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GENETICALLY ENGINEERED *BACILLUS THURINGIENSIS* VAR-*ISRAELENSIS* (BTI) FOR THE BIOLOGICAL CONTROL OF MOSQUITO—A REVIEW

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Abstract

The number of pathogenic microorganisms that are capable of killing insect pests is extremely large. Some bacteria are more attractive to researchers developing microbial biological control agents because their genomes are simple enabling easier study and manipulation. Several bacterial species are already used on a large scale as control agents against some insects. Commercial preparation of these bacteria in the form of insecticidal powders are available for use in the field. Although these preparations are somewhat more expensive and also less efficient than chemical insecticides, for several reasons including recent awareness of environmental safety, development of biological control agents has recieved increased attention of biologists.

Several varieties of Bacillus thuringiensis *have been well studied for their toxic effects on insects. These bacteria produce proteinaceous parasporal crystals during their sporulation. When these crystals are ingested by insect larvae protein protoxins are solubilised in the alkaline environment of the insect midgut releasing polypeptides toxic to the epithelial cells resulting in quick death of the larvae.*

Toxicity of Bacillus thuringiensis *var.* israelensis is *specific to mosquito and blackfly larvae. However, large scale use of this bacterium for the control of mosquitoes is limited by its low persistenee in the field after application. Four genes coding for 4 different toxic polypeptides have been isolated from this bacterium and all these have been cloned in* E. coli *and studied. Research is being done to study the posible manipulation of these genes in order to obtain potentially much more efficient bacterial strains for the control of insects.*

Key words : genetically engineered, Bti, mosquito control

1. General Comments

The number of pathogenic microorganisms that are capable of killing insects is extremely large. Among them are several families of bacteria, viruses, fungi, protozoa and nematodes. Bacteria, fungi and viruses are more attractive for exploitation as biological control agents because their genomes are

quite simple in comparison with those of higher organisms making them easier to study and manipulate. Genetic modifications of these microorganisms have become day to day activities of molecular geneticists especially after the development of the recent technology of genetic engineering. Many strains of bacteria, fungi and viruses have already been produced commercially as insecticides and their formulations, applications and modes of action have been studied Strains of the bacterium *Bacillus thuringiensis* have undergone industrial manufacture as a biological insecticide for world wide use against Lepidoptera on field crops, trees, ornamentals, vegetables, stored grain and grain products(31)

Attempts are made to introduce the genes responsible for the toxins of bacteria into crop plants. Successful experiments on this line have already been performed. Tobacco plants into which d-endotoxin gene of *Bacillus thuringiensis* has been inserted using Ti plasmid of *Agrobacterium tumifaciens* are protected against larvae of *Manduca sexta* (43).

Use of bacteria as biological control agents is not new. However, until recently their cost of production and relatively low effectiveness in the field in comparison with chemical insecticides prevented the growth of strong incentives to industry to make big investments for research and development of biological insecticides. Advantages of biological insecticides over chemical insecticides are many. They are highly specific towards target species and are harmless to man and environment. Since they are not effective against the natural insect predators of the target species even the natural control methods remain intact after the treatment. They leave no harmful residues. Because they are living organisms they have the potential for amplification in the field by reproduction and for reaching a steady state of eluilibrium for continued existence with target species. Although produced by industry today they are products of evolution and have been in contact with insects for a very long time in history. It appears that insects cannot easily develop resistance to these biological agents. Insects have, however, shown remarkable abilities to develop resistance towards chemical agents.

Use of bacterial insscticides in the field still remain low in contrast to the overwhelming use of chemicals because bacterial insecticides are less effective for several reasons. They do not kill insects upon contact but must be ingested either by feeding on the bacterium or by feeding on leaf tissues containing the bacterium. Sometimes it is necessary or helpful to incorporate feeding attractants with the agent. All developmental stages of the insects are not equally succeptible to the pathogen and as a result the user often has to time the application well. Some biological agents are, although highly efficient in the laboratory, not very effective in the field. The lack of potency may be due to reasons such as poor storage capability, requirement of specific ecological nitches or wash off by rain. Biological activity of toxic crystal inclusions of bacteria generally decays with a half life of about 8 days (20). Very narrow host range of the biological agent has its disadvantages. Many worldwide

crops have several different important insect pests with different behaviour patterns. As there can be no biological agent that can control all of them there is the necessity of multiple application bringing economic obstacles. Manufacturing costs of biological agents, specially those which require expensive growth media or *in vivo* cultres of reared insect larvae, are high.

Increased concern of the public and regulatory organisations regarding pollution and environmental safety in reeent years has caused a more intense attention towards biological control methods. Recent advances in recombinant DNA technology provide exceptionally powerful tools for generating entitely new and much more effective biological control agents than naturally occuring ones. General acceptance that genetically engineered bacteria can be used in the fields with safety has also justified the investments on new research ventures. The development of safe effective and profitable biological insecticides, however, require extensive research. Information gathered during last few years about the structure and modes of action of the toxins produced by insect pathogens are of utmost importance and these will lead to the development of genetically engineered biological insecticides in the near future.

2. Insecticidal bacteria

Over 100 entomopathogenic bacteria have been identified. Most of these belong to the families Bacillaceae, Micrococcaceae, Lactobacillaceae and Pseudomonadaceae (31). Some species of the family Bacilliaceae such as *Bacillus sphaericus*, *Bacillus thuringiensis*, *Bacillus lentimorbus* and *Bacillus popilliae* and their many subspecies have received highest attention and have been extensively studied. These bacteria produce proteinaceous parasporal crystal inclusions, -endotoxins, during sporulation. Protein protoxins are deposited in the inclusion and sometimes also on the surface of the spore. When the inciusion is ingested by the insect the protoxin is solubiiised in the alkaline and reducing environment of the midgut of the larvae. The proteases of the gut convert the protoxins into toxins which act on the recepters of the midgut epithelial cells causing swelling and rupture of the cells. Feeding of the insect is inhibited followed by death ocurring within few minites after ingestion or in some cases few days after ingestion. *In vitro* studies of Thomas and Ellar (39) has shown that the toxin binds to phosphatidyl choline, spingomyelin and phosphatidyl ethanolamine containing unsaturated fatty acids suggesting that in the midgut of the mosquito larvae the toxin interacts with specific plasmam- membrane lipids leading to the disruption of members integrity and eventual cytolysis.

The hemolymph of the insect larvae also provides an excellent nutritional environment for the proliferation and sporulation of these bacteria (9). Toxicity of the crystals are specific. For example crystals of *Bacillus thuringiensis* var *kurstaki* are toxic to certain Lepidoptera while crystals of *Bacillus thuringiensis* Var. *israelensis* are toxic to certain Diptera (mosquito and black fly) and have no effect on Lepidoptera. Besides *Bacillus thuringiensis* Var. *israelensis* at least two other subspecies of *B. thuringiensis* which are specifically active

against mosquito larvae have been isolated. These are *Bacillus thuringiensis* Subsp. *darmstadiensis* and *Bacillus thuringiensis* subsp. *morrisoni* (7). *Bacillus sphaericus* also harbour inclusions which are highly toxic to mosquito larvae(12). Aronson et al (4) gives a comparative account of the toxicity of different subspecies of *Bacillus thuringiensis.*

3. **Bacillus thuringiensis** var. **israelensis** (Bti)

Bacillus thuringiensis Var. *israelensis* was discovered in Israel in 1977 (17) and was defined as a new serotype H 14 of *Bacillus thuringiensis* in 1978 (13). Its specific toxicity towards larvae of many species of mosquitoes and black fly has been well established. In a comparative study made with laboratory-rared and field-collected mosquito larvae of several species (38) it has been shown that 2nd instar larvae of *Aedes aegypti* to be the most sensitive with a LC50 in 24h to be 2.5×10^2 organisms/ml. 2nd instar larvae of *Culex quinquefaciatus. Aedes albopictus Culex mimulus, Cules vishnui* are also very sensitive. 3rd instar larvae of *Anopheles dirus*, 2nd instar and 3rd instar larvae of *Anopheles vagus*, 3rd instar larvae of *Anopheles masculatus* and *Armigeres sabalbatus*, 2nd instar larvae of *Culex tritaentiorthynchus* were relatively less sensitive. 3rd instar larvae of *Toxorhynchites splendens* and 4th instar larvae of *Mansonia uniformis* and *Mansonia indiana* were not suseptible.

Bacillus thuringiensis produced by fermentation can be formulated as a stabilised emulsion, wettable powder or dust containing a mixture of spores, toxin crystals and particulate debris. Toxicological tests of these preparations on humans have been performed using large does applied by all conventional routes such as oral respiratory, subcutaneous, skin surface, eye cavity etc. (9)

Inspite of its high potential, use of Bti in the field remain limited. Main reason for this is because of its low persistence in the field after application. The bacterium has not been found to reproduce in natural bodies of water and does not reach environmental steady states with lowered populations of the target species as in case of many other biological control agents. Its toxic effect disappear from mosquito breeding habitats within 24-48 h of application due to absorption of spores to silt particles or due to other reasons (29)

In a field experiment (30) repeated biweekly application of Bti to ditches has effectively controlled the population of *Culex quinquefaciatus.* In this study newly hatched larvae have been observed one day after the treatment of the ditches with Bti indicating that Bti spores remain in the feeding zone of larvae only about one day after application.

Arieh Zaritsky et al. (2) have shown in laboratory experiments that minimal number of Bti spores need to be ingested to kill one 3rd instar larvae of *Aedes aegypti* is about 1000. However, after the death of the larva the spores germinate and multiply in the carcass and several days later 3-4 $x10^6$ spores accumulate in a single carcass. Spores are observed inside the carcass microscopically 30 h after the death although not after 24 h (3). Thus some amount

of amplification of the pathogen can take place in nature after treatment. Arieh Zaritsky, et al have also shown that in the laboratory condition larvae of Aedes aegypti feed on the carcass of its own species killed by Bti spores causing cycles of deaths among larvae. Their observations suggested the possibility of using Bti killed larvae as an encapsulating agent and a more effective delivery agent for Bti spors. However the extent to which the mosquito larvae feed on the carcass of deead mosquito lavae in their natural habitats is unknown.

4. Plasmids of Bti

Bti has been variously reported to contain 5, 7, 8 or 9 plasmids. Their sizes range from 3 to 130 MDa. Ward and Ellar (45) by analysis of over 100 isolates cured of one or more of the 9 plasmids, revealed that the loss of 72 MDa plasmid was invariably accompanied by loss of the ability to synthesise - endotoxin protein. Deletion of one or more of any other plasmid did not have any effect on the endotoxin production. Thus the genes responsible for the production of - endotoxins are carried in a 72 MDa plasmid and not in others. However, according to one report a 135 MDa plasmid seem to be necessary for the transfer of 72 MDa plasmid (18).

Apart from the 72 MDa plasmid the functions of the other plasmids are not known. Clark et al (11) have studied three of these plasmids of the sizes 5.4 kb, 6. 51 kb and 7.53 kb. and prepared restriction endonuclease site maps of them. Small plasmids of Bti may be useful in the construction of shuttle vectors for *E. coli*, *B. subtilis* and Bti. Miteva and Grigorova prepared restriction maps of three small plasmids of sizes 3.65, 4.65 and 5.5 MDa (33) and constructed a 7.4 MDa plasmid by the recombination of the 3.65 MDa plasmid with the E. coli plasmid pJH 101 (32). Mahillon et al (28) have cloned two Bti cryptic plasmids of 9.2 kb and 10.6 kb in their entirety in pBR322.

5. Protoxin

Bacillus thuringiensis and its various subspecies and varieties produce potent insoluble crystal toxins when the cells sporulate. In *Bacillus thuringiensis* Var. *israelensis* -endotoxin crystals are deposited outside exosporium during stage II and III of sporulation (41). There are 2 to 4 inclusions per cell which are cuboidal, bipyramidal, ovoid or amorphous (46). They are 0.1 to 0.5 um in size and are visible in sporulating cells under phase contrast microscope. The crystals account for 20-30% of the dry weight of the sporulated cells (1).

Protoxins of different varieties of *Bacillus thuringiensis* appear to have different shapes of crystals, molecular weights, peptide maps, aminoacid compositions, carbohydrate contents and immunological cross reactivities (31).

-endotoxin genes of *Bacillus thuringiensis* var *berliner* 1715 have been transferred to Bti through *B. subtilis*(21). In the resulting strains both *berliner* toxin active on Lepidoptera and *israelensis* toxin active on Diptera were expressed in normal amounts confirming that two different toxins can be harboured in the same bacterial strain.

It is believed that in the midgut of the insect, where alkaline conditions prevail, the crystals are solubilised releasing soluble glycoprotein protoxins which are then activated by proteolytic cleavage to yield toxic polypeptides of smaller sizes (1,27,31). Most important product appear to be a polypeptide of 60-70 kDa which can account for most of the toxicity associated with the crystal. Treatment of purified protoxin with trypsin or insect midgut juice produces an active toxin of similar size.

It the invitro studies of the crystals three main components with approximate molecular weights 28 kDa, 68 kDa and 130 kDa have been identified and studied. Molecular weights calculated by different workers for these components seem to be slightly different. Although all these components appear to have mosquitocidal activity each has different LC50 values. The 28 kDa component is responsible for cytolytic and hemolytic activities. 130 kDa polydeptide appear to be processed further in the midgut of the larvae releasing 60-70 kDa polypeptides (5, 25, 44, 46). In some reports 4 major polypeptides with molecular sizes of about 135 kDa, 70 kDa, 38 kDa and 28 kDa and some minor species have been identified (42).

Pfannenstial et. al (34) tested the immunological relatedness between different polypeotides of the crystal and found that although 135, 70 and 28 kDa fractions are immunologically distinct 38 kDa species is derived from 70 kDa protein by proteolysis (34).

Cheung et al., (10) isolated a 25 kDa protein by solubilising the crystals at pH 11 followed by anion exchange chromatography. This protein, although it had strong cytolytic activity showed no mosquitocidal activity either in the solubilissd form or when the protein was absorbed into latex beads. This suggested that cytolytic component of the crystal is not insecticidal and that different components do not act alone.

Most of the studies favours the view that there are probably three gene products in the crystal. The three genes appear to code for products of the ranges 55-70, 125-135 and 25-30 kDa. Garduno et. al, cloned a toxin gene from Bti in *E. coli* and in *Bacillus subtilis*. A 58 kDa gene product was mosquitocidal to larvae of *Aedes aegypti* (40). When this gene was introduced into a crystal negative mutant of Bti partial toxicity was restored (16). This 58 kDa component of the crystal is structurally related to 130 kda component. The gene for the 130 kDa component has also been cloned and expressed (37). Evidence has been found (6) to indicate that the two- genes coding for the two polypeptides of 135 akDa and 58 kDa are both found in the 72 MDa plasmid of Bti and are separated physically by as few as 2000 base pairs. The smaller gene of the two is perhaps derived from the bigger one.

6. Endotoxin genes.

The isolation, cloning and characterisation of protoxin genes could result in the development of more potent Bti toxins. Protoxin genes of most of the Bacillus species appear to be located in plasmids of various sizes. All such plasmids are however greater than 35-MDa. There seems to be conserved regions of DNA in plasmids of different subspecies. The location of the

protoxin genes in plasmids also seems to be related to the presence of inverted repeat elements providing a mobilising capacity. Gonsalez and Carlton (18) have found from the study of plasmid profiles of var. *israelensis* that some spontaneous derivatives lost the protoxin encoding plasmid of 75-MDa. However, sequences hybridising with the plasmid were found in the chromosomal DNA. The derivatives were still -oxic to larvae indicating that protoxin gene expression takes place after integration to the chromosome. Derivatives of this strain were the toxic sequences where found in new plasmids of 65-80 MDa were also found. These results and subsequent studies with sequence probes indicated that protoxin genes are located on a transposable element. Detailed mapping and electron microscopic analysis have shown that in the host plasmid the toxin gene is flanked by multiple copies of two repeated DNA elements (23-26-8).

Protoxin genes of *Bacillus thuringiensis* var *kustaki* (19,36), var. *berliner*(22), var. *israelensis* as well as *B. sphaericus*(15) have been cloned. Partial nucleotide sequence also have been elucidated in some cases. It is believed that the promoter of the toxin gene is conserved in different subspecies of Bt. It has been shown that transcription is initiated from two overlapping promoters. One of them is utilised in early stages of sporulation while the other one, located upstream, is utilised four hours after the onset of sporulation (35). Terminator region of the crystal protein gene stabilises the m-RNA. This stability is partly responsible for the abundant synthesis of the protein (23).

One important approach to the study would be the location of the nucleotide sequence for the ultimate active toxic polypeptide within the larger protoxin gene. To achieve this goal it is necessary to study the complete nucleotide of the gene, aminoacid sequence and the molecular weight of the polypeptide required Restriction enzymes and endonucleases can be used to fractionate the genes and each fraction can be cloned in expression vectors which can be used to transform bacteria. Extracts of the transformed bacteria can be screened for toxicity in bioassays. Cloning of the active region of the gene will provide a probe that can be used to scan the total plasmid DNA of different strains. In a study of this nature done with the protoxin gene of the variety kurstaki (24), a single 732 bp restriction fragment has been used as a hybridisation probe for analysing 17 insecticidal strains of 9 subspecies of *Bacillus thuringiensis*. The probe has hybridised with DNA from strains toxic to Lepidoptera but failed to hybridise with DNA from subspecies toxic to Diptera. Hybridisation experiments conducted with 28 kDa protein gene and the 130 kDa protein gene of the Bti have shown that these two genes are probably present in the plasmid DNA of *Bacillus thuringiensis* subsp. *morrisoni* which is also highly active against mosquito larvae (7)

Another line of study would be to compare the aminoacid sequence of protoxins produced by different varieties having different host ranges or to compare the nucleotide sequences of the genes coding for them. This type of

study will elucidate the presence of conserved and variable regions within toxin genes of different subspecies. These in turn will relate the structure to biological activity. Conserved and variable regions of the genes can also be elucidated in nucleic acid hybridisation studies. A study of structure-function relationships of the toxin polypeptide will enable genetic redesigning of the molecules to obtain a more potent toxin. Site directed mutagenesis could be used to make precise changes required and intragenic hybridisation between different strains could be used to obtain combination of characters found in different genes to produce more potent combinations (20)

The synthesis of toxic crystal takes place naturally only at the onset of sporulation of the bacterium. By fusing the structural gene sequence to a strong constitutive promoter it may be possible to make bacteria to produce the toxin throughout their life cycle in large amounts making the process more economical. Such cells may not lyse spontaneously to liberate toxin to environment. An artificcial lysis step can then be introduced in the manufacturing processes (20)

Much information has been gathered during the last few years on the toxin genes and their function. Much research remains to be done. Most of the rzsearch are directed towards creation of genetically redesigned genes and plasmids capable of producing much more potent mosquitocide toxins than those found in nature. When the researchers reach this success it would be another important achievement in the field of recombinant DNA technology. The developing countries like Sri Lanka, and others in the South East Asia and those in Africa and South America where mosquito borne diseases are a major health problem could benefit immensely from such studies.

References :

1. Andrews, R. E., Bulla, L. A. 1981, Toxins of spore forming bacteria. In *spores* VIII. pp. 57-63. Washington Am. Soc. Microbiol.

2. Arieh Zaritsky, Kamal Khawaled, Zeev Barak, David M. Chipman, Tavassa Rabi, 1986 Biological control of mosquitoes by larvidical activity of *Bacillus thur ingiensis var. israelensis* delta endotoxin. ACTA Microbiologica Polonica. 35; 207-214

3. Arieh Zaritsky, Kamal khawaled. 1986. Toxicity in carcasses of *Bacillus thuringiensis* var. *israelensis*-killed *Aedes aegypti* larvae against scagenging larvae; implications to bioassay, J. of Amer. Mosq. Control Assoc., 2:555-559

4. Aronson, A. I., Beckman, W., Dunn, P. 1986. *Bacillus thuringiensis* and related pathogens. Microbiol. Rev. 50: 1-24

5. Armstrong, G. L., Rohrmann, G. f., Beaudreau, G. S. 1985. Delta endotoxin of *Bacillus thuringiensis* subsp. *israelensis* j. Bacteriol. **161**: 39-46.

6. Bourgouin, C., Klier, H., Rapoport, 1986. Characterisation of the genes encoding the hemolytic toxin and the mosquitocidal delta endotoxin of *Bacillus thuringiensis israelsis* Mol. Gen. Genet, 205; 390-397

7. Bourguoin, C., *et al.* 1986. Characterisation of the genes encoding the hemolytic toxin and the mosquitocidal delta-endoloxin of *Bacillus thuringiensis israelensis*. Mol Gen. Genet, **205**:390-397.

8. Bourguoin, C., *et al* 1988. A *Bacillus thuringiensis* subsp. *israelensis* gene encoding a 125-kilodalton lervicidal ploypeptide is associzted with inverted repeat sequences. J. Bacteriol, **170**:3375-83

9. Burges, H. D., 1982. Control of insects by bacteria. Parasitology **84**:79-117.

10. Cheung, P. Y. K., Bustar, D., Hammock, B.D. 1987. Lack of mosquitocidal act'vity by the cytolytic protein of the *Bacillus thuringiensis* subsp. *israelensis* parasporal crystal. Current Micrology **15**:21-23.

11. Clark, B. D. et al. 1985, Restrlction endonuclease mapping of three plasmids from *Bacillus thuringiensis* var. *israelensis* Gene. **36**:169-171

12. Davidson, E. W., Myers, P. 1981, FEMS Microbiol. lett. **10**: 261-65

13. Des Rochers, B., Gercia, R. 1984, Evidence for persistence and recycling of *Bacillus spheericus* Mosq. News. **44**-160.

14. Fast, P. G. Martin, W. G. 1980, Bacillus thuringlensis per asporal crystal toxin; dissociation into toxic low molecular weight peptides. Biochem. Biophysic, Res. Commun. **95**: 1314K20

15. Ganesan, S., Kamdar, H., Jayaraman, K., Szulsmajster, J. 1983. Cloning and expres - ion in *Escherichia coli* of a DNA fragment from *Bacillus sphaericus* coding for the biocidal act i v ty against mosquito larvae. Mol Gen. Genet. **189**¼ 181-83

16. Garduno, F., Thorne, L., Walfield, A. M., Pollock, T. J. 1988. Structural relatedness between mosquitocidal endotoxins of *Bacillus thuringiensis* subsp. *israelensis* Applied and Environmental Microbiology **54**- 277-279.

17. Goldberg, L., Margalit, J. 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii, Uranyteania unguiculata,* Culex univittatus Aedes aegutpti and *Culex pipiens* Mosq. News, **37**:353.

18. Gonzalez, J. M., Jr., Carlton, B.C. 1984, A large transmissible plasmid is required for crystal toxin production in *Bacillus thuringiensis* variety *israelensis*. Plasmid **11**:28-38.

19. Held, G. A., Bulla, L. A. Jr., Farrari, E., Hoch, J. Aronson, A. I., Minnich, S. A. 1982. Cloning and localisation of the lepidopteran protoxin gene of *Bacillus Thuringiensis* subsp. *kurstaki*, Proc. Natl. Acad Sci. USA **79**: 6065-69.

20. Kirschbaum, J. B. 1985. Potential implication of genetic engineering and other biotechnologies to insect control. Ann. Rev. Entomol. **30**:51-70.

21. Klier, A., Bourgouin, C., Rapaport, G. 1983, Mating between *Bacillus subtilis* and *Bacillus thuringiensis* and transfer of crystal genes. Mol. Gen. Genet. **191**:257-262.

22. Klier, A., Fargette, F., Ribier, J., Raport, G. 1982. Cloning and expression of crystal protein genes from *Badillus thuringiensis* strain *berliner* 1715, EMBO J. **1**:791-99

23. Klier, A., Rapaport, G. 1987. *Bacillus* larval toxin crystal protein, Microbiol. Sciences **4**:274-276.

24. Kronsted, J. W., Schnepf, H. E., Whiteley, H. R. 1983, Diversity of locations for *Bacillus thuringiensis* crystal protein genes. *J. Bacteriol.* **154**: 419-28

25. Lee, S. G., Eckbled, W., Bulla, L. A. Jr. 1985, Diversity of protein inclusion bodies and identification of mosquitocidal protein *Bacillus thuringiensis* subsp. *israelensis* Biochem. Biophs, res. Commun. **126**:953-60

26. Lereclus, D. et al. 1984. EMBO J. 3:2561-7.

27. Lilley, M., Ruffel, R. N., Somerville, H. J. 1980, Purification of the insecticidal toxin in crystals of *Bacillus thuringiensis*. J. Gen. Micrbiol. **118**: 1-11

28. Mahillon, J. et al. 1988, Cloning and partial characterisation of three small cryptic plasmids from Bacillus thuringiensis. Plasmid. **19**: 169-173

29. Margalit, J., Zomer, E., Eral, Z., Barak, Z. 1983, Development and application of *Bacillus thuringiensis* va. *israelensis* serotype H-14 as an effective biological control agent against mosquitoes in Israel. 1983. Biotechnology **1**:74

30. McLaughlin, R. E., Fukuda, T. 1982. Effectiveness of *Bacillus thuringiensis* serotype H-14 against *Culex quingiesfasciatus* in small ditches. Mosqueto news. **42**: 158-162

31. Miller, L. K., Ling, A. J., Bulla L. A. Jr. 1983, Bacterial, viral and fungal insecticides. Science **219**:715-21

32. Milteva, V. I., Grigorowa, R. T. 1988. Construction of a bifunctional genetically labelled plasmid for *Bacillus thuringiensis* subsp. *israelensis*, Arch. Microbiol, **190**: 496-98

33. Milteva, V. I., Grigorowa, R. T. 1986. Restriction analysis of plasmids of *Bacillus thuringiensis* subsp. *israelensis* H 14. Lett. in Appl. Microbiol. **3**: 85-88

34. Pfannenstiel, M.A., Couche, G. A., Ross, E. J. Nickersonk. W. 1986, Immunological relationships among proteins making up of the *Bacillus thuringiensis* subsp. *israelensis* crystaline toxin. Appl. Environ. Microbiol. **52**: 644-649

35. Schnepf, H. E. et al. 1985. J. Biol. Chem. **260**: 6264-72

36. Schnepf, H. E., Whiteley, H. R. 1981. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Excherichiacoli* Proc. Netl. Acad. Sci. USA **78**: 2893-97

37. Sekar, V., Carltoh, B.C. 1985. Molecular cloning of the delta-endotoxin gene of *Bacillus thuringiensis* subsp. *israelensis*. Gene 33:151-158.

38. Somsak Pantuwatana, Amporn Youngvanitsed, 1984, J. Sci. Soc. Thailand **10**: 101-108.

39. Thomas, W. E., Ellar, D. J. 1983. Mechanism of action of *Bacillus thuringiensis* var. *israelensis* insecticidal d-endotoxin, FEBS letters. **154**: 362-367

40. Thorne, L. et al. 1986. Structural similarity between the Lepidoptera-and Diptera-specific insecticidal endotoxin gens of *Bacillus thuringiensis* subsp. *kustaki and israelensis* J. Bacteriol. **166**: 801-811

41. Tyrell, D. J., Davidson, L. I., Bulla, L. A. Jr., Ramoska, W. A. 1979. Toxicity of parasporal crystals of *Bacillus thuringiensis* subsp. *israelensis* to mosquitoes. Appl. Environ. Microbiol. **38**: 656-58.

42. Tyrell, D. J. et al. 1981. Comparative biochemistry of entomocidal parasporal crystals of selected *Bacillus thuringiensis* strains. J. Bacteriol. **145** 1052-1062

43. Vaeck, M. et al. 1987. Nature **328**: 33-37

44. Visser, B. Et al. 1986, FEMS Microbiol. lett. **30**: 211-214

45. Ward, E. S., Ellar, D. J. 1983. Assignment of the d-endotoxin gene of *Bacillus thuringiensis* var., *israelensis* to a specific plasmid by curing analysis F×BS lett **158**: 45-49.

46. Yamamoto, T., Lizuka, T., Aronson, J. N. 1983, Mosquitocidal protein of *Bacillus thuringiensis* subsp. *israelensis:* iden ification and partial isolation of the protein. Curr. Microbiol, **9**: 279-841