GENETICALLY MODIFIED BACULOVIRUSES: A HISTORICAL OVERVIEW AND FUTURE OUTLOOK

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ABSTRACT

The concept of using genetic engineering to improve the natural insecticidal activity of baculoviruses emerged during the 1980s. Both academic and industrial laboratories have since invested a great deal of effort to generate genetically modified (GM) or recombinant baculoviruses with dramatically improved speeds of kill. Optimal production methodologies and formulations have also been developed, and the safety and ecology of the recombinant baculoviruses have been thoroughly investigated. Unfortunately, the initial excitement that was generated by these technologies was tempered when industry made a critical decision to not complete the registration process of GM baculoviruses for pest insect control. In this chapter, we summarize the developments in the field from a historical perspective and provide our opinions as to the current status and future potential of the technology. We will argue that GM baculoviruses are valuable and viable tools for pest insect control both alone and in combination with wild-type viruses. We believe that these highly effective biopesticides still

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have a bright future in modern agriculture as public awareness and acceptance of GM organisms, including GM baculoviruses, increases.

I. INTRODUCTION

During the last century, the development of synthetic chemical insecticides and other advances have transformed agriculture from small, family-run operations to large, global-scale operations. With this dramatic increase in scale, damage by pest insects has surged, and this in turn has sometimes led to problems associated with the overuse (and in some cases unnecessary use) of pesticides. The primary problems associated with synthetic chemical pesticides include cost, detrimental effects on nontarget organisms, and the development of resistance. In terms of the natural control of insect populations, written records of pathogens that decimate insect populations have existed for centuries (Tanada and Kaya, 1993). More specifically, the application of insect pathogenic microorganisms has been an environmentally benign method of pest insect control. Baculoviruses, for example, have been used against insect pests of forests since the 1930s (Bird and Burk, 1961).

During the 1980s, Keeley and Hayes (1987), Maeda (1989), Menn and Borkovec (1989), Miller *et al.* (1983), and others developed the concept of genetically modifying the baculovirus to improve its endogenous insecticidal activity. Enthusiasm in both academia and industry quickly moved the concept from an "idea" to a fully developed product, a one of a kind bioinsecticide. Our laboratory has taken part in this process starting from the conceptual beginnings to the time when industry, at least in the United States, made the decision to abandon their efforts to register and implement this class of green insecticide. In this chapter, we summarize from a historical point of view recombinant baculovirus technology as it pertains to improving the endogenous insecticidal activity of the baculovirus. We also provide an analysis of what we believe are the most significant developments in the field and discuss how these developments might be implemented under the current status of the technology.

Several reviews have covered the use, development, and ecology of natural and genetically modified (GM) baculoviruses as biopesticides (Black *et al.*, 1997; Bonning and Hammock, 1996; Bonning *et al.*, 2002; Copping and Menn, 2000; Cory and Myers, 2003; Hammock *et al.*, 1993; Harrison and Bonning, 2000a; Inceoglu *et al.*, 2001; Kamita *et al.*, 2005a; McCutchen and Hammock, 1994; Miller, 1995; Wood, 1996).

II. BIOLOGY OF BACULOVIRUSES

Insect pathogenic viruses are classified into 12 viral families of which *Baculoviridae* is the most intensely studied (Blissard *et al.*, 2000; Tanada and Kaya, 1993). Baculoviruses are the most ubiquitous of the more than 20 known groups of insect pathogenic viruses. The baculovirus nucleocapsid is rod shaped and enveloped, and contains a single large, covalently closed, double-stranded DNA genome. Baculoviruses are classified into two genera, nucleopolyhedrovirus (NPV) and granulovirus (GV). The NPVs are further segregated into groups I and II based on the phylogenetic relationships of 20 distinguishing genes (Herniou *et al.*, 2001). The baculovirus produces two types of progeny, the budded virus (BV) and the occluded virus (OV) (Granados and Federici, 1986; Miller, 1997). The OVs of the NPV and GV are termed polyhedron (plural = polyhedra) and granule, respectively. Each granule occludes a single virion, whereas the polyhedron occludes multiple virions. Additionally, the NPV virion can contain a single (S morphotype) or multiple (M morphotype) nucleocapsids. BVs are produced during an early stage of infection as the nucleocapsid buds through the plasma membrane. BVs are responsible for the systemic or cell-to-cell spread of the virus within an infected insect. OVs are produced during a late stage of infection when the progeny nucleocapsids are directed to the nucleus (or maintained in the cytoplasm in the case of GVs), obtain an envelope, and are subsequently occluded. OVs are responsible for the horizontal or larva-to-larva transmission of the virus. Although relatively stable against environmental factors, the OVs are sensitive to the alkaline insect gut fluid that contains enzymes that break down the crystalline protein matrix, resulting in release of the occlusion-derived virions (ODVs). The extraordinary characteristic of producing two types of progeny (BVs and OVs) makes the baculovirus adept at swiftly infecting and taking over insect cells and then remaining dormant in the environment for extended periods of time following release from the dead host. Fortunately, not many mammalian viruses are as successful and prevailing as baculoviruses in infecting insects.

Baculoviruses are naturally found on leaves and in the soil. For example, a typical portion of cole slaw composed of 100 cm^2 of cabbage from an epizootic plot may contain around 1.12×10^8 OVs (Heimpel *et al.*, 1973). The NPV replication cycle begins when a susceptible host ingests a polyhedron or polyhedra resulting in the release of hundreds of ODVs in the gut. The released ODVs then pass through the peritrophic matrix and enter midgut cells. Following direct fusion to the midgut cell, the nucleocapsids are released, uncoat, and either initiate

viral replication in the cell or pass directly through the cell (Keddie *et al.*, 1989) and infect other cells such as tracheal epithelium cells, or enter the hemocoel (Bonning, 2005). About 5–7 days following the ingestion of NPVs (generally 7 to greater than 14 days in the case of GV infection), the infected host continues to feed and finally succumbs to the virus and dies. Just prior to death, the infected caterpillar exhibits enhanced locomotory activity that is activated by light (Kamita *et al.*, 2005b), a behavior that putatively enhances the dispersal of the virus (Cory and Myers, 2004; Goulson, 1997). Prior to death, the infected caterpillar also appears swollen due to the immense quantities of progeny baculoviruses that are produced. Genetic modification of baculoviruses has been performed almost exclusively using NPVs. This is because NPVs show faster speeds of kill in comparison to GVs, and continuous cell lines that support high-level production of progeny are available only for the NPVs. Detailed information on the biology of baculoviruses has been reviewed elsewhere (Bonning, 2005).

III. Baculoviruses as Insecticides

The single most important task in agricultural pest control is the ability to sustain pest insect population levels below the economic injury threshold in a cost-effective manner so that the costs of pest control operations justify the income generated. With this criterion, few biocontrol agents have so far been deemed successful. Baculoviruses have several inherent advantages as biological pesticides. They are naturally occurring pathogens that are highly specific to insects and closely related arthropods. They are safe in terms of pathogenicity against vertebrates (e.g., mammals, birds, fish, amphibians, reptiles). Moreover, they are benign in terms of pathogenicity against beneficial organisms that naturally suppress pest insect populations. Baculoviruses clearly play an important role in the natural control of insect populations. Despite a great diversity of viruses infecting insects, currently registered products are exclusively from the *Baculoviridae* family. Due to their inherent insecticidal activities, natural baculoviruses (both NPVs and GVs) have been registered, and successfully used as safe and effective biopesticides for the protection of field and orchard crops and forests in the Americas, Europe, and Asia (Black *et al.*, 1997; Copping and Menn, 2000; Hunter-Fujita *et al.*, 1998; Lacey *et al.*, 2001; Moscardi, 1999; Vail *et al.*, 1999). In the early 1970s, several natural baculovirus-based pesticides were available from commercial (Elcar, Spod-X, Cyd-X, and so on) and governmental (Gypcheck, TM BioControl-1, and Neocheck-S) sources (Black *et al.*,

1997). Elcar and Spod-X were based on NPVs that are pathogenic against the heliothines and *Spodoptera* spp., respectively, whereas Cyd-X was based on a GV that is pathogenic against the devastating codling moth. Gypcheck, TM BioControl-1, and Neocheck-S were produced by the US Forest Service and based on NPVs pathogenic against *Lymantria dispar, Orgyia pseudotsugata*, and *Neodiprion sertifer*, respectively, all excellent insecticides for forest ecosystems. Natural baculovirus-based biopesticides have been especially effective for the protection of soybean and forests in South and North America, respectively. In Brazil, an NPV that is pathogenic against the velvet bean caterpillar *Anticarsia gemmatalis* (AgMNPV), the major pest of soybeans, is used for the protection of over a million hectares of this crop (Moscardi, 1999). Although successfully used for soybean and forest protection, natural baculoviruses are imperfect insecticides when judged from an agroindustrial perspective. Many crops can tolerate only minimal fruit or foliar damage. With a natural speed of kill of 5 to greater than 14 days, natural baculoviruses are no match to synthetic pyrethroids, which kill within hours of exposure.

The slow speed of kill has been addressed by modifying the baculovirus using recombinant DNA technology. Baculoviruses are also susceptible to degradation by UV light and have short field stability potentially necessitating frequent applications. In the field, several baculovirus species may also need to be coapplied to control multiple pests because of the narrow host specificity of baculoviruses. Additionally, there are potential problems with high costs associated with production and limited shelf life. All of these potential problems have been addressed, or can easily be addressed, by current technologies. The safety of baculoviruses against nontargeted organisms has been demonstrated on multiple occasions (Cory and Hails, 1997). However, there is a clear need to gain wider public acceptance of GM baculovirus biopesticides. Appropriate governmental registration is also required prior to implementation. Clearly, implementation of GM baculovirus biopesticides will require an organized team effort that can address and solve multiple problems at the administrative, laboratory, and field levels.

IV. INTEGRATION OF IDEAS, RECOMBINANT BACULOVIRUSES FOR PEST CONTROL

The baculovirus offers several unique advantages as a vector for the expression of a foreign gene within insect cells and insect larvae. This potential was initially discovered during the 1980s with exciting research in the laboratories of Max Summers (Summers and Smith, 1987) and Lois Miller (1988) (see chapter by Summers, this

volume, pp. 1–73). In order to express high levels of protein, these researchers took advantage of the promoter of the polyhedrin gene (*polh*) to drive expression of the foreign gene. The two groups also utilized the product of *polh*, polyhedrin (the major protein found in polyhedra), as a visual selection marker to identify recombinant baculoviruses and used cultured insect cells to isolate the recombinant baculoviruses. Both the Summers and Miller laboratories used the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (Vail *et al.*, 1973, 1999) as the parental baculovirus for their baculovirus expression vector systems (BEVS). AcMNPV is the baculovirus type species and basic knowledge about the biology of AcMNPV was highly instrumental in the development of BEVS. The methods for the construction and use of recombinant baculoviruses for the expression of heterologous genes have been thoroughly described and are identical to those that were later used to generate recombinant baculovirus insecticides (Merrington *et al.*, 1999; O'Reilly *et al.*, 1992; Richardson, 1995; Summers and Smith, 1987).

Carbonell *et al.* (1988) were the first to attempt to improve the insecticidal activity of a baculovirus by expressing biologically active scorpion toxin, insectotoxin-1, of *Buthus eupeus*. Three recombinant AcMNPV constructs were generated that expressed the *BeIt* gene under control of the *polh* promoter. One of the constructs (vBeIt-1) carried only the *BeIt* coding sequence, whereas the other two carried fusions of *BeIt* and a human signal peptide (vBeIt-2) or a sequence coding for the 58 N-terminal amino acid residues of AcMNPV polyhedrin (vBeIt-3). All three constructs produced high levels of *BeIt*-specific transcripts, but only the vBeIt-3 construct produced significant amounts of peptide. Unfortunately, biological activity (BeIt-specific activity) was not detected in insect bioassays using larvae of *Trichoplusia ni, Galleria mellonella*, and *Sarcophaga* with any of the constructs.

Playing critical roles in the excretion and retention of water in insects, diuretic and antidiuretic hormones regulate insect responses to changes in their environment (Coast *et al.*, 2002; Gade, 2004; Holman *et al.*, 1990). Maeda (1989) was the first to integrate the contemporary knowledge and to successfully generate a recombinant baculovirus expressing a diuretic hormone gene that disrupted the normal physiology of larvae of the silkworm *Bombyx mori*. Maeda generated a synthetic gene encoding a 41-amino acid neuropeptide hormone of the tobacco hornworm *Manduca sexta* that was designed on the basis of the codon usage of *polh* of *Bombyx mori* nucleopolyhedrovirus (BmNPV). A signal sequence for secretion from a cuticle protein (CPII) of *Drosophila melanogaster* (Meigan) (Snyder *et al.*, 1982) was also included in this gene construct. The *polh*-negative strain of the NPV from *B. mori* was used to ensure biological

containment since resulting virus is unstable, of very poor oral activity, and is not known to infect wild hosts. By bioassays based on injection of BV into fifth instar larvae (as opposed to oral infection of earlier instars with polyhedra), Maeda (1989) showed that the recombinant virus (BmDH5) caused mortality about 1 day faster than the wild-type BmNPV. Although a roughly 20% improvement in speed of kill was obtained, biologically active DH was not detected in the hemolymph. This improvement in the speed of kill of BmDH5 is modest in comparison to more recent recombinant baculovirus constructs (see later), however, these studies established the groundwork for subsequent efforts. With the proof of concept by Maeda and his colleagues, the field accelerated by implementation of multiple approaches to kill insects faster using baculoviruses.

From 1989 to 1991, genes encoding juvenile hormone esterase (JHE) (Hammock et al., 1990a), Bt endotoxins (Martens et al., 1990; Merryweather et al., 1990), and eclosion hormone (EH) (Eldridge et al., 1991) were successfully expressed in recombinant baculoviruses. Of these recombinant viruses, only those expressing JHE showed an improvement in speed of kill. Our laboratory targeted JHE expression for improving the insecticidal activity of the baculovirus on the basis of two key points. First, we had established that the regulation of the titer of JH (a critical hormone for the regulation of insect development and behavior) was dependent on a JH-specific esterase and/or epoxide hydrolase (Hammock, 1985). At the time, our laboratory had more than 30 years worth of experience studying these enzymes. Second, the *jhe* gene had been cloned from the tobacco budworm *Heliothis virescens* (Hanzlik et al., 1989) and was available in our laboratory. Thus, it was only natural to engineer a recombinant, polyhedrin-positive AcMNPV that would secrete JHE into the insect hemolymph in order to disrupt the normal physiology of the insect. Using an authentic, insect-derived protein to combat the insect is conceptually elegant and potentially safer. Conceptually, this reduction in JH titer should halt insect feeding.

As anticipated, our first generation recombinant baculovirus carrying the *jhe* gene expressed biologically active JHE. Larvae of *M. sexta* and *H. virescens* infected with this virus showed reduced feeding and weight gain and subsequently died slightly more quickly in comparison to control larvae infected with the wild-type AcMNPV (Eldridge et al., 1992a; Hammock et al., 1990a,b). Although this was a promising beginning, we later understood that JHE is rapidly cleared from the hemolymph by pericardial cell uptake (Booth et al., 1992; Ichinose et al., 1992a,b). It is now clear that this removal process occurs by a receptor-mediated, endocytotic, saturable mechanism that does not

involve passive filtration (Bonning *et al.*, 1997a; Ichinose *et al.*, 1992a,b). Once the JHE is taken up by the pericardial cells, it is presumed to be directed to and degraded in lysosomes (Booth *et al.*, 1992). The unusually short half-life (measured in minutes) of JHE in the hemolymph is obviously a limiting factor in the insecticidal efficacy of JHE expressing recombinant baculoviruses. Several laboratories including ours are continuing to improve the *in vivo* stability of the overexpressed JHE. Although considerable effort went into expressing the JHE of the major target species, *H. virescens*, this may have been a mistake in retrospect. Some foreign proteins are quite stable when injected into caterpillars. Thus, a *trans*-specific JHE may be more active than the natural JHE of the target species.

The bacterium *Bacillus thuringiensis* (Bt) produces two major types of lepidopteran-active toxins (Aronson and Shai, 2001; Bravo *et al.*, 2005; Gill *et al.*, 1992; Schnepf *et al.*, 1998). During the early period of the development of recombinant baculoviruses for pest insect control, genes encoding the Bt protoxin were placed under a very late gene promoter and expressed by recombinant AcMNPVs (Martens *et al.*, 1990; Merryweather *et al.*, 1990). Although these recombinant AcMNPVs expressed high levels of the Bt protoxin that was subsequently cleaved in insect cells into the biologically active form, these recombinant AcMNPVs did not show improved insecticidal activity. Similar results were found in later studies that used AcMNPV (Martens *et al.*, 1995; Ribeiro and Crook, 1993, 1998) or *Hyphantria cunea* NPV (Woo *et al.*, 1998) to express biologically active Bt toxin. Considering that the site of action of the toxin is the midgut epithelial cell, these results may not be so unexpected. The use of alternative strategies in which the Bt toxin is expressed as a toxin-polyhedrin fusion that results in incorporation of the toxin into the polyhedron is discussed later. The Bt toxin also is an antifeedant. Thus, if the Bt toxin is expressed even at low levels in the polyhedron, then feeding could be reduced.

Eldridge *et al.* (1991, 1992b) hypothesized that the expression of EH at an inopportune time would induce the premature onset of eclosion and molting. Thus, a recombinant AcMNPV that expressed biologically active and secreted EH of *M. sexta* was generated. The recombinant AcMNPV, vEHEGTD, carried the *eh* gene at the ecdysteroid UDP-glucosyltransferase (*egt*) gene locus of AcMNPV. The serendipitous insertion of the *eh* gene into the *egt* locus would later prove to be uniquely advantageous as will be discussed later. Larvae of *Spodoptera frugiperda* that were injected with vEHEGTD showed median survival times ($ST_{50}s$) that were reduced by ~30% in comparison to

control larvae injected with AcMNPV, although this improvement was most likely attributed to the deletion of the *egt* gene.

V. A New Era in Recombinant Baculoviruses, Insect-Selective Peptide Toxins

Insect-specific toxins expressed and delivered by baculoviruses defined a new era in the field of recombinant baculovirus insecticides, beginning with the first successful results obtained by the expression of a paralytic neurotoxin from the insect predatory straw itch mite *Pyemotes tritici* (Tomalski et al., 1988, 1989). TxP-I induces rapid, muscle-contracting paralysis in larvae of the greater wax moth *G. mellonella* (Tomalski et al., 1988, 1989). Although the mode of action of TxP-I is unknown, it is selectively toxic to lepidopteran larvae at an effective dose of about 50 ng per larvae but it is not toxic to mice at a dose of 50 mg/kg. A recombinant, occlusion-negative AcMNPV (vEV-Tox34) carrying the TxP-I–encoding gene *tox34* under a modified polyhedrin promoter P_{LSXIV} (Ooi et al., 1989) was shown to paralyze or kill fifth instar larvae of *T. ni* by 2 days postinjection (Tomalski and Miller, 1991). Tomalski and Miller (1991, 1992) constructed other recombinant AcMNPVs that expressed the *tox34* gene under early (vETL-Tox34) or hybrid late/very late (vCappolh-Tox34) gene promoters. In bioassays, the ET_{50} of these recombinant AcMNPVs in neonates of *S. frugiperda* and *T. ni* was reduced by ~45% in comparison to control larvae infected with AcMNPV (Lu et al., 1996; Tomalski and Miller, 1992). One surprise from these experiments was that the yield of polyhedra was reduced by ~40% in comparison to AcMNPV-infected control larvae. The authors promptly suggested that this reduction in yield may cripple the virus in terms of its ability to compete effectively with the wild-type virus in the environment (Tomalski and Miller, 1992).

By the beginning of the 1990s, the field of insect toxinology was sparsely populated. However, scientists involved with isolation of toxins from venomous animals made a major discovery, the insect-selective toxins. At the time there were no particular applications for these astonishing peptides. *Androctonus australis* insect toxin (AaIT) was the first scorpion peptide toxin that was successfully expressed by baculoviruses (Maeda et al., 1991; McCutchen et al., 1991; Stewart et al., 1991). AaIT was originally isolated from the desert scorpion *Androctonus australis* by Eliahu Zlotkin's group (Zlotkin et al., 1971). Zlotkin et al. (2000) have extensively reviewed this superb toxin. The AaIT peptide is a 70 amino acid, highly folded peptide with four

disulfide bridges. The primary advantage of AaIT is its specificity for the insect voltage-gated sodium channel (and conversely lack of specificity against the mammal sodium channel) (Zlotkin *et al.*, 2000). The second advantage of AaIT is its potency. The potency of AaIT is at least 20-fold better than TxP-I, resulting in an effective concentration of several nanograms per insect larva. AaIT captured the scientific headlines for years as the best model peptide neurotoxin for improving the insecticidal activity of the baculovirus, although it is far more toxic to dipteran than to lepidopteran larvae.

Maeda *et al.* (1991) constructed a recombinant BmNPV carrying a synthetic *aait* gene (Darbon *et al.*, 1982) that was linked to a bombyxin signal sequence for secretion and placed under the *polh* gene promoter. Again, the *B. mori* host and virus were used for biological containment in early studies. This recombinant virus, BmAaIT, expressed biologically active AaIT that was detected in the hemolymph of BmAaIT-infected silkworm larvae. The BmAaIT-infected larvae displayed symptoms that were consistent with larvae that are injected with authentic, purified AaIT. These symptoms included body tremors and dorsal arching that are consistent with the blockage of the voltage-gated sodium channels. The BmAaIT-infected larvae ceased feeding and were paralyzed beginning at roughly 40 hours postinfection (h p.i.). By 60 h p.i., death was observed. This timing corresponds to a 40% improvement in speed of kill in comparison to control larvae infected with BmNPV.

The study by Maeda *et al.* (1991) opened the doors for the development of AaIT-expressing baculoviruses that target pest insects and two groups independently published the expression of AaIT under the very late baculoviral *p10* promoter in recombinant AcMNPVs. The two constructs, AcST-3 by Stewart *et al.* (1991) and AcAaIT by McCutchen *et al.* (1991) showed very similar efficacy with AcAaIT showing a slightly lower LD_{50} and being slightly faster in speed of kill (Fig. 1). However, such small changes in improved speed of kill could simply be due to experimental design. The defining parameter, ST_{50}, of AcAaIT-infected larvae was reduced by about 30% in comparison to control larvae infected with AcMNPV. AcAaIT is more efficient when administered by droplet feeding to the neonate larvae of *H. virescens*, with an improvement of about 45% in ST_{50} (Inceoglu and Hammock, unpublished data). By bioassay using third instar larvae of *M. sexta* (an unnatural host of AcMNPV), McCutchen *et al.* (1991) also observed that larvae infected with AcAaIT typically were paralyzed and stopped feeding many hours prior to death. The cessation of feeding, and consequent reduction in feeding damage, is important because it directly translates into increased pesticidal efficacy. The paralytic effect of AcAaIT was further
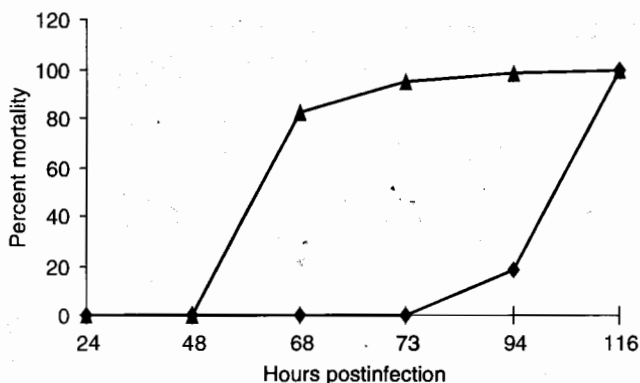
FIG 1. Time–mortality curves of wild-type AcMNPV (◆) or recombinant AaIT-expressing AcMNPV, AcAaIT (▲). The speed of kill of AcAaIT is about 40% faster than the wild-type counterpart in neonate *H. virescens*.

characterized by Hoover *et al.* (1995) using third instar *H. virescens*. They found that AcAaIT-infected larvae fall from the plant 5–11 h before death, much earlier than larvae infected with wild-type AcMNPV. Since this "knockoff" effect occurred before the induction of feeding cessation, the amount of leaf area consumed by the AcAaIT-infected larvae was 60–70% less than that consumed by AcMNPV- or mock-infected larvae. Thus, one of the key conclusions of Hoover *et al.* was that the survival time should not be the sole quantitative measure to assess the efficiency of the recombinant viruses. The increased efficiency of AaIT-expressing baculoviruses due to knockoff effects was further observed in field trials by Cory *et al.* (1994) and Sun *et al.* (2004). Another implication of the knockoff effect as pointed out by Cory *et al.* (1994) and Hoover *et al.* (1995) is that larvae falling off the plants early would lead to reduced foliage contamination because the wild-type virus-infected larvae tend to remain and die on the plant. This is yet another competitive disadvantage of the recombinant virus ensuring GM virus titer will be quickly reduced in the field.

VI. ERA OF MULTILATERAL DEVELOPMENT

By the mid 1990s, the recombinant baculovirus field had attracted still more attention and as would be expected expanded in multiple directions. Figure 2 depicts the improvements in speed of kill of

Improvement over wild type (%)

70
60
50
40
30
20
10
0

WT, BeIt, Bt, PTTH, EH, JHE, Enhancin, DTX9.2, Gelatinase, orf603D, Chitinase, DH, c-MYC, TalTX-1, PBAN, JHE-KK, AaIT, egtD + AaIT, egtD, LqhIT1/LqhIT2, LqhIT2, URF13, egtD + tox34, cv-PDG, Cathepsin L, As II, Sh I, μ-Aga-IV, LqhIT2(ie1), AaIT + pyrethroid, tox34.4, Pol + Bt
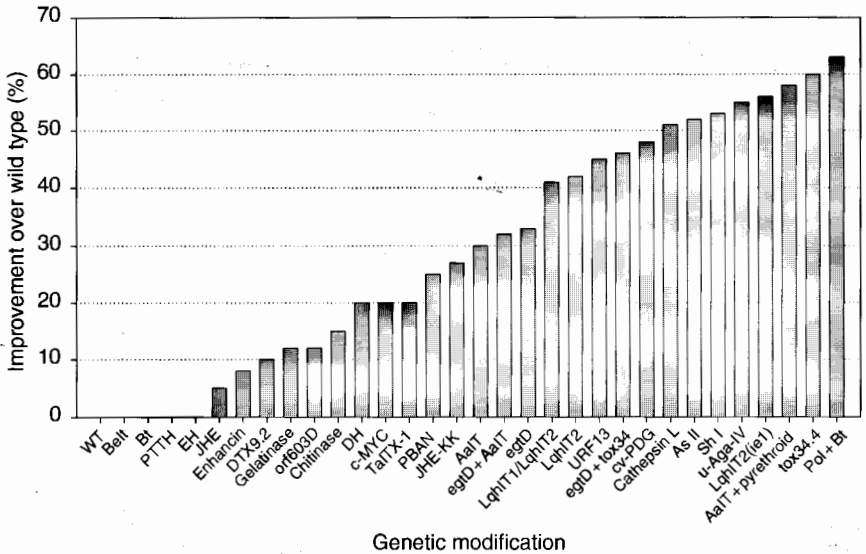
Genetic modification

FIG 2. The speed of kill of the wild-type baculovirus can be dramatically improved by genetic modification. The genetic modification (insertion of a foreign gene or deletion of an endogenous gene) and percent improvement in speed of kill (or paralysis) relative to the wild-type virus or control virus is given. Because of differences in the parent virus, promoter, secretion signal, host strain and age, virus dose, and inoculation methods that were used, comparison between the different virus constructs is not possible. Abbreviations and reference(s): WT, wild type; BeIt, insectotoxin-1 (Carbonell *et al.*, 1988); Bt, *Bacillus thuringiensis* endotoxin (Merryweather *et al.*, 1990); PTTH, prothoracicotropic hormone (O'Reilly *et al.*, 1995); EH, eclosion hormone (Eldridge *et al.*, 1992b); JHE, juvenile hormone esterase (Hammock *et al.*, 1990a); enhancin, MacoNPV enhancin (Li *et al.*, 2003); DTX9.2, spider toxin (Hughes *et al.*, 1997); gelatinase, human gelatinase A (Harrison and Bonning, 2001); orf603D, deletion of AcMNPV *orf603* (Popham *et al.*, 1998); chitinase (Gopalakrishnan *et al.*, 1995); DH, diuretic hormone (Maeda, 1989); c-MYC, transcription factor (Lee *et al.*, 1997); TalTX-1, spider toxin (Hughes *et al.*, 1997); PBAN, pheromone biosynthesis-activating neuropeptide (Ma *et al.*, 1998); JHE-KK, stabilized JHE (Bonning *et al.*, 1997b); AaIT, scorpion *Androctonus australis* insect toxin (McCutchen *et al.*, 1991; Stewart *et al.*, 1991); egtD + AaIT, insertion of *aait* at the *egt* gene locus (Chen *et al.*, 2000); egtD, deletion of ecdysteroid UDP-glucosyltransferase (*egt*) gene (O'Reilly and Miller, 1991); LqhIT1/LqhIT2, simultaneous expression LqhIT1 and LqhIT2 (Regev *et al.*, 2003); LqhIT2, scorpion *Leiurus quinquestriatus* insect toxin 2 (Froy *et al.*, 2000); URF13, maize pore-forming protein (Korth and Levings, 1993); egtD + tox34, insertion of *tox34* under the early *DA26* promoter at the *egt* gene locus (Popham *et al.*, 1997); cv-PDG, glycosylase (Petrik *et al.*, 2003); cathepsin L (Harrison and Bonning, 2001); As II, sea anemone toxin (Prikhodko *et al.*, 1996); Sh I, sea anemone toxin (Prikhodko *et al.*, 1996); μ-Aga-IV, spider toxin (Prikhodko *et al.*, 1996); LqhIT2 (ie1), LqhIT2 under the early *ie1* promoter (Harrison and Bonning, 2000b); AaIT + pyrethroid, coapplication of low doses of AcAaIT and pyrethroid (McCutchen *et al.*, 1997); tox34.4, mite toxin (Burden *et al.*, 2000; Tomalski *et al.*, 1988); Pol + Bt, double expression of authentic polyhedrin and polyhedrin–Bt–GFP fusion proteins (Chang *et al.*, 2003) (from Kamita *et al.*, 2005a).

recombinant baculoviruses. One major direction was the identification of new insect-selective toxins with greater lepidopteran potency that could enhance speed of kill. An extension of this research direction was the development of methodologies to improve the level and timing of toxin expression, and as such crucial and significant advances in the understanding of baculovirus promoters were realized. Although numerous studies have uniformly concluded that insect-selective toxins are not dangerous, the use of genes encoding insect regulatory genes was viewed as a safer alternative to the expression of insect-selective toxins. Thus, another line of academic effort was directed toward the expression of regulatory insect hormones and enzymes with improved *in vivo* stability. This approach later expanded to include protease, chitinase, gelatinase, and other enzymes. Another simple but effective line of research was directed at deletion of endogenous baculovirus genes that kept the host insect feeding. The basis for this approach is that the baculovirus encodes genes that help the larvae to generate more mass (i.e., keep the larva feeding) such that there are more resources available for generating viral progeny. Thus, by removing these genes, the larva should prematurely stop feeding.

A. Improved Toxins

The rapid expansion of the recombinant baculovirus biopesticide field was driven by the early and significant success of the use of insect-selective toxins to improve the speed of kill. The process starts with mining the venom of scorpions, spiders, wasps, and other venomous animals to identify and characterize insect-selective peptides. As a natural and abundant source, arthropod-derived peptide toxins are still the first choice for this purpose. The corresponding genes of these peptide toxins are then transferred to baculovirus genomes under the control of a variety of promoters ranging from early/weak to very late/ very strong expression characteristics.

The venoms of arthropods, such as scorpions, spiders, and parasitic wasps, are composed of a mixture of salts, small molecules, proteins, and peptides that are used to rapidly immobilize prey (Gordon *et al.*, 1998; Loret and Hammock, 1993; Possani *et al.*, 1999; Zlotkin, 1991; Zlotkin *et al.*, 1978, 1985). Over the years, most characterized toxins have been shown to act on major ion channels such as the Na^+, K^+, Ca^{2+}, and Cl^- channels. These channels are convenient and efficient targets for peptides because their blockage generally results in immediate paralysis. Initially, insect-selective toxins were separated into two classes based on the symptoms they produced when injected into fly larvae.

Excitatory toxins, such as AaIT from the North African scorpion *A. australis*, cause paralysis that is immediate and contractive (Zlotkin, 1991; Zlotkin *et al.*, 1971, 1985). On the other hand, depressant toxins, such as LqhIT2 from the yellow Israeli scorpion *Leiurus quinquestriatus hebraeus*, cause transient (e.g., until 5 min postinjection) contractive paralysis, followed by sustained, flaccid paralysis (Zlotkin, 1991; Zlotkin *et al.*, 1985). This classification upheld after the amino acid sequences of these peptides became available.

The popular insect-selective toxin AaIT continued to be the subject of numerous studies once it was established as a potent model peptide toxin with the pioneering work of Maeda *et al.* (1991). The *aait* gene expressed under the control of various promoters has been inserted into several baculovirus vectors, including the NPVs of the mint looper *Rachiplusia ou* (RoMNPV) (Harrison and Bonning, 2000b), cotton bollworms *Helicoverpa zea* (HzNPV) (Treacy *et al.*, 2000), and *H. armigera* (HaSNPV) (Chen *et al.*, 2000; Sun *et al.*, 2002, 2004). Insertion of *aait* into the baculovirus genome resulted in moderate to dramatic improvement in speed of kill. For example, the expression of *aait* under the late *p6.9* promoter of AcMNPV by a recombinant RoMNPV resulted in 34%, 37%, and 19% improvements in speed of kill in comparison to control larvae infected with RoMNPV when tested on neonates of *O. nubilalis, H. zea*, and *H. virescens*, respectively (Harrison and Bonning, 2000b).

The yellow Israeli scorpions *L. quinquestriatus hebraeus* and *L. quinquestriatus quinquestriatus* have also been popular sources of highly potent insecticidal toxins. The venoms of these scorpions contain both excitatory (e.g., LqqIT1, LqhIT1, and LqhIT5) and depressant (e.g., LqhIT2 and LqqIT2) insect-selective toxins (Kopeyan *et al.*, 1990; Moskowitz *et al.*, 1998; Zlotkin, 1991; Zlotkin *et al.*, 1985, 1993). Gershburg *et al.* (1998) have generated recombinant AcMNPVs expressing the excitatory LqhIT1 toxin under the very late *p10* and early *p35* gene promoters and the depressant LqhIT2 toxin under the *polh* gene promoter. These recombinant AcMNPVs show improvements in the speed of kill of up to 32% in comparison to AcMNPV. In similar experiments by Harrison and Bonning (2000b), the expression of LqhIT2 fused to a bombyxin signal sequence under the late *p6.9* or very late *p10* gene promoters resulted in ~34% decrease in median survival times compared to control larvae in neonate *H. virescens* larvae. Expression under these two promoters results in equal efficiency in terms of survival times. Similarly, when LqhIT2 gene constructs were expressed in recombinant RoMNPVs under the control of the *p6.9* or *p10* promoters of AcMNPV, these recombinants also showed similar

improvements ($\sim$40%) in speed of kill in comparison to the wild-type virus when larvae of the European corn borer *O. nubilalis* Hübner and *H. zea* were used for bioassay (Harrison and Bonning, 2000b).

The expression of the insect-selective spider toxins μ-Aga-IV from *Agelenopsis aperta* (Prikhodko et al., 1996), and DTX9.2 and TalTX-1 from the spiders *Diguetia canities* and *Tegenaria agrestis* (Hughes et al., 1997) all result in improved speeds of kill. Likewise, two insect-selective toxins As II and Sh I from the sea anemones *Anemonia sulcata* and *Stichadactyla helianthus* resulted in 38% and 36% improvements in speed of kill in neonate *T. ni* and *S. frugiperda* larvae. Korth and Levings (1993) used their available toxin, URF13, from maize to improve the speed of kill of the baculovirus. URF13 is a mitochondrially encoded protein from maize that forms pores in the inner mitochondrial membrane (Korth et al., 1991). Two recombinant occlusion-negative AcMNPVs expressing authentic or mutated URF13-encoding genes under the *polh* promoter were generated. When larvae of *T. ni* were injected with either of these viruses, all died by 60 h postinjection, however, this $\sim$45% improvement in speed of kill apparently was not linked to the ability of the URF13 to form pores. The mechanism of this improved speed of kill appeared to involve interference of normal cellular functions (Korth and Levings, 1993).

1. Synergy Between Toxins and with Pyrethroids

The molecular target of a broad range of neurotoxins is the voltage-gated sodium channel. The sodium channels of insects and mammals are composed of at least six distinct receptor sites. The insect sodium channel has at least two additional receptors sites that are the molecular targets of insect-selective excitatory and depressant scorpion toxins (Cestele and Catterall, 2000). Several studies (Cestele and Catterall, 2000; Gordon et al., 1992; Zlotkin et al., 1995) have shown that depressant scorpion toxins bind to two noninteracting-binding sites (one showing high affinity and the other low affinity) on the insect sodium channel. The excitatory toxins bind only to the high-affinity receptor site (Gordon et al., 1992). Herrmann et al. (1995) were the first to show that when excitatory and depressant toxins are simultaneously coinjected into larvae of the blowfly *Sarcophaga falculata* or *H. virescens*, the amount of toxin required to give the same paralytic response is reduced 5- to 10-fold in comparison to the amount required when only one of the toxins is injected. On the basis of this synergism, they suggested that the speed of kill of recombinant baculoviruses could be further increased by coinfecting with two or more recombinant baculoviruses each expressing toxin genes with synergistic

properties or by simultaneously expressing two or more synergistic toxin genes. This hypothesis was tested by Regev *et al.* (2003) when they generated a recombinant AcMNPV (vAcLqIT1-IT2) that expressed both the excitatory LqhIT1 and depressant LqhIT2 toxins under the very late *p10* and *polh* promoters, respectively. Time-response bioassays (at an LC_{95} dose) using neonate *H. virescens* showed that the ET_{50} of vAcLqIT1-IT2 is reduced by roughly 20% in comparison to recombinant AcMNPV expressing each toxin alone or by 40% in comparison to the wild-type AcMNPV. Similar or decreased levels of synergism were found with a recombinant AcMNPV (vAcLqαIT-IT2) expressing both excitatory and depressant scorpion toxins in orally and hemocoelically infected larvae of *H. virescens, H. armigera*, and *S. littoralis.*

In addition to being the target of insect-selective scorpion toxins, the voltage-gated sodium channel is also the major target of the well known and commonly used pyrethroid class of insecticides. Although AaIT induces a neurological response similar to that evoked by the pyrethroid insecticides, the binding site on the channel of AaIT and the pyrethroids do not overlap. In fact, AaIT and other scorpion toxins that act on the voltage-gated sodium channel are synergized by pyrethroids *in vivo* allowing both recombinant baculoviruses expressing insect-selective scorpion toxins and pyrethroids to be used simultaneously (McCutchen *et al.*, 1997). Such combinations could be useful in the field. The heliothine complex is often the most resistant of the target pests to pyrethroids. If this complex can be controlled by a recombinant baculovirus, then much lower pyrethroid rates in a tank mixture can be used to control secondary pests. These lower levels of pyrethroid may be adequate to synergize the recombinant baculovirus.

2. *Improving Expression*

Following experiments that showed that the expression of the *tox34* gene from the predatory mite *P. tritici* improves insecticidal efficacy of AcMNPV, Miller's group attempted to improve TOX34 expression by placing the *tox34* gene under the late *p6.9* gene promoter. The recombinant AcMNPV (vp6.9tox34) that carried the *tox34* gene under the *p6.9* promoter expressed TxP-I at least 24 h earlier compared to expression of the *tox34* gene under the very late *p10* gene promoter. Higher toxin yield was also obtained by the earlier expression of TxP-I under the *p6.9* promoter. Lu *et al.* (1996) analyzed the occlusion-positive vp6.9tox34 virus at an LC_{95} dose and showed that the ET_{50} of this virus in neonate larvae of *S. frugiperda* and *T. ni* was reduced by nearly 60% in comparison to AcMNPV. This represents

20–30% faster paralysis in comparison to expression under the very late gene promoter. Furthermore, a variant of the TxP-I–encoding gene was recloned and expressed by Burden *et al.* (2000) under the *p10* promoter. The recombinant virus expressing this gene showed a similar reduction in the mean time to death and showed 85–95% lower yields of polyhedra per unit weight in comparison to control larvae infected with wild-type AcMNPV.

3. Delivery of Bt Toxins

As discussed earlier, high levels of biologically active Bt toxin are produced in insect cells by recombinant baculoviruses. However, recombinant baculoviruses expressing Bt toxin showed no improvement in virulence or decrease in the ST_{50} (Martens *et al.*, 1990; Merryweather *et al.*, 1990). This lack of efficacy most likely resulted from the site of action of Bt toxins on the surface of the midgut epithelial cell, whereas the recombinant Bt protoxin was present in the cytoplasm of cells within the insect body. Chang *et al.* (2003) have overcome the problems associated with efficiently delivering the Bt toxin genes using recombinant baculoviruses. They expressed the Bt toxin as the fusion product, polyhedrin–Cry1Ac–green fluorescent protein (GFP), in which the toxin is fused with both the polyhedrin and GFP proteins using trypsin-sensitive linkers. The recombinant AcMNPV (ColorBtrus) expressing this fusion product as well as authentic polyhedrin produced polyhedra that occlude Bt toxin and GFP, and released toxin and GFP proteins in the insect midgut. Bioassays using second or third instar larvae of the diamondback moth, *Plutella xylostella*, showed that the LD_{50} of ColorBtrus was reduced 100-fold and the ST_{50} was reduced by 60% in comparison to control larvae infected with AcMNPV (Chang *et al.*, 2003).

B. Improved Enzymes

1. JHE Stability

The speed of kill of AcMNPV was improved by about 20% by the expression of an insect-derived juvenile hormone-specific esterase (JHE) (Hammock *et al.*, 1990a). Following the initial observation of the short half-life of recombinant JHE that is injected into the hemolymph (Ichinose *et al.*, 1992b), we have improved the *in vivo* stability of JHE by mutating two lysine residues belonging to an amphipathic helix of the JHE of *H. virescens*. The mutant protein (JHE-KK) is more stable because of decreased lysosomal targeting resulting in reduced

removal and/or degradation from the hemolymph (Bonning *et al.*, 1997b). Bioassays using first instar larvae of *H. virescens* or *T. ni* showed that insects infected with AcJHE-KK (AcMNPV expressing mutant JHE-KK under a strong very late viral promoter) died ~20% faster than control larvae infected with a recombinant AcMNPV expressing the authentic JHE (AcJHE) (Bonning *et al.*, 1999). However, in older instars the ST_{50} of AcJHE-KK–infected insects was only marginally reduced (Bonning *et al.*, 1999; Kunimi *et al.*, 1997).

2. Protease Expression

The baculovirus faces several barriers within the insect midgut. The various pathways by which infectious virions circumvent the midgut are illustrated by Bonning (2005). The final midgut-associated barrier to systemic infection is the basement membrane (BM) or basal lamina, a fibrous matrix composed primarily of glycoproteins, type IV collagen, and laminin that are secreted by the epithelial cells (Ryerse, 1998) with functions including structural support, filtration, and differentiation (Yurchenco and O'Rear, 1993). Harrison and Bonning (2001) have constructed recombinant AcMNPVs expressing three different proteases (rat stromelysin-1, human gelatinase A, and cathepsin L from the flesh fly *Sarcophaga peregrina*) that are known to digest BM proteins. Among these recombinant baculoviruses, the one expressing cathepsin L under the late baculovirus *p6.9* gene promoter generates a 51% faster speed of kill in comparison to AcMNPV in neonate larvae of *H. virescens*. So far, expression of BM-degrading proteases is one of the most impressive improvements in speed of kill of recombinant baculoviruses.

3. Other Enzymes

Petrik *et al.* (2003) have generated a recombinant AcMNPV, vHSA50L, that expresses an algal virus pyrimidine dimer-specific glycosylase, cv-PDG (Furuta *et al.*, 1997), that is involved in the first steps of the repair of UV-damaged DNA in an attempt to reduce UV inactivation of baculoviruses. Sunlight is known to be a major factor in the inactivation of baculoviruses in the field (Black *et al.*, 1997; Dougherty *et al.*, 1996; Ignoffo and Garcia, 1992; Ignoffo *et al.*, 1997). Although the polyhedra of vHSA50L showed no differences in UV inactivation in comparison to AcMNPV, the BV of vHSA50L were threefold more resistant. Bioassays showed that the LC_{50}s of vHSA50L and AcMNPV were significantly different in neonates of *S. frugiperda* (16-fold lower) but not *T. ni*. Consistent with the reduction in LD_{50}, LT_{50} of vHSA50L in neonates of *S. frugiperda* was reduced by ~40%.

C. Gene Deletion

Deleting an endogenous gene that results in improved speed of kill is a simple and elegant approach to improving insecticidal efficacy of the baculovirus. However, in practice, decreases in yields of the viral progeny may be a limitation of this approach. Ecdysteroids are key hormone molecules that regulate larval–pupal molting and other physiological events. Thus, the prevention of their action results in the interruption of growth or causes abnormal development and potentially death. A baculovirus-encoded enzyme called ecdysteroid UDP-glucosyltransferase (EGT) catalyzes the conjugation of sugar molecules to ecdysteroids, a process that renders the ecdysteroid inactive (O'Reilly, 1995; O'Reilly and Miller, 1989). The baculovirus gene (*egt*) that encodes EGT is found in ~90% of baculovirus genomes that have been characterized (Clarke *et al.*, 1996; Tumilasci *et al.*, 2003). Despite this wide presence, the *egt* gene is not essential for either *in vitro* or *in vivo* replication of AcMNPV (O'Reilly and Miller, 1989, 1991). Infection of larvae of *S. frugiperda* or *T. ni* with vEGTDEL, an *egt* deletion mutant of AcMNPV, gave rise to earlier mortality and reduced feeding damage (by about 40%) in comparison to AcMNPV infection (Eldridge *et al.*, 1992a; O'Reilly and Miller, 1991; Wilson *et al.*, 2000). A concurrent reduction in progeny virus yield was also found. In general, the deletion of *egt* gene homologs from the NPV genome resulted in none to moderate improvements in the speed of kill (e.g., Chen *et al.*, 2000; Pinedo *et al.*, 2003; Popham *et al.*, 1997, 1998; Slavicek *et al.*, 1999; Treacy *et al.*, 1997, 2000). This improvement in speed of kill was also dependent on the larval stage that was used for the bioassay (Bianchi *et al.*, 2000; Sun *et al.*, 2004). The *egt* minus virus was used in early field tests in Oxford, England, as an example of a virus that had a small improvement in efficacy but no novel gene added.

D. Choice of Parental Strain

There are several wild-type viruses that are infectious against a variety of pest species that can be used as the parental strain for genetic manipulations. It is possible to use a wild-type virus with strict host specificity to generate a highly host-selective insecticide, or conversely start with a baculovirus with a wider host range. This choice is also very likely to be based on the target pest. Furthermore, once a wild-type virus is selected, it is feasible to initiate or continuously implement a screening effort to isolate a natural mutant that is faster than the parent by conducting time-mortality bioassays. It should be noted

though that a laboratory bioassay primarily focused on speed of kill might not reveal undesirable characteristics that may later hinder industrial manufacturing. However, early investment in such a screening effort may easily translate to advantages during the commercialization phase as exemplified by the discovery of a more virulent HzSNPV strain by DuPont scientists (Dr. L. Flexner, personal communication).

VII. Implementation of a Recombinant Baculovirus Insecticide

A. Field Trials

As improvements in the potency of baculoviruses were realized, the efficacy of these constructs was tested in the field. The earliest field trials of a GM baculovirus (occlusion-negative AcMNPVs carrying junk DNA or a *lacZ* marker gene) were performed in England during the mid to late 1980s before the faster killing recombinants became available (Black *et al.*, 1997; Levidow, 1995). In the United States, the first field trial (a 3-year study) of a GM baculovirus (a *polh* gene-deleted AcMNPV that was co-occluded with wild-type AcMNPV) had begun in 1989 (Wood *et al.*, 1994). The early field trials were designed to determine the environmental persistence of the virus. These trials revealed that the persistence of occlusion-negative constructs is exceptionally low. This, however, is less relevant from a practical point of view because occlusion-negative constructs are not likely to be applied in the field. In 1993, Cory *et al.* conducted the first field trial to test the efficacy of an occlusion-positive recombinant baculovirus that was expected to have a dramatically improved speed of kill. The AcMNPV construct that expressed AaIT, that is, AcST-3 was used for these studies (Cory *et al.*, 1994). The results of these field trials were in agreement with the laboratory experiments. Cabbage plants treated with AcST-3 and artificially inoculated with third instar larvae of *T. ni* showed 23–29% lower feeding damage in comparison to wild-type AcMNPV-treated control cabbage plants. However, the reduction in feeding damage ($\sim$50% reduction) was not as impressive as observed in the laboratory trials (Stewart *et al.*, 1991). On further investigation, Cory *et al.* (1994) determined that this resulted from a tenfold lower yield of AcST-3 progeny in comparison to AcMNPV. These studies also revealed a key biological observation, namely that the AcST-3–treated larvae were knocked off the host plant. The knockoff effect is perhaps more important than the speed of kill because larvae that are not on the plant cannot feed on the plant. Second, if the recombinant

baculovirus is unable to propagate as efficiently as the wild-type virus, it will be outcompeted by the wild-type virus, resulting in an additional layer of safety. In China, multi-year-long field trials have been conducted to test the ability of an occlusion-positive *Helicoverpa armigera* NPV carrying the *aait* gene at the *egt* gene locus (HaSNPV-AaIT) to protect cotton (Sun *et al.*, 2002, 2004). These field trials showed that the yield of cotton in HaSNPV-AaIT treated plots was nearly 20% higher in comparison to wild-type HaSNPV-treated plots and similar to plots treated with chemical insecticides such as λ-cyhalothrin, endosulfan, and β-cypermethrin.

All of the field trials to date indicate that GM baculoviruses are safe and effective biological pesticides that can compete with chemical pesticides in terms of protection from pest insects and maintenance of crop yields (Fig. 3) (Kamita *et al.*, 2005a). These trials also show that
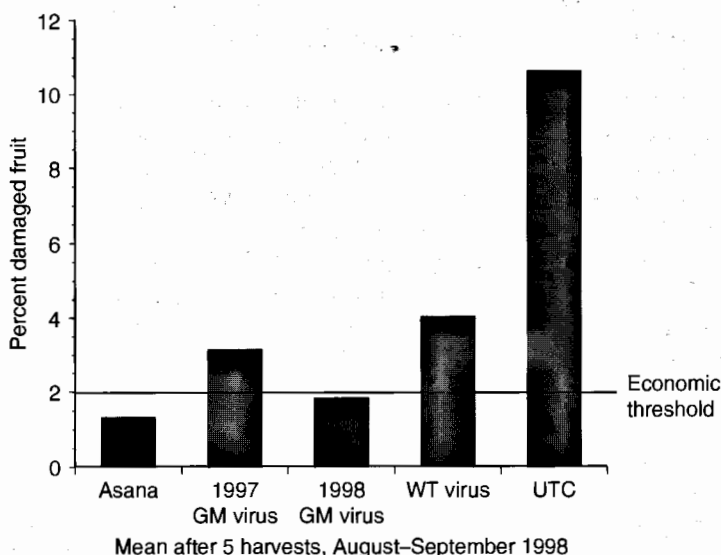

Mean after 5 harvests, August–September 1998

FIG 3. Tomato field trial evaluation from North Carolina (conducted by DuPont, United States). Untreated (UTC) or wild-type baculovirus-treated (WT virus) tomato plots received extensive feeding damage that was well above the economic injury threshold. The application of a synthetic pyrethroid (Asana, esfenvalerate) protected the tomato plants against insect damage. Whereas the first generation of GM baculovirus (1997 GM virus) did not profitably control against pest damage, further improvements (e.g., new parental strain, early promoter, transactivator technology, improved formulation) in the second generation (1998 GM Virus) rendered these GM baculoviruses competitive with synthetic pyrethroid treatment.

GM baculoviruses will quickly disappear from the environment and will have very little to no adverse effects against beneficial insects.

B. Production

A detailed discussion of the numerous issues regarding the commercialization of GM baculovirus insecticides including marketing, *in vivo* and *in vitro* production, formulation, storage, and public acceptance can be found elsewhere (Black *et al.*, 1997). Here, we will briefly discuss a key component of a successful biological control program: the production system. Currently, two choices are available for the production of GM baculovirus insecticides: *in vivo* production using field or insectary-reared insects and *in vitro* production through fermentation of insect cells. Production will most likely become a major factor contributing to the cost of a GM baculovirus insecticide. Therefore, the choice of *in vivo* versus *in vitro* manufacturing is undoubtedly important. *In vitro* systems are sterile, easily scalable, and afford higher predictability in product yield in addition to having the flexibility of allowing one to regulate gene expression. The end product is relatively pure and the process is inexpensive at high volumes. The maintenance of sterility, however, is a disadvantage with microbial contamination being an obvious pitfall for cell culture. The *in vitro* system requires higher initial capital and is expensive at low volumes. Appropriate cell lines that can easily be cultured in large-scale bioreactors may not be available for some key insect viruses. *In vivo* methods on the other hand afford much lower initial investment and have significantly lower operational costs. This is well exemplified by the production of baculoviruses in Brazil where the process occurs in the field. In this case sterility is less of an issue. The system, although labor intensive, involves a relatively simple operation and it is a well-proven production method for several viruses. The disadvantages of *in vivo* production include the necessity to reliably maintain and rear sufficient quantities of host insects, the occurrence of disease in the colony, limitations in scalability, impurity of the final product, and the smaller scale of the operation. An *in vivo* system may also suffer from the lack of flexibility in the regulation of gene expression. Nonetheless, as technologies advance, solutions to the limitations of both systems are likely to emerge (Fig. 4).

Data from field trials conducted by DuPont scientists clearly show that the expression of an insect-selective toxin reduces yield of virus and may have other effects on the final product (Dr. L. Flexner, personal communication). In order to circumvent these effects, DuPont scientists
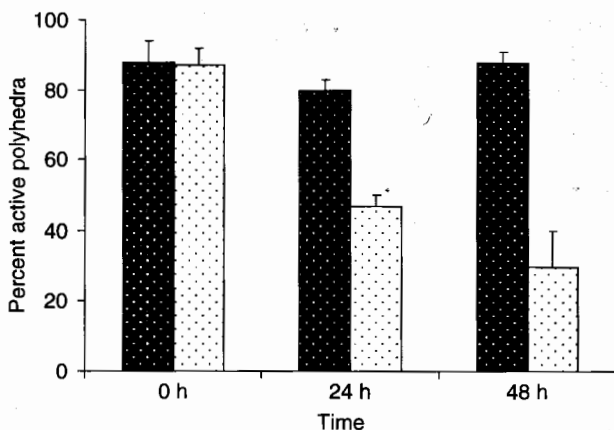
Fɪɢ 4. Improving field stability through formulation research. Polyhedra of HzSNPV formulated with titanium dioxide coaccervation and spray dried remain viable significantly longer (white bar) than the unformulated product (black bar) (figure provided by Dr. L. J. Flexner, DuPont).

have exploited the advantages of a two-phase system by incorporating an "on–off" switch based on a tetracycline transactivator gene placed into the genome of the host cells (McCutchen, US Patent No. 6322781). Briefly, the transgenic host line (insects or cultured cells) produces a protein that is able to suppress expression of the toxin gene under the control of a hybrid promoter in the recombinant baculovirus genome that regulates toxin expression. In the presence of tetracycline (i.e., during the production phase), the recombinant toxin gene is silent and infection results in normal yields of progeny virus. However, the toxin gene becomes active as soon as tetracycline is withdrawn (i.e., within the pest insect host) and the toxin gene is expressed. McCutchen disclosed that the LT_{50} of recombinant baculoviruses expressing the LqhIT2 toxin under *ie-1* promoter was increased by 45–60% under the control of transactivator repression, essentially resulting in the production of normal numbers (i.e., equivalent to the wild-type virus) of polyhedra by the recombinant virus (Fig. 5).

C. Technology Stacking

The expectation of an ideal insect control agent is that it should cost effectively and specifically kill the pest insect within several hours of application. Recombinant baculovirus technology is thoroughly
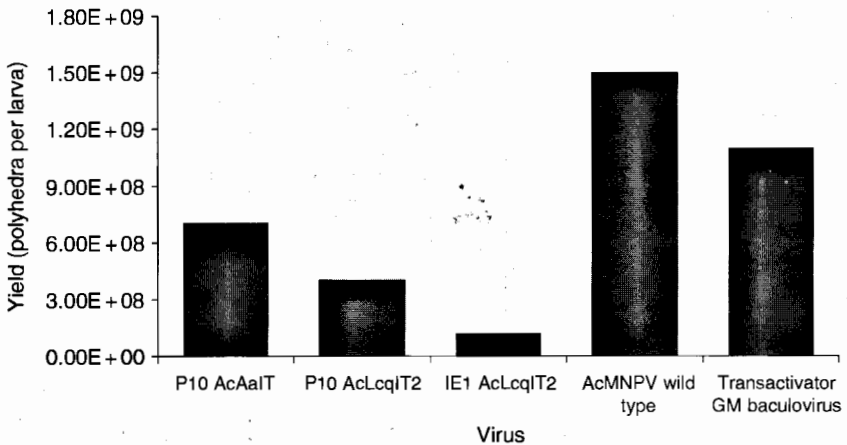
FIG 5. The yield of insect-selective toxin expressing baculovirus in the absence or presence of a tetracycline-controlled transactivator system. The speed of kill of the recombinant baculovirus is inversely correlated with the yield of polyhedra; the wild-type virus produces the highest number of polyhedra per larvae. By turning off toxin expression during the production phase, the yield of polyhedra dramatically improves (figure provided by Dr. L. J. Flexner).

validated and the speed of kill is approaching that of synthetic insecticides in the latest field trials. Improving the technology continuously should lead to faster and more efficient recombinant baculoviruses. As reviewed previously and here, there have been many advances to generate recombinant baculoviruses that kill insects considerably faster than the wild-type parent baculovirus. Therefore, the technology to generate a single baculovirus incorporating numerous exciting developments already exists, although this has not yet been attempted. In this section, we will summarize a "dream" baculovirus, one that theoretically includes several modifications so as to make it competitive against synthetic insecticides.

The dream recombinant baculovirus undoubtedly requires an efficient backbone, the baculovirus genome. This genome is required to contain at least all of the essential genes for replication but may also be supplemented with genes that increase the resistance of the virus to environmental effects such as sunlight. A smaller genome may theoretically result in a faster rate of replication in the host insect and may reduce the fitness of the recombinant, which are both desirable qualities. The deletion of the *egt* gene alone, for example, has been shown to make a faster killing recombinant baculovirus. As an alternative, one

can even exclude some essential genes from the genome of the ultimate baculovirus and compensate these by providing the products of these genes in the manufacturing phase. An immediate example would be the production of a *polh*-deficient virus under the control of the tetracycline switch so that the recombinant baculovirus is packed within polyhedra when being produced but cannot form OVs in the field. This concept of supplementing genes can further be extended to other genes including the recombinant toxin genes where expression of the toxic protein can be switched off in the production phase as exemplified by DuPont technology. An obvious industrial advantage is that this *polh*-negative virus would have low environmental persistence and would need to be reapplied, making it more attractive to the manufacturers. It will be important then to assess the ability to manufacture a crippled virus profitably because these approaches commit industry to a more difficult multistage production process. Undoubtedly, many baculovirus genes are not needed for viral growth and host kill. Potentially large sections of the baculovirus genome can be removed for use of a baculovirus as a green insecticide, although this procedure would be deleterious for a biological control agent.

The potential competitive advantage of selecting a good parental virus is reviewed above in Section VI.D. Simply, a virus with a tenfold lower LD_{50} means about ten times more area can be sprayed with the same quantity of baculovirus. Once a desired genome is selected, it is then a matter of incorporating or mixing and matching the available (and of course yet undiscovered) improvements into this genome. Among the modifications that increase infectivity are enhancins and BM-degrading proteases. Although the expression of enhancins merely increases the speed of kill of baculoviruses, they significantly increase the lethality. Combining an enhancin gene with a cathepsin L from the flesh fly *Sarcophaga peregrina* that resulted in a 51% reduction in speed of kill then seems appropriate. The effect of this increased speed of kill was due in part to the ability of this recombinant to colonize the insect host more rapidly due to damaged BM.

Incorporation of a Bt toxin product into the baculovirus polyhedra has proven to be a very attractive strategy for both improving virulence and the speed of kill of the baculovirus. Even though it is unclear if this strategy can be combined with the expression of other factors, such as peptide toxins, it would be worthwhile to consider stacking this modification, at least in this theoretical section. Clearly, a recombinant virus with a very low LD_{50} is advantageous and incorporation of Bt toxins is one of the best-known ways to attain this effect. An obvious advantage of incorporating the enhancin and cathepsin genes is that

their expression is not expected to impact the expression of lethal factor genes. The currently used scorpion-derived toxins largely are misfolded in insect cells. A systematic study of folding of these toxins could improve expression over tenfold. Similarly, approaches to enhance the stability of the toxin mRNA and translation is certain to improve efficacy. Therefore, in addition to modifications mentioned above, the expression of a pair of synergistically acting insect-selective scorpion toxins could be considered for our dream baculovirus. Although many other excellent insect-selective toxins have been identified, expressed in and tested as recombinant baculovirus insecticides, positive cooperativity has only been shown with a limited number of peptides. Furthermore, the cooperativity among the toxins is dependant on the insect species and thus needs to be fine-tuned. The synergy between toxins belonging to the excitatory and the depressant peptide family could be exploited for this purpose. Another important consideration is the choice of promoter for the expression of all foreign genes in recombinant baculovirus insecticides. The very late and strong *polh* or *p10* promoters have traditionally been used for most studies. However, the benefits of using weaker and earlier promoters have lately become apparent. More potent toxins or toxin combinations allow the evaluation of a wider range of promoter systems. The last group of modifications that can be incorporated into our baculovirus is auxiliary features, such as UV resistance enhancement, related to field stability and formulation. The expression of algal virus pyrimidine dimer-specific glycosylase, cv-PDG (Furuta *et al.*, 1997), involved in the repair of UV-damaged DNA improved both virulence and speed of kill. Therefore, we will recommend this modification as the last component of the GM baculovirus.

To summarize our suggestions, a genome with extensive deletions to remove all nonessential genes, expressing enhancin and cathepsin proteins combined with a pair of synergistically acting peptide toxins under the control of an early weak promoter, a Bt–polyhedrin fusion protein and expressing the algal virus pyrimidine dimer-specific glycosylase may make a better recombinant baculovirus.

VIII. Concluding Thoughts

Natural baculoviruses show poor speed of kill and limited effective range in comparison to synthetic chemical pesticides such as the pyrethroids, thus, one might think that these biological pesticides may not provide sufficient protection for crops. However, there are

cases, such as forest ecosystems, in which the use of a natural baculo-virus may seem less efficient in the short term but is more effective, cost efficient, and less destructive for the ecosystem over the long term. In these situations, the natural baculovirus should be the biopesticide of choice. Analyzing the pest problems in a region and implementing a customized approach that includes baculoviruses, however, is not an easy task. The best example of how this was effectively performed is in Brazilian soybean agroecosystems (Moscardi, 1999). The decision to use or to not use a biological (or chemical) pesticide should be based on the level of tolerance that a particular crop has to pest damage. In general, as the economic injury threshold of a particular crop decreases so does the likelihood that biopesticides will be used for its protection. This is a function not only of crop physiology but also of the value of the crop. As an example of physiology, the baculovirus-based pest manage-ment system is more likely to be ineffective in tropical areas because of the more rapid speed of development of the pest.

During the last 15 plus years, a large number of GM baculovirus constructs have been generated, which show variable improvements in speed of kill (Fig. 2). Undoubtedly, numerous other constructs have been generated in commercial and academic laboratories that are not found in the scientific literature. Some of the best constructs to date induce feeding cessation as early as 24 h p.i., a period of time that is sufficient to make these GM baculovirus pesticides as effective as synthetic chemical insecticides (Kamita et al., 2005a). Our opinion is that the efficacy and safety of GM baculoviruses have been sufficiently proven to make GM baculovirus pesticides a viable tool in our crop protection toolbox. The benefits to society of GM baculoviruses far outnumber risks, especially in comparison to the potential risks posed by many synthetic chemical pesticides and GM crop plants that are routinely used now.

The development of GM baculoviruses for pest insect control is an element of the era of genetic engineering, an era in which the avail-ability of potent, orally active insect-selective toxins from Bt bacteria has driven the field toward GM crops expressing Bt toxins (Aronson and Shai, 2001; Bravo et al., 2005; Gill et al., 1992; Schnepf et al., 1998). Of course, this emphasis on one gene family may in the future prove to be problematic due to the occurrence of insect resistance. In fact, there are now alarming reports on the development of resistance against Bt-expressing plants (Gunning et al., 2005). However, there is so far surprisingly little impact of this resistance considering the widespread use of the Bt gene. Whether resistance will become a major problem for Bt toxin-expressing GM plants remains to be seen.

We believe that the availability of alternative technologies including GM baculoviruses will be an essential part of successful pest management strategies. The generation of resistant insects occurs relatively quickly with synthetic chemical insecticides and has been observed with Bt toxins (Gunning *et al.*, 2005). Chemical insecticides and other agents, such as Bt toxins, are small molecules that often act on a single defined target site potentially making them more vulnerable to target site insensitivity or metabolic enzymes. In contrast, the baculovirus is a much larger agent that takes over the insect host. In both laboratory and field studies, in which insects were continuously exposed to baculoviruses, no significant and consistent level of insect resistance is found (Fuxa, 1993; Fuxa *et al.*, 1988). Although baculovirus-resistant populations may emerge in the long term, the time frame required for this should be immensely longer than that of a synthetic chemical agent.

As transfection systems improve, it becomes technically possible to put Bt toxin genes into a wider variety of crops. However, the economic and regulatory barriers to transgenic crops will limit GMOs to major crops for the foreseeable future. Thus, an advantage of the recombinant baculovirus is that it could be used on a variety of crops including high-value vegetables with much lower economic barriers. However, the greatest value of the recombinant viruses may be in developing countries. In developing countries, they can replace earlier generations of synthetic chemicals that have serious human health and environmental risks rather than compete with modern pesticides with superior green properties. There are also strategies to produce recombinant virus locally in developing countries that would avoid either importing expensive chemicals or reliance on major industrial infrastructure.

Several of the technologies that have emerged from GM baculovirus pesticide development have universal applicability in medical and general biological research. The field of toxinology, for example, has benefited greatly from the isolation and characterization of novel peptide toxins for use in improving the insecticidal efficacy of the baculovirus. The synergy between the fields of pest control and toxinology has not only caused an explosive increase in the number of novel insect-selective peptides isolated from a variety of venomous animals but also led to increased knowledge in toxin mechanism of action, selectivity, cooperativity, and target ion channel diversity in insects and mammals over the last two decades (Fig. 6). Today, most publications involving the isolation and characterization of insect-selective toxins generally mention the potential for use of these peptide toxins to further improve recombinant baculovirus insecticides.
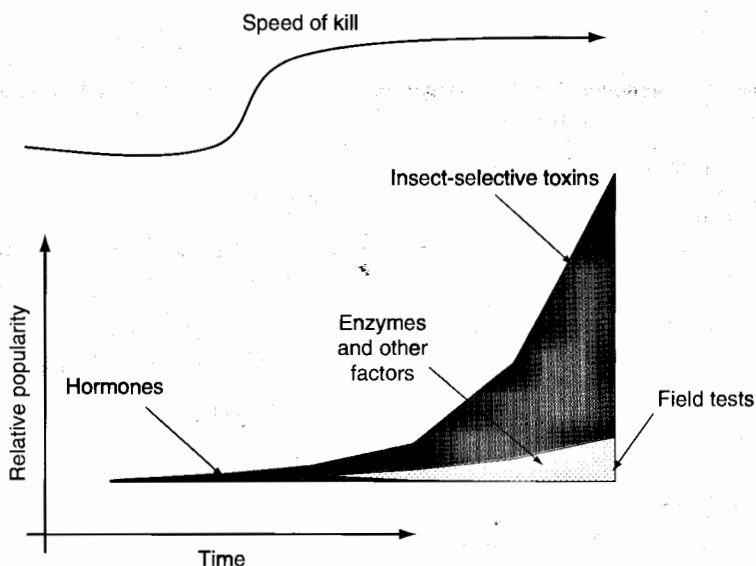
FIG 6. Trends in GM baculovirus research. Although the first generation of GM baculoviruses expressing insect hormone genes showed significant improvements in speed of kill in comparison to the wild-type parent, the expression of genes encoding enzymes that disrupt insect physiology and subsequently insect-selective toxins now dominate the field. The identification of new genes, improvements in expression technology, and advances in manufacturing and formulation have contributed dramatically to improving the speed of kill of GM baculoviruses.

The idea of expressing foreign genes by recombinant baculoviruses has undoubtedly progressed toward a superb expression system which has had and continues to have a wide impact on biological and medical research. Another outcome, also the subject of this chapter, was fine bioinsecticides. All of the studies to date indicate that GM recombinant baculoviruses can become an integral part of modern pest insect control strategies. Therefore, it is now a matter of when and who will take advantage of this technology.

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