Transfer of Toxin Genes to Alternate Bacterial Hosts for Mosquito Control

Sergio Orduz+, Nora Restrepo, Maria M Patiño, William Rojas

Unidad de Control Biológico, Corporación para Investigaciones Biológicas, Apartado Aéreo 7378, Medellin, Colombia

Mosquitoes are vector of serious human and animal diseases, such as malaria, dengue, yellow fever, among others. The use of biological control agents has provide an environmentally safe and highly specific alternative to the use of chemical insecticides in the control of vector borne diseases. Bacillus thuringiensis *and* B. sphaericus *produce toxic proteins to mosquito larvae. Great progress has been made on the biochemical and molecular characterization of such proteins and the genes encoding them. Nevertheless, the low residuality of these biological insecticides is one of the major drawbacks. This article present some interesting aspects of the mosquito larvae feeding habits and review the attempts that have been made to genetically engineer microorganisms that while are used by mosquito larvae as a food source should express the* Bacillus *toxin genes in order to improve the residuality and stability in the mosquito breeding ponds.*

Key words: biological control - *Bacillus thuringiensis* - *Bacillus sphaericus* - crystal proteins - recombinant insecticides - aquatic bacteria

From the medical point of view, mosquitoes are among the most important insects due to their capacity to transmit human and animal diseases. As vectors of debilitating and sometimes lethal diseases, they represent a threat to near 3,000 million people in the tropical and subtropical world (Becker & Margalit 1993). The importance of anophelines in transmitting malaria is widely recognized, while genera such as *Culex*, and *Aedes* are important vectors of filariosis and other diseases caused by viruses. Despite concerted efforts to reduce vector born diseases in high risk areas, diseases transmitted by mosquitoes still represent an important public health problem. During the past 30 years, the main vector control strategy was based on the use of chemical insecticides which produced benefits to the human health. However development of vector resistance to insecticides and the increased environmental concern due to the poor specificity demonstrated by these insecticides, specially those with recalcitrant molecules, were the reasons for their elimination from many of the mosquito control programs worldwide.

Mosquito larvae have several natural enemies, and amongst those, the most relevant are bacteria belonging to the genus *Bacillus*. Bacterial strains that produce toxins to mosquito larvae belong to the subspecies *israelensis* of *B. thuringiensis*, strain PG-14 of *B. thuringiensis* subsp. *morrisoni*, *B. thuringiensis* subsp. *medellin*, a few strains in other *B. thuringiensis* serotypes, and several serotypes of *B. sphaericus*. They produce different proteic toxins to mosquito larvae which are packed in the parasporal crystal as protoxins.

B. thuringiensis subsp. *israelensis* and *B. sphaericus* are currently used to control mosquito larvae populations (Becker & Margalit 1993), and several of the most promising strains of *B. sphaericus* are actually being tested under field conditions in Cameroon, Colombia, France, and Germany. However, the main limitation of these bacteria for their use in mosquito control programs is their short residuality caused by the rapid sedimentation of the spore-crystal complex in the ponds, and therefore requiring frequent applications during the mosquito season in temperate regions and all year round applications in the tropics (Silapanuntakul et al. 1983, Davidson et al. 1984, Ohana et al. 1987). In this paper we will review the mosquito larvae feeding habits as well as briefly summarize the attempts that have been made to genetically engineer larval food with the *Bacillus* toxin genes in order to improve the efficacy of these mosquito larvae active toxins and finally we outline some recommendations to have in mind to select alternative host for the *Bacillus* toxin genes.

Part of the information presented in this article received financial support from Cociencias and World Bank/WHO/TDR.

+ Corresponding author

Received June 24, 1994
Accepted 14 September 1994

PROPERTIES OF MOSQUITOCIDAL *BACILLUS* TOXINS AND GENES

Properties of B. thuringiensis *mosquitocidal proteins and toxin encoding genes* - The diversity of crystal toxins of *B. thuringiensis* has resulted in thousand of isolates and multiple efforts to obtain new strains with a wider host range and potency. Several thousand of isolates have been obtained and are classified in 45 subspecies according to their flagellar antigen. Nevertheless, their serovar status does not reflect their pathogenic characteristics, that are ultimately defined by the crystal protein composition. Most *B. thuringiensis* strains produce more than one crystal protein, and this composition is reflected in some way in crystal morphology (Lerechus et al. 1993). Therefore microscopic examination of the *B. thuringiensis* crystal could suggest insect specificity. The type of protein within the parasporal crystal also provides information on the possible insect target, if it is known. The size of the proteins deposited in *B. thuringiensis* crystals vary between 150 and 14 kDa. Höfte and Whitely (1989) proposed a classification system of the delta-endotoxins based on their insecticidal properties and molecular relationships. To date seven mayor classes of delta-endotoxins (CryI to VI) and cytolysins (Cyt) are known. The CryI type is generally active against Lepidoptera, the CryII type is active against Lepidoptera or Diptera, CryIII produce mortality in coleopteran insects, and CryIV type is lethal to mosquitoes. A controversial activity for CryV proteins towards coleopteran and lepidopteran insects has been reported (Gleave et al. 1993). The Cyt type, which has been found exclusively in mosquito active *B. thuringiensis* strains is also cytolytic to mammalian cells. We have recently identified a new subspecies of B.t. with activity to mosquito larvae (Orduz et al. 1992a). Its major crystal proteins investigated, and found in the range of 100, 68, 42, and 30 kDa. Immunoblot analysis of these proteins with purified antibodies against the major component of the *B. thuringiensis* susbp. *israelensis* crystal revealed that the anti 28 kDa recognized a 30 kDa crystal protein in *B. thuringiensis* subsp. *medellin*, opening the possibility that at least the 68 and 94 kDa proteins could correspond to new Cry proteins (Orduz et al. 1994). A list of the major genes active against mosquito larvae can be found in Table I.

Recent screening programs have obtained numerous *B. thuringiensis* and strains producing atypical delta-endotoxins, which probably have not been described in the Cry-Cyt classification system. In addition to the large variety of delta-endotoxins, some *B. thuringiensis* strains produce heat stable toxins that are named beta-exotoxins (Lecadet & de Barjac 1981). These compounds are nucleotide analogues, and their insecticidal activity is due to inhibition of RNA polymerase by competition with ATP. Although these compounds are not insect specific, they may contribute to the overall insect mortality.

TABLE I

Insecticidal genes of *Bacillus* for mosquito larvae control

Type of gene	Host [a]	No. of amino-acids	Molecular weight (kDa)	Reference
cryIIA	L/D	633	70.9	Donovan et al. 1980
cryIVA	D	1.180	134.4	Ward & Ellar 1987
cryIVB	D	1.136	127.8	Chunjatupornchai et al. 1988
cryIVC	D	675	77.8	Thorne et al. 1986
cryIVD	D	643	72.4	Donovan et al. 1988
cry?	D	?	100	Orduz et al. unpublished
cry?	D	?	68	Orduz et al. unpublished
cytA	D/cytol	248	27.4	Waalwijck et al. 1985
cytB	D/cytol	259	29.2	Koni & Ellar 1993
51kDa	D	448	51.4	Baumann et al. 1988
42kDa	D	370	41.9	Hindley & Berry 1987
100kDa	D	879	100	Thanabalu et al. 1991

[a]: specificity of host range: L, Lepidoptera; C, Coleoptera; D, Diptera; N, Nematode cytol.,cytolytic

Most of *B. thuringiensis* strains contain plasmids of different size (1.4 to 180 MDa), their copy number and size is particular to each strain, and is not related to serotype or pathotype. Despite the genes they contain and their size, the plasmids are stably inherited, suggesting that maintenance and replication functions are very important. Most of the plasmids in *B. thuringiensis* are cryptic and the main function known is the production of the entomocidal crystal proteins. Evidence for the role of this type of plasmids came from cloning the structural genes, which were then used for localization of the genes in many of the known strains, and were found normally in plasmids of 40 to 150 MDa (Whiteley & Schnepf 1986, Lereclus et al. 1989). It is possible that some of the crystal protein genes could be located in the chromosome, but the difficulty to differentiate between chromosomal genes and genes encoded by large plasmids (150 MDa) make this possibility difficult to be probed. It has been established that several plasmid genes encoding delta endotoxins are part of complex structures that include mobile genetic elements. Some of these genes are flanked by insertion sequence elements and by transposons (Lereclus et al. 1993).

Properties of B. sphaericus *mosquitocidal proteins and toxin encoding genes* - The toxicity produced by *B. sphaericus* in mosquito larvae is associated with the assembling of parasporal crystals proteins at the time of sporulation (Yousten & Davidson 1982, Kalfon et al. 1984). The toxic proteins of the most potent *B. sphaericus* strains (2362, 2297, 1593, and IAB 59) have been characterized and their molecular weight determinated (51.4, known as 51 kDa and 41.9, known as 42 kDa) (Table I). These proteins does not have to be synthesized as parasporal inclusion to produce their lethal effect on mosquito larvae, and their toxicity can be observed from the 12th hr of growth, although in minor degree than when the cultures are fully sporulated. A recent finding suggests that contrary to what was believed, the 42 kDa protein produced as a recombinant protein was toxic to *C. pipiens* larvae by itself (LC_{50}, 300 ng/ml), and the recombinant 51 kDa protein was not toxic (Nicolas et al. 1993). *B. sphaericus* strain SSII-1 another mosquito active strain produces a 100 kDa protein (Thanabalu et al. 1991) which is 1000 times less toxic than the binary toxin produced by strains 2362, 2297 and 1593. The 100 kDa protein of strain SSII-1 is labile to treatments such as cell disruption methods, heat, freezing and thawing (Myers & Yousten 1978, Myers et al. 1979). All three genes have been cloned and sequenced, however the location of these genes has been a matter of debate. Singer (1987), have demonstrated the presence of large and small plasmids in most of the larvicidal and nonlarvicidal strains of *B. sphaericus*, but in the case of strain 1691, the presence of plasmids was not observed, suggesting that at least in this strain, the genes for the binary toxin (51-42 kDa) could be located in the chromosome. Recently Liu et al. (1993) have reported new mosquitocidal strains of *B. sphaericus* from Singapore which do not contain the 100 kDa toxin gene, and Orduz et al. (1992b, 1993) have reported very potent strains isolated from Nigeria and Colombia.

POTENTIAL HOST FOR THE *BACILLUS* TOXIN GENES

Mosquito larvae feeding habits - In order to select potential host for the *Bacillus* toxin genes, it is important to understand the mosquito larval feeding behavior. During the first part of the century and before the discovery and use of chemical insecticides, a large body of information regarding mosquito larvae food and feeding habits was collected (Atkin & Bacot 1917, Coggeshall 1925, Hinman 1930, Howland 1930, Rozeboom 1934). In recent years, a new interest in bacteria inhabiting mosquito larvae breeding ponds has appeared, and this interest is growing mainly due to its possible use them as potential hosts for the *Bacillus* toxin genes in order to improve the efficacy of the entomopathogenic *Bacillus* in the mosquito control programs (Ameen & Iversen 1978, Walker et al. 1988, Thiéry et al. 1991, 1993, Merritt et al. 1992).

Based on studies of mosquito larvae feeding behaviour, Merritt et al. (1992) have proposed four types of feeding modes 1. collecting-filtering, 2. collecting-gathering, 3. scraping, and 4. shredding. The *Anopheles*, *Culiseta*, *Culex*, *Mansonia*, *Coquilletidia*, and some *Aedes* species are collect-filtering. The feeding zone of these collecting-filtering species varies vertically from the plant root surfaces in *Coquilletidia* and *Mansonia* to the water column in *Culex* and *Culiseta*, and at the top water-air interface where most of the *Anopheles* species feed (for a complete description of all the categories see Merritt et al. 1992).

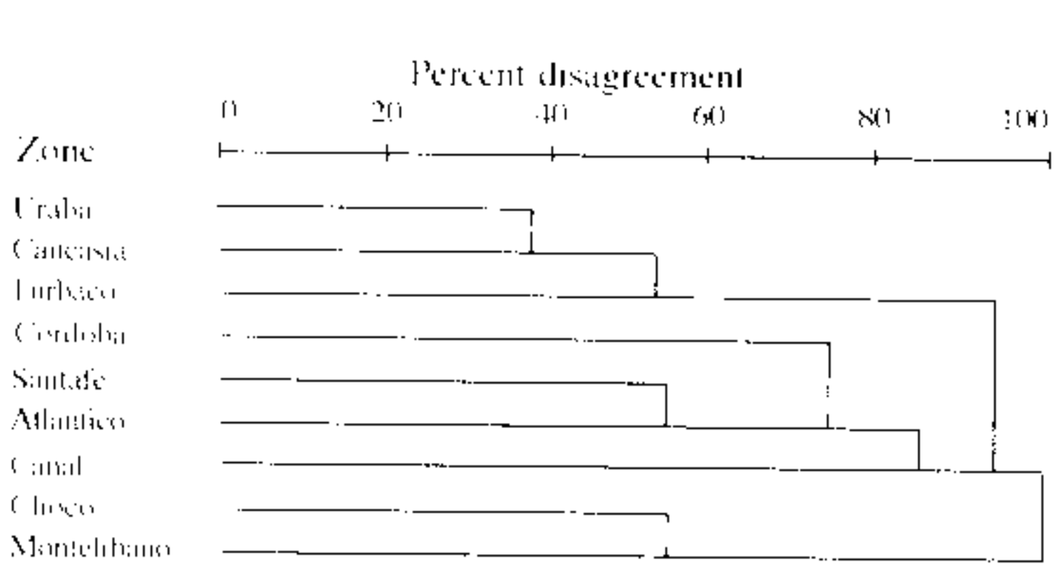
Microorganisms from the mosquito larvae breeding ponds - There are several bacterial species that colonize the upper layers of water bodies that could be potential hosts for mosquito larvae toxins. As indicated by Merritt et al. (1992) the principal components of the mosquito larvae diet are microorganisms and detritus. The microorganisms that have been identified as part of the diet of the collecting-filtering mosquito larvae species vary with the type of pond, the methods used to collect the water samples, the methods used to isolate them as well as with the type of intestinal content analysis. Although literature reports indicate that most of the mosquito species

does not have particular preferences for the kind of food they ingest, it has been observed that the size of the particles is a limiting factor (Dadd 1971, Wallace & Merritt 1980). It has been observed in many studies that cyanobacteria, cocci, rods, spirochetes, unicellular and filamentous algae, protozoans, small metazoans, rotifers, crustaceans and detritus, have been found in the gut content analysis of several mosquito species (Hinman 1930, Howland 1930, Laird 1988, Walker et al. 1988). In order to quantitate the amount of bacteria ingested by mosquito larvae, DNA binding fluorochromes in combination with epifluorescence microscopy was used by Walker et al. (1988) and found that the larval food bolous of *A. triseriatus*, *A. quadrimaculatus* and *C. perturbans* contained an average of 2.2 x 10^6, 2 x 10^6 and 0.9 x 10^6 bacteria respectively. Although, Thiéry et al. (1993) have indirectly demonstrated that in the case of cyanobacteria, fourth instar *C. pipiens* larvae can digest up to 95% of ingested cells, and 100% in the case of *A. gambiae* fourth instar larvae, direct demonstration of the ability of the mosquito larvae to use the bacteria as a nutrient source is required. A problem derived from the gut content analysis, as reported by Laird (1988), is that undigested material could be more easily found in the gut content than the more fragile protists that are readily digested and enter to form the amorphous type of material usually found in the larval gut. Experiments growing mosquito larvae using microorganisms obtained from the natural breeding ponds have demonstrated that larval mortality was not observed, when larvae were reared in water from the pool and bottom substrate containing dinoflagelates and *Chlamydomonas* were added (Ameen & Iversen 1978). Recent studies indicate that cyanobacteria, one of the leading hosts candidates for the mosquito larvae toxin genes, can change its location in the water ponds (Howard 1993), and it is suggested that changes in vertical position could be related to changes in the amount of carbohydrate ballast stored in the cells, and this cell ballast mechanism is controlled by the photosynthetic rate of the cyanobacteria (Kromkamp & Mur 1984); therefore, vertical movements of these bacteria locate them in and out of the anopheline feeding zone which is also an important consideration for the selection of the potential hosts for the *Bacillus* toxin genes.

Literature on the bacterial composition of the tropical pond ecosystems is scarce. In temperate areas, bacterial composition of lakes and small water bodies has been reported. Wolters and Schwartz (1956) described the bacterial inhabitants of oligotrophic water bodies in which *Pseudomonas* spp., *Alcaligenes* spp., and *Azotobacter* spp., were the main genera found. *Caulobacter* spp., and *Galionella* spp., were also reported at lower frequency. In eutrophicated ecosystems they observed mainly *Pseudomonas fluorescens*, and species belonging to the Bacillaceae family and gram negative enterobacteries. In 1980, Konda and Yasuhito studying the water surface bacterial population of the Motosu-ko lake found that 84.1% was gram negative bacteria, and the main genera were *Flavobacterium*, *Pseudomonas*, and *Moraxella*, and when studying the sediments found that 73.9% of the bacterial population was gram positive *Bacillus*. Between 1990 and 1991, we have conducted an inventory of bacteria living in the water-air interface in 27 mosquito larvae breeding ponds in nine localities of northwestern Colombia, and have found 33 bacterial species. We have studied the bacterial distribution of these species and have observed that the most frequent bacterial species isolated were *B. mycoides*, *Aeromona hydrophyla*, *Enterobacter agglomerans*, *B. cereus* and *B. thuringiensis* as shown in Table II. The relationship between each one of the study areas, according to the bacterial species composition found is shown in Figure.

Richness index of Margaleff (1958) was used to study the bacterial populations of the mosquito breeding ponds, and indicates that the most diverse areas in this study were Choco 3.49, Cordoba 3.45, Turbaco 3.27, and Uraba 2.68.

Quality of nutrients - Atkin and Bacot (1917), Hinman (1930), and Rozeboom (1934) could obtain adults from mosquito larvae reared in water containing bacteria and algae from the mosquito breeding ponds. From the bacteria found in the mosquito breeding ponds in Colombia, we selected five bacterial species in order to conduct the feeding and assimilation experiments. When tested with *C. quinquefasciatus*, 22% and 35.4% survived from second to third instar when grown with suspensions of 5x10^6 cells/ml of cultures of *B. mycoides* and *Citrobacter freundii* respectively, while when grown with cultures of *B. megaterium*, *B. pumilus*, and *E. agglomerans*, less than 10% survived to six days, and none



Hyerarchical tree of the nine zones sampled in northwestern Colombia generated by cluster analysis using complete linkage and percent disagreement, according to the presence or absence of bacterial species.

TABLE II

Main bacterial species found in 27 mosquito breeding ponds in nine zones of northwestern Colombia

Bacterial species	Positive ponds[a]	Positive zones[a]
Bacillus mycoides	15(55.5)	8(88.9)
Aeromona hydrophila	14(51.8)	7(77.8)
Enterobacter agglomerans	13(48.1)	6(99.7)
Bacillus cereus	12(44.4)	5(55.6)
Gram positive cocci	7(25.9)	4(44.4)
Bacillus thuringiensis	5(18.5)	4(44.4)
Acinetobacter anitratus	5(18.5)	3(33.3)
Bacillus pumilus	4(14.8)	3(33.3)
Citrobacter freundii	4(14.8)	3(33.3)
Chromobacterium violaceum	4(14.8)	3(33.3)
Acinetobacter Iwoffi	4(14.8)	3(33.3)
Enterobacter cloacae	4(14.8)	2(22.2)
Pseudomona paucimobilis	3(11.1)	1(11.1)
Pseudomona cepacea	3(11.1)	3(33.3)
Pseudomona putida	3(11.1)	3(33.3)
Sarcinas	3(11.1)	2(22.2)
Serratia marcescens	2(7.4)	1(11.1)
Klebsiella oxytoca	2(7.4)	1(11.1)
Enterobacter aerogenes	2(7.4)	1(11.1)
Pseudomona maltophila	2(7.4)	1(11.1)
Pseudomona vesiculare	2(7.4)	2(22.2)
Klebsiella ozonae	2(7.4)	1(11.1)
Pseudomona fluorescens	2(7.4)	1(11.1)
Pleisomona shigelloide	2(7.4)	1(11.1)
Klebsiella pneumoniae	1(3.7)	1(11.1)
Pseudomona aeruginosa	1(3.7)	1(11.1)
Serratia liquefasciens	1(3.7)	1(11.1)
Pseudomona putrefasciens	1(3.7)	1(11.1)
Bacillus sphaericus	1(3.7)	1(11.1)
Hyphomicrobium vulgare	1(3.7)	1(11.1)
Bacillus macerans	1(3.7)	1(11.1)
Pseudomona sp.	7(25.9)	4(44.4)
Bacillus sp.	9(33.3)	5(55.6)
Shigella sp.	1(3.7)	1(11.1)
Salmonella sp.	1(3.7)	1(11.1)
Flavobacterium sp.	2(7.4)	1(11.1)
Moraxella sp.	4(14.8)	2(22.2)

[a]: numbers in braquets represent percent of occurence

reached the third instar. In the case of *A. albimanus*, survivorship was limited to 4-5 days with a 6.8, 32, and 50.4% when grown on cells of *B. mycoides*, *C. freundii* and *B. thuringiensis* 4Q2-81 a *cry*⁻ strain, and similarly, mosquito larvae changes in this period of time from 2nd to 3rd instar.

CLONING THE *BACILLUS* TOXIN GENES

Genetic improvement of the insecticidal activity has been approached in two different ways: (1) transfer of the toxin genes in to non-homologous isolates to combine expression of toxic proteins to produce additive or synergistic effects, and (2) transfer the toxin genes in to baculovirus in order to improve *B. thuringiensis* activity on host and age.

Recombinant Escherichia coli *with* B. thuringiensis *genes* - Haider et al. (1987) cloned the *cryII* gene from *B. thuringiensis* subsp. *aizawai* in *E. coli* TGI, and the recombinants were able to produce and crystallized the CryII protein, and toxicity, was observed as expected in lepidopteran and dipteran larvae.

The *cryIVA*, *cryIVB*, *cryIVC*, *cryIVD*, and *cytA* genes from *B. thuringiensis* subsp. *israelensis* have been expressed in *E. coli*, and the different protein yield and toxicity were probably caused by differences in host strains, vectors, bioassays, protein stability, and/or a variation in the promoter and ribosome binding site efficacy (Waalwijck et al. 1985, Thorne et al. 1986, Ward et al. 1986, Angsuthanasombat et al. 1987, 1992, Chungjatupornchai et al. 1988, Delécluse et al. 1988, Ward & Ellar, 1988). The 128 kDa (CryIVB) toxin expressed in *E. coli* JM107, and purified was highly toxic (LC_{50} 43 ng ml) to third-instar larvae of *A. aegypti* (Chungjatupornchai et al. 1988). Recently, Koni and Ellar (1993) have cloned in *E. coli* the new *cytB* protein gene of 29 kDa of *B. thuringiensis* subsp. *kyushuensis* CytB has shown to have 39% identity and 70% similarity with the CytA proteins. Unlike *cytA*, cloned *cytB* when expressed in *E. coli* was assembled in amorphous cytoplasmic inclusions without the intervention of helper proteins. We have recently cloned and expressed the 100 kDa crystal protein gene of *B. thuringiensis* subsp. *medellin* reported by Orduz et al. (1994) in *E. coli* SOLR. Expression of this probably novel *cry* gene produced significant mortality when whole cells were given to third instar *C. quinquefasciatus* larvae.

Recombinant Bacilli with B. thuringiensis *genes* - All the major crystal proteins of *B. thuringiensis* have been cloned and expressed in bacilli. Expression of the 128 kDa protein corresponding to the *cryIVB* gene of *B. thuringiensis* subsp. *israelensis* in *B. sphaericus* 2362 and 1593, produced an increased activity against *A. aegypti* larvae, which was comparable to the normal toxicity of *B. thuringiensis* subsp. *israelensis* (Trisrisook et al. 1990). In contrast, cloning of the *cryIVD* and *cytA* in *B. sphaericus* produced only a 10 fold increase in activity to *A. aegypti* larvae (Bar et al. 1991). Delecluse et al. (1993)

obtained expression of *cryIVA* and *cryIVB* genes in an acrytalliferous *B. thuringiensis* strain. Mortality was observed in *A. aegypti*, *A. stephensi*, and *C. pipiens* when the transformants containing the CryIVA protein were used in the bioassays. Mortality range was reduced to *A. aegypti* and *A. stephensi* when these mosquito species were challenged with recombinants expressing the CryIVB protein.

The *cryIVD* crystal gene of *B. thuringiensis* subsp. *israelensis* cloned in *B. megaterium*, produced the corresponding 70 kDa protein, and transformants were toxic to *A. aegypti* larvae; although toxicity was not equivalent to that caused by *B. thuringiensis* subsp. *israelensis* (Donovan et al. 1988). When the cryIVD gene from *B. thuringiensis* subsp. *morrisoni* PG-14 was cloned in *B. thuringiensis* subsp. *kurstaki*, recombinants produced comparable toxicity to that of the donor strain (Chang et al. 1992).

The *cytA* gene of *B. thuringiensis* subsp. *israelensis* has been cloned and expression of the 27 kDa crystal protein has been observed in *Bacillus subtilis* (Thorne et al. 1986, Ward et al. 1986, 1988, Ward & Ellar 1988). Expression of *B. thuringiensis* subsp. *israelensis* toxins in *B. sphaericus* have also been obtained (Trisrisook et al. 1990, Bar et al. 1991). The *cytA* and *cryIVD* toxins genes have been cloned and expressed in *B. subtilis* and in *B. sphaericus* strain 2362 (Bar et al. 1991). The expression of the CytA and CryIVD proteins of *B. thuringiensis* subsp. *israelensis* in a recombinant *B. sphaericus* 2362 produced an increase of toxicity toward *A. aegypti* compared to that produced by the parental strain 2362, but lower than that of *B. thuringiensis* subsp. *israelensis* (Bar et al. 1991).

Recombinant E. coli *with* B. sphaericus *genes - E. coli* has been used as hosts for the mosquitocidal toxin genes of *B. sphaericus* (Baumann et al. 1987, 1988, Baumann & Baumann 1989, de la Torre et al. 1989, Davidson et al. 1990, Oei et al. 1990, Thanabalu et al. 1991). Expression of the 51 and 42 kDa toxin genes from strain 2362 in *E. coli* was observed, but its toxicity was lower than any of the high toxicity strains. However, Sebo et al. (1990) observed that *E. coli* GM2199 harboring the same binary toxin gene from *B. sphaericus* 1593M had an LC_{50} of 10 ng ml against *C. pipiens*, similar to the values obtained for either the spore-crystal complex of *B. sphaericus* 2362 or the purified mixture of 51 and 42 kDa proteins (Baumann et al. 1991).

Recombinant Bacilli with B. sphaericus *genes* - Expression of the binary toxin gene in bacilli has been evaluated. Recombinant nontoxic *B. sphaericus* 718 harboring the binary toxin genes, produced crystalline inclusions during sporulation, and toxicity to *C. pipiens* was as high as toxicity caused by strain 2362. *B. sphaericus* SSII-1 transformed with the binary toxin genes also produced inclusions but it was not as toxic as recombinant *B. sphaericus*, and the spores of the recombinant strain *B. subtilis* DB104 contained amorphous inclusions and were three times more toxic to *C. pipiens* larvae than was *B. sphaericus* 2362 (Baumann et al. 1985, 1991, Broadwell et al. 1990a, b).

The binary toxin of *B. sphaericus* has also been expressed from its own promoter using the shuttle vector (pGSP10) in *B. thuringiensis* subsp. *israelensis* 4Q2-72 and a crystal minus strain of *B. thuringiensis* subsp. *israelensis* (4Q2-81) (Bourgouin et al. 1990). Crystals of *B. sphaericus* toxins were deposited with the spores of both strains. Unexpectedly, the crystal minus strain expressing only the *B. sphaericus* toxins was equally toxic to *A. stephensi* and *A. aegypti* larvae, in contrast to the parental *B. sphaericus*, which was about 25 times less active against *A. aegypti* than against *A. stephensi*. In the recombinant *B. thuringiensis* subsp. *israelensis* 4Q2-72(pGSP10) there was no detectable additive or synergistic effect when expressing both classes of toxins in one cell compared with wild type *B. thuringiensis* subsp. *israelensis* (Bourgouin et al. 1990).

B. sphaericus SSII-1 produces a 100 kDa protein that is responsible for the low toxicity to mosquito larvae. Its gene has been cloned and expressed in *E. coli* under the control of its own promoter, and toxicity of the recombinant *E. coli* was lower to *A. aegypti* and *C. quinquefasciatus* larvae than *B. sphaericus* SSII-1 (Thanabalu et al. 1991). More recently, a truncated version of the 100 kDa toxin lacking the N-terminal signal sequence has been produced in *E. coli* (Thanabalu et al. 1992a). Its LC_{50} determined against *C. quinquefasciatus* larvae was similar to the value obtained for the 51 and 42 kDa toxins from *B. sphaericus* 2362 expressed in *B. subtilis* (Baumann et al. 1991), indicating that specificity of the 100 kDa toxin was not the limiting factor found by Thanabalu et al. (1991).

EXPRESSION OF TOXIN GENES IN AQUATIC MICROORGANISM

The mosquitocidal toxin genes of *B. thuringiensis* and *B. sphaericus* have been introduced in several species of aquatic bacteria such as *Caulobacter* species that occur in virtually every aquatic habitat and are found predominantly in regions where several species of mosquito feed (Pointdexter 1981, Meritt et al. 1992) and in cyanobacteria (Table III).

Thanabalu et al. (1992b) cloned separately the binary toxin genes from *B. sphaericus* 2297, the 100-kDa toxin gene from *B. sphaericus* SSII-1,

TABLE III

Recombinant aquatic bacteria with mosquitocidal genes from *Bacillus thuringienensis* subsp. *israelensis* and *Bacillus sphaericus*

Bacterial species	Toxin genes	Toxicity
Caulobacter crescentus	binary	low in *C. quinquefasciatus*
	100kDa	low in *C. quinquefasciatus*
	cryIVB	low in *A. aegypti*
Agmenelum quadruplicatum	*cryIVA*	low in *A. aegypti*
	cryIVD	low in *C. pipiens*
Anabaena sp.	binary	low in *C. pipiens*
	binary	low in *A. sinensis*
Anacystis nidulans	binary	low in *C. pipiens*
Synechococcus sp.	*cryIVB*	low in *A. aegypti*

and the 128 kDa *crylVB* gene from *B. thuringiensis* subsp. *israelensis* in *Caulobacter crescentus*. All recombinants displayed toxicity to mosquito larvae, and the most toxic constructs of the binary toxin gene were lethal to *C. 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 (Thanabalu et al. 1991, 1992b). *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 *C. quinquefasciatus* and *A. aegypti* larvae, respectively. Protein levels were not quantitated but were believed to be low (Thanabalu et al. 1992b).

Various species of cyanobacteria have been investigated as vehicles for prolonged delivery of insecticidal toxins to the larval feeding zone (de Marsac et al. 1987, Angsuthanasombat & Panyim 1989, Chungjatupornchai 1990, Murphy & Stephens 1992). Like *Caulobacter* species, the ubiquitous photosynthetic cyanobacteria exist at or near the water surface. These photoautotrophic bacteria are adaptable to both freshwater and saltwater environments. They have a wide temperature tolerance and limited nutritional requirements (Rippka et al. 1979).

Toxin genes from *B. thuringiensis* subsp. *israelensis* or *B. sphaericus* were cloned in cyanobacteria, but in all cases expression levels and toxicity were very low (de Marsac et al. 1987, Angsuthanasombat & Panyim 1989, Chungjatupornchai 1990). *Agmenelum quadruplicatum* (*Synechococcus* sp. strain PCC 7002) cells were transformed with the 134 kDa toxin gene from *B. thuringiensis* subsp. *israelensis*, cell extracts were weakly toxic to *A. aegypti* larvae (Angsuthanasombat & Panyim 1989).

The 128 kDa CrylVB protein of *B. thuringiensis* subsp. *israelensis* has been expressed in *Synechococcus* sp. strain PCC 6803 after its integration into the chromosome. Cell extracts but not live cells were weakly toxic to 20 to 30% of *A. aegypti* larvae, equivalent to 1 to 2 µg of toxin protein per ml (Chungjatupornchai 1990). Live cells of the cyanobacterium *A. quadruplicatum* PR-6 which had been transformed with the *cryIVD* gene from *B. thuringiensis* subsp. *israelensis*, were reported to be toxic to mosquito larvae (Murphy & Stephens 1992). The *cryIVD* gene was sufficiently well expressed to form phase-bright inclusions and there was a delay in the onset of toxicity of the recombinant *A. quadruplicatum* cells from 1 to 3 days, and 100% mortality occurred 5.5 days after the cells were first fed to the larvae (Murphy & Stephens 1992).

The binary toxin gene of *B. sphaericus* was used to transform *Anacystis nidulans* R2 (also called *Synechococcus* sp. strain R2) (de Marsac et al. 1987). Cell extracts produced low mortality against *C. pipiens* larvae. Recently, Xudong et al. (1993) cloned the binary toxin gene of *B. sphaericus* 2297 in the cyanobacteria *Anabaena* sp. PCC 7120. Mortality of *C. pipiens* by the re-

combinant cyanobacteria was 94% at a concentration of $5x10^5$ cells/ml, and 92% in *A. sinensis* at a concentration of $7x10^5$ cells/ml. Expression of the binary toxin was also observed after 30 generations under nonselective conditions.

The *cryIVD* gene from *B. thuringiensis* subsp. *morrisoni* PG-14 has been inserted in a baculovirus vector, and recombinant viral particles produced infection in *Spodoptera frugiperda* cells and *Trichoplusia ni* larvae; synthesized polyhedrin-CryIVD protoxin fusion protein crystallized as cuboidal inclusions in the cytoplasm (Pang et al. 1992). The infected cells and purified inclusions were toxic to mosquito larvae (LC_{50} was close to 250 ng ml) (Pang et al. 1992).

Increasing host range - By using electroporation, Crickmore et al. (1990), introduced a plasmid containing the *cryIIIA* gene of *B. thuringiensis* subsp. *morrisoni* pathotype *tenebrionis* into *B. thuringiensis* subsp. *israelensis*, and the resulting transgenic bacterium demonstrated activity against coleopteran and dipterans, and surprisingly also showed activity against the lepidopteran *Pieris brassicae*. Similarly, when the *cryIA(b)* gene was introduced in to a coleopteran active strain, toxicity was observed not only in coleopteran and lepidopteran insects, but also in mosquitoes, introducing the new and revolutionary concept that the *B. thuringiensis* toxins can interact synergistically to expand the host range.

Therefore one of the critical points is to select the adequate microorganism in which several of the following parameters should be observed: (1) persistence in the mosquito larvae feeding zone, which is particular for the mosquito species, (2) to be ingestible and digestible by the mosquito larvae in order to uncover the hidden toxic proteins expressed and deposited in the cytoplasm of the recombinant organism, and make them available for activation and binding to the larval midgut receptors, (3) non toxic to mammals, (4) to be widely distributed (ubiquitous), (5) to be compatible with other larvicides, specially with chemicals, and finally (6) to be able to express the recombinant proteins at the required levels to suppress the mosquito larvae population.

In order to avoid recombination regulations it has been proposed to "encapsulate" the spore-crystal complex in *Tetrahymena pyriformis*, an unicellular protozoan. The protozoan normally ingest particulated material they find. Under field simulated conditions, *T. pyriformis* fed with *B. thuringiensis* spore-crystal complex increases the activity of the *B. thuringiensis* subps. *israelensis* toxins from 24 to 71 hr (Zaritsky et al. 1991).

The genetic stability of a cloned toxin gene in a recombinant microorganism has a paramount importance due to the high cost and effort involved in the design of a genetic engineer organism. Plasmid instability could be due to segregational and structural phenomena, and is also caused by incompatibility with other plasmids (Novick 1987, Bron 1990). The variable levels of expression obtained in most of the recombinant events performed with the *Bacillus* toxin genes are due to problems with the promoter, the ribosome binding site, the transcriptional terminator signals, codon usage and the stability of the plasmids used to transform the host cells (Porter et al. 1993). With different degrees of difficulty, most of these factors can be solved, however, the design of stable plasmids is perhaps the most laborious tasks to be undertaken by the scientist. An alternative for the design of stable plasmids for recombinant mosquito larvae food, is chromosome integration of the *Bacillus* toxin genes (Youngman 1990, Hoch 1991), where the recombination events are approximately 1,000 times less frequent than in plasmids (Niaudet et al 1984, Jannière et al. 1990). For a complete review of the attempts that have been made to improve the genetic stability of recombinant bacteria see Porter et al. (1993).

REFERENCES

Ameen M, Iversen TB 1978. Food of *Aëdes* larvae (Diptera, Culicidae) in a temporary forest pool. *Arch Hydrobiol 83*: 552-564.

Angsuthanasombat C, Chungjatupornchai W, Kertbundit S, Luxanabil P, Settasatian C, Wilairat P, Panyim S 1987. Cloning and expression of 130-kd mosquito-larvicidal delta-endotoxin gene of *Bacillus thuringiensis* var. *israelensis* in *Escherichia coli. Mol Gen Genet 208:* 384389.

Angsuthanasombat C, Crickmore N, Ellar DJ 1992. Comparison of *Bacillus thuringiensis* subsp. *israelensis* Cry IVA and CryIVB cloned toxins reveals synergism in vivo. *FEMS Microbiol Letters 94:* 63-68.

Angsuthansombat C, Panyim S 1989. Biosynthesis of 130-kilodalton mosquito larvicide in the cyanobacterium *Agmenellum quadriplicatum* PR-6. *Appl Environm Microbiol 55:* 2428-2430.

Atkin EE, Bacot A 1917. The relation between the hatching of the eggs and the development of thr larvae of *Stegomyia fasciata* (*Aëdes calopus*) and the presence of bacteria and yeast. *Parsitology 9:* 482-536.

Bar E, Lieman-Hurwitz,J, Rahamim E, Keynan A, Sandler N 1991. Cloning and expression of *Bacillus thuringiensis* delta-endotoxin DNA in *B. sphaericus. J Invertebr Pathol 57:* 149-158.

Baumann L, Baumann P 1989. Expression in *Bacillus subtilis* of the 51 and 42 kilodalton mosquitocidal toxin genes of *Bacillus sphaericus. Appl Environ Microbiol 55:* 252-253.

Baumann L, Broadwell AH, Baumann P 1988. Sequence analysis of the mosquitocidal toxin genes encoding 51.4 and 41.9 kilodalton proteins from *Bacillus sphaericus* 2362 and 2297. *J Bacteriol 170:* 2045-2050.

Baumann P, Baumann L, Bowditch RD, Broadwell AH 1987. Cloning of the gene for the larvicidal toxin of *Bacillus sphaericus* 2362: evidence for a family ot related sequences. *J Bacteriol 169:* 4061-4067.

Baumann P Clark MA, Baumann L, Broadwell AH 1991. *Bacillus sphaericus* as a mosquito pathogen: properties of the organism and its toxin. *Microbiol Rev 55:* 425-436.

Baumann P, Unterman BM, Baumann L, Broadwell A, Abbene HSJ, Bowditch RD 1985. Purification of the larvicidal toxin of *Bacillus sphaericus* and evidence for high molecular-weight precursors. *J Bacteriol 163:* 738-747.

Becker N, Margalit J 1993. Use of *Bacillus thuringiensis israelensis* against mosquitoes and blackflies, p. 147-170. In PF Entwistle, JS Cory, MJ Bailey, S. Higgs (eds). Bacillus thuringiensis, *an environmental biopesticide: Theory and Practice.* PF Entwistle, JS Cory, MJ Bailey, S Higgs (eds.). John Wiley and Sons, Chichester, United Kingdom.

Bourgouin C, Delécluse A, de la Torre F, Szulmajster J 1990. Transfer of the toxin protein genes of *Bacillus sphaericus* into *Bacillus thuringiensis* subsp. *israelensis* and their expression. *Appl Environ Microbiol 56:* 340 344.

Broadwell AH, Baumann L Baumann P 1990a. Larvicidal properties of the 42 and 51 kilodalton *Bacillus sphaericus* proteins expressed in different bacterial hosts: evidence for a binary toxin. *Curr Microbiol 21:* 361 366.

Broadwell AH, Baumann L Baumann P 1990b. The 42 and 51-kilodalton mosquitocidal proteins of *Bacillus sphaericus* 2362: construction of recombinants with enhanced expression and in vivo studies of processing and toxicity. *J Bacteriol 172:* 2217-2223 .

Bron S 1990. Plasmids, p. 75-139. In CR Harwood & SM Cutting (eds). *Molecular biology methods for* Bacillus. John Wiley & Sons Ltd., Chichester, United Kingdom.

Chang C, Dai SM, Frutos R, Federici BA, Gill SS 1992. Properties of a 72-kilodalton mosquitocidal protein from *Bacillus thuringiensis* subsp. *morrisoni* PG-14 expressed in *Bacillus thuringiensis* subsp. *kurstaki* by using the shuttle vector pHT3101. *Appl Environ Microbiol 58:* 507-512.

Coggeshall LT 1926. Relationship of plankton to Anopheline larvae. *Am J Hyg 6:* 556-569.

Chungjatupornchai W 1990. Expression of the mosquitocidal protein genes of *Bacillus thuringiensis* subsp. *israelensis* and the herbicide resistance gene *bar* in *Synechocystis* PCC6803. *Curr Microbiol 21:* 283-288.

Chungjatupornchai W, Höfte H, Seurinck J, Angsuthanasombat C Vaeck M 1988. Common features of *Bacillus thuringiensis* toxins specific for Diptera and Lepidoptera. *Eur J Biochem 173:* 9-16.

Crickmore N, Nicholls C, Earp DJ, Hodgman TC Ellar DJ 1990. The construction of *B. thunngiensis* strains expressing novel entomocidal deltal endotoxin combinations. *Biochem J 270:* 133-136.

Dadd RH 1971. Effects of size and concentration of particles on rates of ingestion of latex particulates by mosquito larvae. *Ann Entomol Soc Amer 64:* 687-693

Davidson EW, Oei C, Meyer M, Bieber AL, Hindley J Berry C 1990. Interaction of the *Bacillus sphaericus* mosquito larvicidal proteins. *Can J Microbiol 36:* 870-872.

Davidson EW, Urbina M, Payne J, Mulla MS, Darwazeh H Dulmage HT Correa JA 1984. Fate of *Bacillus sphaericus* 1593 and 2362 spores used as larvicides in the aquatic environment. *Appl Environm Microbiol 47:* 125-129.

De la Torre F, Bennardo T, Sebo P Szulmajster J 1989. On the respective roles of the two proteins encoded by the *Bacillus sphaericus* 1593M toxin genes expressed in *Escherichia coli* and *Bacillus subtilis. Biochem Biophys Res Commun 164:* 1417 1422.

De Marsac NT, de la Torre F Szulmajster J 1987. Expression of the larvicidal gene of *Bacillus sphaericus* 1593M in the cyanobacterium *Anacystis nidulans* R2. *Mol Gen Genet 209:* 396 398.

Delécluse A, Bourgouin C, Klier A, Rapoport G 1988. Specificity of action on mosquito larvae of *Bacillus thuringiensis israelensis* toxins encoded by two different genes. *Mol Gen Genet 214:* 42-47.

Delécluse A, Poncet S, Klier A, Rapoport G 1993. Expression of *cryIVA* and *cryIVB* genes, independently or in combination, in a crystal-negative strain of *Bacillus thuringiensis* subsp. *israelensis. Appl Environm Microbiol 59:* 3922-3827.

Donovan WP, Dankocsik CC, Gilbert MP 1988. Molecular characterization of a gene encoding a 72-kilodalton mosquito-toxic crystal protein from *Bacillus thuringiensis* subsp. *israelensis. J Bacteriol 170:* 4732 4738.

Gleave AP, Williams R, Hedges RJ 1993. Screening by polymerase chain reaction of *Bacillus thuringiensis* serotypes for the presence of *cryV*-like insecticidal protein genes and characterization of a *cryV* gene cloned from *B. thuringiensis* subsp. *kurstaki. Appl Environm Microbiol 59:* 1683-1687.

Haider MZ, Ward ES, Ellar DJ 1987. Cloning and heterologous expression of an insecticidal delta-endotoxm gene from *Bacillus thuringiensis* var *aizawai* ICI toxic to both Lepidoptera and Diptera. *Gene 52:* 285-290.

Haider MZ, Ellar DJ 1987. Analysis of the molecular basis of the insecticidal specificity of *Bacillus thuringiensis* crystal delta-endotoxins. *Biochem J 248:* 197-201.

Hinman EH 1930. A study of the food of mosquito larvae (Culicidae). *Am J Hyg 12:* 238-270.

Hoch JA 1991. Genetic analysis in *Bacillus subtilis. Methods Enzymol 204:* 305-320.

Höfte H, Whiteley HR 1989. Insecticidal crystal proteins of *Bacillus thuringiensis. Microbiol Rev 53:* 242-255.

Howard A 1993. SCUM-simulation of cyanobacteria underwater movement. *CABIOS 9:* 413-419.

Howland LJ 1930. Bionomical investigation of English mosquito larvae with special reference to their algal food. *J Ecol 18:* 81-125.

Jannière L, Bruand C, Ehrlich SD 1990. Structurally stable *Bacillus subtilis* cloning vectors. *Gene 87:* 53-61.

Kalfon A, Charles J-F, Bourgouin C, de Barjac H 1984. Sporulation of *Bacillus sphaericus* 2297: an electron microscope study of crystal-like inclusion biogenesis and toxicity to mosquito larvae. *J Gen Microbiol 130:* 893-900.

Konda T, Yasuhito T 1980. Bacterial flora in the water and sediment of lake Motosu-ko, an oligotrophic lake in central Japan. *J Ecol 29:* 202.

Koni PA, Ellar DJ 1993. Cloning and characterization of a novel *Bacillus thuringiensis* cytolytic delta-endotoxin. *J Mol Biol 229:* 319-327.

Kromkamp J, Mur LR 1984. Bouyant density changes in the cyanobacterium *Microcystis aeruginosa* due to changes in the cellular carbohydrate content. *FEMS Microbiol Lett 25:* 105-109.

Laird M 1988. *The natural history of larval mosquito habitats*. London:Academic Press, 555 pp.

Lecadet MM, de Barjac H 1981. *Bacillus thuringiensis* Beta exotoxin, p. 293-316 In E Davidson, *Pathogenesis of Invertebrate Microbial Diseases*. Allanheld, Osmun and Co. New Jersey.

Lereclus D, Delecluse A, Lecadet MM 1993. Diversity of *Bacillus thuringiensis* toxins and genes, p 37-70. In PF Entwistle, Cory JS, Bailey MJ, Higgs Seds Bacillus thuringiensis, *an environmental biopesticide: Theory and Practice. John Wiley and Sons, Chichester, United Kingom.*

Lereclus D, Arantes O, Chaufaux J, Lecadet MM 1989. Transformation and expression of a cloned delta-endotoxin gene of *Bacillus thuringiensis*. *FEMS Microbiol Lett 60:* 211-218.

Liu EW, Hindley J, Porter AG, Priest FS 1993. New high-toxicity mosquitocidal strains of *Bacillus sphaericus* lacking a 100-Kilodalton-toxin gene. *Appl Environm Microbiol 59:* 3470-3473.

Margaleff R 1958. Information theory in ecology. *Gen System 3:* 36-71.

Merritt RW, Dadd RH, Walker ED 1992. Feeding behaviour, natural food, and nutritional relationships of larval mosquitoes. *Annu Rev Entomol 37:* 349 376.

Murphy RC, Stephens Jr SE 1992. Cloning and expression of the *cryIVD* gene of *Bacillus thuringiensis* subsp. *israelensis* in the cyanobacterium *Agmenelum quadruplicatum* PR-6 and its resulting larvicidal activity. *Appl Environ Microbiol 58:* 1650-1655.

Myers P, Yousten AA 1978. Toxic activity of *Bacillus sphaericus* SSII-l for mosquito larvae. *Infect Immun 19:* 1047 1053.

Myers P, Yousten AA, Davidson EW 1979. Comparative studies of the mosquito-larval toxin of *Bacillus sphaericus* SSII-l and 1593. *Can J Microbiol 25:* 1227-1231.

Niaudet B, Jannière L, Ehrlich SD 1984. Recombination between repeated DNA sequences occurs more often in plasmids than in the chromosome of *Bacillus subtilis*. *Mol Gen Genet 197:* 46-54.

Nicolas L, Nielsen-Leroux C, Charles J-F, Delécluse A 1993. Respective roles of the 42- and 51-kDa components of the *Bacillus sphaericus* toxin overexpressed in *Bacillus thuringiensis*. *FEMS Microbiol Lett 106:* 275-280.

Novick RP 1987. Plasmid incompatibility. *Microbiol Rev 51:* 381-395 .

Oei C, Hindley J, Berry C 1990. Analysis of the genes encoding the 51.4- and 41.9-kDa toxins of *Bacillus sphaericus* 2297 by deletion mutagenesis: the construction of fusion proteins. *FEMS Microbiol Lett 72:* 265 274.

Ohana B, Margalit J, Barak Z 1987. Fate of *Bacillus thuringiensis* subsp. *israelensis* under simulated field conditions. *Appl Environm Microbiol 53:* 828-831.

Orduz S, Rojas W, Correa MM, Montoya AE, De Barjac H 1992a. A new serotype of *Bacillus thuringiensis* from Colombia toxic to mosquito larvae. *J Invertebr Pathol 59:* 99-103.

Orduz S, Diaz T, Thiéry I, Charles J-F, Rojas W 1994. Crystal proteins from *Bacillus thuringiensis* subsp. *medellin*. *Appl Microbiol Biotechnol 40:* 794-799.

Orduz S, Díaz T, Rojas W, Yousten AA 1992b. Isolation and characterization of four new isolates of *Bacillus sphaericus* from Nigeria toxic to mosquito larvae. *J Invertebr Pathol 60:* 107-108.

Orduz S, Restrepo N, Díaz T, Rojas W 1993. Isolation and characterization of thirteen new strains of *Bacillus sphaericus* from Colombia toxic to *Culex quinquefasciatus* larvae. *Mem Inst Oswaldo Cruz 88:* 629-632.

Pang Y, Frutos R, Federici BA 1992. Synthesis and toxicity of full-length and truncated bacterial CryIVD mosquitocidal proteins expressed in lepidopteran cells using a baculovirus vector. *J Gen Virol 73:* 89-101.

Pointdexter JS 1981. The caulobacters: ubiquitous unusual bacteria. *Microbiol Rev 45:* 123-179.

Porter AG, Davidson E, Liu JW 1993. Moquitocidal toxins of Bacilli and their genetic manipulation for effective biological control of mosquitoes. *Microbiol Rev 57:* 838-861.

Rippka R, Deruelles J, Waterbury JB, Herman M, Stanier RY 1979. Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol 111:* 1-61.

Rozeboom L 1935. The relation of bacteria and bacterial filtrates to the development of mosquito larvae. *Am J Hyg 21:* 167-179.

Sebo P, Bennardo T, de la Torre F, Szulmajster J 1990. Delineation of the minimal portion of the *Bacillus sphaericus* 1593M toxin required for the expression of larvicidal activity. *Eur J Biochem 194:* 161-165.

Silapanuntakul S, Pantuwatana S, Bhumiratana A, Charoensiri K 1983. The comparative persistence of *Bacillus sphaericus* strain 1593 and *Bacillus thuringiensis* serotype H-14 against mosquito larvae in different kinds of environments. *J Invertebr Pathol 42:* 387-392.

Singer S 1987. Current status of the microbial larvicide *Bacillus sphaericus*, p. 133-163. In K Maramorosch. *Biotechnology in invertebrate pathology and cell culture*. Academic Press, Inc., San Diego, Ca.

Thanabalu T, Hindley J, Berry C 1992 a. Proteolytic processing of the mosquitocidal toxin from *Bacillus sphaericus* SSII-l. *J Bacteriol 174:* 5051-5056.

Thanabalu T, Hindley J, Brenner S, Oei C, Berry C 1992 b. Expression of the mosquitocidal toxins of *Bacillus thuringiensis* subsp. *israelensis* by recombinant *Caulobacter crescentus*, a vehicle for biological control of aquatic insect larvae. *Appl Environm Microbiol 58:* 905-910.

Thanabalu T, Hindley J, Jackson-Yap J, Berry C 1991. Cloning, sequencing, and expression of a gene encoding a 100-kilodalton mosquitocidal toxin from *Bacillus sphaericus* SSII-l. *J Bacteriol 173:* 2776-2785.

Thiéry I, Nicolas L, Rippka R, Tandeu de Marsac N 1991. Selection of cyanobacteria isolated from mosquito breeding sites as a potential food source for mosquito larvae. *Appl Environm Microbiol 57:* 1354-1359.

Thiéry I, Sinègre G, Tandeu de Marsac N 1993. Occurrence and abundance of cyanobacteria in a brackish marshland and their ingestibility by mosquito larvae. *Bull Soc Vector Ecol 18:* 164-173.

Thorne L, Garduno F, Thompson T, Decker D, Zounes M, Wild M, Walfield AM, Pollock T 1986. Structural similarity between the Lepidoptera- and Diptera-specific insecticida endotoxin genes of *Bacillus thuringiensis* subsp. *kurstaki* and *israelensis*. *J Bacteriol 166:* 801-811.

Trisrisook M, Pantuwatana S, Bhumiratana A, Panbangred W 1990. Molecular cloning of the 130-kilodalton mosquitocidal delta-endotoxin gene of *Bacillus thuringiensis* subsp. *israelensis* in *Bacillus sphaericus*. *Appl Environ Microbiol 56:* 1710-1716.

Waalwijck C, Dullemans AM, Van Workum MES, Visser I 1985. Molecular cloning and nucleotide sequence of the Mr 28000 crystal protein gene of *Bacillus thuringiensis* subs *israelensis*. *Nucleic Acids Res 13:* 8207-8217.

Walker ED, Olds EJ, Merritt RW 1988. Gut content analysis of mosquito larvae (Diptera: Culicidae) using DAPI stain and epifluorescence microscopy. *J Med Entomol 25:* 551-554.

Wallace JB, Merritt RW 1980. Filter-feeding ecology of aquatic insects. *Annu Rev Entomol 25:* 103-132.

Ward ES, Ellar DJ 1988. Cloning and expression of two homologous genes of *Bacillus thuringiensis* subsp. *israelensis* which encode 130-kilodalton mosquitocidal proteins. *J Bacteriol 170:* 727-735.

Ward ES, Ellar DJ, Chilcott CN 1988. Single amino acid changes in the *Bacillus thuringiensis* var. *israelensis* delta-endotoxin affect the toxicity and expression of the protein. *J Mol Biol 202:* 527-535.

Ward ES, Ridley AR, Ellar DJ, Todd JA 1986. *Bacillus thuringiensis* var. *israelensis* delta-endotoxin: cloning and expression of the toxin in sporogenic and asporogenic strains of *Bacillus subtilis*. *J Mol Biol 191:* 13-22.

Whiteley HR, Schnepf HE 1986. The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. *Annu Rev Microbiol 40:* 549-576.

Wolters N, Schwartz W 1956. Untersuchungen uber vorkomer und verhalten von mikroorganismen in reine gundwassen. *Arch Hydrobiol 51:* 500-514.

Xudong X, Renqiu K, Yuxiang H 1993. High larvicidal activity of intact recombinant cyanobacteria, *Anabaena* sp PCC 7120 expressing gene 51 and gene 42 of *Bacillus sphaerius* sp. 2297. *FEMS Microbiol Lett 107:* 247-250.

Youngman P 1990. Use of transposons and integrational vectors for mutagenesis and construction of gene fusions in *Bacillus* species, p. 221-266. In CR Harwood, SM Cutting (eds), *Molecular biology methods for* Bacillus. John Wiley & Sons Ltd., Chichester, United Kingdom.

Yousten AA, Davidson EW 1982. Ultrastructural analysis of spores and parasporal crystals formed by *Bacillus sphaericus* 2297. *Appl Environ Microbiol 44:* 1449-1455.

Zaritsky A, Zalkinder A, Ben-Dov E, Barak Z 1991. Bioencapsulation and delivery to mosquito larvae of *Bacillus thuringiensis* H14 toxicity by *Tetrahymena pyriformis*. *J Invertebr Pathol 58:* 455-457.