CHAPTER 1

INTRODUCTION

Blood sucking insects such as mosquitoes and blackflies have plagued man since prehistoric times. They are the vectors for a multitude of diseases of man and animals through the transmission of pathogenic viruses, bacteria, protozoa and nematodes (Priest, 1992). A number of tropical vector borne diseases such as malaria, filariasis, Japanese encephalitis, dengue fever and yellow fever are transmitted by mosquito vectors. Among these, malaria is the most common debilitating disease being caused by four species of Plasmodium. The incidence of malarial infection is high in the tropical countries and nearly one million children die annually in Africa alone (Cowley et al., 1993). The annual incidence of malaria is approximately 270 million cases and nearly 350 to 450 million people live in highly endemic areas (Crickmore et al., 1990; Lacey et al., 1990).

Filariasis is another tropical disease caused by two nematode parasites namely Wuchereria bancrofti and Brugia malayi. The annual incidence of filariasis is 110 million cases and among them two thirds live in India, China and Indonesia (WHO, 1987). Onchocerca volvulus is another human filarial parasite restricted to parts of Africa and Central America where it is transmitted by blackflies belonging to the genus Simulium. This nematode is the causative agent for river blindness which is caused by accumulation of the dead nematodes in the eye (Walsh, 1986). Nearly 90% of the malarial, filarial, yellow fever and dengue fever cases are reported
from the tropics where the environmental conditions are conducive to vectors as well as pest insects which are responsible for the transmission of these diseases (Rawlins, 1989).

The medically important mosquitoes (*Culex, Anopheles, Monsonia* and *Armigeres*) are classified under *Anopheles* and Culicine species. *Anopheles stephensi* and *Culex quinquefasciatus* are the two species of mosquitoes which transmit malarial and filarial parasites respectively. The *C. tritaeniorhynchus* mosquitoes transmit a viral disease (Japanese encephalitis) and the two other viral diseases (dengue fever and yellow fever) are transmitted by members of the genus *Aedes* (Service, 1986).

### 1.1 CONTROL OF MOSQUITOES

The history of chemical insecticides deployed to control insect pests which cause severe damage to agriculture and human health care, dates back to many centuries. Broad spectrum of chemical pesticides have been used abundantly in the containment or eradication of pests related to medical, veterinary and agriculture (Kline *et al.*, 1985). After the initial success of large scale pest control using chemical pesticides, increasingly complex and important problems have been confronted because of non-degradability, lethality to non-target organisms and development of resistance by target insects (McGaughey, 1985). Therefore, the continued reliance on chemical insecticides to control insects and particularly mosquitoes has greatly diminished since most of the mosquito species have evolved resistance against most of these chemical pesticides (Walsh, 1986; Rawlins, 1989). This has necessitated the search for an alternative and a holistic integrated pest control approach. Insect populations succumb to a variety of infections caused by many pathogenic microorganisms such as
bacteria, fungi, protozoans, and viruses (Payne, 1988). The abilities of certain microorganisms to infect and decimate the predominant insect pests have been capitalised upon and has led to the development of the field of biopesticides.

1.1.1 Insects pathogens as biocontrol agents

Biopesticides derived from microorganisms are now proving to be effective alternatives to chemical pesticides (Moffat, 1991). The use of insect pathogens to control target pests was favoured because of their unique features such as biodegradability, target specificity and high potency. A large number of insect pathogens ranging from viruses to nematodes have been identified and studied (Payne, 1988). Among them only a few have been recognized as being true pathogens or have been favoured for commercial exploitation. These natural pathogens vary greatly in their mode of infection, multiplication and mechanism of pathogenicity (Roberts and Strand, 1977; Roberts and Castillo, 1980).

1.1.2 Viruses and Protozoans as biocontrol agents

Baculovirus, cytoplasmic and nuclear polyhedrosisvirus, entomopox virus, enterovirus, adenovirus, and small RNA virus are the six main group of viruses mainly causing diseases to insects and mites (Granados et al., 1986). Among the viruses which infect insects, only baculoviruses have gained attention as microbial control agents for agricultural pests (Huber, 1986).

Insect-parasitic protozoa are of potential interest for pest control. The infection of susceptible hosts by protozoans is caused by the spores
which are ingested by the hosts. Although a number of protozoans have been identified as pathogens to insects, only a few such as *Nosema pyrausta* and *N. locustae* have received attention. Multitudes of these protozoans are reported from blackflies and mosquitoes. Since the microspodia production require *in vivo* cultivation and the expenses associated with such systems, they are unlikely to be used as microbial insecticides for blackfly and mosquito control in the near future (Lacey and Undeen, 1986).

1.2 BACTERIA AS BIOPESTICIDES

Bacteria are the most successful group of all insect pathogens because of their efficiency and economical production (Aronson *et al.*, 1986). Insecticidal bacteria are broadly classified as true pathogens, opportunistic or facultative and food poisoning organisms (Davidson *et al.*, 1984). The number of insect species that can be controlled by these bacteria is quite large. Most medically important insects are vectors of diseases in their adult stage; their larvae are small and live in aquatic conditions. Thus, microbial pesticides based on Bacillus species particularly *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* play a major role in vector control (Davidson *et al.*, 1984; Lysanko, 1985; Margarlit and Dean, 1985; Khachatourians, 1986; Porter *et al.*, 1993).

These entomopathogenic *Bacilli* have several attractive features over chemical pesticides and other natural pathogens. The requirement of an inexpensive medium for their cultivation (Luthy, 1981), biodegradability of the toxins, narrow host range, lack of toxicity against non-target organisms, high potency and ease of production on a large scale are some of the favourable characters of these bacteria (Aronson, 1986; Davidson, 1982;
Laird et al., 1990). Among the various strains of *B. thuringiensis*, *B. thuringiensis* var. *israelensis*, and *B. thuringiensis* var. *morrisoni* (Aronson et al., 1986) are two strains which have gained much attention as potential candidates in the mosquito control program (Porter et al., 1993).

1.2.1 *Bacillus thuringiensis* as biopesticides

*B. thuringiensis* strains show narrow as well as wide range of specificity against target insects (Aronson, et al., 1986). During sporulation insecticidal strains of *B. thuringiensis* produce insecticidal proteinaceous crystals (β-endotoxins), which are toxic to larvae of a number of insect species. These are rarely active against *Diptera* (specifically mosquitoes and blackflies) or *Coleoptera* (e.g. Colorado potato beetle) species of agricultural insect pests. However they are active against many species of *Lepidoptera*. These insecticidal crystal proteins (ICPs) are synthesised during the sporulation stage and they polymerize as parasporal crystal inclusions. The ICPs are encoded by *cry* genes and consist of several classes of proteins (Hofte and Whiteley, 1989). The shape of the ICPs vary depending upon the type of the gene encoding the crystal protein. For example, the Lepidopteran crystal proteins of the crylA class are bi-pyramidal; dipteran crystal proteins of the crylV class are amorphous; coleopteran-active crystals of the crylIIA class are flat and rectangular and the cryllA class, which are active against both *Lepidoptera* and *Diptera* are cuboidal (Dean and Adang, 1992).

Mosquito larvicidal strains of *B. thuringiensis* are commonly associated with serotype 14 (subsp. *israelensis*) and rarely with serotype 8 (subsp. *morrisoni*). The strain *B. thuringiensis israelensis* shows greater toxicity against *Aedes*, *Culex*, *Anopheles*, *Armigeres* and blackflies (Aronson et al., 1986). The lethality of *B. thuringiensis israelensis* to these
target insects is due to the presence of crystals synthesized during sporulation (Whiteley and Schnepf, 1986). This crystal complex is composed of five different peptides. The molecular weight of the peptides ranges from 134, 128, 58, 70 to 27 kDa. When tested for antigenicity of these peptides, it has been found that these peptides are immunologically distinct from each other (Federici et al., 1990).

1.2.2 Characterization of *B. thuringiensis* insecticidal toxins

The genes coding for the ICPs have been cloned and sequenced from different *B. thuringiensis* strains. The genes coding for the ICPs are located on plasmids and it has been shown that these plasmids may be transmitted to other bacilli by conjugation. On the basis of the sequences of the *cry* genes several genetic nomenclatures have been adopted. Hofte and Whiteley, (1989) have suggested a genetic nomenclature utilising *cry* as the mnemonic. The comparison of amino acid sequences of the ICPs has helped in locating the functional domains of the proteins. The ICPs are classified into two subtypes namely Type I which include ICPs approximately 130 kDa (*cry1* series, and *crylVA* and B) and Type II ICPs that comprises of 65 kDa toxins (*cryII* series; *cryIIIA*; *cryIVC* and D).

Genes coding for the mosquito larvicidal proteins of *B. thuringiensis israelensis* are also known to be localised in the mega plasmid. Bioassays carried out using individual peptides expressed in heterologous hosts revealed that 134 kDa (*CrylVA*), 128 kDa (*CrylVB*), 58 kDa (*CrylVC*) and 70 kDa (*CrylVD*) peptides are independently toxic to *A. aegypti* larvae, but, at varying levels (Chilcott and Ellar, 1988; Delecluse et al., 1988; Ibarra et al., 1986). *CrylVD* alone showed moderate toxicity to *C. pipiens* larvae whereas *CrylVA* or *CrylVC* did not show any toxicity against *C. pipiens* (Donovan et al., 1988; Intara et al., 1988). However, The 27 kDa
(CytA) protein showed hemolytic activity and its contribution to mosquito larvicidal activity is negligible (Delecluse et al., 1991). A second cytolytic protein, CytB from *B. thuringiensis* subsp. *kyushuensis* has been identified with a molecular mass of 29.2 kDa. The nucleotide sequence of *crylVA, crylVB, crylVC, crylVD* and *cytA* genes have been determined. Among these *crylVA, crylV* and *crylVC* genes sequences show significant homology which are scattered throughout the coding sequence.

1.2.3 Mode of action of ICPs

The mode of action of ICPs has been studied in some depth only for Lepidoptera and Coleoptera active toxins belonging to the CryI and CryIII classes and to a lesser extent for Lepidoptera and Diptera specific cryll class. A model for the mechanism of action of ICPs has been suggested by Milne et al., (1990). According to the proposed model, the following steps are involved in the mechanism of Cry toxins

- The gene product occurs as an inactive protoxin (CryI, 130 kDa) in the crystal.
- The crystal and the subunits are dissolved at alkaline pH of the insect midgut, cleaved by insect gut proteases and reduced to activated toxins.
- The columnar epithelial cells of the midgut and goblet cells are attacked and damaged by the toxin. This causes an immediate loss of active potassium transport systems and ultimate lysis. In susceptible insects, damage to the midgut allows an avenue for the
invasion of the haemolymph by *B. thuringiensis* from the germinated spore (Heimpel and Angus, 1959)

The elucidation of three-dimensional structure of CryIIIa by X-ray crystallography (Li *et al.*, 1991) has greatly aided to further investigate the proposed mode of action of ICPs using site-directed mutagenesis. The three dimensional structure revealed that there are three structurally distinct domains; a cluster of seven α-helices, three antiparallel β-sheets, and a β-sandwich (Fig. 1.1). The core structure of the CryIIIa is built upon the sequence segments conserved among Cry proteins and this CryIIIa structure has been proposed to represent a general structure for proteins in the family (Li *et al.*, 1991). It has been suggested that these three regions carry out different functions. For example, domain I may play a role in membrane insertion and pore formation (Gazit and Shai, 1993). Mutations in the conserved block 1 of domain I affected or destroyed the toxicity. This is because the mutant toxin failed to inhibit potassium dependent leucine transport into gut membrane vesicles indicating that domain I is involved in membrane insertion. Three surface-exposed loops of the domain II may be involved in the receptor binding and insect specificity (Ge *et al.*, 1991; Lee *et al.*, 1992). Rajamohan *et al.*, (1995) have reported that loop II region of domain II (CryIAb toxin) is involved in the receptor binding and even a single amino acid change in domain II affected irreversible binding of the toxin. This has also been demonstrated by voltage clamp experiments that there is a positive correlation between receptor binding and inhibition of short-circuit current for CryIAb toxin. Several *in vitro* membrane binding studies have been performed with brush border membrane of the midgut of larvae of susceptible insects to demonstrate the binding of the ICPs to the receptors (Rajamohan *et al.*, 1995). The domain III may play a role in structural stability and integrity of ICPs (Chen *et al.*, 1993). Often a positive correlation has
Figure 1.1 Schematic ribbon representation of CryIII A endotoxin illustrating the domain organisation and three surface-exposed loops (Wu et al., 1996).
been observed between the binding affinity of the processed toxin and the insect toxicity. Recently, the mode of action of the activated insecticidal toxins has been revised by Dean et al (1996). The receptor for CryIAc toxin has been identified as aminopeptidase-N, which is a GalNAc bearing glycoprotein (Knight et al., 1994).

The amino acid sequence and secondary structure of the CrylVA and CryVIB show certain degree of similarity to other Lepidopteran toxin (CryI and CryIII) of B. thuringiensis (Thorne et al., 1986). Since the mode of action of Lepidopteran toxins (CryIAa and CryIAb) have been studied considerably, it is assumed that the CryIV toxins may also act in a similar manner (Li et al., 1991). Preliminary studies carried out using brush border membrane vesicles and cultured cells of mosquito larvae (Knowles and Ellar, 1987) suggested that the following steps are involved in the mode of action of B. thuringiensis toxins. Initially the mosquito larvae ingest the crystal-spore complex followed by solubilisation of crystal complex at the alkaline pH in the midgut. These solubilised crystals undergo proteolytic processing followed by binding of the processed toxins to specific receptors present in the brush border membranes vesicles (BBMV) of susceptible larvae (Rie et al., 1990). Eventually, the larvae die because of the formation of pores which lead to leakage of channels, cell swelling and colloid osmotic lysis.

1.3 BACILLUS SPHAERICUS AS A MOSQUITO CONTROL AGENT

B. sphaericus is an aerobic, rod-shape, endospore-forming, saprophytic bacterium widely distributed in different environments. The major steps of developments involved in Bacilli is illustrated in figure 1.2. This Bacilli has several advantageous characters which are favourable for
Figure 1.2 Schematic representation of different stages of sporulation in Bacilli

A: Simplified life cycle of *B. subtilis* illustrating the major steps in development.

B: Sporulation of *B. sphaericus* 2297 studied by Electron microscopy (Kalfon et al., 1983).
use as bio-control agents (Singer, 1980). *B. sphaericus* has been known to be pathogenic to mosquitoes for the last three decades. The first pathogenic strain reported was Kellen K isolated from moribund larvae of *Culiseta incidens* in California (Kellen *et al*., 1965). It was found that this was one of the least lethal among all the pathogenic strains of *B. sphaericus* discovered subsequently (Yousten and Davidson, 1982; Yousten *et al*., 1984). This was followed by identification of a number of new isolates with far greater larvicidal activity. Notable among them are 2297, 1593 and 2362 (Davidson, 1982).

### 1.3.1 Taxonomy

Among the 186 strains of *B. sphaericus* isolated until recently, only 45 strains showed significant toxicity to mosquito larvae (WHO, 1985). The classical biochemical identification methods (biotyping) were not successful in distinguishing between the insecticidal and non-insecticidal varieties of *B. sphaericus*. The DNA homology studies provided five homology groups with homology group II being divided into groups IIA and IIB. Group IIA is composed of seven strains that are pathogenic to the *C. quinquefasciatus* mosquito larvae. Yousten (1984) has developed a bacteriophage typing protocol which allows grouping of the insecticidal strains into seven phage types. The strains 1593 and 2362 are classified into 3 groups which contain most of the strains showing higher insecticidal activity. An another potent mosquito larvicidal strain, 2297, is included in group 4. On the basis of the H-flagellar antigen, 47 serotypes of *B. sphaericus* have been characterised, only 5 out of these serotypes are known to include mosquito pathogenic strains. They include serotypes H5a, H5b, H25, H2, H1a, H26a and H26b, in decreased order of toxicity towards *C. pipiens* larvae (de Barjac
et al., 1985; 1990). A comparative properties of some mosquito larvicidal strains of *B. sphaericus* has been presented in Table 1.1.

### 1.3.2 Mosquito larvicidal proteins (MLPs) of *B. sphaericus*

So far, five proteins with molecular weights 51, 42, 100 (Mtx), 30 kDa (Mtx2) and 35.8 Kda (Mtx3) have been identified as MLPs in *B. sphaericus* (Charles *et al.*, 1996; Thanabalu and Porter, 1996; Liu *et al.*, 1996). Among these the 51 and 42 kDa are needed together for larvicidal activity and they act as binary toxins (Baumann *et al.*, 1985; Broadwell *et al.*, 1990). The amino acid sequence of these two proteins do not have any homology with the known bacterial toxins (Baumann *et al.*, 1991). However, the 51 and 42 kDa proteins share four segments of sequence homology, the significance of which remains unclear (Charles *et al.*, 1996). The binary toxins are synthesised during third phase of sporulation.

The other two proteins, 100 kDa and 30 kDa are synthesised during vegetative phase and are independently toxic to mosquito larvae (Thanabalu *et al.*, 1991). These two proteins carry an N-terminal signal sequence which is characteristic of grampositive bacteria. The N-terminal (27 kDa) region of 100 kDa shows ADP-ribosylating activity (Thanabalu *et al.*, 1993). The 100 kDa and 30 kDa toxins do not show any similarity to each other or to any other insecticidal proteins. However, the 30 kDa toxin shares about 30% homology to ε-toxin and cytotoxin of *Clostridium perfringens* and *Pseudomonas aeruginosa* (Thanabalu and Porter, 1995). Figure 1.3 shows a diagrammatic summary of structural and functional domains of *B. sphaericus* toxins.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Source</th>
<th>Flagellar serotype</th>
<th>Phage type</th>
<th>DNA group</th>
<th>RNA group</th>
<th>Fatty acid group</th>
<th>Mosquitocidal activity</th>
<th>Crystal genes</th>
<th>Mix genes</th>
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<td>1</td>
<td>IIA</td>
<td>NA</td>
<td>AII</td>
<td>L</td>
<td>−</td>
<td>+ NA</td>
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<tr>
<td>Kellen Q</td>
<td>USA</td>
<td>Culicoides incidens</td>
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<td>1</td>
<td>IIA</td>
<td>NA</td>
<td>AII</td>
<td>L</td>
<td>−</td>
<td>+ NA</td>
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<td>1a</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>H</td>
<td>+</td>
<td>NA NA</td>
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<td>2a2b</td>
<td>2</td>
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<td>RIIA</td>
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<td>M</td>
<td>−</td>
<td>+ − −</td>
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<td>Ghana</td>
<td>Culex breeding site</td>
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<td>M</td>
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<td>AIV</td>
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</table>

Table 1.1. Comparative properties of some mosquitocidal strains of *B. sphaericus*. +, Present; − absent; NA, No information available (Charles et al., 1996).
Figure 1.3. Diagrammatic summary of structural and functional domains of mosquito larvicidal proteins.

A: Genes encoding the *B. sphaericus* toxins (left) and schematic representation of corresponding polypeptides (right). (Charles et al., 1996).

B: *B. sphaericus* mosquitocidal endotoxins (protoxins) with functional domains (Porter et al., 1993).
1.3.3 Identification of DNA sequences coding for MLP

The lethality of *B. sphaericus* to mosquito larvae is largely due to the presence of 51 and 42 kDa peptides. Initial efforts were made to identify the genes coding for the binary toxins from different strains of *B. sphaericus*. Genomic libraries were constructed in *E. coli*, oligonucleotide probes based on the N-terminal amino acid sequence of the 43 kDa peptide were used to identify the positive clones (Baumann *et al.*, 1985). A 3.5 kb *HindIII* fragment was identified from the strain 2362 and sequenced by Baumann *et al.*, (1988). The sequence data revealed that this fragment has the coding sequence for the 51 and 42 kDa peptides. They also showed that similar DNA fragments were present in other highly toxic strains, but absent in the sub toxic strain SSII-1. The nucleotide sequence of this fragment seems to be highly conserved and they are identical in the strains 1593, 2317-3 and 2362. The MLP genes from the strains 2297 and IAB59 differed by 25 and 7 nucleotides respectively from the other strains (Berry and Hindley, 1987; Hindley and Berry, 1988; Arapinis *et al.*, 1988; Berry *et al.*, 1989). Table 1.2. represents a comparison between the nucleotide and amino acids sequence of binary toxins of five highly toxinogenic strains. Souza *et al.*, (1988) reported the cloning and expression of two dissimilar toxin genes from *B. sphaericus* 1593M. One of these fragments has similar restriction pattern as the one reported by Baumann *et al* (1987).

The second gene of *B. sphaericus* coding for 100 kDa (SSII toxin) MLP has been cloned and sequenced from the strain SSII-1. This gene is known to be present in various strains of *B. sphaericus* strains (Thanabalu *et al.*, 1991). Recently, two more genes coding for a 30 kDa and 35 kDa proteins have also been identified from *B. sphaericus* SSII-1 and this toxin shows independent toxicity against mosquito larvae (Thanabalu and Porter,
### Table 1.2

The table shows a comparison of the nucleotide and deduced amino acid sequences of binary toxins of five highly toxinogenic strains of *B. sphaericus* (Berry et al., 1989).

<table>
<thead>
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<th>Region</th>
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<th>Amino Acid change 1593, 2362, 22317.3</th>
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<td>91</td>
<td>G T T</td>
<td>Lys Asn Asn</td>
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<td>700</td>
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<td>Thr Thr</td>
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<td>1859</td>
<td>-</td>
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<td>2417 A A T</td>
<td>2490</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2643 C C G</td>
<td>2745</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2813 G G A</td>
<td>3336</td>
<td>41.9 kDa</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.2**
1995; Liu et al., 1996). Schematic representation of *B. sphaericus* MLP genes and their corresponding polypeptides have been shown in figure 1.3.

### 1.3.4 Genetic control of Binary toxin genes

Analysis of the sequences of the genes encoding the 51.4 and 41.9 kDa peptides from various strains of *B. sphaericus* revealed the following features.

a) Ten nucleotides upstream of the initiation codon of both the peptides, a putative RBS (Shine Dalgarno sequence) complementary base pair (bp) the 3' terminus of the 16s RNA characteristic for a number of *Bacilli* genes is present (Berry and Hindley, 1987; Arapinis et al., 1988; Hindley and Berry, 1988)

b) The 51.4 kDa peptide is terminated by an amber codon followed by six additional stop codons in the same reading frame. The 41.9 kDa peptide is terminated by an Ochre codon followed by seven additional stop codons in the same reading frame (Hindley and Berry, 1987, 1988; Baumann et al., 1988; Arapinis et al., 1988).

c) There was only one region containing an inverted repeat which is complementary at 14 to 15 nucleotides downstream of the region coding for the 42 kDa peptide, suggesting a transcriptional termination signal, followed by a T-rich sequence. No other region that could form 'hairpin' structures could be detected in the whole of the 3.6 kb sequence, including the 171 bp intervening region between the ORFs coding for the 51.4 kDa and 41.9 kDa peptides. This suggested that the two genes might reside within a single transcriptional operon.
1.4 MODE OF ACTION OF MLPs
1.4.1 Mode of action of binary toxin

Studies of the mode of action directly in the larvae are difficult because of the small size of and the aquatic nature of the mosquito larvae. For this reason, a cell culture assay utilizing mosquito cell lines have been approached. Based on the studies carried out using wild type and deletion derivatives of binary toxins, a model for the mode of action of these binary toxin has been hypothesized (Oei et al., 1990; Baumann et al., 1991). This model states that the binary toxins are solubilized in the midgut of mosquito larvae due to the alkaline pH followed by proteolytic cleavage of the 51 and 42 kDa proteins. This results in the processing of 51 kDa and 42 kDa into 43 kDa and 39 kDa respectively. These processed proteins bind to the epithelial cells of the midgut and lead to internalization of both toxins which results in larval death (Porter et al., 1993; Charles et al., 1996).

The proteolytic conversion of 51 and 42 kDa proteins purified from heterologous host using trypsin and chymotrypsin enzymes has been demonstrated (Broadwell and Baumann, 1987). The processed 39 kDa protein contained all the determinants for toxicity to cultured mosquito cells but showed increased toxicity in the presence of intact 51 kDa or its derivative 43 kDa (Baumann and Baumann, 1991). The structure-function relationship of the binary toxins have been studied using fluorescent dye labelled 51 and 42 kDa proteins separately. Mosquito larvae fed with 51 kDa protein showed localised binding to posterior midgut (PMG) and gastric caecum (GC) whereas the 42 kDa protein showed nonspecific binding throughout the gut and GC. Localised binding of 42 kDa proteins to GC has been observed in the presence of 51 kDa (Oei et al., 1992). Internalization
of 51 and 42 kDa has been observed only in the presence of both the proteins and absence of one protein abolished the internalization process. These studies suggest that the 51 kDa protein acts as a cell binding component and aids the 42 kDa peptide to bind to target sites. It has been earlier reported that the 51 and 42 kDa strongly associate with each other resulting in the modification of the toxin complex before internalization take places (Davidson et al., 1990).

*In vitro* binding studies using BBMV isolated from the larvae of *C. pipiens* have showed the presence of specific receptors to binary toxins (Nielsen et al., 1992). This study also showed that the mixture of 51 and 42 kDa proteins bind to a single receptor with the dissociation constant (*Kd*) of 20nM. To support the observations, using fluorescent labelled 51 and 42 kDa, it has been demonstrated that the specific binding was lost when these proteins were challenged with (BBMVs) isolated from *Aedes* mosquito larvae. The results indicated that the absence of toxicity of *B. sphaericus* against *A. aegypti* larvae may largely be due to absence of specific receptors for binary toxins (Davidson, 1988; 1989).

The contribution of individual amino acids to overall toxicity has yet not been studied in detail (Porter et al., 1993). The amino acids localised around 100 position in 42 kDa has been shown to be responsible for toxicity against *Aedes* larvae. Moreover, the 42 kDa has been shown to be responsible for the host range. This has been evidenced by substitution of a single amino acid in the 42 kDa (serine to alanine at 104 position or from phenylalanine to valine at 99 position) affected overall toxicity to *Aedes* larvae. In contrast, a single amino acid change in the 51 kDa did not affect host range (Berry et al., 1993). It has been envisaged that the elucidation of X-ray crystal structure of binary toxins together with site-directed
mutagenesis may aid in the identification of amino acids essential for receptor binding, binary toxin interactions and internalization of toxin complex. Baumann et al. (1992) have used site-directed mutagenesis to generate deleted versions of 51 and 42 kDa toxins similar to mosquito larval gut proteases processed toxins. But their study was not aimed at identifying the role of individual amino acids towards biological activity.

1.4.2 Cytopathological and physiological effects of binary toxins

Unlike *B. thuringiensis israelensis* toxin the binary toxin of *B. sphaericus* does not break down mid gut epithelium of larvae. After the ingestion of spore-crystal complex alterations in the mid gut has been observed (Davidson, 1981; Charles, 1987). These alterations are mainly formation of large vacuoles in the case of *C. pipiens* and formation of low electron density in the midgut of *Anopheles* larvae. Though the neural tissues and skeletal muscles of midgut are damaged, the major target for the binary toxins is posterior stomach and GC (Singh and Gill, 1988). The ultrastructural studies using cultured cells of *C. quinquefasciatus* larvae indicate that toxin interaction of the toxin with the target cells results in the swelling of mitochondrial and endoplasmic redicules (Davidson and Titus, 1987). This leads to the inhibition of oxygen uptake by mitochondria as observed in the toxin treated *C. quinquefasciatus* cells and rat liver cells (Narasu and Gopinathan, 1988).

1.4.3 SSII toxin (Mtx)

Compared to binary toxins a very few literature are available regarding mechanism of action of the 100 kDa toxin. The amino acids sequence of Mtx toxin shows homology to known bacterial toxins such as
pertussis and diptheria. These toxins act as ADP-ribosylating protein of cellular proteins. Hence, it has been suggested that the 100 kDa toxin also act as ribosylating peptide. It has been observed that the 100 kDa protein is cleaved into N-terminal 27 kDa and C-terminal 70 kDa by mosquito gut juices containing proteases such as trypsin and chymotrypsin (Thanabalu et al., 1992). However, these two processed peptides failed to ADP-ribosylate the proteins in the cultured mosquito larvae.

In contrast, a 57 kDa N-terminal peptide containing the sequences similar to bacterial toxins showed ADP-ribosylation but failed to produce toxicity against mosquito larvae. The 70 kDa processed peptide was able to induce morphological changes to the cultured mosquito larvae. These studies suggest the presence of two functional domains in the 100 kDa toxins. The N-terminal region is essential for ADP-ribosylation whereas the C-terminal region (70 kDa) is involved in inducing cytopathological effects and both the regions are essential to exert toxicity against mosquito larvae.

The cytopathological modifications observed in cultured mosquito cells induced with 100 kDa toxin has been different from that observed with cells exposed to binary toxins (Thanabalu et al., 1993). This study suggests that the mechanism of action of 100 kDa is different from that of the binary toxin and there may be different receptors for this toxin. Further investigation is required to identify the presence of specific receptor for this toxin in susceptible mosquito larvae and also to study the nature of the receptor. Interestingly, Hazes and Read (1995) have further analysed the sequence of Mtx and found that the processed 70 kDa toxin sequence carries a ricin-like cell-binding domain (Fig.1.4) similar to pertussis and cholera toxin. Since the AB toxin (class of cholera toxin) acts via binding to glycoproteins or glycolipids it is predicted that the 70 kDa processed toxin of Mtx binds to
Figure 1.4. Diagram showing the ricin like galactose-binding domain of Mtx toxin. The 70 kDa C-terminal region of Mtx shows homology to the ricin like galactose-binding domain (Hazes and Read, 1995).
specific glycoproteins or glycolipids on the insect gut epithelium and then facilitate the entry of the toxic domain into the cytosol.

Based on the properties of other AB toxins it has also been suggested that the carbohydrate specificity of Mtx determines the range of cells and insects that are susceptible to the toxin. Modification of the carbohydrate specificity of Mtx by genetic manipulation could thus potentially alter the range of susceptible insects (Hazes and Read, 1995).

The mechanism of action of the recently identified 30 kDa (Mtx2) and 35 kDa (Mtx 3) toxins is yet to be understood.

1.5 EXPRESSION OF B. SPHAERICUS TOXINS IN HETEROLOGOUS HOSTS

1.5.1 Expression of MLPs in E. coli

E. coli has been used as the primary host for construction of genomic libraries of different B. sphaericus strains for identifying the genes of MLPs. A number of attempts have been made to express the MLP genes in E. coli mainly to study the structure-function relationships of MLPs (Baumann et al., 1988; Davidson et al., 1990; Thanabalu et al., 1991; Suresh et al., 1992). These genes have been cloned and expressed under the control of various constitutive and inducible E. coli promoters such as lac, lacUV, ‘tac’, T7, using different vectors systems (Baumann et al., 1991; Rajamohan et al., 1992).

The binary toxin genes have been cloned under the control of putative and also E. coli promoters and ribosome binding sequences (RBS). Different strains of E. coli have been used as hosts to express these
proteins and also to analyze the biological activities of the MLPs. Variations in the level of toxicity against mosquito larvae have been observed and it has been suggested that they could be due to use of different strains of *E. coli*, vector systems, and larval stages (Porter *et al.*, 1993). In addition to wild type toxins, deletion derivatives and mutant binary toxins, generated by site-directed mutagenesis have also been expressed and purified from *E. coli*.

### 1.5.2 Expression of MLPs in other Bacilli

The binary toxins genes of *B. sphaericus* has been expressed in various strains of Bacillus such as *B. subtilis*, *B. thuringiensis* and *B. thuringiensis israelensis*. In *B. subtilis* the MLP genes have been over-expressed using sporulation promoters (Broadwell *et al.*, 1990). In order to increase the host range, the binary toxin genes have been transferred to both *B. thuringiensis israelensis* 4Q2-72 and crystal minus *B. thuringiensis israelensis* (4Q2-81). The expression of binary toxins in *B. thuringiensis israelensis* did not affect the synthesis of its own MLPs and unexpectedly its expression did not enhance toxicity. In other words, synergism between binary toxins and other MLPs of *B. thuringiensis israelensis* has not been observed (Bourgouin *et al.*, 1990).

### 1.5.3 Novel hosts to deliver MLPs

One of the limitations of parental *B. sphaericus* strains which has discouraged its successful application in field conditions is the rapid settlement of its spores to the bottom from the surface of mosquito larval breeding places (Davidson *et al.*, 1984) To circumvent this, problem genetic engineering approach was used to transfer the genes coding for the MLPs to
novel hosts which are permanently present in the surface of aquatic environments (Angsuthanasombat and Panyim, 1989). Towards this, the first effort was made to transfer the binary toxin genes to different species of cyanobacteria for prolonged delivery of MLPs. But the binary toxin genes transferred to Anacytis nidulans showed a very low level of expression and less toxicity to mosquito larvae (de Marsac et al., 1987).

Efforts have been also made to transfer the binary toxins and 100 kDa toxin of *B. sphaericus* and CryIVB toxin genes of *B. thuringiensis israelensis* to another ubiquitous and surface dwelling bacterium namely, *Caulobacter crescentus*. As observed in the case of Cyanobacterium species the expression of the binary toxin was lethal to *C. quinquefasciatus* larvae but at low levels, whereas the expression of 100 kDa gene showed toxic to *C. quinquefasciatus* at levels similar to obtained with native *B. sphaericus* SSII-1 strain (Thanabalu et al., 1992).

Recently, the binary toxin genes has been transferred to another novel host namely *Astitacaulis excentricus*. Like cyanobacterium and *Caulobacter* species, this bacterium is also ubiquitous in nature, and exist on the surface of aquatic environments. In contrast to results obtained with previous hosts, a very high level of binary toxin expression has been observed (Liu et al., 1996).

1.6 APPLICATION OF *B. SPHAERICUS* IN THE ENVIRONMENT FOR THE CONTROL OF MOSQUITOES

The economic production of *B. sphaericus* depends on strain selection, inexpensive raw materials for medium development, and optimization of cultivation conditions for growth, sporulation and MLP
synthesis. There have been several studies made on the nutrition of the nonpathogenic strains of *B. sphaericus*. Amino acids such as arginine, aspartate, glutamate, glycine, histidine, and proline serve (*B. sphaericus* 1593) efficiently as both carbon and nitrogen sources (Yousten, 1984). Russell *et al.* (1989) demonstrated the lack of glucokinase, hexokinase, phosphokinase isomerase, phosphofructokinase and glucose 6-phosphokinase and glucose 6-phosphate dehydrogenase which are early enzymes of Embden -Myerhof and hexose monophosphate pathways.

Based on the nature of the pathogenic bacteria, biological control is directed at the larval stage of these insects and the diversity of habitats and feeding habits is an important consideration in the formulation and application of these biocontrol agents. Nowadays, *B. sphaericus* and *B. thuringiensis israelensis* are formulated as wettable powders, liquid flowable concentrates and granules (Lacey, 1984; Mulla, 1990; Yap, 1990). Since the mosquito larvae are filter feeders, the ingestion of toxin depends on rate of feeding and the rate at which the toxin sediments to the bottom.

The efficacy of *B. sphaericus* has been tested in laboratory and field conditions and a good control over *Culex* and *Anopheles* larvae has been achieved (Mulligan *et al.*, 1980). No adverse effects of *B. sphaericus* on non-target fauna occupying the same environmental niche as that of mosquito larvae have been reported (Lacey and Undeen, 1986, Mulla, 1990, Yap, 1990).

1.7 SCOPE OF THE PRESENT INVESTIGATION

As a non-sugar utilizing organism, the high cost of production is a major limiting factor for large scale production and application
of *B. sphaericus* (Russell *et al*., 1989). Therefore, economic production of mosquito larvicidal proteins (MLP) can be achieved if the genes coding for the toxins are transferred to a sugar utilizing bacterium like *E. coli*.

1. The first objective of the present work was to over-express the mosquito larvicidal binary toxin genes in *E. coli* under an efficient, specific and regulatable T7 promoter and T7 RNA polymerase system.

2. The second objective was aimed at investigating the mechanism of action of binary toxins using mutant toxins generated by site-directed mutagenesis. Attempts were made to analyze the effect of alanine substitutions in N- and C-terminal regions of binary toxins towards biological activity.

3. The third objective was aimed at monitoring the *B. sphaericus* released in environment by applying Polymerase Chain Reaction and non-radioactive gene probes.