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Mosquitocidal Toxins of Bacilli and Their Genetic Manipulation for Effective Biological Control of Mosquitoes

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INTRODUCTION

Classification of Mosquitoes and the Diseases They Spread

Mosquitoes transmit some of the world's most serious diseases. The most important disease vectors are members of the subfamilies Anopheliae and Culicidae (Fig. 1). *Anopheles* mosquitoes transmit malaria, filarial parasites (*Wucheria bancrofti* and *Brugia malayi*), and a few arboviruses (176). There are 30 genera in the Culicidae subfamily, but the medically important mosquitoes are *Culex*, *Aedes*, *Mansonia*, and *Armigeres*. *Culex quinquefasciatus* is an important vector of filariasis, and *C. tritaeniorhynchus* transmits Japanese encephalitis. Members of the genus *Aedes* are vicious biters and transmit yellow fever and dengue viruses as well

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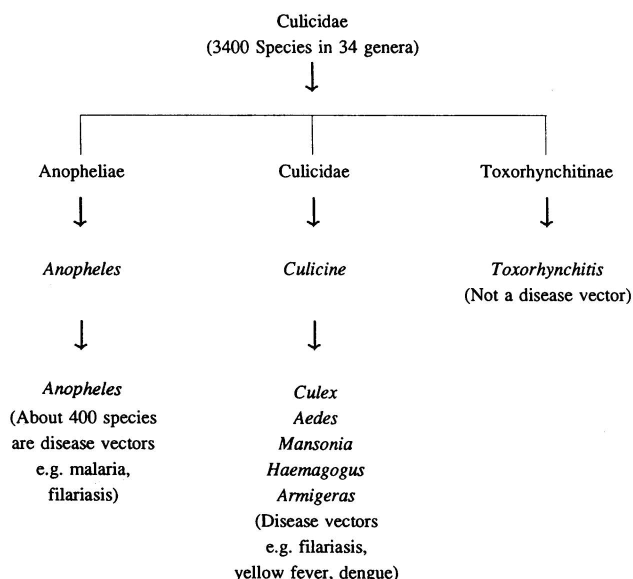


FIG. 1. Summary of mosquito classification (176).

as filariasis in several parts of the world (176). In 1984, it was estimated that there were over 90 million cases of human lymphatic filariasis worldwide.

Four *Plasmodium* species cause malaria, the most common and debilitating disease transmitted by mosquitoes. Approximately 350 to 450 million people live in highly malarious areas, and malaria has an annual incidence of about 270 million cases and kills over 1 million children in Africa alone (45, 110). In 1992 Brazil recorded 600,000 new cases of malaria (45).

Attack against the mosquito is recognized as the only hope for eradication of the malarial parasite (128). Over the last 45 years, the use of chemical pesticides such as dichlorodiphenyltrichloroethane (DDT), gammaxane, malathion, and chlordane has been the method of choice for mosquito control, and the antimalarial drugs chloroquine and the affordable pyrimethamine-sulfadoxine combination have proved successful in lowering morbidity and mortality. However, malaria and other mosquito-borne diseases are on the rise again in many tropical areas (128). In addition, the emergence of pesticide- and drug-resistant mosquitoes, coupled with a clearer appreciation of the long-term detrimental effects of powerful chemicals to nonpest insects and concern about accumulation of pesticides in the food chain and environment, has highlighted the need to quickly develop an alternative. A promising alternative is biological control (129, 156).

Biological Control of Insects

There are many naturally occurring predators, parasites, and pathogens of vector insects including viruses, rickettsiae, fungi, and bacteria, which vary greatly in their mode of infection, site of replication, and mechanism of pathogenicity (167–169). Certain entomopathogenic bacteria, particularly members of the genus *Bacillus* (e.g., *Bacillus thuringiensis* and *Bacillus sphaericus*) produce protoxin crystals during sporulation. These proteins are deposited as inclusions alongside the spore and are highly toxic to susceptible insects which ingest them (for reviews see references 10, 18, and 92). The protoxins dissolve in the alkaline pH of the insect midgut, where they are proteolytically activated and bind to epithelial cell membranes in the brush border. The cells are destroyed, and the larva stops feeding and dies. Depending on the particular strain of *B. thuringiensis*, the protein toxins may be specific for members of the Lepidoptera (butterflies, caterpillars), Coleoptera (beetles), or Diptera (mosquitoes and blackflies), while some are both Lepidoptera and Diptera specific. Each class of toxin effectively kills a narrow range of target insects, and in recent years *B. thuringiensis* strains have been discovered with a target range including organisms other than insects (65).

For nearly 30 years, various *B. thuringiensis* strains have been successfully used as biopesticides, mostly against agriculturally important caterpillar pests (65, 129). The spore/toxin preparations are applied at 10 to 50 g acre^{-1}, and the molecular potency is high compared with that of chemical pesticides (about 300 times higher than that of synthetic pyrethroids or 80,000 times higher than that of organophosphates). The toxins degrade in a few days after their action is complete, and the bacteria appear to be safe for other insects, animals, and the environment (47, 112).

Strains of bacteria which produce mosquitocidal toxins are restricted to *B. thuringiensis* subsp. *israelensis*, a few strains in other *B. thuringiensis* serotypes, *B. sphaericus*, and *Clostridium bifermentans* serovar *malaysia*. Field trials with various formulations of *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* against *Aedes* and *Culex* mosquitoes have demonstrated their safety and potential for controlling mosquito larvae (47, 53, 110, 132, 216). However, operational success against the three major mosquito genera, *Culex*, *Anopheles*, and *Aedes*, has been limited and confined mainly to temperate regions of the world, where these insects are merely a nuisance. Mosquitocidal bacteria currently represent a tiny fraction of the biopesticide market, which in turn is still only a small fraction of the annual worldwide pesticide market.

In this review these issues will be addressed by focusing on the narrow target range of the mosquitocidal bacilli and their lack of persistence in the larval feeding zone. The importance of a detailed understanding of the structure and function of mosquitocidal toxins in the derivation of recombinant strains with a broad mosquito host range will be emphasized. Recent advances with novel types of recombinant microorganisms and new cloning strategies, which together have the potential to provide effective control of a wider range of mosquito species for a longer duration than the naturally occurring bacilli, will be described.

CHARACTERIZATION OF MOSQUITOCIDAL BACILLI

B. sphaericus

B. sphaericus is widespread in soil and aquatic environments (22, 151). The most widely studied mosquitocidal strains of *B. sphaericus* belong to serotypes H5a5b (e.g., strains 1593 and 2362), H25 (e.g., strain 2297), and H6 (e.g., strain IAB 59), which are all in the highly toxic group, and serotype H2 (e.g., strain SSII-1), which falls in the low-toxicity group (55, 79, 104, 157, 182). In general, members of highly toxic groups have certain positive characteristics which are relevant to their use as microbial insecticides: their toxic crystals are protected within the exosporium, they are stable over a range of temperatures, and they may remain insecticidal in polluted water (18, 22, 216).

B. thuringiensis

Compared with *B. sphaericus*, *B. thuringiensis* comprises a much greater diversity of known insecticidal strains. Furthermore, whereas certain *B. sphaericus* strains are toxic exclusively to dipteran pests, particular *B. thuringiensis* strains may be toxic to a wide or narrow range of insect, nematode, or protozoan pests (10, 65). Some *B. thuringiensis* strains kill mosquitoes as well as certain lepidopteran larvae (e.g., *B. thuringiensis* subsp. *kurstaki* HDI) but there are several strains (e.g., *B. thuringiensis* subspp. *kyushuensis*, *darmstadiensis*, and *morrisoni* PG-14) that are toxic primarily for members of the Diptera (e.g., mosquitoes, blackflies, and midges). The morphologies of the protein crystals in *B. thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *morrisoni* are very similar (10), but only in the case of *B. thuringiensis* subsp. *israelensis* have field trials against mosquitoes been carried out (92, 120, 132). *B. thuringiensis* subsp. *israelensis* has significant insecticidal activity against *Aedes* mosquitoes and blackflies, but its toxicity appears to persist for a shorter time than the toxicity of *B. sphaericus* against *Culex* mosquitoes. In addition, *B. thuringiensis* subsp. *israelensis* does not survive long in highly polluted water and is particularly prone to UV inactivation in strong sunlight (132, 134). Nevertheless, as will be discussed in the following sections, the different protein toxins of *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* each have potentially useful properties and specificities.

ISOLATION AND PROPERTIES OF MOSQUITOCIDAL TOXINS

Purification and Characterization

B. sphaericus binary toxin. There is now ample evidence that the mosquito larvicidal activity of the highly toxic strains of *B. sphaericus*, such as 2362, 2297, and 1593, is due to a large extent to the presence of approximately equal amounts of antigenically distinct 51.4- and 41.9-kDa protein toxins, produced during sporulation (99, 220), both of which are required for toxicity (18, 19, 26, 36, 157). These studies also demonstrated that the 42- and 51-kDa proteins do not have to be deposited as crystals to be toxic, although it seems that the noncrystalline and purified forms of these proteins have much lower toxic activity (18). This raises the intriguing question of whether the spore itself plays a role in toxicity or whether the toxins interact synergistically with an as yet unidentified component of the spore.

At variance with the findings that both the 42- and 51-kDa proteins are required for toxicity to mosquito larvae is a more recent report (140a) that inclusions containing only the 42-kDa protein purified from a recombinant *B. thuringiensis* strain were toxic to *Culex pipiens* larvae (50% lethal concentration [LC_{50}], 300 ng/ml). The reason for the discrepancy is unclear, but it should be noted that the 42-kDa protein was expressed in a novel host, *B. thuringiensis* SPL407. Perhaps the 42-kDa toxin folded differently and was stabilized in this strain, or, alternatively, the 42-kDa toxin may have interacted synergistically with a spore component present in the inclusion from this novel host strain.

B. sphaericus SSII-1 toxin. The weakly toxic strains of *B. sphaericus* have received less attention than the highly toxic strains which produce the binary toxin (the 42- and 51-kDa proteins). *B. sphaericus* SSII-1 differs in many respects from the high-toxicity strains such as 1593 and 2362 (137). SSII-1 does not sporulate well. Its toxicity, which is about 1,000-fold lower than that of the high-toxicity strains, appears during vegetative growth and is cell associated. Compared with strain 1593, SSII-1 has a relatively unstable toxin, whose activity is destroyed by heat, refrigeration, freezing and thawing, and methods of cell disruption which do not affect the toxicity of strain 1593 (136, 137). The conclusion that a different toxin is present in SSII-1 from that in the highly toxic strains was supported by the finding that DNA probes to the 42- and 51-kDa toxin genes failed to hybridize to total DNA from the weakly toxic strains SSII-1, 1889, 2173, and Kellen K (17, 117). Proof of a distinct mosquitocidal toxin came from the cloning of a gene for a 100-kDa toxin and its expression in *Escherichia coli* (190) (see below). The high-toxicity strains 1593M, 2297, and 2362 all appear to have the gene for the 100-kDa toxin, and it appears to be expressed during their vegetative growth (190).

B. thuringiensis toxins. Unlike *B. sphaericus*, both *B. thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *morrisoni* PG-14 produce three major inclusion types during sporulation which are toxic for mosquito larvae (64, 208). The crystals within the inclusions are composed of or contain at least five polypeptides of approximately 134, 128, 58 (a minor component), 70, and 27 kDa, whose size and antigenicity clearly distinguish them from the toxins of *B. sphaericus* (64). It should be borne in mind that the sizes of these proteins reported in the literature lie in the range 98 to 145, 93 to 135, ~58, 65 to 72, and 25 to 28 kDa, respectively (64, 92) (Table 1).

Before the *B. thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *morrisoni* toxin genes were cloned, the diversity of polypeptides and uncertainty about the presence of contaminants and breakdown products hampered identification of the proteins responsible for larvicidal activity. Now a better, although still incomplete, picture of their mosquitocidal activity has emerged. The 27-kDa CytA protein is hemolytic and has no or marginal mosquitocidal activity (see reference 58 and references therein). In contrast, the 134-, 128-, and 70-kDa proteins are not hemolytic, and individually they show significant toxicity to *Aedes* mosquito larvae (38, 57, 95, 152, 160). This has been confirmed with the individual recombinant proteins expressed in heterologous cells which are free of all other toxins. Thus, the 134-kDa (CryIVA), 128-kDa (CryIVB), ~58-kDa (CryIVC), and 70-kDa (CryIVD) proteins (Table 1) are all toxic to *A. aegypti* larvae to various degrees, and the 70-kDa protein has moderate activity against *C. pipiens* (7, 57, 61, 155, 193, 194, 204). In contrast, neither the 134-kDa CryIVA protein nor the *cryIVC* gene product is by themselves toxic to *C. pipiens* mosquitoes (57).

One report indicates that *B. thuringiensis* subsp. *israelensis* toxins are glycoproteins containing approximately 1.0% neutral sugars and 1.7% amino sugars and that *N*-acetylglucosamine-containing oligosaccharides may be important for full activity of the δ-endotoxins (161), but little subsequent work in this direction has been done, and the findings remain unconfirmed.

None of the crystal proteins of *B. thuringiensis* subsp. *israelensis* is as toxic as the intact crystal complex containing all the proteins (64, 92). One explanation is that synergism may be important for toxicity. There are several reports indicating synergism of toxicity between the 27-kDa CytA protein and some of the other proteins in the crystal (38, 95, 214, 221). Of particular interest is a mathematical analysis (186) which demonstrated that there is a positive synergism between the 27-kDa CytA protein and either the 70-kDa toxin or a mixture of the 128- and 134-kDa toxins (38). This

TABLE 1. Mosquitocidal proteins[a] and their genes

Bacterium and gene type (92)	Reported designation of gene (18, 92, 190)	Predicted mol mass (kDa)	Predicted no. of amino acids	Reported mol mass of protoxin (kDa)	Reported mol mass of gut processed or activated toxin (kDa)	Reference[b]
B. sphaericus	51-kDa gene	51.4	448	51	43	16
B. sphaericus	42-kDa gene	41.9	370	42	39	88
B. sphaericus	100-kDa gene	100.6	870	~100	?	190
B. thuringiensis subsp. *israelensis cryIVA*	125- to 140-kDa, ISRH3, Bt303	134.4	1,180	98–145	53–67	203
B. thuringiensis subsp. *israelensis cryIVB*	125- to 140-kDa, ISRH4, Bt8	127.8	1,136	93–135	53–67	40
B. thuringiensis subsp. *israelensis cryIVC*	ORF1	77.8[c]	675	? 58[c]	?	193
B. thuringiensis subsp. *israelensis cryIVD*	*cryD*, 65- to 72-kDa	72.4	643	65–72	30–38	61
B. thuringiensis subsp. *israelensis cytA*	26- to 28-kDa gene	27.4	248	25–28	25	201

[a] Only the better-characterized toxins are given.

[b] Reference of the first submitted publication describing the gene type. The predicted molecular masses and number of amino acids are derived from these publications.

[c] A protoxin of 77.8 kDa, the predicted product of ORF-1, has not been found in *B. thuringiensis* subsp. *israelensis* crystals. However, ORF-1 was translated into a protein of ~58 kDa in recombinant *E. coli* and *B. subtilis* which corresponds in size to a protein (protoxin?) of 58 kDa found in *B. thuringiensis* subsp. *israelensis* crystals (69, 193).

analysis is in agreement with results of other experiments showing that mixtures of the 27-kDa protein and either the 70-kDa toxin or the 128- and 134-kDa toxins were more toxic than would have been predicted from their individual activities (95, 214, 221). At variance with these findings are the results of a more recent study showing that deletion by in vivo recombination of the 27-kDa gene from *B. thuringiensis* subsp. *israelensis* does not affect the mosquitocidal activity of this strain toward *Culex* and *Aedes* mosquitoes (58). There have been two papers, both describing experiments with the 128-kDa CryIVB toxin, suggesting a synergistic effect not involving the 27-kDa protein (8, 57). The toxicity of a mixture of the 128- and 58-kDa (CryIVC) proteins of *B. thuringiensis* subsp. *israelensis* to *Culex* larvae was much greater than that of either protein alone, but synergism was not demonstrated mathematically (57). The contribution of the individual *B. thuringiensis* subsp. *israelensis* toxins toward overall toxicity and the importance of their crystalline form must be clarified before synergistic combinations can be predictably engineered in new recombinant strains.

Cloning and Comparison of Genes

***B. sphaericus* binary toxin.** The genes encoding the 51- and 42-kDa toxins have been cloned from several high-toxicity strains and are found to encode proteins of predicted molecular masses 41.9 and 51.4 kDa (16, 17, 20, 23, 59, 88, 89) (Table 1). All strains so far examined contain both genes within a similar ~3.0- to 3.5-kb *Hin*dIII fragment (116a), whereas strains of low larvicidal activity do not appear to contain either gene (17, 18). The nucleotide sequences in the coding regions of strains 1593, 2317.3, and 2362 are identical, and the greatest variation is shown by strain 2297 (a difference of 25 nucleotides from strain 2362 (23). The region upstream of the open reading frame (ORF) for the 51-kDa protein clearly contains a promoter (14, 26) but does not contain sequence motifs which may be readily identified as being similar to any of the *B. subtilis* sporulation promoters (131).

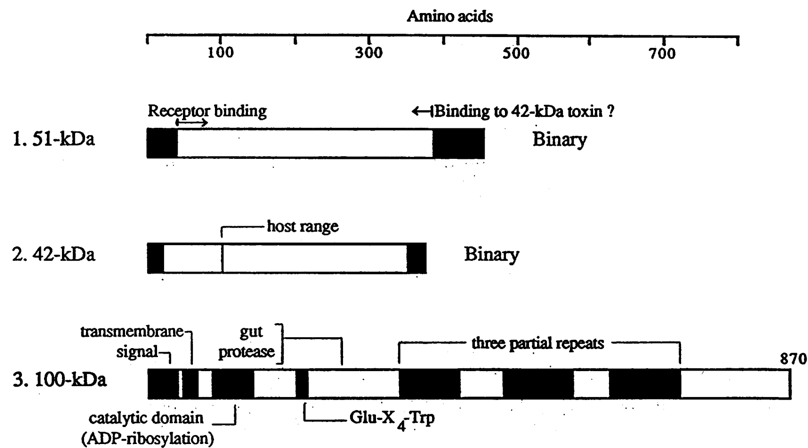
It is now clear that the binary toxin genes of some strains are located on the bacterial chromosome (116a). Strain 1691 lacks detectable plasmids, implying that its toxin genes are chromosomal (181). Complete linkage of toxin sequence to the H-antigen serotype further suggests that these genes are chromosomal (23, 54, 55).

***B. sphaericus* SSII-1 toxin.** A gene coding for a protein of 100.6 kDa and 870 amino acids and having no significant homology with the 51- and 42-kDa genes has been cloned from the low-toxicity strain *B. sphaericus* SSII-1 (190). Immediately upstream of the ORF is a ribosome-binding site with a 10-base homology to the 3′ terminus of *B. subtilis* 16S rRNA. The ribosome-binding site is preceded by a putative promoter which shows good homology to the consensus sequence for the σ^{55} vegetative promoter of *B. subtilis* (−10 TATAAA and −35 TTGACA) (130). The 100-kDa gene codes for a mosquitocidal toxin, since expression of this gene in *E. coli* under the control of its own promoter results in significant larvicidal activity (190). A DNA clone with a similar restriction enzyme map to that of the 100-kDa toxin gene was isolated from *B. sphaericus* 1593M, but the DNA was not sequenced (184).

***B. thuringiensis* subsp. *israelensis* toxins.** *B. thuringiensis* subsp. *israelensis* and its close relatives express a heterogeneous group of mosquitocidal crystal proteins (CryIV class) under the control of sporulation-dependent promoters (92) (Table 1). These genes, together with the gene coding for the 27-kDa cytolytic protein (CytA class), are all located on the same 72-MDa plasmid. None of these genes shares any homology with the 51-, 42-, or 100-kDa toxins of *B. sphaericus*.

The *B. thuringiensis* subsp. *israelensis cryIVA*, *cryIVB*, *cryIVC*, and *cryIVD* genes encode proteins with predicted molecular masses of 134.4, 127.8, 77.8, and 72.4 kDa, respectively. Table 1 summarizes the relationships of these ORFs to those of the known crystal proteins isolated from the crystal complexes. Briefly, the *cryIVA* and *cryIVB* genes each encode different ca. 130-kDa proteins (designated 134 and 128 kDa, respectively, in Fig. 2B), which are proteolytically converted into toxic N-terminal core fragments of 53 to 67 kDa. The exact cleavage sites are not known (38, 40). The *cryIVC* gene encodes an initial translation product of 77.8 kDa (Table 1; Fig. 2) and has some homology to the 5′ half of the *cryIVA* and *cryIVB* genes (193). When introduced into *B. subtilis* or into a nontoxic mutant of *B. thuringiensis* subsp. *israelensis*, the gene directs the synthesis of a toxic

A. ***Bacillus sphaericus*** **toxins**



B. ***Bacillus thuringiensis*** **toxins**

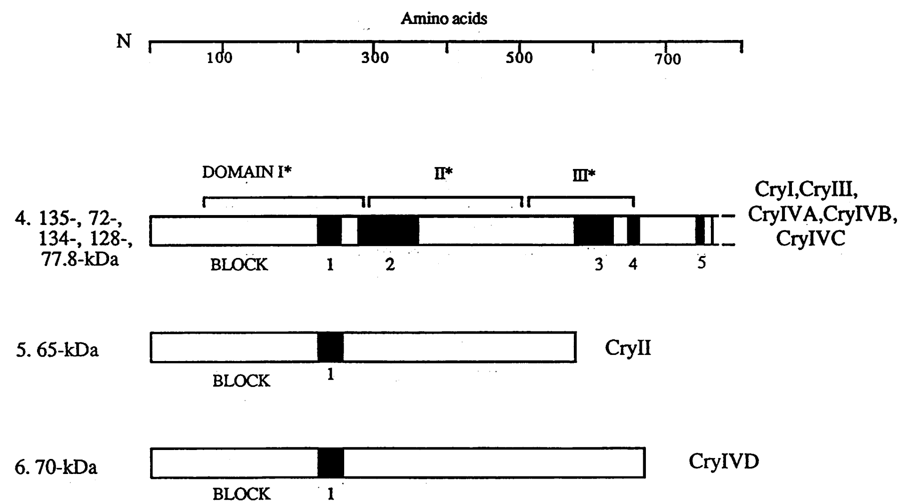


FIG. 2. Diagrammatic summary of structural and functional domains. (A) *B. sphaericus* mosquitocidal δ-endotoxins (protoxins). The shaded N- and C-terminal regions in the 51- and 42-kDa toxins are not essential for toxicity. The various identified or putative functional regions (or motifs) of the 100-kDa toxin are indicated and shaded. (B) *B. thuringiensis* insecticidal δ-endotoxins. The amino acid numbering is based on the CryIV class of toxins and is slightly different in the CryI, CryII, and CryIII toxin class; 134-, 72-, 134-, 128- and 77.8-kDa refer to the CryI, CryIIIA, CryIVA, CryIVB, and CryIVC classes of toxins, respectively. In the class of toxins denoted 4, only the N-terminal (~760 amino acid) toxic core fragment is shown. *, Domains I, II, and III correspond to the three structural domains proposed for the beetle-active CryIIIA toxin based on its three-dimensional structure (116). The shaded areas show the locations of the partially conserved blocks 1 to 5 (92). The protein sizes in kilodaltons given to the left of each represented toxin class are intended to identify each protein and are approximate.

protein of ca. 58 kDa (Table 1) (69, 193), which corresponds in size to a minor protein of 58 kDa found in *B. thuringiensis* subsp. *israelensis* crystals (69). The *cryIVD* gene encodes a 72.4-kDa protein (Table 1; designated 70-kDa in Fig. 2B), which is a major component of the toxic crystal complex along with the *cryIVA*, *cryIVB*, and *cytA* gene products (61, 64). The 72.4-kDa protein is proteolytically activated to a fragment of ca. 30 kDa, but its exact location in the intact protein is not known (38, 95). The *cryIVA*, *cryIVB*, and *cryIVC* genes have significant homology to one another which is scattered throughout their coding regions, whereas the similarity between the *cryIVD* gene and the other crystal genes is much more restricted (Fig. 2; see below).

The ORF for the *cytA* gene from *B. thuringiensis* subsp. *israelensis* encodes a hydrophobic protein (CytA) with a predicted molecular mass of 27.4 kDa (Table 1), which corresponds to the 26- to 28-kDa crystal protein cytolytic to a variety of invertebrate and vertebrate cells (204, 206). A second cytolytic protein, CytB from *B. thuringiensis* subsp. *kyushuensis*, has been described with a deduced molecular mass of 29.2 kDa and 30% identity with the 27.4-kDa CytA protein from *B. thuringiensis* subsp. *israelensis* (103a). The larger size of CytB compared with CytA appears to be due to an additional sequence in CytB after the CytA C terminus (103a).

Mechanism of Action and Structure-Function Relationships

***B. sphaericus* binary toxin.** The weight of evidence strongly suggests that the 51- and 42-kDa proteins of *B. sphaericus* act together as a binary toxin and that both are required for toxicity to susceptible mosquito larvae (26, 27, 29, 42, 52, 175). This has been most clearly demonstrated in mixing experiments with recombinant strains of *E. coli*, *B. sphaericus*, or *B. subtilis* which express only one or other of the toxins (18, 26, 27). The maximal toxicity of the recombined crystal proteins was found at a 1:1 ratio (15, 52), and radiolabeled toxins bound in approximately equal amounts to the gastric cecum (GC) and posterior midgut (PMG) of *C. quinquefasciatus* larvae (52). In the report (140a) which showed that the 42-kDa protein expressed in *B. thuringiensis* is toxic to *C. pipiens* larvae, the toxicity was significantly lower than was previously found for inclusions or mixtures of the 42- and 51-kDa proteins (18). Moreover, the toxicity of the 42-kDa protein was greatly stimulated by the addition of the 51-kDa protein (140a).

Purified recombinant 42-kDa toxin is "toxic" to cultured mosquito cells (15), but this phenomenon may not be a true reflection of all the events occurring at the epithelium in the larval gut. Cultured cell lines do not have the peritrophic membrane barrier, and they may have different, lower-affinity receptors or another internalization mechanism for the toxin (see below).

Many studies have established that the action of the crystal toxins on susceptible *C. quinquefasciatus* larvae involves the following series of steps: (i) ingestion of the crystal by the filter-feeding larva; (ii) solubilization of the crystal in the midgut by the alkaline pH; (iii) proteolytic processing of the 51- and 42-kDa protoxins to ~43- and 39-kDa proteins, respectively; (iv) binding of the processed proteins to epithelial cells of the GC and PMG; (v) internalization of both toxins, as a result of which toxicity is exerted by an unknown mechanism involving the appearance of areas of low electron density, vacuolation, and mitochondrial swelling; and (vi) lysis of the cells (147; see reference 18 and references therein).

It was originally shown that the 42-kDa protein is slowly converted into a stable ~39-kDa derivative in the gut while the 51-kDa protein is rapidly converted into a stable ~43-kDa protein (19, 27, 28). Treating the purified or recombinant 42-kDa protein with protease-containing larval gut preparations from several mosquito species or with trypsin or chymotrypsin led to the appearance of a 39-kDa protein with a toxicity greater than 50-fold higher than that of the 42-kDa protein for tissue culture-grown cells of *C. quinquefasciatus* (15, 28). The 39-kDa processed form of the 42-kDa toxin has all the determinants for toxicity to cultured mosquito cells, and its toxicity is enhanced neither by the 51-kDa toxin nor by the 43-kDa product of its proteolysis (15).

These observations supported the idea that the 39-kDa processed form of the 42-kDa protein is the toxic component, but they did not answer the question of which role the 43-kDa processed form of the 51-kDa toxin plays in the toxicity of the binary toxin towards mosquito larvae. Binding studies with the 51- and 42-kDa proteins which had been separately purified from recombinant *E. coli* cells and labeled with a fluorescent dye have shed light on the individual roles of and structure-function relationships in these toxins (147). There was localized binding to the PMG and GC when the 51-kDa protein was fed alone to *Culex* larvae, whereas the 42-kDa protein alone showed nonspecific binding throughout the gut and GC (Fig. 3, top). In the presence of the 51-kDa protein, the binding pattern of the labeled 42-kDa protein changed and became localized to the PMG and GC, i.e., where the 51-kDa protein alone bound (Fig. 3, bottom) (147). Moreover, internalization of the 42-kDa protein was observed only in the presence of the 51-kDa protein, and conversely the 51-kDa protein was internalized only in the presence of the 42-kDa protein. It was proposed that the 51-kDa protein acts as the cell-binding component of the binary toxin and directs the binding of the 42-kDa protein to the sites where it exerts toxicity, i.e., the PMG and GC (49, 50, 147). Since it has been shown that the 51- and 42-kDa proteins associate very strongly with each other (52, 138, 178), it is plausible that the 42-kDa protein binds simultaneously to sites on the 51-kDa protein and the cell membrane, which together form a high-affinity site and/or result in a configuration necessary for internalization of both proteins. As mentioned above, each toxin is internalized only in the presence of the other, so a dual role for the 51-kDa protein in cell binding and toxic action cannot be ruled out.

The importance of localized toxin binding to the putative cell surface receptor of the gut epithelium was demonstrated by a correlation between the absence of specific binding of the toxin proteins to the PMG and GC and a lack of toxicity to larvae of toxin-resistant mosquitoes (49, 50). The 51-kDa toxin did not bind at all to the gut of nonsusceptible *Aedes aegypti* larvae, and the 42-kDa protein did not bind specifically to the PMG or GC in these larvae, even when mixed with the 51-kDa protein (147). Moreover, in four *Anopheles* species, the binding of the binary toxin was not localized to the PMG and GC and the toxin was not internalized. This correlates with the finding that *Anopheles* mosquitoes are ~4- to 40-fold less sensitive to the binary toxin than are *Culex* mosquitoes (50). Very recently, the presence of specific receptors for the purified *B. sphaericus* 1593 binary toxin on brush border membrane fractions from *C. pipiens* larvae was demonstrated by an in vitro binding assay (141). Mixtures of the 51- and 42-kDa proteins bound equally well to a single class of toxin receptors with a dissociation constant (K_d) of 20 nM, which is similar to the values obtained for the Lepidoptera-active δ-endotoxins (66, 91, 196). No specific binding of the binary toxin was detected with *A. aegypti* larval gut membranes (141), in agreement with the results of the fluorescence-labeling studies (49, 50, 147). Since the putative receptor(s) for the *B. thuringiensis* δ-endotoxins in certain lepidopteran larvae are glycoproteins (68, 101, 103), it is surprising that no evidence could be found for the participation of sugars or proteins in recognition and binding of the binary toxin to the receptor in *C. pipiens* larvae (141).

The idea that the presence or availability of specific membrane receptors is a crucial determinant of toxin susceptibility is consistent with results of other experiments which show that the differences in susceptibility to the binary toxin between *Aedes* and *Culex* larvae are not due to differences in rates of ingestion of the cells, dissolution of the toxin, or specificity of the gut proteases (5). The mode of action of the toxins is unknown, but in a toxin-resistant cell line of *C. quinquefasciatus*, binding and internalization of the toxin can occur without cell death, implying that the lethal action of the toxin on cultured cells occurs after initial interactions with the cell membrane and internalization (174).

In studies of the gut binding of the fluorescent deletion derivatives of the 51- and 42-kDa proteins (Fig. 4), it was found that the presence of the N terminus of the 51-kDa

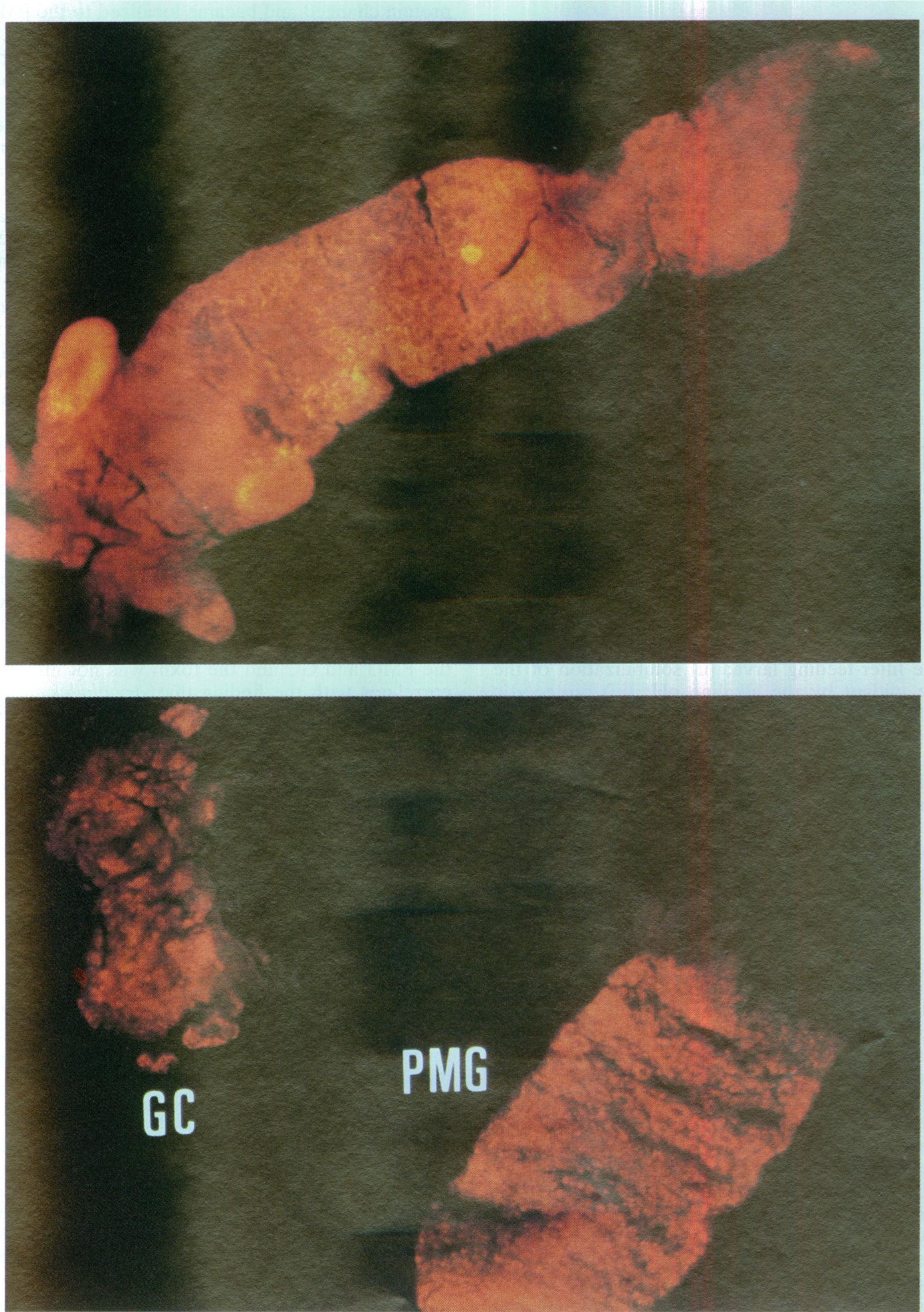


FIG. 3. (Top) Midgut of *C. quinquefasciatus* larvae fed fluorescently labeled 42-kDa protein from *B. sphaericus* 2297. Strong fluorescence is observed over the entire midgut (middle and right) and gastric caecum (left). Multiplication, ×400 (147). (Bottom) Gut of *C. quinquefasciatus* larvae fed a combination of fluorescently labeled 42-kDa protein and unlabeled 51-kDa protein. Note the strong localized binding of the labeled 42-kDa protein to the GC and PMG in the presence of the 51-kDa protein. Multiplication, ×250 (147). Reprinted with permission of the publisher.

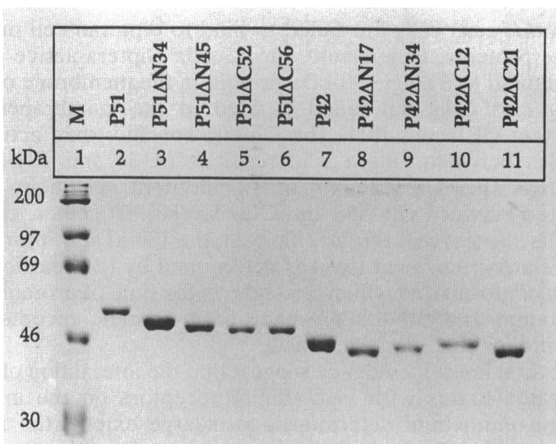


FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified 51-kDa protein (P51) and 42-kDa protein (P42) from *B. sphaericus* 2297 and their N-terminal (N) and C-terminal (C) deletion derivatives. Proteins were expressed as fusions in *E. coli*, cleaved, and purified as described previously (147). The gel was stained with Coomassie Blue. Molecular size markers are at left. P51ΔN34, 51-kDa protein lacking 34 N-terminal amino acids; P42ΔC12, 42-kDa protein lacking 12 C-terminal amino acids (147). Reprinted with permission of the publisher.

protein between amino acids 39 and 45 was required for its localized binding to the PMG and GC (147). In contrast, the nontoxic C-terminally truncated derivatives of the 51-kDa protein exhibited localized binding but were unable to direct the localized binding of the 42-kDa protein and its internalization into the epithelium (Fig. 2). The data suggest that the presence of the N-terminal region of the 51-kDa protein is required for receptor binding, the presence of the C-terminal region is required for binding the 42-kDa protein, and internalization of the 42-kDa protein following its interaction with the 51-kDa protein is important for toxicity (147). The results are also compatible with the above model, which proposes that a high-affinity binding site for the 42-kDa protein is formed by a juxtaposition of a site on the 51-kDa toxin (involving the C terminus) and a site on the cell surface.

Much less has been done to probe the function of the individual amino acid residues in the binary toxin. The 51- and 42-kDa toxins of *B. sphaericus* 1593, 2362, and 2317.3 in serotype H5a5b are identical and differ in a total of eight amino acid positions from the binary toxin of strain 2297 (23). Since 2297 is nontoxic to *Aedes* mosquitoes (unlike the H5a5b strains), any or all of the eight amino acid positions could contribute to the toxicity to *Aedes* mosquitoes. There is evidence that the specificity of the *B. sphaericus* binary toxin from strains 2362, 2317.3, and 1593 for *Aedes* mosquitoes may be due largely to amino acids centered around position 100 in the 42-kDa protein (21). This is based on the gain in two functions, overall *A. aegypti* mortality and growth retardation, caused by the change from serine to alanine at position 104 or from phenylalanine to valine at position 99 in the 42-kDa protein of strain 2297. Single changes in the 51-kDa protein did not affect the host range, although some did affect toxicity (21). These findings are in accord with the idea that the 42-kDa protein is responsible for the host range whereas overall toxicity is the function of both proteins.

Ultimately, the elucidation of the X-ray crystal structure of the cell receptor for the binary toxin, together with the structure of the binary toxin itself, will provide important insights into the molecular interactions with the cell surface leading to toxicity. To this end, the 51- and 42-kDa proteins have recently been expressed at high levels in recombinant *E. coli* cells and purified to homogeneity (Fig. 4, lanes 2 and 7) (147).

***B. sphaericus* SSII-1 toxin.** As previously mentioned, the 100-kDa mosquitocidal toxin of *B. sphaericus* SSII-1 shows no homology with any known insecticidal toxin (190). However, the following amino acid motifs may be identified (Fig. 2), beginning from the N terminus: (i) a potential hydrophobic signal sequence (amino acids 1 to 30) and transmembrane domain (amino acids 43 to 60); (ii) a motif (amino acids 95 to 148) resembling a sequence essential to the toxicity of the S1 subunit of pertussis toxin and the A subunits of cholera toxin and *E. coli* heat-labile toxin; (iii) a short signature (Glu-X-X-X-X- Trp; amino acids 219 to 224) also present in the exotoxin of *Pseudomonas aeruginosa* and the A subunit of diphtheria toxin; and (iv) three long partially repeated sequences (amino acids 330 to 704).

The presence of the putative transmembrane domain is essential for toxicity, whereas the signal peptide is dispensable (188). Since the toxicity of *B. sphaericus* SSII-1 is cell associated, it is possible that either the transmembrane sequence or the signal peptide (or both) anchors the toxin to the inner or outer membrane.

The bacterial toxins such as pertussis toxin and diphtheria toxin exert their actions by ADP-ribosylation of cellular proteins. The homologies between the 100-kDa toxin of SSII-1 and these other bacterial toxins strongly suggest that the 100-kDa toxin likewise acts by ADP-ribosylation. The following results support this suggestion. Both trypsin and mosquito larva gut proteases cleave the 100-kDa toxin into very similar ~27-kDa N-terminal and ~70-kDa C-terminal fragments (188). Both the 27-kDa peptide and a 97-kDa peptide (i.e., the 100-kDa toxin lacking the nonessential N-terminal signal sequence) are by themselves unable to ADP-ribosylate proteins in cultured *Culex quinquefasciatus* mosquito cell extracts. However, a nontoxic 57-kDa peptide (amino acids 30 to 493), which contains all the regions of homology with the catalytic peptides of the ADP-ribosyltransferase toxins (41, 60), appears to ADP-ribosylate proteins of 38 and 42 kDa in extracts of mosquito cells (187). Interestingly, the 42-kDa cellular protein corresponds in size to 41- to 42-kDa mammalian regulatory G proteins, which are ADP-ribosylated by pertussis toxin, cholera toxin, and *E. coli* heat-labile toxin (142). The 42-kDa protein is also similar in size to mammalian nonmuscle actin, a known target of *Clostridium botulinum* C2 toxin (2).

Both the 97-kDa toxin and the 70-kDa C-terminal peptide, but not the 57-kDa N-terminal peptide, are able to rapidly induce dramatic morphological changes in *C. quinquefasciatus* cells in culture, but the ~70-kDa C-terminal peptide lacks ADP-ribosylation activity (187). Thus, there are at least two functional domains, both of which are required for toxicity to larvae. These are a C-terminal (70-kDa) peptide which causes the dramatic morphological changes to cultured cells and an N-terminal peptide which has ADP-ribosylation activity (187). More work must be done to elucidate the mechanism of cleavage activation and to determine whether the effects on cultured cells are relevant to the binding to the gut epithelium and subsequent toxic action. The pathology of mosquito cells treated with the 100-kDa toxin from SSII-1 is very different from that described for cells treated with the 51- and 42-kDa binary toxin of *B.*

sphaericus 1593 (48), suggesting distinct mechanisms of action for these two *B. sphaericus* toxins. This finding may have significance for enhancing the potency of mosquitocidal toxins by expressing the 100-kDa and binary toxins together in one recombinant cell (see below).

***B. thuringiensis* toxins.** The mechanism of toxic action of the crystal proteins from *B. thuringiensis* has been studied in some depth only for the Lepidoptera- and Coleoptera-active toxins belonging to the CryI and CryIII classes and, to a less extent, for the Lepidoptera- and Diptera-specific *cryII* class (74, 92). However, because the mosquitocidal toxin genes, particularly those in the CryIVA and CryIVB classes, show significant amino acid sequence and secondary-structure homology with the *cryI* and *cryIII* gene products (Fig. 2), it is likely that their mechanisms of action will all turn out to be similar. In fact, there are similarities in the actions of the *B. thuringiensis* toxins and *B. sphaericus* mosquitocidal toxins (see above). Therefore, the following discussion will not only cover what is known about the *B. thuringiensis* mosquitocidal toxins but will also speculate about their mechanisms of action and structure-function relationships based on comparisons with the better-studied CryI, CryII, and CryIII classes of *B. thuringiensis* toxins.

The effects of *B. thuringiensis* toxins have been studied both on brush-border midgut membranes (171) and on cultured insect cells (102). In the latter cells, several *B. thuringiensis* strains were used, including *B. thuringiensis* subsp. *israelensis*. These studies, together with results obtained with susceptible larvae, have established the following series of steps: (i) ingestion of the crystal-containing spore; (ii) solubilization of the crystal in the midgut by the alkaline pH; (iii) cleavage by gut proteases of the ~70- to 135-kDa protoxins to release the active δ-endotoxins; (iv) high-affinity binding ($K_D \approx 0.1$ to 20.0 nM) of the activated toxins to specific brush border membrane proteins (91, 197); (v) induction of nonspecific pores (0.5 to 1.0 nM) which function as leakage channels, resulting in the net influx of ions and inflow of water (102); and (vi) cell swelling and colloid osmotic lysis.

Which factors determine the often extreme insect specificity of the *B. thuringiensis* mosquitocidal toxins? Three obvious factors which may govern the host range of toxins are (i) differences in the larval gut which affect the solubility of the crystal complex, (ii) the efficiency with which the protoxin is processed, and (iii) the expression or availability of putative membrane receptors for the toxins in the gut of the insect.

The efficiency of solubilization of inclusion proteins can depend on protoxin composition and is a factor in toxicity to certain insects (11). However, dramatic differences in insect specificity are maintained even after solubilization of the crystal complex (92).

Can variations in pH and the spectrum of gut proteases in different insect groups generate toxins of different potencies and specificities? In one case, a 130-kDa protoxin from *B. thuringiensis* subsp. *aizawai* was processed to a 55-kDa protein by trypsin digestion, and this preparation was toxic to lepidopteran (*Choristoneura fumiferana* CFI) cells but not to dipteran (*A. albopictus*) cells. In contrast, when the protoxin was activated by sequential treatment first with trypsin and then with *A. aegypti* gut proteases, the resulting 53-kDa polypeptide was now toxic only to dipteran (*A. albopictus*) cells (80, 82). Moreover, results of affinity-labeling experiments indicated that the 55-kDa Lepidoptera-active protein had specific affinity for 120- and 68-kDa membrane proteins in lepidopteran (*C. fumiferana* CFI and *Heliothis zea*) cells but failed to bind to dipteran cell membrane proteins. Conversely, the 53-kDa Diptera-active protein bound to a single 90-kDa protein in the membrane of *A. albopictus* cells but failed to bind to the membranes of resistant CF1 cells (81). The Diptera-specific toxin activity resides between amino acid residues Ile-30 and Gly-558, whereas the determinants of Lepidoptera specificity are located between Gly-558 and Glu-595 (83). Together, these results suggest that the specificity of the 130-kDa *B. thuringiensis* subsp. *aizawai* toxin is determined by (i) a particular toxin conformation which depends on the type of proteolytic activation and (ii) the presence of membrane receptor(s) specific for that conformation.

Several lines of evidence suggest that the interaction of the activated toxins with high-affinity receptors on the insect midgut epithelium determines, to a large extent, the host range of the *B. thuringiensis* crystal proteins (91, 92, 196, 197). This is well illustrated for certain lepidopteran insects, in which resistance to the CryI class of toxins was associated with much reduced receptor binding affinity or the complete absence of high-affinity sites (66, 198). In other studies, the toxin sensitivity of insects was not proportional to receptor-binding affinity, suggesting that the presence of binding sites does not necessarily lead to insecticidal activity (68, 76, 211). Apart from the 130-kDa dual-specificity toxin from *B. thuringiensis* subsp. *aizawai*, the relationship of toxin sensitivity and receptors has not been studied for the mosquitocidal toxins.

The toxic domain of the CryIVA, CryIVB, CryIVC, and CryIVD protoxins resides in the N-terminal half of each protein and is equivalent to the gut-activated core toxin, whereas the more highly conserved C-terminal half of each protoxin is not required for toxicity or host range (40, 57, 155, 193) (Table 1). It is probable that the high conservation of C-terminal amino acid sequences is required for some other function such as the formation of crystalline inclusions. No other information has emerged about the function of this C-terminal region, and it will not be considered further.

When the amino acid sequences in the N-terminal half of the CryIV mosquitocidal toxins are compared, it may be seen that the sequences of the CryIVA, CryIVB, and CryIVC toxins share significant homology, which is restricted largely to five distinct regions named blocks 1 to 5 (92). Figure 2 shows diagrammatically the positions of these blocks within the N-terminal half of the protoxins (i.e., the toxic core fragments) and also shows other important structural or functional domains (see below). The CryIVD toxin has extensive homology with the other classes of mosquitocidal toxins, including CryII, only within block 1 (Fig. 2). The five sequence blocks are also conserved in the Lepidoptera-active *cryI* and Coleoptera-active *cryIII* gene products, which likewise show significant homology to the CryII toxins only within block 1 (92, 158). Alignment of representative sequences of the CryI, CryII, CryIII, CryIVA, and CryIVB toxins, together with secondary-structure predictions from various algorithms, revealed a striking structural homology not just in one or a few of the conserved blocks but throughout the entire N-terminal half of each protoxin (44, 158). The N-terminal and C-terminal halves of the toxic core fragments of seven classes of toxins were predicted to consist mainly of similarly located α-helices and β-sheets, respectively (44). It was proposed that the gut-processed insecticidal δ-endotoxins of *B. thuringiensis* have a general fold consisting of at least two domains, an α-helical N-terminal domain and a C-terminal domain of β-sheets (43, 44).

The three-dimensional structure of a CryIIIA beetle-active toxin, determined by X-ray crystallography, generally agreed with these predictions and clearly showed that the processed toxin consists of three structural domains (116) (Fig. 2). These are domain I (amino acids 67 to 290), a seven-helix bundle including long hydrophobic and amphipathic segments well equipped for pore formation in the insect membrane and thus in the cytolytic activities; domain II (amino acids 291 to 500), a three-β-sheet domain proposed to participate in receptor binding and host specificity determination; and domain III (amino acids 501 to 644), a β-sandwich region likely to be essential for maintaining the structural integrity of the whole molecule (116).

Because the CryIIA, CryIVA, CryIVB, and CryIVC mosquitocidal toxins of *B. thuringiensis* have similar predicted secondary structures to the Lepidoptera- and Coleoptera-specific toxins (including CryIIIA) and the three-dimensional structure of a CryIIIA toxin reveals that its core is built of the conserved sequence blocks 1 to 5 (Fig. 2), it is likely that most if not all the insecticidal Cry δ-endotoxins of *B. thuringiensis* adopt similar tertiary conformations. This suggests that it is valid to model the mosquitocidal toxins on the known structure of the CryIIIA toxin. Regions important for toxicity and mosquito host range are likely to be found in the sequences corresponding to the CryIIIA toxin domains I and II, respectively. Attention should be focused on block 1 within domain I (Fig. 2), since it contains sequences critical for eliciting toxicity in the Lepidoptera-active toxins and is well conserved in all insecticidal toxins so far sequenced.

Mutations of specific amino acids within the highly conserved block 1 of several CryI toxins (Fig. 2) reduced or destroyed toxicity without affecting the ability of the mutants to bind to receptors on the plasma membrane of susceptible cells (1, 213). The mutations in block 1, which gave rise to nontoxic proteins unable to inhibit potassium-dependent leucine transport into gut membrane vesicles (213), coincide with an amphipathic α-helical region in domain I of the CryIIIA toxin structure (116). Thus, the results provide some experimental evidence for the prediction that domain I is required for membrane insertion and the inhibition of certain ion-dependent transport processes. Support for the idea that domain II determines host specificity comes from several site-directed mutagenesis studies. A determinant of dipteran (*A. aegypti*) toxicity in a CryIIA toxin was mapped between amino acids 307 and 382 (209), which corresponds to domain II in the three-dimensional structure of the CryIIIA toxin (Fig. 2) (116). Likewise, the specificity-determining regions of various CryI toxins mapped largely within domain II (70, 172). It remains to be determined whether domain II serves only to position domain I to initiate pore formation without any subsequent signal transduction (116). If this is so, the membrane proteins to which domain II binds should be considered binding proteins and not receptors in the classical sense.

ASSESSMENT OF TOXICITY

The assessment of the toxicity of *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* strains in the laboratory and in the field has been covered in a number of articles (22, 67, 110, 132, 151, 216) and will be briefly reviewed here mainly with a view to emphasizing the limitations of existing strains and methods.

Formulation

Formulations of *B. sphaericus* and *B. thuringiensis* which have been successfully used in laboratory trials and in the field include liquid concentrates; dry, primary, technical, or wettable powders; granules; briquettes; and encapsulated forms (22, 132, 216). The powders have a longer shelf life, but the liquid concentrates, although bulky, are easier to prepare. These formulations generally cause high rates of mortality in susceptible mosquito species, but the rapid settling of the bacterial spores leads to a relatively short duration of control under normal field conditions (~1 to ~4 weeks).

There are several possible ways to overcome the settling problem and enhance larval mortality. The more readily dispersible *B. sphaericus* and *B. thuringiensis* powders show lower settling rates, but this approach only goes part of the way to solving the problem. Bacterial particles which leave the surface region are not available for ingestion by mosquito larvae, particularly *Anopheles* larvae, which feed at the water surface (126). The development of floating formulations is desirable, and several types have been tested in the laboratory and in the field (6, 37, 109, 119). With the microlipid droplet encapsulation method, *B. thuringiensis* subsp. *israelensis* was made equally effective against *Anopheles* and *Culex* larvae, unlike the nonencapsulated method, which gave much lower activity against *Anopheles* larvae (37). Similarly, other floating-type formulations had significantly increased activity against *Anopheles* larvae (6). Extensive trials are urgently needed to determine whether floating-type formulations are also effective against *Anopheles* mosquitoes in the field.

Mosquitocidal toxin genes have been expressed in bacteria which normally exist at or near the water surface, such as cyanobacteria and *Caulobacter* species (9, 39, 59, 135, 189) (see below). This promising approach provides a possible solution to the settling problem and may circumvent the need to develop costly floating formulations of bacteria.

Slow-release formulations of *B. thuringiensis* subsp. *israelensis* have been tried with limited effectiveness (144), whereas slow-release floating pellets of *B. sphaericus* 1593 reduced larval populations of *C. quinquefasciatus* for over 8 weeks (111). The cells in slow-release and floating formulations will be more susceptible to UV radiation, which causes a rapid loss of spore viability but not necessarily toxicity (32). Therefore, such formulations may have to include screening agents to block the effects of UV light.

Laboratory Bioassay

For the bioassay of *B. sphaericus* and *B. thuringiensis* subsp. *israelensis*, the most accurate expression of the killing power of the preparation is the LC_{50}, the concentration (or the dilution of final whole culture) of sample in the surrounding water that will theoretically kill 50% of the mosquito larvae in a certain time (48 h for *B. sphaericus*; 24 h for *B. thuringiensis* subsp. *israelensis*) (54). Although different methods of laboratory assay have been used and conflicting results have sometimes been obtained, some general trends concerning the relative efficacy of some mosquitocidal bacilli against mosquitoes of public health importance have been observed. For *B. thuringiensis* subsp. *israelensis* the relative efficacy in decreasing order was *C. quinquefasciatus* and *C. tarsalis*, *Aedes aegypti*, and *Anopheles quadrimaculatus*. It should be noted that *Aedes aegypti* was close to both *Culex* species in susceptibility, while

Anopheles quadrimaculatus was 2- to 15-fold less susceptible than *C. quinquefasciatus* (132). The susceptibility of mosquito larvae to *B. thuringiensis* subsp. *israelensis* also depends on the mosquito species within a genus (67); for example, *B. thuringiensis* subsp. *israelensis* was more toxic to *Anopheles albimanus* than to *Anopheles quadrimaculatus* (132).

There is some variation among *B. sphaericus* strains in their toxicities toward a given mosquito species, and not all species of mosquito within a particular genus are equally susceptible to a given toxin (21, 216). *B. sphaericus* 2362, IAB59, and 2297 were found to be equally effective against *C. quinquefasciatus* and *Aedes atropalpus* (21), and strain 1593 was toxic to *Aedes nicromaculis* (133). However, strains 2297 and IAB59 are nontoxic and strains 2362, 2317.3, and 1593 are only weakly toxic to *Aedes aegypti* (21, 216).

Very little information is available regarding the relative susceptibility of mosquito species to the low-toxicity strain *B. sphaericus* SSII-1. The 100-kDa toxin from SSII-1 was found to be toxic to *Aedes aegypti* and *C. quinquefasciatus* mosquitoes, and the LC_{50} of the purified protein against *C. quinquefasciatus* was similar to that of the purified binary toxin from strain 2362 (188). There have been no reports of bioassays of either SSII-1 or the 100-kDa toxin against *Anopheles* mosquitoes.

Field Use and Safety

Most small-scale field trials of *B. sphaericus* have been carried out against *C. quinquefasciatus* or *C. pipiens* mosquitoes. Although the picture is far from complete, the relative field efficacy against other genera of mosquitoes in decreasing order of susceptibility was similar to the laboratory findings, with several *Aedes* species being the most resistant (216). The residual effects (persistence and recycling in dead larvae) of *B. sphaericus* were noted in several studies of *Culex* species. Since good control was obtained over short periods of about 2 to 6 weeks, it was impossible to determine the relative contributions of the initial inoculum and the recycled spores, particularly since the larval cadavers tended to sediment in about 48 hours and so would be removed from the larval feeding zone (22). Wide variations in habitat, rainfall, temperature, pollution, competing larva food sources, and larval density have most probably been responsible for the different results reported in various studies (151, 216). However, it is clear that *B. sphaericus* 2362 has the potential to control some *Culex*, *Mansonia*, and *Psorophora* species, whereas results with certain *Aedes* and *Anopheles* species have been variable. All the trials were small-scale pilot studies and have not addressed the longer-term effect of *B. sphaericus* on the fluctuation of mosquito populations and the consequent impact on mosquito-borne diseases.

Field activity of *B. thuringiensis* subsp. *israelensis* against some *Aedes*, *Psorophora*, and *Culex* mosquito species has generally been found to be rather variable, as was the case with *B. sphaericus* (132). Again, the different habitats infested by these mosquitoes and the wide variation in environmental factors make comparison difficult. *B. thuringiensis* subsp. *israelensis* concentrate formulations have sometimes provided excellent initial control (90 to 100%) of larvae at rates as low as 0.1 to 0.2 kg of cells per hectare (107, 132). However, mosquito larvae of asynchronous species generally reappeared within 3 or 4 days of application. The efficacy of *B. thuringiensis* subsp. *israelensis* against *Anopheles* mosquitoes appears to be somewhat lower. In all cases, a significant increase in spore dose was required to control mosquitoes in polluted or brackish water. In contrast to laboratory studies, field evaluation has indicated that *B. thuringiensis* subsp. *israelensis* has the potential to control *Culex* in addition to *Aedes* mosquitoes, but long-term studies are needed, particularly of formulation methods and the frequency of application, to generate a clearer picture of whether this bacterium is a suitable agent to reduce the incidence of mosquito-borne diseases in a cost-effective manner.

The safety of both *B. sphaericus* and *B. thuringiensis* subsp. *israelensis* against the vast majority of nonpest insects, invertebrates, and vertebrates has been well documented (see references 22, 108, and 132 for reviews). The tests included several naturally occurring predators of mosquito larvae, which are believed to restrict the expansion of larval populations in many habitats. Thus, biological control with bacilli may enhance natural forms of biological control. The safety of both *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* for various mammals has been demonstrated, and it has been concluded that the larvicidal bacilli are highly unlikely to be hazardous to humans (22, 179).

Limitations of Natural Strains

The main limitations of natural strains of *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* which have discouraged their development are the high costs of fermentation, lack of persistence due to rapid settling of the spore-crystal complexes (53, 148), and narrow host range compared with that of the chemical insecticides. The last problem can be addressed by the genetic engineering of strains expressing combinations of new and existing mosquitocidal toxins, and the settling-out problem may eventually be solved by a combination of formulation technology and new host microorganisms such as *Caulobacter* species or cyanobacteria (see below). The engineering of increased potency, host range, and persistance will also have an impact on the cost of application.

EXPRESSION OF TOXINS IN RECOMBINANT MICROORGANISMS

Expression in *E. coli* and Bacilli

***B. sphaericus* toxins.** Several *Bacillus* species and various strains of *E. coli* have been used as recipient hosts for the expression of mosquitocidal toxin genes from *B. sphaericus*. In some studies, these hosts were used to evaluate the toxicities of the individual proteins or to study their structure-function relationships, and high levels of expression were not sought (14, 16, 17, 52, 56, 146, 190).

In general, it has proved difficult to compare the activities of the cloned toxins from different laboratories owing to the difficulty in quantitating expressed protein, the different degrees of toxin purity, and variations in the larval instar and the type of assay system used. However, a series of cloning experiments from a single laboratory has enabled a comparison of the expression levels of the recombinant binary toxin gene in *E. coli* JM105, *B. subtilis* DB104, nontoxic *B. sphaericus* 718, and *B. sphaericus* SSII-1 (18). Expression of the binary toxin gene from strain 2362 on plasmid pKK382 in *E. coli* was placed under the control of the *tac* promoter, whereas expression in the bacilli was driven by using plasmid pUE382 together with the *aprE* (subtilisin) promoter,

which is transcribed early in sporulation (26, 27). Several important observations were made in these experiments. First, the toxin-minus strain *B. sphaericus* 718 harboring pUE382 produced crystalline inclusions during sporulation and was as toxic to *C. pipiens* as was the high-toxicity strain 2362. *B. sphaericus* SSII-1(pUE382) also produced inclusions but was less toxic than the recombinant *B. sphaericus* 718. Second, the spores of the protease-negative strain *B. subtilis* DB104 harboring pUE382 contained large amorphous inclusions and were threefold more toxic to *C. pipiens* larvae than was *B. sphaericus* 2362. Third, *E. coli* JM105(pKK382) was more than 10-fold less toxic than any of the high-toxicity or recombinant bacilli, and the difference was apparently due to a reduced level of expressed proteins. In all cases, the results of the larvicidal assays correlated with the amounts of proteins detected in immunoblots (18, 19, 26, 27). The low expression in *E. coli* JM105(pKK382) was unlikely to be due to an inherent difficulty in expressing the binary toxin in *E. coli*, since *E. coli* GM2199 harboring the same binary toxin gene from *B. sphaericus* 1593M had an LC_{50} of 10 ng/ml against *C. pipiens* (175), which is comparable to the values obtained for either the spore-cell-crystal complex of *B. sphaericus* 2362 or the purified mixture of 51- and 42-kDa proteins (18).

The binary toxin gene of *B. sphaericus* has been expressed from its own promoter by using a *B. subtilis* plasmid (pGSP10) in both *B. thuringiensis* subsp. *israelensis* 4Q2-72 and a crystal-minus strain of *B. thuringiensis* subsp. *israelensis* (4Q2-81) cured of all resident plasmids (24). Crystals of *B. sphaericus* toxins were deposited alongside the spores of both strains. Unexpectedly, the crystal-minus strain expressing only the *B. sphaericus* toxins was equally toxic to *Anopheles stephensi* and *Aedes aegypti* larvae, in contrast to the parental *B. sphaericus*, which was about 25-fold less active against *Aedes aegypti* than against *Anopheles stephensi*. The discrepancy was not explained, but it is possible that *B. thuringiensis* subsp. *israelensis* activity against *Aedes aegypti* is determined in part by proteins other than the crystal toxins (24). Expression of the binary toxin and the formation of crystalline inclusions did not affect the production or deposition of the *B. thuringiensis* subsp. *israelensis* crystal toxins in the recombinant *B. thuringiensis* subsp. *israelensis* 4Q2-72(pGSP10). Surprisingly, there was no detectable additive or synergistic effect of expressing both classes of toxins in one cell compared with wild-type *B. thuringiensis* subsp. *israelensis* (24). The expression levels of the binary toxin in recombinant *B. thuringiensis* subsp. *israelensis* and wild-type *B. sphaericus* were not, however, compared, and the crystals of the 51- and 42-kDa proteins were smaller in *B. thuringiensis* subsp. *israelensis* than in *B. sphaericus*. Thus, it is possible that the binary toxin was less well expressed in 4Q2-72(pGSP10) than in *B. sphaericus*.

The 100-kDa toxin from the low-toxicity strain *B. sphaericus* SSII-1, which is expressed during the vegetative phase of growth, has been cloned and expressed in *E. coli* under the control of its own constitutive promoter (190). Recombinant *E. coli* was about 10 times less toxic to *Aedes aegypti* and *C. quinquefasciatus* larvae than was *B. sphaericus* SSII-1, but it was unclear whether this difference resulted from lower protein expression, polypeptide instability, or protein misfolding in *E. coli* (190). More recently, a truncated version of the 100-kDa toxin lacking the nonessential N-terminal putative signal sequence has been produced in recombinant *E. coli* and purified to homogeneity by affinity chromatography (188). The LC_{50} of this protein was determined to be 15 ng/ml against *C. quinquefasciatus* larvae, which is comparable with the value obtained for the 51- and 42-kDa toxins from *B. sphaericus* 2362 expressed in *B. subtilis* (18). Thus, the low larvicidal activity of SSII-1 (190) is more likely to be due to poor expression or enzymatic degradation than to a low specific activity of the 100-kDa toxin. There have been no reports of the expression of the 100-kDa toxin in recombinant bacilli during either the vegetative or sporulation phase of growth, and the host range of this toxin remains to be measured.

***B. thuringiensis* toxins.** All the major crystal toxins of *B. thuringiensis* subsp. *israelensis* have been expressed with various efficiencies in *E. coli* or *B. subtilis* (7, 8, 25, 40, 57, 124, 193, 201, 204, 207). Although the variation in assay systems, plasmids, host strains, and toxin purity makes a meaningful comparison difficult, some general conclusions can nevertheless be drawn. The *cryIVA*, *cryIVB*, *cryIVC*, *cryIVD*, and *cytA* genes from *B. thuringiensis* subsp. *israelensis* have all been individually expressed in *E. coli*, and the highly variable yields of protein were probably due to different host strains, dissimilar polypeptide stabilities, or a range of promoter and ribosome-binding site strengths (7, 8, 40, 57, 193, 201, 204, 207). The *cryIVB* gene is one of the few *B. thuringiensis* subsp. *israelensis* crystal genes to have been well expressed in *E. coli* (40, 204). The 128-kDa protein (Table 1) has been synthesized as a *lacZ* transcriptional fusion in *E. coli* TG1 at a level sufficient to produce phase-bright inclusions, which were purified and shown to be weakly toxic to *A. aegypti* larvae (204). Similarly, the 128-kDa (CryIVB) toxin has been overexpressed in *E. coli* JM107, and the purified protein was highly toxic (LC_{50}, 43 ng/ml) to third-instar larvae of *A. aegypti* (40).

The dual-specificity 130- to 135-kDa CryII toxin from *B. thuringiensis* subsp. *aizawai* was also apparently synthesized in large amounts in recombinant *E. coli* TG1, since microscopically visible inclusion bodies were observed in the cytoplasm, and lysates of these cells were found to be toxic to both lepidopteran and dipteran (*A. aegypti*) larvae (84).

B. subtilis has been used as a host for expressing some of the crystal genes and the *cytA* gene of *B. thuringiensis* subsp. *israelensis* (193, 204, 205, 207). Good expression of the *cryIVA*, *cryIVC*, and *cytA* genes was obtained, although protease-deficient strains of *B. subtilis* were not used in these experiments (193, 204, 205, 207). In some cases, phase-bright inclusions were observed during late sporulation (204, 207).

The *cryIVD* crystal gene of *B. thuringiensis* subsp. *israelensis* (Table 1) has been cloned into the nonlarvicidal bacterium *Bacillus megaterium*. Significant amounts of the 70-kDa protein were produced, and the cells were toxic to *A. aegypti* fourth-instar larvae, but the toxicity was much lower than that of *B. thuringiensis* subsp. *israelensis* (61). There has been only one other report of good expression of a cloned *cryIVD* gene in *B. thuringiensis*. The 70-kDa *cryIVD* gene from *B. thuringiensis* subsp. *morrisoni* PG-14 was inserted into the shuttle vector pHT3100, and an acrystalliferous nontoxic mutant of *B. thuringiensis* subsp. *kurstaki* was transformed with the recombinant plasmid. Parasporal inclusions of the 70-kDa protein that were comparable in size, shape, and toxicity to those produced by the parent strain, *B. thuringiensis* subsp. *morrisoni* were observed (35). This system may prove useful for evaluating the toxicities and host range of the individual toxins from *B. thuringiensis* subsp. *israelensis* and its relatives.

Two laboratories have reported expression of *B. thuringiensis* subsp. *israelensis* toxins in *B. sphaericus* in an attempt

to increase the larvicidal activity and host range of the mosquitocidal bacilli (12, 194). The 27-kDa (CytA) and 70-kDa (CryIVD) toxins have been cloned and expressed in both *B. subtilis* and the high-toxicity strain *B. sphaericus* 2362 (12). Although plasmid loss or instability occurred in some transformed *B. sphaericus* clones, others retained toxicity which was stable for up to 20 generations in the absence of antibiotic selection (12). Remarkably, the evidence in the case of the stable clones pointed to a transposition of the toxin gene(s) from the plasmid to the chromosome by an unknown mechanism. The expression of the 27- and 65-kDa toxins of *B. thuringiensis* subsp. *israelensis* in *B. sphaericus* 2362 led to a 10-fold enhancement of toxicity toward *A. aegypti* compared with that of the parental strain 2362, but it was still lower than that of *B. thuringiensis* subsp. *israelensis* itself (12). This enhancement was attributed to the 27-kDa hemolytic protein. It is possible that the 27-kDa protein synergized with a component of *B. sphaericus*, since it is known that the 27-kDa protein by itself is only weakly toxic (124, 207) and that it contributes nothing toward the toxicity of *B. thuringiensis* subsp. *israelensis* to *A. aegypti* (58, 64). Overall, the expression of *B. thuringiensis* subsp. *israelensis* toxins was higher in *B. subtilis* than in *B. sphaericus*, and crystalline inclusions were seen accompanying the spores of *B. subtilis* only; however, the larvicidal activity and crystals were unstable in the *B. subtilis* transformants even with antibiotic selection (12).

The other report of cloning in *B. sphaericus* showed that the expression of the 128-kDa *cryIVB* gene product of *B. thuringiensis* subsp. *israelensis* in *B. sphaericus* 2362 led to a 100-fold increase in activity against *A. aegypti* larvae, which was comparable to the toxicity of *B. thuringiensis* subsp. *israelensis* (194). Thus, the characteristically high activity of *B. thuringiensis* subsp. *israelensis* against *A. aegypti* was transferred to *B. sphaericus*. Was the 128-kDa toxin from *B. thuringiensis* subsp. *israelensis* alone responsible for this dramatic increase in toxicity to *A. aegypti*, or was there synergy between the 128-kDa toxin and endogenous component of *B. sphaericus*? This question cannot yet be answered since the authors did not also express the 128-kDa toxin in nontoxic strains of *B. sphaericus*.

In conclusion, the binary toxin of *B. sphaericus* has been expressed in recombinant *B. subtilis* and *B. sphaericus* at levels comparable to those in the natural high-toxicity strains (18). Good expression of several *B. thuringiensis* CryIV toxins in *E. coli* or heterologous bacilli has been achieved, and in certain cases highly active toxins have been deposited as crystalline or amorphous inclusions. However, the data have not shed much light on the genetic stability of cloned toxin genes (see below) and the possible interactions between new combinations of toxins which might produce synergistic effects.

Expression in Novel Hosts

***Caulobacter* species.** Bacteria belonging to the genus *Caulobacter* were chosen as hosts for the heterologous expression of mosquitocidal toxins since *Caulobacter* species occur in virtually every aquatic habitat and are found predominantly in regions at or close to the water surface (162), where larvae of many mosquito species feed (126). In the flagellate swarmer stage *Caulobacter* cells are motile, thus allowing their distribution throughout the habitat, and in both the swarmer and stalked cell stages they are capable of attachment to solid particles. *Caulobacter* species are able to persist and grow in adverse environments low in nutrients (162).

The genes encoding the binary toxin from *B. sphaericus* 2297, the 100-kDa toxin from *B. sphaericus* SSII-1, and the 128-kDa CryIVB toxin from *B. thuringiensis* subsp. *israelensis* were separately inserted into the broad-host-range plasmid pRK248 (191) and electroporated into *Caulobacter crescentus* CB15 (189). The resultant recombinants all exhibited toxicity to mosquito larvae. The most toxic constructs of the binary toxin gene were lethal to *Culex quinquefasciatus*, with LC_{50}s of about 2×10^5 cells per ml, which is similar to the toxicity of the natural strain, *B. sphaericus* SSII-1, from which the 100-kDa toxin gene was originally cloned (189, 190). *E. coli* JM109 cells transformed with the same plasmid were about 1,000-fold less toxic, but the reason for this difference was not investigated. *Caulobacter* cells containing either the *B. sphaericus* 100-kDa toxin gene or the *B. thuringiensis* subsp. *israelensis* 128-kDa toxin gene were weakly toxic to *Culex quinquefasciatus* and *A. aegypti* larvae, respectively. Protein levels were not quantitated but were believed to be low (189). There is scope for increasing expression in *Caulobacter* by the use of stronger promoters and ribosome-binding sites (200).

Cyanobacteria. Various species of cyanobacteria have been investigated as vehicles for prolonged delivery of insecticidal toxins to the larval feeding zone (9, 39, 59, 135). Like *Caulobacter* species, the ubiquitous photosynthetic cyanobacteria exist at or near the water surface. These bacteria are adaptable to both freshwater and saltwater environments, they have a wide temperature tolerance, and, being protoautotrophs, they have limited nutritional requirements (166).

The first attempts at cloning toxin genes from *B. sphaericus* or *B. thuringiensis* subsp. *israelensis* in cyanobacteria were successful, but expression levels and toxicity were very low (9, 39, 59). The binary toxin gene of *B. sphaericus* was inserted into a shuttle vector suitable for cloning in both *E. coli* and cyanobacteria (105) and transformed into *Anacystis nidulans* R2 (also called *Synechococcus* sp. strain R2) (59). Cell protein extracts gave low mortality against *Culex pipiens* larvae. A similar low expression of the 134-kDa CryIVA toxin from *B. thuringiensis* subsp. *israelensis* was observed in the cyanobacterium *Agmenellum quadruplicatum* PR-6 (*Synechococcus* sp. strain PCC 7002) (9). Cell extracts were much less toxic to *Aedes aegypti* larvae and contained less of the ~130-kDa protein than did extracts of *E. coli* transformed with the same plasmid (9). A low copy number of one plasmid per cell (33) may have accounted, at least in part, for the poor expression in *Agmenellum quadruplicatum*.

Expression of the 128-kDa CryIVB protein of *B. thuringiensis* subsp. *israelensis* has also been achieved following its integration by homologous recombination into the chromosome of *Synechococcus* sp. strain PCC 6803 (39). Cell extracts but not live cells were weakly toxic to 20 to 30% of *Aedes aegypti* larvae, equivalent to 1 to 2 μg of toxin protein per ml. Live cells of the cyanobacterium *Agmenellum quadruplicatum* PR-6 which had been transformed with the *cryIVD* gene from *B. thuringiensis* subsp. *israelensis* under the transcriptional control of the strong phycocyanin (*cpcB*) promoter derived from PR-6 were reported to be toxic to mosquito larvae (135). The *cryIVD* gene was sufficiently well expressed to form phase-bright inclusions, but the toxicity of whole cells to *Culex pipiens* larvae was not quantitated. There was a delay in the onset of toxicity of the recombinant *Agmenellum quadruplicatum* cells from 1 to 3 days, and

TABLE 2. Suitability of various bacteria as hosts for the delivery of engineered toxins to mosquito larvae

Property	Suitability[a] of:					
	E. coli	*B. thuringiensis* subsp. *israelensis*	*B. sphaericus*	*B. subtilis*	*Caulobacter* spp.	Cyanobacteria
Good capacity to express foreign proteins	Y	Y?	Y?	Y	?	?
Good genetic maps and mutants available	Y	N	N	Y	N	N
Structurally and segregationally stable plasmids	Y	?	?	Y	Y	N
Persists at larva feeding zone	?	N	N	N	Y	Y
Known food source for larvae	Y?	Y	Y	Y	Y	Y
Survival in diverse environments	?	N	Y	Y	Y	Y
Experience in						
Production	Y	Y	Y	Y	N	N
Formulation	N	Y	Y	N	N	N
Mammalian toxicity	Y	N	N	N	?	?

[a] Y, yes; N, no.

100% mortality occurred 5.5 days after the cells were first fed to the larvae (135).

Baculoviruses. The nuclear polyhedrosis viruses are large double-stranded viruses that cause fatal disease mainly in lepidopteran insects (77, 122). Several baculoviruses belonging to this group are already in use as microbial insecticides (94, 156). They have particular advantages over chemical insecticides, such as lack of toxicity toward nonpest insects, which are very similar to those of the insecticidal bacilli. One disadvantage is that these viruses often take a week or longer to kill lepidopteran larvae advanced in development, by which time substantial damage to a crop has occurred (94, 156).

To investigate the potential of baculoviruses to deliver insecticidal toxins to larvae, a 133-kDa CryIA protoxin specific to lepidopteran insects was expressed in vitro in cells of the fall army worm *Spodoptera frugiperda* by using baculovirus vectors (118, 163), and the recombinant CryIA protoxin was shown to be biologically active (121, 127). The virulence of the recombinant baculovirus was not, however, enhanced by the expression of the δ-endotoxin (121, 127).

The *cryIVD* gene from *B. thuringiensis* subsp. *morrisoni* PG-14 (35) has been inserted in a baculovirus vector, and recombinant virus has been obtained; infection of *S. frugiperda* cells and *Trichoplusia ni* larvae with the recombinant virus resulted in the synthesis of a polyhedrin-CryIVD protoxin fusion protein, which crystallized into cuboidal inclusions in the cytoplasm (153). The infected cells and purified inclusions were toxic to mosquito larvae (LC_{50}, ≈250 ng/ml) (153).

Recombinant baculoviruses are clearly capable of expressing active δ-endotoxins, but at present it seems unlikely that this approach will supersede the bacterial delivery of toxins.

POTENTIAL OF ENGINEERED STRAINS

Desirable Features of a Recombinant Strain

The diversity of bacteria and other organisms which have been successfully transformed with plasmids encoding expressible toxin genes (24, 26, 59, 135, 153, 189) has raised the question of which types of recombinant microorganisms are the most suitable for delivering long-lasting and effective combinations of toxins to the larval feeding zone (154).

Table 2 provides an evaluation of six types of bacteria in terms of various desirable features, which together might produce a recombinant strain superior to *B. thuringiensis* subsp. *israelensis* or the known *B. sphaericus* strains. All the bacteria listed in Table 2 are aquatic and, with the exception of *E. coli*, occur naturally in diverse environments in many parts of the world. These bacteria can exist in polluted environments to various degrees, although *B. thuringiensis* subsp. *israelensis* is sensitive to certain pollutants (134). The bacilli are advantageous owing to industrial experience in their production and formulation (132, 216). *B. subtilis* has important attributes not shared by any of the other bacteria—there is an excellent and comprehensive genetic map, and useful strains including protease-deficient mutants have been developed (86, 100, 215). The organism has proved on many occasions its capacity to overexpress foreign proteins (86). Recombinant strains of *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* are able to express their own and foreign toxin genes carried on plasmids. Although much less is known about *Caulobacter* species and cyanobacteria, they have a particular advantage since they dwell at the surface zone, where many mosquito larvae feed (126). However, regulatory agencies in many countries will not allow recombinant *Caulobacter* species or cyanobacteria. *E. coli* has many positive attributes, but it should not be seriously considered for environmental release owing to mammalian pathogenicity.

It is evident from Table 2 that none of the bacteria is ideal and possesses all the desirable features of an effective mosquitocidal microorganism; however, apart from *E. coli*, they are all worthy of further investigation.

Improving Potency and Persistence

The potency of *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* 2362 is already very high (LC_{50}s of about 10^3 and 10^2 spores per ml, respectively) because expression levels of the toxins during sporulation are high (18, 92).

An effective way to further enhance potency would be to engineer synergism of toxicity, although there are as yet no reports of this. A recent paper showed that the 134-kDa (CryIVA) and 128-kDa (CryIVB) cloned toxins of *B. thuringiensis* subsp. *israelensis* interacted synergistically in vivo (8). There are clear examples of synergism between the purified 27-kDa protein and the purified 70-kDa (or 128- and 134-kDa) toxins of *B. thuringiensis* subsp. *israelensis* (38, 186), but the 27-kDa protein is hemolytic and neurotoxic (202, 206) and might not be acceptable in a recombinant organism. Mixtures of the 128-kDa CryIVB toxin and the 58-kDa CryIVC toxin of *B. thuringiensis* subsp. *israelensis*

were far more toxic than expected, but the effect was restricted to *Culex* species (57). Expression of the *B. sphaericus* binary toxin in an acrystalliferous mutant of *B. thuringiensis* subsp. *israelensis* unexpectedly enhanced toxicity to *Anopheles stephensi* mosquitoes (24), and conversely, the expression of the 27-kDa protein of *B. thuringiensis* subsp. *israelensis* in *B. sphaericus* led to the appearance of significant toxicity to *Aedes aegypti* (12). In none of these studies, however, was the in vivo potency greater than that of the parent organism. In the future, it will be important to carefully and systematically analyze the interactions between new and known toxins (186).

Recombinant DNA technology has the potential to enhance the expression levels of the toxins in many bacterial species by the use of stronger promoters either alone or in tandem (86, 164, 180), by optimizing the strength of ribosome binding sites (200), and by downstream placement of bacterial enhancer/terminator sequences (71, 212). Promoters which are silent unless a chemical inducer is added cannot be used to secure toxin production in cells released into the environment. Therefore, the challenge will be to avoid toxicity to the host cell as a result of either constitutive overexpression of a heterologous protein or untimely expression of a crystal protein during the vegetative phase of growth.

As mentioned above, potential alternatives to the bacilli are the gram-negative *Caulobacter* species and the cyanobacteria, which are able to survive in diverse environments and exist near the water surface (135, 162, 189). Although good expression of the 70-kDa *cryIVD* gene was reportedly obtained in a cyanobacterium (135), it is still unclear whether stable high expression of foreign proteins can be routinely achieved with these microorganisms.

The 27-kDa hemolytic protein from *B. thuringiensis* subsp. *israelensis* was expressed equally well in both sporogenic and certain asporogenic strains of *B. subtilis* (207). The OJ lesion of one asporogenic strain, which prevents the morphological manifestations of the sporulation process, did not appear to affect the temporally regulated transcription of the 27-kDa gene in the stationary phase of growth. Inclusions from the OJ mutant were as toxic to *A. aegypti* larvae as those from the sporogenic strain (207). *B. sphaericus* asporogenous mutants blocked at early stages of sporulation were nontoxic, whereas mutants blocked at later stages were as toxic as the parental strain (36). The use of asporogenic mutants of *B. subtilis* or *B. sphaericus* may reduce the problem of spore-toxin complexes settling away from the larval feeding zone, although the ability of these mutants to survive and recycle would have to be determined.

Plasmid Stability and Chromosomal Integration

The genetic stability of a cloned toxin gene in a recombinant cell will obviously have an important bearing on the potency of the strain and the persistence of toxicity. Plasmids are not universally stable. There are at least two types of plasmid instability, segregational and structural (30). Segregational instability refers to the loss of the entire plasmid population from the cell, and structural instability refers to plasmid rearrangements, most frequently deletions. Another phenomenon, termed plasmid incompatibility (145), exists when two different plasmids cannot stably coexist in one cell in the absence of selection pressure. The random selection of plasmid copies for replication will eventually result in the appearance of homoplasmid segregants.

In the following section, the suitability of plasmids currently used for expression in gram-positive and gram-negative hosts and the advantages of a new generation of stable plasmids for gram-positive bacteria will be examined. In addition, the engineering of genetic stability by homologous recombination and chromosomal integration of cloned toxin genes will be discussed.

Gram-positive bacteria. Various toxin genes from *B. sphaericus* and *B. thuringiensis* subsp. *israelensis* (or *B. thuringiensis* subsp. *morrisoni* PG-14) have been expressed in heterologous *B. subtilis*, *B. sphaericus*, or *B. thuringiensis* subsp. *israelensis* and its relatives (12, 18, 24, 35, 194). Extremely variable levels of expression of larvicidal activity were observed, and the most likely explanations are variations in promoter and ribosome-binding site strength, the presence or absence of strong transcription terminators, and the stability of the expression cassette in the recombinant plasmid. Of these factors, the long-term stability of the plasmid is the most difficult to engineer and has been investigated in only a few cases (12, 194). The 128-kDa *cryIVB* toxin gene of *B. thuringiensis* subsp. *israelensis* was cloned in *B. sphaericus* by using a derivative of pBC16, a plasmid from *B. cereus*, but most transformants had suffered deletions which included parts of either the toxin gene or the vector (194). Nonetheless, other clones were stable and maintained the recombinant plasmid intact for 4 weeks of daily subculture without antibiotic selection, and only a slight reduction in the number of plasmid-free cells was observed (194). A similar phenomenon occurred in the cloning of the 27- and 70-kDa crystal genes of *B. thuringiensis* subsp. *israelensis* in *B. sphaericus* when a derivative of the *Staphylococcus aureus* plasmid pUB110 was used (12). Only about 8% of transformants had stably maintained the plasmids after 20 passages of culture in the absence of antibiotic selection. The remainder had either lost the plasmid or deleted parts of it (12). By using the pHV33 bifunctional vector derived from *S. aureus* plasmid pC194, it was found that the toxicity of the *B. sphaericus* binary toxin expressed in *B. subtilis* gradually declined over time. The loss of toxicity was attributed to the known instability of pC194-based vectors (59).

The plasmids used for cloning toxin genes in *B. subtilis*, *B. sphaericus*, or other bacilli have all been derived from *S. aureus*, *B. cereus*, or related bacteria and are known to replicate via single-stranded DNA (ssDNA) intermediates, probably by rolling-circle replication (78). The generation of ssDNA intermediates, which may be exacerbated in plasmid vectors lacking a minus-strand origin of replication (MO), greatly increases the opportunity for deletions or rearrangements to occur in *B. subtilis* (structural instability) (30). Moreover, the segregational instability of pUB110 derivatives in *B. subtilis* became markedly more pronounced as the size of the insert increased; and in the absence of the MO, the segregational instability was further enhanced (30).

There have been few investigations of plasmid stability in *B. sphaericus*. One study illustrates the disadvantage of using plasmids which replicate via ssDNA intermediates. The plasmid pUB110 (4.5 kb) and its derivatives pLDT103 (7.6 kb) and pLDT117 (9.3 kb) were able to recycle during spore germination, vegetative growth, and sporulation in larvae of *C. quinquefasciatus*, but only pUB110 and pLDT103 were segregationally stable (177). pLDT117 exhibited 23% segregational instability, which was suggested to be due to the larger size of the plasmid or the deletion of the MO (177). The segregational stability of pUB110 during recycling in mosquito larvae implies that the *S. aureus* MO is functional in *B. sphaericus* as well as in *B. subtilis*. Derivatives of

B. cereus plasmid pBC16 were shown to replicate and be stably maintained in *B. sphaericus* 1593 without affecting the larvicidal activity of this organism (143). These plasmids were segregationally stable in both *B. sphaericus* and *B. subtilis*, but one plasmid had suffered a spontaneous insertion of foreign DNA of unknown origin (143). Overall, these results show that plasmids from *S. aureus* and *B. cereus* can coexist with the large resident plasmids of *B. sphaericus*, and there was no evidence for plasmid incompatibility (143, 177). However, all the stable plasmids were small (≤ 7.6 kb), and stability was not examined after additional pressure was exerted on the cell by the expression of nonessential foreign proteins (e.g., toxins) (143, 177).

Two families of plasmid vectors have been developed on the basis of either the small cryptic plasmid pTA1060 from *B. subtilis* (85) or the *Enterococcus faecalis* plasmid pAMβ1 (185), which are significantly more structurally and segregationally stable in *B. subtilis* than are the *S. aureus* and *B. cereus* ssDNA plasmids (30). pTA1060 can be stably maintained in *B. subtilis* with inserts up to ~16 kb in length, even though it replicates by a rolling-circle mechanism (85). A 5.65-kb plasmid pHPS9, generated from pTA1060, is useful as a selection vector since recombinants can easily be identified as white colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates. pHPS9 can accept inserts up to ~30 kb in size (30).

The natural *E. faecalis* plasmid pAMβ1 has been found to replicate by a theta-type mechanism, which is distinct from rolling-circle replication. Its structural stability is partly because single-stranded DNA intermediates are not produced (31, 62). A series of high-copy-number cloning vectors have been constructed from pAMβ1, including pHV1431 and pMTL500E res$^+$ (96, 185). The segregational stability of these vectors is conferred by a resolvase gene, which is absent from the *S. aureus* and *B. cereus* ssDNA plasmids (185). Plasmid pAMβ1 has been transformed into *B. sphaericus* 1593 (150). Either of these families of plasmids, which are best represented by pHPS9 and pHV1431, may prove suitable for use in *B. sphaericus*. However, the stability of these plasmids remains to be tested in the context of recombinant vectors expressing mosquitocidal toxins in *B. subtilis*, *B. sphaericus*, or other suitable bacilli. Thus the various forms of plasmid stability are vital to the genetic stability of the cloned mosquitocidal toxin genes carried on plasmids, particularly since antibiotics cannot be used to override segregational instability and incompatibility in cells released into the environment.

An attractive alternative to plasmid-encoded toxin gene expression is homologous recombination and chromosomal integration in bacilli (90, 219). Plasmid instability has been largely avoided by inserting foreign genes into the *B. subtilis* chromosome, where recombination is approximately 1,000-fold less frequent than in plasmids (97, 139). Following integration, stable amplification of the inserted sequences as tandem repeats up to about 50 to 250 copies per chromosome can be induced and may allow enhanced expression of the integrated gene comparable to that obtained with plasmids (3, 98, 159, 217). Many different methods of chromosomal integration and amplification exist, although not all integrated genes are stable as tandem repeats (217). A *Bacillus* alkaline protease gene was integrated into the genome by (i) homologous recombination, resulting in a strain with two tandemly arranged genes, and (ii) illegitimate recombination, resulting in a strain with a second copy of the protease gene at a distant locus from the originally present gene. Only the nontandemly duplicated gene was completely stable (195).

There have been few reports of chromosomal integration of insecticidal toxin genes. An attempt was made to integrate the binary toxin genes of *B. sphaericus* 1593M in the *B. subtilis* chromosome, and integrants expressing the 42-kDa toxin gene were isolated (56). The integrated gene(s) was not mapped or characterized further. In a new approach to in vivo recombination in *B. thuringiensis* subsp. *israelensis*, the *cytA* gene encoding the 27-kDa hemolytic protein was deleted from the resident 72-MDa plasmid and replaced by the erythromycin resistance gene (*erm*) of the transposon Tn*1545* (58).

In the majority of the standard techniques used to promote chromosomal integration of a plasmid-borne gene, an antibiotic resistance marker and the vector sequences including the plasmid origin of replication are cointegrated with the gene (58, 90, 159, 219). The problem is that antibiotic resistance genes, especially in multiple copies, would be undesirable for environmental applications of mosquitocidal bacteria. In addition, the presence of a replication origin and *rep* gene on the amplified DNA can cause high levels of instability under certain circumstances (218).

An impressive demonstration of homologous recombination and transfer of an insecticidal δ-endotoxin gene without plasmid vector sequences resulted in the expansion of the insecticidal host range of a strain of *B. thuringiensis* (114). This strain, toxic only to members of the Lepidoptera, was transformed with a plasmid thermosensitive for replication bearing a Coleoptera-specific *cryIIIA* gene and an *erm* gene as a selectable marker. At the nonpermissive temperature, plasmid replication was abolished and only clones bearing a copy of the thermosensitive plasmid integrated on the large resident plasmid were selected. The thermosensitive plasmid, together with the *erm* gene, was deleted by a second recombination event, leaving only the *cryIIIA* gene integrated in the stable resident plasmid. The new recombinant *B. thuringiensis* strain carried both the *cryIAc* (Lepidoptera-specific) and *cryIIIA* (Coleoptera-specific) genes and was toxic to members of both Lepidoptera and Coleoptera. The expression of the endogenous *cryIAc* gene was unaffected by the high expression of the *cryIIIA* gene, which appeared to be structurally and segregationally stable (114). There is no reason why this technique cannot be applied to *B. sphaericus*, *B. thuringiensis* subsp. *israelensis*, or other bacilli to integrate new combinations of toxin genes without plasmid vector sequences and obtain their expression during the vegetative and/or sporulation phases of growth.

Gram-negative bacteria. Recombinant plasmids of *E. coli* and other gram-negative organisms generally suffer less frequently from rearrangements and deletions than do the ssDNA plasmids of gram-positive cells, since they replicate by a theta-type mechanism (192). In the large broad-host-range plasmids RK2 and RP4, the highly efficient partitioning system (*par*) is present and maps to a complex region of about 2.2 kb, which contains three ORFs (72, 170). However, the small ColE1-type plasmids commonly used in *E. coli*, such as pBR322, are not segregationally stable owing to the absence of various stabilizing loci (e.g., *par*) (191, 192, 210).

The plasmid pRK248 used for expression of the binary toxin, the 100-kDa toxin of *B. sphaericus*, and the 128-kDa CryIVB toxin of *B. thuringiensis* subsp. *israelensis* in *Caulobacter* species (189) lacks the *par* locus (191) and is rapidly lost in the absence of selection pressure. Cloning the 2.2-kb *par* region of RK2 back into pRK248 would very probably stabilize the plasmid, as was found for the general-purpose vector pXL1635 (46).

Other broad-host-range plasmids have been constructed as cloning vectors for *Caulobacter crescentus*; their stability and utility in expressing cloned toxin genes remains to be determined (63, 173).

Homologous recombination and chromosomal integration in the gram-negative bacteria *E. coli* (165, 183) and *C. crescentus* (13, 63, 149) are well-established phenomena. Integrational vectors have been developed for the introduction of single-copy sequences into the chromosome of *E. coli* without the presence of plasmid sequences and antibiotic resistance genes (165) as described for *B. thuringiensis* (114). The same principle can be applied in the development of similar integrational vectors for *C. crescentus* (63).

Integrational vectors and systems to study genetic exchange in cyanobacteria (33, 87, 105) have not so far been used for the expression of chromosomally integrated toxin genes in this organism.

Increasing the Range of Susceptible Mosquitoes

There are various strategies to enlarge the insecticidal spectrum of a bacterium: (i) transformation or transduction of toxic strains with plasmids encoding toxin genes of insecticidal specificity which is different from that of the recipient (12, 24, 65, 113, 194); (ii) transformation of nontoxic strains with combinations of toxins producing broader insecticidal specificity than each toxin alone (189); (iii) integration of toxin genes of different specificities into the bacterial chromosome or large resident plasmids (56, 114); and (iv) engineering of mutant toxins with a new or enlarged insecticidal specificity (21, 34, 93).

Several examples of transformation of high-toxicity strains of *B. thuringiensis* subsp. *israelensis* or *B. sphaericus* with heterologous toxin genes have been presented, but only in one case was the strain potentially useful (194). However, the expression plasmid was based on an unstable ssDNA plasmid from *B. cereus* and would not be expected to be stable in the environment for many generations (30, 194).

The search for new mosquitocidal toxins with new specificities must continue. The validity of the screening approach to identify toxins with new specificities and potencies is demonstrated by the discovery of *B. thuringiensis* strains with very diverse activities against not only new species of insects but also protozoan pathogens, animal-parasitic nematodes, and liver flukes (65). The screening of bacteria need not be confined to the bacilli. A mosquitocidal strain of *Clostridium bifermentans* serovar *malaysia* was discovered to have excellent *Anopheles stephensi* activity, followed by activity for certain *Culex* and *Aedes* species in decreasing order (140). This is the first mosquitocidal bacterium which shows greater toxicity to *Anopheles* mosquitoes than to the other mosquito species, and the toxin appears to be safe for nonpest insects and mammals. The toxicity of *Clostridium bifermentans* is labile except in the presence of EDTA, and the organism is anaerobic and likely to prove difficult to ferment on the large scale (140).

Insect resistance to any insecticide can be expected (66, 76, 123, 198). Therefore, combining various toxins of different specificities in one recombinant cell may prevent or delay the onset of resistance. There are a few documented cases which suggest, however, that resistance to mosquitocidal toxins used in the field does not develop easily (51). In laboratory and field trials, the resistance of *Aedes aegypti* larvae to *B. thuringiensis* subsp. *israelensis* was found to be only twofold or less than that of sensitive *Aedes aegypti* larvae (73, 75). Laboratory populations of *Culex quinquefasciatus* were treated with *B. thuringiensis* subsp. *israelensis* for 32 generations, producing only a ca. five- to sevenfold increase in resistance, which reverted to complete susceptibility after three generations without selective pressure (199). Selection of *C. quinquefasciatus* with *B. sphaericus* toxins over 2 years at 80 to 90% mortality led to threefold resistance to the purified toxins, but less than twofold resistance to the spores of this organism (51).

With a better understanding of the specificity determinants of the mosquitocidal toxins (21, 116, 209), it may in the future be possible to broaden insect specificity by protein engineering (125). Initial attempts to enlarge the insecticidal spectrum in this way have focused on the CryIA Lepidoptera-specific toxins. Two truncated *B. thuringiensis* crystal protein genes, both coding for insecticidal N-terminal fragments of crystal proteins with different insect specificities, were translationally fused (93). Expression of the gene fusion in *E. coli* resulted in a chimeric protein with a toxicity spectrum that overlapped those of both contributing proteins (93). This method ensures the expression of the active protein fragments in a 1:1 ratio but otherwise does not offer particular advantages over the expression of the proteins individually. Hybrids between two *cryIA* toxin genes were also produced by in vivo plasmid recombination. Some hybrids had a narrower host range in their insecticidal action; several hybrids were significantly more potent than the parental toxins; and one had surprisingly acquired a completely new, albeit weak, biological activity against *Spodoptera littoralis* (34). A new insecticidal activity (against *Pieris brassicae* larvae) was also gained when a *cryIIIA* toxin gene from *B. thuringiensis* subsp. *tenebrionis* was expressed in recombinant *B. thuringiensis* subsp. *israelensis* (45a). It remains a mystery why the new insecticidal activities were only transiently expressed in some of the recombinant strains (34, 45a).

Deliberate Release into the Environment

The release of "beneficial" genetically engineered organisms into the environment raises a whole set of extremely contentious and difficult issues (154). However urgent the need for biological control, there must be exhaustive research and consideration before an engineered microorganism is released into the environment. A genetically engineered mosquitocidal microorganism must be effective over a period of weeks or even months, but it is the very persistence of the microorganism which raises genuine concerns about the unforeseen detrimental effects it may have on the biosphere.

The arguments for and against the deliberate release of recombinant mosquitocidal microorganisms are listed below. The arguments for are as follows.

(i) There is an urgent need. Over 1 million children die of malaria in Africa alone (110), and malaria infects 270 million people annually (45).

(ii) Genetic exchange creating new arrangements of genes and genomes by conjugation, transduction, transformation, or splicing via transposons and insertion sequences is perfectly natural and is going on all the time in the biosphere.

(iii) The mosquitocidal genes originally came from natural soil and aquatic bacteria in the biosphere. They can be reintroduced by chromosomal integration without cointegration of extraneous vector sequences or antibiotic resistance genes.

(iv) Chromosomal integration maximizes genetic stability (97, 139).

(v) The engineered organism is unlikely to survive for more than a few months, because spontaneous mutations which delete the toxin genes or block their expression will probably confer a growth advantage on the altered strain, which will eventually replace the original strain.

(vi) Tests to evaluate the survival and possible detrimental effects of the organism can be carried out on islands which are a long distance from the mainland.

(vii) The natural strains of *B. sphaericus* and *B. thuringiensis* subsp. *israelensis* have been applied in many localities in different countries without any apparent detrimental effects or reports of significant resistance arising in mosquito populations. In the Onchocerciasis Control Program in West Africa, *B. thuringiensis* subsp. *israelensis* has been successfully used to control blackflies without a single report of insect resistance (106).

The arguments against are as follows.

(i) Once introduced into the biosphere, the microorganism cannot be withdrawn, especially if the need should arise because of resistance developing in mosquito populations (51) or unforeseen consequences.

(ii) Genes can be transferred across the boundaries of species and genera. No segment of DNA is considered absolutely stable, even in the chromosome.

(iii) There is a lack of useful information on survival, genetic exchange, and possible detrimental effects of engineered microorganisms in the environment. Results of simulated laboratory tests must be treated with caution.

(iv) Plasmids which are considered nonconjugative and poorly mobilizable can be transferred from cell to cell. For example, pBR322 can be transferred to other enteric bacteria by triparental matings (115).

(v) There may be subtle effects, for example, on the viability of nontarget organisms which were not measurable in laboratory tests. This might result in the reduction of populations of natural mosquito larval predators. Important mosquito predators vary from region to region, and it is unlikely that all the predators have been identified.

(vi) Performing field tests on an island is no guarantee of containment; spores can be carried to the mainland by wind or water. Likewise, spores can be carried to a neighboring country (which prohibits their release).

(vii) Some bacteria (e.g., cyanobacteria) form "blooms" and may pollute the water (166).

(viii) Introduction of a toxin in a persistent form may hasten the onset of insect resistance, which renders the toxin useless.

At present, the decision to release genetically engineered organisms is made in many countries on a case-by-case basis after very careful consideration, but few engineered bacteria have been approved for release. It is hoped that the results of research in the coming years will be positive and allay genuine fears about the possible detrimental effects of recombinant microorganisms in the environment.

CONCLUSIONS

The characterization of mosquitocidal toxins and the cloning of their genes has led and will continue to lead research and development in several directions.

(i) The identification of mosquitocidal toxins from various sources has allowed an appreciation of the diversity of their structures, mechanisms of action, and insecticidal host range. Thus, there are groups of vegetatively produced toxins, binary toxins, and single toxins which are unrelated in sequence. The vegetative toxin of *B. sphaericus* SSII-1 probably has a distinct mode of action from the toxins produced during sporulation. The search for new genes must continue, particularly to discover toxins effective against *Anopheles* mosquitoes and to create new synergistic combinations. It is probable that a greater diversity of mosquitocidal toxin genes exists.

(ii) A start has been made in elucidating the polypeptide determinants of toxicity and toxin specificity. The availability of purified recombinant toxins may allow their X-ray crystal structures to be solved, and may lead to the cloning of membrane receptor genes.

(iii) Field and laboratory tests have shown that the toxins and the bacteria which produce them are generally safe for mammals and nontarget organisms. Sophisticated floating and slow-release formulations have been developed and tested on a limited scale.

(iv) Toxins of *B. sphaericus* and *B. thuringiensis* subsp. *israelensis* have been cloned and expressed in diverse foreign cells, including recombinant high-toxicity strains of bacilli. The results suggest that expansion of insecticidal host range while maintaining toxicity is possible. In addition, active toxins have been produced in *Caulobacter* species and cyanobacteria, hosts which have the potential for long persistence at the larva feeding zone. The challenge is to engineer good and stable expression in these novel hosts.

(v) The tools of bacterial genetics, especially homologous recombination, have been applied to engineer genetically stable toxin expression and to expand the insecticidal range of strains of *B. thuringiensis*.

(vi) The development of floating and slow-release formulations of genetically stable recombinant microorganisms with an expanded mosquitocidal host range, combined with the ability to persist at the larval feeding zone, should lead to biological insecticides which are effective enough to have a real impact on mosquito-borne diseases.

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