GENETIC ENGINEERING OF MICROBIAL PESTICIDES

Bruce C. Carlton

Professor of Molecular and Population Genetics, University of Georgia, Athens, Georgia 30602 U.S.A.; and Vice President of Research and Development, Ecogen Inc., Princeton, New Jersey 08540 U.S.A.

Recent advances in genetics and molecular biology make possible the cloning and genetic manipulation of genes for insecticidal activities from natural insect pathogens. Using recombinant DNA methods and site-directed mutagenesis of specific gene regions, production of new and improved biorationals should be possible.

In recent years the agricultural pesticide industry, currently generating the equivalent of more than 13 billion U.S. dollars in world-wide sales, has been fraught with a number of problems that in the aggregate have led both manufacturers and consumers to seriously examine the future trends of the industry. These problems include the acute and chronic toxicities of many pesticides to man and other animals, lack of specificity (killing of desirable insect species and natural predators), rapid development of resistant insects under intensive usage, rapidly escalating production costs, and disposal problems of often-toxic residues and by-products. Increased pressures from environmental groups and governmental regulatory agencies have led to the banning of chemicals such as DDT, 2-4-D, Mirex, EDB and various others (especially chlorinated hydrocarbons) from the pesticide marketplace. One solution to these problems is to develop new pesticides which are safer to use, more specific to target insects, weeds, and plant disease organisms, and non-polluting to the environment. The most obvious alternative to chemical pesticides is the so-called biorationals. Such a class of pesticides has been known for some years and includes a variety of insect-pathogenic bacteria, viruses, and fungi. In addition, there is a variety of protozoans, predators and parasites, growth regulators, and pheromones which show potential as biological pesticides.

Despite their many desirable properties, the list of biological pesticides and related biorationals currently registered for use is distressingly small (Miller et al, 1983). The major product, by far, is Bacillus thuringiensis (BT), a sporulating bacterium that produces a crystalline toxic protein active on many lepidopterous (caterpillar) insects. United States sales of BT in 1982 were about $10M -- world-wide they approach ~$20M. A variant of BT, called BTI, has recently been licensed for control of several species of disease-carrying mosquitos and blackflies. Three other related bacteria, Bacillus popilliae, Bacillus sphaericus, and Bacillus penetrans, are of more limited use. B. popilliae (sold in the U.S. under the trade name Doom) has activity on Japanese beetles and related species and has a small market. B. sphaericus is highly pathogenic to mosquitos but to date has only been used experimentally. B. penetrans has potential for control of root-knot nematodes and root lesions, but so far has only been tested in greenhouse conditions. Biopesticides of viral origin include Elcar, used for control of Heliothis larvae, Gypchek, a virus for control of gypsy moth, and an as-yet unregistered virus which has considerable potential for control of codling moths on a variety of fruit and nut trees, as well as for tip moth control on pine trees. Fungal agents for insect control include a single product in the United States (Mycar, registered for citrus mite control in Florida) and two Verticillium species registered in the United Kingdom for control of aphids and white flies on glasshouse crops. Two fungal products have recently been licensed for weed control in the U.S.: Devine, used for control of strangler weed in Florida citrus groves, and Collego, a fungus licensed for use on northern jointvetch in soybeans and corn.

The only other biorational products of any significance in the agrichemical market are pheromones, attractants, and hormones. Gossyplure is a pheromone of Heliothis (cotton bollworm) that is used fairly extensively as a mating confusion spray in the southwestern United States, Mexico, Brazil, and Egypt. Dimilin is a growth inhibitor that has recently been registered for use on cotton and on forest trees.

Although all of these products are effective when used properly, they have distinct drawbacks which limit user acceptability. The bacterial and viral agents must be ingested to be active, and their killing action, especially the viruses, is slower than conventional chemical insecticides. These agents are also subject to rapid inactivation by exposure to sunlight and are readily washed off the foliage by rain. Viral products are expensive to produce since current methods require propagation in living insect larvae. Fungi are very intolerant of low humidity conditions or high temperature, and thus are generally used only in greenhouses or in cool climates. All of these agents require more user sophistication to be effective than is commonly encountered among growers.

Clearly, the array of effective biological pesticides developed to date is not extensive. While many of these biorationals have been known for some time, their aggregate market share of the total pesticide industry has remained relatively insignificant, for several reasons. Chief among them has been a lack of understanding of their basic properties, including their synthesis and mode of action. As an example, consider the bacterial insecticide BT. Although BT has been marketed as an insecticide since the late-1950's, the nature of its insecticidal toxicity came under study only several years later and is still not fully understood. Genetic analyses of the production of the insecticidal toxin were not

even initiated until the late 1970's, and are just now beginning to reveal where the toxin genes are localized and how they are expressed. For other biologicals the level of our basic understanding of their inheritance and mode of action is considerably less. Without detailed knowledge of their mechanisms, it is not surprising that efforts to develop these agents as alternatives to more conventional pesticides have been sporadic at best.

Despite the limitations and disadvantages of these biorational products, there is currently a great deal of interest, both from the basic science viewpoint as well as from the commercial side, in agents such as BT. A number of laboratories in the U.S. and abroad are currently focusing their attention on the genetics and molecular biology of BT and other biological pathogens, including other bacteria, viruses, and fungi. In addition, a number of new start-up biotechnology companies have announced plans to direct significant fractions of their efforts to development of new and improved biological pesticides. One such company is Ecogen, Inc., founded just a few months ago in Princeton, N.J., and with which I am especially familiar since I have recently agreed to become their full-time director of research and development beginning on June 1 of this year. The major thrust of Ecogen, Inc. will be to apply a multidisciplinary, integrated approach to the development of novel biopesticides, using modern technologies of genetics, protein chemistry, immunology, and cell biology.

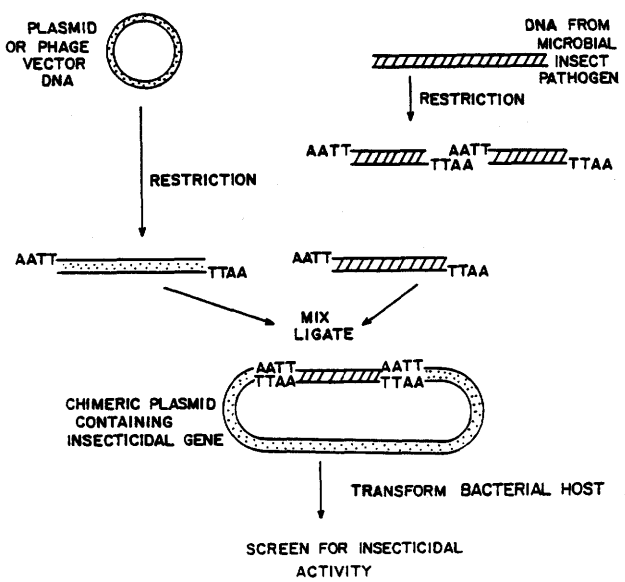

Fig. 1 Gene-splicing technology for molecular cloning of insecticidal genes.

What factors have led to the resurgence in interest in biological pesticides? Other than economic and environmental considerations, probably the major technological advance that has contributed to interest in biorationals is that of recombinant DNA-gene splicing methodology (Fig. 1). There are two factors of importance in this methodology. First is the ability to isolate a gene (or cluster of genes) controlling a given activity (e.g. pesticide synthesis) from the vast background of other genes and activities in any organism, regardless of its genetic complexity. Thus, in principle, a gene from a fungal cell can potentially be isolated (cloned) and its structure and regulation studied as easily as that of a bacterial gene. Second, once a gene has been isolated by molecular cloning, its structure can be readily altered (mutated) by a variety of molecular tricks that would be impossible by conventional mutagenesis procedures using intact organisms. For example, digestion of cloned genes with a combination of restriction endonucleases (enzymes which recognize and cleave specific 4- or 6-base sequences in DNA) identifies sites which can be specifically mutated by one of several methodologies recently developed for site-specific mutagenesis (Figs. 2 and 3).

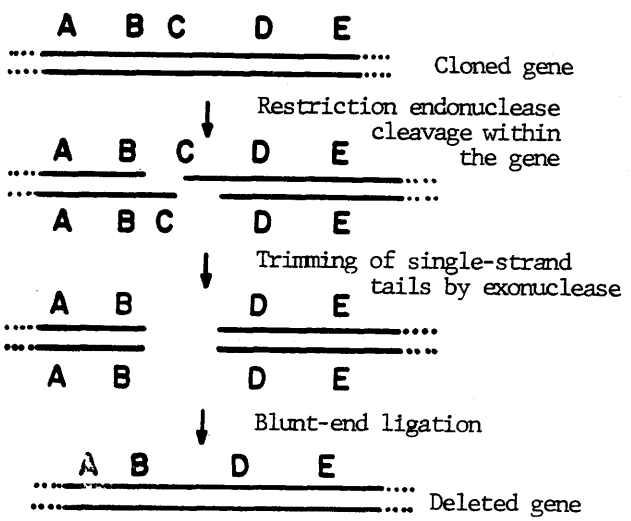

Fig. 2 Generation of deletions in an insecticidal gene.

These treatments can be carried out on a cloned gene in vitro (i.e., in a test tube) with much greater efficiencies than could be accomplished in vivo (in the intact organism). By these techniques one can thus change single bases in a DNA gene, delete blocks of bases, or add segments of DNA at will and observe the effects on expression of the gene. While these changes might also occur spontaneously in vivo for a non-cloned gene, their frequencies would be orders of magnitude less and their detection would be exceedingly difficult. Thus, the techniques of

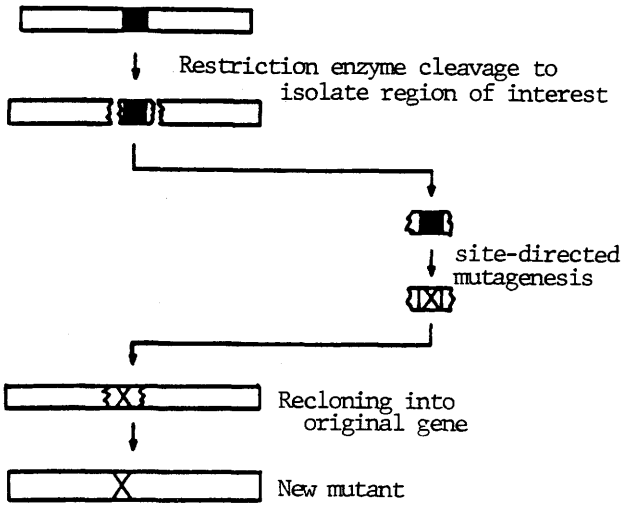

Fig. 3 Site-directed in vitro mutagenesis.

modern molecular cloning serve to greatly enhance the genetic sensitivity of mutational approaches. The isolation of individual genes by molecular cloning also makes possible the enhanced production of their products (usually enzymes or other proteins) by genetically fusing them to highly-efficient regulatory sequences (promoters). In certain instances, this manipulation can elevate a gene's expression several hundred-fold above its normal level of activity. Further, a gene which normally produces its protein product inside the cell, often a self-limiting process, can be fused to a small signal peptide coding sequence which allows the protein to be transported outside the cell. This construction can both increase the level of production of a particular gene product as well as facilitate its recovery because it is transported to the external medium.

How can the types of genetic manipulation described above be applied to the improvement and commercialization of biological pesticides? The possibilities are numerous. For example, molecular cloning of a gene which specifies an insecticidal toxin protein makes it amenable to yield enhancement by attaching the gene to a high-effiency promoter sequence, thereby markedly increasing the yield. The gene can also be altered by site-directed mutagenesis procedures to either broaden the spectrum of targets (e.g., different insects it will attack) or to increase its activity on a specific target. Cloning of two or more genes for distinct pesticide activities into the same host cell could generate a multi-purpose pesticide, having activities against two or more very different targets (e.g., two different insects, an insect and a weed, an insect and fungal disease,etc.). The relative ease with which such different gene combinations can be potentially constructed by recombinant DNA technology makes the possibilities virtually unlimited. The limitations fall to the imagination of the researcher and to the sensitivity of detection methods for identifying new gene combinations of interest amongst a background of hundreds, or even thousands, or new variants. In Bacillus thuringiensis, the discovery in our laboratories (Gonzaléz et al 1982) of a natural, highly-efficient plasmid exchange system opens the way for constructing strains having novel combinations of insecticidal genes from different strains of BT, or other related strains. This plasmid transfer system has already been used to construct strains of BT having two toxin-coding plasmids from different parental origins, as well as to convert strains of normally non-toxic B. cereus into BT-like variants. These and other methodologies offer the possibility for producing new pesticides that can be rapidly tailored to individual locales and situations. For example, predictions of an outbreak of a particular plant disease and concomitant infestation of a particular insect on some important crop could dictate the construction of a bacterial, viral or fungal strain having the capabilities to control both pests. For some other crop or locale, a different combination might be called for. This type of rapid response to changing pest problems would be a significant departure from current planning in the pesticide field, which must be done years in advance due to the time required to develop new products.

Another future innovation in biological pesticides, as previously mentioned, is the possibility for creating pathogens having two different activities in the same strain. Smith, et al (1983) have recently shown that the genome of AcNPV, a baculovirus with insecticidal activity, can be used as a cloning vector into which foreign genes can be inserted and subsequently propagated and expressed in cultured insect cells. In the first experiments the gene they cloned was human β interferon, an anti-viral protein which is being clinically tested for activity on a variety of disease-causing viruses as well as for possible anti-cancer activity. Remarkably, the amount of interferon produced by the insect cell clones was much higher than that produced by E. coli bacterial cells, the most common host for propagation of cloned genes. This virus may be potentially useful in the future for cloning genes for BT toxins, generating a bifunctional pathogen having two distinctly different pathogenic activities directed at the same target insects.

Perhaps the ultimate accomplishment in developing new biological pesticides would involve incorporating their genes directly into the genome of a susceptible organism, thereby conferring the ability to synthesize its own pesticide and totally eliminating the usual delivery system. For example, it may be possible to transfer genes for bacterial insecticide toxins directly into the genetic backgrounds of various plants, enabling them to synthesize their own insecticidal material and bypassing the costly need for repeated sprayings. Genetic manipulations of this type may already be technically possible.

The soil bacterium Agrobacterium tumefaciens harbors Ti plasmids with potential as natural

vectors for the transfer of foreign DNA into plant cells (Chilton, 1983). Upon infection of a susceptible plant the bacterium induces formation of a crown gall tumor. Crown gall tissues synthesize a group of novel metabolites called opines, which are specified by the high molecular weight Ti plasmids in the Agrobacterium strain. Using modified Ti plasmids deleted in many or all of their tumorigenic functions it is now possible to molecularly clone a variety of homologous and heterologous gene functions and to express them in plant cells (Zambryski et al, 1983). Advances in the regeneration of whole plants from callus tissue derived from transformed plant cells promise to greatly expand the spectrum of plant and foreign gene combinations which can be constructed. The introduction of toxin-specifying genes from Bacillus thuringiensis and other microbial pathogens directly into plants may thus be possible in the very near future. Both plant viruses and bacterial vectors are already known which show promise for transporting foreign genes into plant cells. While many technical difficulties still remain to be overcome in accomplishing many of the suggested goals, it seems clear that the pesticide industry is poised on the threshold of a new era in its history.

Literature Cited

Chilton, M.D. A vector for introducing new genes into plants. Scientific American 248:51-59; 1983

Gonzaléz, J.M., Jr., Brown, B.J., Carlton, B.C. Transfer of Bacillus thuringiensis plasmids coding for δ-endotoxin among strains of B. thuringiensis and B. cereus . Proc. Nat. Acad. Sci., USA 79:6951-6955; 1982.

Miller, L.K., Lingg, A.J., Bulla, L.A., Jr. Bacterial, viral, and fungal insecticides. Science 219:715-721; 1983.

Smith, G.E., Summers, M.D., Fraser, M.J. Production of human beta interferon in insect cells infected with a baculovirus expression vector. Mol. Cell Biol. 3:2156-2165; 1983.

Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M., Schell, J. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO Journal 2:2143-2150; 1983.