Chapter 18

Development of Improved Bioinsecticides Based on *Bacillus thuringiensis*

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Research on the genetics and molecular biology of *Bacillus thuringiensis* (BT) during the past 10-12 years has led to a reasonably clear understanding of the genetic organization and diversity of the insecticidal crystal protein (ICP) genes responsible for the bioactivity of BT strains. This research has led to the recognition that ICP genes are primarily localized to extrachromosomal plasmids, that many strains harbor multiple plasmids for these genes, and that certain of these plasmids are self-transmissible between strains of BT by a conjugation-like process. The molecular cloning and characterization of individual ICP genes has further emphasized the diversity of ICP insecticidal activities. This information has provided both the knowledge base and the methodologies for the systematic construction of new strains of BT having improved properties over their naturally-occurring counterparts. Utilizing both non-recombinant and recombinant genetic approaches, new BT-based products have been developed that are not only superior to older products based on naturally-occurring strains but are competing effectively with synthetic chemical insecticides for many applications.

The development of alternatives to synthetic chemical pesticides is an ever-accelerating activity around the world today. Despite the reassurances from the manufacturers and distributors of chemical pesticides as to their safety, the lesson taught by Rachel Carson in her 1962 book, Silent Spring (*1*) is more than ever apparent. The perception of chemical pesticides by the consuming public is that their safety is questionable, and there is an increasing demand for safer alternatives. Among these alternatives are such approaches as organic farming, employment of farming practices that encourage the control of pests through non-intervention, and the use of biological pesticides. Each of these alternatives has its own merits, and this discussion will concentrate on the latter of these approaches. Of all of the biological agents that have been discovered and evaluated as potential pesticides, very few have achieved a significant penetration into the pest control market in a commercial sense. In fact, of the current annual world-wide pesticide expenditures of greater than

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$25 billion, less than $150 million (0.6%) represents non-chemical pest control products.

If biologically-derived products are indeed safer, why then have they received so little acceptance within the pest-control marketplace? There are several reasons for this situation, some obvious and some rather subtle. A major historical problem with biological products has been their lack of consistency in field efficacy as compared to their synthetic chemical competition. Over the last fifty years users of chemical pesticides have come to expect high levels of consistency and efficacy in pesticides, and this has not been a strong feature of biologicals. Another major problem with biologicals has been that many of them, especially those based on complex organisms (e.g., fungi, protozoans, and viruses that are obligate parasites of living cells) could not be produced, packaged, and delivered to the user in a cost-effective way. Third, and perhaps less obvious, is that in contrast to the synthetic chemicals, many of which are derived from less effective naturally-occurring progenitor compounds, biologicals have undergone little or no improvement over the years. For example, one of the first biologicals to be marketed commercially in the U.S., *Bacillus popilliae*, a bacterium that generates milky spore disease in white grubs, is still produced today by the same labor intensive *in vivo* production process as it was when first introduced in the late 1940's. Thus a product that potentially could address a $100 million turf insecticide market remains relegated to a sales volume of less than $50 thousand due to the very limited availability of product. Similarly, the expansion of markets for products based on insect viruses has been held back for the same basic reason- the only commercial method currently available for producing the viruses is *in vivo* within the host insects, thus drastically constraining their productivity and cost-competitiveness.

A fourth limitation in the acceptance of biological alternatives to chemical pesticides has been the perceived (and sometimes real) delayed action of biologicals. When a farmer has a problem pest that is endangering his crop, he wants to apply a pesticide that literally stops the pest "in its tracks." Many biologicals, especially those that function as infective agents of pests, require days or even weeks for their effects to be expressed on the pest organism. Insect viruses, for example, must be disassembled in the infected insect target, and then express a complex sequence of functions that may require many days to be fully expressed. During much of this activity by the virus the host insect does not even recognize that it has been infected and thus goes on feeding normally, and continuing to cause crop damage.

For the reasons stated above, biologicals have for the most part received only minor attention as alternatives to chemical pesticides, primarily by environmentally-conscious growers and for home garden and organic farming activities. Probably the one exception to this lack of attention is the bacterial insecticide *Bacillus thuringiensis*, or BT as it is commonly called. BT products have been sold commercially since the late 1950's, and now exceed $125 million in worldwide sales- increasing at the rate of about 20 per cent annually. Part of this expanded usage has resulted from the introduction of new products for different insect applications. The original BT products marketed in the 1950's, '60's, and '70's were only for caterpillar (Lepidopteran) insect control. Following the discovery in 1977 of a new subspecies named subsp. *israelensis*, an array of new products were developed for control of certain Dipteran insects such as mosquitos and blackflies, many of which are important vectors of human diseases such as malaria, Dengue fever, and river blindness disease.

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More recently, the discovery in 1982 of the subspecies *tenebrionis* strain active on certain beetle (Coleopteran) insects has produced an array of products directed at Colorado potato beetle on potatoes, eggplant, and tomatoes.

These and other developments at the basic research level have contributed to the general popularity of BT as a bioinsecticide, and the remainder of this chapter will focus on this agent. For an earlier summary of this topic please refer to reference (*2*).

To begin this discussion, a few statements are in order about BT and how it acts as a bioinsecticide . BT is a common gram-positive bacterium, closely related to *B. cereus*, and is found in soils, grain dusts, the bodies of dead insects, and on many plant leaf surfaces. Considerable effort has been put into examining the diversity of BT, and it is now recognized that this bacterial species is indeed very diverse. One criterion that has been used extensively to characterize and categorize BT was developed by de Barjac and associates (*3*) at the Pasteur Institute, and involves the immunological characterization of antigens on surface flagellae; appendages that act as locomotion structures for the bacterium. Currently there are nearly 30 subspecies of BT that are recognized based on this criterion. Another approach to assess the diversity of BT in nature has been described by Baumann *et al* (*4*), who evaluated a wide array of biochemical properties of BT's in an attempt to provide additional criteria for classification. As useful as these approaches have been, neither of them has been definitively correlated with insecticidal activity, which at present can only be determined by *in vivo* bioassay, a very time-consuming and labor intensive procedure. Thus, the characterization of BT strains remains complicated by the different criteria which are used. Regardless of which parameters are used to assess diversity however, the basic conclusion that BT is complex is consistent throughout.

As an insecticide, BT acts as a stomach poison to susceptible insects. As the bacterium grows in culture, it undergoes a pseudo-developmental change resulting in the formation of a spore, which encapsulates the essential genetic information of the cell. When this process (sporulation) is complete the "mother cell" dies and disintegrates, releasing the mature spore into the surrounding medium. The major property that distinguishes BT from its close Bacillus relatives is the production, in sporulating cells, of a prominent proteinaceous crystal, termed the parasporal body. This structure contains most of the insecticidal activity of BT, in the form of insecticidal crystal proteins, or ICP's as they are commonly referred to. When eaten by a susceptible insect, the parasporal body is first solubilized in the insect midgut. Then, the crystalline protein which usually, although not always, exists in the form of an inactive protoxin is converted into an active toxin by a proteolytic activation process. The active toxin binds to either specific or non-specific receptors on the insect mid-guts, and an ion channnel imbalance is created which ultimately leads to swelling and bursting of the mid-gut epithelial cells. In some insects, the movement of the spore into the hemolymph leads to a secondary septicemia which contributes to the pathogenic response. Recently there has been a great deal of interest in the nature and specificity of the toxin receptors, particularly with regard to the management of the potential for insects to develop resistance to BT toxins. At the moment what is known about this situation is that there are apparently different receptors for BT toxins in given insects, and that different toxins may bind to the same or different receptors (*5*). The next year or two will undoubtedly reveal many new and important aspects of this situation as ongoing studies on the nature of these receptors are completed and reported in the literature.

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The diversity of BT has also been well-documented with respect to the various types of different toxin proteins that can be expressed in BT, and the fact that many strains of BT are capable of producing multiple toxin proteins. This situation has served to complicate the understanding of the activity diversity of BT, since a given strain may be expressing widely-varying levels of several different ICP's, each with its own unique activity. The cloning and characterization of individual genes, for which some 25 or so have now been reported, has helped to clarify this situation. Two significant conclusions can be drawn from the information obtained. First is that even very closely-related ICP's may have widely different levels of activity on different insects, even those in the same genus. Second, the activity of a specific ICP varies over a range of as much as 1000-fold on different insects. Both of these observations undoubtedly reflect the specificity of the mode of action of the ICP proteins, which at this writing cannot yet be pinpointed to a specific region or site on the ICP protein.

The ICP proteins can be grouped into families that are closely related in actual amino acid sequence and with respect to insecticidal specificity. Based on these criteria a BT toxin classification system was recently proposed by Hofte and Whiteley (*6*) that allows the placement of ICP proteins into the classification system. For example, the Cry I proteins are grouped together from two perspectives; they all are of the 130-145 kd size range, they are all primarily active only on Lepidoptera and they all have at least 50% identity at the level of amino acid sequence homology. Cry II proteins, in contrast, are much smaller (70-75 kd), some (e.g., Cry IIA) have bifunctional activities on Lepidopterans and Dipterans, and their sequence homology to Cry I proteins is very low. Cry III proteins are those having Coleopteran insect activity, are very similar in size to the Cry II proteins, and have greater inter-group sequence homology than with proteins in other groups. While there will undoubtedly arise exceptions to the clear assignment of toxin proteins to specific groups, particularly as more and more different insecticidal activities are discovered, the system has nevertheless provided a useful beginning to separate the various proteins in a systematic way.

As both genetic and biochemical techniques have become more sophisticated in recent years, the complexity of naturally-occurring BT strains has been clearly documented. From the earlier indications of Krywienczyk and Angus (*7*) that BT crystals contained different antigenic determinants, it is now very clear that a single strain may contain multiple toxin proteins, either of the same family or of different types. Analyses of purified crystals by techniques such as gel electrophoresis have confirmed that a bipyramidal crystal may be composed of different but related toxin proteins. It has also been shown that a single strain may produce more than one crystal type, as exemplified by the highly-studied strain HD-1, the basis for most existing caterpillar control products. This strain produces a large bipyramidal crystal comprised of three Cry I proteins, and a smaller, cuboidal crystal containing a Cry II protein (reviewed in *2*). Through a combination of protein and genetic analyses, using gene probes to identify various toxin genes, it has been well-documented that many BT isolates are multi-genic for these insecticidal proteins, and in some instances may contain as many as six identifiable genes. What is less-well understood at present is how these multi-genic situations are regulated with respect to the individual gene products. While it is evident that the protein products of closely-related genes are expressed to different levels and that transcriptional regulation plays an important role in differential gene expression (*8*), it is not yet clear whether translational or post-translational

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factors may also play an important role in the ultimate levels of the various proteins expressed by a given strain.

Over the past several years much effort has been expended in developing an understanding of the genetic organization of BT toxin systems. One of the major conclusions from these efforts is that the genes for these proteins are located, for the most part, not on the essential chromosome but instead on an array of non-essential extrachromosomal plasmids (see reference (*9*) for review). Different strains of BT may harbor anywhere from one or two such plasmids to as many as 12-14 different species. A given strain of BT may contain several such plasmids harboring up to five or six different insecticidal toxin genes, including some localized on the same plasmid. The second important conclusion from BT genetic studies is that many of the toxin-encoding plasmids are capable of self-transmission from one cell to another by a naturally-occurring process called conjugation (*10*). This is a type of sexual gene transfer previously described in great detail for *E. coli* and other bacteria and for which Joshua Lederberg shared the Nobel Prize in Physiology or Medicine. In BT, this process has been observed to permit reciprocal transfer of toxin-encoding plasmids at high efficiency (up to 75%) in matings between different parental strains.

A second property of significance to BT toxin plasmids is their ability to be spontaneously lost ("cured") from cells that harbor them. Variants can be readily recovered that have lost one or more of their plasmid complement, and if the cured plasmid contains an insecticidal toxin gene the resulting mutant strain becomes crystal-negative (unless it harbored another toxin-encoding plasmid). Regardless, there are molecular techniques that can readily distinguish such cured variants. If derivatives are identified that have been cured of toxin-encoding plasmids that contribute relatively little to the activity against a particular insect target, the typical result is that the remaining toxin gene products are expressed in relatively higher amounts. This is because there is an apparent built-in dosage compensation effect that allows the production of a certain level of toxin protein, within some as yet undefined range of ICP genes. Although this regulatory system is not yet well understood, the data from various mutant strains are sufficiently convincing that this conclusion appears valid.

Thus, one way of improving a naturally-occurring BT strain is to isolate cured variants that have lost plasmids encoding inactive or poorly-active toxins. A variation of this approach that exploits the natural transmissibility of BT toxin plasmids involves the mating of a partially-cured variant of an otherwise desirable strain with a second strain having a toxin plasmid with some desired property. The resulting transconjugants will typically yield some derivatives which have acquired the desired toxin plasmid from the donor strain, effectively replacing the undesired or poor activity with a better activity. Depending upon how many toxin plasmids were originally present, this first transconjugant may be mated with still another plasmid donor strain to transfer in another toxin plasmid. This process allows the accumulation, in a single strain background, of an array of BT toxin plasmids derived from several different parental origins. By the selective choice of parental donor strains possessing either more potent insecticidal activities, or different insecticidal activities, new strains can be constructed that are more active or that have broader target insect spectra. In addition, the properties of such genetically-derived strains provide the basis for the filing of patents to provide a means of proprietary protection.

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Development of Improved BT Products by Non-Recombinant Approaches

Based upon the foregoing considerations Ecogen Inc. set about several years ago on a BT improvement program. This program was designed with several integrated components to ensure the most rapid development of new BT-based products.

First was an aggressive new strain discovery program to expand its library of BT insecticidal activities. Samples were collected from all over the world and carefully examined to identify new and different strains of BT, using a variety of criteria such as plasmid arrays, insecticidal activities, toxin gene profiles, etc. Through these activities we have amassed a collection of over 8000 novel BT isolates having a broad array of characteristics. From the strains identified as having desired characteristics, a strain development program was instituted to utilize the procedures of plasmid curing and conjugal transfer described previously to generate new gene combinations directed at specific crop and insect applications. From many hundreds of such constructs a small number of candidate strains was identified with the desired levels of activity and/or insecticidal spectrum. These strains were subjected to an intensive evaluation program, including fermentation process behavior, greenhouse performance in on-plant assays, formulation behavior, and field performance. From among those having acceptable properties were selected three strains of different genetic compositions for development as new products. One of these products, Condor® bioinsecticide, was specifically designed for improved performance on soybean caterpillars, cotton caterpillars (the so-called *Heliothis* complex), and caterpillars that attack forest trees (gypsy moths and spruce budworms). Condor also has excellent performance against an important vegetable insect pest, the diamondback moth. A second product, Cutlass® bioinsecticide, was developed for improved efficacy against a broad array of vegetable crop pests, including cabbage loopers, beet armyworms, webworms, imported cabbageworms, and others. Although the BT strains contained in the two products are genetically distinct, the nature of the gene compositions in the two strains provides for significant overlap in their insecticidal activities. The third product, Foil® bioinsecticide is very different in its activity spectrum, however, since it combines in a single strain activities against both beetle and caterpillar insects. This is a truly novel construct that we have not observed in natural BT isolates, and is the only product of its type that has ever been commercialized to our knowledge. Foil® was developed to control a complex of beetle and caterpillar pests on crops such as potato, tomato, and eggplant. U.S. patents have been received on all three of these strains and are pending in foreign countries. Condor®, Cutlass®, and Foil® are all fully-registered for sale in the United States, and registrations are currently being pursued in other countries. It is important to note in the context of the registration of BT products that the U.S. Environmental Protection Agency, which is responsible for the regulatory oversight and approval for sale of all pesticide products in the U.S., has determined that genetically-altered strains of BT generated by plasmid curing or conjugal transfer are considered the same as naturally-occurring strains with respect to risk assessment and environmental impact considerations. Obtaining registrations for new BT products developed by these techniques has thus been greatly facilitated. The registrations for Condor®, Cutlass® and Foil® were received following only a few months in the review process.

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Development of Improved BT Products By Recombinant Approaches

Despite the advances in BT strain development that have been possible through the non-recombinant methods described, there are significant limitations to the genetic improvements that can be made with these approaches. For example, the conjugal transfer approach requires that the gene to be transferred be located on a transmissible plasmid. Although many BT plasmids are capable of transfer, either on their own or through mobilization by other plasmids, some are very difficult to move from one cell to another or do not transfer at all. A second limitation of the non-recombinant approaches is a consequence of the frequent localization of two or more insecticidal genes on the same plasmid. If one of these is a desired gene and the other encodes a poorly-active protein, the curing or conjugal transfer techniques only allows moving the two genes together. Thus, for greater flexibility in new strain construction it is highly desired that techniques be available to transfer any gene from any donor source into any desired recipient. Such a technique is obvious through the use of recombinant DNA, or enzyme-mediated gene splicing.

In choosing a strategy for BT product improvement utilizing recombinant DNA techniques, we elected to utilize, as the expression and production system, the BT bacterium itself, for several reasons. First is that BT is already a very efficient production system. BT cells synthesize typically greater than 30 per cent of their cell mass in the form of the insecticidal crystal proteins, and have clearly evolved very efficient expression systems for these proteins. Second, was the objective of developing products containing multiple insecticidal activities, much as naturally-occurring BT strains typically have. Since BT is known to maintain in a genetically-stable way such multigenic situations, we believe that the choice of BT as the host organism will minimize stability problems in the future.

The objective of multigenic constructs has three significant advantages. First is that these constructs allow one to take advantage of the potency synergies that frequently occur between different insecticidal proteins. In addition, the use of multigenic strains having activities with different receptor binding properties or other aspects of their mode of action helps to minimize the potential for the target insects to develop resistance to the products. Lastly, the choice of BT as an expression host for the generation of new insecticidal gene combinations we believe will create less of a regulatory concern than if these activities were expressed in a heterologous host.

For a cloning vector, we chose to isolate replication origins from native BT plasmids. This choice was based on two considerations. First was to maintain an all-BT nature of the recombinant constructs. Second was that we reasoned that native BT plasmids, particularly those that naturally harbor insecticidal toxin genes, should be stable when such genes are spliced onto them. From these considerations a number of replication/maintenance fragments were initially cloned from a BT strain of subspecies *kurstaki*. It turned out in subsequent analyses that these replication fragments had a number of very interesting properties. First, of the three replication origins derived from a single BT parent, all had extremely different nucleotide sequences (which presumably explains why they could naturally co-exist within the same cell). Second, although two of these origins were derived from plasmids capable of high efficiency transfer by conjugation, the replication fragments derived from them had drastically reduced transfer efficiencies, either by themselves or in the presence of mobilizing plasmids. Third, when many different BT strains were probed with these replication origins it was

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observed that they were ubiquitous, in that one or more were found to exist in many of the strains examined. For each of these replication origins we constructed shuttle cloning vectors by adding an *E. coli* replicon, a Gram-positive antibiotic selection marker that is expressed in BT, and a small synthetic fragment of DNA containing a number of unique restriction enzyme cleavage sites that serves as a multi-insertion site for a variety of different ICP genes. These vectors are uniquely constructed such that either the *E. coli* DNA or the antibiotic resistance marker, or both, can be conveniently removed by cleavage of the shuttle vector with specific restriction enzymes whose sites are not present within the BT replication fragment or the ICP genes to be cloned. Thus, with this cloning system, initial cloning and characterization of ICP genes can be done in *E. coli*, which has a number of important advantages. The desired clones can then be transferred to BT, in which their stability can be examined both in the presence and absence of the selected antibiotic. Ultimately, for a final product the recombinant plasmid can be finally modified to remove all of the foreign DNA, leaving only the BT replication origin, the cloned ICP gene, and the small amount of synthetic DNA from the multi-cloning site (see reference *(11)* for a review of this cloning and expression system).

Utilizing this system, we have constructed recombinant strains with improved potencies and increased fermentation productivities. One of the prime examples of this approach is illustrated by a derivative of our current Foil® product that was field-tested both in 1991 and in 1992. The original Foil® product contains a strain that, by the conjugal transfer system, has two copies of a caterpillar-active gene and one copy of a beetle-active gene. During fermentation, the strain produces roughly two-thirds of the insecticidal toxin of caterpillar type and one-third of the beetle toxin. We inserted into this strain a recombinant plasmid containing a new beetle-active toxin gene that we isolated on one of our proprietary plasmid vectors. Due to the fact that the original Foil strain carried a toxin-encoding plasmid having the same replication origin as that of the recombinant plasmid, transformants were recovered in which one of the native plasmids containing a caterpillar-active toxin gene was replaced by the related replication origin of the recombinant plasmid containing the novel beetle-active gene. Thus the recombinant derivative now contains two different beetle-active genes, and only one caterpillar-active gene. During fermentation this switching of gene ratios is basically reflected in a similar reversal of the relative amounts of production of the beetle-active and caterpillar-active proteins. This new recombinant (EG7618) was field-evaluated in 1991 at two locations and showed excellent performance in control of Colorado potato beetle (caterpillar pest control was not evaluated in these trials). More extensive field trials are being conducted as this is being written. The importance of this recombinant strain is two-fold. First, by changing the gene ratios of the original Foil® strain we have essentially achieved a two-fold increase in the production of the beetle-active protein per volume of fermentation broth. Second, since the added beetle-active gene is different than the one originally present in Foil®, it has certain important differences in the activity of its ICP toxin on young adult larvae of Colorado potato beetle, thus offering a potentially important advantage over similar products that lack adulticide activity (see reference *(12)* for a more complete description of this strain).

Similar constructs utilizing a variety of new gene combinations are currently under evaluation against other important target insects. It is our expectation that the additional advantages of this BT-based plasmid vector system, employed in concert with our strain discovery program, will allow us to

make significant and dramatic improvements in BT-based insecticide products within the very near future, and we are optimistic in obtaining permission to field-test such strains and to register them for commercial production. It is our firm belief that the future is extremely promising for a broad new array of BT-based insect control products, that will not only be highly effective but will be both cost-competitive with current chemical insecticides and much less likely to cause problems with resistance.

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