

Overview of the Basic Biology of *Bacillus thuringiensis* with Emphasis on Genetic Engineering of Bacterial Larvicides for Mosquito Control

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Abstract: The insecticidal bacterium, *Bacillus thuringiensis*, consists of a wide variety of subspecies, most of which are insecticidal for either lepidopteran, coleopteran, or dipteran insect larvae. Subspecies such as *B. thuringiensis* subsp. *kurstaki* have been used with remarkable safety for more than forty years to control lepidopteran pests in agriculture and forestry, and over the past thirty years, *B. thuringiensis* subsp. *israelensis*, has proven to be a safe and effective larvicide for controlling mosquito and black fly larvae. Studies of the basic biology of *B. thuringiensis* have shown that it produces a variety of insecticidal proteins produced during vegetative growth and sporulation that determines its activity for insect species belonging to different orders, with the most important of these being the Cry proteins active against lepidopteran and coleopteran pests, and a combination of Cry and Cyt proteins for mosquitoes and blackflies. After intoxication by these proteins, spores typically germinate and invade larvae, contributing to insect mortality. Whereas strains of many wild type isolates have been commercialized and are now used worldwide, the use of recombinant DNA techniques, i.e., genetic engineering, has been used over the past decade to recombine the proteins of different *B. thuringiensis* strains with those of *B. sphaericus* to generate recombinant larvicides as much as ten-fold more toxic than the parental strains. In this chapter, we begin with a general overview of the basic biology of *B. thuringiensis* and *B. sphaericus*, then show how studies of its molecular genetics combined with recombinant DNA techniques have been used to generate highly improved bacterial larvicides for control of nuisance and vector mosquitoes.

Keywords: Insecticidal proteins, insect-pathogenic bacteria, mosquitocidal bacteria, mosquitocidal proteins, bacterial endotoxins, synergism, cry proteins, Cyt proteins.

INTRODUCTION

The species that we recognize today as *Bacillus thuringiensis* Berliner was originally discovered in Japan over a century ago by Shigetane Ishiwata [1] as the cause of the sudden (“sotto”) death disease of silkworms, larvae of the silkworm moth, *Bombyx mori*. A little over a decade after Ishiwata’s discovery, the German bacteriologist Ernst Berliner [2], unaware of Ishiwata’s paper, described a similar bacterium as the cause of disease in larvae of the flour moth, *Ephestia kuehniella*. The species name “*thuringiensis*” is derived from Thuringia, the German state where the diseased flour moth larvae were found. In his description, Berliner noted the presence of parasporal inclusions, as did several subsequent researchers, but their role in disease remained unknown until the 1950’s (Fig. 1). Even though little was understood about the basic biology of *B. thuringiensis* in the 1930’s, it was shown to be highly pathogenic for larvae of certain species of lepidopterous pests. As synthetic chemical insecticides had not yet been developed, a preparation of sporulated *B. thuringiensis* cells known as “Sporeine” was used in France for insect control just prior to the

outbreak of World War II. There was little interest in using *B. thuringiensis* as an insecticide during WWII, however, after the war Edward Steinhaus [3] of the University of California began the modern era of research on this species showing that it had potential for controlling the alfalfa caterpillar, *Colias eurytheme*. His studies were followed soon thereafter by others which demonstrated that the bipyramidal parasporal body produced by *B. thuringiensis* was responsible for the rapid death of caterpillars [4, 5].

Fermentation studies during the 1950’s determined that large-scale culture of *B. thuringiensis* was possible at relatively low cost, accelerating its development as an insecticide. After many failures, an isolate known as HD1 of *B. thuringiensis* subsp. *kurstaki* (Fig. 2) was cultured by Howard Dulmage of the USDA and shown to have broad spectrum of activity against lepidopterous pests, while being safe for most non-target invertebrates including bees and beneficial predatory and parasitic insects, as well as for humans and other vertebrates [6, 7]. By the early 1970’s, commercial formulations of *B. thuringiensis* subsp. *kurstaki* (HD1) with names such as Dipel and Thuricide were in use to control many lepidopterous pests including the cabbage looper (*Trichoplusia ni*), corn earworm (*Helicoverpa zea*), and tobacco budworm (*Heliothis virescens*) in vegetable and field crops, and major forest pests, mainly the gypsy moth

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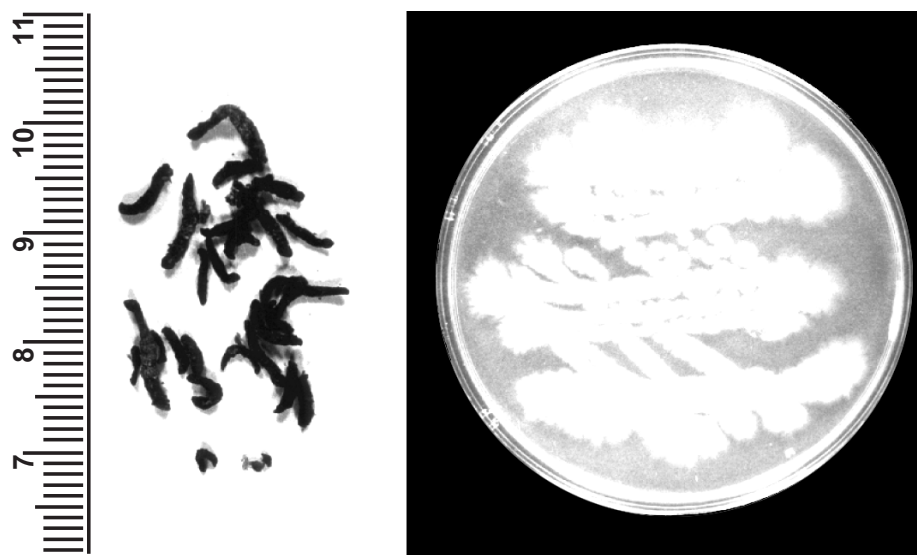


Fig. (1). Natural epizootic of *Bacillus thuringiensis* in a colony of the navel orangeworm, *Amyelois transitella* (family Pyralidae). On the left, larvae killed and colonized by a strain of *B. thuringiensis* subsp. *aizawai* (H 7). On the right, a virtually pure culture of *B. thuringiensis* subsp. *aizawai* isolated from a small piece of a dead larva and cultured on nutrient agar. The most productive ecological niche for most isolates of *B. thuringiensis* appears to be lepidopteran larvae.

(*Lymantria dispar*) and spruce budworm (*Choristoneura fumiferana*). During the late 1970's and early 1980's, years after its initial commercial success, the application of new molecular biological techniques to research on *B. thuringiensis* demonstrated that HD1's broad spectrum of insecticidal activity was due to its complex parasporal body, which was shown to consist of two crystal types (Fig. 2B) that together contain four proteins, Cry1Aa, Cry1Ab, Cry1Ac in a bipyramidal crystal, and Cry2Aa in a cuboidal or "cushion-shaped" crystal, each protein having a slightly different lepidopteran target-spectrum and specific activity.

By the mid-1970's, thirteen subspecies of *B. thuringiensis* had been described based on an analysis of hundreds of isolates. None of these had any significant insecticidal activity to insect species outside the order Lepidoptera. Then in 1976, Goldberg and Margalit [8] discovered a new subspecies, subsequently named *B. thuringiensis* subsp. *israelensis*, in the Negev desert of Israel, which proved highly toxic to larvae of a wide range of mosquito species. This subspecies was subsequently shown to also be insecticidal for larvae of other species of flies in the dipteran suborder Nematocera, including blackflies and chironomid midges. The parasporal body of this subspecies is spherical and composed of four major proteins, Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa, and thus considerably different from the bipyramidal crystals produced by subspecies toxic to lepidopteran larvae (Fig. 2C). Although their parasporal bodies differ, comparative studies of *B. thuringiensis* subsp. *kurstaki* and *israelensis* showed that strong sporulation-dependent promoters accounted for the large amount of insecticidal protein produced during sporulation.

The broad spectrum of activity of *B. thuringiensis* subsp. *israelensis* against biting flies led to its rapid commercialization, and products such as VectoBac, Bactimos, and

Teknar for control of nuisance and vector mosquitoes and black flies. Owing to their high efficacy and narrow target spectrum, these products replaced many broad-spectrum chemical insecticides used for mosquito and black fly control in developed countries, and are currently under development for control of the major anopheline vectors of malaria in Africa, Asia, and South America. Moreover, products based on *B. thuringiensis* subsp. *israelensis* proved of particular importance in the Onchocerciasis Control Program in West Africa, which significantly reduced onchocerciasis, commonly known as "River Blindness", a debilitating human eye disease caused by *Onchocerca volvulus*, a nematode trans-mitted by blackflies of the *Simulium damnosum* complex [9].

Not long after the discovery of *B. thuringiensis* subsp. *israelensis*, a third pathotype highly toxic to larvae and adults of coleopterous insects, i.e., beetles, was discovered in Germany [10]. This isolate, which produces a thin rhomboidal parasporal crystal composed of Cry3A, was originally named *B. thuringiensis* subsp. *tenebrionis*, but is known formally as *B. thuringiensis* subsp. *morrisoni*, strain *tenebrionis*. Due to its toxicity to certain important coleopteran pests, such as the Colorado potato beetle (*Leptinotarsa decemlineata*), this isolate was developed as a bacterial insecticide for control of beetle pests. Unlike *B. thuringiensis* subsp. *kurstaki* and *israelensis*, however, the efficacy of commercial products based on the original and similar isolates of *B. thuringiensis* toxic to beetles was not as effective as new chemical insecticides, such as the neonicotinoids, and thus these have not been commercially successful.

Insecticides based on the above *B. thuringiensis* isolates are the most successful of the various bacterial, fungal, viral, and other pathogens developed as insecticides, with current worldwide annual sales estimated to be \$100 million.

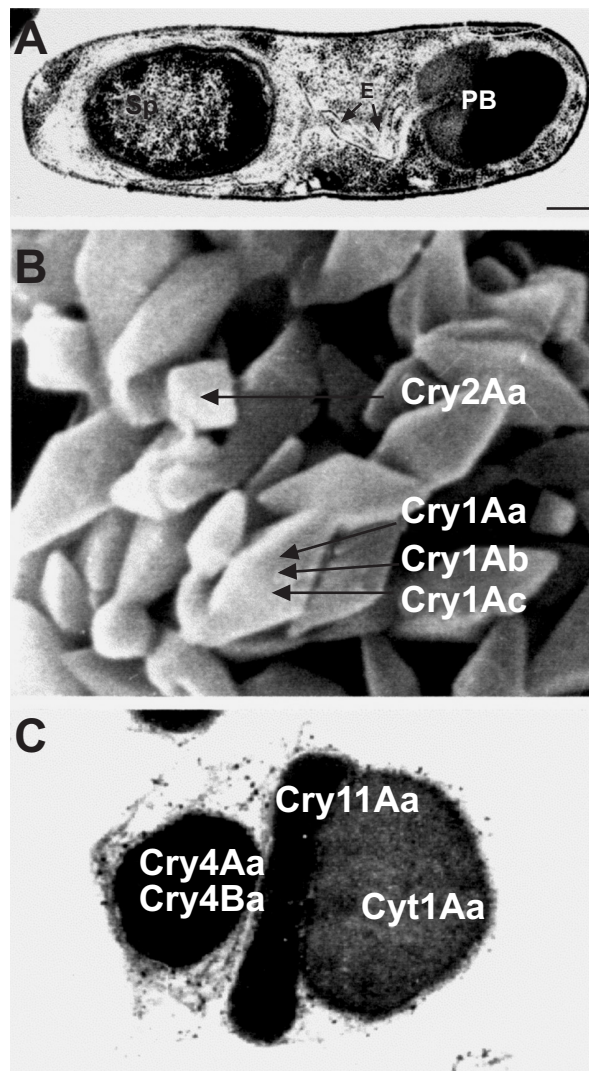


Fig. (2). Sporulation of *Bacillus thuringiensis* and associated production of parasporal bodies containing crystals of insecticidal proteins. **A**, Sporulating cell of *B. thuringiensis* subsp. *israelensis*. **B**, Scanning electron micrograph of purified crystals produced by the HD1 isolate of *B. thuringiensis* subsp. *kurstaki*. **C**, Transmission electron micrograph of the parasporal body of *B. thuringiensis* subsp. *israelensis*. The arrows identify the Cry proteins found in the insecticidal crystals. Those in B are toxic to lepidopteran larvae, whereas those in C are toxic to dipteran larvae of the suborder Nematocera.

Although successful, the most significant scientific, technological, environmental, and commercial success resulting from research on the parasporal crystals of *B. thuringiensis* is the development of insecticidal “Bt” transgenic crops based on Cry proteins. These crops, primarily Bt cotton (based on Cry1Ac) and Bt maize (based on Cry1Ab or this and related proteins combined with Cry3 proteins for root worm control), first released for commercial use in the United States in 1996, and later in countries such as Australia, Argentina, China and India, have annual revenues in the range of \$5-6 billion. Environmental benefits are derived from the insecticidal specificity of these crops, which unlike synthetic chemical insecticides, kill only target species and closely related insects, as well as from

reductions in usage of the latter chemicals [11], the reductions of which now amount to millions of kg annually in the U.S. and other countries. In the U.S., approximately 80% of the maize and cotton are now Bt crops. Moreover, insecticidal transgenic crops are expected to increase in sales worldwide at a rate of 10-20% per year as new varieties of Bt crops are developed [12]. Use of these crops remains controversial in many countries, especially in Europe, and while this chapter is not the place to discuss this new pest control technology, it should be realized that Bt as well as other types of transgenic crops originated with the study of the crystal proteins produced by *B. thuringiensis*.

BASIC BIOLOGY OF *BACILLUS THURINGIENSIS*

As noted above, *B. thuringiensis* is a common Gram-positive, spore-forming aerobic bacterium that can be readily isolated on simple media such as nutrient agar from a variety of environmental sources including soil, water, plant surfaces, grain dust, dead insects, and insect feces [13]. Its life cycle is simple. When nutrients and environmental conditions are sufficient for growth, the spore germinates producing a vegetative cell that grows and reproduces by binary fission. Cells continue to multiply until one or more nutrients, such as sugars, amino acids, or oxygen, become insufficient for continued vegetative growth. Under these conditions, the bacterium sporulates producing a spore and parasporal body, the latter, as noted above, composed primarily of one or more insecticidal proteins in the form of crystalline inclusions (Fig. 2). These are commonly referred to in the literature as insecticidal crystal proteins or endotoxins (formally, δ -endotoxins), and can compose as much as 40% of the dry weight of a sporulated culture. The proteins are actually protoxins that must be activated by proteolytic cleavage to be toxic, discussed in more detail later. There are two types of insecticidal crystal proteins, Cry (for crystal) and Cyt (for cytolytic) proteins, and variations on each of these types. Genes encoding more than 150 Cry proteins and 12 Cyt proteins have been cloned and sequenced. Most Cry proteins are active against lepidopteran insects, with a few being toxic to dipteran (flies) or coleopteran (beetles) insects, or nematodes. Cyt proteins are only moderately toxic to mosquito and black fly larvae, and a few beetle species, and occur typically in what are referred to as mosquitocidal subspecies, such as *B. thuringiensis* subsp. *israelensis*.

Systematics of *Bacillus thuringiensis* and its Insecticidal Proteins

The insecticidal crystals formed by Cry and Cyt proteins are the principal characteristic that differentiates *B. thuringiensis* from *B. cereus* as well as other species of the *B. cereus* group. As far as is known, most, if not all Cry and Cyt proteins are encoded on plasmids present in Bt, i.e., not on the bacterial chromosome. Thus, if these plasmids are lost from a strain, or deliberately eliminated by plasmid curing, the resulting strain would be identified as *B. cereus*. Several earlier as well as recent studies of the phenotypic and genomic properties of *B. thuringiensis* and *B. cereus* provide strong evidence that the former is essentially the latter species bearing plasmids encoding endotoxins [14-16]. Despite this, *B. thuringiensis* presently remains considered a valid species due to a combination of tradition and practical

value, and this is unlikely to change, at least in the near future. In some studies, it has been suggested that *B. cereus*, *B. thuringiensis*, and *B. anthracis* are all members of the same species [17]. Whereas there is ample evidence that *B. cereus* and *B. thuringiensis* are members of the same species, the idea that *B. anthracis* is a member of this same species is not supported by the evidence. Among other features, although it has been shown that Bt plasmids can be transmitted to and replicate in *B. cereus*, the two plasmids that encode the toxins of *B. anthracis* do not occur naturally in Bt or *B. cereus*, nor have parasporal bodies containing Bt Cry proteins been found naturally in *B. anthracis*. This implies that there are probably natural barriers, currently not understood, to plasmid mobilization and transmission that exist among these species, and probably “cross-talk” between their different toxin-encoding plasmids and chromosomal genes of their normal host species, that control toxin production. At present, this supports maintaining *B. anthracis* as a species different from *B. cereus* and *B. thuringiensis*.

As a species, Bt is subdivided into more than 70 subspecies, which are not based on insecticidal protein complements or target spectrum, but rather on the antigenic properties of the flagellar (H) antigen [18]. Each new isolate that bears a flagellar antigen type that differs detectably from the others in immunological assays is assigned a new H antigen serovariety number and subspecific name. Thus, for example, of those used commonly in bacterial insecticides, there are four main subspecies (Table 1): *Bacillus thuringiensis* subsp. *kurstaki* (H 3a3b3c) and *B. thuringiensis* subsp. *aizawai* (H 7) used against lepidopteran pests; *B. thuringiensis* subsp. *israelensis* (H 14) used against mosquitoes and blackfly larvae; and *B. thuringiensis* subsp. *morrisoni* strain *tenebrionis* (H 8a8b), used against certain coleopteran pests, such as the Colorado potato beetle, *Leptinotarsa decemlineata*. Target spectrum is frequently correlated with flagellar serovariety (also referred to as serotype). However, the correlation is far from absolute because this identification is not based on insecticidal protein complements, which can vary markedly, even within the same subspecies/serovariety. For example, within the subspecies/serovariety *B. thuringiensis* subsp. *morrisoni* (H 8a8b), isolates exist that are toxic to lepidopteran, dipteran,

or coleopteran larvae. Because the plasmid complements, and therefore the insecticidal protein complements, can vary within a subspecies/serovariety, isolates that have distinctive target spectra and/or toxicity are typically given specific designations. The most widely used Bt isolate in agriculture and forestry, for example, is the HD1 isolate of *B. thuringiensis* subsp. *kurstaki* (H 3a3b3c), which is toxic to many different important lepidopteran pests of field and vegetable crops, as well as many forest pests. This isolate, the active ingredient of commercial products such as DiPel and Foray 48B, produces four major endotoxin proteins, Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa, which together account for its broad target spectrum. Interestingly, this isolate has served as the genetic source of the Cry proteins used most extensively in Bt crops to control lepidopteran pests, specifically, Cry1Ac used in Bt cotton, and Cry1Ab used in certain types of Bt maize. However, there are numerous other isolates of this subspecies that produce fewer Cry proteins, for example, HD73, which has a plasmid complement that only produces a single Cry protein, Cry1Ac. As a result, HD73 has a very limited target spectrum. Alternatively, the ONR 60A isolate of *B. thuringiensis* subsp. *israelensis* and PG14 isolate of *B. thuringiensis* subsp. *morrisoni*, both bear a large 128 kb plasmid (pBtoxis) that encodes a different set of insecticidal proteins, namely Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa, responsible for the mosquitocidal activity of these isolates [19]. Regardless of the subspecies/serovariety, the only way to be certain of the target spectrum of a new isolate is to conduct bioassays against a range of insect species, and combine this information with the cloning, sequencing, and analysis of genes encoding the insecticidal proteins. In general, each subspecies/serovariety has the capability of encoding a range of Cry genes, and, correspondingly, many of these genes occur in different subspecies/serovarieties.

This brief background demonstrates how the insecticidal protein complexity can vary within and among various isolates and subspecies of *B. thuringiensis*. Suffice it to say that there is enormous variation among the plasmids and insecticidal protein complements that occur among the collections of Bt isolates, now estimated to be about 100,000, grouped together under the more than 70 subspecies of *B. thuringiensis*. As noted above, more than

Table 1. Important Subspecies of *Bacillus thuringiensis* Used in Bacterial Insecticides

Subspecies/ Serovariety ^a	H-Antigen	Major Endotoxin Proteins (Mass in kDa)	Insect Spectrum (Target Group)
<i>kurstaki</i>	3a3b3c	Cry1Aa (133), Cry1Ab (131)* Cry1Ac (133)*, Cry2Aa (72) ^c	Lepidoptera
<i>aizawai</i>	7	Cry1Aa (133), Cry1Ab (131) Cry1Ca (135), Cry1Da (133)	Lepidoptera
<i>morrisoni</i> ^b	8a8b	Cry3Aa, Cry3Bb (73)*	Coleoptera
<i>israelensis</i>	14	Cry4Aa (134), Cry4Ab (128) Cry11Aa (72), Cyt1Aa (27)	Diptera ^d

^aFrom [18].

^bStrain *tenebrionis*, commonly referred to as *B. t.* subsp. *tenebrionis* (or previously, *san diego*).

^cAlso toxic to larvae of nematoceran dipterans (e.g., mosquitoes and blackflies).

^dOnly toxic to species of the dipteran suborder Nematocera (e.g., mosquitoes and blackflies).

*Used to construct insect-resistant transgenic crops.

150 different types of genes encoding Cry proteins, and at least 12 different types of genes encoding Cyt proteins have been cloned and sequenced.

As a group, the Cry protein family contains considerable diversity, enabling Bt strains to kill different hosts under appropriate conditions (Table 2). Most Cry proteins are of the Cry1 type, a class of molecules in which the overwhelming majority are toxic to lepidopteran insects [20, 21]. These molecules are typically in the range of 133-150 kDa in mass. Cry2 molecules, depending on the specific protein, are also toxic to lepidopterans, but some, such as Cry2Aa, are toxic to both lepidopterans and dipterans (mosquito larvae, in this case). Cry2 molecules are generally about half the mass, i.e., 65 kDa, of Cry1 proteins, and in essence are naturally truncated molecules consisting of the N-terminal half of the latter (the portion of the molecule that contains the active protein). Cry3 proteins are similar in mass to Cry2 proteins, but they are only insecticidal to coleopteran insects. The other major Cry type used in bacterial insecticides, the Cry4 proteins, are, like Cry1 molecules, in the 135 kDa range, but are toxic to nematoceran dipterans, the suborder that contains the mosquitoes and black flies. Phylogenetic studies indicate that all of the above Cry types evolved over millions of years from the same ancestral molecule, the diversity in host spectra being selected for when mutant strains wound up in the midguts of insect species belonging to different orders.

Although each type of Cry protein has a limited target spectrum – typically lepidopteran, dipteran, or coleopteran insects (or nematodes) – the target spectrum of a specific protein, for example, Cry1Ac, is always much narrower than the type as a whole. In addition to the spectrum, the toxicity of each Cry protein within a type can vary significantly from one insect species, even in cases where insect species are closely related (Table 2). For example, two different lepidopteran species of the family Noctuidae can differ markedly in their sensitivity to Cry1Ac, from being highly sensitive (*Heliothis virescens*) to being essentially insensitive (*Spodoptera exigua*). It is for this reason that different Cry

proteins are used in different Bt crops for insect resistance management, i.e., to provide a high level of control for different insect pest species, or that two different Cry proteins would be used in the same crop to control different pest species. Examples of the latter are new maize varieties that produce both Cry1A proteins for control of lepidopteran larvae, and Cry3 proteins for control of corn rootworms, which are coleopteran insects.

During the past decade, the number of Cry protein types has expanded dramatically as a result of the search for new proteins with novel target spectra. The current list of Cry proteins includes more than 50 different holotypes (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/), Cry1 through Cry 50, most, but not all of which are related phylogenetically, i.e., appear to have evolved from the same ancestral molecule. In addition to Cry protein types, there are nine holotypes of Cyt proteins. These proteins have a mass in the range of 26-28 kDa and are phylogenetically unrelated to Cry proteins [20, 21], i.e., they share no significant degree of amino acid identity/similarity, and have a spectrum of activity limited to certain dipteran and coleopteran species. Data on the toxicity of the most important Cry and Cyt proteins can be found at <http://www.glf.cfs.nrcan.gc.ca/bacillus>, a website maintained by the Canadian Forest Service.

Toxicity and Mode of Action

Knowing the precise complement of insecticidal proteins produced by a specific isolate of *B. thuringiensis* can go a long way to explaining its toxicity and lethality to a particular insect or nematode species. However, several Bt components other than endotoxins contribute to the activity of a particular isolate against a specific insect species (Table 3). Owing to the overwhelming interest in Cry proteins, most of these other factors have received relatively little attention. Among the most important of these are the spore, β -exotoxin, antibiotics such as zwittermicin, vegetative insecticidal proteins (Vip's), phospholipases, chitinases, and various proteases. In some target insects, Cry proteins alone are sufficient to intoxicate larvae by destroying enough

Table 2. Toxicity of *Bacillus thuringiensis* Cry Proteins to First Instars of Various Pest Insect Species^a

Cry Protein ^d	LC ₅₀ in ng/cm ² of Diet or Water ^{b,c}				
	Tobacco Hornworm	Tobacco Budworm	Cotton Leafworm	Yellow Fever Mosquito	Colorado Potato Beetle
Cry1Aa	5.2	90	> 1,350	> 5,000	> 5,000
Cry1Ab	8.6	10	> 1,350	> 5,000	> 5,000
Cry1Ac	5.3	1.6	> 1,350	> 5,000	> 5,000
Cry1Ca	> 128	> 256	104	> 5,000	> 5,000
Cry11Aa	> 5,000	> 5,000	> 5,000	60	> 5,000
Cry3Aa	>5,000	>5,000	>5,000	>5,000	<200

^aTobacco hornworm (*Manduca sexta*), Tobacco budworm (*Heliothis virescens*), Cotton Leafworm (*Spodoptera littoralis*), Yellow Fever Mosquito (*Aedes aegypti*), Colorado Potato Beetle (*Leptinotarsa decimlineata*). Modified from [21].

^bValues of > 5,000 indicate a lack of toxicity at high doses, doses equivalent to field applications rates that would not be economical. Lack of toxicity at these rates illustrates the high degree of insect specificity characteristic of Cry proteins.

^cFor insecticidal activity of other Cry proteins see: www.glf.cfs.nrcan.gc.ca/bacillus

^dFor updates of Cry taxonomy see: www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/

Table 3. Insecticidal Components Produced by *Bacillus thuringiensis*

Cry Proteins	β -Exotoxin
Cyt proteins	Zwittermicin
Spores	Phospholipases
Vegetative insecticidal proteins (Vips)	Chitinases

midgut epithelial cells to allow the alkaline midgut juices to flow into the hemolymph and raise the blood pH, which causes paralysis and cessation of feeding [22]. This is typically followed by death in a few days due to either the toxicity of the insecticidal protein(s) alone, as in the case of mosquitoes and black flies, or a combination of these and infection and colonization of the larva by *B. thuringiensis*, the latter being the typical cause of death in most lepidopteran species. For example, in highly susceptible species, such as grain-feeding lepidopteran larvae of the family Pyralidae, as paralysis sets in due to intoxication by Cry proteins, Bt spores germinate in the midgut as the alkaline pH (8-10) drops to around 7. The resulting vegetative cells invade the larva, colonize the hemolymph and other tissues, and reproduce to an extent that the cadaver becomes virtually a pure culture of Bt (Fig. 1, right panel). In other species, such as most *Spodoptera* species, death appears to depend on a combination of factors including Cry proteins, Vips, β -exotoxin (a competitive inhibitor of mRNA polymerase, which is not allowed in bacterial insecticides in the U.S. and Europe, as it is teratogenic at high levels), and various enzymes that help break down midgut barriers to infection by Bt and other bacteria present in the midgut lumen. In some species, such as larvae of the gypsy moth, *Lymantria dispar*, naturally occurring midgut bacteria may also be the cause of death [23], but this is an exception to the rule. In lepidopteran species with low sensitivity to Cry toxins, death may be brought about by a combination of Bt and enteric bacteria, but in the end it is the former species that effectively colonizes dead larvae. In lepidopteran hosts that are not natural hosts for Bt, where reproduction is restricted, such as in the gypsy moth, there appears to be less benefit to killing these species.

Although these other factors are important to Bt's insecticidal activity, regardless of the target insect, Cry proteins are the most important of the insecticidal components found in commercial Bt formulations. Without these, for example, when endotoxin plasmids are eliminated from Bt strains by curing, the resulting spores, which lack a parasporal body containing endotoxins, are in essence not toxic or pathogenic to insects that eat them.

To account for the complexity of the toxicity factors that occur in many Bt isolates, it appears that the various components other than Cry proteins evolved to optimize the chances that the bacterium could overcome host defenses, kill the insect, and then use the dead insects for reproduction. The evidence suggests that this set of components evolved in grain-feeding and other pyralid insects, specifically in larvae of species such as the southern European sunflower moth, *Homoeosoma nebulella* (a grain pest), the navel orangeworm, *Amyelois transitella*, which feeds on rotting

fruit and tree nuts), and the Mediterranean flour moth, *Ephestia kuehniella*, from which the Bt type species, *B. thuringiensis* subsp. *thuringiensis*, was isolated by Ernst Berliner in 1911. Larvae of these moths, all members of the family Pyralidae, are the only species of the order Lepidoptera in which periodic natural epizootics of *B. thuringiensis*, spreading as an infectious disease, are known to occur [24, 25]. In such species, larval cadavers filled with Bt spores and insecticidal crystals resulting from infection and colonization of the body serve as the source of inoculum for epizootics. The intoxication and infection processes are initiated by Cry proteins, after which vegetative growth and invasion of the hemocoel occur, possibly with the aid of one or more of the other toxicity components noted above. The reason that other types of lepidopterans, which are not known to be "natural" hosts for Bt subspecies, are sensitive to Bt's is that they contain the same "receptors" for Cry proteins that occur in the larvae of grain-feeding moths. The degree of sensitivity will depend on the species, specifically on the number and affinity of midgut microvilli receptors for various Cry proteins. For insect species recalcitrant to Bt, such as most *Spodoptera* species (family Noctuidae), the components of toxicity other than Cry proteins play an important role in bringing about death, even if the vegetative cells are not successful in colonizing the larva. The importance of these other toxic components, for example, Vip3, a protein toxin that also targets midgut epithelial cells, has been demonstrated for larvae of *Agrotis ipsilon* and *Spodoptera frugiperda*. When the Vip3 gene was deleted from *B. thuringiensis*, its pathogenicity was reduced markedly against these species [26]. Another example of a contributing toxic component is β -exotoxin, which synergizes the activity of Cry proteins and other proteins produced as spores germinate. The β -exotoxin is an inhibitor of mRNA polymerase, and appears to act by preventing intoxicated midgut epithelial cells from recovering, and regenerative midgut cells from developing. Thus, although Bt apparently evolved in the larvae of grain-feeding moths, the common occurrence of receptors, i.e., docking molecules, for Cry proteins in many lepidopteran species makes them susceptible to many Bt's, but mortality in species not highly sensitive to Cry proteins requires other toxic components. Nevertheless, even if eventually killed by Cry proteins in combination with other factors, Bt may not colonize the body of some species, making these species poor hosts for Bt reproduction. Interestingly, as Bt crops produce only the endotoxins, i.e., not spores, enteric bacteria likely accelerate death of larvae feeding on these.

Mode of Action of Cry Proteins

Owing to their widespread occurrence and importance to the efficacy of Bt insecticides used in agriculture, forestry, and vector control, Cry proteins have been the subject of numerous mode of action studies over the past two decades. Prior to this, it was known that Cry proteins are not contact poisons, as are most synthetic chemical insecticides, but rather insecticidal proteins that acted on the midgut, and being proteins, had to be ingested to be effective. It was also known that these proteins had to be cleaved by midgut proteases to be active – cleavage releases the active toxin, which then binds to specific receptors on the microvilli of the target insect's midgut epithelium (stomach). If the

appropriate receptors are not present, there is little if any binding and thus toxicity. These studies in combination with resolution of the three-dimensional structure of several Cry proteins [27, 28] have provided the following basic understanding of the mode of action Cry proteins produced by Bt.

Analysis of *cry* gene sequences combined with the three-dimensional structures of Cry3A, Cry1Aa, and Cry2A showed that the active portion of Cry toxins is a wedge-shaped molecule of three domains, and typically consists of approximately 600 amino acids (residues 30-630; see [28] for a review). The active toxin contains five blocks of conserved amino acids distributed along the molecule, and a highly variable region within Domain II. This region is the primary region responsible for the insect spectrum of activity, as demonstrated through domain-swapping studies [29], with the sensitivity of a specific insect species to a particular Cry toxin being directly correlated with the number and affinity of binding sites on the midgut microvillar membrane [30-33]. Resolution of Cry3Aa crystal structure [27] and other Cry proteins [28] showed that Domain I of this protein is composed of amino acids 1-290, and contains a hydrophobic seven-helix amphipathic bundle, with six helices surrounding a central helix. This domain contains the first conserved amino acid block and a major portion of the second conserved block. Theoretical computer models of the helix bundle show that after insertion and rearrangement, aggregations of six of these domains likely form a pore through the microvillar membrane [27, 28]. Domain II extends from amino acids 291-500 and contains three antiparallel β -sheets around a hydrophobic core. This domain contains most of the hypervariable region and most of conserved blocks three and four. The crystal structure of the molecule together with recombinant DNA experiments and binding studies indicate that the three extended loop structures in the β -sheets are responsible for initial recognition and binding of the toxin to binding sites on the microvillar membrane [29-32]. Domain III is comprised of amino acids 501 to 644 and consists of two antiparallel β -sheets, within which are found the remainder of conserved block number three along with blocks four and five. The Cry3Aa structure indicated that this domain provides structural integrity to the molecule. Site-directed mutagenesis studies of conserved amino acid block 5 in the Cry1 molecules show that this domain also plays a role in receptor binding and pore formation [28].

To cause toxicity after activation, Cry proteins must cross the peritrophic membrane and bind to proteins on the surface of midgut microvilli before they can insert to form a pore. The first proteins identified as receptors in the mid-1990's were aminopeptidases [33]. These extended into the midgut lumen but were tethered to the microvillar membrane. Subsequently, other molecules including cadherins and glycolipids were also shown to be midgut receptors for Cry proteins [34]. Studies of these receptors showed that even more important than the type of protein or lipid receptor was the surface glycosylation on these, which provides the specific surface sugars that the Cry molecule recognizes and binds to. Importantly, recent studies have shown that invertebrates, but not vertebrates, have a glycosylating enzyme, BL2, which creates the specific sugar residues on the glycolipid microvillar receptor recognized by Cry

proteins [34]. The lack of this enzyme in vertebrates provides a possible explanation for why activated Cry proteins do not appear to bind to cells lining the stomach and intestines of vertebrates [35]. Just prior to entry or after, individual Cry molecules oligomerize forming a complex of three, four or six molecules that form the actual pore [36, 37]. These findings are based on *in vitro* experiments, and it must be realized that the actual structure of the pore *in vivo* is not known. Nevertheless, a variety of evidence indicates this pore is a cation-specific channel [37, 38]. Once a sufficient number of these channels have formed, a surplus of cations, K⁺ for example, enter the cell. This causes an osmotic imbalance within the cell, and the cell compensates by taking in water. This process, referred to as colloid-osmotic induced lysis, continues until the cell ruptures and exfoliates from the midgut microvillar membrane. When a sufficient number of cells have been destroyed, the midgut epithelium loses its integrity. This allows the alkaline gut juices and bacteria to cross the midgut basement membrane, resulting in death, the latter caused by *B. thuringiensis* bacteremia and tissue colonization in lepidopteran species. In mosquito and black fly larvae, midgut bacteria do not cross the midgut epithelium until after death, thus in these the cause of paralysis and death is apparently due only to the insecticidal Cry and Cyt proteins.

This overview of toxin structure, receptors, and binding requirements constitutes a series of steps that account for the specificity and safety of Bt insecticides and Bt crops, as summarized below.

1. Endotoxin crystals must be ingested to have an effect. This is the reason sucking insects and other invertebrates such as spiders and mites are not sensitive to Cry proteins used in Bt insecticides or Bt crops.
2. After ingestion, Bt endotoxin crystals active against lepidopterous insects must be activated. Activation requires that crystals dissolve. This typically occurs in nature under alkaline conditions, generally in digestive juices in the midgut lumen, where the pH is 8 or higher. Most non-target invertebrates have neutral or only slightly acidic or basic midguts. Under the highly acidic conditions in stomachs of many vertebrates, including humans, Cry and Cyt protein crystals may dissolve, but once in solution they are rapidly degraded to non-toxic peptides by gastric juices, typically in less than 2 minutes.
3. After dissolving into midgut juices, Cry proteins must be cleaved by midgut proteases at both the C-terminus and N-terminus to be active.
4. Once activated, the toxin must bind to glycoprotein or glycolipid "receptors" on midgut microvillar membrane. Most chewing insects that ingest toxin crystals, even those with alkaline midguts, including many lepidopterans, do not have the appropriate receptors, and thus they are not sensitive to activated Cry proteins. This is because the activated Cry molecule typically requires a specific arrangement of sugar residues on the receptor to bind effectively. As a result, even insects sensitive to one class of Bt proteins, such as larvae of lepidopteran species sensitive to Cry1 proteins, are not sensitive to Cry3 proteins active against coleopterans - they lack

receptors for these. A high degree of specificity is even apparent within each order of sensitive insects. For example, larvae of *Heliothis virescens* are highly sensitive to Cry1Ac (hence its use in Bt cotton), but larvae of *Spodoptera* species, such as the beet armyworm, *S. exigua* and fall armyworm, *S. frugiperda*, are typically insensitive to this protein at rates encountered in nature or when treated with Bt insecticides. Cry1Ac is activated in these insensitive species, but binding to receptors is inefficient. Of relevance to vertebrate safety, no significant binding of Cry proteins has been detected in mammalian stomach epithelial cells.

5. After binding to a midgut receptor, the toxin must enter the cell membrane and form a cation-selective channel. This requires a change in the conformation of the active Cry molecule and oligomerization to form the channel.

With respect to level 5, at present, the specific conformational changes and details of the oligomerization process that must take place to exert toxicity are not known. It is known, however, that high affinity irreversible binding can occur in some insects, yet not lead to toxicity. This implies that a specific type of processing, i.e., another level of specificity, may be required for toxicity that occurs as or after the toxin inserts into the membrane.

An important aspect of specificity and safety is the route by which an organism is likely to encounter a toxin. Even though pulmonary (inhalation) and intraperitoneal injection studies are done with microbial Bt insecticides and proteins, their normal route of entry by target and non-target organisms is by ingestion. In comparison to most synthetic chemical insecticides, which as contact poisons kill many non-target organisms when used in any crop, forest, or aquatic ecosystem, Cry proteins used in Bt insecticides and Bt crops as well, are inherently much safer due to their specificity and targeted dissemination in the environment.

Mode of Action of Cyt Proteins

Cyt proteins have received little study in comparison to Cry proteins, as they typically only occur in mosquitocidal strains of Bt. Nevertheless, these proteins, based primarily on studies of Cyt1Aa, are extremely important in the biology of mosquitocidal strains because they synergize mosquitocidal Cry proteins, such as Cry4Aa, Cry4Ba, and Cry11A, and delay the phenotypic expression of resistance to these ([39-41]; see chapter by Wirth, MC in this volume for a detailed review). Cyt1A has also been shown to delay the evolution of resistance to the *B. sphaericus* binary toxin [42]. Cyt proteins likely play a similar role in other strains in which they occur, such as the PG14 isolate of *B. thuringiensis* subsp. *morrisoni*. As far as is known, Cyt proteins do not require a protein receptor, but instead bind directly to the non-glycosylated lipid portion of the microvillar membrane. Once within the membrane, they appear to aggregate, forming lipid faults that cause an osmotic imbalance that results in cell lysis [43].

BASIC BIOLOGY OF BACILLUS SPHAERICUS

Since the mid-1960s it has been known that many isolates of *Bacillus sphaericus* (Bs) are toxic to mosquito species. Over the past three decades, three isolates have been evaluated for mosquito control, 1593 from Indonesia, 2297 from Sri Lanka, and 2362 from Nigeria. The 1593 and 2297

isolates were obtained from soil and water samples at mosquito breeding sites, whereas 1593 was isolated from a dead adult black fly [44].

The toxicity of Bs, like Bt, is the result of protein endotoxins that are produced during sporulation and assembled into a parasporal body. Bs is unusual in that the main toxin is a binary toxin, i.e., composed of two protein subunits (BinA and BinB). These are proteolytically activated in the mosquito midgut to release peptides of, respectively, 43 and 39 kDa, that associate to form the binary toxin, with the former protein constituting the binding domain, and the latter the toxin domain. The toxins bind to microvilli of the midgut epithelium, causing hypertrophy and lysis of cells, destroying the midgut and killing the mosquito larva [44-46].

Recently, a commercial product known as VectoLex (Valent BioSciences, Libertyville, Illinois) has come to market for control of *Culex* mosquito larvae, and certain species of *Anopheles* mosquitoes. Although *B. sphaericus*, especially strain 2362 and similar strains, is an excellent control agent for many mosquito species, it has the drawback of acting as a single toxin, and thus is prone to the rapid evolution of resistance. Where it has been used intensively in China and Thailand, level of resistance ranging from 22,000-50,000-fold have been reported [47, 48]. However, as is shown below, this problem can largely be overcome by engineering bacterial strains that combine the *B. sphaericus* Bin toxin with other mosquitocidal proteins, especially the Cyt1A protein.

GENETIC ELEMENTS REGULATING INSECTICIDAL PROTEIN SYNTHESIS

The primary genetic factors affecting insecticidal protein synthesis in *B. thuringiensis* are promoters, a 5' mRNA stabilizing sequence and 3' transcriptional termination sequences.

Promoters

In *Bacillus* species, the endospore develops in a sporangium consisting of two cellular compartments, the mother cell and the forespore. In *B. subtilis*, the developmental process is temporally regulated at the transcriptional level by the successive activation of six σ factors that by binding to RNA polymerase determine which gene promoters are recognized [49]. These factors are σ^A , the primary sigma factor of vegetative cells, and five factors that are activated during sporulation, σ^E , σ^F , σ^G , σ^H and σ^K , in order of their occurrence during sporulation. The σ^A and σ^H factors are active in the pre-divisional cell, σ^E and σ^K are active in the mother cell, and σ^F and σ^G are active in the forespore. In *B. thuringiensis*, two genes encoding sigma factors, σ^{35} and σ^{28} , which show, respectively, 88 and 85% amino acid sequence identity with σ^E and σ^K of *B. subtilis*, have been cloned [50]. In *B. thuringiensis*, there are two primary sporulation-dependent promoters, BtI and BtII. The BtI promoter is transcribed by σ^{35} complexed with the RNA polymerase [51], whereas the BtII promoter is transcribed by the σ^{28} complexed with the RNA polymerase [52].

Over the years, several *cry* promoters have been identified and their sequences determined. Consensus sequences for promoters recognized by *B. thuringiensis* RNA polymerase containing σ^E -like or σ^K -like factors have been deduced from alignment of the promoter regions of these genes [53]. The results indicate that the transcription of many other *cry* genes is likely to be σ^E - or σ^K -dependent. Unlike BtI and BtII, the *cry3A* promoter is similar to promoters recognized by σ^A . The expression of *cry3A* is not dependent on sporulation-specific σ factors in either *B. subtilis* or *B. thuringiensis* [54, 55].

5' mRNA Stabilizing Sequence

The 5' region of the *cry3Aa* transcript beginning at nucleotide position 129 contains a region that stabilizes this mRNA [54]. Fusion of this region to the 5' region of the *lacZ* gene transcribed from a promoter inducible in *B. subtilis* increased the stability of the *lacZ* fusion mRNA and resulted in a 10-fold increase of both steady-state mRNA and β -galactosidase synthesis [55, 56].

The determinant of stability appears to be a consensus Shine-Dalgarno (SD) sequence, designated STAB-SD, close to the 5' end of the *cry3Aa* mRNA [56]. Mutations introduced into this region suggest that this sequence provides stability through interaction with the 3' end of the 16S rRNA. Therefore, the binding of a 30S ribosomal subunit to the SD sequence located in the 5' untranslated region of *cry3Aa* apparently stabilizes the corresponding transcript by protecting it against 5'-3' ribonuclease activity. Such SD sequences are also present in similar positions in at least two other members of *cry3* gene family, *cry3Ba1* and *cry3Ba2* [20, 56].

3' Transcriptional Termination Sequence

Wong and Chang [57] showed that a non-coding region near the 3' terminus of *cry1Aa* from *B. thuringiensis* subsp. *kurstaki* HD-1 acts as a positive retroregulator, i.e., serves as a *cis*-acting element that regulates a target gene from a distance. The fusion of this fragment with the 3' end of heterologous genes increased transcript half-life and consequently the amount of Cry protein synthesized.

The activity of 3'-5' exonucleases is affected by RNA secondary structure. In particular, their rate of mRNA degradation is impeded by 3' stem-loop structures. Therefore, it is likely that *cry* and *cyt* gene terminators are involved in mRNA stability by protecting the mRNA from exonucleotic degradation from the 3' end. The putative terminator sequences downstream from various *cry* genes are widely conserved. It has been shown that the orientation of the *cry3Aa* transcription terminators was important to enhance truncated *cry1Ca* transcript stability and protein synthesis [58].

CONSTRUCTION OF RECOMBINANT LARVICIDAL BACTERIA

Due to their high toxicity and specificity, *cry* and *cyt* protein genes of *B. thuringiensis* have been introduced into *B. thuringiensis* and several other bacterial species to improve efficacy using either plasmids that can replicate in the host or by integrating genes into host chromosomal DNA. Although *B. thuringiensis* is still the most successful

organism used as a host to synthesize mosquitocidal proteins, other bacterial species discussed below also have been used. Beginning with the use of *B. thuringiensis* as the host, we provide examples of how several bacterial species were genetically engineered to improve the efficacy of bacterial larvicides for control of nuisance and vector mosquitoes. Although none of these has yet been commercialized, as they are recombinant organisms and require further testing, they should be in operational use within the next five years or so.

Bacillus thuringiensis

Transfer of plasmids into *B. thuringiensis* was first reported via cell mating, also known as conjugation [59, 60]. Using this method, transformation efficiency was low, and as these plasmids lacked a selectable marker, screening cells for transformants was slow and cumbersome. Several years later, improved protocols for transformation of *B. thuringiensis* using electroporation were published independently, and these new methods accelerated research on the construction of recombinant strains of *B. thuringiensis* [61, 62]. These protocols provided high transformation efficiency and made transformants easy to recognize and recover by using antibiotics as selectable markers; their development greatly facilitated basic research and engineering of *B. thuringiensis*.

The most common strategy for constructing recombinant *B. thuringiensis* strains is using a shuttle expression vector, such as pHT3101 [62] that contains replication origins for both *B. thuringiensis* and *E. coli*, genes for resistance, for example to ampicillin and erythromycin, for easy selection of transformants, and a multi-cloning site. A shuttle vector containing the gene of interest is amplified in *E. coli*, isolated, and subsequently introduced into the desirable *B. thuringiensis* strain by electroporation.

In many cases, *cry* and *cyt* genes of *B. thuringiensis* inserted into shuttle vectors were expressed under the control of their own promoters, which typically resulted in a high yield of the encoded protein. In terms of promoter strength, *cyt1Aa* promoters are the strongest known among *cry* and *cyt* genes [53, 63, 64]. In addition, as mentioned above, the *cry3Aa* upstream 5' mRNA stabilizing sequence (STAB-SD) improves stability of *cry3A* transcripts and concomitantly the yield of certain *Cry3Aa* [56]. Therefore, to optimize *Cry* and *Cyt* protein yields in *B. thuringiensis*, a recombinant expression vector, pSTAB was developed [65, 66]. This vector was constructed by inserting the 660-bp DNA fragment containing *cyt1Aa* promoters combined with the STAB-SD sequence into the multi-cloning site of pHT3101 (Fig. 3). Using the pSTAB expression vector that combined these different genetic elements, we significantly increased yields of several *Cry* proteins. For example, by expressing the *cry3Aa* gene using this vector, we were able to obtain yields twelve-fold greater than those obtained with the wild type strain of *B. thuringiensis* subsp. *morrisoni* (isolate DSM2803) from which this gene was cloned [65]. The yield of *Cry3Aa* obtained per unit medium using *cyt1Aa* promoters alone, i.e., lacking the STAB-SD sequence, was only about two-fold higher than that of the wild-type DSM280 strain (Fig. 4). This demonstrates that most of the enhancement was due to inclusion of the STAB-SD sequence.

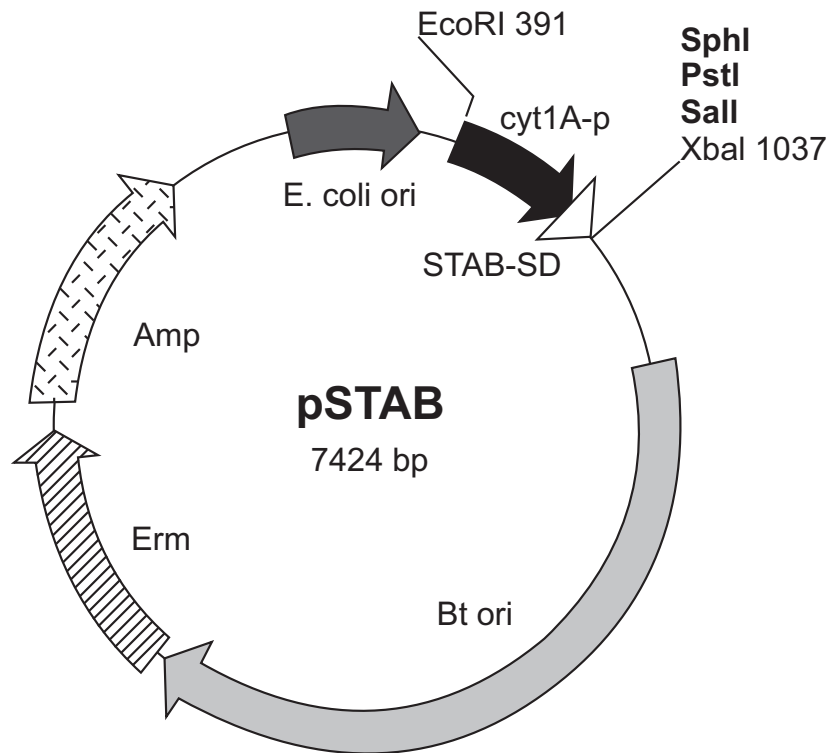


Fig. (3). The *Bacillus thuringiensis* expression plasmid, pSTAB. Physical map of pSTAB. Amp, ampicillin-resistant gene; Erm, erythromycin-resistant gene; E. coli ori, *E. coli* replication origin, Bt ori, *B. thuringiensis* replication origin; cyt1A-p, *cyt1Aa* promoters.

The significant increase in Cry3Aa yield obtained using *cyt1Aa* promoters combined with the STAB-SD sequence led us to test this expression vector for enhancing synthesis of other insecticidal proteins in *B. thuringiensis*. Results of these later studies showed that the level of enhancement using this expression system varies depending upon the candidate protein. For example, yields of Cry11Ba and the binary toxin of *B. sphaericus*, as discussed in the following sections, were increased substantially, as much as eight-fold, whereas yields of proteins such as Cry11Aa and Cry2Aa increased only 1.5 to two-fold [66-68].

Bacillus thuringiensis* subsp. *israelensis

As our research is primarily directed toward improving mosquitocidal bacteria, our best examples of the successful use of pSTAB come from engineering recombinant strains of *B. thuringiensis* subsp. *israelensis*. We have used this vector to produce several different recombinant strains that vary in complexity, ranging from a strain that produces only a single endotoxin to strains that produce as many as five endotoxins. In the simplest case, we used pSTAB to express the binary (Bin) toxin operon of *B. sphaericus* 2362 in the acrySTALLIFEROUS strain 4Q7 of *B. thuringiensis* subsp. *israelensis* [68]. The Bin toxin of *B. sphaericus* [69] consists of a 51-kDa binding domain (BinA) and a 42-kDa toxin domain (BinB). Using the pSTAB vector to express the *bin* operon alone (under control of *cyt1A* promoters), synthesis of Bin was eight-fold higher than that obtained with wild type *B. sphaericus* 2362 (Fig. 5). Whereas wild type *B. sphaericus* typically has an LC₅₀ in the range of 8 – 12 ng/ml against fourth instars of *Culex quinquefasciatus*, the 4Q7 strain that produces the Bin toxin has an LC₅₀ of 1.4 ng/ml

[68]. However, as this recombinant, like wild type *B. sphaericus*, only produces a single toxin, it is likely its use would lead the development of resistance in target populations.

To improve toxicity while at the same time preventing or delaying the development of resistance, we made several strains in which we increased toxin complexity and added the Cyt1Aa protein for resistance management, the efficacy of which we established in several papers [40-42]. Previously, Li *et al.* [70] attempted to make a similar strain. They used a shuttle expression vector, pBU-4 to synthesize the Bin toxin of *B. sphaericus* C3-41 along with the Cyt1Aa protein of *B. thuringiensis* subsp. *israelensis* in an acrySTALLIFEROUS strain of *B. thuringiensis*. However, the recombinant strain producing the Bin toxin and Cyt1Aa showed very poor toxicity against both sensitive (LC₅₀ = 1.12 µg/ml) and resistant (LC₅₀ = 2,116.33 µg/ml) colonies of *Cx. quinquefasciatus*. In our studies, one of the first strains we constructed using this strategy was a recombinant that synthesized the Bin toxin, Cyt1Aa and Cry11Ba [71]. In this recombinant, which again used the 4Q7 strain of *B. thuringiensis* subsp. *israelensis* as the host cell, the mosquitocidal proteins were from three different species; (1) Bin from *B. sphaericus* 2362, (2) Cry11Ba from *B. thuringiensis* subsp. *jegathesan*, and (3) Cyt1Aa from *B. thuringiensis* subsp. *israelensis*. The Cry11Ba protein is 58% identical to Cry11Aa but more toxic than the latter, the most toxic mosquitocidal protein produced by *B. thuringiensis* subsp. *israelensis* [72]. This recombinant was constructed using a dual-plasmid expression system with two different plasmids, each with a different antibiotic resistance gene for

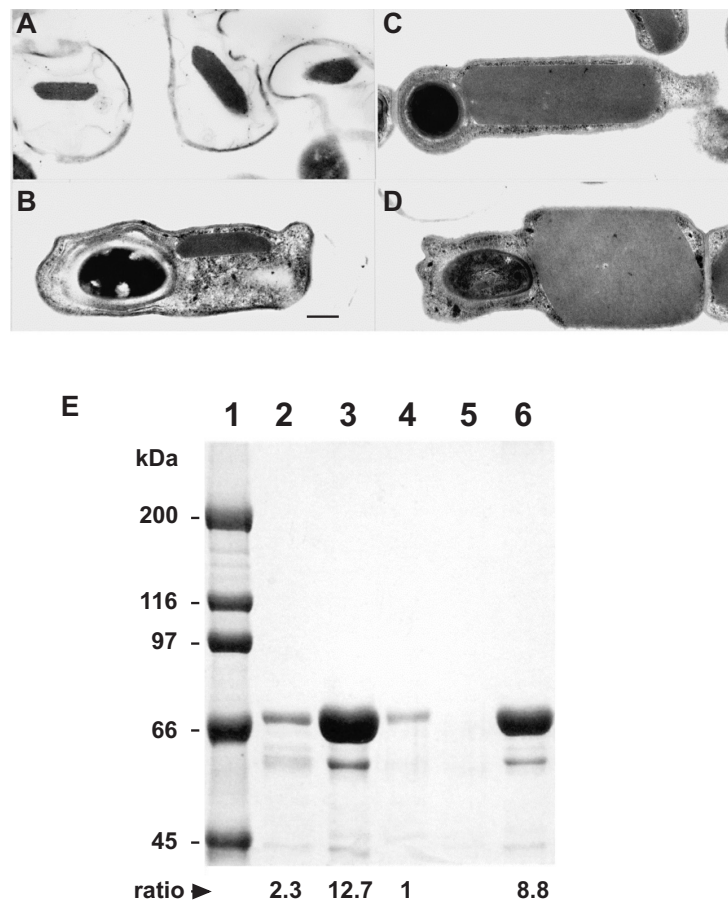


Fig. (4). Transmission electron micrographs of wild type and recombinant *Bacillus thuringiensis* strains producing Cry3Aa. (A) Wild-type *B. thuringiensis* subsp. *morrisoni* (strain *tenebrionis*) DSM 2803. (B) AcrySTALLIFEROUS strain (4Q7) of *B. thuringiensis* subsp. *israelensis* transformed with pPFT3A (*cry3Aa* without the STAB-SD sequence under the control of *cyt1Aa* promoters). (C and D) Cross section (C) and sagittal section (D) through 4Q7 cells transformed with pPFT3As (*cry3Aa* with the STAB-SD sequence under the control of *cyt1Aa* promoters). All micrographs are at the same magnification. E. SDS-PAGE gel showing amounts of Cry3Aa produced by different constructs. Lane 3 is shows Cry3Aa produced using the STAB-SD expression vector, whereas lane 4 shows the amount produced by the wild-type isolate. Bar, 300 nm. From Park *et al.* (1998).

selection. The resulting recombinant *B. thuringiensis* produced three distinct crystals (Fig. 4), apparently one for each of these proteins, i.e., Cyt1Aa, Cry11Ba, and the Bin toxin, and was significantly more toxic ($LC_{50} = 1.7$ ng/ml) to *Cx. quinquefasciatus* fourth instars than either *B. thuringiensis* subsp. *israelensis* IPS-82 ($LC_{50} = 7.9$ ng/ml) or *B. sphaericus* 2362 ($LC_{50} = 12.6$ ng/ml).

To construct a recombinant with an even greater range of endotoxins for both increased toxicity and resistance management, we transformed the IPS-82 strain of *B. thuringiensis* subsp. *israelensis*, which produces the complement of toxins characteristic of this species, with pPHSP-1, the pSTAB plasmid that produces a high level of the *B. sphaericus* Bin toxin (Fig. 6). When mortality was obtained after 48 h of exposure, LC_{50} s of this recombinant were 0.014 and 3.8 ng/ml, respectively, against *Cx. quinquefasciatus* and *Cx. tarsalis*, whereas those of *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* 2362 were 3.2 and 37.7 ng/ml, and 11.9 and 24.6 ng/ml, respectively [68]. Aside from high efficacy, as noted above, this new bacterium is much less likely to induce resistance in target populations, as it combines Cyt1Aa with *B. thuringiensis*

subsp. *israelensis* Cry toxins and the *B. sphaericus* Bin toxin. The resistance management properties of this bacterium are currently under evaluation. The markedly improved efficacy and resistance-delaying properties of this new bacterium make it an excellent candidate for development and use in vector control programs, especially to control *Culex* vectors of West Nile and other viruses as well as species of this genus that transmit filarial diseases. Moreover, the larvae of certain species of *Anopheles* mosquitoes that are important malaria vectors, such as *An. gambiae* and *An. arabiensis*, should be highly sensitive to this recombinant, as they are not only sensitive to the toxins of *B. thuringiensis* subsp. *israelensis*, but are also highly sensitive to the *B. sphaericus* Bin toxin [73].

Bacillus sphaericus

Mosquitocidal strains of *B. sphaericus* produce several protein toxins. Those referred to as Mtx toxins (of 34-36 or 100 kDa) are produced during vegetative growth, whereas the so-called Bin (for binary) toxin is produced during sporulation [44]. The Bin toxin forms a crystal on the inner surface of the exosporium membrane, and this toxin

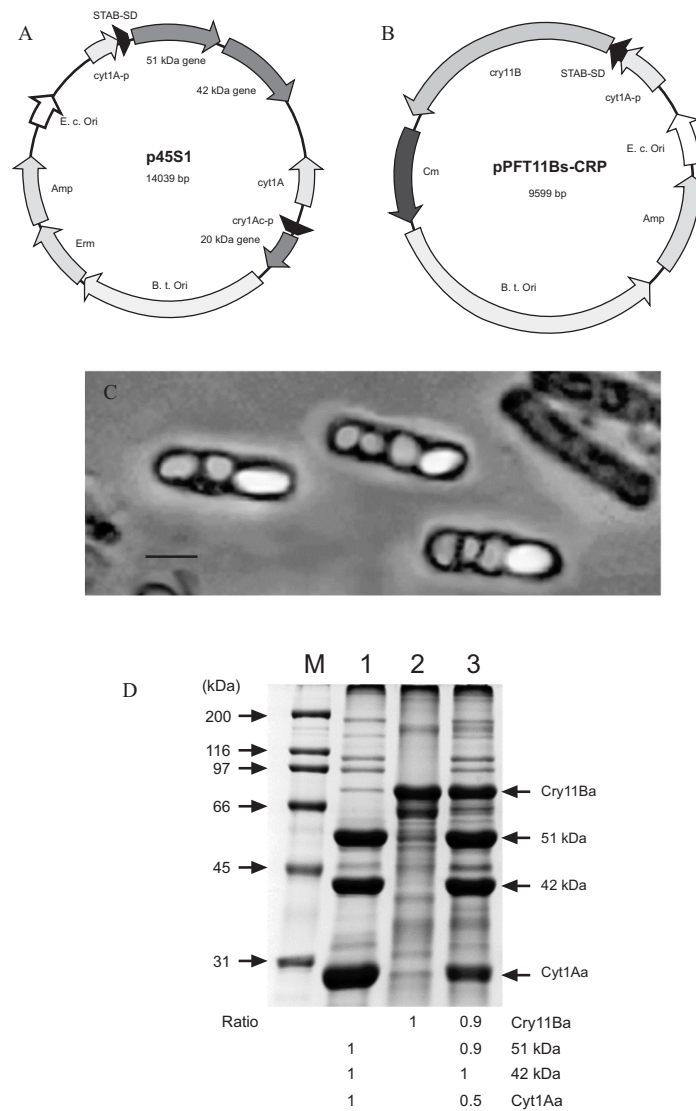


Fig. (5). Expression vectors use to produce a recombinant strain of *Bacillus thuringiensis* that synthesizes Cyt1Aa, Cry11Ba, and the *B. sphaericus* binary toxin. **A & B,** Maps of recombinant plasmids for producing Cyt1Aa, Cry11Ba, and the Bs2362 binary toxin. **A,** p45S1 containing *cyt1Aa* from *B. thuringiensis* subsp. *israelensis* and a binary toxin gene from Bs2362. **B,** pPFT11Bs-CRP containing *cry11Ba* from *B. thuringiensis* subsp. *jegathesan*. Amp, ampicillin-resistant gene; Erm, erythromycin-resistant gene; Cm, chloramphenicol-resistant gene; *cyt1A-p*, *cyt1Aa* promoters; *cry1Ac-p*, *cry1Ac* promoters; *E. c. ori*, *E. coli* replication origin; *B. t. ori*, *B. thuringiensis* replication origin. **C,** Light micrograph of the recombinant strain showing the three crystals adjacent to the spore. **D,** SDS-Page profiles of the different constructs. Lane 3 shows the amounts of proteins produced by the recombinant containing both expression plasmids. From Park *et al.* [70].

accounts for most of this species activity, whereas the Mtx toxins are soluble and degrade quickly after synthesis. Highly toxic strains of *B. sphaericus* such as 2362 exhibit activity against *Culex* species equal to or slightly better than *B. thuringiensis* subsp. *israelensis*. In addition, *B. sphaericus* has longer residual activity, by at least several days, than *B. thuringiensis* subsp. *israelensis* in various larval habitats, including polluted water. However, the Bin toxin is the only major crystal toxin produced by *B. sphaericus*, and as a result, mosquitoes have developed resistance quickly in the field where this bacterium was used intensively [47, 48].

To improve the efficacy of *B. sphaericus*, there have been several attempts using different transformation strategies to introduce into this species mosquitoicidal Cry

and Cyt proteins of *B. thuringiensis* subsp. *israelensis* and other subspecies. Trisrisook *et al.* [74] reported Cry4Ba production in strains 1593 and 2362 using protoplast transformation. Bar *et al.* [75] expressed *cry4Ba* and *cyt1Aa* genes independently or in combination in strain 2362. Similarly, Poncet *et al.* [76] synthesized Cry4Ba and Cry11Aa independently in strain 2297. A few years later, *cyt1Ab* gene from *B. thuringiensis* subsp. *medellin* was introduced into several *B. sphaericus* strains and a reasonable amount of Cyt1Ab was produced only in strain 2297 [77]. In all cases, *cry* and *cyt* genes were under the control of their own promoters and the level of synthesis of introduced Cry proteins was very poor due to instability of introduced plasmids.

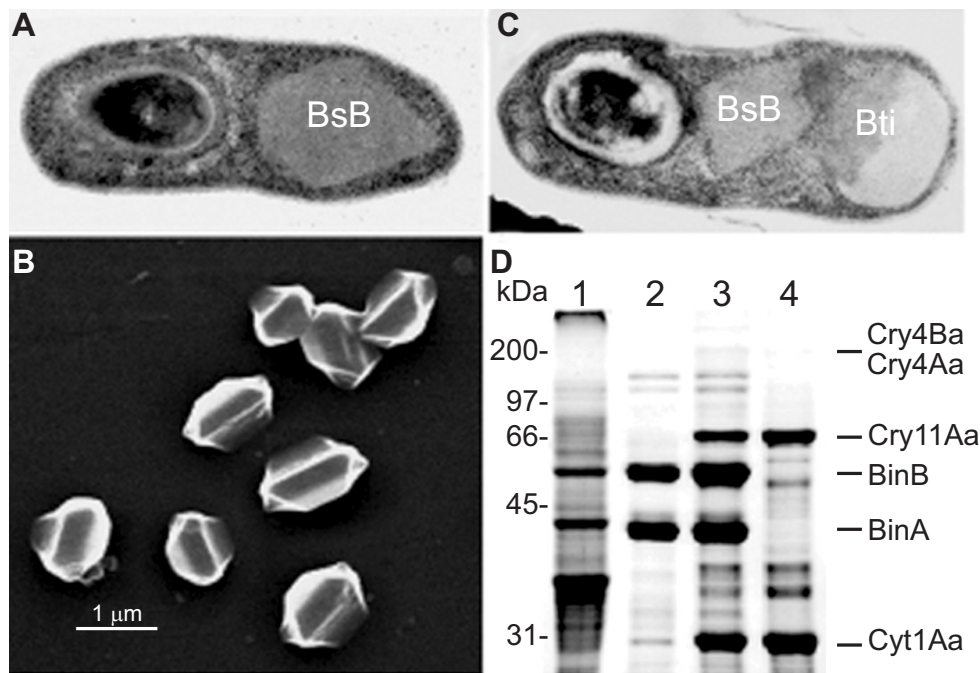


Fig. (6). Synthesis and purification of *Bacillus sphaericus* 2362 binary toxin in *B. thuringiensis* subsp. *israelensis*. (A) Transmission electron micrograph of the recombinant acryalliferous strain, Bti4Q7/BsB, engineered to synthesize the *B. sphaericus* binary toxin. The single large crystal (BsB) is adjacent to the spore. (B) Scanning electron micrograph of purified *B. sphaericus* 2362 binary toxin crystals produced in the recombinant strain, Bti4Q7/BsB. The bar equals 1.0 µm. (C) Transmission electron micrograph of the recombinant crystalliferous strain, BtiIPS-82/BsB, engineered to synthesize the *B. sphaericus* binary toxin. This recombinant strain produces the typical IPS-82 parasporal body (Bti) and Bs2362 binary toxin crystal (BsB). (D) Comparative endotoxin yields produced per unit medium by wild type *B. sphaericus* strain 2362, and wild type and engineered strains of *B. thuringiensis* subsp. *israelensis* constructed to synthesize the Bs2362 binary toxin. Lanes: 1, wild type control *B. sphaericus* 2362 strain; 2, recombinant Bti4Q7 strain, Bti4Q7/BsB, engineered to produce Bs2362 toxin; 3, recombinant BtiIPS-82 strain, BtiIPS-82/BsB, that produces the Bs2362 toxin and typical Bti parasporal body; 4, wild type control Bti IPS-82 strain. From Park *et al.* [68].

Subsequently, a stable and improved level of synthesis of Cry11Aa in *B. sphaericus* 2297 was obtained using a new approach [78], *in vivo* homologous recombination. In this method, the gene of interest is inserted into the target sequence located on the chromosome without including any other unnecessary sequences such as antibiotic-resistant genes and replication origins. Toxicity of the recombinant strain against *Anopheles stephensi* was enhanced, although against *Cx. quinquefasciatus*, the toxicity was similar to the wild type. The same protocol was used to produce both Cry11Aa and Cry11Ba in *B. sphaericus* 2297 [79]. Although Cry11Aa and Cry11Ba production was poor in the recombinant strain for unknown reasons, it was toxic to *Aedes aegypti* to which the wild type does not show activity. However, it did not increase the toxicity to *Cx. pipiens*.

More recently, an erythromycin resistance-marked pBtoxis, the major toxin-coding plasmid of *B. thuringiensis* subsp. *israelensis* was transferred to the restriction-negative strains of *B. sphaericus* 1593 and 2362 by conjugation [80]. To construct the recombinant *B. sphaericus*, triparental mating was performed using the wild-type VectoBac strain of *B. thuringiensis* subsp. *israelensis* that contains a natural conjugative plasmid, pXO16 to mobilize the pBtoxis::erm plasmid from strain 4Q5::erm. The resulting recombinant *B. sphaericus* strains, which produced Cry11Aa of *B. thuringiensis* subsp. *israelensis*, were significantly more toxic to *Ae. aegypti* and were able to overcome resistance to

B. sphaericus in a resistant colony of *Cx. quinquefasciatus*. However, the introduced pBtoxis::erm plasmid (erythromycin resistant) in both recombinants was lost after serial culturing in the absence of selective antibiotics.

Despite the numerous attempts, researchers have not been able to identify the molecular factors that prevent a high level of foreign gene expression in *B. sphaericus*. Determination of these factors could lead to improved mosquitoicidal strains of *B. sphaericus*. Whether these would be more toxic and more persistent than existing recombinant strains of *B. thuringiensis* subsp. *israelensis* awaits future development of improved *B. sphaericus* strains.

Cyanobacteria

Use of formulations of *B. thuringiensis* subsp. *israelensis* for mosquito control requires frequent application because in most habitats these remain near the water surface where larvae feed for only a day or so, or they are inactivated by sunlight. A potential approach to circumvent this problem is to genetically engineer microorganisms living in the upper layers of the water to synthesize Cry endotoxin proteins of *B. thuringiensis* subsp. *israelensis*. Cyanobacteria are strong candidates for this type of genetic engineering owing to their photosynthetic capability and resultant simple nutritional requirements, and because they are widely distributed in the upper layers of water.

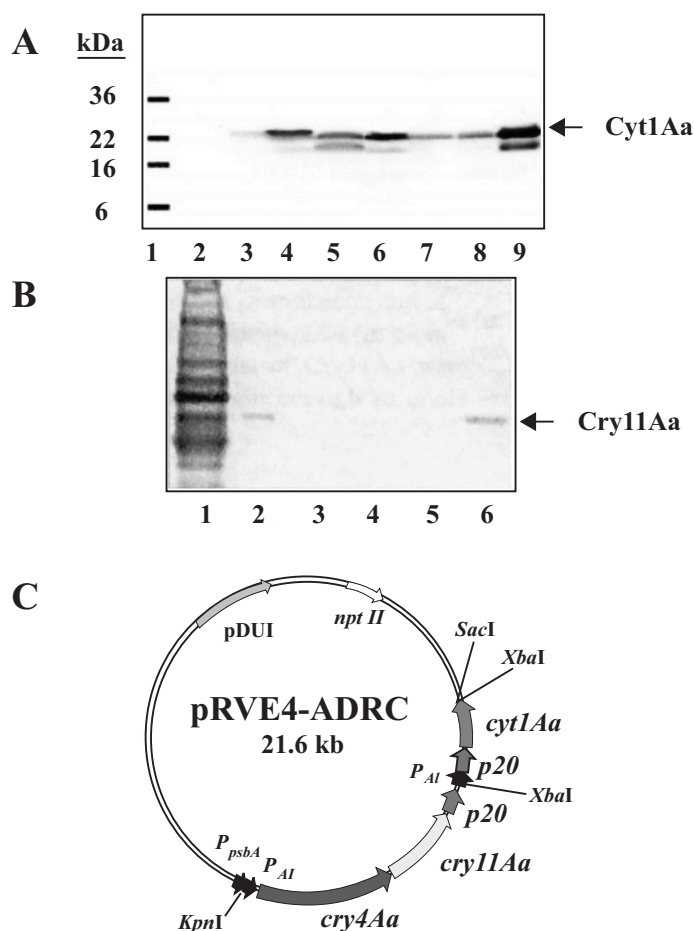


Fig. (7). Western blot analysis of recombinant *Anabaena* and *E. coli* strains that synthesize Cyt1Aa (A) and Cry11Aa (B). Anti-Cyt1Aa and antiserum against whole *B. thuringiensis* subsp. *israelensis* crystals were used, respectively, in (A) and (B). (A) Lane 1, molecular size marker; lane 2, *Anabaena* PCC 7120; lane 3, *Anabaena* PCC 7120 containing *cyt1Aa* under control of the *psbA* and T7 promoters; lane 4, *E. coli* XL-Blue MRF' containing *cyt1Aa* under control of the *psbA* and T7 promoters; lane 5, *Anabaena* PCC 7120 containing *cyt1Aa* and the 20-kDa protein gene under control of the *psbA* and T7 promoters; lane 6, *E. coli* XL-Blue MRF' containing *cyt1Aa* and the 20-kDa protein gene under control of the *psbA* and T7 promoters; lane 7, *Anabaena* PCC 7120 containing *cry4Aa* and *cry11Aa* under control of the *psbA* and T7 promoters, and *cyt1Aa* and the 20-kDa protein gene under control of the T7 promoter; lane 8, *E. coli* XL-Blue MRF' containing *cry4Aa* and *cry11Aa* under control of the *psbA* and T7 promoters, and *cyt1Aa* and the 20-kDa protein gene under control of the T7 promoter; lane 9, *B. thuringiensis* subsp. *israelensis*. (B) Lane 1, *B. thuringiensis* subsp. *israelensis*; lane 2, *Anabaena* PCC 7120 containing *cry4Aa*, *cry11Aa* and the 20-kDa protein gene under control of the T7 promoter; lane 3, *Anabaena* PCC 7120; lane 4, *Anabaena* PCC 7120 containing *cyt1Aa* under control of the *psbA* and T7 promoters; lane 5, *Anabaena* PCC 7120 containing *cyt1Aa* and the 20-kDa protein gene under control of the *psbA* and T7 promoters; lane 6, *Anabaena* PCC 7120 containing *cry4Aa* and *cry11Aa* under control of the *psbA* and T7 promoters, and *cyt1Aa* and the 20-kDa protein gene under control of the T7 promoter. (C) Physical map of the pRVE4-ADRC used to synthesize *cry4Aa*, *cry11Aa* and *cyt1Aa* of *B. thuringiensis* subsp. *israelensis* in *Anabaena*. *P_{psbA}*, cyanobacterial *psbA* promoter; *P_{AI}*, *E. coli* T7 promoter; *p20*, *B. thuringiensis* subsp. *israelensis* 20-kDa protein gene. Modified from Khasdan *et al.* [88].

Toward this goal, the cyanobacterium, *Agmenellum quadruplicatum* strain PR-6, was engineered in separate studies to synthesize either Cry4Ba [81], or Cry11Aa [82] of *B. thuringiensis* subsp. *israelensis*. This species was selected because it has a natural mechanism for uptake and integration of exogenous DNA, and therefore an efficient transformation procedure was developed more than twenty-five years ago [83]. In both of the engineering studies, the phycocyanin operon promoter was used to express *cry* genes. In the first study [81], approximately 1.5×10^2 *A. quadruplicatum* transformants per μg of plasmid DNA were obtained. However, the level of Cry4Ba synthesized by the recombinant was extremely low. Concomitantly, the recombinant *A. quadruplicatum* showed only 45% mortality

against the second instars of *Ae. aegypti* after 48 h of incubation using 10 mg ml^{-1} of total protein concentration. A few years later, improved Cry protein synthesis was obtained using *cry11Aa* and a translational gene fusion technique [82]. Though 100% mortality with 3 – 5 μl of recombinant cells against neonates of *Cx. pipiens* after 6 days of treatment was reported, the amount of toxin per unit volume was not quantified, making it impossible to assess the efficacy per unit volume, and thus compare this recombinant to others.

Subsequently, species of cyanobacteria belonging to the genus *Synechococcus* strains PCC 6803 [84] and PCC 7942 [85, 86] were used to produce Cry4Ba. To enhance Cry4Ba yield, the *cry4Ba* gene was placed under control of either the tobacco *psbA* promoter [84], the *lacZ* promoter combined

with the endogenous *cry4Ba* promoter, or the ferredoxin (*petF1*) promoter [85, 86]. Of these expression systems, the *lacZ* promoter combined with the *cry4Ba* promoter resulted in the highest Cry4Ba yield in the *Synechococcus* strain. However, even with this recombinant, larval mortality using neonates of *Cx. restuans* was only approximately 70% after 3 days of incubation when a mid- to late-log phase of culture was used [84].

More recently, in two different studies *Anabaena* sp. strain PCC 7120 was used to express either *cry4Aa*, *cry11Aa* and the 20-kDa protein gene [87] or *cry4Aa*, *cry11Aa*, *cyt1Aa* and the 20-kDa protein gene [88]. In both cases, greater insecticidal protein synthesis was achieved using a dual promoter system - a cyanobacterial *psbA* promoter and an *E. coli* T7 promoter, and pRL488p, in an *E. coli* - *Anabaena* shuttle vector [88]. In a former study [87], the recombinant *Anabaena* strain producing Cry4Aa, Cry11Aa and the 20-kDa protein was approximately 60-fold more toxic to third instars of *Ae. aegypti* compared with that producing only Cry4Aa [LC_{50} (10^5 cells ml^{-1}) = 53 vs. 0.9]. The recombinant strain harboring a plasmid that contained *cry4Aa* under the control of the *psbA* promoter alone did not show any toxicity against the same mosquito species. In the later study [88], the recombinant *Anabaena* strain producing Cry4Aa, Cry11Aa, Cyt1Aa and the 20-kDa protein [Fig. 7] showed approximately 2.4-fold more toxicity to fourth instars of *Ae. aegypti* compared with the strain producing Cry4Aa, Cry11Aa and the 20-kDa protein [LC_{50} (10^5 cells ml^{-1}) = 0.83 vs. 0.35].

Caulobacter crescentus

The gram-negative bacterium, *Caulobacter crescentus*, another species found commonly near the water surface [89], has also been used as a host for producing the Cry protein of *B. thuringiensis* subsp. *israelensis*. This species exhibits two distinct cell cycles, a nonmotile stalked cell phase and a monoflagellated swarmer cell phase. The flagellated swarmer stage of this bacterium is motile, and thus distributed throughout the habitat. Therefore, it could be an ideal carrier for biological toxins aimed at the surface-feeding larvae of mosquitoes.

To test this possibility, the *cry4Ba* gene of *B. thuringiensis* subsp. *israelensis* was placed under the control of *tac* promoter in the presence of the lactose repressor gene and transformed into *C. crescentus* by electroporation [90]. Recombinant *C. crescentus* cells producing Cry4B were tested against *Ae. aegypti* larvae using a concentration of 3.2×10^8 cells ml^{-1} . Only 32.5% mortality was obtained after 48 h of incubation. To improve Cry synthesis in *C. crescentus*, two recombinant regulatory sequences that affect transcription were investigated to determine their effect on Cry4Ba synthesis in *C. crescentus* strain CB15 [91]. The *cry4Ba* gene was placed under control of either the (1) *tac* promoter and the putative ribosome binding sequence (RBS) of the *C. crescentus* 130-kDa surface layer protein gene, or the (2) *bin* toxin promoter of *B. sphaericus* 2297 and its putative RBS. The *lacZ* gene was placed under control of both expression systems to determine the transcriptional efficiency in *C. crescentus*. The former resulted in approximately 1.3-fold higher β -galactosidase activity than the latter (2,199 vs. 1,711 Miller units). When the *C. crescentus* recombinants producing Cry4Ba were tested

against second instars of *Ae. aegypti*, the former was 18-fold more toxic than the latter ($LC_{50} = 4.0 \times 10^7$ vs. 2.2×10^6 cells ml^{-1}). As the two studies mentioned above used different mosquito bioassay procedures, direct comparison of bioassay data to determine the level of improvement obtained with the latter recombinants was not possible.

CONCLUSIONS AND FUTURE PROSPECTS

As shown above, several different types of bacterial species have been used to construct recombinant bacteria for producing insecticidal proteins of *B. thuringiensis* and *B. sphaericus* depending on the purpose of application. Although *B. thuringiensis* remains the best host to synthesize endotoxin proteins, other bacteria also hold some potential. Major disadvantages of most of the other bacterial species we discussed as hosts were the low level of toxin protein yields and/or instability of the toxin gene(s) after introduction to these species. As molecular biology and genetic engineering techniques advance, we expect that researchers will overcome these barriers and develop much better recombinant bacteria with improved efficacy for insect pest control. Ideally, the design of recombinant bacteria should take into consideration the key principles of resistance management, namely, mixtures of toxins are better than single toxins, especially where the toxins have different modes of action, and where specific proteins are known that delay resistance, such as Cyt proteins in the case of mosquitoicidal bacteria, these should be included in the constructs.

The application of recombinant DNA techniques to improving insecticidal bacteria, which began more than two decades ago, was initially met with a high degree of enthusiasm, followed by the establishment of many small biotechnology companies. At the same time, techniques were developed for generating transgenic crops resistant to insects based on the Cry proteins of *B. thuringiensis*. These crops, such as Bt cotton and Bt corn have been an enormous success, and currently constitute a multi-billion dollar industry. Many of the recombinant bacterial insecticides under development in the 1980's and 1990's targeted the same pests on the same crops. In addition, new insecticides, such as imidacloprid and the spinosids came to market. Due to a combination of these events, most of the new biotechnology companies focusing on recombinant bacteria failed. Nevertheless, the extension of the use of *B. thuringiensis* endotoxins in crop plants must be considered one of the key advances, if not the key advance, in pest control technology of the last half of the 20th century. While clearly this success has dimmed interest in recombinant bacterial insecticides (and many other microbial pesticides), there remains an enormous number of crops and markets where these may be useful, and thus justify continued research and development. For example, the market for bacterial insecticides to control nuisance and vector mosquitoes continues to expand, and, as we have shown, recombinants based on *B. thuringiensis* and *B. sphaericus*, are much more efficacious than the wild type species used in current commercial products. With respect to crop pests, there are many crops that have not been transformed with endotoxin genes, including lettuce and cabbage, tomatoes, celery, fruit crops and grapes where lepidopterous insects continue to be major pests. Thus, though the economic

prospects may not be as large as they were twenty years ago, many opportunities remain for the development of new and more efficacious recombinant bacterial insecticides. The higher specificity and environmental safety of the recombinants compared to synthetic chemical insecticides, along with increases in efficacy that reduce the cost of production, provide reasons for optimism that these bacteria will play a significant role in future pest and vector control programs.

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