM breaching (Hoover et al., 2010).

Homologues of 11K protein are conserved in most baculoviruses and can be present in multiple copies (Dall et al., 2001). These proteins are characterized by the presence of C_6 chitin-binding motifs or peritrophin-A domains that are also present in chitin binding proteins such as mucins, peritrophins and chitinases of insect guts and basal laminae of trachea (Dall et al., 2001; Tellam et al., 1999). The AcMNPV 11K proteins were found to associate with BVs and OBs (Lapointe et al., 2004) but not with the ODVs themselves (Braunagel et al., 2003; Zhang et al., 2005) and were shown to enhance oral infection (Lapointe et al., 2004; Zang et al., 2005) but not hemocoelic infection. Although the function of Ac150 and Ac145 is not yet known (Lapointe et al., 2004; Zhang et al., 2005), they are important mediators of primary infection where they impact infectivity differentially in two different hosts of AcMNPV, *T. ni* and *H. virescens* (Lapointe et al., 2004).

3.3 Entry in midgut cells

The midgut tissue consists principally of midgut epithelial cells that are prone to apoptosis (Uwo et al., 2002) and slough off regularly (Engelhard & Volkman, 1995) which, when considering the obligate nature of a viral infection, makes them a sub-optimal environment for viral replication. The process of midgut epithelial cell infection is carried out by ODVs. Comparatively, BVs are 10,000 fold less efficient than ODVs at infecting midgut cells (Volkman & Summers, 1977; Volkman et al., 1976). On the other hand, AcMNPV ODVs have been shown not to infect or even penetrate insect tissue culture cells (Volkman & Summers, 1977; Volkman et al., 1976). In a cell culture assays, 2.3 x 10^5 ODVs were shown to be required to infect a single *T. ni* cell (TN-368) making them 1700 to 1900 times less infectious than BVs (Volkman et al., 1976). ODV infectivity to TN-368 cells was improved, however, in the presence of midgut juices from *Heliothis zea* and *Estigmene acrea* larvae (Elam et al., 1990), indicating that midgut lumen factors are important to the primary infection process.

Due to the complexity of *in vivo* studies and the lack of midgut cell lines that would simplify studies of ODV host cell entry, the exact mechanisms directing the primary infection processes are still relatively unclear. Once across the PM, ODVs bind to ODV-specific receptors and fuse to the brush border microvilli of the columnar epithelial cells (Haas-Stapleton et al., 2004; Horton & Burand, 1993). Nucleocapsids are then released into the cell cytoplasm to initiate primary infection of the midgut epithelium (reviewed in Slack & Arif, 2007). ODV-associated proteins of both NPVs and GVs are numerous (Braunagel et al., 2003; Wang et al., 2011) and include structural proteins responsible for encapsidation, unique ODV-envelope proteins involved in tissue tropism and proteins involved in viral gene expression and DNA replication (Braunagel et al., 2003). The ODVs unique association with proteins involved in viral gene expression and DNA replication is thought to optimize viral replication in midgut cells. Over 40 proteins were found to be associated with the ODVs of the alphabaculovirus AcMNPV (Braunagel et al., 2003), the betabaculovirus *Pieris rapae* GV (Wang et al., 2011) and the deltabaculovirus CuniNPV (Perera et al., 2007). Most of the proteins found in both AcMNPV ODVs and BVs are nucleocapsid proteins while most of the tissue-specific envelope proteins are different for each phenotype (Braunagel et al., 2003; Wang et al., 2010).

ODV-envelope proteins are highly complex and over 10 envelope proteins have been found to be associated with the ODVs of AcMNPV (reviewed in Slack & Arif, 2007; Braunagel & Summers, 2007). Only few of these ODV proteins, termed the *per os* infectivity factors (PIFs), have been shown to be essential for the AcMNPV *per os* infection process (Braunagel et al, 1996; Fang et al., 2009; Faulkner et al., 1997; Kikhno et al., 2002; Pijlman et al., 2003; Ohkawa et al., 2005; Sparks et al., 2011; Xiang et al., 2011) but more are expected to be identified by *in vivo* infectivity assays. Recently, three of these PIFs have been shown to form a very stable core complex (PIF-1, PIF-2 and PIF-3), held together by non-covalent bonds, that contributes to efficient entry and nucleocapsid delivery into midgut cells (Peng et al., 2010). The lepidopteran midgut is rich in trypsin and chymotrypsin (Johnston et al., 1995; Terra et al., 1996) and it has been suggested that the tight conformation of this PIF-complex might protect the active, internal PIF domains from the harsh chemical conditions of the midgut. In contrast, P74 (PIF-0) is only loosely associated with the core PIF-complex and actually requires the midgut environment to undergo functional activation by protease cleavage (Slack et al., 2008) by an ODV-associated host alkaline protease and by a host midgut trypsin

(Peng et al., 2011). Binding of ODVs to the tip of the midgut microvilli most likely occurs through a protein receptor binding mechanism (Horton & Burand, 1993; Yao et al., 2004) that relies upon PIF-0, PIF-1 and PIF-2 (Haas-Stapleton et al., 2004; Ohkawa et al., 2005). Supporting ODV adaptation to the midgut environment, binding efficiency has been shown to be optimal at alkaline pH (Horton & Burand, 1993).

Studies suggest that ODV binding proteins from different viruses interact with cell receptors that are specific to different virus/host systems (Haas-Stapleton et al., 2003, 2004, 2005, Horton & Burand, 1993; Ohkawa et al., 2005). Larvae of the fall armyworm *Spodoptera frugiperda* are highly resistant to oral infection by AcMNPV but are susceptible to infection by BVs when injected directly into the hemocoel (Haas-Stapleton et al., 2003). The resistance to *per os* infection is due to a lower level of ODV binding to the midgut cells (Haas-Stapleton et al., 2005) indicating that ODV interaction with specific midgut receptors is necessary for productive primary infection of midgut columnar epithelial cells. In addition, co-inoculation studies demonstrated that AcMNPV did not compete with SfMNPV for receptor binding indicating further that midgut receptor specificity is important for baculovirus host range determination.

As early as 30 min after BVs enter the cell, AcMNPV infection stimulates the formation of filamentous F-actin cables (Charlton & Volkman, 1993) and nucleocapsids are transferred to the nucleus by active actin polymerization (Lanier & Volkman, 1998; Ohkawa et al., 2010). The motile nucleocapsids encounter the nucleus and quickly enter through nuclear pores (Ohkawa et al., 2010) where the viral DNA genome is released. The nucleus is the site of replication for all baculoviruses and viral assembly for all NPVs is also carried out fully in the nucleus. In GVs, however, the occlusion step occurs following the disintegration of the nuclear envelope and the merging of the nucleoplasm and the cytoplasm (reviewed in Federici, 1997; Rohrmann, 2011; Winstanley and Crook, 1993). *Spodoptera frugiperda* and *T. ni* cells lines, Sf9 and High Five™, respectively, are permissive to AcMNPV but not to *Bombyx mori* NPV (BmNPV). Infection by BmNPV BVs was shown to be restricted, in part, by defective nuclear import of BVs where the virions entered the cell cytoplasm but were not able to enter nuclei of the cells (Katou et al., 2006). Mammalian cells lines are non-permissive to baculoviruses. AcMNPV ODVs were shown to bind to human carcinoma cells lines, A549 and HepG2, at the very low efficacy of 3×10^6 and 6×10^6 ODVs per cell, respectively (Mäkelä et al., 2008). Where binding occurred, the ODVs were inefficiently internalized into cytoplasmic vacuoles and were not released into the cytoplasm for intracellular trafficking and nuclear entry (Mäkelä et al., 2008). Thus, the non-permissive infection of HepG2 and A549 cells by AcMNPV ODVs is caused by ineffective binding to and internalization in the cell.

3.4 Disease progression in gammabaculoviruses

In hymenopteran sawfly NPVs, virions are singly enveloped and the host range of each virus is restricted to a single species (Federici, 1997). ODVs initiate midgut cell infection and once infected, the midgut cell nuclei become the site of viral gene expression and DNA replication in a manner that is consistent with that described for alphabaculoviruses (Duffy et al., 2007). In infected balsam fir sawfly larvae, *Neodiprion abietis* NPV (NeabNPV) viral DNA increased over 200% within the first 2 hours post infection (hpi) (Duffy et al., 2007). Progeny virions were occluded directly without the production of BVs (reviewed in

Federici, 1997; Slack & Arif, 2007; Rohrmann, 2011). Sequence data from gammabaculoviruses failed to identify homologues to the alphabaculovirus fusion proteins, GP64 or F-protein (Duffy et al., 2006; Lauzon et al., 2006), indicating the absence of the BV phenotype. Also lacking in gammabaculoviruses are viral fibroblast growth factor (vfgf) homologues (Jehle et al., 2006), that have been shown to accelerate the establishment of systemic infections in alphabaculoviruses (Detvisitsakun et al., 2007; Passarelli, 2011). (See section 3.7.) The NeabNPV replication cycle is rapid and efficient, with over 100 nucleocapsids being occluded in each OB (Duffy et al., 2007). Rather than using the BV phenotype to propagate the initial infection to other midgut cells, the released OBs have been suggested to serve as inoculum for other gut cells (Rohrmann, 2011). Midgut cell lysis in sawflies, rather than being a hindrance to gammabaculoviruses, is utilized as a mechanism for dispersal and only hymenopteran baculoviruses encode a trypsin-like protein (Duffy et al., 2006; Lauzon et al., 2006) that is thought to aid this process (Rohrmann, 2011). Much of the horizontal transmission occurs while infected larvae are still alive and feeding because OBs are excreted in a virus-laden diarrhea that is infectious to other larvae (Federici, 1997).

3.5 Disease progression in deltabaculoviruses

The replication of deltabaculoviruses is restricted to the cells of the posterior midgut and gastric caeca (Moser et al., 2001). Virions are singly enveloped prior to occlusion and the host range is restricted within suborders (Andreadis et al., 2003). While the primary infection is initiated by ODVs, the amplification of virus progeny within larvae occurs by cell-to-cell transmission that is carried out by BVs (Moser et al., 2001) and sequence data from CuniNPV identified an homologue to the F-protein of LdMNPV (Afonso et al., 2001). Restriction to the midgut is further corroborated by the lack of vfgf homologues in CuniNPV (Jehle et al., 2006). The production of OBs is rapid (14-48 hpi) and occluded progeny are released upon larval death (Becnel, 2007) for horizontal transmission to permissive hosts.

3.6 Disease progression in betabaculoviruses

Betabaculoviruses are only known to infect lepidopteran larvae. Primary infection is by OBs called granules and is initiated in midgut cells. The granules occlude a single virion and the host range of GVs is very specific (Cory, 2003). Some GV infections are restricted to the midgut (Type III), others cause systemic infections that progress either only to fat body tissues (Type I) or extend to most organs and tissues (Type II) (reviewed in Federici, 1997; Rohrmann, 2011). The time to host death caused by Type II GVs is similar to that of alphabaculoviruses (Lacey et al., 2002) and Type I GVs take the longest to kill the host larva (Federici, 1997). The transmission of Type II GVs occurs following the death and liquefaction of the infected larvae (Federici, 1997). The only identified Type III GV, *Harrisina brillians* GV (HabrGV) is transmitted horizontally by the release of OBs into the gut lumen of the infected larvae and out of the larva in the frass (Federici & Stern, 1990), similar to gammabaculoviruses. With the exception of the Type III midgut tropic HabrGV, the infection of the midgut epithelium is transient and does not produce OBs (Federici, 1997; Hess & Falcon, 1987). The cytopathology of GVs differs from that of other baculoviruses in that, although viral replication occurs and BV particles are produced in the nucleus of

infected cells, OBs are formed in an area where the nucleoplasm and cytoplasm merge following the dissolution of the nuclear membrane (Hess & Falcon, 1987; Winstanley & Crook, 1993). Sequence data from GVs identified homologues of the F-protein and fgf orthologues (Liang et al., 2011; Miele et al., 2011) that are conserved in baculoviruses that cause systemic infections (Rohrmann, 2011).

3.7 Disease progression in alphabaculoviruses

The most studied baculovirus systems are those of lepidopteran alphabaculoviruses where ODVs enter midgut cells and BVs distribute the infection to other tissues in the hemocoel. In the well characterized system, AcMNPV-infected *T. ni* larvae, the infection progresses sequentially from midgut epithelial cells (4 - 12 hpi) to midgut-associated tracheoblasts and tracheal epithelial cells (12 - 24 hpi) (Engelhard et al., 1994). The infection then proceeds to hemocytes and tracheoblasts (36 hpi), later to fat body tissues (48 hpi) and finally to the majority of the remaining larval tissues by 70 hpi (e.g. cuticular epidermis, gonads, Malpighian tubules, midgut epithelia and salivary glands) (Engelhard et al., 1994). Although alphabaculovirus-infected midgut cells do not typically yield OBs (Granados & Lawler, 1981), they are crucial to the establishment of the systemic infection either by re-channeling ODV nucleocapsids into BV (without transiting through the nucleus) or by producing *de novo* BVs after a cycle of replication in infected midgut cell nuclei (reviewed in Rohrmann, 2011). While all other infected organs remain infected and produce viral particles, the infected midgut recovers by 48 hpi by sloughing off infected cells (Engelhard et al., 1995) and replacing them with new, healthy midgut cells (Keddie et al., 1989). Normally permissive to AcMNPV, *T. ni* larvae become resistant to AcMNPV infection as the larval development progresses. Active midgut cell sloughing is thought to play an important role in the developmental resistance that occurs in fourth-instar *T. ni* larvae (Engelhard & Volkman, 1995).

Once in the nucleus, the baculovirus genome utilizes the host transcriptional machinery to initiate the regulatory events that will result in the initial production of nucleocapsids that bud out of the cell by interacting with the cell plasma membrane and acquiring envelope fusion protein (EFP). Group I alphabaculoviruses (e.g. AcMNPV) encode for two main EFPs, GP64, and F-protein while group II alphabaculoviruses (e.g. LdMNPV) encodes only F-protein (Pearson et al., 2000). Deltabaculoviruses encode F-protein while gammabaculoviruses do not encode a discernable EFP (Miele et al., 2011). These EFPs are essential for BVs to exit infected cells and for cell-to-cell transmission (Monsma et al., 1996; Oomens & Blissard, 1999). An alternate mode for exiting the midgut cells rapidly has been described for AcMNPV (Washburn et al., 1999). In infected cells, some of the co-enveloped nucleocapsids enter the nucleus to initiate a round of replication while others, initially, remain in the cytoplasm. GP64 is produced early and modifies the basal membrane of the cell to mediate exit of BVs (Keddie et al., 1989). Even before *de novo* BVs are produced, ODV nucleocapsids in the cytoplasm bypass replication by reaching GP64-modified basal membranes and budding directly to initiate systemic infection of non-midgut tissues (Washburn et al., 2003a; Zhang et al., 2004). The fast shuttle of nucleocapsids through midgut cells is thought to accelerate the establishment of secondary infections before the midgut cells are sloughed off thereby potentially contributing to the wider host range of MNPVs (Washburn et al., 1999; Washburn et al., 2003b) and counteracting host defense

mechanisms. This process of direct transformation of ODV to the BV phenotype has not been observed in SNPVs (Rohrmann, 2011).

ODVs must cross the PM to establish primary infections but BVs need to breach the basal lamina (BL) at the base of the midgut epithelium to initiate secondary infections. The BL is an extracellular layer of protein sheets that are secreted by epithelial cells lining the midgut trachea and other organs (Rohrbach & Timpl, 1993). The BL serves as structural support for regenerating epidermal cells that replace senescing cells that were sloughed off during development or physical assaults to the gut and as a separation between the sterile hemocoelic organs and the midgut, thus preventing the passage of natural microbiota and pathogens acquired during feeding. A model for AcMNPV breaching of the BL has recently been proposed (Means & Passarelli, 2010; Passarelli, 2011). The tracheal system is the insect respiratory system and the first cells to be infected by BVs are the tracheoblasts (Engerhard et al., 1994; Kirkpatrick et al., 1994; Washburn et al., 1995) which are highly motile, single-cell tracheal projections that respond to signaling from oxygen deficient cells and organs. One essential component to this response are the fibroblast growth factors (FGF) that, through a cascade of activation involving fibroblast growth factors receptors (FGFR), trigger tracheal cell motility. To branch to other cells and tissues, tracheoblasts are thought to degrade the BL by secreting enzymes. Baculoviruses are the only viruses known to encode FGF signaling molecules (Passarelli, 2011). Conserved only in alphabaculoviruses and betabaculoviruses, but absent in midgut-restricted gammabaculoviruses and deltabaculoviruses, viral FGF (vFGF) has been shown to accelerate BV exit from midgut cells and secondary infection by rerouting the host respiratory system to the midgut epithelium by mimicking host FGF. Although not essential for host infection *per se*, the difference in speed of establishment of systemic infection and speed of kill (Detvisitsakun et al., 2007; Katsuma et al., 2006) is thought to impact the capacity of any given virus to infect different hosts (Passarelli, 2011).

BV entry into insect cells is effected by GP64 via a clathrin-mediated, low-pH dependent, endocytic process (Long et al., 2006). Insect cells have different receptors for GP64 and F-protein and these two proteins appear to act separately (Hefferon et al., 1999; Westenberg et al., 2007; Wickham et al., 1999). GP64 tropism is so broad in fact, that BVs from AcMNPV and other baculovirus species such as BmNPV have been shown to be taken up by numerous non-lepidopteran cell lines including mammalian and dipteran cell lines (Carbonell et al., 1985; Shoji et al., 1997). Given the broad cellular tropism of GP64, receptors are thought to be common molecules present in invertebrates and vertebrates such as phospholipids (Tani et al., 2001). Therefore, GP64-mediated BV entry into cells is most likely not a restricting event. Receptor specificity for F-protein, however, is more restricted. Mammalian cells were shown not to possess F-protein receptors and could not to be transduced with a gp64-null AcMNPV pseudotyped with baculovirus F-protein (Westenberg et al., 2007). The lack of the *gp64* gene in LdMNPV, might contribute to its narrow host range (Barber et al., 1993; Glare et al., 1995).

Through their distribution in the hemolymph and systemic infection of a variety of tissues, BVs are largely responsible for the amplification of the virus within infected host larvae. Although the primary infection process is essential to the infectivity of baculoviruses in lepidopteran baculoviruses the systemic infection and ultimate death of larvae are dependent upon production of BVs. Horizontal transmission to neighboring larvae is

dependent upon the release of OBs that occurs following larval death. Expression of viral proteases (cathepsin) and chitinase, late post infection, ensures that, in most alphabaculoviruses and in some betabaculoviruses, progeny OBs are released in the environment by lysing larval tissues and the exoskeleton of larvae following death (Hawtin et al., 1997; Hom et al., 2002). In addition, in silkmoth and gypsy moth, virally-produced proteins, tyrosine phosphatase (ptp) (Kamita et al., 2005) and ecdysteroid uridine 5´-diphosphate (UDP)–glucosyltransferase (egt) (Hoover et al., 2011), are responsible for behavioral changes that occur during the infection process where infected larvae leave their normal sheltered habitats and climb to exposed surfaces. This alteration in larval behaviour is thought to assist in virus distribution by facilitating predation by animals and by increasing exposure to elements.

4. Replication cycle

For all baculoviruses, once inside the nucleus, genome replication events follow a strictly-controlled cascade of temporal and sequential events (reviewed in Friesen, 2007; Rohrmann, 2011). Baculovirus genes are transcribed in three temporal phases (early, late and very late) where later steps of each phase are dependent on occurrence of earlier molecular events (Carstens et al., 1979; Guarino & Summers, 1986; Guarino & Summers, 1987).

4.1 Early phase

Upon viral DNA release into the cell nucleus, cellular transcription is harnessed for the expression of viral immediate early genes (Carstens et al., 1979; Guarino & Summers, 1986, 1987) and host-cell transcripts are decreased progressively from 12 - 18 hpi until complete shut down by 36 hpi (Nobiron et al., 2003). Baculoviruses depend on the cellular RNA polymerase II, to initiate the cycle of replication (Fuchs et al., 1983; Huh & Weaver, 1990) by recognizing and initiating transcription of viral early promoter sequences (Fuchs et al., 1983; Hoopes & Rohrmann, 1991; Huh & Weaver, 1990). Early viral transcripts can be detected as early as 0.5 hpi and through to 6 - 9 hpi in AcMNPV-infected susceptible cells (Chisholm & Henner, 1988; Guarino & Summers, 1986). The early promoter regions are conserved throughout baculoviruses and mimic those of the host RNA polymerase II with a consensus TATA element at about 30 bp upstream from the RNA start site (Pullen & Friesen, 1995) and a CAGT motif that acts as an initiator element (Blissard et al., 1992; Pullen & Friesen, 1995). Early genes encode mainly for polypeptides (IE1, IE0, IE2 and PE38) that have regulatory functions which are responsible for the transcriptional regulation of other viral genes, for the initiation of viral DNA replication (Lu & Carstens, 1991; Stewart et al., 2005; Todd et al., 1995), or to take control of the host cell for the purpose of viral multiplication (Possee & Rohrman, 1997). Early baculovirus gene expression is mostly regulated at the level of transcription and transfected viral DNA is infectious to permissive cells (Burand et al., 1980; Cartens et al., 1980) indicating that initial transcription of early genes does not require viral factors to be present at the start of the infection. The lack of requirements for viral proteins has been substantiated recently by proteomic analysis of AcMNPV BV particles that have been shown not to contain regulatory proteins (Wang et al., 2010). Larvae of *Anticarsia gemmatalis* (velvetbean caterpillar) are highly resistant to AcMNPV. Infection was shown to be blocked at early stages of replication where, even though ODVs successfully entered the midgut cells and were rechanelled to tracheal cells, the immediate early gene, *ie-1*, was not

transcribed (Chikhalya et al., 2009). The inhibition of this essential transactivator resulted in the disruption of the gene expression cascade resulting in a failure to produce infectious viral particles.

4.2 Late phase – DNA replication

Baculovirus early gene products are required for viral DNA replication. In AcMNPV-infected cells, genome replication occurs between 6 hpi and to 18 hpi after which time it starts to decline (Erlandson et al., 1985; Erlandson & Carstens, 1983). Six AcMNPV genes were found to be essential for DNA replication in transient DNA replication assays (Kool et al., 1994; Lu & Miller, 1995a). The genes directly involved in DNA replication (reviewed in Rohrman, 2011) encode for a homologous-region (hr) binding protein and transcriptional activator (*ie-1*) (Leisy et al., 1995; Rodems & Friesen., 1995), a single-stranded DNA binding protein (*lef-3*) (Hang et al., 1995), DNA binding helicase (*p143*) (McDougal & Guarino, 2000), a putative primase (*lef-1*), a primase-associated protein (*lef-2*) (Mikhailov & Rohrmann, 2002) and a DNA polymerase (*dnapol*) (Hang & Guarino, 1999; McDougal & Guarino, 1999). Four of these genes, *dnapol*, *p143*, *lef-1*, and *lef-2* are core genes found in all baculoviruses (Okano et al., 2006). Other genes such as *ie-2*, *lef-7*, *pe38*, and *p35*, stimulate viral DNA replication in transient assays (Chen & Thiem, 1997; Lu & Miller, 1995a; Milks et al., 2003) and were found to be differentially required in cell lines from various lepidopteran origins as well as *in vivo* (Chen & Thiem, 1997; Lu & Miller 1995b; Milks et al., 2003; Prikhod'ko et al., 1999).

BmNPV and AcMNPV genomes are highly homologous (Gomi et al., 1999) but their host ranges are very different (Gröner, 1986). *Bombyx mori* larvae and cell lines such as BmN, are fully permissive to BmNPV but their infection by AcMNPV is non-permissive (Morris & Miller, 1993). Though delayed in BmN cells, AcMNPV temporal gene expression occurs as in the permissive *Spodoptera* cells until very late times post infection (Iwanaga et al., 2004; Morris & Miller, 1993). AcMNPV DNA replication also takes place in BmN cells but the infection is arrested before BV or OBs are produced (Morris & Miller, 1993). AcMNPV-infected BmN cells also showed marked cytopathic effects (Maeda et al., 1993) which led to a drop in gene expression. Though DNA replication seemingly occurred as in the permissive Sf-21 cells, the defect in AcMNPV-BmN cells was shown to be caused by differences in the *DNA helicase* gene (*p143*) (Maeda et al., 1993). A few amino acid changes in AcMNV P143 were sufficient to overcome the defect in *B. mori* cells and larvae (Argaud et al., 1998; Croizier et al., 1994). The cytotoxicity and block in AcMNPV infection of *B. mori* cells are suggested to stem from aberrant DNA replication (reviewed in Thiem & Cheng, 2009; Rohrmann, 2011).

4.3 Late phase – Late gene expression

The final step in the replication cycle of baculoviruses is the expression of late and very late genes that mainly code for structural proteins. AcMNPV genes encoding structural proteins of nucleocapsids and BVs are transcribed at their peak during the late phase (6 - 24 hpi), while occlusion-related genes are transcribed at very late times post-infection (18 - 76 hpi) (Thiem & Miller, 1990; Wu & Miller, 1989). The increase in late viral transcription parallels the decline in host and early viral transcription (Nobiron et al., 2003). Late promoter sequences are conserved in baculoviruses with the TAAG sequence being the essential

component for the recognition of late and very late promoters by viral RNA polymerase factors with cis-acting sequences dictating the differential levels and temporal expression of late and very late genes (Ooi et al., 1989). In AcMNPV, 19 genes were found to be required for optimal transcription of late (*vp39* and *p6.9*) and very late promoters (*polh* and *p10*) but not early promoters (*etl* and *pcna*) (Li et al., 1993; Lu & Miller, 1995b; Passarelli & Miller, 1993a, 1993b, 1993c; Rapp et al., 1998; Todd et al., 1995). Being required for the transcription of late and very late genes, these 19 genes have been defined as late expression factors (lefs). Since the late transcriptional events are dependent upon early transcription and DNA replication, nine of these lefs have indirect effects on late transcription by being involved in early gene transcription and DNA replication (*ie-1*, *ie-2*, *lef-1*, *lef-2, lef-3, p143*, *dnapol*, *p35*, and *lef-7*) (Rapp et al., 1998). Only four of these lefs (*p47, lef-4, lef-8,* and *lef-9*) have been shown to form the viral RNA polymerase that is directly responsible for the *in vitro* transcription of baculovirus late and very late promoters (Guarino et al., 1998; Rapp et al., 1998). A unique feature of baculoviruses is the hyperexpression of the very late genes, *polyhedrin* and *p10*. Increases in transcription levels of RNA polymerase occurs through the binding of very-late expression factor (VLF-1) (McLachlin & Miller, 1994; Ooi et al., 1989; Yang & Miller, 1999) to very late promoters stimulating expression of the high levels of polyhedrin required for OB formation.

Gypsy moth Ld652Y cells are semi-permissive to AcMNPV and, although all temporal classes of virus genes are transcribed and viral DNA replication is detected, translation of both viral and host proteins is arrested by about 12 hpi (reviewed in Thiem & Cheng, 2009; Guzo et al., 1992; McClintock et al., 1986; Morris & Miller, 1993). An LdMNPV gene, named the host range factor (*hrf-1*) was found to rescue the translational arrest in AcMNPV-infected Ld652Y cells (Du & Thiem, 1997a; Thiem et al., 1996). AcMNPV does not encode for a *hrf-1* homologue. Gypsy moth *in vivo* resistance was also overcome by a hrf-1-bearing, recombinant AcMNPV (Chen et al., 1998).

5. Registration of baculoviruses

Agencies responsible for pesticide product regulations were initially put in place by governments to evaluate the efficacy and non-target safety of synthetic chemical pesticides. Their extension into environmental safety assessments came in response to concerns related to the increasing number of reports of environmental damage due to pesticide toxicity and the accumulation of chemical residues such as those from DDT (Hauschild et al., 2011). Gradually, mounting public pressure and the implementation and enforcement of stricter rules and regulations led to the ban and rejection of many chemical pesticides and the need for lower-impact pesticides such as those classified as biological control agents (BCA) and microbial pest control agents (MPCA). MPCAs are those products that have a microorganism (i.e., virus, bacterium, fungus, protozoan or nematode) as the active ingredient. When submitted for registration, MPCAs were initially evaluated using existing regulatory processes that were developed for broad-spectrum chemical pesticides. Since then, a trend has emerged that now favours the development of lower-risk products such as BCAs and MPCAs and specific regulations have been developed that are better suited to the requirements of MPCAs. To facilitate the registration process for MPCAs, many countries have established departmental branches that specialize in the registration of MPCAs and other low-risk product submissions (Hauschild et al., 2011; Kabaluk et al., 2010). Despite

these efforts, the MPCA registration process in many countries still requires a number of toxicity tests that might not be necessary based on the biology of the microbe that acts as the active ingredient of a MPCA. Compared to chemical pesticides, MPCAs typically target small niche markets and unnecessary registration requirements can impose burdensome costs on the biopesticide industry (Chandler et al., 2011).

The most important function of any pesticide regulatory agency is to insure that unsafe pesticides are not registered for use. Although the registration procedures, costs and processing times differ between countries, data requirements are generally similar (Hauschild et al., 2011). Typically, numerous categories of data are required for the registration of MPCAs. These categories are designed so that applicants provide 1) product identity, physical, chemical and technical properties; 2) methods of analysis, manufacturing and quality control; 3) toxicological studies and exposure data geared towards human and veterinary health and safety; 4) product residue data; 5) product fate and behaviour in the environment; 6) environmental and non-target toxicity and 7) efficacy data (Hauschild et al., 2011).

The majority of the topics included in registration packages are essential and desirable. For baculoviruses in particular, it is essential that the active ingredient has been identified as belonging to the family, *Baculoviridae* and that the species of its primary host is known (e.g. *Neodiprion abietis* gammabaculovirus) (category 1), that the method of manufacturing, analysis and quality control is appropriate and reliable (category 2) and that it has been proven to be efficacious (category 7) for the purpose intended. The regulations as applied to some of the more recently registered baculovirus-based products may, however, demonstrate the redundancy of certain data requirements involving vertebrate toxicity (categories 3 and 6) and some aspects of environmental and non-target effects (categories 4 and 5). The Pest Management Regulatory Agency (PMRA) in Canada and the United States Environmental Protection Agency (EPA) accept that certain data requirements can be met using "waivers" that provide scientific arguments based on published, peer-reviewed, scientific literature and data while, in the European Union (EU), less formal procedures allow for similar science-based evidence to replace the actual, newly-generated data (Hauschild et al., 2011). A combination of fulfilling specific data requirements and the use of waivers were used in the successful registration of the baculovirus-based product, Abietiv™, for the suppression of balsam fir sawfly populations in Canada (Lucarotti et al., 2006, 2007).

5.1 Identity of baculovirus products

MPCAs are usually registered at the strain level where the active ingredient is derived from a single host, colony or spore. While this selection method is mostly feasible for MCPAs such as bacteria and fungi, it may not be feasible or desirable for baculovirus-based products. In nature, baculoviruses consist of mixtures of different genotypes of the same species (Cory et al., 2005) and it has been shown that this diversity is naturally favored in wild type virus populations (Clavijo et al., 2011) where these genomic variants are known to impact virulence in the target organism (López-Ferber, 2003; Simón et al., 2008). The different viral genotypes may compensate for variations that occur in the larval host and/or its environment (Berling et al., 2009; Hitchman et al., 2007; Hodgson et al., 2002). For this

reason, the evaluation of baculoviruses should be carried out at the species level rather than at the level of a single isolated genotype (Hauschild et al., 2011).

5.2 Human toxicity and infectivity

The scientific literature on the health and environmental safety of baculoviruses is extensive and has been well reviewed (see reviews by Black et al., 1997; Burges et al., 1980a, 1980b; Gröner, 1986; Ignoffo, 1975; OECD, 2002) and more recently by the Food and Agriculture Organization of the United Nations (FAO) (McWilliam, 2007) and the European Food Safety Authority (EFSA) (EFSA, 2009; Leuschner et al., 2010). The host range of baculoviruses is restricted to terrestrial arthropods (Barber et al., 1993; Doyle et al., 1990; Cory, 2003; Cory & Hails, 1997; Miller & Lu, 1997; Thiem & Cheng, 2009). Baculovirus products that are commercially available for biological control of insect pests have been extensively tested to determine effect on humans and other non-target animals (Hauschild et al., 2011).

Data required for assessment of human infectivity and toxicity typically involve mammalian toxicological studies of the product in laboratory test mammals (e.g. mice, rats, rabbits) *in vivo* as well as in mammalian cell cultures. Baculovirus active ingredients and end products have been ingested and inhaled by, injected (intravenous, intraperitoneal, intramuscular) into, and applied to the skin and eyes of test animals without detrimental effects that could be attributed to the baculovirus tested (Table 1) (Ashour et al., 2007; Gröner, 1986; Ignoffo, 1975; Lightner et al., 1973). Many species of baculoviruses have been tested on numerous species of animals at doses that are many times those that could be acquired in the field. For example, for the registration of Abietiv™ (NeabNPV), typical toxicity data were presented where rats had been fed single dose of 1 $x10^8$ NeabNPV OBs (Health Canada PMRA, 2009; Lucarotti et al., 2006). All of the rats survived to the end of the observation period and showed no adverse clinical effects. At the application rate given on the Abietiv™ product label (1 x 10^9 OBs in 2.5 L of 20% aqueous molasses/ha), this would be the equivalent of a 70-kg man ingesting 16 L of the tank-mixed product. Thus, taking into account the label application rates and volumes at which the products are applied, the concentrations used for toxicity tests are well beyond what could be expected to be acquired in the field.

In vitro, mammalian and other vertebrate cells lines are non-permissive to baculoviruses (reviewed in Gröner, 1986; OECD, 2002). Although BV uptake has been observed, there has been no evidence that viral DNA replication, production of viral proteins or cytopathological effects have occurred. *In vivo*, the uptake of baculovirus OBs by various animals did not lead to the production of baculovirus-specific antibodies (reviewed in Gröner, 1986). Human carcinoma cell lines, HepG2 and A549, were recently challenged with AcMNPV ODVs that had been chemically extracted from OBs (Mäkelä et al., 2008). The non-permissive infection of HepG2 and A549 cells by AcMNPV ODVs was shown to be caused by the inefficient binding and internalization in the cell. The ODV-derived nucleocapsids did not reach the nucleus to release the viral genome. In addition to the lack of infection and replication in vertebrate cells, no evidence for baculovirus induced cytogenic, carcinogenic, mutogenic or teratogenic effects has ever been found (Gröner, 1986; Ignoffo, 1975; McWilliam, 2007; OECD, 2002).

Alphabaculovirus	**Vertebrate Test Animals**
Amscacta albistriga NPV	chicken
Autographa californica NPV	rat, guinea pig, rabbit, shrimp[a], fish[b]
Choristoneura fumiferana NPV	rat, rabbit, duck, quail, rainbow trout, white sucker
Erranis tilliara NPV	mouse
Galleria mellonella NPV	mouse
Heliothis zea NPV	mouse, rat, guinea pig, rabbit, dog, monkey, man, quail,chicken, sparrow, mallard, killifish, spotfish, rainbow trout, black bullhead, white sucker, sheepshead minnow
Lymantria dispar NPV	mouse, rat, guinea pig, rabbit, dog, blackcap chickadee,duck quail, sparrow, bluegill, brown trout
Malacosoma disstria NPV	guinea pig, rabbit, chicken
Mamestra brassicae NPV	mouse, guinea pig, pig, chicken
Orgyia pseudotsugata NPV	mouse, rat, rabbit, mule deer, duck pheasant, sparrow, chinook salmon, coho salmon, steelhead trout
Spodoptera exempta NPV	rat
Spodoptera exigua NPV	mouse, guinea pig
Spodoptera frugiperda NPV	mouse, guinea pig
Spodoptera littoralis NPV	Rat, fish[b]
Spodoptea litura NPV	chicken
Thymelicus lineola NPV	mouse, sheep, goldfish
Trichoplusia ni NPV	mouse, guinea pig, sparrow
Betabaculovirus	
Cydia pomonella GV[c]	mouse, rabbit
Estigmene acrea GV	mouse, guinea pig
Gammabaculovirus	
Neodiprion abietis NPV[d]	rat, mouse, rabbit
Neodiprion lecontei NPV	rat, hamster, rabbit, chicken, turkey, rainbow trout
Neodiprion sertifer NPV	rat, guinea pig, rabbit, duck, quail
Neodiprion swainei NPV	mouse, rat, guinea pig, rabbit, duck, quail

Table 1. List of vertebrate test animals exposed to baculoviruses from a variety of Lepidoptera and sawflies to which no adverse effects of exposure to the baculovirus could be attributed. (from Gröner, 1986; Ignoffo, 1975). a, b, c, d: Data obtained from Lightner et al. (1973), Ashour et al. (2007), and Health Canada PMRA Regulatory Notes REG2000-10 and RD2009-05, respectively.

Currently, all baculovirus-based MCPs are produced, *in vivo*, in permissive larval hosts. While baculovirus OBs are inert and non-allergenic, the larvae in which they are produced can produce dermatitis and contact urticaria where larval setae cause mechanical irritation or contain histamine or other irritating substances (Hossler, 2010a, 2010b). Although anaphylactic shock has not been reported to be caused by lepidopteran insects (Hossler, 2010a, 2010b), eye irritation studies on rabbit (Reardon et al., 2009) and limited skin eruptions have been reported from human exposure to gypsy moth larvae during heavy infestations (Tuthill et al., 1984). As a result, the Gypchek product label warns of potential eye irritation. While this is the case for some of the baculovirus products, the majority of products are not considered as sensitizers (Hauschild et al., 2011: Ignoffo, 1975).

5.3 Baculoviruses and biomedical applications

Additional evidence of the safety of baculoviruses to humans comes from their use in biomedical applications. The unique baculovirus properties, coupled with recent advances in molecular and cell biology, have broadened the scope of their application in basic and applied biomedical fields. To date, the prototype baculovirus, AcMNPV, is the most widely used baculovirus for the production of biologics for therapeutic purposes (Aucoin et al., 2010; Cox & Hollister, 2009; van Oers, 2006). This has been accomplished in part by use of baculovirus expression vector systems (BEVs) for heterologous recombinant protein production and gene transfer. The principle behind the use of BEVs is based on use of *polyhedrin* and *p10* promoters to drive the expression of foreign genes in cell culture or *in vivo* (van Oers, 2011; Summers, 2006). BEVs continue to evolve to new and robust systems. For instance, the latest vectors (flashBACultra/BacMagic3) can be semi-automated for high quality, yield, and stable recombinant protein (van Oers, 2011). Many advantages of using BEVs over other expressions systems have been reviewed in previous reports (Airenne et al., 2009, 2010; Hu, 2005). Also, there are available insect cell lines such as those derived from *S. frugiperda* (Sf9 cells) and *T. ni* (High Five™ cells), which have been extensively characterized for optimal, high quality recombinant protein production (Aucoin et al., 2010). Some of these cell lines have been adapted to grow as continous suspension cultures in serum-free media thus, allowing for high-throughput scale-up production in bioreactors (Elias et al., 2007; Feng et al., 2011). Furthermore, use of serum-free cell cultures have been recognized by regulatory agencies including the United States, Food and Drug Administration (FDA), and European Medicines Agency (EMA) as a standard approach for limiting potential adventitious agents in therapeuatic products (FDA, 2010). Many advantages of using insect cell substrate compared to embryonated eggs have been reported leading to simplified regulatory avenues for licensing baculovirus-based biologics (Cox & Hollister, 2009, Treanor et al., 2007). In addition to cell substrates, insect larvae such as *T. ni* (Chen et al., 2011), and *B. mori* (Kato et al., 2010) have been reported as potential biofactories for *in vivo* therapeutic production. For instance, *in vivo* production of antiviral agents including human interferon-γ against influenza virus H1N1 in *T. ni* larvae have been demonstrated (Chen et al., 2011; Gomez-Casado et al., 2011). Nevertheless, insect cell cultures and BEVs platforms continue to expand the applications of baculoviruses as novel tools for vaccine development, drug screening, and gene therapy (Airenne et al., 2010, 2011; Cox & Hollister, 2009, Kost et al., 2005, van Oers, 2011). The recent initiative of having a standard baculovirus reference material repository (BRM) will further boost their application and perhaps hasten the regulatory process for registering new baculovirus products (Kamen et al., 2011). This initiative was mainly proposed in order to have a proper standard that is acceptable to all researchers in academic institutions, regulatory agencies, and industries (Kamen, et al., 2011).

5.4 Baculoviruses and vaccine development

To date, there are different baculovirus-based vaccines for human and veterinary use (van Oers, 2011). Also, vaccines targeting highly pathogenic viruses that are transmitted by arthropod vectors (arboviruses) are being developed (Metz & Pijlman, 2011). Characteristics of baculovirus-based human vaccines that are currently approved or are in later phases of clinical trials are given in Table 2. The different strategies employed in the production of baculovirus-based vaccines include: (i) BEVs-based subunit vaccines; here, recombinant viral proteins or peptides are produced using BEVs in cell culture. Subunit vaccines can be

efficiently produced in insect cells and have additional safety advantages over live attenuated vaccines (Madhan et al., 2010). A good example is the influenza vaccine (FluBlok), which is based on recombinant Hemagglutin (HA) proteins selected from three influenza virus strains as determined by the World Health Organization (WHO) and the Centre for Disease Control (CDC) (Airenne, 2009; Cox & Hollister, 2009). Subunit vaccines developed in BEVs have been approved based on the standards stipulated by various regulatory agencies especially clinical data on toxicology and efficacy assessment (Cox & Hollister, 2009; Cox & Hashimoto, 2011; FDA, 2009). (ii) BEVs-based virus like particles (VLPs); for example, a prophylactic, bivalent human papillomavirus vaccine for cervical cancer (Cervarix) consisting of C-terminally truncated HPV-16/18 L1 proteins is produced using BEVs in *T. ni* High- Five™ cells and assembled as VLP (Harper et al., 2006). VLPs mimic the real virus but are non-infectious due to lack of viral genome, and are safe for human use. Detailed safety data for human papillomavirus types 16 and 18 recombinant vaccine have been outlined by USA and Canada health regulatory agencies (FDA, 2009; Health Canada, 2010). (iii) Active cellular-based vaccine; a classical example and the first vaccine of this kind to be approved by FDA is Provenge® for prostate cancer. This vaccine is composed of fusion proteins consisting of a prostate cancer marker, prostatic acid phosphatase (PAP), linked to granulocyte macrophage colony stimulating factor (PAP-GM-CSF) and generated in insect cells via BEVs. The fusion protein is in turn loaded *ex vivo* in dendritic cells, the most potent antigen presenting cell (APC), leading to stimulation of cytotoxic T-cell immune response against patients cancer cells (Small et al., 2006; Vergati et al., 2010). The prostate cancer cells expressing these recombinant proteins are recognized by the patient's cell-mediated immune system. In addition to the aforementioned baculovirus strategies for vaccine development, there are other baculovirus technologies, such as baculovirus surface display technology, that are being considered for production of vaccines. Here, the desired foreign antigens are displayed on the surface of the baculovirus envelope or capsid (Mäkelä & Oker-Blom, 2006; Oker-Blom et al., 2003). More recently, a novel system based on the use of a defective baculovirus vector incapable of self assembly has been developed (Marek et al., 2010). In this approach, the baculovirus vector is engineered to produce biologics that are free from contaminating BVs and ODVs.

Vaccine	Producer	Disease	Status	Reference
Cervarix™	GlaxoSmithKlines, Rixensart, Belgium	Cervical cancer	Approved	Harper et al., 2004; 2006
Provenge	Dendreon Inc., Seattle, WA, USA	Prostate cancer	Approved	Kantoff et al., 2010 ; Small et al., 2006;
Chimigen	Virexx Medical Corp., Calgary, Canada	Hepatitis B and C	Clinical trial	
FluBlok	Protein Biosciences Corp., CT, USA	Influenza virus	Clinical trial	Cox & Holister, 2009, Cox & Hashimoto, 2011
Dyamid	Diamyd Medical AB, Stockholm, Sweden	Type-1 diabetes mellitus	Clinical trial	

Table 2. List of baculovirus-derived vaccines.

5.5 Baculovirus and mammalian gene delivery/ therapy platforms

Although baculoviruses replicate in the nucleus of specific insect hosts, mammalian cells have been shown to internalize baculoviruses, but no progeny virions are produced (Volkman & Goldsmith, 1983). Similarly, recombinant baculoviruses carrying a reporter gene under the control of human cytomegalovirus (CMV) and Rous sarcoma virus (RSV) promoters were shown to efficiently transduce mammalian cells and express foreign proteins (Boyce & Bucher 1996; Hofmann et al., 1995). These studies showed varying levels of reporter gene expression in mammalian cells of different origins. Although all cells were reported to internalize the same amount of virus, the block to expression or low expression observed in epithelial cells compared to human and rabbit hepatocytes was attributed to a specific receptor on the hepatocyte cell membranes and inhibition of endosomal maturation. Additional blocks have been linked to poor cytoplasmic transport or entry of nucleocapsid to the nucleus (reviewed in Airenne et al., 2009).

The basis of baculovirus gene delivery/ transfer in mammalian cells has been accomplished using BacMam vectors (Invitrogen Corporation, Carlesbad, CA). Unlike BEVs, which relies on baculovirus late promoters, the gene of interest in BacMam vectors is placed under the transcriptional control of mammalian active-promoters such as those of CMV, RSV, chicken beta-actin (CAG), among others (Madhan et al., 2010). Various cellular and viral promoters have been shown to affect transduction efficiency in different mammalian cells implying that promoter selection is critical to efficient use of baculovirus vectors (Kim et al., 2007; Shoji et al., 1997). Nonetheless, safety of baculovirus vectors in gene delivery is supported by extensive safety data that show lack of toxicity or pathogenicity in various mammalian species (reviewed in Airenne et al., 2009). Based on this, baculoviruses have been considered as ideal candidate for future gene therapy. Gene therapy is a novel approach for treating various forms of genetic diseases through the use of viral or non-viral shuttle vectors. This has been successfully demonstrated through *in vivo*, and *ex vivo* (human-derived tissues) studies as previously reviewed (Airenne et al., 2009, 2010; Hu, 2005). To date, viral-based vectors including those of DNA and RNA animal viruses are increasingly being tested as potential agents for gene therapy. Health Canada, like other regulatory bodies, recognizes various viral-based vectors for *in vivo* and *ex-vivo* gene therapy. Baculoviruses are included in these lists due to recent studies on their potential as tools for gene therapy and requirements for extensive preclinical studies. Baculovirus-based vectors remain promising candidates for gene delivery primarily due to the following attributes: (i) natural occurrence, (ii) host specificity (iii), well characterized genomes (Cohen et al., 2009), (iv) genetic stability due to lack of reversions or genome integration, (v) rapid and relatively low cost of production to high titers (~ 10^{12} pfu/ml) (Airenne, 2010), (vi) lack of cell substrates associated with animal serum, (vi) large transgene capacity (~100 kb), and allowance for multiple gene inserts, (viii) ability to transduce a myriad of dividing and non- dividing cells (Airenne et al., 2011; Kost & Condreay, 2002) and, (ix) lack of pre-existing immunity.

Although baculovirus gene therapy technology is relatively recent, there is a wealth of safety data in animal models (Airenne et al., 2009) and preclinical trials based on *ex-vivo* experiments (Georgopoulos et al., 2009). Their safety is augmented by early toxicity studies using intravenous, oral, intracerebral, and intramuscular inoculation of animal models, and feeding tests on voluntary humans (Gröner, 1986; Ignoffo, 1973, 1975). Similarly, techniques to assess the toxicity and transformation potential of baculovirus in mammalian cells have

been developed (Hartig et al., 1989, Gonin & Gaillard, 2004). Here, quantitative PCR (qPCR), using SYBR Green or TaqMan probes is viewed as the standard tool for assessing the biodistribution of the transgene and expression of shuttle vectors (Gonin & Gaillard, 2004).

5.6 Environmental toxicity

The environmental and ecological impacts of baculovirus products are mirrored by characteristics of their pathogenesis and host range. In essence, every study that is required to assess their potential for environmental toxicity will be influenced by their limited host range and lack of infectivity to non-target animals. Data required for assessment of environmental toxicity typically involve environmental fate and environmental toxicological studies on birds, fish, plants, microorganisms, aquatic arthropods and non-target insects including beneficial insects. Baculoviruses are ubiquitous and persistent in aquatic, terrestrial and forest ecosystems (England et al., 2004; Hewsen et al., 2011; Podgwaite et al.,1979) yet, there has been no report of negative impact of baculoviruses on ecosystems other than the effect on the target host insect (Black et al., 1997; Cory, 2003; FAO, 2007; OECD, 2002). As a matter of fact, when applied in the context of pest control, the persistence and amplification of baculoviruses in the larval host population has been recognized as being an essential component of plant protection, in particular for forestry (Moreau et al. 2005; Moreau & Lucarotti 2007). Field application of baculoviruses into the environment, such as occurs when LdMNPV is applied for the control of gypsy moth, does not increase virus levels beyond those that would occur naturally (Reardon et al., 2009). Also, through water run off or direct deposit of contaminated material (insects, frass, etc.), aquatic systems are recipients of baculoviruses (Hewsen et al., 2011). None of the non-target arthropods, such as shrimps, *Daphnia* spp. and *Notonecta* spp., or fresh-water, estuarine and marine fishes that have been tested by exposure to several NPVs have shown evidence of infection, toxicity or mortality (Table 1) (Dejoux & Elouard, 1990; Lightner et al., 1973; Couch et al., 1984).

Non-target insect toxicity studies are complex and the results usually depend upon the natural host range of any given baculovirus. Fundamental studies that were aimed at determining putative host range factors generally found that a different barrier to infection occurred for every virus and non-host system examined (See sections 2 and 3). The determination of the host range for any given baculovirus has, therefore, proven to be difficult to predict. Most baculoviruses, however, are very specific to their host species or closely-related ones (reviewed in Miller & Lu, 1997; Thiem & Cheng, 2009) and cross-order infections do not occur. In addition, SNPV alphabaculoviruses, betabaculoviruses and gammabaculoviruses appear to be the most restricted in host range, while some of the MNPV alphabaculoviruses (e.g. AcMNPV and *Mamestra brassicae* NPV [MabrNPV]) can infect over to 30 species crossing over 10 families of Lepidoptera (reviewed in Miller & Lu, 1997; Thiem & Cheng, 2009). Even within the MNPVs, however, those infecting Lymantriidae hosts such as LdMNPV in the gypsy moth, *L. dispar,* appear to be truly specific to a single host (Barber et al., 1993; Cory, 2003; Cory & Myers, 2003; Glare et al., 1995). Most *in vivo* host range studies have been carried out in the laboratory and at extremely high baculovirus dosage rather than at a range of concentration that might allow for the determination of a range of lethal doses (e.g., LD_{50} – LD_{95}). This artificial system does not accurately reflect the field situation and additional caution must be given to older toxicology

– host-range studies, where the evaluation of permissiveness is based only on mortality rates. Often these studies lack confirmation of productive infections which could lead to an overestimate of the host range of a given viral isolate. Therefore, confirmation of infectivity and host range through the use of molecular techniques to identify patent infections is recommended (Cory, 2003; OECD, 2002; Thiem & Cheng, 2009). Unfortunately, the insect species that have been selected for non-target toxicity tests have often been ones that have been shown not to be susceptible to baculoviruses. Only Lepidoptera, hymenopteran sawflies and a few species of Diptera have been confirmed to host baculoviruses. There is no cross-infection of baculoviruses between these orders. Baculoviruses do not infect cockroaches, grasshoppers, aphids, neither have they been shown to infect non-phytophagous beneficial and predatory insects such as lady beetles, parasitoids and honey bees (Doyle et al., 1990; Huang et al., 1997; Ignoffo, 1975). Although not infecting parasitoids, baculoviruses can cause the premature death of the larval host and competition for resources that can affect the fitness and survival of parasitoids (Hochberg, 1991; Nakai & Kunimi, 1997). Parasitoids are often generalists and while the depletion of virally-treated insect populations will occur, the lack of non-target effects on other potential lepidopteran hosts would presumably provide alternate hosts for the parasitoids (Strazanac & Butler, 2005). In addition, some studies suggest that some parasitoids such as *Cotesia melanoscela*, *Parasetigena silvestris* and *Apanteles melanoscelus* transmit baculoviruses (e.g., LdMNPV) and contribute to the viral epizootic (Reardon & Podgwaite, 1976).

6. Summary

Viruses in the family *Baculoviridae* are host specific, infecting only one or a few closely related species of insects. They are ubiquitous in the environment and are known to be an important contributor to insect population regulation. These characteristics make them good candidates for management of crop and forest insect pests with minimal or no off-target impacts. Commercial production of baculoviruses for use as biological control agents of insect pests is carried out worldwide at different scales depending on the market. Over 50 baculovirus products have been used worldwide as microbial insecticides. Five viruses are registered for use in Canada, mostly for the control of forest insect pests. As is the case in other industrialized countries, the commercialization of baculoviruses as microbial insecticides in Canada is dependent upon the submission of a number of scientific studies that establish proof that the products are efficacious and safe. Given the extensive and long standing use of synthetic pesticides, regulatory policies are often geared toward chemical pesticides requiring extensive safety testing that could be considered to be superfluous and unwarranted given the long history of safe and efficacious use of baculoviruses. Most recently, baculovirus safety has been substantiated further by fundamental research geared towards understanding the molecular basis for the events that regulate baculovirus life cycles, pathogenesis, and host range and by the increased application of baculoviruses for pharmaceutical and therapeutic use.

The safety of baculovirus products is innately linked to the pathogenesis and host range of this family of viruses. For a productive baculovirus infection to occur, the viral replication process must successfully cross multiple environmental, temporal and organism-specific barriers. Every step in the life cycle of baculoviruses is challenged beginning with the external environment and the long periods between host availability. Once in contact with a

potential host, viral particles must be released from the OBs, enter permissive cells and successfully take over the host cell transcriptional machinery to initiate the viral replication cycle. Dependence on the host-cell molecular machinery is reduced over the course of the infection as baculovirus gene expression and regulatory proteins take over. However, host- and/or tissue-specific interactions continue to play a role as the infection progresses within the infected host which will determine whether or not a patent infection will occur.

Prompted by the publication of the OECD consensus document on the "Assessment of Environmental Applications involving Baculovirus", the Regulation of Biological Control Agents (REBECA) entered baculoviruses in the positive list of "low risk" candidate microbial pest control agents (Strasser et al., 2007). In addition, baculoviruses have recently been included in the qualified presumption of safety (QPS) list authorized by the European Food Safety Authority (EFSA) (Leuschner et al., 2010) panel on Biological Hazards (BIOHAZ) (EFSA, 2009). Following a review of literature, EFSA concluded that baculoviruses are safe for animal and human consumption and are, therefore, acceptable for use in the control of insects that cause damage to plants (EFSA, 2010). Given that all published reviews unequivocally state that baculoviruses are safe and support their use as low-risk biological control agents for the control of insect pests, we propose that human and environmental toxicity tests and studies related to the residual fate of baculoviruses not be required for the registration of baculoviruses.

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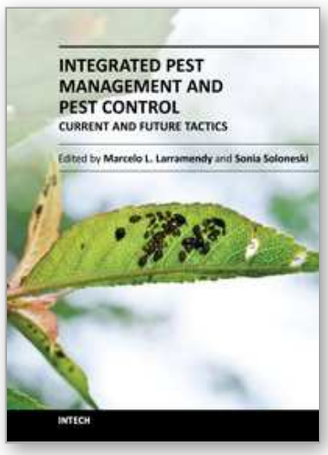

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