BIOLOGY AND ECOLOGY OF BACULOVIRUSES

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ABSTRACT

Baculoviruses are arthropod specific pathogens which are commonly found in insect populations. Their restricted host range, high pathogenicity and lack of effect on beneficial insects has led to their development as microbial insecticides for a range of pests from both forestry and agriculture. Baculoviruses have features which promote their persistence outside their hosts and dispersal in the environment, enabling them to spread rapidly in susceptible insect populations. These characteristics can be utilized in the development of longer term pest control strategies. Baculoviruses have not been developed to their full potential for a variety of commercial and practical reasons. Some of these problems are now being addressed through genetic engineering.

INTRODUCTION

Insects are hosts to a wide variety of pathogens which include representatives from all the major groups of microorganisms. Some have been studied in detail, but the majority remain uncharacterized, providing a reservoir which continues to be raided in the search for effective, new, naturally occurring pest control agents, or increasingly, for material for genetic modification. One group of pathogens which attracted a considerable amount of interest early on was the insect baculoviruses. Part of the reason for this attention was no doubt due to the dramatic effects that these viruses have on their hosts which turn into a liquefying bag of virus. This effect is often made even more obvious by an accompanying behavioural change where the infected insects climb up their host plant to the end of branches or leaves to die.

Baculoviruses have only been isolated from arthropods, primarily moths and butterflies (Lepidoptera), but also, *inter alia*, beetles (Coleoptera), sawflies (Hymenoptera) and prawns (Crustacea) (Entwistle and Evans, 1985). In the insects, their distribution is limited to plant-feeding species and they have no effect on groups of beneficial invertebrates, such as, bees, parasitoid wasps or predatory beetles. This specificity, combined with their high pathogenicity, were key reasons for their promotion and early development as biological pest control agents.

Many of the observations and studies on the ecology of baculoviruses in the field have centred on temperate forest insects, in which virus epizootics appear to be relatively common and undoubtedly play a role in the regulation of their host (Stairs, 1972; Evans, 1986). This had led to speculation that baculovirus diseases could be

involved in the population cycles that are often observed in forest insect species (Anderson and May, 1980; Myers, 1988). Baculovirus epizootics are not limited to forest insects and have also been recorded in species which exploit agricultural systems, however, long term studies in these less permanent habitats are more limited (Fuxa, 1982; Evans, 1986).

The first well documented examples of using baculoviruses as pest control agents date from the 1940s (Entwistle and Evans, 1985). Since then they have been used to control pest species in virtually every crop situation, from forests and orchards to agricultural crops, greenhouse plants and stored products. Good results have been obtained in all types of crop, but particularly successful examples of their use include, the velvet bean caterpillar, *Anticarsia gemmatalis*, on soybean in Brazil, where over 1 million hectares are now protected with a baculovirus (Moscardi and Sosa-Gomez, 1992), the palm rhinoceros beetle, *Oryctes rhinoceros*, which has been successfully controlled using its non-occluded virus in the Pacific region (Bedford, 1981, Zelanzy *et al.,* 1990) and the pine sawfly, *Neodiprion sertifer*, a major pest of pine in Europe and North America, which is now routinely controlled using a baculovirus (Doyle and Entwistle, 1988). Although various strategies can be adopted in the use of baculoviruses for pest control, the majority of applications fall into one category, inundative release, where large quantities of virulent virus are applied to high density pest populations. In general, baculoviruses have been found to be particularly successful on forest pests, and other species found in more permanent habitats, or in other systems where the damage threshold of the crop is relatively high, such as the soybean. Thus has meant that viable control systems have been developed for most of the major temperate forest pests, including, the Douglas fir tussock moth, *Orgyia pseudosugata* (Otvos *et al.,* 1987) and gypsy moth, *Lymantria dispar* (Lewis, 1981) in the US and Canada and the pine beauty moth, *Panolis flammea,* and the pine sawfly in Europe (Doyle and Entwistle, 1988; Cory and Entwistle, 1990).

BACULOVIRUS CHARACTERISTICS AND BIOLOGY

Baculoviruses are large, double-stranded DNA viruses with a genome in the range of 90-230 kilobase pairs (kbp) (Francki *et al.* 1991; e.g. *Autographa californica* NPV - 134 kbp). They can be divided into several groups on the basis of the presence of an occlusion body and the number of virions it contains. There are two occluded groups; the nuclear polyhedrosis viruses or NPVs, which contain many virions, each of which is capable of initiating infection, and the granulosis viruses or GVs, which usually contain a single virion. The NPV occlusion body or polyhedra is large enough to be visible under a light microscope which has aided their identification in a wide range of species. GVs are smaller and need to be viewed under an electron microscope for positive diagnosis. The protein occlusion body is a key feature of the NPVs and GVs as it greatly enhances their survival outside the host and thus their use as pest control agents. Viruses in the third group lack the occlusion body and are known as the non-occluded baculoviruses. The best known example is the non-occluded baculovirus from the rhinoceros beetle.

Baculovirus isolates have been collected from hundreds of different species of insects, although few isolates have been studied in detail. Most are named after the host of isolation, although this procedure is not entirely satisfactory as similar, or even, identical strains, have been isolated from unrelated species. Baculovirus host range can vary from a single species to several species in different families within an insect Order, although only species which are closely related to the host of isolation are usually highly sensitive. Not all stages of the host are susceptible to baculovirus infection. In most species only the larval stage, which is the major feeding stage of the host, is vulnerable. There are exceptions to this, for example, the rhinoceros beetle non-occluded virus is also infective to the adult beetles. However, as this virus is not able to survive outside its host except under limited conditions, the cycle of transmission is different from that of the occluded baculoviruses. Baculoviruses tend to be highly pathogenic, usually causing lethal infections in their hosts. The dose needed to initiate infection usually increases with size of the larvae. The dose needed to kill half the test larvae (LD_{50}) can be as little as 3 or 4 polyhedra in first instars, rising to more than 10,000 polyhedra in later instars (Smits and Vlak, 1988). This rapid decrease in susceptibility to infection has important implications for pest control. Virus applications ideally must be timed to infect the young larvae in order to produce infection without using uneconomically large quantities of baculovirus inoculum.

The main route of infection of a permissive species by an occluded baculovirus is by ingestion: unlike entomopathogens such as fungi, the virus cannot enter the insect through the chitin body wall. Under normal circumstances the larva ingests the occlusion body when feeding or browsing on foliage. The occlusion body is then rapidly broken down in the alkaline mid-gut. This liberates the virions into the gut lumen; these pass through the peritrophic membrane and enter the mid-gut cells by fusion thereafter initiating the replication cycle.

In NPVs it appears that two genetically identical, but morphologically distinct, forms of the virus exist. The occluded polyhedra are the agents of horizontal transmission and infection of the mid-gut cells. However, spread within the body of the host is conducted by a non-occluded form, "extracellular virus" or ECV (Volkman and Keddie, 1990). In the early stages of infection only the ECV form is produced, budding from infected cells and spreading to new tissues in the haemocoel. In the later stages, polyhedra are found in the nuclei of nearly all the cells of the body, causing whitening of the larva. Cell lysis and rupturing of the integument then releases the occlusion bodies into the environment where they are available to infect other larvae. Baculovirus infections are extremely productive, with, for example, a single, late instar, noctuid larva producing between 10^9 and 10^{10} polyhedra (Evans, 1986).

Although few baculoviruses have been well characterized, recent progress on the molecular biology of NPVs has given us an insight into the way in which baculoviruses interact with their hosts. For example, the discovery of genes coding for products which affect insect development, such as the ecdysteroid UDP-glucosyl transferase (*egt*) gene, which interferes with insect moulting (O'Reilly and Miller, 1989) or the apoptosis gene, which blocks cell death (Clem *et al.*, 1991), should allow investigation of the selective advantage that genes of this nature confer on the virus. In the absence of the *egt* gene, for example, the virus kills the host more rapidly; thus when it is present it prolongs the

life, and therefore the feeding time, of the larva (O'Reilly and Miller, 1991). Although the full effects of this gene product are not yet known, it would appear that by possessing this gene, the NPV maximizes its use of host resources (the larva) by increasing the number of progeny produced.

BACULOVIRUS ECOLOGY

The biology and ecology of baculoviruses make a strong contribution to their success as pest control agents because they will spread after they are released in the field, often causing epizootics in their host. This is in contrast to chemicals and other entomopathogens, such as the bacterium *Bacillus thuringiensis,* which have considerably less, if any, epizootic potential. In most inundative release biocontrol programmes, epizootics tend to be condensed into a single generation or season: in natural epizootics this process can be spread over several generations and years.

In order to spread in the environment baculoviruses need to persist and disperse. NPVs and GVs are able to persist outside their hosts because they possess the protein occlusion body. Although virus exposed to sunlight will be rapidly broken down by ultraviolet radiation, any virus which is protected, such as, within a plant canopy (e.g. Carruthers *et al.,* 1988) or in the soil (e.g. Thomas *et al.*, 1972), can persist for a considerable amount of time, even years. Environmental persistence of this type is thought to be the major route whereby virus is maintained in insect populations (Murray and Elkinton, 1990). The existence and role of vertical transmission of virus via the adults, although common in non-occluded baculovirus systems, has been less well studied in the NPVs and GVs. However, it appears to occur at relatively high levels in some species (Fuxa and Richter, 1990). The precise mechanism by which this happens and its importance in virus:host dynamics is, as yet, poorly understood.

Baculovirus dispersal can be achieved by various means. Abiotic events (rain, irrigation or wind) can serve to move the virus in the local environment. Baculoviruses can also be dispersed by passive vectors which either feed on infected larvae or come in contact with the virus-killed cadaver. For example, insect parasitoids can become contaminated with virus when they oviposit in infected larvae and subsequently transmit virus to other susceptible individuals (e.g. Hochberg, 1991). However, the main means of biotic dispersal is probably via predators. Because the guts of many predators are not alkaline, the polyhedra do not break down and instead pass through the gut unharmed. When predators have fed on a virus-killed caterpillar they ingest virtually pure virus so the levels excreted can be high enough to infect younger, susceptible larvae. Over small areas, this type of dispersal is achieved by a range of invertebrate predators, such as carabid beetles, harvestmen and predatory bugs (e.g. Young and Yearian, 1987). However, the most important vectors over a wider area are likely to be birds, which readily feed on baculovirus infected larvae and have the opportunity to carry the virus for considerable distances (Entwistle *et al.*,1993).

The combination of these persistence and dispersal mechanisms, plus the great capacity that the virus has to increase in susceptible hosts, means that baculoviruses can spread rapidly after application. This is not only of benefit in programmes of inundative

release, but also means that longer term control strategies which use these characteristics can be developed. One tactic, which utilizes the persistent capacity of the virus, is to apply NPV or GV to the pest population before it reaches outbreak levels so as to build up a reservoir of virus. This approach has worked for control of the Douglas-fir tussock moth in Canada (Shepherd *et al.*, 1984). Another technique is to manipulate the environment so as to enhance the contact between virus and host. This has been successfully utilized in the control of a group of lepidopteran pasture pests (*Wiseana* spp.) in New Zealand. In this situation it was found that deep ploughing of the pastures removed the virus from the host's environment and thus reduced virus mortality. The instigation of a low tillage regime combined with increased virus dispersal, via passive movement by animal stock, was found to maintain the pest at sub-economic levels (Crawford and Kalmakoff, 1977). It is also not difficult to see how knowledge of the ecology of baculoviruses has obvious and important implications for assessing the fate of genetically modified baculoviruses after release (Cory, 1991).

LIMITATIONS TO THE USE OF BACULOVIRUSES

The use of baculoviruses has not matched up to their potential, so what has limited their development? There are several factors involved, both commercial and practical. Restricted host range is a double-edged sword. It is excellent from an environmental standpoint but less attractive in terms of market size; the target pest would have to be very economically important or the virus would need to kill a whole complex of pests, on a widely planted crop, to make development and registration of a baculovirus insecticide product a worthwhile process. This has meant that the commercialization of baculoviruses has not really taken off, and that most of the large-scale control programmes cited above, such as, the rhinoceros beetle, the velvetbean caterpillar or most forest pest management programmes, have been funded by government or other non-profit-making organizations. Virus production is also a problem because *in vivo* culture is by far the most productive and reliable way of producing the virus. Insect production in the laboratory, or even collection of infected insects from the field, is very labour intensive, and thus expensive, at least for the developed countries. Cell culture systems have been developed, and continue to be so, but, as yet, the yield is too low for large scale commercial production and the virus can lose viability in continual culture. The problem of patenting a natural organism has also been regarded as a drawback. However, the recent patenting of a naturally occurring NPV from the celery looper, *Anagrapha falcifera*, in the US, may pave the way ahead for other newly isolated baculoviruses with pest control potential (Hostetter and Puttler, 1991).

On the practical side, one of the key problems with using baculoviruses, in agricultural systems in particular, is their slow speed of kill as compared with chemical insecticides. Baculovirus infections need time to develop within the host so there is inevitably a gap between ingestion of the virus and death from the disease, although feeding can be significantly reduced during the later stages of the incubation period. Crops where cosmetic damage is important will thus be particularly vulnerable, especially at high pest densities, and so the widespread use of baculoviruses in these situations has been less developed. Some of the problems associated with the delay before cessation of feeding could be obviated by careful timing and targeting of the

baculovirus application. However, this requires more careful pest monitoring and a detailed knowledge of the pest's behaviour, which obviously requires more effort on the part of the farmer. Although the development of naturally occurring baculoviruses still has some way to go, specific problems attached to their use, such as increasing their speed of kill, might be usefully overcome by genetic engineering. This alternative approach will be discussed in other papers in this volume.

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GENETIC ENGINEERING OF BACULOVIRUSES

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ABSTRACT

Baculoviruses are insect pathogens with great potential as biological insect control agents, due to their safety for non-target organisms and natural role in insect ecology. Major drawbacks are the slow speed of action as compared to chemical insecticides, their restricted host range, which makes these viruses less attractive commercially, and in some case their low virulence. The purpose of this contribution is to give an overview of the 'state of the art' with respect to the engineering of baculoviruses with improved insecticidal properties. Furthermore, the potential risks associated with release of genetically modified baculoviruses in the field as well as strategies to improve their biological safety will be discussed.

INTRODUCTION

About a third of the loss in agricultural production in the world is caused by insects and their control is of prime importance to secure food production. Insect control usually includes the excessive use of chemical insecticides. However, the rapid build-up of resistance to insecticides and the hazards associated with the use of such chemicals in the environment have led to a search for alternative methods. One of the alternatives is the use of insect pathogenic baculoviruses (Payne, 1988).

Baculoviruses are viral pathogens, that cause fatal diseases in insects, mainly in members of the families Lepidoptera, Diptera, Hymenoptera and Coleoptera (Granados & Federici, 1986). More than 600 baculoviruses isolates have been described (Martignoni & Iwai, 1986), categorized in two subfamilies: the nuclear polyhedrosis viruses and the granulosis viruses (Francki *et al.*, 1991). Baculoviruses are highly specific for insects at the species level. In nature baculoviruses can cause epizootics which reduce the size of insect populations. Therefore, they are recognized as attractive biological control agents of insect pests in agriculture and forestry as alternative to chemical insecticides. Baculoviruses have been successfully used in the control of a variety of insects, including codling moth, pine beauty moth, Douglas fir tussock moth, beet armyworm, fall army worm, cabbage moth and many other insect pests, on all continents.

The major limitation to a more wide-spread use of baculoviruses as insect control agents is their relatively slow speed of action, in particular in crops with low damage thresholds (Payne, 1988). Upon infection, insects stop feeding only after a few days (Figure 1), whereas immediate insecticidal effect is often required. With the advent of genetic manipulation techniques it has become possible to engineer baculoviruses with improved insecticidal properties (see Vlak, 1993; Hammock *et al.*, 1993, for review).

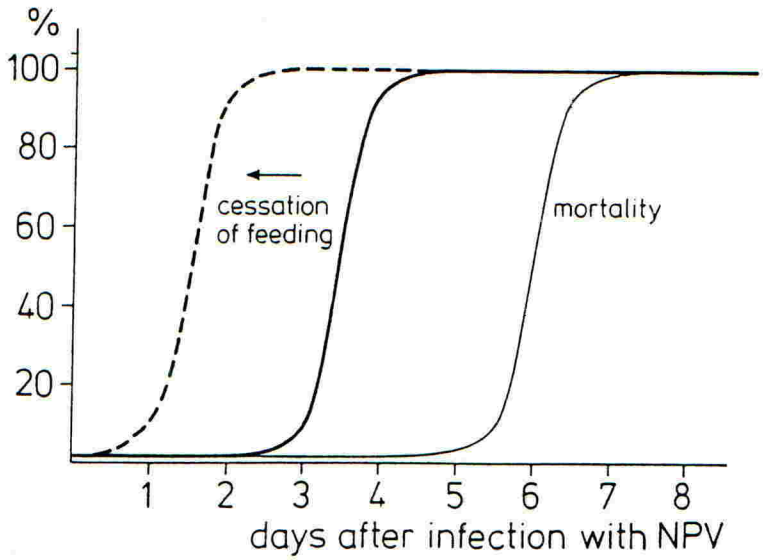

Figure 1. Relation between larval mortality (thin line) and cessation of feeding (thick line) in percentage versus time after infection (days). The dotted line (cessation of feeding and/or mortality) represents the anticipated effect of a recombinant baculovirus with enhanced insecticidal effect.

BACULOVIRUSES

Baculoviruses are characterized by the presence of rod-shaped (baculum = rod) nucleocapsids, that are enveloped singly or in bundles by a unit membrane. The virus particles are usually embedded into large protein capsules or occlusion bodies (OB), also called polyhedra c.q. granula. These OBs, 0.1 - 10 µm in diameter, provide protection of the virus particle and enhance the persistence of the virus in the environment. The occluded virions (OVs) are the infectious entities of the particle.

The major constituent of OBs is a single protein (polyhedrin c.q. granulin) with a subunit molecular weight of approximately 30 kilodalton (kDa). The amino acid sequence is highly preserved among baculoviruses (Vlak & Rohrmann, 1985). Baculoviruses contain a double-stranded, circular DNA molecule as their genetic element. This DNA varies in size between 100 and 200 kilobase pairs (kbp) and is able to code for more than 70 average-sized proteins. Physical maps of various baculovirus DNAs have been established, the most detailed one being of the prototype baculovirus *Autographa californica* multiple-nucleocapsid nuclear polyhedrosis virus (AcMNPV) (134 kbp). About forty genes have been mapped on the AcMNPV genome, including polyhedrin and the nucleotide sequence has been determined (see Rohrmann, 1992; Kool & Vlak, 1993, for review).

REPLICATION IN VIVO

In nature, OBs enter the larvae via ingested food and dissolve in the midgut as a consequence of the local alkaline conditions. The released virus particles pass

the peritrophic membrane of the insect gut and infect the midgut epithelium, in particular in columnar and regenerative cells. The virus replicates in the nucleus of these cells and progeny nucleocapsids bud through the midgut cell membrane at the basolateral side into the insect hemolymph thereby acquiring a membrane. This extracellular virus (ECV) circulates in the hemolymph and causes the systemic spread of the virus to other organs of the insect, such as the fat body (see Volkman & Keddy, 1990, for review).

In cells of these organs a second round of virus replication occurs (secondary infection), whereby the produced ECVs are responsible for the cell-to-cell spread of the infection in these organs. Late after infection of these cells virus particles are occluded in large quantities in newly synthesized OBs. In summary two phenotypically distinct forms of infectious virus exist: the OV form causing infection of individual insects and the ECV form which is responsible for the systemic spread of the virus in the organism. Genetically, these forms are identical as they contain the same genetic information.

Initial signs of infection in the insect appear about three days after ingestion of virus via food. As a result of the infection, the insects lose appetite, become sluggish and finally die from what is known as polyhedrosis or granulosis. In more advanced stages of the disease, six to seven days after infection, the larval body usually develops a creamy yellow color due to accumulation of OBs in the infected tissues. Subsequently, the larvae become flaccid and hang by the prolegs from leaves or branches in a characteristic inverted position, or detached from the plants on the soil. Larvae die three to eight days after the initial signs of infection, after which the cuticle ruptures liberating masses of OBs (about 10^9 - 10^{10} per larva) being about one-third of the insect's body weight. This clinical syndrome was already recognized in insects about two centuries ago (Steinhaus, 1956). The OBs are released into the environment and can be taken up by other insects and hence cause an epizootic in the insect population. For effective control about 10^{11} OBs (100 larval equivalents) sprayed per hectare are required. Only larvae are susceptible to baculovirus infection.

REPLICATION *IN VITRO*

The ECV form of the virus is able to infect efficiently cultures of insect cells and this has allowed detailed molecular studies on baculovirus replication, in particular of the nuclear polyhedrosis virus of *A. californica* (AcNPV) in *Spodoptera frugiperda* (Sf) cells being the best studied example (Faulkner, 1981; Kelly, 1982; Granados & Federici, 1986). The ECV enters the cell by adsorptive endocytosis. Via endosomes the nucleocapsids are partially uncoated and transported to the nucleus, where virus replication takes place in so called virogenic stroma. In the first phase of the infection newly-synthesized nucleocapsids move to the cell membrane, where they bud into the medium. These second generation (passage) ECV are able to infect other cells in the culture. In the second phase of the infection the newly-synthesized nucleocapsids are enveloped inside the nucleus and occluded into OBs. Finally, the cells lyse and release the OBs. The availability of permissive virus-cell systems has also facilitated the engineering of baculoviruses with improved insecticidal properties.

BACULOVIRUS MOLECULAR GENETICS

The replication of baculoviruses is temporally regulated by a coordinate expression in a cascade of four classes of genes (Blissard & Rohrmann, 1990).

Upon infection of cells, viral genes of the *immediate early* class are transcribed by host factors, including RNA polymerases. Products of these genes possibly in concord with host factors turn on an array of *delayed early* genes, including virus-coded RNA and DNA polymerases, and turn off some host functions. Transcription of these delayed early genes precedes (by definition) the onset of DNA replication. *Late* genes are switched on concurrently with the onset of DNA replication and their expression is promoted by *immediate early* and *delayed early* gene products. The *late* genes code for structural proteins of the virus particles. The class of *very late* genes codes for proteins that are involved in the late stages of virus infection and OB morphogenesis. Two of these *very late* genes, coding for polyhedrin and a protein of 10 kDa, are hyperexpressed late after infection. Genes of each of the four temporal classes are not clustered, but randomly distributed along the virus genome (see Kool & Vlak, 1993, for review).

Mutations or deletions in the very late genes do not affect NOV replication and this, together with the strong expression of every late genes, forms the basis for the use of baculoviruses as vectors for the expression of foreign genes in a eukaryotic environment (Smith *et al.*, 1983; Luckow & Summers, 1988). These viral expression vectors are capable of synthesizing foreign gene products in copious quantities. In many cases these products are antigenically, immunologically and functionally similar to the authentic proteins, and hence attractive for diagnostic, therapeutic and prophylactic use in human and veterinary medicine (Vlak & Keus, 1990). On the basis of the baculovirus expression vector system a recombinant Human Immunodeficiency Virus subunit vaccine to combat AIDS has been developed, which is now being tested in a phase II trial in the U.S.A. The development of baculovirus expression vectors as well as the increased knowledge of the molecular genetics of baculoviruses (Blissard & Rohrmann, 1990) has aided in the engineering of baculoviruses for insect control.

GENETIC ENGINEERING OF BACULOVIRUSES

Late after infection two viral proteins accumulate to very high levels, polyhedrin and a protein of 10 kDa. Their genes are hyper-expressed due to the presence of very strong promoters upstream from these major late genes. In infected cells these two proteins can amount to about 30% of the total cell protein by weight. The 10 kDa protein is the major component of fibrillar structures in infected cells; the function of which is unknown. These strong promoters can be exploited to express the insecticidal genes in substantial amounts (see for details, King & Possee, 1992; O'Reilly *et al.*, 1992).

The size of the baculovirus genome (AcMNPV 134 kbp) prevents the direct manipulation of the genome. Therefore, in order to insert foreign genes into the baculovirus genome, transfer vectors have been designed. These transfer vectors contain, in addition to bacterial plasmid sequences, a baculovirus DNA fragment with the polyhedrin or p10 5' and 3' flanking sequences. The integrity of the 5' flanking sequences (promoter) is important for high-level expression of the insecticidal genes to be inserted. A unique cloning site is present behind the polyhedrin or p10 promoter for insertion of the foreign gene in the transfer vector. The foreign genes are transferred to the baculovirus genome by homologous recombination of transfer vector and wild-type baculovirus DNA during replication in insect cells. The foreign gene is targeted to the correct location in the viral genome by the 5' and 3' flanking sequences in the transfer vector.

In the case of transplacement of polyhedrin, the recombinant viruses are recognized in the light microscope by the absence of OBs or by the presence of the

foreign gene itself (as indicated by Southern blot hybridization, enzymatic activity, immunofluorescence) or by coexpression of enzymes such as beta-galactosidase, glucoronidases or luciferase. The circular viral genome is expansible in size and can accommodate up to 25 kbp of additional DNA, while still being properly assembled in rod-shaped virus particles.

The insecticidal genes are inserted either at the polyhedrin site or, preceded by the p10 promoter, 100 bp upstream from the polyhedrin gene. When the polyhedrin gene is replaced by the insecticidal gene, the recombinant cannot survive in the field for long periods of time as it does not produce OBs. In case the insecticidal gene is inserted upstream or at the p10 gene locus, the polyhedrin gene is preserved and OBs will maintain to be produced.

BACULOVIRUS RECOMBINANTS WITH IMPROVED INSECTICIDAL PROPERTIES

The identification of genes interfering with the insect metabolism is pivotal for the construction of baculoviruses with improved insecticidal properties (see Vlak, 1993; Hammock *et al.* 1993, for review). Possible strategies include the over-expression in insects of peptide hormones regulating diuresis (diuretic hormone) or insect metamorphosis (prothoracicotropic hormone, eclosion hormone, allatostatin, allatotropin), or overproduction of enzymes (juvenile hormone esterase, chitinase) in insects through baculovirus recombinants. Other candidate genes include insect-specific toxins, such as those from the bacterium *Bacillus thuringiensis*, or neurotoxins of scorpions and mites. The products of these genes may, when expressed at high level through baculovirus vectors, interfere with the insect metabolism resulting in, among others, early cessation of feeding (Table I).

Initial attempts to engineer viruses with increased virulence were unsuccessful. The first recombinant carrying a gene coding for a toxin of the scorpion *Buthus eupeus* failed to produce sufficient toxin to cause detectable biological activity (Carbonell *et al.*, 1988). The toxin of *B. thuringiensis* was produced in insect cells and insects in large quantities and was equally active against target insects as the toxin from the bacterium (Martens *et al.*, 1990; Merryweather *et al.*, 1990). However, the presence of this bacterial toxin in these recombinants did not enhance the insecticidal activity as compared to wild-type virus. The introduction of the diuretic hormone gene (Maeda, 1989) and the juvenile hormone esterase gene from *Heliothis virescens* (Hammock *et al.*, 1990) in recombinant baculoviruses resulted in a slight increase in virulence to host insects as compared to control viruses, but insertion of the eclosion hormone gene (Eldridge *et al.*, 1992) had no effect.

A major breakthrough was achieved recently with the expression of neurotoxin genes from the arthropods *Androctonus australis* (Stewart *et al.*, 1991) and *Pyemotes tritici* (Tomalski & Miller, 1991), respectively, using baculovirus recombinants. These recombinants secreted the toxin into the insect and caused a considerable reduction in time required to incapacitate the host insect due to acute paralysis. The added benefit is that feeding is greatly reduced. Recently, expression of the maize mitochondrial protein URF13 conferred insecticidal activity in larvae (Korth & Levings, 1993). This observation calls for a re-evaluation of recombinants, which express heterologous proteins. The combination of a highly specific insecticidal protein with a highly selective group of insect viruses presents an attractive approach to crop protection (Wood, 1991; Vlak, 1993).

Table I. Engineering of baculoviruses with potential improved insecticidal activity.

Recombinant expressing	Intrinsic effect	Effect on insect	Reference
Butheus eupus toxin	paralysis	none	Carbonell *et al.* 1988
Diuretic hormone	water balance	20% reduction in LT_{50}	Maeda 1989
Bacillus thuringiensis toxin	paralysis gut	none	Martens *et al.*, 1990 Merryweather *et al.* 1990
Heliothis virescens juvenile hormone esterase	regulates molt	decrease weight gain	Hammock *et al.*, 1990
Androctonus australis toxin	paralysis	20% reduction in LT_{50}, reduced feeding	Stewart *et al.*, 1991 McCutchen *et al.*, 1991
Pyemotes tritici toxin	paralysis	toxic to insects	Tomalski & Miller, 1991
Manduca sexta eclosion hormone	eclosion	none	Eldridge *et al.*, 1992
Maize mitoch. protein URF13	male sterility in plants	toxic to insects	Korth & Levings, 1993

BIOSAFETY OF (RECOMBINANT) BACULOVIRUSES

Baculoviruses have been used as insecticides at large scale for almost half a century in various regions of the world. In terms of safety they have a perfect track record (Summers *et al.*, 1975). Their specificity provides an inherent safety for use in the environment. AcMNPV has the most widely known host-range, and infects 28 insect species (Bishop *et al.*, 1988). Most baculoviruses, however, infect a single or a few related insect species. They do not adversely affect non-target hosts including vertebrates, plants and beneficial invertebrates and therefore impose no ecological hazard on the environment.

The risks of recombinant baculoviruses are partly similar to those associated with the deliberate release of wild-type baculoviruses such as the effects on non-target hosts (Summers *et al.*, 1975). The additional risks with genetically engineered viral insecticides may involve alteration of the host range, the spread of the engineered virus from the field site to other ecosystems, the physical instability of the viral genome, and the possible exchange of genetic information, in particular the insecticidal gene, with other organisms. The fundamental question is to what extent genetically-modified baculoviruses behave differently from wild type viruses (J. Cory, this volume).

A few of these fundamental problems have been addressed through extensive laboratory and field testing of a number of model AcMNPV recombinants at the Institute of Virology and Environmental Microbiology at Oxford (Bishop *et al.*, 1988; Bishop, 1989; Possee *et al.*, this volume; H.A. Wood, personal communication). In 1986 an AcNPV was engineered containing a non-coding 'marker' sequence with translational stop codons in all possible reading frames. This recombinant behaved identical in laboratory tests as compared to wild-type virus. Subsequently, a polyhedrin-minus recombinant was constructed in 1987, followed by a recombinant where the polyhedron gene was replaced by beta galactosidase. The polyhedrin-minus viruses were quickly inactivated in the field as they do not form OBs. They persisted in the environment for only very short periods of time, i.e. in soil, on vegetation, or in caterpillar corpses (Bishop, 1989). This type of recombinant is thus not suitable for insect control in the field. However, information was assembled on the behavior of recombinants and it was concluded that they behave as expected. Field releases are in progress with recombinants carrying a toxin gene and also having polyhedra.

A concern is the possibility of alteration of the virus host range incurred by the foreign gene. Recombinant baculoviruses have been tested against an extensive range of susceptible and non-susceptible hosts. From these experiments there is no evidence that the host range has been altered. There may be also the possibility of transfer of the foreign gene to other related baculoviruses or to non-target hosts. Although baculoviruses most likely have a similar genomic organization (Blissard & Rohrmann, 1990), the over-all nucleotide sequence homology is low and illegitimate recombination in insects between two distantly related baculoviruses has never been observed. Transfer of baculovirus genes to non-target insects has not yet been observed. In the unlikely event that it occurs, it would only effect the insect population if the foreign genetic element was incorporated into the germ line.

Natural isolates of baculoviruses show genetic heterogeneity. Also during evolution baculoviruses appear to have acquired host genes. The presence of insect transposon-like elements in baculoviruses assumes genetic flow from insects to viruses. In the event of gene transfer results in a more virulent virus it will be counter-selected (see below).

The engineering of baculovirus recombinants is aimed at increasing the insecticidal activity (virulence, potency) of the virus. In nature virus persistence and virulence are in equilibrium with the host population. In natural systems there appears to be a selection against highly virulent strains during an epizootic. Probably the quick kill of a host reduces the amount of virus produced and thus the virus load in the environment. Consequently, introduction of a virulent trait would reduce virus persistence. The arthropod toxins produced by recombinants described Stewart *et al.* (1991) are specific for insects and cause no toxicity in mice at 50 mg/kg dose, equivalent to the production of 1.5×10^5 recombinant virus-infected larvae. The toxins derived from *B. thuringiensis* are insect-specific and non toxic to vertebrates.

In the case of genetically engineered baculoviruses for insect control it is desirable to have recombinants with increased insecticidal activity. Despite the fact that no adverse effects of baculovirus recombinants are to be expected, it remains desirable for safety reasons to provide recombinant viruses with traits that reduce their ability to survive for long periods of time in the environment. They should also preferably have reduced biological fitness as compared to the wild-type virus. Several strategies can be followed to reach this goal.

CONSTRUCTION OF BIOSAFE RECOMBINANT BACULOVIRUSES

The first strategy is to produce viruses with reduced biological fitness, while maintaining their virulence. To this end, genes can be deleted from a baculovirus following a similar strategy as with the insertion of genes (via transfer vectors). These deletions may result in reduced virus persistence or progeny virus production and this will greatly limit their epizootiological potential (Table II). OB-minus recombinants with an insecticidal gene in lieu of the polyhedrin gene can be disseminated in the environment after co-occlusion into an OB of a wild-type baculovirus during a co-infection (Miller, 1988). Upon release in the environment OB-minus genotypes have a selective disadvantage (quickly inactivated) over wild-type baculoviruses, since only OBs are infectious and persistent. The OB-minus genotype will gradually disappear from the genotype pool. A field release with this type of virus has been carried out in 1990 in the United States by Dr H.A. Wood, Boyce Thompson Institute of Plant Research at Cornell, Ithaca, New York State. The results indicated that the recombinant, in this case a polyhedrin deletion mutant, disappeared rapidly from the ecosystem (Table III). A recent development is that intracellular virus particles, that await occlusion (pre-occluded virus), can be formulated and sprayed. This avoids the co-occlusion process which is not easy to control.

OB-positive recombinants can be provided with deletions in genes that affect persistence or reduce virus yield, such as the polyhedral envelope gene, p10 or ecdysteroid UDP-glucosyltransferase (egt) gene (Table II). Deletion of the egt gene has the added benefit that such recombinants also exhibit increased insecticidal activity (O'Reilly & Miller, 1991). Larvae infected with a mutant lacking the egt gene showed reduced feeding and a one day earlier mortality, by one day, than wild-type virus-infected larvae.

Table II. Deletion mutants with reduced potential for survival.

Gene affected	Biological consequence	Environmental consequences
polyhedrin	no polyhedra	low persistence
p10	lack of fibrillar structures fragile polyhedra; impaired polyhedra release	reduced stability and spread
polyhedron envelope gene (34 kDa)	no polyhedron envelope	reduced stability; alkali sensitivity
ecdysteroid UDP-glucosyl transferase	accelerated molt	increased LT_{50}; reduced virus yield
chitinase	impaired insect lysis	reduced spread

A second strategy can be envisaged based on the built-in suicide mechanisms in the virus. The "suicidal" baculovirus for example can produce polyhedra in laboratory-reared insects, but not in field populations. Such a virus could contain the *Escherichia coli* LacI repressor sequences adjacent to the

transcriptional start site of the polyhedrin gene. The Lac I coding sequences can be inserted, for example, under the control of the p10 promoter upstream from the polyhedrin gene. The binding of the Lac I protein to repressor sequences should prevent polyhedrin transcription. This process can be prevented upon addition of a repressor analog, thus permitting the production of OBs. When released as polyhedra in the field the recombinant virus will infect and kill insect larvae, but only produce non-occluded virions which are unable to survive in the environment. Alternatively, the insecticidal gene can be introduced into baculovirus recombinants sandwiched between duplicated flanking sequences. During virus replication the insecticidal gene will be gradually eliminated due to intramolecular recombination.

Table III. Survival of occlusion body-minus deletion mutants in the field when applied as co-occluded virus.

Year applied	Polyhedra released/ha	% OB deletion mutant
1989	1×10^{14}	42%
1990		13.8%
1991		4.5%

A third strategy may encompass the generation of inducible promoters upstream from the insecticidal gene, such as temperature sensitive, metal- or hormone inducible promoters. This may allow the polyhedra being produced only under 'induced' condition, such as during production, but not in the field. The drawback of the second and third strategy is that the recombinant may revert to wild-type in the field.

CONCLUSIONS AND PROSPECTS

In this contribution the biology and molecular genetics of baculoviruses have been shortly reviewed and the possibilities and strategies for genetic engineering to improve the insecticidal activity of these pathogens have been outlined. The present knowledge about baculovirus gene structure, function and regulation allows the manipulation of the viral genome and the development of the baculovirus expression vector and delivery systems. This technology can now be exploited and tailored for the construction of recombinants with novel insecticidal properties for the combat of insect pests.

The construction of baculovirus recombinants which cause quick cessation of feeding has been achieved. In addition, by deletion mutagenesis these recombinants can be made less persistent in the environment. The recombinants can be produced in insects, when the insecticidal action does not cause immediate

mortality. Otherwise, cell culture is an attractive alternative since inexpensive media and large-scale bioreactors are now available. Development of additional strategies for improvement of baculoviruses requires a more detailed understanding of insect biochemistry and physiology. There is a strong quest for additional insecticidal genes for a variety of insect species in order to tailor baculoviruses.

The ideal improved baculovirus insecticide should have a broad but defined host-range and cause upon infection cessation of feeding without killing the host instantaneously. Only baculovirus insecticides with these specifications are commercially attractive to develop. The availability of genetically engineered baculovirus insecticides with genes coding for insect-specific toxins, hormones or metabolic enzymes are not likely to impose additional environ-mental risks. These proteins are specific for insect species and they are all natural elements of the insect biosphere. Recombinant baculovirus insecticides containing genes of this nature may therefore be considered as natural, insect-specific biocontrol agents producing biorational compounds. A reduced persistence and host specificity are important benefits as they reduce the risks associated with the deliberate release of genetically modified viral insecticides in the environment.

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FIELD TESTS OF GENETICALLY ENGINEERED BACULOVIRUSES

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ABSTRACT

Baculoviruses may be modified using genetic engineering techniques to improve their efficacy as biological insecticides. Laboratory studies have shown that the insertion of functional insect-specific hormone, enzyme and toxin genes into the virus genome can reduce the time required to kill the larval host and decrease the feeding damage inflicted on the plant. The next step in this research programme is to assess the effectiveness of these agents in small scale field trials. As a prelude to such experiments, field trials were performed between 1986 - 1989 with genetically modified *Autographa californica* nuclear polyhedrosis viruses (AcNPV). The first virus used contained a small, unique genetic marker to facilitate monitoring. The second virus contained another genetic marker, but also had a polyhedrin gene deletion which prevented the production of virus occlusion bodies (polyhedrin-negative), which protect the virus in the environment. The third virus was also polyhedrin-negative but contained a bacterial beta-galactosidase gene, under the control of a virus gene promoter, to serve as an innocuous foreign "reporter" gene. These experiments demonstrated that small scale field trials with recombinant baculoviruses presented minimal risk to the environment. The virus did not spread from the initial release site and could be rapidly inactivated using chemical agents. The key results from these trials will be described. In the fourth stage of this programme, we are planning to release a genetically modified AcNPV containing an insect-specific scorpion toxin gene. The aim of the experiment is to compare its effectiveness with the unmodified, wild type AcNPV under controlled conditions. Details will be provided of some of the safety assessment carried out prior to seeking permission to perform the release experiment.

INTRODUCTION

Baculoviruses are invertebrate-specific agents with a large (e.g., 130,000 nucleotides), circular DNA genome. Most baculoviruses have been isolated from insects, which has resulted in their use as biological control agents of pest populations (Cory, 1993). Many baculoviruses have proteinaceous virus occlusion bodies (polyhedra) which protect the infectious virus particles in the period after spraying and before they encounter a susceptible host. The polyhedra largely comprise a single polyhedrin protein (28 kDa) which is produced in large quantities in the latter stages of the virus replication cycle (see Blissard and Rohrmann, 1990). The polyhedra of such viruses allow them to persist in the environment between successive hosts. This is an important feature of the baculoviruses life cycle, due to the discontinuous nature of insect populations, particularly in temperate climates.

While baculoviruses have been used as alternatives to chemical agents to control insect pests, their principal disadvantage is the time required to kill the target insect. This may take several days. In this incubation period, the insect larva continues to feed on the crop, often causing unacceptable damage. Various approaches have been investigated to reduce this time. Wood *et al.* (1981) increased the virulence of *Autographa californica* nuclear polyhedrosis virus (AcNPV) by replication of the virus in the presence of 2-aminopurine. The lethal time 50 (LT_{50}) of a mutant, designated AcNPV HOB, was significantly lower than the parental virus. Fifth instar larvae infected with AcNPV HOB gained weight at a lower rate than the unmodified virus. Hughes *et al.* (1983) compared the time-mortality response of *Heliothis zea* to 14 isolates of *H. zea* NPV. Some isolates had significantly different LT_{50} values.

The most effective and predictable method for improving baculovirus insecticides, however, appears to be the insertion of genes encoding virus-specific toxins, hormones or enzymes into the virus genome. When the appropriate gene product is synthesised in virus-infected insect larvae, it can reduce the feeding activity of the insect and ultimately bring about premature death. Successful examples of this approach have been the use of an insect juvenile hormone esterase gene (Hammock *et al.*, 1990), an insect-specific scorpion toxin gene (Stewart *et al.*, 1991) and an insect-specific mite toxin gene (Tomalski and Miller, 1991). Each of these genes was inserted into the AcNPV genome. The recombinant viruses produced in each study offered significant improvements in insecticidal activity when compared with the unmodified, parental baculovirus.

All tests with improved, genetically modified baculoviruses have been conducted in contained laboratory conditions. The results, while encouraging, have yet to be confirmed in field experiments. Such experiments represent a major advance and require careful planning and thorough assessment of their safety. In anticipation of these field trials, a programme was initiated in Oxford to investigate many of the perceived problems associated with such work.

PAST UK FIELD RELEASE EXPERIMENTS WITH MODIFIED BACULOVIRUSES

In 1986, an experiment was undertaken to monitor the persistence of a genetically modified AcNPV. A very simple addition was made to the virus genome. This consisted of the insertion of a short, unique, non-coding genetic marker into the 3' non-coding region of the AcNPV polyhedrin gene. This marker did not affect the replication of the virus in insect cells in culture or insect larvae. The identity of the recombinant virus, however, could be readily confirmed by hybridizing virus genomic DNA with a radioactive probe specific for the added genetic marker.

Prior to the field release experiment, a number of risk assessment analyses were performed. One of the most important was to establish whether the addition of the genetic marker affected the host range of the baculovirus. A full account of these tests is provided by Bishop *et al.* (1988). Briefly, no change in the host range of AcNPV was detected in species from 5 families of butterflies and 11 families of moths. Attempts to infect species such as ants, or honey bees (Hymenoptera), lacewings (Neuroptera), hoverflies (Diptera) or beetles and ladybirds (Coleoptera) were unsuccessful, as expected.

The marked AcNPV was genetically stable, as evidenced after passage for about 50 replication cycles in insect cells. It was also phenotypically stable, with no alteration in infectious virus particle or polyhedra formation. Furthermore,

laboratory studies showed that the polyhedra were stable in soil collected from the proposed field release site.

These and other data were reviewed by the appropriate regulatory authorities and other interested parties (Bishop *et al.*, 1988) prior to the field release experiment. This experiment was conducted in the summer of 1986. The site chosen was located in a field of light loam bounded by agricultural land at the Oxford University field station at Wytham, Oxfordshire. The site was about 0.5 km away from the nearest houses. A 10 m square, netted, insect-proof enclosure was erected and surrounded by a 2 m high wire fence to exclude large herbivores and other animals. The netted, inner enclosure prevented arthropods from entering or leaving the enclosure. Within this area, plexiglass sub-enclosures were erected to provide further containment of the virus-infected insect larvae.

In field trials with unmodified baculovirus insecticides, the virus preparation is sprayed onto leaf surfaces infested with the pest larvae. In the field trial with the genetically marked AcNPV, the host insect (small mottled willow, *Spodoptera exigua*) was infected with virus in the laboratory, prior to release on sugar beet plants. This ensured infection of all of the experimental larvae and limited spread of the virus which might have resulted from spray application. One week later, all of the insect larvae had succumbed to virus infection. In a control plot, uninfected insect larvae continued to feed over a three week period.

When virus-infected insects were returned to the laboratory, it was demonstrated that they contained the genetically marked baculovirus. Sugar beet leaves collected at the site were also shown to be contaminated with the modified AcNPV by feeding them to the highly sensitive *Trichoplusia ni* larvae, which subsequently succumbed to virus infection. Soil was also found to be contaminated with the genetically marked virus. In the laboratory, cabbage seeds were germinated in soil samples collected at the site and fed to *T. ni* larvae. These individuals died with typical virus symptoms and yielded genetically marked virus in subsequent tests.

The field trial initiated in 1986 was terminated in February 1987 by disinfecting the site with formalin. This inactivated the infectious baculovirus remaining in the soil. This procedure was monitored by propagating cabbage seedlings in soil samples and subsequent feeding on these plants by *T. ni* larvae as described above. Three applications of formalin to the soil reduced infectious baculovirus to undetectable levels.

The results from the first field trial, with a genetically marked AcNPV, demonstrated that this virus could persist in the environment for at least six months. The stability of the baculovirus is attributed to the virus occlusion body. The assembly of this structure is dependent on the synthesis of the polyhedrin protein in virus-infected cells. Deletion of the polyhedrin gene from the AcNPV genome does not affect the production of infectious virus particles (Smith *et al.*, 1983). Virus can still be propagated both in cell culture and insects.

The ability of a polyhedrin-negative or crippled baculovirus to persist in the environment was tested in a further field trial in 1987 (Bishop *et al.*, 1988). The aim of the trial was to determine whether the polyhedrin-negative phenotype was a suitable vehicle for testing the efficacy of a recombinant baculovirus containing a foreign gene encoding an insecticidal protein. The complete polyhedrin gene promoter and coding sequence were removed from AcNPV and replaced with a second, unique genetic marker. The host range of the modified virus was tested as before. In these experiments, however, the absence of polyhedra meant that budded virus from *in vitro* cultures was fed to the insect larvae to be tested. The insertion of the genetic marker did not alter the host range of the virus. The virus

was also shown to be genetically and phenotypically stable after 50 cycles of replication in insect cells. Unlike the polyhedrin-positive phenotype, the polyhedrin-negative virus was not stable when mixed with soil. After 7 days, less than 0.1 % of the virus remained infectious. This suggested that, unlike the parental, polyhedrin-positive virus, this virus mutant would not persist in the environment after release.

The risk assessment data, acquired from laboratory studies, was reviewed as for the first field release study, before permission was granted to perform the second field trial. The field enclosure was the one used in the 1986 study with the marked, polyhedrin-positive virus. Insect larvae were infected with the marked, polyhedrin-negative virus in the laboratory prior to release onto sugar beet plants in the field. After seven days, all the larvae had died. One week later, infectious virus could not be recovered from plant surfaces, soil samples or dead larvae. This was in marked contrast to the first field release with a genetically marked AcNPV, where infectious virus persisted in the soil for six months, and until the site was disinfected.

Further experiments in 1988 and 1989 utilized a polyhedrin-negative AcNPV with a complete β-galactosidase coding region under the control of a functional polyhedrin gene promoter. Although conducted in exactly the same manner as the 1987 field trial, with a marked, polyhedrin-negative virus, these results were less satisfactory. Very few of the insects which had been fed the polyhedrin-negative virus died from virus infection. This was attributed to the field trial being conducted in early September, when early frosts and damp weather precluded normal insect development.

The preliminary field trials with genetically modified baculoviruses described above served to highlight a number of important issues: 1. Polyhedrin-positive viruses, while very stable in soil, could be effectively inactivated by formalin-treatment of the field site at the end of the release experiment. 2. A polyhedrin-negative virus, while very susceptible to inactivation in the field, was an unreliable vehicle with which to monitor the effectiveness of a recombinant baculoviruses containing a foreign gene. 3. The choice of host insect for the field trial was very important. Preferably, a highly susceptible insect, such as *T. ni* should be used in place of *S. exigua*, which required quite high doses of virus to cause mortality. In conclusion, it was decided that future, small scale field trials with genetically modified baculoviruses should use a polyhedrin-negative AcNPV (containing a foreign gene encoding an insecticidal protein) with *T. ni* larvae as the "pest" species.

A field trial with a genetically modified AcNPV has also been performed in the USA (H.A. Wood, personal communication). In this experiment a "co-occluded" AcNPV was sprayed onto cabbages infested with *T. ni* larvae. The co-occluded virus was produced by co-infection of insect cells with polyhedrin-positive and polyhedrin-negative AcNPVs. The polyhedrin-positive virus packaged a proportion of the virus particles produced by the polyhedrin-negative recombinant. This allowed the co-occluded mixture of viruses to be sprayed in the field, without the problems associated with the use of the polyhedrin-negative phenotype. With successive rounds of replication in insect larvae the proportion of the polyhedrin-negative genotype decreased, providing an effective way to limit the persistence of a genetically modified baculovirus in the environment. The polyhedrin-negative phenotype may be readily distinguished in a simple plaque assay titration in cell culture. The significance of this approach is that it could allow the delivery of a polyhedrin-negative AcNPV, containing a foreign gene, as a "passenger" within a polyhedrin-positive AcNPV population. The polyhedrin-negative phenotype would not persist in the environment for more than few virus replication cycles. The disadvantage of the method is that production of the virus

for spraying requires coinfection of cells in culture or insect larvae with two viruses. It is also unlikely in the longer term to be an economically viable way of producing recombinant baculovirus insecticides.

FUTURE UK FIELD TRIALS WITH GENETICALLY MODIFIED BACULOVIRUSES

A field trial has been proposed to test the effectiveness of the recombinant AcNPV containing the scorpion toxin gene (Stewart *et al.*, 1991). This virus (AcST-3) was constructed by inserting a copy of the *Androctonus australis* Hector insect-specific neurotoxin (AaHIT) coding region under the control of a duplicated p10 gene promoter, upstream of the polyhedrin gene in AcNPV. This arrangement retained the function of the polyhedrin gene and permitted the production of normal polyhedra. The toxin coding region was fused, in frame, with a copy of the AcNPV gp67 signal peptide coding sequence, to facilitate secretion of the toxin from virus-infected cells. The AaHIT acts by causing specific modifications to the Na^+ conductance of neurons, producing a presynaptic excitatory effect leading to paralysis and death. When neonate *T. ni* larvae were infected with AcST-3, they died 25% earlier than insects infected with the parental AcNPV. There was also a slight reduction in the lethal dose 50 (LD_{50}). Furthermore, feeding damage to cabbages by AcST-3-infected larvae was reduced by 50% (Stewart *et al.*, 1991).

HOST RANGE STUDIES WITH AcST-3

The improved insecticidal properties of AcST-3 suggested that previously non-permissive insect species might now be susceptible to the modified virus. This possibility was tested by challenging a variety of insect species with AcNPV or AcST-3. Given the logistical problems associated with obtaining and handling many different insects, it was not possible to perform normal bioassays to determine the LD_{50} in each species. For example, each virus requires 50 insect larvae per dose and 7 doses per bioassay. In each experiment the recombinant virus must be compared with the unmodified AcNPV at the same time. A compromise was reached where two virus doses (10^3 or 10^5 polyhedra) were used as a challenge. These are equivalent to 25 and 2500 LD_{50}s respectively, as determined in a highly susceptible species such as *T. ni*.

Various insect species were collected in the field, returned to the laboratory and allowed to produce eggs. These were surface sterilised with formalin to inactivate associated viruses, fungi or bacteria. Neonate larvae were transfered to natural diet, or to semi-synthetic diet if this was acceptable to the species. When the insects had progressed to the second instar, they were assigned to separate groups, which were fed small portions of diet contaminated with 10^3 or 10^5 polyhedra (unmodified AcNPV or AcST-3) or water as an uninfected control. After 24 hours, those insects which had consumed all of the diet were transferred to fresh, virus-free diet and incubated until death or pupation. Deaths were diagnosed as a conseqence of virus infection by simple Giemsa staining of smeared larvae to identify polyhedra and hybridization of purified DNA with radioactive DNA probes specific for the AaHIT coding region or the AcNPV polyhedrin gene. The results are summarized in Table 1. The various insect species were described as permissive for virus replication if they were readily infected with a dose of 10^3 polyhedra, semi-permissive if virus deaths occured after infection with 10^5 polyhedra and non-permissive if no virus deaths resulted after this higher dose was given. The results showed that the host ranges of the parental AcNPV and recombinant AcST-3 were very similar. Differences in the percentages of insect deaths in a single species after infection with each virus are well within the

normal experimental variation expected for this method. The insect species tested which are known to occur naturally at the proposed field site at Wytham were, with the exception of *A. gamma*, only semi-permissive or non-permissive for virus infection. *A.gamma* is an occasionally immigrant pest in the UK.

In summary, the host range data presented in Table 1 supported the conclusion that the recombinant virus, AcST-3, had a similar host range to the unmodified parental AcNPV. Its use in the environment would not constitute a significant risk to indigeneous insect species.

RISK ASSESSMENT OF THE TOXIN TO HUMAN HEALTH AND SAFETY

While the host range of the genetically modified AcST-3 was the same as that of the parental AcNPV, there remained the possibility that the recombinant toxin would have an effect on non-target species. The available data on the mode of action of the AaHIT was reviewed and additional experiments performed to investigate its safety.

Mode of action of the toxin

The symptomology (paralysis of injected insects), low doses required and rapid onset of symptoms point to AaHIT's major mode of action being on the insect nervous system. Experiments have shown that AaHIT causes repetitive firing of the insect's motor nerves resulting in massive and uncoordinated stimulation of skeletal muscle. These experiments have been carried out with several insect species including *Musca domestica* (Loret *et al.*, 1991), *Locusta migratoria* (Walther *et al.*, 1976), and *Periplaneta americana* (D'Ajello *et al.*, 1972).

The selectivity of AaHIT for the insect nervous system was demonstrated by showing that no effect was observed on similar muscle preparations from members representing the Crustacea (Rathmayer *et al.*, 1977), Arachnida Ruhland *et al.*, 1977), and mammals (guinea-pig) (Tintpulver *et al.*, 1976).

In experiments using isolated single insect nerve fibres, voltage clamp experiments were performed which showed that the repetitive firing induced by AaHIT is due to a unique modification of the sodium channel conductance of the insect neuronal membrane. It was manifested as an increase of the sodium current and a slowing of its turn-off (Lester *et al.*, 1982; Gordon *et al.*, 1985; Zlotkin *et al.*, 1985).

In binding experiments using synaptosomes, membrane vesicles prepared from isolated insect nervous tissue, ^{125}I-labelled AaHIT has been shown to bind to preparations from insects (*L. migratoria*, *Gryllus bimaculatus* and *M. domestica*) but not to those of crustaceans or mammals (Teitelbaum *et al.*, 1979; Gordon *et al.*, 1984; 1985). The toxin binds to a single class of non-interacting binding site with high affinity ($K_d = 1.2\text{-}3$nM) and low capacity (0.5-2.0 pmol/mg membrane protein). Comparative assays with saxitoxin (which is known to bind to the sodium channels due to its displacement by tetradotoxin [Walther *et al.*, 1976]) shows that membrane binding capacity for both toxins is the same. The binding site on the sodium channel is different from that of other well characterized toxins. AaHIT is not displaced by veratridine, tetrodotoxin, sea anemone toxin or the α and β scorpion toxins which are specific for vertebrates (Gordon *et al.*, 1985).

Host range of the toxin and route of application

Injecting purified AaHIT into various insect species has revealed considerable variation in toxicity (De Dianous *et al.*, 1987). These LD_{50} data are

summarized in Table 2. Lepidopteran species, such as *Spodoptera littoralis*, were about 650-fold less sensitive than a Dipteran (*Musca domesticus*). The relative lack of sensitivity (Table 3) of AaHIT by injection in lepidopterous larvae representing some 6 species has recently been shown to be a consequence of nonspecific (ineffective) binding and proteolytic degradation of AaHIT. It is not due to a reduction in the high affinity binding to neurons since the toxin binds efficiently to lepidopterous neuronal preparations (Herrmann *et al.*, 1990). In our own experiments, no lethal effect was recorded after injection of 300 ng of the natural toxin into 3rd instar *T. ni* larvae.

The route of application of AaHIT to insects also affects toxicity. The toxin was 500-fold less effective after topical application to *M. domestica* than by injection (De Dianous *et al.*, 1988). Our own studies have shown that feeding 340 ng of toxin to 1 mg second instar *T. ni* larvae produced no obvious toxic effect. All larvae continued to develop normally and eventually pupated. This equates to an LD_{50} of $>$ 35,000 ng/100mg.

These results support the conclusion that for the recombinant toxin to have an effect on insects, it must be synthesised *in vivo* by the baculovirus.

Effect of the toxin on carabid beetles (*Pterostichus madidus*)

After release into the environment, *T. ni* larvae are susceptible to predation by beetles. The effect of the scorpion toxin on predatory beetles was assessed by feeding virus-infected larvae to *Pterostichus madidus* in the laboratory. Adult beetles were collected from the field and fed on insect larvae until required for experimental use. They were observed to be active hunters, rapidly locating any live material within their containers. They were kept separately to prevent fighting.

The virus-infected larvae were prepared by droplet-feeding neonate *T. ni* larvae with AcST-3 or AcNPV ($2x10^6$ polyhedra/ml). Two days later, 40 fully developed first instar larvae were fed to each beetle. The beetles were monitored 1 and 20 hours after feeding, but demonstrated no abnormal behaviour and continued to hunt. The beetles were kept for a further 10 days but remained completely normal.

To quantify the amount of toxin within the virus-infected larvae fed to the beetles, 40 larvae at 75 hours post-infection were homogenised and dilutions assayed for toxin activity by dorso-lateral injection of *Musca domestica* (adult flies). This species is highly susceptible to the toxin after injection. The response to the recombinant toxin produced in the AcST-3 infected larvae was compared with that produced after injection of known amounts of the natural toxin, purifed from total venom using HPLC. It was estimated that each *T. ni* larva contained 2.25-4.5 ng toxin. Since each beetle consumed 40 larvae (28 mg), they would have received 225-450 ng toxin.

In a similar experiment 8 beetles were fed approximately 20 mg of moribund AcST-3 -infected *T. ni* larvae. The next day, a further 20 mg of dead, AcST-3 infected larvae were fed to the beetles. The beetles accepted the cadavers. The beetles were maintained for a further 10 days, hunted actively and showed no behavioural changes.

TESTING THE RECOMBINANT VIRUS AND TOXIN IN SMALL MAMMALS

Virus infectivity

Although the safety of baculoviruses in small mammals is well accepted, it was considered prudent to repeat some of these tests with the recombinant baculovirus. Three separate trials were undertaken in rats and guinea pigs.

Acute toxicity test in rats

Three groups of experimental rats were injected subcutaneously with 0.5 ml of sterile water, AcNPV (unmodified virus) or AcST-3 containing 10^6 polyhedra (equivalent to 10^4 LD50 in 2nd instar *Trichoplusia ni* larvae). In the subsequent 28 day test period. individual bodyweights, general appearance, feeding and drinking habits were recorded. There was no difference between the 3 groups, with all animals remaining healthy. At the end of the 28 days, animals were humanely killed (anaesthetic overdose) and necropsies performed. There were no differences in the organs in animals from each of the 3 groups. The sera derived from the animals before and after the toxicity tests did not contain antibodies to AcST-3.

Acute oral test in rats

Three further groups of rats were intubated with 1.0 ml of sterile water, AcNPV or AcST-3 containing 10^6 polyhedra. The animals were observed for 28 days as described above and showed no abnormal symptoms. Subsequent autopsy also confirmed that there were no differences in the appearance of the organs in animals from each experimental group. The sera derived from these animals did not contain virus-specific antibodies.

Acute dermal toxicity tests in guinea pigs

Guinea pigs were randomly assigned to 3 experimental groups as for the tests with the rats described above. The test material was applied to an area of shaved back (6 cm^2) which was divided into 4 sub-areas, 2 of which were abraided using a 26g needle. Each group received either 0.1 ml distilled water, 0.1 ml AcNPV or 0.1 ml AcST-3 containing 10^6 polyhedra. The treated areas were re-covered with gauze for a 12 hour period. Daily observations were recorded for individual animals over a 14 day period, namely, skin reactions-erythma and oedema, body weight, general appearance, feeding and drinking habits. There were no differences between the control and experimental groups of animals, all remained normal. On day 14 the animals were humanely killed, blood samples were taken and necropsies performed. The appearance of the organs from animals in all groups was normal. The blood samples were analysed for antibodies to the AcST-3 virus, with negative results.

Effect of insect-specific toxin in mice

Acute oral test in mice

Twenty adult mice were randomly assigned to 2 groups. Mice in the first group received 0.1 ml of sterile distilled water. Mice in the second group mice received 0.1 ml containing 1µg AaHIT. Daily observations were recorded for individual animals over a 14 day period, i.e. skin reactions-erythma and oedema, body weight, general appearance, feeding and drinking habits. There were no differences between the control and experimental groups of animals, all remained normal. At the end of the experiment the animals were humanely killed (anaesthetics overdose) and necropsies performed. The appearance of the organs from animals in all groups was normal.

Acute nasal test in mice

In this experiment with adult mice, AaHIT (1µg) or water were applied via the intra-nasal route. All mice remained normal throughout the trial period and showed no abnormalities in internal organs.

RECOMBINATION BETWEEN AcST-3 AND OTHER BACULOVIRUSES

The transfer of the AaHIT coding region and associated transcription regulatory elements into another baculovirus might confer the improved insecticidal properties of AcST-3 on the recipient. The potential for transfer of the viral DNA sequences into other species, however, is realistic only for recipients representing other AcNPV strains, or a limited number of other, closely-related baculoviruses, co-infecting the same cells in the same insect host. Such recombination has been demonstrated experimentally between AcNPV and *Galleria mellonella* NPV (Croizier and Quiot, 1981) and AcNPV and *Rachiplusia ou* NPV (Croizier *et al.*, 1988). These 3 viruses are regarded as different strains of AcNPV. In addition, RoNPV/AcNPV recombinants have been isolated from a wild type stock of RoNPV using plaque purification (Smith and Summers 1980). The frequency with which recombination occurs in cells infected with two baculoviruses is difficult to determine accurately. This is particularly true for closely related baculoviruses where recombinant characterisation depends on laborious analyses of virus DNA genomes with restriction enzymes and electrophoresis. Co-transfecting insect cells with infectious AcNPV DNA and plasmid transfer vectors containing a marker gene (beta-galactosidase or polyhedrin) estimated a recombination frequency of approximately 1% (Kitts *et al.*, 1990). In similar experiments, the AcNPV transfer vector was substituted with one derived from the *Mamestra brassicae* NPV, which shares 2% sequence similarity with AcNPV. Although there was some evidence for transient gene expression from the MbNPV transfer vector in AcNPV-infected cells, stable recombinant viruses were never obtained in the virus progeny (R.D. Possee, unpublished data).

Baculoviruses which are closely related to AcNPV have never been isolated in the UK. The likelihood of recombination between a genetically modified AcNPV and other, unrelated UK baculoviruses such as MbNPV or *Panolis flammea* NPV is extremely low. In the proposed field trial with AcST-3, coinfection of the *T. ni* larvae to be employed by additional viruses will be minimal, as both insects and plants will be introduced from clean stocks and removed at the end of the study. Netted sub-enclosures on the site will also prevent access to the AcST-3-infected *T. ni* by other insects.

SUMMARY

The preliminary results described in this paper support the safe use of genetically modified baculoviruses in the environment. The introduction of an insect-specific scorpion toxin gene into AcNPV does not result in alteration of the host range of the virus. The recombinant toxin has no effect on small mammals or insect predators of virus-infected larvae. The potential for recombination and transfer of the foreign gene to other, distantly related baculoviruses is very small. Furthermore, the procedures adopted for the field trial may be designed to prevent the access of other insects which may harbour baculovirus infections. The successful completion of a field trial with a recombinant baculovirus with improved insecticidal properties is an essential step in our evaluation of whether these agents offer significant advantages over natural baculovirus or chemical insecticides.

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Table 1. Host-range of parent AcNPV and genetically modified AcST-3*

PERMISSIVE SPECIES (doses of $\leq 10^3$ PIBs)

Noctuidae	**AcNPV**	**AcST-3**
Autographa gamma (Silver Y)	100%	71%
Spodoptera exigua (Small Mottled Willow)	100%	42%
Trichoplusia ni (Cabbage looper)	100%	100%
Yponomeutidae		
Plutella xylostella (Diamond-back Moth)	100%	100%
Arctiidae		
Estigmene acrea (Salt Marsh Caterpillar)	98%	94%

SEMI-PERMISSIVE SPECIES (doses of $> 10^3$) e.g., % deaths at doses of 10^5 PIBs

Noctuidae	**AcNPV**	**AcST-3**
Agrotis segetum (Turnip Moth)	32%	24%
Agrotis puta (Shuttle-shaped Dart)	88%	92%
Apamea epomidion (Cluded Brindle)	33%	58%
Aporophyla nigra (Black Rustic)	90%	67%
Autographa jota (Plain Golden Y)	26%	10%
Caradrina morpheus (Mottled Rustic)	63%	13%
Ceramica pisi (Broom Moth)	22%	18%
Colocasia coryli (Nut tree Tussock)	10%	60%
Diarsia mendica (Ingrailed Clay)	2%	2%
Heliothis armigera (American Bollworm)	67%	28%
Heliothis zea (Cotton Bollworm)	77%	25%
Lacanobia w-latinum (Light Brocade)	69%	37%
Mamestra brassicae (Cabbage Moth)	63%	2%
Noctua pronuba (Large Yellow Underwing)	30%	33%
Noctua janthina (Lessr Broad-Bord'd Yellow U'Wing)	36%	7%
Mythimna separata (Rice Army Worm)	23%	14%
Panolis flammea (Pine beauty Moth)	42%	14%
Orthosia stabilis (Common Quaker)	45%	30%
Rusina ferruginea (Brown Rustic)	33%	10%
Spodoptera frugiperda (Fall Armyworm)	88%	66%
Spodoptera littoralis (Mediterranean Brocade)	12%	NP
Geometridae		
Idaea aversata (Riband Wave)	4%	NP
Sphingidae		
Laothoe populi (Poplar Hawk Moth)	NP	12%
Mimas tilae (Lime Hawk Moth)	88%	92%
Nymphalidae		
Melitaea cinxia (Glanville Fritillary)	80%	80%
Polygonia c-alba (Comma)	15%	5%

NON-PERMISSIVE SPECIES (doses of $>10^5$ PIBs)

Noctuidae	**AcNPV**	**AcST-3**
Acronicta megacephala	NP	NP

Acronicta rumicis (Knot Grass)	NP	NP
Dicestra triflolii (The Nutmeg)	NP	NP
Hada nana (The Shears)	NP	NP
Hoplorina ambigua (Vine's Rustic)	NP	NP
Lacanobia oleracea† (Bright-line Brown-eye)	NP	NP
Ochropleura plecta (Flame shoulder)	NP	NP
Orthosia gothica (Hebrew Character)	NP	NP
Polia nebulosa (Grey Arches)	NP	NP
Xestia c-nigrum (Setaceous Hebrew Character)	NP	NP
Notodontidae		
Furcula furcula (Sallow kitten)	NP	NP
Geometridae		
Ourapteryx sambucaria (Swallow-tailed Moth)	NP	NP
Pelurca comitata (Dark Spinach)	NP	NP
Lymantriidae		
Euproctis similis (Yellow-tail)	NP	NP
Pieridae		
Pieris brassicae (Large White)	NP	NP
BUTTERFLIES		
Nymphalidae		
Aglais urticae (Small Tortoiseshell)	NP	NP

*Only data for species tested simultaneously with both viruses are included, specifically focussing on those species that earlier tests had indicated were likely to be infected at some dose of AcNPV (plus some other non-permissive species, as controls, and previously indicated as not infectible by AcNPV). Not shown are the uniformly negative results of tests involving non-lepidopteran insects (bees, ants, beetles, lacewings, ladybirds, sawflies etc). Other lepidoptera and other non-lepidoptera insects (in sum >70 other species) that previous studies indicated were not infectible by AcNPV were not tested. In these tests, second instar larvae were fed virus polyhedra (PIBs) in a 24 h period and then placed onto virus-free diet. The larvae were observed until death or pupation. The % mortalities at the indicated doses for virus-confirmed infections are given. NP indicates that significant numbers of insects (<2%) were not susceptible to virus infection.
While subjective, experience indicates that the likelihood of any "semi-permissive" species attaining high LD_{50} doses under field conditions is, at best, remote. The likelihood of such infections leading to epizootics in those species is considered to be improbable. Semi-permissive species, like the permissive species, may contribute to virus maintenance, although for such semi-permissive species there is no evidence to support this view.

Table 2. Responses of various species to AaHIT†

Test animal (route)	LD_{50} ng/100mg	95% confid.
DIPTERA (injection)		
Musca domestica	2	1.3-2.4
Sorcophaga argyrostoma	15	13.7-17.2
DICTYOPTERA (injection)		
Blattela germanica	26	24.3-28.0
Periplaneta americana	46	36.5-57.5
ORTHOPTERA (injection)		
Gryllus domesticus	289	224-373
Gryllus domesticus	375	384-400
LEPIDOPTERA (injection)		
Spodoptera littoralis	1310	1180-1460
MOUSE		
(sub-cutaneous injection)	>50 mg/kg	
(intra-cerebral injection)	>2.5 mg/kg	

† Data summarized from De Dianous et al. (1987)

Table 3. Responses of lepidopterous larvae to AaHIT†.

Insect	Dose µg/100mg	Response*
Spodoptera littoralis	0.65 µg/100mg	0/5
	2.0	5/5
Heliothis peltigera	2.5	0/3
	5.0	1/3
	10.0	3/3
Galleria mellonella	0.8	1/3
Bombyx mori	0.35	2/3
	0.65	5/5
Ocnogyna loewi	0.65	0/3
Cydia pomonella	0.8	0/3

† Data summarized from Herrmann *et al.*, (1990).
* Lethality was determined by inability of the insect to pupate.

GENETICS OF *BACILLUS THURINGIENSIS* TOXINS

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ABSTRACT

Bacillus thuringiensis produces during sporulation large amounts of crystal proteins with toxic activity against insect larvae. The *cry* genes encoding these proteins can be divided into several groups which share sequence similarities, thus probably constituting a family of genes.

The broad diversity of *cry* genes, their cloning and the characterization of their products, the modification of their insecticidal specificities by *in vitro* mutagenesis and the understanding of the regulation of their expression, promise to contribute to the construction of genetically engineered *B. thuringiensis* strains with improved properties for pest control. Specific plasmid vectors and *in vivo* recombination mediated by homologous DNA sequences are two potentially valuable tools for introducing DNA into *B. thuringiensis*. Recombinant strains harbouring various combinations of insecticidal crystal protein genes and displaying broader but defined activity spectra could thereby be constructed.

INTRODUCTION

The insecticidal properties of *Bacillus thuringiensis* are highly variable. In recent years this has resulted in extensive searches for new strains with different target spectra. Several thousand natural strains have been isolated from various geographical areas and from different sources. These isolates can be classified into about 30 serotypes based on biochemical properties and flagellar antigens (de Barjac and Frachon, 1990). However, this classification does not reflect the pathotype of the bacteria, which is essentially defined by the delta-endotoxins that make up the characteristic crystalline inclusion of the *B. thuringiensis* strains.

Most *B. thuringiensis* strains can synthesize more than one crystal, which may itself be formed by different, although related delta-endotoxins. Depending on their delta-endotoxin composition, the crystals have various forms and a partial correlation between structure and protein composition of the crystals can be established. Moreover, a system of nomenclature and classification of the delta-endotoxins according to their insecticidal properties and sequence similarities has been proposed (Höfte and Whiteley, 1989; Lereclus *et al.*, 1989b).

Five major classes of delta-endotoxins (CryI, II, III, IV and V) and a cytolysin (Cyt) have been described to date (Lereclus *et al.*, 1993). The different delta-endotoxins belonging to the Cry classes contain homologous domains and presumably constitute a single family of proteins. Generally these proteins are active against Lepidoptera (CryI), both Lepidoptera and Diptera (CryII), Coleoptera (CryIII), Diptera (CryIV) or both Lepidoptera and Coleoptera (CryV). They are in fact protoxins which are activated by insect midgut proteases to yield toxic fragments.

These insecticidal proteins are synthesized during sporulation and accumulate in the mother cell as a crystal which can account for up to 25 % of the dry weight of sporulating cells. The very high level of crystal protein synthesis in *B. thuringiensis* and its coordination with sporulation is a remarkable and interesting system for the investigation of genetic regulation in sporulating Gram-positive bacteria. Several mechanisms of transcriptional or post-transcriptional molecular regulation have been found to be devoted to the synthesis of crystals in *B. thuringiensis*. Moreover, the level of toxin production in a given strain is dependent on the *cry* gene copy-number.

THE TOXIN GENES

About twenty different crystal protein genes, isolated from various *B. thuringiensis* strains, have been cloned and sequenced. They have been designated as *cry* genes. The determination of both the amino acid sequence similarities and the insecticidal activities of the encoded polypeptides has allowed the classification of these genes into five classes (Lereclus *et al.*, 1993). Moreover, a *cyt* gene encoding a cytolytic toxin is found in the *B. thuringiensis* strains active against mosquitoes. A simplified classification of the toxin genes isolated from *B. thuringiensis* is shown in Table 1.

TABLE 1. Classification of the *B. thuringiensis* toxin genes

Class	Subclass	Size of the protoxin (kDa)	Susceptible insects
cryI	*A - G*	130 -140	Lepidoptera
cryII	*A*	71	Lepidoptera and Coleoptera
	B, C	71	Lepidoptera
cryIII	*A, B, D,*	70 - 73	Coleoptera
	C	129	Coleoptera
cryIV	*A, B*	130	Diptera
	C	78	Diptera
	D	72	Diptera
cryV	*A*	81	Lepidoptera and Coleoptera
cyt	*A, B, C*	28	non specific

The *cryI* gene class (the most frequent class in *B. thuringiensis*) contains at least seven subclasses (*cryIA - G*). The encoded polypeptides (130 - 140 kDa) are active against Lepidoptera and have related amino acid sequences. The *cryIA* subclass has itself been divided into three subgroups (*cryIA(a), (b)* and *(c)*) sharing more than 80 % amino acid identity.

Many *B. thuringiensis* strains produce 71 kDa proteins in addition to the CryI polypeptides. Some of these 71 kDa proteins are toxic for both Lepidopteran and Dipteran larvae. The genes encoding these polypeptides belong to the *cryII* class. The product of the *cryIIA* gene is active against Lepidoptera and mosquitoes, whereas CryIIB and CryIIC toxins are only active against Lepidopteran species.

A third class of toxin genes encodes Coleopteran-specific proteins (CryIII). Four genes belonging to this class have been characterized : *cryIIIA, B, C* and *D*. CryIIIA, B and D toxins are polypeptides of 70 - 73 kDa. The *cryIIIC* gene product is a 129 kDa protoxin active against Coleoptera only after *in vitro* solubilization and activation (Lambert *et al.*, 1992).

The crystal protein genes belonging to the *cryIV* class were isolated from *B. thuringiensis israelensis* which is toxic for Dipteran larvae (mosquitoes). This class contains four genes (*cryIVA, B, C* and *D*) that encode crystal proteins with predicted molecular masses of 135, 128, 74 and 72 kDa. The comparison of the deduced amino acid sequences of these toxins indicates that the CryIVD protein is distantly related to the other toxins of this class. Another polypeptide found in *B. thuringiensis*

israelensis crystals is a cytolytic protein of 28 kDa, encoded by the *cytA* gene. Two related proteins (CytB and C) have been found in the crystals of other *B. thuringiensis* subspecies active against mosquitoes (Yu *et al.*, 1991; Koni and Ellar, 1993).

Finally, a novel gene (*cryVA*) encoding a 81 kDa protein has recently been characterized (Tailor *et al.*, 1992). The product of this gene presents a dual specificity, active against both Lepidopteran and Coleopteran larvae.

LOCALIZATION OF THE TOXIN GENES

Although DNA sequences related to *cry* genes were recently found in the chromosome of several *B. thuringiensis* strains (Carlson and Kolstø, 1993), most of the *cry* genes are located on large plasmids (50 - 250 kb). These plamids generally possess conjugative properties (González *et al.*, 1982) and it was shown that plasmids harbouring *cry* genes are able to transfer from one *B. thuringiensis* strain to another within insect larvae (Jarrett and Stephenson, 1990).

Most *B. thuringiensis* isolates harbour more than one toxin gene. Some strains (for example: *kurstaki* HD1, *israelensis*, *aizawai* 7-29) harbour at least four different crystal protein genes. The *aizawai* strain contains *cryIA(a)*, *cryIA(b)*, *cryIC* and *cryID* genes (Sanchis *et al.*, 1988). A second example is the *israelensis* strain in which the four toxin genes, *cryIVA-D*, and the *cytA* gene are present on a single 125 kb plasmid.

Most of the plasmid genes encoding crystal proteins are parts of composite structures including several transposable elements. The *cryIA* genes are flanked by two sets of insertion sequences, IS*231* and IS*232*, and by the transposon Tn*4430*. The *cryIVA* gene of the *israelensis* strain is flanked by two copies of the insertion sequence IS*240*. The genetic organization of these *cry* genes and their related mobile genetic elements was recently reviewed (Lereclus *et al.*, 1993). The conjugative transfer between *B. thuringiensis* strains and the presumed mobility of the *cry* genes (mediated by transposable elements) may have contributedto the diversity of the crystal protein genes within the *B. thuringiensis* species.

REGULATION OF TOXIN SYNTHESIS

The high level of crystal protein synthesis in *B. thuringiensis* and its coordination with sporulation constitute an interesting system for the study of genetic expression of secondary metabolites in sporulating Gram-positive bacteria. At least four distinct mechanisms of transcriptional or post-transcriptional regulation are involved in the synthesis of crystals in *B. thuringiensis*. The *cry* gene copy-number also probably plays an important role in the differential expression of the various *cry* genes present in a given strain.

Temporal and spatial control of toxin gene expression

In the Lepidopteran-specific *B. thuringiensis* strains, crystal protein gene expression starts in the mother cell compartment at t_2 of sporulation (t_0 is the onset of sporulation and t_n indicates the number of hours after t_0). Sporulation negative (Spo^-) mutants blocked at t_0 do not produce crystals, whereas those blocked after t_2 can produce crystals (Ribier and Lecadet, 1981). However, in wild type strains the crystals increase in size until about t_{12} (Ribier and Lecadet, 1973). Since these preliminary observations, it was shown that a *cryIA(a)* gene was transcribed from two overlapping promoters (BtI and BtII) used sequentially (Wong *et al.*, 1983). BtI is active between about t_2 and t_6 of sporulation, and BtII from about t_5 onwards. Similar regions (containing only one or both types of promoters) are located upstream of the *cryI* genes, the *crylVA* and *B* genes and the *cytA* gene (Ward and Ellar, 1986; Brizzard *et al.*, 1991; Yoshisue *et al.*, 1993).

Two sigma factors (σ^{35} and σ^{28}) which specifically direct the transcription of *cryI* genes from BtI or BtII have been isolated (Brown and Whiteley, 1988; Brown and Whiteley, 1990). The genes encoding σ^{35} and σ^{28} have been cloned and sequenced (Adams *et al.*, 1991). They are homologous to the genes encoding the σ^{E} and σ^{K} factors of *B. subtilis*, respectively. This homology is in agreement with the fact that in *B. subtilis* both types of sigma factors are successively functional in the mother cell compartment from t_2 to about t_7 of sporulation (Stragier and Losick, 1990).

Promoter regions of several toxin genes do not share sequence identity with σ^{35} or σ^{28} specific promoters. This is for example the case of the *cryIII* genes (Sekar *et al.*, 1987; Donovan *et al.*, 1992; Teixeira de Souza *et al.*, 1993). The upstream DNA region of these genes is dissimilar to the promoter region of the *cryI* genes. The activity of the *cryIIIA* promoter is detectable during the vegetative phase, and transcription increases as early as the start of the stationary phase to reach a plateau at t_6 (Teixeira de Souza *et al.*, 1993). The transcription of the *cryIII* genes may therefore require a RNA polymerase with a sigma factor other than σ^{E} or σ^{K}. However, the temporal and spatial control of expression of all *cry* genes occurs at the transcriptional level. The common and major feature of these control systems is to limit the *cry* gene expression to the stationary phase of *B. thuringiensis* growth: *cry* gene expression during the vegetative phase is zero or strongly repressed.

mRNA stability

The high level of toxin synthesis is partly due to the stability of toxin mRNA (Glatron and Rapoport, 1972). The half-life of these transcripts was estimated to be about 10 minutes. It has since been shown that the putative transcriptional terminator of a *cry* gene (a stem-loop structure) increases the mRNA half-life (Wong and Chang, 1986). The potential terminator sequences are relatively well conserved downstream of the different *cry* genes. The presence of the stem-loop structure at the 3' end of the mRNA may protect it from exonuclease degradation.

Post-transcriptional effect

It has been reported that polypeptides are involved in increasing crystal production by presumably acting as a molecular chaperone either to direct folding of the toxins into a stable conformation or to protect them from proteolytic degradation. A 20 kDa polypeptide found in the crystals of *B. thuringiensis israelensis* enhances the production of CytA and CryIVD when the corresponding genes are cloned in *E. coli* (Adams *et al.*, 1989; Visick and Whiteley, 1991). However, high level *cytA* and *cryIVD* gene expression in *B. thuringiensis* does not require the 20 kDa polypeptide (Chang *et al.*, 1993).

In *B. thuringiensis kurstaki* HD1, the *cryIIA* gene is the distal gene of an operon which is comprised of three open reading frames. The second (*orf2*) encodes a polypeptide with an unusual amino acid sequence: a 15 amino acid motif is repeated 11 times in tandem (Widner and Whiteley, 1989). This polypeptide contributes to the efficient expression of a cloned *cryIIA* gene in *B. thuringiensis* (Crickmore and Ellar, 1992).

A second type of post-transcriptional event has recently been reported: DNA molecules were found specifically associated with the non toxic part of a CryI protoxin (Bietlot *et al.*, 1993). It is proposed that DNA binds to the COOH-terminal half of the crystal protein and is essential for maintaining the conformational integrity required for crystal formation and generation of toxin. Although these post-transcriptional mechanisms are still unclear and controversial, they could play an important role in the stability of the crystals in *B. thuringiensis*.

Toxin gene copy number and differential expression

In addition to the mechanisms of transcriptional and post-transcriptional regulation, the copy number of the plasmids carrying the *cry* genes could be determinant for the relative expression of the different toxin genes present in a given strain. Consequently, copy number could determine the insecticidal activity spectrum of the strain.

Two different *cryIA* genes are present in the strain *B. thuringiensis* 407. The *cryIA(a)* gene is poorly expressed in comparison with the *cryIA(b)* gene. However, CryIA(a) production was high when the gene was cloned alone in a *B. thuringiensis* Cry⁻ strain using the low copy-number plasmid pHT3101 (Lereclus *et al.*, 1989a). Its very low expression in the strain 407 may therefore be due to a difference in the copy-number of the two *cry* genes present in this strain. Similarly, when a *cryIA(c)* gene is cloned in a strain harbouring other *cryI* genes, significantly less CryIA(c) toxin is synthesized than when cloned into a Cry⁻ strain (Baum *et al.*, 1990). When the *cry* genes are cloned on high copy-number plasmids and transferred into a *Bacillus* Cry⁻ strain, they reduce the ability of the cells to form spores. This disturbance of sporulation is probably due to titration effects (increased number of sporulation specific promoters and subsequent competition for specific sigma factors).

The effect of plasmid copy-number on toxin production was tested by cloning a *cryIIIA* gene into vectors varying only by their copy-number (Arantes and Lereclus, 1991). The production of CryIIIA toxin was saturated at an intermediate copy-number (15 copies per equivalent chromosome). These results strongly suggest that the *cry* gene copy-number could be partly responsible for their differential expression in a *B. thuringiensis* strain. It is therefore important that the relative *cry* gene copy-number should be considered when constructing recombinant *B. thuringiensis* strains harbouring several toxin genes.

ENGINEERING OF *B. THURINGIENSIS*

Conjugation is currently used to construct recombinant *B. thuringiensis* strains with new combinations of *cry* genes. Strains can thereby be constructed with properties appropriate for the biological control of a broad spectrum of insect larvae. However, this procedure is limited to the *cry* genes carried by conjugative plasmids. Moreover, conjugation does not allow the association of *cry* genes located on plasmids belonging to the same incompatibility group .

The development of an efficient host-vector system now make feasible the construction of genetically engineered *B. thuringiensis* strains. Most strains can be easily trasformed by plasmid DNA using electroporation procedures and shuttle vectors have been constructed using replication regions of *B. thuringiensis* resident plasmids (Lereclus *et al.*, 1989; Baum *et al.*, 1990). These plasmids are segragationally and structurally stable, thus allowing the cloning of several *cry* genes and the production of crystals in the *B. thuringiensis* transformants.

The scope for genetic manipulation was increased recently when it was shown that *in vivo* recombination can occur in *B. thuringiensis* between homologous sequences carried by a non-replicative or thermosensitive plasmid and a resident plasmid. The *cytA* gene of *B. thuringiensis israelensis* was disrupted in the 125 kb resident plasmid by *in vivo* recombination. The *cytA* gene was replaced by a disrupted copy of the gene by forcing recombination between homologous regions in a non-replicative plasmid used to transform the *B. thuringiensis* strain, and the 125 kb resident plasmid (Delécluse *et al.*, 1991). The resulting strain produces all the crystal proteins (CryIVA, B, C and D) but not CytA; its mosquitocidal activity did not differ significantly from that of the wild strain and it would therefore be economically useful.

Similarly, expansion of the insecticidal host range of a *B. thuringiensis* strain was obtained by insertion of a toxin gene into a resident plasmid (Lereclus *et al.*, 1992). A gene encoding a coleopteran-specific toxin was inserted into a fragment of an insertion sequence (IS232) cloned into a plasmid thermosensitive for replication. The plasmid was used to transform a *B. thuringiensis* strain toxic to Lepidoptera. The transformants were then selected at non-permissive temperature for clones in which the vector had integrated into a copy of IS232 present on a resident plasmid (Fig. 1a). A second recombination event was selected such that the vector was eliminated and the newly introduced toxin gene was conserved (Fig. 1b). The resulting strain containted only DNA of *B. thuringiensis* origin, and displayed insecticidal activity against both Lepidoptera and Coleoptera.

In conclusion, genetic manipulation of *B. thuringiensis* strains can improve their insecticidal properties and thus contribute to their development as biopesticides. There are three possible approaches: conjugation, transformation with plasmid vectors and *in vivo* genetic recombination. The latter allows the deletion of disadvantageous genes, the addition of new genes or the modification of genes present in the strain. Furthermore, *in vivo* recombination results in genetically engineered strains that only contain DNA of *B. thuringiensis* origin. Therefore, problems of regulatory approval and acceptance in the marketplace can be minimized.

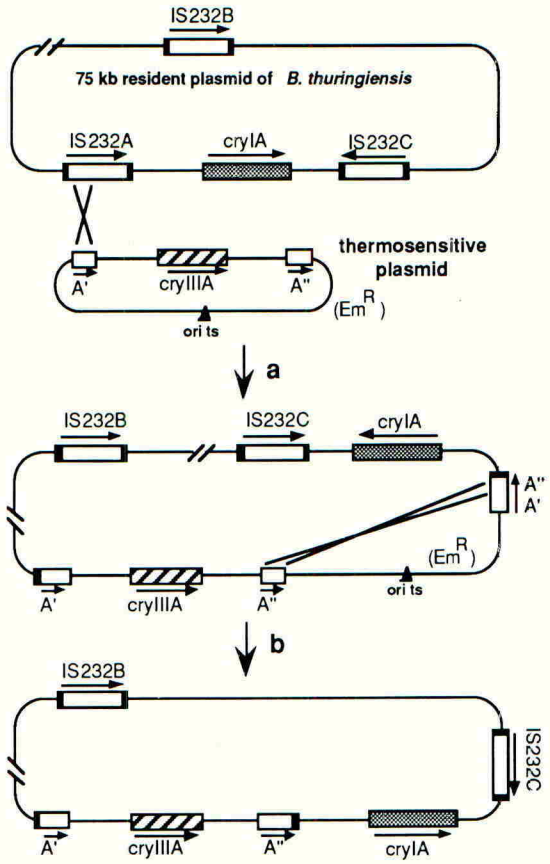

FIGURE 1. Introduction of a *cryIIIA* gene into IS232 by homologous recombination. **a)** The thermosensitive plasmid integrates into the resident 75 kb plasmid by recombination between homologous A' regions of IS232. Em^R clones were selected at non-permissive temperature (37°C). **b)** A second recombination event occurs between the two A" regions of IS232. The resulting Em^S clones produce both CryIA and CryIIIA toxins.

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INSECTICIDAL ACTIVITY OF *B. THURINGIENSIS* TOXIN

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No paper submitted

ENGINEERING *BACILLUS THURINGIENSIS* TOXINS INTO OTHER BACTERIA

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ABSTRACT

Current application of *B.thuringiensis* is mainly restricted to the use in high value markets like cash crops, disease vector control and forestry. Application of recombinant DNA technologies can expand the markets for *B.thuringiensis* toxins by engineering them into other hosts. Transgenic microorganisms can improve the efficacy of *B.thuringiensis* toxins by (*i*) expanding the persistence of *B.thuringiensis* toxin in the field, (*ii*) enlarging the spectrum of susceptible insect species and (*iii*) delivery of the toxin at places inaccessible for products based on natural *B.thuringiensis* preparations.

INTRODUCTION

Increasing concern regarding the environmental impact of agrochemicals has resulted in growing pressure in many countries to reduce the use of these chemicals in agriculture. At present microbial insecticides occupy only a small portion (0,5 -1%) of the total insecticide market, but in the near future it is likely that the use of biopesticides will increase dramatically.

Microbial control agents have a number of advantages over chemical insecticides. First of all microbial agents demonstrate highly specific antagonistic activities. In general they will only affect the target pest and will be innoxious towards beneficial insects and natural antagonists. Secondly there is little or no environmental impact associated with the use of microbial agents and they generally will not persist over extended periods of time after application in the field. Thirdly, most microbial agents are safe for workers handling them.

By far the most prominent microbial insecticide is *Bacillus thuringiensis*. Following its discovery at the beginning of this century *B.thuringiensis* has gained interest from a few entomologists and microbiologists and its commercial use was restricted to a narrow range of lepidopteran (caterpillar) pests. During the last two decades, however, with the discovery of strains with specificity for dipteran as well as coleopteran pests, *B.thuringiensis* has penetrated into other research areas like (bio)chemistry, ecology, molecular biology and physiology. Moreover the exploitation of its use as a microbial insecticide has increased exponentially in the 1980s. The general features of biopesticides mentioned above all apply to *B.thuringiensis*. In addition,

B.thuringiensis is pathogenic to pests in some of the major crops grown throughout the world; it can be cultured easily in large fermenters, making it relatively inexpensive to produce and it has an acceptable shelflife. In addition, as compared to chemical insecticides, the development of resistance against *B.thuringiensis* in target insects is a rare occasion.

Mode of action

B.thuringiensis is a Gram-positive, rod-shaped bacterium. Under starvation conditions sporulation is induced and simultaneously proteinaceous parasporal crystals are formed. These crystals that may comprise up to 30-40 percent of the cell dry weight provide *B.thuringiensis* with its unique insecticidal activity. When *B.thuringiensis* crystals are ingested by insect larvae, they are solubilized by the high pH in the larval midgut and proteins of 27 000 to 140 000 kDa are liberated. These polypeptides are processed by gut enzymes to release toxin molecules that range from 25 000 to 65 000 kDa. These activated toxins bind to specific receptors on the membranes of epithelial midgut cells in susceptible insects where non-specific pores are formed that disrupt the semipermeability of the membrane. Subsequently the epithelial cells will swell and lyse. These factors, solubilisation, proteolytic processing, membrane binding and pore formation, all contribute to the specific activity and the insecticidal spectrum of a given *B.thuringiensis* isolate. At present many strains with different insecticidal spectra have been identified.

Why improving naturally occurring *B.thuringiensis*?

Despite the fact that *B.thuringiensis* is by far the most successful biopesticide, comprising 80 - 90 percent of the total market, the use of *B.thuringiensis* based products could certainly be expanded to other areas of application. At present *B.thuringiensis* is mainly applied in several cash-crops, in disease vector control and in forestry. These limitations in the use of *B.thuringiensis* are largely caused by inherent biological characteristics of the bacterium and can be placed within three categories:

1 limitations with respect to host range,
2 restricted persistence in the environment, and
3 inability to reach target insects that feed on roots or on inner parts of plants.

These features add to the criticisms with regard to a worldwide adoption of *B.thuringiensis*-based products in crop production. In order to enable farmers to switch to *B.thuringiensis*-based protection of their crops it will be essential to improve the efficacy of *B.thuringiensis*. As discussed below the application of recombinant DNA technologies provides possibilities to meet the current disadvantages of natural *B.thuringiensis* preparations.

Transgenic plants

One way of overcoming the problems with persistence of *B.thuringiensis* toxins is the construction of transgenic plants that express their own toxin genes. Genes can be inserted into plants by means of *Agrobacterium tumefaciens* mediated transfer of DNA. This methodology allowed the construction of transgenic dicotyledons, such as tobacco, tomato, potato and cotton. However, the recent development of transformation using ballistics also opens the way to transform a hole range of monocots, eg. corn, rice etc. In the first generation of *B.thuringiensis*-plants the levels of expression of toxin were insufficient to control the most sensitive larval species. Changes in the promotor as well as the structural gene were used to obtain levels of expression sufficient for larval control. These modifications involve the use of a 35S promotor of the cauliflower mosaic virus containing a duplicated enhancer region, resulting in a 5 - 10 fold increase of expression. More dramatic improvement of expression was brought about by changes in the nucleotide sequence that did not interfere with the aminoacid sequence of the protein. These substitutions involved elimination of regions in the bacterial sequence with a hypothetical regulatory role in plants and changes in the codon usage. These changes led to a 1000 fold increase in expression of the *cryIA(b)* gene and these plants were not damaged by *Spodoptera exigua* (Perlak *et al.* 1990).

TRANSGENIC BACTERIA

The use of transgenic plants will be restricted to the major cultivars of most crops, since the production of transgenics of cultivars grown in small areas is economically not feasible. Moreover, although the number of plant species that can be transformed increases constantly, some agronomically interesting crops are at present not (yet) accessible for genetic manipulation because they are recalcitrant for transformation.

An alternative way to improve the host range and efficacy of natural *B.thuringiensis* toxins is the introduction and expression of toxin genes in different genera of microorganisms. On the one hand the ability to construct strains with any given combination of toxin genes expands the host range of *B.thuringiensis* preparations and opens the way to produce tailor-made bioinsecticides. On the other hand the introduction of toxin genes into ecologically adapted bacteria will improve the persistence as well as the delivery of *B.thuringiensis* toxins.

Limited host range

The highly specific activity of *B.thuringiensis* is beneficial to the environment, since toxicity is specific for the pest species and non-target insects as well as other organisms will survive. This can be an advantage under circumstances when a single pest has to be controlled. On the other hand, the narrow host range of *B.thuringiensis* restricts the use of products based on a particular *B.thuringiensis* strain to a limited number of susceptible insects.

Novel insecticidal activities

New products have to be made to control insect species with low susceptibility towards the commercially available *B.thuringiensis* products. To meet the disadvantage that the toxicity of *B.thuringiensis* is confined to a small group of insect species several groups have set out to isolate and screen natural *B.thuringiensis* strains from all over the world to collect strains with novel insecticidal spectra. This approach to make inventory of the natural variability of targets for *B.thuringiensis* toxins has led to the discovery of strains with activity against plant- and animal-parasitic nematodes, whereas strains with activity towards hitherto unsusceptible species like soil grubs (*Coleoptera*) and leafminers (*Diptera*) have also been discovered (Feitelson *et al.*, 1992). These screening programs have also yielded *B.thuringiensis* strains with activity to hitherto unsusceptible lepidopteran pests, like beet armyworm (*Spodoptera exigua*). The toxicity of the individual *B.thuringiensis* strain nonetheless remains usually confined to a small range of insect species. In situations where a complex of pests must be controlled, which is often the case in agriculture, a much broader range of activities would be required, resulting in the simultaneous use of additional (bio)insecticides.

Extension of host range

The activity spectrum of *B.thuringiensis* strains could be expanded by constructing strains that express a combination of different toxins. Indeed many natural *B.thuringiensis* isolates produce more than one toxin. The construction of strains with broader or improved insecticidal activities would require the transfer of *B.thuringiensis* genes into non-homologous *B.thuringiensis* isolates. Three systems of genetic exchange are available in *B.thuringiensis*; generalized transduction mediated by bacteriophages, conjugation-like plasmid transfer and electro-transformation.

Conjugation has proved to be very useful because most toxin genes are located on plasmids (González *et al.*, 1982). Klier *et al.* (1983) have used conjugation between *B.subtilis* and *B.thuringiensis* to construct a *B.thuringiensis* strain with dual insecticidal activity. By introduction of a cloned *cryIA(b)* gene into *B.thuringiensis* subspecies *israelensis* a transconjugant was obtained that was toxic for *Ephestia kuehniella* (*Lepidoptera*) as well as for two dipteran species (*Anopheles stephensi* and *Culex pipiens*).

Conjugation can also be used to improve the efficacy of strains by rearranging the composition of the proteins in the parasporal crystal. This approach involves the curing of plasmids that carry genes coding for toxins with little efficacy against the intended pest and substituting them for more effective ones by introducing plasmids from other strains. This strategy was followed by Carlton *et al.* (1990) to construct strains with improved activity towards specific target insects. Strains with a broader spectrum of activities were produced by introduction of the coleopteran active *cryIIIA* gene into a lepidopteran active strain of *B.thuringiensis* subspecies *kurstaki*. The resulting transconjugant possessed both lepidopteran and coleopteran activity and proved to be effective against the larvae of both the Colorado beetle, *Leptinotarsa decemlineata*, and the European corn borer, *Ostrinia nubilalis*. Because conjugation is considered to be a natural process the regulatory restrictions associated with testing and use of organisms developed by

recombinant DNA technology do not apply to these novel strains and the US Environmental Protection Agency approved the use of this strain in 1990.

The extension of electroporation from eukaryotes to bacteria at the end of the 1980s has dramatically increased the possibilities to manipulate *B.thuringiensis*. This technique of electrotransformation whereby foreign DNA is introduced into organisms during an electrical discharge has enabled several workers to construct *B.thuringiensis* strains with novel insecticidal activities. In general transformants are readily obtained with transformation frequencies between 10^2 to 10^5 transformants per μg of plasmid DNA although in some strains restriction systems prevent transformation with methylated plasmid DNA (Macaluso and Mettus, 1991). The stability of the incoming plasmids however is rather variable, a problem that has been solved in different ways.

Crickmore *et al.* (1990) encountered instability with shuttle vectors based on plasmid pUC and plasmid pC194. The structural instability could be eliminated by replacing the pUC moiety of their shuttle vectors by pBR322 resulting in the shuttle vector pSV1. When the *cryIIIA* gene was inserted in this plasmid, the resulting construct, pSVten replicated in a stable manner in *B.thuringiensis* subspecies *israelensis*. These transformants were toxic both for dipteran and coleopteran larvae.

Several others have constructed new cloning vectors that are based on origins of replication of resident plasmids of *B.thuringiensis* (Gamel and Piot, 1992). It is expected that these vectors will function optimally in the development of commercial strains as they are compatible with resident plasmids and are stably maintained in the absence of antibiotics.

An elegant way of introducing toxin genes into *B.thuringiensis* has been described by Lereclus *et al.* (1992). These authors have used electroporation to insert the *cryIIIA* gene into a resident plasmid in *B.thuringiensis* strain HD-73. The *cryIIIA* gene was cloned within a fragment of the insertion sequence IS232 and inserted into a plasmid thermosensitive for replication in *B.thuringiensis*. Upon electrotransformation and shift to the nonpermissive temperature the *cryIIIA* gene was integrated into one of the three IS232 elements present on the resident plasmid of HD-73. Integration occurred by homologous recombination and vector sequences were lost. The resulting strain contained only sequences of *B.thuringiensis* origin and displayed insecticidal activity against both *Lepidoptera* and *Coleoptera*.

Interestingly, some of the strains with new combinations of insecticidal activity demonstrate synergistic interactions between the toxins. Crickmore *et al.* (1990) observed some toxicity towards *Pieris brassicae* (*Lepidoptera*) with *B.thuringiensis* subspecies israelensis transformed with *cryIIIA*. This result supports the notion that toxins may act in concert leading to specific activities towards insect species that are insusceptible to either of the individual toxins. Such a synergistic effect has also been suggested for the toxins of *B.thuringiensis* subspecies *israelensis*, where none of the individual toxins is as toxic as the complete array of proteins occurring in the parasporal crystals of natural strains.

Residual activity and delivery of *Bacillus thuringiensis*

There are three different ecological niches into which *B.thuringiensis* has to penetrate in order to control insect pests:

- on the plant surface to control leaf-eating insects
- in aquatic systems to control the water-borne larvae of numerous disease vectors
- in soil for the control insects that feed on roots

Each of these niches poses its own problems with regard to the efficacy of *B.thuringiensis*. Although *B.thuringiensis* is a sporeforming bacterium and spores can survive for many years, the bioinsecticidal half-time of *B.thuringiensis* on the other hand is rather short. Parasporal crystals on leafs can be inactivated by ultraviolet light and other environmental factors, and rain may wash off crystals from their intended site of action. In soil crystals can be degraded by indigenous soil microorganisms and during aquatic applications the crystals may sediment or aggregate with particulate matter. All these factors greatly reduce the persistence of insecticidal activity in the field.

To improve the persistence of *B.thuringiensis* toxins two approaches are imaginable. First, *B.thuringiensis* itself might be improved in its survival under various ecological conditions and second,the toxins genes could be cloned and expressed in microorganisms that are better adapted to the environment. As ecological performance is expected to be the result of many gene functions as well as different (a)biotic factors the first option is not very likely to succeed. The introduction of *B.thuringiensis* genes into other bacteria however appears quiet feasible.

Encapsulated crystals

To increase the residual activity of crystal proteins after foliar application Mycogen Corp. has followed the approach to encapsulate the crystals to reduce the degradation rate of the insecticidal activity. In this system several *cry* genes are introduced and expressed in a non-pathogenic, leaf-colonizing isolate of *Pseudomonas fluorescens*. Using appropriate plasmid vectors crystals were produced in *P.fluorescens* that account for 10 -30 percent of total cell protein. This system was originally intended to be used as a living delivery system for *B.thuringiensis* toxins, by regulatory restrictions on the release of transgenic microorganisms forced the development of an alternative strategy. As a final step in the production of the encapsulated crystals the transgenic *P.fluorescens* cells were killed by fixation. This resulted in crystals protected from external factors by the bacterial cellwall (Gelernter, 1993).

Aquatic delivery

The crystals of *B.thuringiensis* subspecies *israelensis* are highly toxic to certain dipteran pests and *B.thuringiensis* subspecies *israelensis* has proved valuable as a biological agent for the control of larvae of mosquitoes and blackflies, that transmit several diseases. These larvae are found in various aquatic systems and most mosquito and blackfly larvae feed at the air/water

interface. The effectivity of *B.thuringiensis* subspecies *israelensis* is limited by short residual activity because spores and crystals tend to sediment quiet rapidly. Adsorption to organic matter even adds to reduce the longevity of crystals are these feeding sites. Improvement of aquatic activity and delivery have been pursued using microorganisms that naturally occur at larval breeding sites. Cyanobacterial species have been used to develop a delivery system for dipteran active genes. Initial experiments demonstrated that *Agmenellum quadruplicatum* and *Synechocystis spp.* are capable of expressing the *cryIVB* gene but toxin levels were not sufficient to obtain effective mortality rates. These problems were caused both by the low level of expression as well as by the substantial level of proteolytic degradation of the toxin by endogenous proteases of the cyanobacteria (Angsuthanasombat and Panyim, 1989; Chungjatupornchai, 1990) More recent experiments involved the expression of the *cryIVD* gene. This gene was translationally fused to the open reading frame of the homologous phycocyanin ß gene.(Murphy and Stevens, 1992).Freshly hatched larvae of *Culex pipiens* readily ingest both wild-type and transformant cyanobacteria. Larvae feeding on transformants however stopped feeding within 2 days and showed 100 percent mortality after 6 days. In contrast, more than 90 percent of the control larvae remained alive and continued feeding.

Inability to reach target insects

One of the major problems of applying toxins at the site where they can be ingested by insect larvae is encountered in situations where the larvae are hidden for standard applications. These situations include pests that have soilborne larvae as well as pests with larval that occur *in planta.* To face these complications several delivery systems have been developed.

Endophytic delivery

Another example of the ecological constraints of *B.thuringiensis* is the European corn borer. The larvae of the insect species feed inside the corn ear and stalk and therefore are difficult to target. Crop Genetics International has selected the endophytic bacterium *Clavibacter xyli* subspecies *cynodontis* to deliver the *B.thuringiensis* toxin. Although closely related to various phytopathogenic Clavibacter species *C.xyli* subspecies *cynodontis* does not cause disease symptoms in Bermuda grass, where it was originally isolated. Using seed infiltration the bacteria can colonize the xylem system of a large variety of grasses, including various cultivars of corn. Due to its precise nutritional and environmental requirements the survival of *C.xyli* subspecies *cynodontis* outside living plants is brief. The *cryIA(c)* gene was cloned onto a vector consisting of pUC19, a tetracycline resistance gene and three fragments of the *C.xyli* subspecies *cynodontis* chromosomal DNA. This construct was electroporated into *C.xyli* subspecies *cynodontis* and tetracycline resistance was used to select transformants with the plasmid integrated into the chromosome. To ensure loss of vector sequences tetracycline sensitive segregants were isolated. Corn plants inoculated with the highest producing transformant were able to reduce corn borer damage by more than 80 percent in the greenhouse (Turner *et al.* 1991).

Delivery in soil

The inefficacy of *B.thuringiensis* against pests that feed on roots is largely due to the fact that these pests cannot be reached by normal application methods. After spraying a *B.thuringiensis* product a large fraction of the crystals will no reach the rhizosphere or the rhizoplane. In order to control these larvae with *B.thuringiensis* strategies have been developed to ensure that the toxin is delivered on site. More than bulk soil, rhizosphere and rhizoplane are heavily infested with saprophytic microorganisms, that are well adapted to compete under low nutrient levels and these bacteria might form suitable delivery systems.

The first report on the production of transgenic bacteria containing *B.thuringiensis* genes originates from 1985 when the *cryIA(b)* gene was engineered into corn root colonizing isolates of *P.fluorescens*. A large series of different transgenics was produced to ensure stability of the gene and to avoid horizontal transfer of the gene to other bacteria. These experiments included integration of the toxin gene into the chromosome of P.fluorescens. To allow by homologous recombination to occur a mutant of transposon Tn5 was inserted in the chromosome of *P.fluorescens* to provide a target for recombination. Subsequently a suicide vector was introduced, that consisted of a Tn5 copy with a 324 bp deletion is the gene coding for the transposase and the *cryIA(b)* gene. By hybridisation it was shown that the resulting transgenic pseudomonads contained the *cryIA(b)* gene between two defective borders of Tn5 (Obukowicz, 1987). Expression of *cryIA(b)* was evident as the toxin comprised approximately 1 percent of total protein and 100 percent mortality was observed when transgenic *P.fluorescens* cells were added to the artificial diet of larvae of tobacco hornworm (*Manduca sexta*)

In our laboratory similar experiments were conducted to control the soilborne larvae of crane flies (*Tipula oleracea*). It was demonstrated that the larvae of these insects that cause severe damage in grasslands, are susceptible to the CryIVB protein of *B.thuringiensis* subsp. *israelensis* (Waalwijk *et al.*, 1992) This finding led us to engineer this gene into bacteria isolated from the rhizosphere of grass. As horizontal transfer of heterologous DNA is one of the major objections for the release of genetically engineered organisms, we selected endogenous DNA fragments as targets for homologous recombination. Introduction of these constructs by triparental mating as well as by electroporation led to the integration the *cryIVB* gene and the flanking selection marker *nptII*. DNA hybridisation showed that no vector sequences were integrated into the genome. We could show that the transgenic pseudomonads express the *cryIVB* gene and although considerable degradation was evident these recombinants proved toxic for *Anopheles stephensi* as well as for laboratory reared *Tipula oleracea*. (Waalwijk *et al.*, 1991)

Another innovative way of delivering toxins directly in roots was followed by Skøt *et al.* (1990). These authors used Rhizobium leguminosarum biovars to deliver the *cryIIIA* gene into the nodules of pea (*Pisum sativum*) that are invaded and eaten by larvae of pea weevil, Sitona spp. The crystal protein gen was cloned onto the broad host range plasmid pKT230 and the resulting construct was introduced into *R.leguminosarum*. Peas nodulated by toxin gene containing rhizobia provokes mortality among Sitona larvae, whereas those plants were significantly less damaged that control plants nodulated by

rhizobia containing only the pKT230 vector(Skøt *et al.*, 1990).

RISK ASSESSMENT

Recombinant DNA technologies offer prospects for creating new varieties of organisms that can have enormous benefits for agriculture, health and environment. Three major concerns relating the introduction of genetically engineered microorganisms (GEMs) can be distinguished.

Firstly, the GEM may have unexpected properties once released in the field. With respect to engineered *B.thuringiensis* there could be changes in virulence, host range or survival. In view of the intensive efforts to isolate natural *B.thuringiensis* strains with enhanced insecticidal activity or broader host ranges it is unlikely that changes in virulence or host range will occur unintentionally. The survival of wild-type *B.thuringiensis* strains in the environment is rather limited due to low levels of available nutrients (West, 1985). These authors also showed that little or no multiplication of *B.thuringiensis* occurs in soil.

Improving delivery and residual activity by constructing GEMs is inherently in conflict with the demand that deliberately released GEMs do not disperse, because they may have an adverse environmental impact. To solve this contradiction killed *P.fluorescens* (Gelernter, 1993) and hosts that survive only in limited ecological niches (Turner, 1991) have been used as delivery systems.

A second area of concern involves the horizontal transfer of introduced DNA to indigenous microorganisms. This topic has been addressed by many researchers in various ways. Engineered *B.thuringiensis* strains were constructed that only contain *B.thuringiensis* DNA and with respect to the origin of DNA sequences resemble strains modified by conjugation (Crickmore *et al.*, 1990; Lereclus *et al.*, 1992). When *B.thuringiensis* genes are cloned into other bacterial hosts the change of horizontal transfer of toxin gene should be reduced to a minimum. This was established by Obukowicz *et al.* (1987) by using a disarmed transposon Tn5, defective for the endogenous transposase enzyme. In these strains transposition can only take place when the transposase enzyme is provided *in trans* which is unlikely to occur since Tn5 and Tn5 like sequences are rare in nature. Others, including our own group, have isolated DNA sequences from the host bacterium to be used as sites for integration in the chromosome (Chungjatupornchai, 1989; Waalwijk *et al.*, 1991)

Finally, the major concern involves the development of resistance against *B.thuringiensis* and resistance specialists view resistance as the single most important threat of continued application of *B.thuringiensis* in insect control. Natural *B.thuringiensis* strains often contain more than one toxin gene and that can well explain that despite the large scale application of *B.thuringiensis* the reports on resistance are rare. The application of single gene based transgenic plants or the use of transgenic bacteria expressing single genes is indeed a serious concern. It has been shown that resistance in

the laboratory is readily emerged when the test insects are exposed to single toxins. Stone *et al.* (1989) observed a 24 fold increase in resistance when *Heliothis virescens* was challenged with *P.fluorescens* expressing the *cryIA(b)* gene. In our own laboratory resistance readily developed in *A.stephensi* towards an acrystalliferous mutant of *B.thuringiensis* subspecies israelensis transformed with the *cryIVD* gene (Feldmann *et al.* , unpublished results)

FUTURE DEVELOPMENTS AND PROSPECTS

The development of transgenic bacteria expressing *B.thuringiensis* genes bears great promises, since delivery systems can be applied to several cultivars of the same crop or even to a variety of crops. Moreover, recombinant DNA technologies allow researchers to react swiftly to newly emerging pests with the construction of custom-made biopesticides. At present, however, there are some problems to be solved before engineered biopesticides will be used on a large scale.

A majority of the transgenics discussed above demonstrated toxicity towards the intended pests in bioassays performed in the laboratory. Whether these strains will be effective under field conditions remains to be seen. This subject could be addressed in the near future by improving the expression of the *cry*-genes by stronger promotors, gene amplification and elimination of degradation of the toxins by host proteases. Another complication is the fact that the expression of *cry*-genes poses a metabolic load to the bacterial host and that these GEMs will probably be outcompeted by their natural counterparts. This would restrict their dispersal, relieving one of the major environmental concerns. On the other hand the longevity of the toxins will be diminished. Elaborating the endophyte delivery system, this subject could be addressed by taking advantage of the close association between some bacterial species and the respective host plants. Biological containment under the regulation of plant derived factors could offers an exciting approach to safety the regulatory authorities.

Resistance against *B.thuringiensis* toxins will emerge inevitably, but this problem can be counteracted by strict management of toxin application. In this area resistant individuals can be combatted by the same bacterial host expressing another crystal protein gene.

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2.
Search for Novel Crop Protection Agents

Chairman: G.K. DIXON

A REVOLUTION IN DRUG RESEARCH: THE IMPACT OF MOLECULAR BIOLOGY ON THE PHARMACEUTICAL INDUSTRY

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ABSTRACT

In just two decades, the application of recombinant DNA techniques has dramatically changed the drug discovery process in the pharmaceutical industry. From the initial impact of providing primary structural information and the drive to produce recombinant proteins for therapeutic purposes, the technology has been used to identify a variety of receptors, ligands and cellular processes which are involved in human diseases. This knowledge has not only provided a plethora of new targets for drug screening, but has also introduced an element of selectivity in the screening process. Construction of cell-based assays, combined with reporter gene end points, has produced test systems which are easy to operate and have capacity to screen large numbers of compounds. The impact of molecular biology on the pharmaceutical industry is already clearly visible and further significant changes in the treatment of human diseases are likely to evolve from technologies such as antisense and gene therapy.

INTRODUCTION

Drug discovery in the pharmaceutical industry has relied traditionally on the skills of pharmacologists and medicinal chemists. Whereas pharmacologists design assays that they believe will be indicative of biological effects in humans, the chemists take compounds with identified biological activity and modify the structure of the compounds until the right balance of pharmacokinetic and pharmacological properties has been achieved. The process often starts with identification of a lead compound, whose activity has been detected either in an established biological assay or in a new assay developed on the basis of an hypothesis about the steps involved in a disease process. The lead compound can arise through random screening of natural products or chemical collections. Alternatively, compounds are made in a more rational manner based on a variety of parameters, prejudices or hunches. Biological assays need to be "calibrated" with compounds either known to be active against the disease in humans or to have biological activity that substantiates the hypothesis being advocated. In many cases, the compounds required are proteins, with potent biological activity, isolated in small amounts from sources of animal or human origin. Such compounds from natural sources are often invaluable tools for hypothesis testing prior to establishing a biological assay.

A particular difficulty facing the pharmacologist is that in the majority of cases, assays are set up with non-human enzymes, cell lines or tissues. There is always a possibility that effects seen in an animal system will not extrapolate to use in humans. Thus, the pharmacologist and medicinal chemist are constantly faced with the dilemma of discovery and development of compounds over periods of many years with the uncertainty of

the effect of the compounds in humans. The pharmaceutical industry has many examples of drugs failing after the compounds had performed adequately in animal test systems. In those cases, the human enzyme or receptor is sufficiently different from the animal system that the compounds were ineffective in humans. When one considers that it now costs £150M, with the efforts of many people over 10 years or more, to bring a drug to the market, getting it wrong can mean disaster for the companies involved. Whereas larger companies can absorb the costs involved, smaller companies usually suffer a severe financial setback.

A report in January 1980 in the journal Nature, which described the impact of biotechnology on the chemical and pharmaceutical industry, suggested that the "new biology" had made an impact on the pharmaceutical industry but had not really revolutionised it. At this time, recombinant DNA technology (molecular biology techniques for manipulating and transferring DNA between organisms) was really in its infancy. The main impact had been on provision of primary structural information for some biologically important proteins. Production of proteins for therapeutic applications had only just begun. Most notably, synthetic genes for human insulin A and B chains were cloned and expressed in Escherichia coli as β-galactosidase fusion proteins (Goeddel et al., 1979). The insulin chains were cleaved from β-galactosidase and purified. When the products were mixed, reduced and re-oxidised, the presence of insulin was detected by radio-immune assay. In the early 1980s Biotechnology was advancing so rapidly that pharmaceutical products based on large proteins were likely to become a reality in the not too distant future. Indeed, it was felt that development time, and cost, would be substantially less than that associated with traditional small molecules. The past decade has seen progress so rapid that it has not been possible to keep abreast of advances in every area. With such developments over the past few years, the impact of recombinant DNA technology has, I believe, truly revolutionised the pharmaceutical industry. It is now virtually impossible to think of a drug discovery programme that has not been influenced by or has made use of materials obtained through recombinant DNA technology. Several proteins, previously only known for their biological activities, have been fully sequenced, characterised and produced in quantity through the technology and over 12 of these have been developed into successful drugs with combined sales in excess of £3B.

A comprehensive review of the impact of recombinant DNA technology on the pharmaceutical industry is beyond the scope of this paper. However, examples of the way the technology has revolutionised the drug discovery process will be presented with an emphasis on the strategy involved.

PROTEIN SUPPLY

As indicated in the Introduction, recombinant proteins have many applications in the drug discovery process both as research tools and for direct use in assays. However, it was the potential for their use as therapeutic agents that fuelled the early work on recombinant protein production. The approaches used to generate a protein for therapeutic applications are the same as those used to generate proteins as tools.

One of the earliest proteins to be exploited by recombinant DNA technology was interferon. From its discovery in 1957, interferon has always held out a promise of important clinical utility, first as an

anti-viral and later in the treatment of cancer. The development of interferon into a series of commercially successful drugs is typical of the way recombinant DNA technology opened new avenues to characterise and produce scarcely available proteins. These proteins were developed, initially, not because of their novelty of action but because no small molecules could mimic their activity. Thirteen years after interferon was first produced by recombinant DNA technology (Nagata et al., 1980) over 12 additional protein products are being sold. They include insulin, growth hormone, various α-interferons, β-interferons, tissue plasminogen activator, hepatitis vaccine, erythropoietin, granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF).

The major steps for development of a recombinant protein therapeutic are summarised in Fig. 1. Although extremely difficult, it is possible to isolate a gene for a protein of known biological activity, without resorting to use of specific probes designed from knowledge of the amino acid sequence of the protein. This was in fact the case with interferon (Taniguchi et al., 1979). From this early work, it was soon realised that the alpha interferons form a group of 13 or more proteins. Many of these have been evaluated in a variety of biological systems and together with β-interferon and γ-interferon developed into therapeutics with combined sales in excess of $500M per year. A review of the methods used to clone genes for the interferons and to synthesise the interferon proteins in micro-organisms is available (Edge & Camble, 1984).

To achieve this level of success with recombinant proteins, suitable expression systems in a variety of bacteria, yeasts, insect cells and mammalian cells have been developed for producing human proteins. Most of the products manufactured today are made in either E. coli or in mammalian Chinese Hamster Ovary cells. For expression in microbial systems, E. coli is the host of choice. Levels of foreign protein of up to 70% of total cellular protein can be produced, usually as insoluble inclusion bodies in the E. coli cytoplasm. These inclusion bodies can be isolated, solubilised, purified and folded to produce active protein with varying degrees of difficulty depending on the nature of the protein being expressed. Some proteins remain soluble in E. coli even when expressed at high level. However, it is possible to manipulate expression and fermentation conditions to achieve a desired level of expression and in some cases solubility of the recombinant protein.

By use of a suitable signal sequence attached to the N-terminus of the recombinant protein, the protein can be translocated across the intracellular membrane to the periplasmic space. Often, under appropriate fermentation conditions this results in the formation of soluble, correctly-folded biologically-active protein which can be recovered from the cell or released into the fermentation medium. Secretion to the periplasm has been used to produce proteins that are difficult to refold in vitro or that comprise multiple chains. Molecules as complex as antibody F(ab')2 fragments, a total of 4 protein chains, can be generated in a biologically active form by secretion to the periplasm (Carter et al., 1992; Better et al., 1993).

The major drawback of E. coli as a host for production of recombinant proteins is its inability to carry out post translation modifications, such as glycosylation. In some cases glycosylation is essential for biological activity, and a mammalian cell system must be used for protein production. Generation of recombinant proteins from mammalian cell systems is less

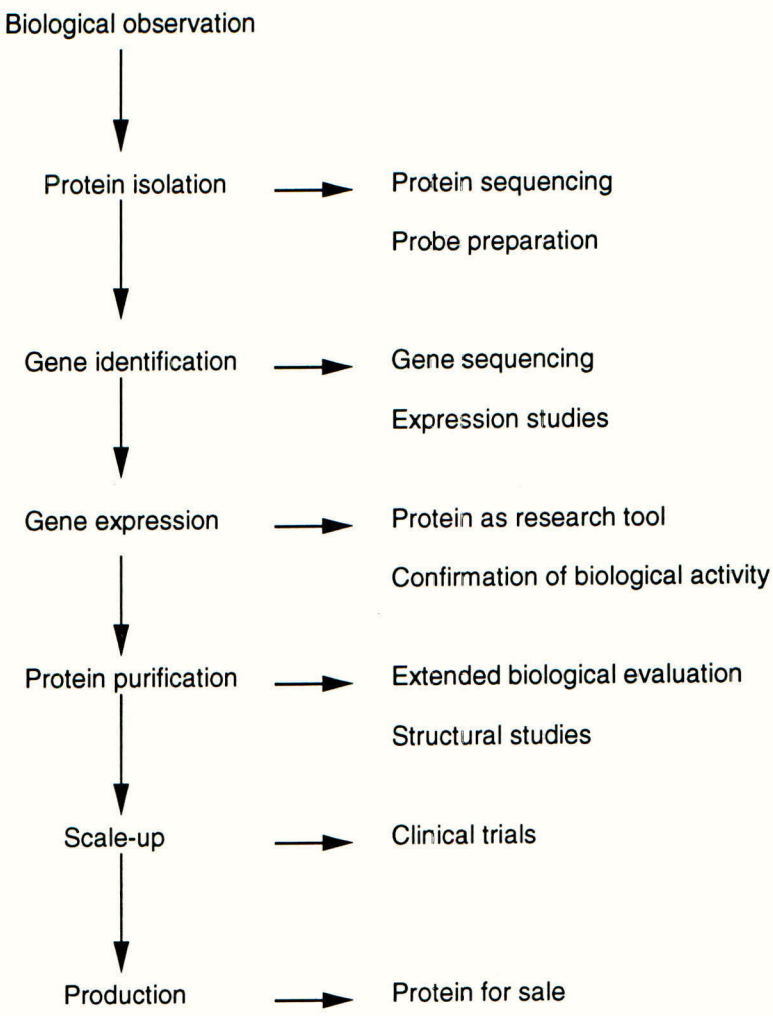

Figure 1 Outline scheme for development of a recombinant protein therapeutic

productive and more costly than from E. coli systems and considerable development work is required to achieve adequate levels of accumulation. However, mammalian cell systems have the advantage of being able to produce complex proteins, glycosylated at the appropriate position in the protein and in a soluble and active form. Tissue plasminogen activator and erythropoietin are examples of proteins produced by mammalian cell systems.

An important application of molecular biology in the study of proteins can again be found in the story of the interferons. Analogues, or variants, of the natural protein can be produced readily by a variety of molecular biology techniques and used to carry out structure-activity studies to understand better the structural basis of biological function (see in Edge & Camble, 1984). Ultimately such knowledge could lead to the design of products with improved profiles of activity or stability for clinical use. These modified proteins are generally referred to as second-generation protein therapeutics. The simplest process for generating protein analogues is to exploit common restriction endonuclease sites on different genes to produce, by in vitro recombination, hybrid genes containing portions from each parent molecule. Site-directed mutagenesis techniques, developed over the past few years, enable any amino acid to be changed to any other genetically coded amino-acid residue. Whole domains may be deleted or sequences added at specific sites. Probably the most versatile approach to analogue design is to employ genes obtained by total chemical synthesis. In principle, any genetically coded amino-acid residue can be substituted at any desired position throughout the entire length of the polypeptide chain. Any combination of changes, substitutions, deletions, additions is possible in a single gene synthesis. Analogues generated by structure-activity studies with interferon-α_2 have shown that the dipeptide sequence Lys112-Glu113 makes an important contribution to the epitope for the NK-2 monoclonal antibody. An exciting outcome of the structure-activity programme was the discovery that the analogue [$Ala^{30,32,33}$]IFN-α_2 can act as a competitive antagonist of the anti-viral and anti-proliferative effects of IFN-α_2 on Madin-Darby bovine kidney (MDBK) cells (for a review of this work see Edge et al., 1986).

Second generation protein therapeutics have been slow in coming to the market, possibly owing to uncertainties over their regulatory approval. However, much of the work on protein engineering has added to our knowledge of the biological properties of the protein being studied and has been the driving force to develop techniques such as site-directed mutagenesis, alanine-scanning mutagenesis (Cunningham & Wells, 1989) and gene synthesis for generation of the analogues. One important strategic issue arises when considering the changes to be made to the parent molecule. By comparing amino-acid sequences of related proteins, or proteins with similar activities (e.g. family of human proteins or proteins from animal species), differences and similarities can be identified. If the outcome of a structure-function programme is to produce an analogue with improved potency or selectivity, amino-acid residues conserved or only conservatively substituted between the group of proteins should be examined. On the other hand, if the end point is a protein with improved pharmacokinetics or increased stability, amino-acid residues conserved between the group should be left untouched as these are likely to be important for biological activity. Instead, amino-acid residues known to be different between the group of proteins should be changed to achieve the desired effect.

TARGET IDENTIFICATION

Traditional pharmacological techniques, based on tissue preparations and organ baths, can be used to measure the functional response generated by a compound binding to its target ligand. Whereas these techniques are useful testing procedures before moving into whole animal studies, they are too complex for the rather routine testing required for structure-function studies. Ideally, the chemist would like to carry out structure-function analyses on isolated protein, especially if the target ligand is an enzyme. If the enzyme is known, the gene for the enzyme can be isolated from the appropriate human tissue, expressed in one or more of the standard systems and purified as for any recombinant protein. In many cases, however, there may only be sequence information on non-human forms of the enzyme. In these circumstances, a variety of techniques can be used to identify and isolate the equivalent human gene. Some pharmaceutically important enzymes exist in more than one form and can be differentially regulated in different tissues or in the same tissue in response to different stimuli. Fortunately, it is now possible to study tissue distribution and individual cell expression of most proteins with antibody or RNA probes. This information becomes important once testing moves away from isolated protein to tissue preparations and whole animals.

In many cases, the target ligand is a receptor, usually bound to a cell membrane. Although it is possible to prepare pure, solubilised recombinant protein, compound testing is best carried out with the receptor sitting in its natural environment. Many receptors are present in the cell membrane as complex seven-helical transmembrane proteins. Other receptors are simpler and have just two domains, one of which is external to the cell, for ligand binding. The extracellular domain is linked via a short transmembrane region to the intracellular domain that elicits the biological response within the cell. A crucial discovery over the past several years has been the identification of receptor subtypes for many pharmaceutically important receptors, including β-adrenergic receptors, adenosine receptors, 5-HT receptors and α-adrenoceptors. These receptor subtypes display specific properties and when compound screening is based on analysis of binding and physiological properties in cell-lines or tissues, the fine properties of a compound are difficult to establish. By expressing the individual receptors in an environment free of its subtype(s), the critical receptor species can be identified and used to generate meaningful structure-function data for compound selection. As with isolated enzymes, human forms can be used and the tissue distribution and cellular expression level can be ascertained in normal and diseased situations.

Cytokines and their receptors have also become important targets for therapeutic intervention (Mire-Sluis, 1993). The cytokines are proteins which are responsible for the differentiation, activation and regulation of growth of the hematopoietic cells responsible for maintenance of the immune system. They are intimately involved in inflammatory processes and probably have an important role in many chronic degenerative diseases. Cytokines were originally identified as signalling proteins within the immune system and were simply called interleukins (of which there are now 13 members). However, the term is now used to encompass a wide variety of signalling molecules which include G-CSF, γ-interferon and tumour necrosis factor (TNF). It has become clear that the receptors for cytokines may be grouped into discrete families based on regions of sequence similarity shared between different receptor genes. As with many receptors, the cytokine receptors exist as different isoforms. These isoforms arise by alternative exon splicing mechanisms at the RNA level. Perhaps the most interesting

discovery in the cytokine area has been the identification of receptor molecules which can be secreted from cells. These so called soluble receptors are essentially the external domains of the intact receptor, lacking a transmembrane region and intracellular domain. Soluble receptors have been identified for IL-1, IL-4, IL-5, IL-6, IL-7, TNF, GM-CSF, G-CSF and γ-interferon. These soluble receptor molecules are likely to play an important role in the regulation of cytokine action, and recombinant forms may have utility as therapeutic agents for immunosuppression and inflammatory conditions.

An important contribution of recombinant DNA technology to cancer research has been the identification of retroviral oncogenes (Bishop, 1985), cellular proto-oncogenes (Chen & Barker, 1985) and the unravelling of the complex signalling pathways within cells (Edgington, 1992). Today, instead of screening for compounds that arrest the growth of cancer cells, many pharmaceutical companies are trying to identify the key intracellular proteins that can be used for compound screening (Nomura et al., 1993). By identifying proteins important as sites of therapeutic drug action, new classes of pharmaceutical products will be identified with new sites of action and new mechanisms of action.

NEW SCREENING SYSTEMS

Many drug discovery programmes are based on the premise that an over-active enzyme or receptor is implicated in the disease process and that inhibitors of the enzyme or receptor (Williams, 1991) will have therapeutic benefit. Similarly, interference with transcriptional activation (Peterson & Baichwal, 1993) and signalling pathways (Nomura et al., 1993) is beginning to offer opportunities for drug discovery. As indicated in the previous section, recombinant DNA technology can be used both to identify which enzyme or receptor subtype, protein in the signalling pathway or protein in the transcription process contributes most to the disease situation and to produce the required protein as target for evaluation of biologically active substances.

In addition to the ability to clone and express specific human genes, recombinant DNA technology can be used to engineer cells. Although isolated proteins, particularly enzymes, can be used for initial screening of compounds, cell-based assays offer a more realistic environment for the screening process. Together with the advantage seen in the general trend away from screening against animal tissues, recombinant whole cell assays offer further benefits. Many intracellular processes involve complex multi-protein interactions which would be difficult to replicate in vitro. Clearly, a lead compound derived from a cell-based screen has a greater chance of being active in a tissue or whole animal model than if the compound had been derived from an isolated protein assay. If, for example, the active compound has to cross the cell membrane to demonstrate activity, time and effort to carry out secondary testing will be eliminated. An obvious disadvantage of primary screening with cell-based assays is that potential lead compounds that inhibit the target, but are unable to cross cell membranes, will be missed. However, in a number of cases cells with permeability mutations can be used to reduce the likelihood of missed leads.

The previous discussion has taken no account of the detection systems used for identification of active compounds in engineered cells. In some cases, cell growth can be used as a simple end point for the assay. In

other cases, turnover of radioactive substrates provide an adequate means to detect active compounds. As more becomes known about the complex intracellular pathways, opportunities increase for further modification of the system to simplify the screening processes. The past 2 to 3 years has seen a dramatic move to reporter gene detection systems in which a biological response at the cell surface, or at a point in the intracellular process, is transmitted through a reporter gene such as the genes for the enzymes β-galactosidase, alkaline phosphatase, luciferase, or chloramphenicol acetyltransferase (Alam & Cook, 1990). The activity of the expressed enzymes can be detected readily by adding substrates that generate coloured products or, in the case of luciferase, emit light. Alternatively, the reporter gene can encode an easily measurable protein such as human growth hormone. Basically, the cell line is engineered to produce the requisite human or animal target protein together with a transactivation system which results in expression of the reporter gene. On addition of a suitable substrate the coloured product or emitted light is measured. Depending on the way the system is set up, active compounds are detected by an increase or decrease in the end signal. Apart from its intrinsic elegance and simplicity, the more significant value of reporter-gene systems is that the whole process of drug screening can be automated to achieve a level of throughput not possible by conventional screening techniques. A combination of recombinant DNA technology and automation has transformed compound screening to allow the rapid search through a company's compound collection and through natural products for new agents as potential leads to new therapeutics. In the past, the ability to screen hundreds of compounds per week was regarded as high throughput screening. Today this term only applies to systems which allow screening at the rate of thousands of compounds per week. Generally, the only limitations are the ability to supply compounds in the format necessary for screening and the number of robotic systems needed to handle reagent transfers and end point detection.

Active compounds derived from assays using human proteins, either in an isolated form or in a cell-based system, have to be tested in animals to ensure adequate bioavailability and for toxicity evaluation. Whereas animal test systems for the major diseases have been developed over the years, a compound found to be active against a human protein may only be partially active or be inactive on the non-human form. In such cases, secondary evaluation with appropriate non-human forms of the protein will be required before committing valuable material to extensive studies in animals. Clearly, toxicity evaluation in animals will only be meaningful if the selected compound(s) function in the species to be used for the toxicity study. Some of these concerns may be addressed by use of transgenic animals for the in vivo studies.

Transgenic animals have foreign DNA which has been incorporated stably into their genome. Expression of the foreign gene depends both on the regulatory regions present in the DNA used to prepare the transgenic animal and on the site of incorporation into the animal genome. Transgenic animals of several species have been produced, including mouse, rat, rabbit, sheep, pig and cow. Although transgenic sheep and cows are being considered for production of recombinant proteins for therapeutic use, by targeting the foreign gene for production of the protein in the milk of the animal, only mice and rats are likely to be of interest for evaluation of active compounds in vivo. It is now possible to generate models of human disease in a transgenic animal and, therefore, have the ability to evaluate the effects of drugs in the whole animal environment. Such diseases include cancer, by incorporation of specific activated oncogenes or mutated

proto-oncogenes, and include viral diseases by introduction of all or part of the viral genome. The many cloned human receptors can be considered for over or under expression in transgenic mice and although not yet in widespread use, the technology offers considerable prospects for development of animal models of human disease for pharmaceutical testing.

FUTURE PROSPECTS

Recombinant DNA technology has become an essential component of the drug discovery process and some examples of how this has occurred have been described in the preceding sections. The technology has opened up new and exciting avenues to drug discovery and therapy, but because of the considerable time required to bring a new drug to the market, is only just beginning to impact on human diseases. So what about the discoveries of the relatively recent past, and where might these impact in the future? Three areas are worth highlighting: combinatorial libraries and generation of molecular diversity on the surface of phage and E. coli; regulation of cellular processes by intervention at the gene expression level; human genome sequencing.

Combinatorial libraries

Following the demonstration that peptides can be displayed on the surface of bacteriophage and that libraries of these bacteriophage can be created by the cloning and expression of random mixtures of oligonucleotides into one of the coat proteins (Scott & Smith, 1990), several reports have appeared on the use of these libraries to identify peptide ligands for antibodies and other proteins. The libraries are usually generated by fusion to the bacteriophage coat protein PIII or PVIII, but can be displayed on the surface of E. coli by, for example, incorporation into outer membrane proteins. Peptide ligands are identified by the process of biopanning, a method of affinity-purifying the phage or E. coli displaying the binding peptide. Phage or cells that bind specifically to the target protein or ligand can be eluted, propagated and again affinity-purified against the target. Following 3 or 4 rounds of selection and growth, rare binders are enriched by several orders of magnitude and their sequence can be determined simply by DNA sequencing the encoded gene. The application of this technology to drug design has not yet been realised, but it is a potentially powerful tool. Libraries of 10^8 to 10^9 peptides can be created. If the library contains hexapeptide sequences, most or all of the possible sequences will be represented. If used to screen against protein targets of pharmaceutical interest, lead ligands may be identified. Although the lead peptides are unlikely to be of any great interest as drug candidates, the structural information deduced from analysis of the binding sequences can be used as a starting point for the design of modified peptide or organic drug molecules.

The technology has recently been extended to include libraries of antibody fragments on the surface of bacteriophage (Marks et al., 1991) and E. coli (Fuchs et al., 1991) with the expectation that such libraries could be used to isolate human antibodies against any antigen, by-passing both hybridoma technology and immunisation.

Intervention at the gene expression level

The identification of transcription factors as the key regulatory

molecules in eukaryotic gene expression, and analysis of their structure and function, has revealed that these proteins are potential targets for therapeutic intervention. These targets, which have been considered in the earlier section on target identification, are likely to result in generation of traditional drug molecules as a result of screening compound collections and compounds from natural sources. Since transcription is a fundamental cellular process, drugs with specificity of action may be difficult to find and alternative therapeutic approaches may need to be considered. For diseases that result from the inappropriate expression of genes, either genes of host cells or those of an invading pathogen, specific reduction of expression of such genes presents a potential therapeutic opportunity. In principle, production of a particular gene product may be inhibited or reduced substantially by hybridisation of a single-stranded oligodeoxyribonucleotide (oligonucleotide) complimentary to an accessible sequence in the mRNA, or to a sequence within the gene itself. This process is often referred to as antisense or antigene inhibition (Wickstrom, 1992). The use of antisense or antigene oligonucleotides to inhibit or modify expression of individual genes is a powerful emerging technology for the therapy of disease. It offers the possibility of developing agents of unprecedented specificity through Watson-Crick base pairing. However, before antisense oligonucleotides can be developed for therapeutic applications there is a need to understand and optimise the transport and controlled delivery of these compounds to their target sites within cells. In most cases, oligonucleotides are designed to produce translation arrest either by direct physical block of ribosomal translation or via stimulation of RNase H-induced degradation of mRNA. The main disease targets for oligonucleotide based therapeutics are viral infections and cancer, but cardiovascular and arthritic diseases may also be amenable to treatment with oligonucleotides. At least three oligonucleotides are in Phase I/II clinical trials. If early results, which indicate very low toxicity in animals, are confirmed in humans, the serious problem of synthesis of the molecules on a multi-kilogram scale will have to be overcome.

Gene therapy, a new approach to the treatment of human disease has emerged during the past two decades and has been put into clinical practice within the last couple of years. Basically, methods that allow stable transfer and expression of foreign DNA sequences in human cells have been applied in attempts to correct genetic defects. Such genetic changes may provide a new and effective means for therapy of human diseases (Roemer & Friedmann, 1992). The foreign genetic material can be introduced directly into the appropriate tissue in vivo, or can first be introduced into cells in vitro which are subsequently grafted to a relevant site in vivo.

Although more than 4,500 human diseases are classified as genetic, most genetic diseases are individually relatively rare. However, it is now realised that some of the common diseases such as most cancers, cardiovascular disease and degenerative diseases have important genetic components and may be amenable to treatment by a gene therapy approach. Gene therapy requires not only the introduction of foreign DNA sequences into eukaryotic cells, but also their stable and appropriately regulated expression in the new environment. Retroviral vectors are commonly used to deliver DNA to host cells, but other viral vectors, such as adenovirus and herpes simplex virus, are also being used. The first gene therapy trials are underway for the treatment of adenosine deaminase (ADA) deficiency, familial hypercholesterolaemia and brain cancer. Over 30 approved human experimental gene protocols have been approved and trials in cystic fibrosis, liver cancer and breast cancer are likely this year.

Whereas it may be possible to deliver genes to major tissues by injection, or by inhalation in the case of delivery to the lung, selective delivery to diseased cells has received less attention. Approaches such as receptor-mediated endocytosis or antibody-directed delivery have the potential to deliver substances specifically to target cells and may have important future applications.

In addition to providing the ability to correct genetic defects, the ability to deliver genetic material to cells has potential therapeutic opportunities in other ways. Genes encoding antisense sequences or ribozymes may have application for down regulation of proteins whose levels are elevated in disease. However, the most exciting potential application of gene therapy techniques is in the area of cancer vaccines. Several human studies are in progress in which retroviral vectors are being used to introduce cytokine genes directly into cells derived from a tumour. The cells are irradiated to prevent replication and returned to the patient to function as a tumour vaccine. This form of immunotherapy generates an immune response that prevents the growth of tumour metastases. The mechanism of the anti-tumour effect is not entirely clear, but recent publications (see review by Travis, 1993) suggest that in addition to the presence of cell surface tumour antigens, the tumour cells must provide a co-stimulant to call into action the critical immune cells called killer T cells. In the work reviewed by Travis, a surface molecule known as B7 was introduced into melanoma cells. It remains to be seen whether general stimulation of tumour cells by a range of cytokines results in production of cell-surface immune co-stimulants that provides a route to general therapy of cancer.

Human genome sequencing

Current efforts to sequence the human genome are likely to impact on the pharmaceutical industry in a dramatic way. It is abundantly clear that many diseases have a genetic component in the sense that the presence of an abnormal protein, inappropriate regulation of a normal protein or abnormal hormone production or response can be traced back to a DNA sequence that has been altered in some way from that found in the non-diseased state. Whereas the genetic defect for several diseases are known, some of these have taken many years of effort to identify. This is because the exact sequence of genes along the human chromosome is not known and much of the research effort goes into the painstaking process of establishing the "local" genetic map. Once the complete sequence of the genome becomes available, the time and effort to home into the gene(s) involved in disease will be reduced to a matter of a few months. Depending on the information gained from the gene sequence or sequence of the control regions, the appropriate pharmaceutical approach to tackling the problem can be mounted with the greater certainty that the treatment derived will work in the clinic.

CONCLUSION

The impact of molecular biology on the pharmaceutical industry has been revolutionary. From the heady days of the 1970s and early 1980s when primary structural information on proteins of pharmaceutical interest was pouring out at an ever increasing rate and protein based drugs were rapidly becoming reality, the science and associated technology has continued to move at alarming speed. Not only have many intracellular pathways been

identified, providing new opportunities for therapeutic intervention, but many new procedures for rapid screening of compounds have also been developed. The future of the new technologies that are just beginning to be evaluated in the clinic is uncertain. Some have the potential to completely change our approach to disease treatment, and if this were to happen the traditional small molecule approach to drug discovery may even become obsolete. This scenario may seem wildly futuristic, particularly when one considers that some of the best drug leads are still derived from natural sources, continuing a trend set thousands of years ago. However, 20 years ago recombinant DNA technology was just beginning and the idea that the technology could develop into a multi-billion pound industry was barely thinkable. The impact of the technology over the past decade has been so dramatic that one cannot be too circumspect about future applications. In the pharmaceutical industry there are always winners and losers but those companies that are not prepared to embrace fully recombinant DNA technology may find the going much harder in the future.

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TOWARDS A RATIONAL DESIGN OF PESTICIDES

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ABSTRACT

As a prerequisite to the biochemical design of pesticides it is important to be certain that the enzymes targeted by this approach are essential to the target organism. We call this evaluation of enzymes "target validation". This paper reviews methods of target validation including: chemical validation, the use of mutants and the use of reverse genetics in plants and plant pathogenic fungi. A literature example of chemical invalidation of a target enzyme, ketol-acid reductoisomerase is given and the use of non-pathogenic mutants of plant pathogenic fungi is described. Specific examples of a reverse genetics approach are taken from our work using transgenic plants expressing antisense RNA for three enzymes: acetolactate synthase, threonine deaminase and glutamate semialdehyde aminotransferase. We also review literature examples of a reverse genetics approach to target validation in plant pathogenic fungi. In these examples the function of the enzyme cutinase is perturbed by gene disruption and the effect this has on pathogenicity is described.

INTRODUCTION

The purpose of this paper is to focus on an area of the biochemically inspired design of pesticides which we believe has been neglected in the past, the validation of target enzymes chosen for biochemical design. We define target validation as any means of confirming that a particular enzyme chosen for a biochemical design project is indeed essential to an organism so that successful inhibition of that enzyme can lead to a commercial pesticide. Recent advances in plant and fungal molecular biology have made target validation possible and it is to these molecular advances that this paper will be addressed.

The concept of the rational, biochemically inspired, design of biocides is not new and there has been some commercial success in the pharmaceutical industry (Bey, 1989). A biochemical approach has been adopted within the agrochemical industry and has been part of our company's approach since the mid-sixties (Baille *et al.*, 1988). We have had scientific successes, but no agrochemical product has been discovered by this approach alone (Pillmoor *et al.*, 1991). Until recently the biochemical approach usually involved input from both chemists and biochemists who would choose a suitable biochemical target (an enzyme for example) and then attempt to design and synthesise potent inhibitors of that target. Experience has taught us that choosing a biochemical target that is essential to the target organism is a key element in this process. This paper will focus on the validation of biochemical targets as the first step toward a biochemical design of pesticides.

Not all enzymes are created equal

The best example of this involves the work undertaken by Schloss and his co-workers (Aulabaugh and Schloss, 1990) on ketol-acid reductoisomerase. This enzyme catalyses the next biochemical step after the established herbicide target, acetolactate synthase, in the biosynthesis of the branched chain amino acids. It could be considered, therefore, as an attractive target in a pathway already validated by the acetolactate synthase inhibitors. A potential reaction intermediate analogue, an oxalyl hydroxamate, was synthesised and found to be a powerful inhibitor both *in vitro* and *in vivo* with an overall *in vitro* affinity of 22pM. This compound, however, had insufficient herbicidal activity to merit its commercialisation. It has been since demonstrated that it is necessary to inhibit ketol-acid reductoisomerase by 95% to achieve a good herbicidal effect, whereas 60-70% inhibition of acetolactate synthase is sufficient to achieve herbicidal activity (Wittenbach *et al.*, 1992).

It is clear that the industry, if it is going to commit chemical synthesis effort to biochemical design projects, needs to focus its attention on validated target enzymes. The next section will review methods of target validation, focusing on the herbicide and fungicide discovery process and emphasize the contribution that molecular biology can make to the process.

METHODS OF VALIDATION

There are three methods of target validation which can be applied to plant and fungal systems, each of which will be discussed separately.

Chemical Validation

It is obvious that new target enzymes can be discovered through mode of action studies of biologically active compounds generated by chemical synthesis or from natural product screens. In addition, biochemically inspired inhibitors can validate a new target enzyme if their biological activity can be correlated unequivocally with *in vitro* activity. In this context, the work described earlier on ketol-acid reductoisomerase could be considered as a chemical validation exercise where the enzyme was shown not to be a valid target.

Classical Mutagenesis

Mutants generated either by chemical mutagens or by electromagnetic radiation can be used to validate target enzymes. For biochemical design, two features are important; the mutants have to be both conditionally lethal and defined at the enzyme level.

Plant Mutants

There are many plant mutants described in the literature (Blonstein, 1986) although they have not been studied with a view to the herbicidal nature of their phenotypes. The most tractable are auxotrophic mutants because they are conditionally lethal and they can be defined biochemically to the enzyme level. From the ketol-acid reductoisomerase evidence, it is important to define the mutant at the enzyme level: a lethal plant mutant for branched chain amino acid biosynthesis would suggest that a good *in vitro* inhibitor of any enzyme in the pathway would be herbicidal, but one may choose the wrong enzyme. It is perhaps too difficult to set up a plant mutagenesis programme *de novo* for new targets, but mutant lines of *Arabidopsis* are now held centrally (The Nottingham *Arabidopsis* Centre, Faculty of Science, Department of Life Science, School of Biological Sciences, University Park, Nottingham NG7 2RD).

Mutants of plant pathogenic fungi

For plant pathogenic fungi classical mutagenesis can be used to generate non-pathogenic mutants, but it is difficult to define the mutant to the biochemical level. Albino mutants of *Pyricularia oryzae*, for example, which are deficient in melanin biosynthesis are also non-pathogenic. Melanin deficient mutants, therefore, could have been used to define a biochemical process essential to pathogenicity. These mutants, however, were generated after the discovery of the melanin biosynthesis inhibiting tricyclazole fungicides (Bell and Wheeler, 1986). Kubo *et al.* (1991) have cloned a melanin biosynthesis gene essential for the pathogenicity of *Colletotrichum lagenarium*, although they have yet to define the biochemical function of the gene product.

It has been assumed that plant pathogenic fungi could acquire their nutritional requirements from their host, so that auxotrophic mutants would still be pathogenic. This may not be the case however. A cytosine auxotroph of *Sclerotinia sclerotiorum*, for example, has been described which had a radically reduced host range (Miller *et al.*, 1989). The pathogenicity of this mutant could be restored by adding cytosine, implying that, when defined to the biochemical level, the enzyme coded by the mutant gene would be a target for fungicide design. From this example auxotrophic, non-pathogenic mutants of plant pathogens could be used to validate biochemical targets if the two phenotypes were correlated.

The problems with classical mutagenesis

The major difficulty of the classical mutagenesis approach is that it is random so that a mutation in a specific gene cannot be generated at will. The problem is then one of defining the mutant at the enzyme level which is akin to discovering a new mode of action of a pesticide and can be equally time-consuming. Another problem is that classical mutagenesis produces mutants with no measurable target enzyme activity. It is unlikely that a pesticide could achieve equivalent levels of inhibition and so classical mutants are not a perfect foundation on which to build a chemical synthesis programme. The next section will discuss methods to obviate the random and absolute nature of classical mutants by using reverse genetics. Reverse genetics is a generic term to describe any process whereby gene function is perturbed by manipulating the target gene itself. In this paper we mean techniques such as gene disruption and antisense RNA expression. Presently these techniques are divided between the two kingdoms so that antisense RNA expression would be the method of choice in plants and gene disruption the method of choice in fungi. These will be discussed separately.

Antisense RNA

There are now many literature examples of transgenic plants expressing antisense RNA. One of the best is the work describing the delay of tomato ripening by antisense suppression of the levels of polygalacturonase (Smith *et al.*, 1988). Other examples of antisense work include creating transgenic plants resistant to viral infection by suppression of viral genome replication (Bejarano and Lichtenstein, 1992), using antisense expression to manipulate starch metabolism (Sonnewald and Willmitzer, 1992), and using the technique to manipulate pigment production (van der Krol *et al.*, 1988). We felt that the generation of transgenic plants expressing antisense RNA for the genes of target enzymes could be used to validate new targets. We have analyzed the effects of antisense RNA expression for three genes:

(a) acetolactate synthase (a known herbicide target),
(b) threonine deaminase and
(c) glutamate semialdehyde aminotransferase.

Acetolactate Synthase

As a method of testing the validation system, we created transgenic potato plants

expressing an antisense potato acetolactate synthase gene. Different regenerants had different phenotypes, but all the phenotypes were consistent with acetolactate synthase inhibition. The most severe phenotypes were lethal but could be protected with branched chain amino acids in tissue culture. Less severe phenotypes showed the classical symptoms of acetolactate synthase inhibition, slow growth and chlorosis. The phenotypes could be correlated with the degree of acetolactate synthase inhibition. Transgenic plants with severe phenotypes still exhibited about 20% of the wild-type levels of acetolactate synthase.

Threonine deaminase

Threonine deaminase is involved in isoleucine biosynthesis, catalysing the conversion of threonine to 2-oxobutyrate. A tobacco mutant, generated by chemical mutagenesis is lethal but can be rescued with isoleucine (Negrutiu *et al.*, 1985) and has no detectable threonine deaminase activity (Wallsgrove *et al.*,1986). These data suggest that threonine deaminase could be a target for biochemical design, if similar lethal effects could be achieved by inhibition of the enzyme. We assumed that inhibitors would only partially inhibit threonine deaminase and that transgenic plants expressing an antisense gene would achieve a similar incomplete inhibition.

A partial cDNA clone for threonine deaminase was isolated from potato and returned to potato and tobacco in the reverse orientation to the CaMV 35S promoter. Transgenic plants were not lethal but did have altered phenotypes when compared to the wild-type. In the homologous system (potato-potato) transgenic plants were shorter, had a slightly altered leaf morphology; the tubers were discoloured and also had an altered morphology. In the heterologous system (tobacco-potato) transgenic plants were shorter, showed a loss of apical dominance and had an altered leaf morphology. We were able to cross tobacco transformants and analyze F1 plants in which we had, presumably, doubled the antisense gene dose. The F1 plants were extremely dwarfed, had a totally changed leaf morphology and no apical dominance. The transgenic plants had severely reduced levels of threonine deaminase activity. Since we were unable to reproduce the extreme phenotype of the lethal mutant described above, our results cast doubt on the suitability of threonine deaminase as a target for biochemical design.

Glutamate semialdehyde aminotransferase

Glutamate semialdehyde aminotransferase (GSA-AT) catalyses the formation of 5-aminolevulinate from glutamate-1-semialdehyde, leading ultimately to the biosynthesis of chlorophyll. Gabaculine is an inhibitor of the enzyme, but this compound has additional biochemical effects *in planta* (Pallett, 1991). The GSA-AT gene was cloned from tobacco leaf cDNA libraries and used in antisense studies (Höfgen *et al.*, 1993). A population of transgenic plants showing reduced levels of chlorophyll were generated. As with the earlier examples, individual plants showed different degrees of antisense-mediated repression of their wild-type phenotype. Reductions in chlorophyll content were not lethal since transgenic plants could be propagated in axenic culture. Greenhouse grown plants with reduced levels of chlorophyll showed leaf damage and, in some cases, death, dependent on the light intensity and chlorophyll content. The symptoms partially resembled the effects of bleaching herbicides and tetrapyrrole- dependent photodynamic herbicides (Höfgen *et al.*, 1993). In the transgenic plants, however, tissue damage occurred at a much slower rate than herbicide treated plants. Enzyme activity in severely affected plants was reduced by antisense RNA expression to about 25% of wild-type levels, with a corresponding reduction in chlorophyll content to about 10%. These data indicate that inhibition of GSA-AT by about 75% would be herbicidal and that GSA-AT is a valid target for biochemical design.

Gene disruption

The present method of choice for target validation in plant pathogenic fungi is by gene disruption. In this method the gene for the target enzyme is cloned from the

pathogen and the majority of the coding region is replaced by a marker gene (usually an antibiotic resistance marker such as hygromycin resistance). The disrupted gene is then returned to the pathogen where homologous recombination between the introduced, disrupted gene and its endogenous copy can take place. This results in integration of the disrupted gene into the chromosome to replace the functional copy. This recombination event is selected for by acquisition of the marker gene by the pathogen. Practically, these homologous events are rare, so further analysis of the transgenic fungus is required to confirm that gene replacement has taken place (van den Hondel and Punt, 1991).

A cutinase gene of *Magnaporthe grisea*, the causative agent of rice blast, has been disrupted using this technique (Sweigard *et al.*, 1992), but the mutant was as pathogenic as the wild-type organism. It was subsequently discovered that there was a second cutinase activity in these "cutinase deficient" mutants, coded for by a second gene. One could assume that it was this activity that preserved the mutant's pathogenicity. A similar strategy was used to disrupt the cutinase gene of *Nectria haematococca*, the causative agent of foot rot in pea. This pathogen was known to have only one copy of the gene, and when this gene was disrupted no cutinase activity could be detected. Cutinase deficient mutants however, were still as pathogenic as the wild-type (Stahl *et al.*,1992). These data suggest, therefore, that for *Nectria* cutinase is not a valid target for biochemical design.

The problems with reverse genetics

Although both antisense RNA expression and gene disruption are very powerful tools with which to validate target enzymes, there are problems associated with these techniques. In both cases the target genes have to be cloned from the species where the validation is to be done. This means that in plants we have to work on transformable species and since most biotechnological effort focuses on crops, we validate targets in these. The best systems for antisense studies, therefore, are tobacco, potato, tomato and the non-crop plant *Arabidopsis thaliana*. By using these plants as model systems we are making the reasonable assumption that whatever is true for the model is also true for weeds.

The best model fungal systems for gene disruption are not plant pathogens at all but the yeasts (such as *Saccharomyces cerevisiae*) and filamentous fungi (such as *Neurospora crassa*). It is essential that this type of validation is done on plant pathogenic fungi since we need to know which enzymes are essential to pathogenicity. Many plant pathogenic fungi are transformable, albeit at low frequencies, and any transformable species should be amenable to gene disruption techniques.

Other problems have been alluded to in the text, namely the existence of multigene families where more than one gene exists for any particular enzyme. This implies that every functioning gene may have to be suppressed before a mutant phenotype can be generated. There are subtle problems with antisense RNA expression where mRNA stability and turnover may influence the effectiveness of the technique. Finally, until we are able to control the effects of antisense RNA expression and gene disruption we are limited to conditional lethal effects such as biosynthetic pathways, for example, where we can correct the deficiency of the mutation by the addition of nutritional supplements.

PROSPECTS FOR THE FUTURE

Control of antisense expression

From the above examples it is clear that antisense expression is a valuable tool for validating new target enzymes for biochemical design. The range of phenotypes correlated to target enzyme levels in the above examples has allowed us to estimate the levels of

inhibition needed for an effective herbicide. In the future it will be important to develop inducible antisense RNA expression systems so that we are more able to control the antisense effect at any stage of the plant's development. There are inducible promoters such as the tetracycline promoter described by Gatz *et al.* (1992) which have been used to demonstrate tetracycline mediated expression of reporter genes. We hope that such inducible systems can be used to control antisense RNA expression so that non-conditional lethal phenotypes will be amenable to study. In addition, the use of inducible antisense RNA expression will allow us to study the most advantageous timing of enzyme inhibition in order to get the best herbicidal effect.

Developing fungal molecular biology

A wide range of plant pathogens can now be genetically transformed albeit at low frequencies and so should be amenable to gene disruption techniques. To create the best reverse genetic systems, however, will require an inducible antisense system similar to that developed for plants. This should not be difficult in principle, and the tetracycline-inducible promoter described earlier has been used in the yeast *Schizosaccharomyces pombe* (Faryar and Gatz, 1992). It seems possible, therefore, that this, or a similar system, could be used with a model plant pathogen in the future.

Developing invertebrate molecular biology

There are two model genetic systems where similar validation strategies could be adopted, *Drosophila melanogaster* and *Caenorhabditis elegans* (Plasterk, 1992). The plant protection industry should be ready to exploit these genetic systems when they become workable.

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DEFINITION OF NOVEL PESTICIDE TARGETS AND MODES OF ACTION BY MOLECULAR GENETIC APPROACHES

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ABSTRACT

Modern molecular genetics, based on efficient techniques to isolate genes, characterise them and assess their function, can provide a powerful complement to existing methods of determining pesticide mode of action and target selection. Studies on resistant organisms already provide valuable information, albeit to date largely of a confirmatory nature. The establishment of detailed gene structure and sequence databases and our developing ability to assess more complex situations through sophisticated analytical studies can, however, be expected to deliver increasingly definitive and rapid information on the mode of action of new pesticides and leads. They should also help in both the selection of specific gene products as new targets and in setting up tailored primary screens for compounds with a chosen mode of action.

INTRODUCTION

At this time it would hardly be controversial to conclude that the direct contribution of molecular genetic studies to agrochemical invention has not been great. The majority of studies to date have been limited to confirming the predictions of biochemical, physiological and electrophysiological analyses.

The intrinsic power of modern molecular genetic technology and the enormous wealth of new basic knowledge and data becoming available through its application should change this situation. Such changes are already influencing pharmaceutical research (e.g. Edge, 1993) and, also as discussed elsewhere in this volume, significant impacts are beginning to be made in some areas of agricultural science, with particular advances in the development of herbicide and insect resistant plants (Mazur & Falco, 1989; Perlak *et al.*, 1990), recombinant biocontrol agents (e.g. Tomalski & Miller, 1991; Stewart *et al.*, 1991) and the analysis of resistance mechanisms (e.g. Martin *et al.*, 1992).

Technical feasibility alone will not of course be sufficient to ensure a role for molecular genetics in agrochemical invention. This will only happen if the technology can help deliver products with more specific and selective activities, which address the environmental and toxicological issues facing existing pesticides, or define new modes of action required to counter the continuing problem of pesticide resistance.

In this paper we will consider the use of molecular genetic techniques to support such pesticide invention. Firstly we will provide a brief historical perspective, commenting on the contributions which have been made so far and the indications they give for the future. Secondly, we will discuss recent technical developments which we believe will have important roles to play in the future: the ability to "describe" genomes in detail through DNA

sequences and genetic maps, and to analyse gene function by sophisticated and efficient new techniques.

A comprehensive review is prohibited by space constraints, so our approach has been to provide illustrative examples to allow a comparison of the situation as it relates to the three disciplines of fungicide, herbicide and insecticide research.

THE HISTORICAL PERSPECTIVE

Mode of action investigations in pesticide science have classically been the responsibility of the biochemist and, in the case of insecticide research, sometimes the electrophysiologist. To achieve their goal these scientists use all appropriate procedures, including: radiolabelling, substrate accumulation and reversal studies. However, these investigations can be time consuming and often only provide an answer after product launch. Target validation, through which a particular mode of action is confirmed as a "valid" means of killing a pest, is usually a retrospective consequence of such investigations when a novel pesticide, discovered through *in vivo* screening programmes, is found to have a new mode of action. Rarely, if ever, has experimental target validation in the absence of an existing pesticide series been a precursor to establishing a targeted *in vitro* screen followed by the discovery and development of *in vivo* active pesticides. As is widely recognised, this situation contrasts markedly with that in our sister effect chemical business: pharmaceuticals.

Within the process of mode of action confirmation, molecular genetic techniques have begun to be one of the tools in our armoury. In particular, examples of studies where analysis of resistance determinants have shed light on the mode of action of compounds can now be cited for each pesticide area: insecticides, herbicides and particularly fungicides.

Fungicide Resistance

The methoxyacrylates represent a new class of fungicides developed through pursuit of the leads provided by the natural product respiration inhibitors strobilurin A (mucidin) and myxothiazol (Godwin *et al.*, 1992). Extensive studies of the target and mode of action of these leads have been undertaken in yeast. These include isolation, biochemical and genetic characterisation of resistant mutants (Subik *et al.*, 1977; di Rago *et al.*, 1989). Genetic studies with such mutants showed that a significant group map to the mitochondrial genome and specifically the cytochrome b gene.

Sequence analyses on resistant cytochrome b genes, in combination with biochemical characterisation of the mutant strains, pinpoint methoxyacrylate resistance to the Q_o site at which ubiquinol mediated reduction of the Rieske protein occurs (Tron *et al.*, 1991). Specifically these studies suggest that the methoxyacrylates might act by mimicking the binding of the methoxy-substituted ring of ubiquinol to cytochrome b.

Given the very high level of sequence conservation between cytochrome b proteins from a wide variety of species, including filamentous fungi (di Rago *et al.*, 1989), such detailed molecular genetic analyses, undertaken with closely co-ordinated biochemical studies, yield important information. They not only confirm the target protein of the methoxyacrylates but also the specific site at which they act. They indicate possible mechanisms by which

resistance could emerge in the field and suggest strategies which might be used to monitor and manage such resistance. With more precise structural information on cytochrome b they might even aid rational design of novel Q_o site active fungicides.

Given the relative genetic tractability of even some genuine plant pathogenic fungi, it is likely that a number of other molecular genetic studies of isolates resistant to a variety of novel fungicides and leads, are presently in hand, with the aim of defining their mode of action. It may, however, be some time before the most interesting examples of this work become public knowledge!

Insecticide Resistance

Although it has long been accepted that cyclodiene insecticides act on a $GABA_A$ receptor (Tanaka *et al.*, 1984), definition of the precise target has until recently remained elusive. ffrench-Constant has, however, now elegantly mapped and isolated a gene likely to encode the genuine *Drosophila melanogaster* (fruit fly) target (ffrench-Constant, 1993). Central to this work was the semi-dominant phenotype displayed by flies heterozygous for the Rdl^R dieldrin resistance locus: Rdl^R/Rdl^R flies are resistant to 30µg dieldrin in a vial; Rdl^R/Rdl^S flies to 0.5µg dieldrin and Rdl^S/Rdl^S flies to <0.07µg. Using these simple phenotypes, it proved relatively straightforward to map the genetic locus at which *Rdl* lies, refine that map with new mutations and isolate overlapping cosmid clones one of which contained DNA encoding a functional Rdl^S gene. Sequence analyses of cDNA clones copied from the specific mRNA encoded by this cosmid reveal a gene with the greatest overall homology to members of the vertebrate $GABA_A$ receptor subunit family and rather less similarity to the glycine receptor family. The *Rdl* locus is however complex and the gene described may encode several, perhaps alternatively spliced, $GABA_A$ subunits.

Thus, although a description of the identity of mutations in *Rdl* which confer cyclodiene resistance is awaited, confirmation has been obtained that the cyclodiene site of action is indeed a $GABA_A$ subunit. Moreover, it shows some interesting distinctions from previously reported vertebrate members of the family. Such distinctions might, in future, be usefully exploited. At a more technical level this study is also significant because it highlights the advantages in working with a model organism such as *Drosophila* with a well developed genetic system. Such sophisticated, rapid and cost effective studies would have been impossible with any major insect pest.

Herbicide Resistance

In herbicide research the photosynthetic cyanobacteria, for example, also provide powerful and rapid model systems for the identification of herbicide targets through the analysis of resistant mutants.

A chromosomal DNA library from *Synechococcus* PCC7942 mutants resistant to the herbicide norflurazon, which was already known to inhibit phytoene desaturase, was constructed and amplified in *E. coli* using a plasmid vector incapable of autonomous replication in *Synechococcus* (Chamovitz *et al*, 1990). Library DNA was transfected into wild-type *Synechococcus* cells and transformants selected with resistance to both the herbicide and the plasmid encoded antibiotic resistance marker. These transformants carried the norflurazon resistance gene, plus an adjacent kanamycin resistance gene and an origin of plasmid replication, integrated into the host chromosome by

homologous recombination. Simple plasmid rescue, by restriction and self ligation of chromosomal DNA and retransformation back into *E. coli*, allowed a plasmid to be isolated which could reproducibly transform wild-type *Synechococcus* to norflurazon resistance. Sequence analysis of the cloned segment of *Synechococcus* DNA later confirmed that the gene did indeed encode phytoene desaturase (Chamovitz *et al.*, 1991).

Thus, it is now clear that pesticide resistance determinants can be cloned directly using genetic methods rather than by using protein based information. We should expect this sort of exercise to become rapid and routine in years to come. Whilst we are not yet aware of any documented examples where this has been the primary route to target identification, it is clear that reliable target identification by this approach only awaits the day when it will be possible to identify a gene conferring resistance by reference to databases of nucleotide sequences. Then perhaps molecular genetics will begin to be a preferred route to determining modes of action of new pesticides and lead compounds.

GENOME PROGRAMMES: DEVELOPMENTS IN DESCRIPTIVE MOLECULAR GENETICS

In light of the obvious advantages which databases can provide in enabling new gene sequences to be recognised, it is relevant to consider how their content and nature are developing and how those developments might influence options for pesticide mode of action definition and target validation.

Currently, protein and nucleotide sequence databases are expanding at an almost exponential rate, with the greatly increased facility of DNA sequencing being the major driving force. The amount of known DNA sequence data available within databases is now doubling every 12-18 months, with approximately 20 million bp of new sequence having been added in 1991/1992 (Higgins *et al.*, 1992; Burks *et al.*, 1992). All of this information is instantly available to any scientist with access to GenBank™ or EMBL databases.

What content do the DNA databases have and how is it evolving with time? Ten to fifteen years ago almost any nucleotide sequence information was novel and the ease with which DNA fragments could be isolated and characterised was a major influence on database content. As a result, the genes of simple organisms tended to be over represented. Subsequently a major emphasis was placed on isolating and characterising genes encoding proteins of known biochemical activity. Often these proteins had medical relevance. Now a series of "genome" research programmes and projects are in hand which have an immediate objective of describing most, if not all, of the expressed genes in species including man (*Homo sapiens*), *D. melanogaster*, *Caenorhabditis elegans*, maize (*Zea mays*), *Arabidopsis thaliana* and yeast (*Saccharomyces cerevisiae*) (Courteau, 1991). Full descriptions of the protein coding sequences of species both closely and distantly related to fungal, plant and insect pests will therefore become available.

Interestingly, at the present time only a small proportion of the sequences obtained through analysis of randomly isolated cDNA clones can be identified as encoding proteins with known biochemical function. For example, only about 8% of rice (*Oryza sativa L.*) cDNA sequences could be recognised (Uchimaya *et al.*, 1992). Although we should expect that the number of genes whose function is known to increase with time, we are clearly already at a point where the rate of acquisition of new sequence data exceeds our ability

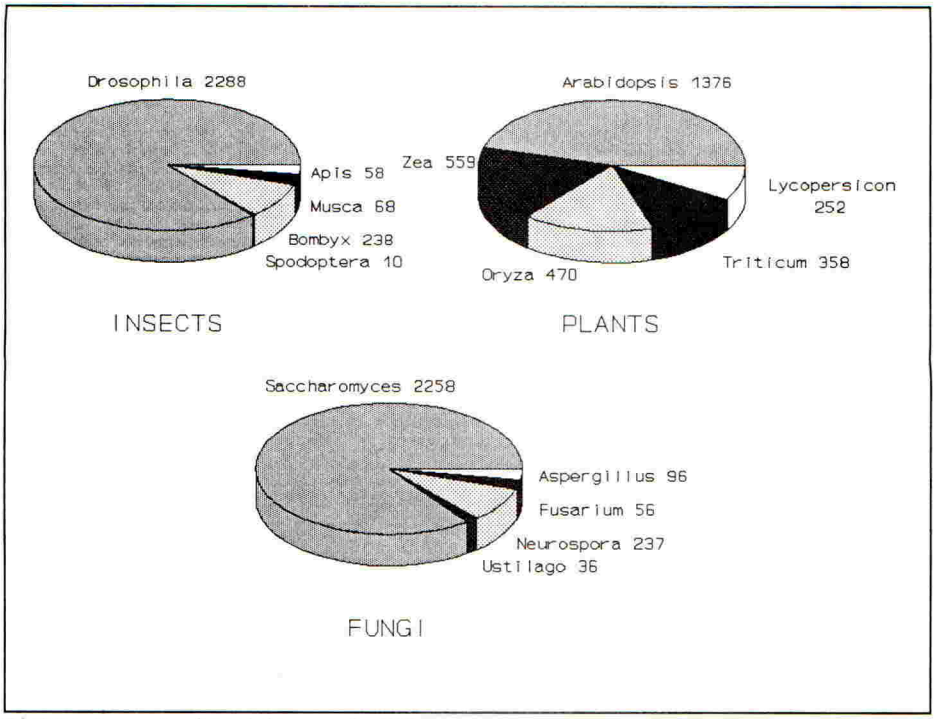

Figure 1. A breakdown of DNA sequences in the EMBL Data Library (version 34) of potential relevance in agrochemical research.

to assign biochemical function.

To provide an indication of the current content of sequence databases of potential relevance to agrochemical research, we have surveyed the latest EMBL Data Library for DNA sequences of insect, plant and fungal origin. These searches were performed simply using generic names to identify the total number of entries. The scores for the best represented genera/species in each group are presented graphically in Figure 1. For clarity only the top five scores for each group have been included. The results should not be taken as a measure of the numbers of potential pesticide targets since we have not made serious attempts to filter out known protein coding sequences from sequences of unknown function, viral sequences, ribosomal and tRNA sequences etc. (The most extreme case of *Autographa californica* (alfalfa looper), where all 62 sequence entries were for the baculovirus AcMNPV, was discounted!)

What can we draw from this simple exercise? For insects and fungi the vast majority of known DNA sequences are from the two model organisms *D. melanogaster* and *S. cerevisiae*. Very few sequences are yet known for agronomically important fungal pathogens or insect pests. It is perhaps also surprising that, given the length of time that both *Neurospora crassa* and *Aspergillus nidulans* have been studied as model filamentous fungi, so few of their genes have been characterised.

The situation with plants is very different. *A. thaliana* represents less than half of the plant sequence entries. Major crop species are already well represented. By contrast, we found only 24 entries for the weed *Avena fatua* (wild oat). Like insect and fungal pests, weeds have clearly received little

attention to date.

In parallel with gene sequencing intitiatives, significant effort is also being invested in genome mapping studies, with an objective of obtaining ordered banks of overlapping clones spanning the entire genome of the source organism and detailed "maps" showing the positions of known genetic loci and the corresponding clones on each chromosome. As progress is made towards this complete integration of genetic maps based on recombination frequencies with physical maps based on restriction fragment length polymorphisms, it should become routine to isolate genes on the basis of mapped positions of informative mutants. ffrench-Constant's studies with the *Drosophila Rdl* locus exemplify how genes conferring pesticide resistance can be isolated by such an approach (ffrench-Constant, 1993). Resistance mutations will, however, be only one entry to map based cloning strategies. For example, though not of immediate relevance to pesticide discovery, the recent isolation of genes encoding the *Arabidopsis* and oil seed rape (*Brassica napus*) fatty acid metabolism enzyme ω3 linoleate desaturase, based only on the map position of the *fad3* locus, shows how more complex phenotypes can also be used to access target genes (Arondel *et al.*, 1992). We anticipate that genes encoding proteins of more direct relevance to agrochemical research and discovery will also be accessible via such approaches.

How might the information and gene sequences available through such genome initiatives be helpful in pesticide mode of action studies and new target validation?

Firstly, as indicated previously, as we approach the stage at which genes encoding most known biochemical functions have been defined, the probability of identifying the mode of action of a new pesticide through the sequence of a resistance determinant will become significantly greater.

Secondly, as databases become increasingly comprehensive, "menus" of readily available recombinant proteins will eliminate the "bottleneck" to protein supply which specific gene cloning so often creates. Given nucleotide sequence data and the polymerase chain reaction (PCR) it is already feasible to clone most genes and express them in a heterologous system in a matter of weeks. Abundant quantities of protein or recombinant cell based functional assays will be available, on a timescale appropriate to current agrochemical research and synthetic chemistry programmes, for use in *in vitro* screening cascades, receptor binding studies, mechanistic enzymology, structural studies etc. Indeed, the rapid establishment of high throughput screens might allow compounds active at a specific, but unproven, target site to be identified within timescales and resource requirements compatible with target validation.

Thirdly gene sequence databases will allow comparisons between related genes and gene products from different species. The prospects of achieving intrinsic selectivity with a pesticide active against a particular target protein should therefore be readily assessed, if necessary through construction of suitable functional assays which can be run in parallel. Alternatively judgements will be able to be made as to whether a gene product from one organism represents a suitable surrogate for the corresponding protein of a target pest. If not, then the databases will provide ideal sources of information for designing strategies to isolate target genes from real pest species.

We anticipate that genome programmes will prove as valuable a resource to agrochemical research as they are expected to be for pharmaceutical

research. One hopes that the intellectual property strategies developed for this area will encourage rather than stifle such applications!

ANALYTICAL TECHNIQUES IN TARGET VALIDATION

Descriptive genome studies cannot always be expected to deliver simple, clear-cut, solutions and guidance on their own. In the majority of cases newly isolated genes are not found to encode proteins of known function when databases are searched. In other instances they encode members of gene families, sometimes when classical biochemical studies have given few grounds to expect such complications. Already such complex stories are emerging for a significant proportion of known insecticide targets (Table 1). It would not be surprising if such discoveries were common.

TABLE 1. Insecticide targets encoded by complex genes or members of multigene familes.

Target	Gene Features	Reference
Nicotinic acetyl-choline receptor	ALS, SAD, SBD, ARD & Dα3 genes in *Drosophila*	Gundelfinger & Hess, 1992
Voltage gated Na^+ channels	*DSC1* & *para* genes in *Drosophila*	Ramaswami & Tanouye, 1989
GABA receptor	Complex locus in *Drosophila*	ffrench-Constant, 1993
Ryanodine receptor	*RYR1*, *RYR2* & *RYR3* genes in mammals	Sorrentino & Volpe, 1993
Ecdysone receptor	Complex locus in *Drosophila*	Koelle *et al.*, 1991

If descriptions of new gene sequences and their expression patterns are to be a help, rather than to be irrelevant or, at worst, add additional uncertainty to pesticide discovery, analytical techniques are required which allow definition of the role played by specific genes and gene family members. Interference with the activity of the gene products might then be assessed for the potential to deliver a valuable pesticidal effect.

Potentially suitable analytical methods include: site-selected or targeted mutagenesis, homologous recombination and antisense techniques. The latter methods, discussed elsewhere in this volume (Foster & Höfgen, 1993), have many attractions and have, for example, been used in a different context very successfully by our colleagues in Zeneca Seeds to construct tomato plants with new and commercially desirable properties (Schuch, 1991). Our current preferences are, however, the former methods.

Site-selected mutagenesis is particularly attractive, when it can be used, because of its ability to allow studies on several genes in parallel and the prospect it offers of assembling "banks" of genetically altered organisms available for rapid analysis as and when new genes and gene products of interest emerge. It is less of a "one gene at a time" method than either antisense technology or homologous recombination and is therefore more suited to asking questions about the role played by genes of uncertain function.

Homologous recombination is the analytical genetic technique which can

deliver the greatest precision and certainty, in those systems where it is feasible.

Site-Selected Mutagenesis

Insertion element or transposon mutagenesis is not of course in itself a new technique: it has been widely used in bacterial genetics for many years and in plant systems the elegant studies initiated by McClintock in maize are now recognised as transposon mediated events (Freeling, 1984). T-DNA mediated gene transfer is also one of the commonest means of genetically manipulating plants and "transposon tagging" is a favoured means of physically isolating genes which have been subject to an insertional mutagenic event.

However, important recent developments extend the usefulness of transposon mutagenesis for efficiently creating mutations in genes which might only be known from their nucleotide sequence. This is achieved by harnessing transposons which are themselves understood in great detail at the molecular level and, sometimes, have been genetically manipulated to provide specific properties. One such system is the P element system of *Drosophila melanogaster*, developed independently as a site-selected mutagenesis tool for analysing gene product function by Benzer and Kaiser (Ballinger & Benzer, 1989; Kaiser & Goodwin, 1990). Such P element based techniques are presently under investigation by ourselves, in collaboration with Kim Kaiser, as a tool to define the precise role played by specific gene products in the fruit fly. This, we anticipate, will help define gene products with the potential to be good insecticide targets.

P Element Mediated Site-Selected Mutagenesis of *Drosophila*

The capacity to create targeted P element insertion mutants of *D. melanogaster* efficiently relies on:

- The sophisticated status of *D. melanogaster* genetics.
- The availability of small mutant P elements, such as are found at approximately 17 loci on the Birm-2 chromosome, which lack their own transposase activity but which can be mobilised by a *trans* acting P transposase.
- Access to a second class of mutant P element, fixed near to a readily identified marker gene *Dr* ("Drop eye"), unable to transpose itself but still encoding P transposase.
- PCR techniques which allow detection of one fly in a thousand carrying a P element within 1 Kbp of a target sequence.

With these tools we have the means to create banks of mutant flies and analyse them in parallel for insertions within a whole range of genes.

As shown diagrammatically in Figure 2, virgin female flies homozygous for the Birm-2 chromosome are mated with male flies carrying the *Dr* linked transposase. Male *Dr* progeny are selected. In the germ line of these flies the transposase acts to mobilise the Birm-2 derived P elements, creating an average of approx. 10 new insertion events per genome. The testes of these F_1 flies therefore effectively comprise a library of sperm carrying an enormous number of new P element insertion mutations. Back crossing these flies to homozygous P element free Oregon-R females and selecting for female progeny lacking the *Dr* phenotype counter selects against the transposase and fixes

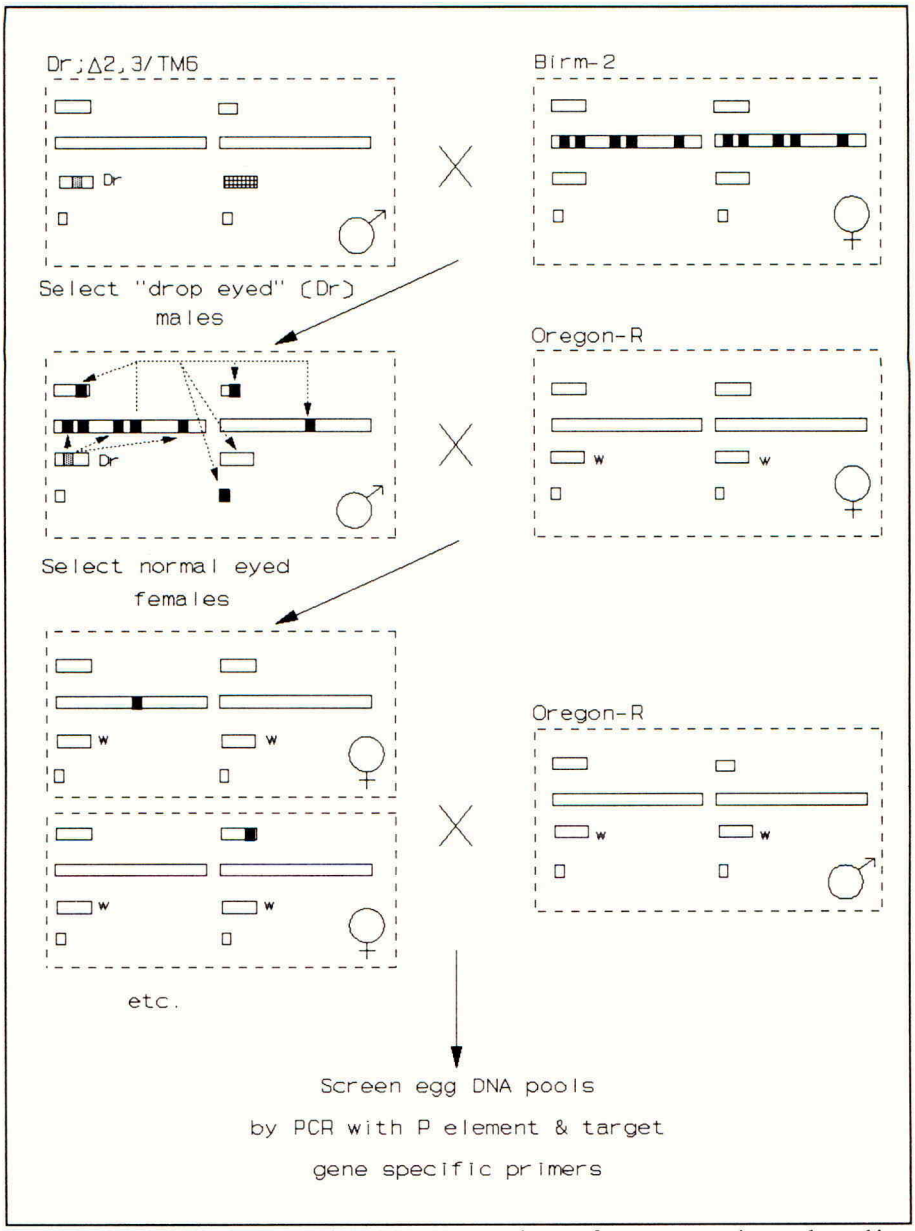

Figure 2. A diagrammatic representation of a convenient breeding programme to create a library of novel P element insertions in *D.melanogaster*.

each new mutation in a heterozygous background. Recessive lethal mutations are not therefore lost. Each female fly is a potential source of the new P element insertions present in the sperm which was one of her progenitors.

To identify F_2 females carrying insertions within a chosen gene they are first mated, in pools of 1000, with Oregon-R males and encouraged to lay eggs.

PCR analysis of DNA derived from these eggs defines those pools containing females with a P element within 1 Kbp of a target sequence. Sib-selection of those females, based on PCR analysis of the DNA in their eggs, can then be used to narrow down to a single fly carrying the selected insertion within, or at least close to, the target gene. Such is the power of this technique that, from a starting population of several 1000 F_2 female flies, a single healthy fly carrying the chosen mutation can be isolated within her egg-laying lifetime: approx. 30 days.

By such means potential knock-out mutations in selected genes can be created with only sufficient nucleotide sequence knowledge to design a PCR primer. Furthermore, since the mutations are selected in a heterozygous background, any deficiency caused by the mutation can be complemented. Brother/sister mating studies on progeny of those flies however allow the impact of the new mutation in the homozygous state to be defined. Formally this is equivalent to asking what impact selective inhibition of the product of the target gene would have? Might it be insecticidal? Would it act only at a particular developmental stage? Could the lesion be readily compensated for? In essence: might the gene product be a useful insecticide target?

Whilst such studies are obviously themselves sophisticated and could only be undertaken in an organism with a highly advanced genetic system they might in fact be only the first stage in the analyses one would like to undertake and which are enabled by P element site-selected mutagenesis. Deletion mutants, which ensure the ablation of gene activity, are more readily recognised or selected when a P element "tag" is available as a marker. Homologous recombination mediated modification of genes can also be achieved in *Drosophila* at present only if the target gene contains a P element (Gloor *et al.*, 1991). Using such approaches, for example, condition (temperature, tissue or developmental stage) specific gene ablation systems might be generated using the yeast FLP-FRT (Golic & Lindquist, 1989) site specific recombination system. By such means the impact of developmental stage, time or tissue specific inhibition of the activity of a particular gene product might be established.

As indicated above, we are working with Dr Kaiser to begin an assessment of this type of analytical molecular genetics as an approach to the validation of novel insecticide targets. The progeny of a breeding programme of the type discussed above are presently being analysed for insertion mutants in a panel of more than 15 genes encoding proteins which might be candidate targets for novel insecticides.

At this time studies of this nature can only be undertaken in specialist laboratories equipped for large scale *Drosophila* breeding programmes. However recent developments in the cryogenic preservation of *Drosophila* embryos might shortly make it possible to store libraries of P element mutant flies ready to be screened when a gene of interest becomes available (Mazur *et al.*, 1992).

Targeted Insertional Mutagenesis in Plants

In principle, similar techniques to those available in *Drosophila* are available in plants - with the added attraction that a library of insertion mutants could be very conveniently stored as seeds.

Unfortunately, however, the basic tools and methodology in plants lag some way behind those available in the insect system. Thus, although *Arabidopsis* mutants have been generated using both T-DNA (reviewed by Walden *et al.*, 1991) and transposon (Bancroft *et al.*, 1992) mutagenesis, major

challenges need to be overcome. For example: only a small number of insertion mutants can be isolated from any one experiment, and, in the case of available transposons, they are only able to jump efficiently to sites closely linked to the original site of integration. It is therefore difficult to envisage how a plant genome could be saturated with new mutation events.

Despite such limitations, several thousand T-DNA tagged lines of *Arabidopsis* have been constructed (Feldmann, 1991) and a spectrum of different mutation types recognised. Many of these affect developmental patterns: size, pigmentation etc., providing a fascinating resource of developmentally interesting isolates. Unfortunately, however, less than 10% of the mutants available are homozygous lethals and, for the most part, these have been left aside as the (academically) least interesting class for detailed study. It is of course precisely those mutants which would be potentially of greatest value to the agrochemical industry!

Homologous Recombination

Homologous recombination relies on the natural capacity of identical or almost identical tracts of DNA sequence to recombine with each other *in vivo* to create new combinations of genetic material. As such it lies at the heart of most classical genetic studies: linkage groups, gene mapping etc. More recently it has been harnessed as the most exact and precisely targeted analytical genetic technique available for use in whole organisms.

Such targeted homologous recombination is achieved by re-introducing DNA fragments which have been manipulated *in vitro* back into the parent organism. Recombination with a corresponding tract of genomic DNA introduces the altered DNA back into the host's genome. At its limit homologous recombination therefore has the capacity to allow deliberate exchange of single nucleotides within the genome of a whole organism. Though rarely used with this level of precision, it nevertheless offers an extraordinarily powerful tool to ask questions about the role played by a particular gene product in determining an organism's behaviour and properties. It therefore has great potential as a target validation method.

Given this potential it is obviously regrettable that homologous recombination cannot yet be used at all in plant systems and is really only just beginning to be available in *Drosophila*! Fortunately the position in many fungal systems is very different. In these homologous recombination is an increasingly commonly and usefully applied technique.

Cloned segments of fungal DNA encoding a gene of interest are manipulated *in vitro* to alter the target sequence in the chosen way: commonly inactivation by introduction of a marker, such as an *argB*, *pyrG* or hygromycin resistance gene, into the middle of the target coding sequence. The altered gene is then returned to its parent fungus. Sometimes it is linearised before re-introduction, in other cases it is left as a circular plasmid. In the latter case only a single cross-over event between the target chromosomal gene and the homologous sequence on the plasmid is required to insert the entire plasmid into the target, potentially disrupting its structure and function (see Figure 3a). With linear DNA a double cross-over event allows the altered DNA to be stably integrated into the host cell chromosome (see Figure 3b). In this situation precise exchange of the resident DNA sequence with that manipulated *in vitro* is achieved.

Homologous recombination dependent processes such as these are a routine

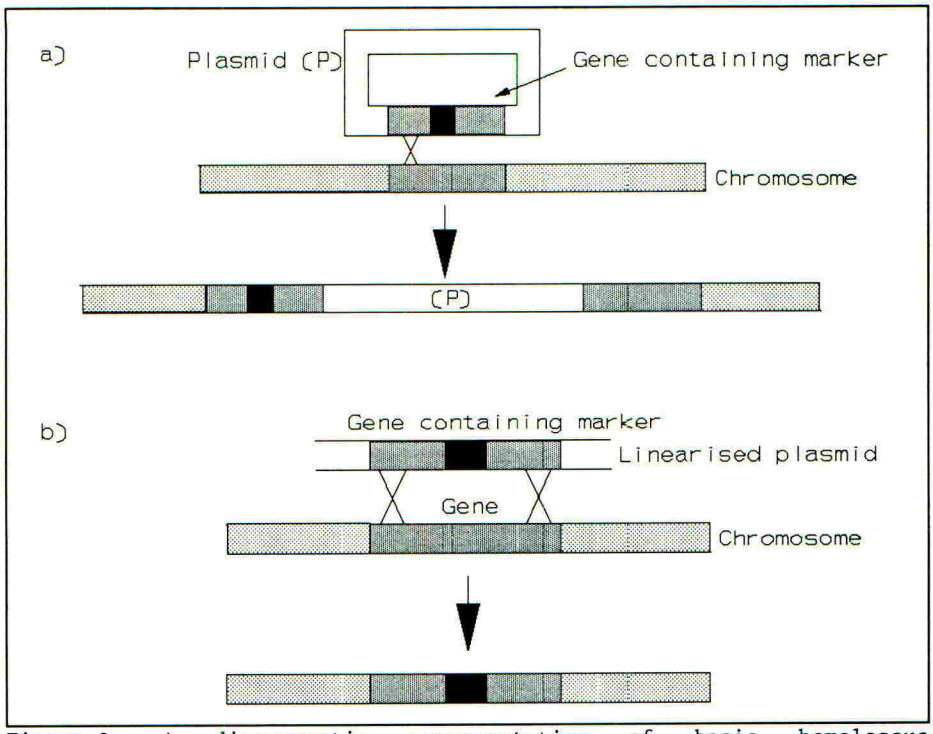

Figure 3. A diagrammatic representation of basic homologous recombination strategies.

part of modern yeast genetics. As shown in Table 2, a significant number of similar analyses have also now been undertaken in filamentous fungal pathogens of both plants and animals, with the aim of establishing if a specific gene product has a role in the pathogenicity of the organism. Targeting such a validated component of a pathogenic process might be a valuable approach to fungicide development.

TABLE 2. Examples of Gene Inactivation in Fungal Pathogens By Homologous Recombination

Fungal Pathogen	Gene/Product	Reference
Nectria haematococca	Cutinase	Stahl & Schaefer, 1992
Magnaporthe grisea	Cutinase	Sweigard *et al.*, 1992
Cochliobolus carbonum	HC-Toxin	Panaccione *et al.*, 1992
Aspergillus fumigatus	Elastase	Tang *et al.*, 1992
Candida albicans	PIG1	Gorman *et al.*, 1991
Ustilago maydis	pyr6	Kronstad *et al.*, 1989
Ustilago maydis	sid1	Mei *et al.*, 1993
Gibberella pulicaris	Tox5	Hohn & Desjardins, 1992

An interesting example is the use of homologous recombination to explore the role of a peptide toxin (HC-toxin) in the pathogenicity of *Cochliobolus carbonum* on maize (Panaccione *et al.*, 1992). Based on previous genetic and

cloning studies it was believed that there were two copies of a gene encoding an enzyme involved in the biosynthesis of the toxin at a specific chromosomal locus. Two rounds of gene disruption by homologous recombination were therefore undertaken. In the first round hygromycin resistance was used as the marker and in the second, *amdS*. Strains with one copy of the gene disrupted had half the enzyme activity but could still produce the toxin and were still pathogenic. Those transformants with both genes disrupted produced no toxin and were not pathogenic. An effective inhibitor of the synthesis or action of HC-toxin might therefore be useful in counteracting the pathogenicity of *C.carbonum*.

A contrasting example is the case of cutinase. Biochemical and mutation studies carried out over a number of years had suggested that this enzyme played a major role in the penetration of plants by a number of plant pathogens, and in particular *Nectria haematococca* (previously known as *Fusarium solani*) (Kolattukudy *et al.*, 1991). More recent work using inactivation of the cutinase gene by homologous recombination in the same fungal strain has however shown clearly that cutinase deficient *N. haematococca* remain pathogenic (Stahl & Schaefer, 1992; reviewed by Chasan, 1992). Cutinase has similarly now been shown not to be necessary for the pathogenicity of *Magnaporthe grisea* by homologous recombination based gene inactivation (Sweigard *et al.*, 1992). Previously this enzyme had been considered a potentially attractive fungicide target (Köller, 1992).

Gene inactivation by homologous recombination should therefore be considered a powerful tool to verify the true potential of specific proteins as fungicide targets.

CONCLUDING REMARKS

In this paper we have sought to provide an exemplified commentary on the contribution molecular genetic investigations can make to pesticide mode of action determination and target validation and discuss how current developments might provide future opportunities.

Several clear messages emerge:-

Firstly, the most useful and readily understood information to date has come from studies of genes capable of conferring pesticide resistance. So far even those studies have, however, provided mainly retrospective confirmation of previous conclusions rather than having themselves defined new modes of action. Over the last few years, however, the relative importance and priority accorded to such studies has begun to rise and it is our expectation that this trend will continue as the methods and results become more familiar to the agrochemical research community.

Secondly, studies with model organisms are attractive if conclusions are to be drawn which might be of value within the real time of a programme of pesticide discovery, optimisation and selection for development. This situation is less marked with fungal systems where at least some real plant pathogens now have manageable genetic systems. With insecticide and herbicide studies there are, however, major technical advantages from working with *Drosophila*, *Arabidopsis* and cyanobacteria. These "advantages" must, of course, be carefully weighed against reservations and concerns about relevance.

Thirdly, the development of detailed physical genetic maps and gene

sequence databases for a range of organisms, from man to yeast, will provide a major resource and should have significant impact in target and lead selection. The stage may in fact soon be reached when it is easiest, most certain and precise to recognize modes of pesticide and lead compound action by identifying resistance determinant genes by reference to such databases.

These databases will also provide rapid access to cloned gene sequences, with straightforward strategies often then available to allow corresponding genes to be isolated from real pest species. The bottlenecks often encountered in cloning genes chosen for their specific biochemical interest will therefore progressively disappear. When genes are available most can now be expressed in heterologous systems and formats designed to facilitate screening, structure function analysis, structure determination etc. In the meantime, before we reach the situation where all genes are available in libraries, it might be valuable for biochemists considering novel potential pesticide targets for use in high throughput screens to refer to a "what cloned genes are available" menu alongside their biochemical pathway charts.

Finally, when target selection or validation issues are being addressed, molecular geneticists' contributions need not be limited to providing protein tools to their biochemist colleagues. Increasingly sophisticated analytical techniques can help pinpoint those gene products, gene product family members, biochemical and physiological processes which represent valid new pesticide targets.

Modern molecular genetic techniques therefore have considerable potential to contribute to pesticide mode of action studies and target selection. Obvious needs exist for more selective and specific agrochemical products, ideally with novel modes of action, to tackle environmental, toxicological and resistance problems. The challenge for molecular geneticists will be to make real, cost-effective, contributions to the solution of these problems.

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MUTATIONAL ANALYSIS AND POSSIBLE APPLICATIONS OF RIBOSOME-INACTIVATING PROTEINS

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ABSTRACT

Ribosome inactivating proteins (RIPs) inhibit protein synthesis by suceptible ribosomes through the depurination of 26/28S rRNA. Two classes of these proteins exist, type I RIPs consisting of a single polypeptide with an N-glycosidae activity, and type II RIPs which consist of a type I RIP attached via a disulphide linkage to a lectin, conferring cytotoxicity upon this second class. Recent research has not only provided information on the functioning of the RIPs themselves but also provided insights into a number of other important biological phenomenon. These proteins are known to be involved in the resistance of plants to a variety of predators and pathogens and thus provide an exciting topic for investigation with much potential for future development.

INTRODUCTION

By definition ribosome-inactivating proteins (RIPs) catalytically and irreversibly inactivate the 60S subunit of eukaryotic ribosomes, rendering it incapable of binding elongation factor 2 (Stirpe *et al.*, 1992). RIPs are widely distributed in Angiosperms and also occur in several species of bacteria and fungi. The best known example is ricin which occurs in the seeds of the castor bean, *Ricinus communis*. Ricin is a toxic lectin consisting of a catalytic A chain (RTA) of 32kDa linked through a disulphide bond to a cell surface-binding B chain (RTB) of 34kDa with a speceficity for galactose and N-acetyl galactosamine residues of glycoproteins and glycolipids. However single chain RIPs resembling the A chain of ricin are more commonly found in nature and these are relatively non-toxic to intact cells. The enzymatic activity of RIPs is, in most cases an rRNA N-glycosidase which removes a single adenine residue from 26S/28S rRNA which forms a highly conserved stem loop structure involved in binding elongation factors (Endo *et al.*, 1987). The target site requirements for RIP action have been determined by using synthetic oligo-ribonucleotide analogues of the conserved stem-loop structure (Endo *et al.*, 1991). In most cases the ribosomes of the RIP-producing plants are sensitive to their homologous RIP. Cytotoxicity is avoided by the co-translational targetting of precursor forms into the ER lumen and subsequently into protein bodies, vacuoles or the cell wall. It is believed that the physiological role of RIPs is that of defense, protecting the plant from attack by predators and pathogens including fungi. An example is the increased resistance of transgenic tobacco plants expressing barley RIP to the soil born pathogenic fungus *Rhizoctonia solani* (Logemann et al., 1992).

A major application of RIPs, particularly ricin is in the construction of immunotoxins in which specific monoclonal antibodies are conjugated with RTA in order to target the toxin to specific cells *in vivo* and destroy them. Targets include neoplastic cells, lymphocytes involved in auto-immune reactions and virally-infected cells (Lord *et al.*, 1991). RIPs are also being increasingly used to probe the structure and functions of ribosomes. In this article we outline some of the research on these proteins currently being undertaken at Warwick.(It should be noted that where possible the single letter amino acid code has been used in the following descriptions).

ANALYSIS OF THE ACTIVE SITE OF RICIN A CHAIN

In order to investigate the catalytic mechanism of RTA various approaches have been taken, including site-directed mutagenesis as described here. The amino acid sequence for many RIPs is now known and a high degree of homology has been observed (Funatsu *et al.*, 1991). Certain residues have been shown to be absolutely conserved in all the sequenced RIPs, probably indicating a conserved role in RIP structure and/or function. X-ray crystal data have been reported for ricin to 2.8Å (Montfort *et al.*, 1987) and subsequently refined to 2.5Å (Katzin *et al.*, 1991). Analysis of the structure has revealed a cleft which is proposed to be the active site of the enzyme. E177 and R180 are two of the absolutely conserved residues in this cleft and were chosen for further examination. Previous analysis of E167 of *Escherichia coli* Shiga-like toxin I A-chain, which is functionally equivalent to the RTA E177, had implicated this residue in the catalytic mechanism of the bacterial toxin (Hovde *et al.*, 1988).

Creation of RTA mutants with altered activity

Utilising a suitable fragment from the cDNA for preproricin (Lamb *et al.*, 1985) as a template for mutagenesis, six mutant active-site constructs were prepared: E177 was mutated to K, A and D; R180 was mutated to Q, A and M. Mutant recombinant RTA (rRTA) constructs were cloned into transcription/translation vectors to allow for initial screening of activity *in vitro*. Using established methods for analysis of RIP depurination activity (May *et al.*, 1989), non-quantitative assessments of N-glycosidase action were made. In the cases of E177K, R180Q, R180A and R180M no detectable activity remained, i.e. N-glycosidase activity was removed by mutagenesis. E177D and E177A both retained activity toward rabbit reticulocyte ribosomes, demonstrating that functional protein had been created in the *in vitro* system. However, the *in vitro* experiments suggested that E177A retained greater depurination activity than the relatively conservative E177D mutation. This observation was also made by Schlossman *et al.* (1989). A possible reason for this apparent anomaly was indicated by Kim *et al.* (1992) following crystallisation and X-ray analysis of E177A. It was demonstrated that E208 rotates into the space left vacant in the E177A mutation to fulfil the role of E177. In the case of E177D however, rotation of E208 into the active site would be repelled by the negatively charged D177. The experimental observations suggest that aspartate at position 177 is less able to substitute for glutamate than rotation of E208.

In vitro experiments therefore suggested that both residues E177 and R180 were indeed important in the catalytic mechanism of RTA. No mutant at the R180 locus could be isolated implying the conservation of arginine was crucial. Later mutagenesis of R180 to K (Frankel *et al.*, 1990) and to Q (Kim *et al.*, 1992) with decreased activities of 4- and 200-fold respectively, demonstrated that retention of the positive charge was of paramount importance at position 180.

In an attempt to investigate the role of the E177 residue in more detail, soluble, active E177D protein was isolated and purified from *E.coli* expressing cultures. Careful kinetic analysis of this mutant using isolated *Saccharomyces cerevisiae* ribosomes was performed, and depurination quantitated following cleavage of the toxin-treated RNA with aniline. Identical analysis of wild-type rRTA prepared in the same way allowed comparisons of activity to be drawn. The K_m and k_{cat} of wild type rRTA were shown to be 1.48μM and 195 per minute. In contrast, E177D revealed a K_m and k_{cat} of 1.79μM and 4 per minute (Chaddock & Roberts, 1993). Since the K_m is proposed to be an estimate of the affinity of rRTA for the ribosomal substrate, this would suggest that the binding interaction was barely altered, therefore indicating that E177 is most likely involved in the rate-limiting step in catalysis. Analysis of the specificity constant (k_{cat}/K_M) suggested that the interaction of E177D with the transition state is 10.3kJ/mol less favourable than wild type rRTA. Comparable work performed by Kim *et al.*, (1992) using purified E177A demonstrated an almost identical figure of 9.9kJ/mol implying that the effectiveness of E177D in performing the catalytic function of E177 is equivalent to partial rescue by E208 in E177A.

The mechanism of RTA action becomes clearer

The limited knowledge available regarding the effects of mutagenesis of RIPs and the study of other related enzymatic mechanisms has led workers in the field of RIPs to propose a tentative mechanism of action (Ready *et al.*, 1991). This assigns E177 to the role of positive charge stabilisation on the ribose ring of the target adenine. R180 may act to bind to the rRNA phosphate backbone, hence the absolute requirement of a positive charge at this locus.

Further mutagenesis of rRTA has been performed in an attempt to identify the roles of other residues in the catalysis. Comparable work is also beginning to appear using other RIPs as a base for mutagenesis. By analysis of the structural requirements of RIPs for catalysis, and using this information together with information relating to the rRNA substrate, it is hoped that the mechanism of action of this important class of proteins will soon become clear.

MUTAGENIC ANALYSIS OF RTB STRUCTURE AND FUNCTION

Mutagenesis of galactose binding sites

RTB is a bilobal molecule thought to have arisen from a gene duplication event, consisting of two structurally similar domains (I and II) each carrying a single galactose-binding site. Each of the two domains is composed of three similar sub-domains, once again thought to be the

product of gene duplication events. The elucidation of the crystal structure of RTB by Robertus and colleagues (Montfort *et al.*, 1987) dramatically increased the feasibility of a mutagenic approach to the study of RTB function. The amino acid residues involved in galactose binding were located to the sub-domains 1a and 2c creating the galactose binding sites on domain I and domain II respectively. In each case one side of the galactose binding pocket is formed by a kink in the polypeptide backbone not present in the non-binding sub-domains caused by the amino acid sequence DVR. The other side of the pocket is created by the side chain of an aromatic amino acid, W37 in the first site and Y248 in the second site. In the first galactose binding site the galactose was shown to form H-bonds to N46 and K40, the N46 being in turn stabilised by a hydrogen bond to the D22 of the DVR kink. In the second galactose binding site a hydrogen bond was found to be formed between the galactose and N255, this residue being stabilised through the formation of an H-bond to D234 of the second DVR kink. Refinement of the crystal structure to 2.5Å (Rutenber & Robertus, 1991) has revealed that (in addition to the H-bonds to N46 and N255) the primary interaction with the sugars is through H-bonds to D22 and D234, these two residues being stabilised by H-bonds to Q47 and N256.

To investigate the effects of the inactivation of these galactose binding sites a number of mutants were created by site directed mutagenesis. The amino acids thought at the time to form H-bonds directly to the sugar were mutated;

1st site: K40 to M, N46 to G
2nd site N255 to G

Also mutated were the amino acids forming the DVR kink. These were altered to the amino acids found in the homologous but non-binding sub-domain thus removing the kink.

1st site: DVR 22-24 to QAN
2nd site: DVR 234-236 to AAT

Both sets of mutants were created singly and as double mutants. The constructs used for the expression of these proteins consisted of the DNA encoding the ricin signal sequence fused directly to that encoding RTB, downstream of an SP6 RNA polymerase promoter sequence. RNA was then produced *in vitro* using SP6 RNA polymerase and the transcripts injected into *Xenopus laevis* oocytes to produce recombinant RTB in a N-glycosylated form. N-glycosylation has been shown to be essential for the stability and correct functioning of RTB from previous work in our laboratory (Richardson *et al.*, 1991).

The RTB molecules containing single galactose binding site mutations at K40/N46 or N255 were found to bind both simple sugars, in the form of immobilised lactose, and complex sugars, in the form of the carbohydrate moieties of the glycoprotein asialofetuin. The double mutant however showed no sugar binding activity thus illustrating the ability of these amino acid changes to inactivate the respective sugar binding sites. The mutant proteins were found to be produced in a soluble, N-glycosylated form and were capable of reassociating with RTA and forming the inter-chain disulphide bond. Scatchard analysis revealed that the strength of binding to asialofetuin by the single galactose binding sites remaining active in the single site mutants was not significantly altered. Similar results were obtained with the DVR mutants although the affinity of the binding sites was not measured (Wales *et al.*, 1991).

The independence of the two binding sites found in these experiments is at odds with the observations of Vitetta and Yen (1990) in which the mutation of N255 to G led to the complete abrogation of sugar binding of recombinant RTB expressed in Cos-M6 cells. However support for the results showing independence of the two sites is provided by observations on deletion mutants of RTB. Clones encoding three deleted forms of RTB were created, encoding the regions A1 to R236, A1 to P139, N94 to F262, and the recombinant proteins produced in *Xenopus laevis* oocytes as described above. Each of the deleted forms of RTB carried both of the native sites for N-glycosylation but only one of the galactose binding sites. In each case the mutant proteins were shown to bind to immobilised lactose supporting the previous data suggesting the independence of the two galactose binding sites (Wales *et al.*, 1992a).

Role of the N-linked glycosylation of RTB

A further observation on the deletion mutants of RTB (Wales *et al.*, 1992a) was that in the case of the two smaller forms of RTB, the proteins still bound to simple sugars even in the absence of glycosylation. This result does not agree with the situation found for full-length RTB (Richardson *et al.*, 1991). To determine the role of the N-linked glycosylation mutations were created in the two glycosylation sites of RTB. In each case the Asn residue (N95 and N135) of the Asn-X-Thr motifs was converted to Gln. The mutated forms of RTB were produced as for the previously described mutants and their sugar binding and solubility properties examined. The single site mutants were found to be unstable upon storage at 4 C in contrast to the wild-type protein, however these mutants did show an initial ability to bind to immobilised lactose. The double glycosylation mutant however became insoluble rapidly, and even when soluble immediately after homogenisation of the oocytes showed no ability to bind to immobilised lactose or asialofetuin. Such results are similar to the observations on the expression of RTB at high level in *E.coli* (Hussain *et al.*, 1989) where the protein was found to be unstable and to rapidly aggregate. Although *E.coli* produced RTB will not bind to immobilised lactose it will bind to asialofetuin although only a small proportion of this binding can be competed by galactose and therefore be assumed to be specific (Richardson *et al.*, 1991, Swimmer *et al.*, 1992). There is an anomaly however in that it might be expected that the competable portion of asialofetuin binding protein would bind to immobilised lactose, however this is not observed. It is concluded therefore that in the absence of the carbohydrate groups of RTB the integrity of the galactose binding sites is not 100%.

Possible explanations for the ability of the deleted non-glycosylated forms of RTB to bind simple sugars are that smaller forms of RTB are intrinsically more stable or that protective factors in the *Xenopus* oocyte homogenate are able to more easily stabilise these smaller forms. To distinguish between these possibilities a number of deletion mutants of RTB were created and expressed at low level into the periplasm of *E.coli*. Only one of six deleted forms of RTB expressed (L132 to F262) was found to bind to immobilised lactose. Included in those not binding were the equivalents of the forms expressed in *Xenopus* oocytes and found to bind. The galactose binding deletion form comprised domain II of RTB and was consistently expressed at higher levels. It is concluded that stable deleted forms of

RTB can be produced in a non-glycosylated yet functional form but the boundaries of such proteins must be very carefully determined.

Potentiation role of RTB

In order to determine if the galactose binding sites of RTB were involved in the intracellular role of RTB the cytotoxicity of holotoxins constructed with the various galactose binding mutants of RTB was assessed on rat bone marrow macrophages (Newton *et al.*, 1992) These cells carry on their surface mannose receptors, in addition to the glycolipids and glycoproteins which provide the normal route of uptake for ricin, which provide a secondary mode of uptake for ricin via its N-linked carbohydrate moieties.

Toxins created by the reassociation of rRTA with wild type RTB produced in *Xenopus laevis* oocytes or with either of the single galactose binding-site mutants (K40/N46 to M/G, or N255 to G) were all found to be toxic to Vero cells and this toxicity was inhibited by lactose. Such holotoxins were also found to be cytotoxic to macrophages in the presence of lactose indicating cytotoxicity via the mannose receptors, this effect being inhibited by free mannose. Holotoxin constructed using the double galactose binding site mutant of RTB however (K40/N46 to M/G, and N255/G) was not found to show any cytotoxicity on either Vero cells or macrophages even in the absence of lactose and mannose. It was concluded from these results that the intracellular role of the RTB in cytotoxicity is mediated via the galactose binding sites, the most likely role being in assisting the routing of RTA through the endomembrane system of the cell to the site of translocation.

Enhancement of holotoxin and RTA cytotoxicity by ER retrieval signals

To attempt rescue of the cytotoxicity of the RTB galactose binding site mutants it was decided to attempt to substitute the role of the galactose binding sites with signals found on a toxin with a similar mode of entry, *Pseudomonas* exotoxin A, which are thought to be involved in intracellular routing. *Pseudomonas* exotoxin A carries at the C-terminus of it's A chain the sequence REDLK (Chaudhary *et al.*, 1990) which bears similarity to the endoplasmic reticulumn (ER) retrieval signal KDEL found at the C-terminus of soluble proteins destined to be retained in the ER of eukaryotic cells (Munro & Pelham, 1987). In support of a role for this motif in toxin entry are the observations that removal of the relevant motif from *Pseudomonas* exotoxin A and replacement with a non-retrievable signal reduces cytotoxicity 100 fold, while replacement with the sequence KDEL enhances cytotoxicity (Seetharam *et al.*, 1991). Also, similar sequences are found on the Cholera toxin (KDEL) and its E.*coli* homologue, heat labile toxin (RDEL).

Mutants of RTA were created in which an ER retrieval motif, KDEL or a non-retrieved motif, KDEA, were added to the C-terminus of RTA, and the mutant protein produced in *E.coli*. After expression and purification the enzymatic activity of the two mutant proteins was measured and they were shown to be of equal activity. To ensure that the mutants were still translocationally competent holotoxin was prepared through the reassociation of RTAKDEL, RTAKDEA and rRTA with RTB purified from plants and the cytotoxicity of the conjugates assessed on mammalian cells.

Unexpectedly the KDEL motif was found to enhance the cytotoxicity of the holotoxin approximately 7-fold while that of the KDEA tagged toxin was found to be similar or slightly less than the holotoxin constructed using rRTA (Wales *et al.*, 1992b). Even more unexpectedly, control experiments to assess the cytotoxicity of the free RTA forms on mammalian cells revealed that the KDEL motif enhanced the cytotoxicity of RTA by 250 fold on Vero cells and 10 fold on Hela cells (Wales *et al.*, in press).

These results implicate the KDEL receptor in the retrograde transport of endocytosed toxins from early in the endocytic pathway to the site of translocation. The results with the free RTA might suggest that the KDEL receptor can recycle proteins from much later in the secretory pathway than has previously been assumed. Indeed the deepest position in the cell that ricin has been observed by microscopic techniques is the trans-Golgi network (van Deurs *et al.*, 1988). The observations that brefeldin, a fungal metabolite which disrupts the golgi apparatus, protects cells against ricin (Yoshida *et al.*, 1991), that another endocytosed toxin, Shiga toxin, can reach the ER under certain conditions (Sandvig *et al.*, 1992) and the time lag before any inhibition of protein synthesis is seen in ricin treated cells combined with the results obtained in our work all points to the ER as the current favourite for the role of translocation compartment (Pelham *et al.*, 1992). Experiments are currently underway to show a direct role for the KDEL receptor in the cytotoxic effect, however the fact that *Pseudomonas* exotoxin A is still cytotoxic after removal of the REDLK sequence, and that ricin does not carry such a sequence at all, suggest that it plays merely an enhancement role in cytotoxicity.

GENETIC ABLATION USING RTA

A powerful tool for analysing developmental processes

The high efficiency with which ricin, (and other cytotoxins), inhibit protein synthesis has been usefully exploited in genetic ablation studies. Traditionally, UV lasers or microdissection have been used to physically destruct target cells and thereby assess their role within complex tissues and developmental processes. Such methods, however, are often limited by the inaccessibility of the cells to be ablated. Genetic ablation overcomes this by expressing a toxin under the tight control of a cell- or tissue-specific promoter so that only those cells in which the promoter is expressed are killed. Using such an approach, exquisite control of cell ablation, both spatially and temporally, can be achieved throughout differentiation.

Genetic ablation has been widely applied in animal systems, but only recently has its potential in plant studies been recognised: Robert Goldberg and colleagues (UCLA) successfully generated male-sterile tobacco plants using a tapetal-specific promoter to drive expression of diphtheria toxin A chain (DTA) (Koltunow *et al.*,1990). In collaboration with Jan Leemans (Plant Genetic Systems, Belgium) they further refined this method of producing male-sterile plants by substituting DTA with the ribonuclease gene, barnase, which could then subsequently be inactivated by barstar to restore male fertility, (Mariani *et al.*, 1990). Recently, elegant experiments using DTA fused to the promoter of the *Brassica* S-Locus Glycoprotein gene have revealed previously unsuspected complexities within

the mechanisms of pollen perception in *Arabidopsis* and *Brassica*, (Thorsness *et al.*, 1993; Kandasamy *et al.*, 1993).

At Warwick, we are using genetic ablation to study the development and physiology of N_2-fixing root nodules in legumes.

The role of the nodule parenchyma in regulating oxygen diffusion within root nodules

Root nodules, formed on legume roots following infection with the soil bacteria *Rhizobia*, provide a unique, favourable environment for the fixation of atmospheric N_2 by the enzyme, nitrogenase. Nitrogenase is irreversibly inactivated by high oxygen concentrations; oxygen diffusion within root nodules, therefore, must be tightly regulated. It has been proposed that a specific layer of densely packed cells within the nodule, the nodule parenchyma, acts as an O_2 diffusion barrier (Van de Wiel *et al.*, 1990). Evidence to support this hypothesis comes from studies using O_2-specific microelectrodes (Witty *et al.*,1987), that demonstrated free oxygen concentrations decreased sharply across the nodule parenchyma to a low and constant value throughout the central infected tissue. *In situ* hybridisation studies have revealed that the early nodulin gene, ENOD2, is exclusively localized within the nodule parenchyma (Van de Wiel *et al.*, 1990). This gene encodes a (hydroxy)proline-rich (glyco)protein which may contribute to the special morphology of the nodule parenchyma and its role as the O_2 diffusion barrier.

We are using genetic ablation to investigate this proposed role of the nodule parenchyma. Using the toxin, ricin, fused to the promoter of the ENOD2 gene we aim to target cell death specifically to cells in the nodule parenchyma and assess the effect this has on subsequent N_2 fixation. We have created recombinant ENOD2-ricin genes using either wild-type RTA or conditional mutants of RTA (Moffat *et al.*, 1992), so that synthesis of active ricin, and therefore cell death, can be switched on at different stages of nodule development. Binary vectors containing these constructs have been introduced into *Lotus japonicus* via an *Agrobacterium tumefaciens* system (Handberg & Stougaard, 1992). Several independent, hygromycin-resistant *L.japonicus* plants have been regenerated for each construct, and nodules from these plants are currently undergoing analysis for altered N_2 fixation levels. Studies of these transgenic nodules should determine whether the nodule parenchyma does indeed function as an O_2 diffusion barrier within root nodules.

ANALYSIS OF THE ANTIVIRAL ACTIVITY OF RIPS

The physiological role of RIPs

It is commonly accepted that the physiological role of RIPs is one of defense protecting the plants or seeds from attack by predators or pathogens (Olsnes & Phil, 1982; Lord *et al.*, 1991). The most widely studied defensive property is the antiviral activity shown by single-chain RIPs towards diverse viruses of both plant and animal origin (Hartley & Lord, 1991). For example, it has been shown that pokeweed antiviral protein (PAP), protects appropriate indicator plants from infection by viruses from seven different groups, including both DNA and RNA viruses (Chen *et al.*,

1991). The inhibitory effect of PAP does not act directly on the virus since it has been shown that cucumber mosaic virus mixed with PAP regains full infectivity on *Chenopodium quinoa* when the virus is separated by centrifugation (Tomlinson *et al.*, 1974). It has therefore been assumed that the antiviral effect is mediated through the inhibition of host cell protein synthesis (Stevens *et al.*, 1981).

PAP and single-chain RIPs from *Dianthus barbatus* and *Chenopodium amaranticolor* are predominantly located in the cell wall matrix of leaf mesophyll cells (Ready *et al.*, 1986; Frotschl *et al.*, 1990). This finding has been incorporated into a hypothesis which states that local damage caused by vectors which transmit viruses to plants (eg. aphids) releases the RIP into the cytosol, resulting in local suicide at the site of infection. However, this hypothesis is at variance with the commonly held view that ribosomes are uniquely resistant to their endogenous RIP (Stirpe and Hughes, 1989; Logemann *et al.*, 1992). Recent work in which rRNA depurination was assayed has shown that this is not the case for six species of dicotyledenous plants (Taylor & Irvin, 1990; Prestle *et al.*, 1992; Kataoka *et al.*, 1992). In contrast, there is strong evidence that cereal seed ribosomes are resistant to the action of endogenous RIPs, this being derived from their *in vitro* resistance and the finding that cereal RIPs genes do not encode precursor forms of the RIP containing targeting information (Taylor & Irvin, 1990; Leah *et al.*, 1991; Walsh *et al.*, 1991). Such RIPs are presumably cytosolic making it imperative that the ribosomes exhibit resistance to RIP-catalyzed depurination. Cereal RIPs have also been shown to have a more restricted ribosome substrate range than most single chain RIPs from dicotelydenous plants.

Correlation between the activities of RIPs in depurination of tobacco ribosomes and inhibition of tobacco mosaic virus (TMV) infection.

We have compared the *in vitro* rRNA depurination activities of five RIPs using yeast and tobacco leaf ribosomes as substrates. All of the RIPs (PAP, dianthin 32, tritin, barley RIP and RTA) were active on yeast ribosomes. PAP and dianthin 32 were highly active and RTA weakly active on tobacco ribosomes, whereas tritin and barley RIP were inactive. PAP and dianthin 32 were highly effective in inhibiting the formation of local lesions caused by TMV on tobacco leaves, whereas tritin, barley RIP and RTA were ineffective. The apparent anomaly between the *in vitro* rRNA depurination activity, but lack of antiviral activity of RTA was further investigated by assaying for rRNA depurination *in situ* following the topical application of the RIP to leaves. No activity was detected, a finding consisitent with the apparent lack of antiviral avtivity of this RIP. These results show that there is a positive correlation betwen RIP-catalysed depurination of tobacco ribosomes and antiviral activity which gives strong support to the hypothesis that the antiviral activity of RIPs works through ribosome inactivation.

Expression of RIPs in transgenic tobacco plants.

The antiviral properties of RIPs makes these proteins ideal candidates for genetically engineering virus resistant plants. If RIPs inhibit virus infection by inactivating the host cell ribosomes, as our results suggest, then transgenic plants expressing RIPs should be resistant to a wide range

of plant viruses. In addition, the viral resistance of these plants should not be overcome by high concentrations of virus.

To investigate the potential of RIPs as antiviral agents in plants we have attempted to express dianthin and RTA in tobacco. As both of these RIPs are active on tobacco ribosomes, it is essential that in the transgenic plants these proteins do not come into contact with ribosomes until the cells are damaged during virus infection. Therefore plants were transformed with RIP genes encoding precursor forms of the proteins which contain the signal peptide, so directing the RIP to the ER. Several *Nicotiana tabacum* lines expressing RTA have been successfully established. Crude extracts prepared from the leaves of the transgenic plants depurinate yeast and tobacco ribosomes *in vitro*, confirming that the plants are expressing active RTA. However these plants are not resistant to infection by TMV at 10 mg/ml. It may be that RTA, which is only weakly active on tobacco ribosomes, is not expressed at sufficient levels in these plants to overcome the TMV infection. Alternatively, RTA readily associates with lipid bilayers (Utsumi *et al.*, 1984), therefore in the transgenic plants RTA may be sequestered by membranes which prevents it from reaching the vacuoles where it would be released into the cytosol during cell damage. The finding that RIP activity is only detected in transgenic leaf extracts prepared in the presence of the detergent triton, is also consistent with the hypothesis that RTA may associate with membranes when it is not linked to RTB in the holotoxin.

Initial attempts to transform tobacco with dianthin, a single chain RIP which is highly active on tobacco ribosomes, were unsuccessful. The results of these experiments suggest that expression of high levels of dianthin may be toxic to the plant. To overcome this potential problem the dianthin construct was placed under the control of a modified CaMV 35S promoter, which can be tightly repressed by the bacterial Tn *10* Tet repressor (Gatz *et al.*, 1992). The construct was then transformed into tobacco plants constitutively expressing the Tet repressor protein. This system allows the regeneration of transgenic plants carrying a repressed dianthin transgene. The expression of dianthin can then be specifically de-repressed by tetracycline and the plants assessed for viral resistance.

Genetic engineering of virus resistant plants has provided new strategies for control of virus disease. At present, the most successful of these has been the expression of viral coat-protein coding sequences in plants. However these plants are only resistant to the virus or strain of virus from which the coat-protein transgene is derived. The use of general inhibitors of plant virus infection, such as RIPs, provides a promising approach to developing transgenic plants which are resistant to a wide range of plant viruses.

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PROSPECTS FOR THE GENETIC MANIPULATION OF ANTIMICROBIAL PLANT SECONDARY PRODUCTS

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ABSTRACT

Phytoalexins are low molecular weight antimicrobial compounds that are synthesized by plant cells in response to infection, elicitation by fungal macromolecules, or metabolic stress. The biochemical pathways leading to the synthesis of the isoflavonoid phytoalexins of the leguminosae have been elucidated, and the genes encoding many of the biosynthetic enzymes have been cloned. It is often observed that a pathogen is more sensitive to the phytoalexins of a non-host species than to those of its natural host, and structure-activity studies predict that alterations of isoflavonoid phytoalexin substitution patterns or stereochemistry may increase the phytoalexin's antimicrobial activity or decrease its degradation by fungal pathogens. These studies, along with an increasing understanding of defence-inducible plant gene promoters, form the basis for the rational manipulation of phytoalexin structures by genetic engineering.

INTRODUCTION

Naturally occurring antimicrobial compounds from plants (phytoalexins) have been the subject of intense study at the physiological, biochemical and molecular genetic levels (Dixon *et al.*, 1983; VanEtten *et al.*, 1989; Dixon and Harrison, 1990). In spite of this, roles for these compounds as causal agents of disease resistance have been inferred, indirectly, in only a few cases (VanEtten *et al.*, 1989). There is, however, sufficient information now available to suggest strategies by which the amounts or structures of phytoalexins could be modified, by molecular genetic techniques, to engineer improved disease resistance against fungal pathogens. This article outlines these strategies with particular emphasis on alfalfa (*Medicago sativa*), a species in which the molecular biology of defence metabolism is reasonably well understood (Dixon *et al.*, 1992). More detailed overviews of this subject have recently appeared (Lamb *et al.*, 1992; Dixon *et al.*, 1993).

BIOCHEMISTRY AND MOLECULAR BIOLOGY OF THE PHYTOALEXIN RESPONSE

Like most legumes, alfalfa responds to microbial attack or exposure to microbial elicitor macromolecules by producing isoflavonoid-derived phytoalexins synthesised by the phenylpropanoid/polymalonate pathway (Fig. 1). The eleven enzymes involved in the formation of the pterocarpan phytoalexin medicarpin from phenylalanine have been characterised, and cDNAs encoding phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (CA4H), 4-coumarate: CoA ligase (4CL), chalcone synthase (CHS), chalcone reductase (CHR), chalcone isomerase (CHI) and isoflavone reductase (IFR) have been cloned (Dixon *et al.*, 1992). Elicitation of the pathway involves increases in the extractable activities of all eleven enzymes, apparently resulting from transcriptional activation of the corresponding genes (Dixon and Harrison, 1990; Dixon *et al.*, 1992). The key regulatory enzymes PAL and CHS are encoded by multigene families in legumes such as alfalfa (Dixon *et al.*, 1992); individual members exhibit differential tissue-specific expression, but most or all are activated by elicitation or infection.

Medicarpin has one of the most simple substitution patterns of the pterocarpan phytoalexins. In bean and soybean, the pterocarpan phytoalexins are isoprenylated, whereas the major phytoalexin of pea, pisatin, has methoxy, methylenedioxy and 6a-hydroxy substitutions.

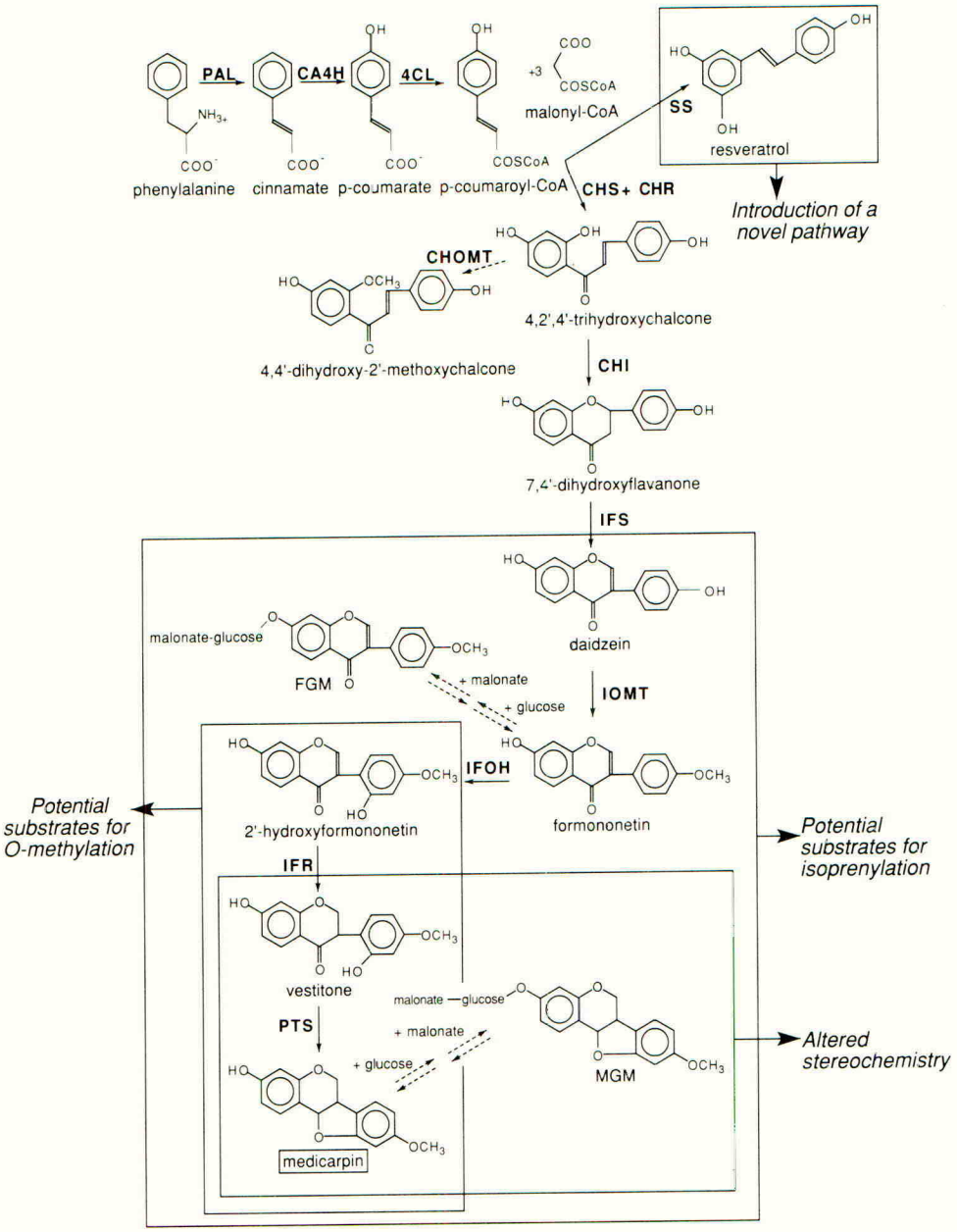

Fig. 1. Biosynthesis of the phytoalexin medicarpin in alfalfa, and potential for altering phytoalexin structures by genetic manipulation. PAL = L-phenylalanine ammonia-lyase, CA4H = cinnamic acid 4-hydroxylase, 4CL = 4-coumarate: CoA ligase, SS = stilbene synthase, CHS = chalcone synthase, CHR = chalcone reductase, CHOMT = chalcone 2'-O-methyltransferase, CHI = chalcone isomerase, IFS = isoflavone reductase, PTS = pterocarpan synthase, FGM = formononetin malonyl glucoside, MGM = medicarpin malonyl glucoside.

Prenyltransferases and O-methyltransferases active against isoflavonoids and pterocarpans have been characterized, but their genes have not yet been cloned.

In addition to isoflavonoids, peanut produces stilbene phytoalexins, which have also been reported in pine and grapevine. Stilbene synthase, an enzyme closely related to chalcone synthase (Fig. 1), has been cloned from several sources. Solanaceous plants produce primarily bi-cyclic sesquiterpene phytoalexins, whereas mono-cyclic and tetra-cyclic diterpenes are found in castor bean and rice respectively. Sesquiterpene and triterpene cyclases, responsible for the initial folding and cyclisation of the farnesyl or geranylgeranyl pyrophosphate building blocks of the complex terpenoids, have been isolated and cloned. Thus, there is now significant information available on the enzymology of phytoalexin synthesis and modification to underpin the design of strategies for the alteration of phytoalexin structures by genetic engineering.

STRUCTURE-ACTIVITY RELATIONSHIPS

Several studies have addressed structure-activity relationships among the isoflavonoid phytoalexins (Adesanya *et al.*, 1986; Delserone *et al.*, 1993), but few general principles have emerged. In many cases, increasing hydrophobicity by addition of prenyl side chains or by O-methylation increases biological activity. Indeed, several fungi have evolved mechanisms to detoxify their hosts' phytoalexins by hydration of prenyl groups or by demethylation (VanEtten *et al.*, 1989). Pathogens that can detoxify their host's phytoalexins are often considerably more sensitive to phytoalexins from non-host species, even if their phytoalexins are structurally related to those of the host. The pterocarpans can exist as (+) or (-) stereoisomers. In most legumes analysed to date, only the (-) forms accumulate although pea produces (+) pisatin and *Sophora japonica* can produce both (+) and (-) maackiain. Field isolates of *Nectria haematococca* from red clover, which produces (-) maackiain, are much more sensitive to (+) maackiain than to (-) maackiain in bioassays (Delserone *et al.*, 1993), apparently due to their inability to degrade the (+) isomer.

STRATEGIES FOR THE GENETIC MANIPULATION OF PHYTOALEXIN STRUCTURES.

The above considerations of structure-activity relationships suggest that resistance to pathogenic fungi could be improved by engineering plants to produce phytoalexins not normally encountered by their pathogens. This could be done by introducing genes that either lead to expression of novel pathways or to altered structures of phytoalexins synthesised via pre-existing biosynthetic machinery.

Synthesis of novel phytoalexins in transgenic plants.

Tobacco produces sesquiterpene phytoalexins, and uses the flavonoid pathway for production of UV-protective pigments and flower colours. Chalcone synthase (flavonoid pathway) and stilbene synthase share the same substrates, malonyl CoA and 4-coumaroyl CoA (Fig. 1). The stilbene synthase gene from grapevine has been transformed into tobacco under the expression of its own promoter, leading to pathogen-inducible production of the novel stilbene phytoalexin resveratrol in tobacco (Fig. 1). The transgenic plants exhibit significantly improved resistance to infection by *Botrytis cinerea* (Hain *et al.*, 1993). It is not known whether the tobacco genes encoding 4-coumarate: CoA ligase and acetyl CoA carboxylase, responsible for the production of the substrates for the stilbene synthase reaction, are inducible by infection as they are in alfalfa (Dixon *et al.*, 1992). If not, further genetic manipulation could be performed to increase substrate supply for formation of the novel phytoalexin. It would also be interesting to express stilbene synthase in a legume such as alfalfa, where both chalcone synthase and the product of the transgene would be infection-inducible and competing for substrates. Depending on the level of stilbene synthase expression, different relative proportions and absolute levels of resveratrol and medicarpin may be produced.

Theoretically, the above strategy could also be used to produce the diterpene phytoalexin casbene in transgenic plants, using the cloned casbene synthase from castor bean (Lois and West, 1990). In this case the substrate would be geranylgeranyl pyrophosphate, an intermediate in the synthesis of gibberellins and carotenoids. A fungal sesquiterpene cyclase, trichodiene synthase, has been expressed in tobacco, leading to production of low levels of trichodiene which, unlike casbene, is not directly antifungal (Hohn and Ohlrogge, 1991). More information on the enzymology of sesquiterpene phytoalexin synthesis will be required before antimicrobial sesquiterpenes can be engineered in plants.

Modification of structures of pre-existing phytoalexins

Position-specific prenyltransferases and O-methyltransferases exist for isoflavone, isoflavan and pterocarpan substrates, and several of these have been purified. The isolation and cloning of the prenyltransferases is somewhat problematical as these are membrane-associated proteins of low abundance (Biggs *et al.*, 1987). Availability of cDNA clones for these modifying enzymes could enable novel phytoalexins to be engineered into alfalfa as indicated schematically in Fig. 1. Genes encoding fungal enzymes could also be used for such metabolite engineering; transfer of a 6a-hydroxylase gene from *Nectria haematococca* and a pterocarpan O-methyltransferase from pea (Preisig *et al.*, 1991) could theoretically lead to the synthesis of homopisatin in alfalfa (H. VanEtten, personal communication), a manipulation which might increase resistance against those pathogens that are insensitive to medicarpin.

To engineer pterocarpan phytoalexins with altered stereochemistry, it is necessary to obtain the enzymes that catalyze the reactions that introduce chiral centres from plants making the opposite stereoisomer to that found in the target plant. In the case of alfalfa, which produces (-) medicarpin, genes could be obtained from pea, peanut or *Sophora japonica*. It was originally believed that the stereochemistry of pterocarpans was determined at the level of the isoflavone reductase (IFR) and pterocarpan synthase reactions (PTS) (Fig. 1); as would be predicted, the isoflavone reductase from alfalfa specifically produces the (-) isoflavanone (Paiva *et al.*, 1991). However, recent studies indicate that the reductase from pea also produces the (-) isoflavanone, suggesting that an epimerase may be involved in the production of (+) pterocarpans (N. Paiva, Y. Sun, R.A. Dixon, H.D. VanEtten, G. Hrazdina, unpublished results). As structure-activity studies indicate the potential efficacy of manipulating stereochemistry, elucidation of the stereospecific interconversion of isoflavone to pterocarpan is now a major priority of our program.

The flavonoid/isoflavonoid pathway is not only defence-related. 4',4'-dihydroxy-2'-methoxychalcone and formononetin malonyl glucoside are potent inducers of Rhizobial nodulation genes, and medicarpin malonyl glucoside is stored in uninfected alfalfa roots. Medicarpin may act as an allelochemical. Modifications to the pathway could therefore also impact on food or forage quality, symbiosis with *Rhizobia* or mycorrhizal fungi, and allelopathy. These and other concerns have been discussed elsewhere (Dixon *et al.*, 1993). Furthermore, it is inadvisable to initiate genetic manipulation of phytoalexin pathways without a full understanding of the antimicrobial activities of pathway intermediates and potential novel products. In a recent study it was shown that intermediates in the biosynthesis of medicarpin are more toxic than medicarpin itself to *Phytophthora* root rot of alfalfa (Blount *et al.*, 1992). It is highly unlikely that a single genetically engineered change in phytoalexin structure would lead to increased resistance against all fungal pathogens of a particular plant. A better strategy, and one which would decrease the prospects of the pathogen overcoming the resistance, would be the co-production of several novel phytoalexins.

If a novel phytoalexin is to be engineered using a gene encoding an enzyme that uses substrates which are present constitutively in the plant cell (e.g. stilbene synthase), it will be necessary to express the transgene under an infection-inducible promoter in order to avoid build up of potentially toxic phytoalexins in uninfected cells. Several such promoters are now available (Dixon *et al.*, 1993). However, it should be borne in mind that many defense-inducible promoters can exhibit exquisite developmentally regulated tissue specific expression, and could thus direct phytoalexin production to a specific cell type in the absence of infection. It is not safe to assume that the tissue specificity of expression of the promoter in the species of origin will be the same as in the transgenic

target plant. For example, the alfalfa isoflavone reductase promoter is expressed in roots, stems, pollen and stigmas in uninfected transgenic tobacco, whereas its constitutive expression appears limited to roots in alfalfa (A. Oommen, R.A. Dixon, N.L. Paiva, unpublished results). Thus, promoters should be carefully evaluated in the transgenic target plant using a readily scored reporter gene. Constitutive promoters (e.g. cauliflower mosaic virus 35S) should only be used if substrates for the transgene's protein product have been conclusively demonstrated to be absent from cells not expressing a defence response, or if the new phytoalexin has been proven to be non-phytotoxic. Similarly, any introduced phytoalexin-modifying genes must be expressed in the same cells and at the same time as the host phytoalexin pathway enzymes in order to have much effect on the product composition. The use of phytoalexin-related promoters from the host would ensure correct coordinate and tissue-specific expression.

Manipulation of flux into antimicrobial pathways

In some cases, the rate limiting step in a phytoalexin pathway may be known, and increased production of this enzyme, perhaps by expressing it under a stronger promoter, could lead to a greater or faster phytoalexin response. For example, it appears that medicarpin accumulation in chickpea is limited by the activity of isoflavone 2'-hydroxylase (IFOH) (Fig. 1), a microsomal cytochrome P450 enzyme (Gunia *et al.*, 1991). Recent studies on genetically manipulated tobacco plants expressing variable levels of PAL (Fig. 1) indicate that the activity of this first committed enzyme in the phenylpropanoid pathway correlates positively with the levels of certain phenylpropanoid end products and resistance to infection by *Cercospora nicotianae* (E. Maher, N. Bate, J. Orr, Y. Elkind, R.A. Dixon, C.J. Lamb, unpublished results), suggesting that manipulation of a single gene encoding an early committed step may in some cases alter flux through a multistep metabolic pathway.

Increasing information is now available on the *cis*-elements which confer elicitor- or infection-induciblity to phytoalexin biosynthetic and other defence response genes, and transcription factors have recently been identified which appear to be involved in signalling the elicitation response to the plant nucleus (Dixon and Harrison, 1990; Lamb *et al.*, 1992; Yu *et al.*, 1993). In the future, modification of combinatorial interactions between different *cis*-elements in a particular defence gene promoter may be used to engineer novel properties leading to improved expression of defence responses, whereas modifications to the structure or regulation of *trans*-factors will allow concerted manipulation of batteries of genes. The latter will become increasingly important as sets of genes encoding enzymes of complex biosynthetic pathways are transferred into new plant species.

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