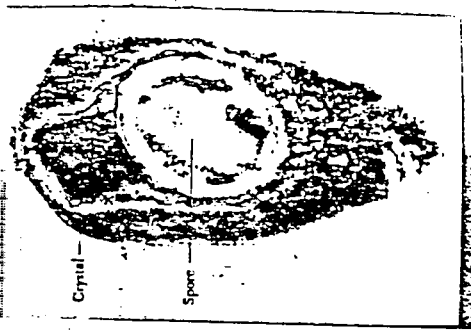
PN-AAZ-809 4.528

PROGRESS REPORT

USAID

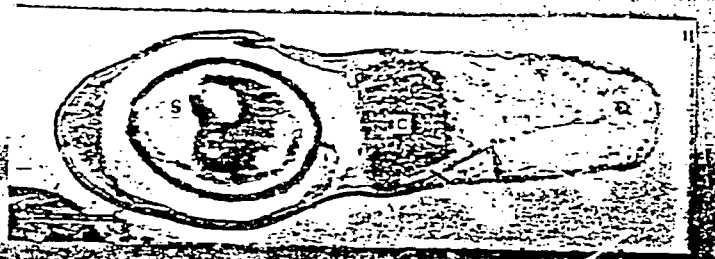
SCIENCE & TECHNOLOGY COOPERATION PROGRAM

BACILLUS THURINGIENSIS
SUBSPECIES
ISRAELENSIS



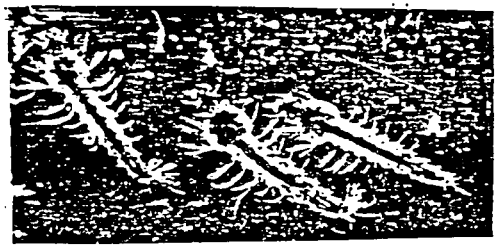
+

BACILLUS SPHAERICUS
STRAIN 1593



?

KILL



MOSQUITO LARVAE

PROGRESS REPORT

1. USAID - PROGRAM IN SCIENCE AND TECHNOLOGY COOPERATION(PSTC)

1.1 Project Number: 936-5542

Grant No. 936-5542(Washington)

Obligation No. 364129

Project Title: Improvement of Bacterial Agents for control of Mosquito Vectors

1.2 Principal Investigator

Dr. Somsak Pantuwatana

Department of Microbiology

Faculty of Science, Mahidol University

Rama VI Road, Bangkok 10400

Thailand

Telephone (02) 245-5197

Telex 84770 UNIMAHI TH

Associate Investigators

Dr. Amaret Bhumiratana	Department of Microbiology
Dr. Sakol Panyim	Department of Biochemistry
Dr. Prapon Wilairat	Department of Biochemistry

Collaborating Scientists

Dr. Elizabeth J. West Davidson	Arizona State University
Dr. Samuel Singer	Western Illinois University

1.3 Period(month/year) covered by this report

From 5/1985 to 11/1985

1.4 Starting date of project

May 17, 1984

2. Project Objectives

To increase the toxicity of bacterial pathogens of malaria-carrying mosquitoes through genetic engineering.

3. Scientific Progress

3.1. Isolation and identification of the delta-endotoxin from Bacillus thuringiensis

3.1.A. From Bacillus thuringiensis serotype H-14

Recombinant plasmids containing delta-endotoxin gene were successfully constructed by ligating Sau 3A1 fragments of *B. thuringiensis* H-14 plasmids into expression vectors, pUR290, pUR291 and pUR292. The ligated product was used to transform *E. coli* strain JM 107 by the DMSO method (Hanahan, D.. J. Mol. Biol. 166:557, 1983) and the transformants were selected on L-agar containing 50 μg/ml ampicillin. Approximately 30,000 transformants were obtained with transformation efficiency of 10^4 per μg DNA.

The colonies of transformant were lifted onto nitrocellulose filter, induced with IPTG for 3 hr at 37°C and lysed with 0.5 M NaOH. They were probed for delta-endotoxin production using the rabbit antibody raised against the delta-endotoxin coupled with ^{125}I-protein A followed the technique described by Kemp et al.(Kemp et al. PNAS, USA 80:3781, 1983).

The positive clones were picked and the proteins analysed by SDS-PAGE, transferred onto nitrocellulose and probed with the antibody and ^{125}I-protein A. From over 30,000 transformants 21 were found to give positive signals when probed for expression products with antibody to the delta-endotoxin proteins. Among the positive clones, some showed β-galactosidase fusion peptides and few gave small peptides.

Results indicated that delta-endotoxin gene from B. thuringiensis serotype H-14 was successfully cloned in E. coli. Further works on detailed analysis of each clones, DNA sequencing of the delta-endotoxin gene, and mosquito larvicidal activity are being carried out.

3.1.B. From Bacillus thuringiensis strain HD-1

The preliminary experiment of conjugation resulted in the successful cloning of mosquito toxin gene from B. thuringiensis strain HD-1 into E. coli strain C-600 suggests the possibility of using this same technique for the introduction of the delta-endotoxin gene into other Bacillus.

Our former objective was aimed at transferring mosquito toxin gene of B. thuringiensis strain HD-1 into B. subtilis. It was shown that transformation in B. subtilis with plasmid DNA was very inefficient by comparison with chromosomal transformation. It was believed that the specific activity of

plasmid DNA in transformation of B. subtilis was dependent on the degree of oligomerization of the plasmid genome. It was shown later that the efficiency of protoplast transformation system could be increased into higher level. However, the protoplast system has disadvantage in terms of difficulty in protoplast formation and regeneration. Thus, we decided to select for mutants of B. thuringiensis strain HD-1 to use as a recipient.

In this cloning experiment, the major part of the program will be concerned with the identification of the transformed recombinant clones. Various methods have been developed to identify clones that carry desired DNA sequences and antibiotic resistance genes is one of them.

In order to speed up the selection of mutants, non-toxic mutants were selected from cultures of B. thuringiensis strain HD-1 which grown on L-agar medium after the treatment of the bacteria with 1% SDS(sodium dodecyl sulfate). The larvicidal activity was done against 2nd instar larvae of Aedes aegypti. The creamy white, dense, opaque colony was observed for the selection of a sporogenic acrystaliferous mutant and the potential mutant was picked and restreaked for the purification on L-agar medium. Only non-toxic mutants of a sporogenic acrystaliferous mutants were selected for the analysis of DNA plasmid. As a result, one strain was obtained and it was named strain HD-7 and this strain did not have any DNA plasmid.

The selection for selective markers or mutants of strain HD-7 which carry antibiotic resistance genes was carried out using spontaneous mutation technique. The mutants were selected from cultures which were grown on the L-agar growth medium containing specific antibiotics. The mutants obtained by this method were suspected to carry drug resistant gene in the chromosomal DNA. Several mutants were obtained by this technique as shown in Table 1.

Table 1. Drug resistant mutants of B. thuringiensis strain HD-1 obtained by spontaneous mutation which were selected by growing the bacteria(strain HD-7) on L-agar growth medium containing specific antibiotics.

Mutant No.	Characteristics
HD-7 Str^R	Streptomycin resistance
HD-7 Tc^R	Tetracycline resistance
HD-7 Km^R	Kanamycin resistance

Mutants of B. thuringiensis strain HD-1 which carry antibiotic resistance genes were also selected using spontaneous mutation technique. The mutants were selected from cultures which were grown on the L-agar growth mediu containing specific antibiotics. So far, two mutants were obtained by this technique. They were strain HD-1Tc^R which was resistant to tetracycline and strain HD-1 Km^R which was resistant to kanamycin. These two strains were still carrying gene(s) which were responsible for the production of delta-endotoxin.

The gene(s) which are responsible for the production of delta-endotoxin from either strain HD-1 Tc^R or strain HD-1 Km^R will be transferred into B. thuringiensis mutants either strain HD-7 Str^R or HD-7 Tc^R or HD-7 Km^R using conjugation-like process. Attempts of gene transferring are still going on.

3.2. Introduction of the delta-endotoxin gene into other B. thuringiensis strains and/or B. sphaericus strains

3.2.A.1 Development of the conjugation-like process for an effective genetic transformation system in B. thuringiensis H-14

The gene manipulation technology for the improvement of B. thuringiensis serotype H-14 has been facing the lack of an effective genetic transformation system. This is based on the difficulty in carrying out transformation in B. thuringiensis H-14 using protoplast transformation or competent cell transformation. Several attempts have been conducted in order to overcome this problem and finally a conjugation-like gene transfer process on millipore filter has been developed. This technique has been shown to be an effective gene transfer system in B. thuringiensis serotype H-14 with the following evidence.

A lysozyme sensitive strain of B. thuringiensis (strain 0 016) was isolated and shown to be effectively transformed with plasmids pC 194 and pHV 33 using the protoplast transformation technique.

The plasmid pC 194 from one successful transformant, strain 0 016-194, was subsequently transferred to B. thuringiensis serotype H-14 by a conjugation like process. The plasmid pBC 16 from B. cereus could also be transferred to B. thuringiensis serotype H-14 with high frequency using the conjugation-like

process. Further, both plasmids, pC 194 and pBC 16, were transferred between strains of B. thuringiensis serotype H-14 to yield transcipient strains that harbored and expressed properties of both plasmids.

3.2.A.2. Selection of mutant with defect(s) in delta-endotoxin

Attempts have been made to obtain non-toxic mutants of B. thuringiensis serotype H-14 with specific selective markers. Mutants were selected from standard strain of B. thuringiensis serotype H-14 using NTG(N-methyl-N'-nitro-N-notrosoguanidine) treatment. After the treatment with NTG, mutants were selected from the drug resistant colonies which appeared as creamy white, dense, and opaque colony. These mutants were purified by restreaking on L-agar medium containing a given antibiotics. The selection of mutant is still going on.

Drug resistant mutants of B. sphaericus strain 1593 were also selected from the standard strain using the same technique described above. So far, one mutant of B. sphaericus strain 1593 which carried a chloramphenicol resistance (Cm^R) was obtained.

3.2.A.3. Introduction of the mosquito-toxin gene into B. thuringiensis H-14 and/or B. sphaericus 1593

Eventhough mutants with defect in delta-endotoxin are not available on hand, but several strains of B. thuringiensis serotype H-14 and B. sphaericus strain 1593 which carried specific drug resistance marker are available. Thus, the conjugation-like gene transfer process on millipore filters was done for the introduction of the mosquito-toxin gene between the selected mutants of B. thuringiensis serotype H-14 and B. sphaericus strain 1593. The mating experiment was carried out between B. thuringiensis H-14 mutant which carried a tetracycline resistance (Tc^R) and B. sphaericus 1593 mutant which carried a chloramphenicol resistance (Cm^R) marker. After mating, tentative transcipient

cells with both drug resistant genes were selected on plates containing 10 μg/ml tetracycline and 6 μg/ml chloramphenicol. Under these conditions, the donor and recipient strains could not multiply even at every high concentrations of cells. Several clones of transcipient cells were obtained by this method. The larvicidal activity and electrophoretic patterns ofthe clones are in progress.

3.2.B. Development of spheroplast and mixed protoplast experiment using B. thuringiensis H-14 and B. sphaericus strains

Spheroplast production and regeneration of spheroplast of B. thuringiensis serotype H-14 and B. sphaericus strain 1593 were carried out with a considerable good yield. Mixed protoplast experiment using B. thuringiensis H-14 mutant which carried Tc^R marker and B. sphaericus 1593 mutant which carried Cm^R marker was carried out in the presence of PEG. After fusion, the tentative fusants with Tc^RCm^R trait were selected on plates containing 10 μg/ml tetracycline and 6 μg/ml chloramphenicol. Under these conditions, the parent strains could not multiply even at every high concentrations of cells. One clone of fusant was obtained. After several purifications had been carried out, the cellular morphology of the fusant was observed under the phase-contrast microscope. It was found that the cellular morphology of the fusant which carried both of a tetracycline and chloramphenicol resistance markers was similar to the cellular morphology of B. thuringiensis H-14 except that some cells developed slightly oval shape spore which was similar to spore of B. thuringiensis and some cells developed spore with round shape which was similar to spore of B. sphaericus as shown in Figure 1. It was interesting to observe that some cells developed one parasporal protein crystal and some cells developed three parasporal crystals. After purification, the tentative fusant was confirmed for the presence to plasmid(s) using agarose gel electrophoresis technique. After plasmid

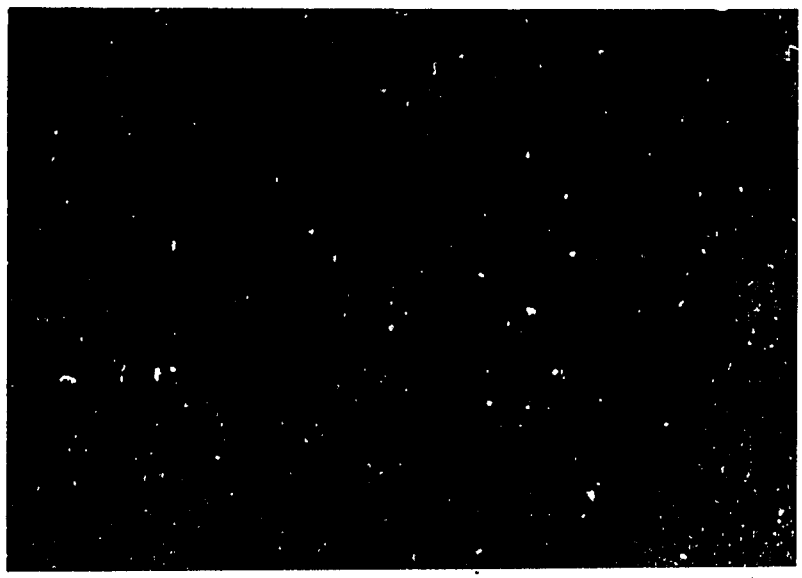


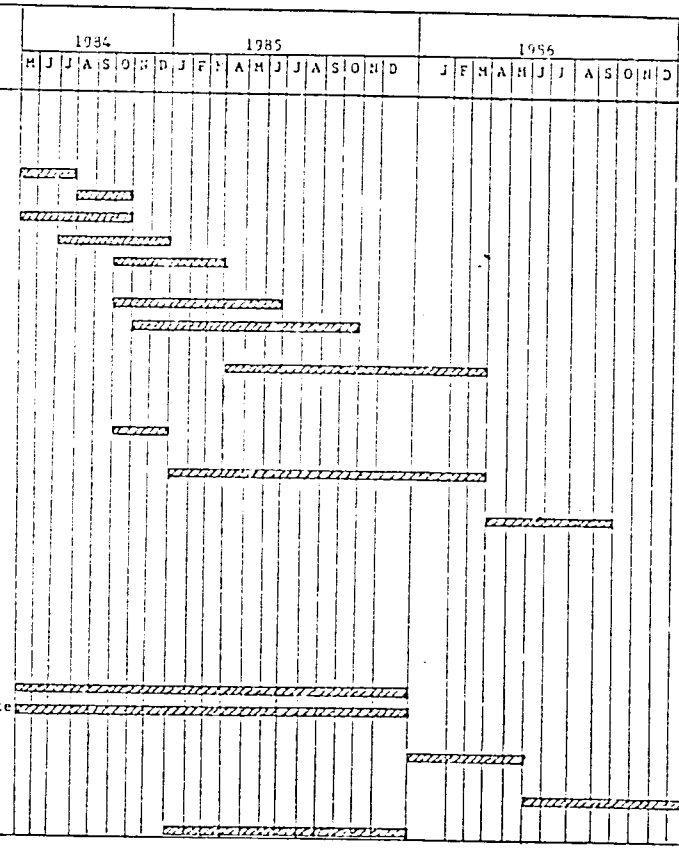
Figure 1. Cellular morphology of tentative fusant which obtained from the fusion between B. thuringiensis H-14 and B. sphaericus 1593 mutants.

extraction, electrophoretic patterns of the transcipients were compared with those of the parent strains. It was found that bands which comigrated with the B. thuringiensis H-14 plasmid could be detected in the plasmid preparations from the fusant clone. This fusant clone was tested for larvicidal activity against larvae of Ae. aegypti, Cx. quinquefasciatus and Anopheles maculatus. It was found that the fusant clone had a very good activity against all three species tested whereas the B. thuringiensis H-14 parent strain was very active against Ae. aegypti larvae and the B. sphaericus 1593 parent strain was very active only against larvae of Cx. quinquefasciatus and An. maculatus . The identification and characterization of the fusant clones are under investigation.

(1st revision)

Revised activities and implementation schedule and financial plan (August 22, 1985)

Activities	Cost US $
1. Isolation and identification of the δ-endotoxin	
A. From B.T. H-14	
A1. isolation of δ-endotoxin	
A2. development of antibodies to δ-endotoxin	7,500
A3. isolation of plasmid	1,000
A4. isolation of total DNA	6,000
A5. In vitro transcription-translation of δ-endotoxin gene	6,000
A6. Cloning of B.T.H-14 DNA into E. coli	4,000
A7. Identification of δ-endotoxin gene cloned into E. coli and characterization	5,000
A8. DNA sequencing of the δ-endotoxin gene	6,000
	6,700
B. From B.T. HD-1	
B1. Development of antibodies to the mosquito toxin of strain HD-1	1,000
B2. Cloning of mosquito toxin gene into B. subtilis	9,000
B3. Characterization of the mosquito toxin gene	5,000
2. Introduction of the δ-endotxin gene into other B. thuringiensis strains and/or B. sphaericus strains	
2A. Development of transformation system in B.T. H-14	
2A.1. Construction of appropriate plasmid	10,000
2A.2. Search for and development of appropriate host	15,000
2A.3. Selection of mutant with defect(s) in δ-endotoxin	2,000
2A.4. Transformation of δ-endotoxin gene into B.T. H-14 mutants	8,000
2A.5. Conjugation	5,000



(1st revision)

Revised activities and implementation schedule and financial plan (August 22, 1985)

Activities	1984 M	J	J	A	S	O	N	D	1985 J	F	M	A	M	J	J	A	S	O	N	D	1986 J	F	M	A	M	J	J	A	S	O	N	D	1987 J	F	M	A	Cost US $
2B. Development of spheroplast and mixed protoplast experiment using B.T. H-14 and B. sphaericus strains																																					
2B.1. Spheroplast production experiment																																					8,000
2B.2. Regeneration of spheroplast																																					1,000
2B.3. Mixed protoplast experiment																																					1,000
2B.4. Screening for fused zygotes																																					4,000
3. Strain selection																																					
3.1. Bioassay of larvicidal activity of genetically engineered strains																																					13,000
3.2. Production and maintenance of the selected strains																																					10,730
4. Scientific meeting																																					10,000
5. Report writing																																					5,000
Cost US$	41,720		5,750		6,450				12,912 .04			12,010				7,250			7,950		15,087 .06			12,400			8,100			9,700		10,600			149,930

Revised financial plan according to item catagory (August 22, 1985)

(1st Revision)

Cost element	Budget Plan												
	1984			1985				1986			1987		Total cost
	M - J	A - O	N-D-J	F - A	M - J	A - O	N-D-J	F - A	M - J	A - O	N-D-J	F - A	
Salary	3,750	3,750	3,750	3,750	4,250	4,250	4,250	4,250	5,100	5,100	5,100	5,100	52,400
Equipment	26,000												26,000
Supplies	6,000	2,000	2,000	6,000	3,900	3,000	3,000	3,000	3,500	3,000	2,000	1,500	38,900
Transportation			700				700				600		2,000
Travel (SEA)				2,000							2,000		4,000
Travel (USA)				1,162.04				4,837.96					6,000
Contingency								3,000				4,000	7,000
Overhead	5,970				3,860				3,800				13,630
Total Cost	41,720	5,750	6,450	12,912.04	12,010	7,250	7,950	15,087.96	12,400	8,100	9,700	10,600	149,930

12

XN-AAZ-809-A

PROGRESS REPORT NO. 5

IMPROVEMENT OF BACTERIAL AGENTS FOR CONTROL OF MOSQUITO VECTORS

A RESEARCH PROJECT
USAID/PSTC PROGRAM
GRANT NO. 936-5542

SUBMITTED BY

SOMSAK PANTUWATANA
PROJECT LEADER

MAHIDOL UNIVERSITY
FACULTY OF SCIENCE
RAMA VI ROAD
BANGKOK 10400

PROJECT PROFILE

Country:	Thailand
Grant No. :	936-5542
Program:	Program on Science and Technology Cooperation
Project Title:	Improvement of Bacterial Agents for Control of Mosquito Vectors
Project Leader:	Dr. Somsak Pantuwatana
Organization:	Mahidol University
Co-investigators:	Dr. Amaret Bhumiratana Dr. Sakol Panyim Dr. Prapon Wilairat
Project Consultants:	Dr. Samuel Singer, Western Illinois University Dr. E. J. W. Davidson, Arizona State University
Authorized Officer:	Dr. Natth Bhamarapravati Rector, Mahidol University
Total Project Budget:	$149,930
Project Duration:	3 years (May 1984 to April 1987)
Reporting Period:	7/1986 to 12/1986
Budget Allocation for this period:	$12,400 (=฿ 322,400)

14

1. BACKGROUND/INTRODUCTION

Recently, the development of microbial agents for using as biological control agents against mosquitoes has been studied intensively by many groups of scientists. This effort is related to the use of long residual action and widespectrum insecticides resulting in insecticide and behavioral resistance and environmental contamination.

The most famous biological control agents for mosquitoes are Bacillus thuringiensis var. israelensis (H-14) and B. sphaericus 1593. Bacillus thuringiensis H-14 is more effective against Aedes and Culex than Anopheles larvae, but even within a species different strains show variation in their susceptibilities. It does not persist in the environment and consequently has to be repeatedly applied. In contrast, Most Culex species, including Cx. quinquefasciatus, are highly susceptible to Bacillus sphaericus, but Aedes larvae are little affected. In general, Anopheles are more susceptible to B. sphaericus than B. thuringiensis H-14 but this varies according to species and even strains of the same species (Lacey , 1985 and Singer, 1985). However, B. sphaericus 1593 has the ability to recycle and appears to persist in the environment better than B. thuringiensis H-14. Thus, it is more interesting to find a good strain of these two bacteria that can persist better in the environment and is more effective against Anopheles larvae than the existing strains.

There are serveral approaches have been used in order to obtain a more effective strain of bacteria than the existing ones. It may be obtained by selection from natural population of by the new methods of genetic engineering. Over many generations, selection can have a powerful effect, and certain organisms have been induced to divert a

major share of their metabolic energy into the production of substances more useful to man than to the organism in its natural setting. Nevertheless, the inescapable limitation of selection is that it can act only on organisms that already exist, favoring those whose genome, or total complement of genes, specifies the synthesis of desired materials. Selection can never introduce new information into the genome.

The new methods of genetic engineering give the biologist direct access to the genome. A gene can be inserted, deleted, altered or duplicated. For example, a gene from one organism can be transferred into the genome of another, and such a transfer of genetic information can be accomplished even across the evolutionary distance that separates a human being from a bacterium. An artificial gene, encoding instructions for making a molecule unknown in nature, can be assembled by chemical means and introduced into a microorganism. It has been shown that the activity of B. thuringiensis H-14 and B. sphaericus is a result of the activity of toxins produced by the bacteria. These toxins are coded by genes found inside the bacterial cell. Thus, it is possible to improve the toxicity of these two bacteria by using the new methods of genetic engineering.

2. OBJECTIVE

To increase the toxicity of bacterial pathogens of malariacarrying mosquitoes through genetic engineering.

3. MATERIALS AND METHODS

In order to improve the toxicity of bacterial agents either B. thuringiensis H-14 or B. sphaericus 1593, the first requirement is that the gene(s) that responsible for the production of mosquito toxin should be identified and isolated. Once the gene is identified, recombinant-DNA will be constructed by inserted into selected vector plasmids that can express in Escherichia coli. The idea is to transfer an individual gene coding for the mosquito toxin into a host microorganism for characterization. So far the bacterium Escherichia coli has served almost exclusively as the host because effective vector plasmids, whose DNA has been mapped in fine detail, are available for this workhorse of molecular biology. When the mosquito toxin gene is characterized, it will be possible to insert this gene into other vector plasmids that can express in Bacillus spp. which probably resulting in a new strain of Bacillus thuringiensis or Bacillus sphaericus than can kill wider hostrange. Two bacterial species, B. thuringiensis strains H-14 & HD-1 and B. sphaericus 1593, were used as models in the study. The procedures were done as follows:

3. A. FROM B. THURINGIENSIS H-14

After the delta-endotoxin was isolated from B. thuringiensis H-14 by centrifugation through renografin gradient, it was solubilized and analyzed by SDS-polyacrylamide gel electrophoresis to determine the major component that responsible for the larvicidal activity of the delta-endotoxin crystal. The larvicidal activity was done by using 18 hrs old Ae. aegypti larvae exposed directly to the suspension of the solubilized component and observed 24 to 48 hr after exposure. Individual protein components obtained from the solubilization of delta-endotoxin were tested against purified rabbit antibodies which prepared

to soluble delta-endotoxin proteins and rabbit antibodies which prepared to the 25 kD protein purified from Sephadex G-100. After the individual protein components reacted with the antibodies, the mixtures were analyzed by SDS-PAGE, followed by Western blot transfer, probing with the antibody and subsequent ^{125}I-protein A coupled with autoradiography. Results indicated that gene (genes) coding for delta-endotoxin crystal resided on plasmids of B. thuringiensis H-14.

Plasmids from B. thuringiensis H-14 were isolated by the alkaline-phenol extraction procedure of Kado and Liu (1981). Recombinant plasmids containing delta-endotoxin gene were constructed by ligating Sau 3A1 fragments of B. thuringiensis H-14 plasmids into expression vectors, pUR290, pUR291 and pUR292. The ligated product was used to transform E. coli strain JM 107 by the DMSO method described by Hanahan (1983) and the transformants were selected on L-agar containing 50 μg/ml ampicillin. The colonies of transformant were lifted onto nitrocellulose filter, induced with IPTG for 3 hr at 37°C and lysed with 0.5 M NaOH. They were probed for delta-endotoxin production using the rabbit antibody raised against the delta-endotoxin coupled with ^{125}I-protein A followed the technique described by Kemp et al. (1983). Recombinant plasmids were also constructed from 110 Kb plasmid of B. thuringiensis H-14 digested with Xba I and ligated to Xba I digested pUC12. The recombinant clone was selected and screened for delta-endotoxin mRNA-enriched RNA. The recombinant clone was further screened by the delta-endotoxin antibody. The DNA inserts and of the protein products which kill Ae. aegypti larvae were analyzed.

The 5 Kb fragments were further subfragmented by partial Sau 3A1 and ligated to Bam HI digested pUC12, followed by transformation. The

transformants were screened for products using antibody to the total crystal proteins and larvicidal activity. The positive clones were used for characterization of the DNA insert and the protein product in order to determine whether this endotoxin gene was related to the 130 kD endotoxin gene. The analysis was done with a series of restriction enzymes, i.e., EcoR I, Pvu II, Pst 1, Xba I, Hind III, Hae III, Bel I, Xho II, Acc I, Msp I, Hpa II, Acy I, Sau 961, Dde I, Cla I, and Bst NI.

After successful transfer the mosquito toxin gene into E. coli, attempts to introduce the delta-endotoxin gene into other Bacillus spp. were done by conjugation. The 130 kD delta-endotoxin gene was inserted into pUB 110 by joining pUB 110 plasmid vector with the recombinant E. coli plasmid containing the 3.8 Kb gene encoding 130 kD delta-endotoxin. The joining was done at Bam HI site. Then this recombinant plasmid was transfered into B. subtilis and E. coli. The production of delta-endotoxin gene was determined using bioassay against Ae. aegypti larvae and observed for the crystal formation under the phase-contrast microscope.

3.B. FROM B. THURINGIENSIS HD-1

From our previous reports, it was shown that the mosquito toxin gene of B. thuringiensis HD-1 was also coded by plasmid. Thus, similar procedures were used for the construction of recombinant plasmid as described for B. thuringiensis H-14. Recombinant plasmids containing mosquito-toxin gene were constructed by ligating fragments of B. thuringiensis HD-1 plasmid which had been restricted with either Hind III, Bam HI or Xba I into expression vectors, pBR 322, pUN 121 and pUC 12. The ligated products derived from pBR 322 and pUN 121 were used to transform

E. coli strains C-600, and MM 294, where as that derived from pUC 12 was used to transform E. coli strain JM 107, and the transformed E. coli with ampicillin resistant and tetracycline resistant markers were selected and screened for the presence of mosquito toxin gene by using enzyme-linked immunosorbant assay (ELISA). Only those transformants obtained from recombinant plasmids containing mosquito-toxin gene ligated with pUC 12 were selected by using ampicillin resistant and beta-galactosidase negative markers. Those clones which yielded ELISA positive reaction were then screened for larvicidal activity towards Ae. aegypti larvae.

3.C. INTRODUCTION OF THE DELTA-ENDOTOXIN GENE INTO OTHER BACILLUS AND/OR B. SPHAERICUS

As reported previously that several transcipient clones were obtained from the conjugation experiment in which selected mutants of B. thuringiensis H-14 and B. sphaericus 1593 were used. Also, several fusants were obtained from the mixed protoplast experiments. The identification and characterization of the fusant clones are being done. The development of enzyme analysis technique using vertical polyacrylamide gel electrophoresis is also being developed.

4. RESULTS AND DISCUSSION (PROGRESS)

4.A. IDENTIFICATION OF THE DELTA-ENDOTOXIN GENE OF BACILLUS THURINGIENSIS H-14

As previously reported, in an attempt to clone the delta-endotoxin genes (genes coding for 25 kD, 65 kD, and 130 kD protein) we had constructed pUN 1.5 whose DNA insert was 5 kb and weakly produced 25 kD protein. E. coli containing pUN 1.5 although producing a weak 25 kD protein did not show toxicity towards Ae. aegypti larvae. Further

subfragmenting of the 5 Kb fragments by partial Sau 3A1 and ligated to Bam HI digested pUC 12, followed by transformation, yieled 2 immunoreactive clones when probed for product using antibody to the total crystal proteins. However, these two clones did not exhibit any larvicidal activity. By using Bal 31 to delete 5 Kb fragment followed by Pst 1 cut and ligated to pUC 12 cut with Pst 1 and Sma I, 2 immunoreactive clones (pMUA 136 and pMUA 231) were obtained which exibited the larvicidal activity.

4.B. CHARACTERIZATIONS OF DELTA-ENDOTOXIN GENE AND THE 130 Kd RECOMBINANT ENDOTOXIN

Previously we reported the cloning of 110 Kb plasmid of *B. thuringiensis* H-14 into *E. coli*, in an attempt to isolate, identify and characterize the delta-endotoxin gene. By using Xba I fragments of 110 Kb plasmid ligated to pUC 12 we succeeded in constructing five *E. coli* clones which showed a stable toxicity to *Ae. aegypti* larvae. Analysis of the clones by restriction enzymes digestions revealed that they had a common DNA insert of 3.8 Kb. A detailed restriction enzymes mapping showed recognition sites by enzymes,EcoRI, Pvu II, Pst 1, Xba I, Hind III, Hae III, Bcl I, Xho II, Acc I, Msp I, Hpa II, Acy I, Sau 961, Dde I, Cla I, and Bst NI. Other *E. coli* clones which were immunopositive but did not showed toxicity to the *Ae. aegypti* larvae had inserts of either 7.5, 2.4 or 1.9 Kb. Futher analysis of the protein product by Western Blot immunoassay showed that all the *E. coli* clones showing toxicity to the larvae produced proteins with 130 kD as the major component. The minor components apparently were degradation products of the 130 kD. The clones giving no toxicity with the inserts of 7.5, 2.4 and 1.9 Kb proteins of 90 kD which were not toxic to mosquito larvae.

21

4.C. INTRODUCTION OF THE DELTA-ENDOTOXIN GENE INTO OTHER BACILLUS AND/OR B. SPHAERICUS

In addition to an attempt to introduce the delta-endotoxin gene into other Bacillus spp. by conjugation, we introduced the 130 kD delta-endotoxin gene into Bacillus subtilis. This was carried out by joining the Bacillus plasmid (pUB 110) to the recombinant E. coli plasmid containing the 3.8 Kb gene encoding 130 kD delta-endotoxin (pMU 388). The joining was successfully done at Bam HI site. The bifunctional plasmid replicated and coded for endotoxin production in both B. subtilis and E. coli. Both exhibited toxicity towords larvae of Ae. aegypti. E. coli was also produced protein crystal when observed under the phase-contrast microscope as shown in Figure 1. The bifunctional plasmid was stably maintained in B. subtilis as well as in E. coli.

Figure 1. Characteristics of E. coli clones that contain endotoxin protein crystal.

4.D. CLONING OF MOSQUITO-TOXIN GENE FROM B. THURINGIENSIS HD-1 INTO E. COLI

Attempts of cloning of mosquito-toxin gene derived from B. thuringiensis HD-1 were carried out in E. coli strains C-600, MM 294 and JM 107. Fourteen E. coli C-600 ELISA positive clones were obtained from the ligated product of Bam HI cut mosquito-toxin gene joined with Bam HI digested pBR 322 plasmid vector, 10797 E. coli C-600 ELISA positive clones were obtained from the ligated product of Hind III cut mosquito-toxin gene joined with Hind III digested plasmid vector pUN 121, and 205 E. coli JM 107 ELISA positive clones were obtained from the ligated product of Xba I cut mosquito-toxin gene joined with Xba I digested plasmid vector pUC 12. Only those ELISA positive clones were used in larvicidal activity screening test against Ae. aegypti larvae. So far, only one out of 205 E. coli clones obtained from the transformation by recombinant plasmid demonstrated a good larvicidal activity towards Ae. aegypti mosquitoes as well as the parent strain, B. thuringiensis HD-1. Unfortunately, this recombinant plasmid was not stably maintained in E. coli JM 107. Further investigation is going on.

4.E. THE IDENTIFICATION AND CHARACTERIZATION OF TRANSCIPIENT CLONES AND FUSANT DERIVED FROM B. THURINGIENSIS H-14 AND B. SPHAERICUS 1593

Attempts to identify and characterize those transcipient clones and fusants derived from B. thuringiensis H-14 and B. shpaericus 1593 are in progress. Since we have to develop the enzyme analysis technique using vertical polyacrylamide gel electrophoresis, it will take some times to use this technique for the identification and characteri-

zation of the trancipient clones and fusants. The development of enzyme analysis is in progress.

5. DISCUSSION/REMARKS

The potential difficulty of gene transfer system is how to transfer an individual gene coding for the desired product into a host microorganism and grow the organism in volume to yield the product. That an individual gene should be stably maintained in a host microorganisms. Neither a copy-DNA gene nor an artificially synthesized gene carries the appropriate promotor and other control signals needed for expression in bacteria. It is necessary, therefore, to splice the artificial gene into the vector DNA at a point where the bacterial control signals on the vector will lead to expression of the artificial gene. That is not the end of the task. Bacteria often digest foreign protein, so that the host cell may have to be modified to keep it from destroying its own novel product, a mutant host may have to be selected that has lost the enzymes for protein digestion. The host must also be selected or modified to grow well as well as to accumulate or secrete large quantities of a protein for which it has no use. Thus, we are facing a lots of difficulties. Another difficulty is the selection of ideal plasmid cloning vehicle, since we know that a cloning vehicle should be as small as possible to reduce the possibility of including undesirable genes and prevent self-transmission by conjugation to another cell. The yield and purity of cloned genes and the efficacy of ligation and transformation are also greater with smaller DNA molecules. This is probably the reason why we obtained lower yield of possitive clones (pMUA 136 and pMUA 231) that exhibit larvicidal activity whereas

several clones did have DNA fragment insert (7.5, 2.4 or 1.9 Kb) with out larvicidal activity. Fortunately, we still have 2 E. coli clones that express larvicidal activity. Thus, further characterization should be carried out.

It was also found that recombinant plasmids obtained from Xba I cut mosquito-toxin gene joined with Xba I digested plasmid vector pUC 12 was not stably maintained in E. coli JM 107 after successful transformation. Further construction of recombinant plasmid is needed from these same set of genes. The chances are very high because we already have experience with these genes and plasmid vector.

6. WORKPLAN FOR THE NEXT PERIOD

A successful transfer of mosquito toxin gene from B. thuringiensis H-14 into E. coli in which 2 clones (pMUA 136 and pMUA 231) exhibited the larvicidal activity towards Ae. aegypti. Works in progress are attempting to characterize the DNA insert and the protein product to find out if this endotoxin gene relates to the 130 kD endotoxin gene described in 4B. And if they are different genes (i.e. gene coding for endotoxin protein other than the 130 kD) a possibility of combining the endotoxin genes to exhibit a greater toxicity will be explored.

Since all the E. coli clones that showing toxicity to the Ae. aegypti larvae produced protein with 130 kD as the major component. Works in progress are focusing on the DNA sequence of the 130 kD endotoxin gene and identification of the antigenic and toxic moieties of the 130 kD protein.

Further attempts on the introduction of delta-endotoxin gene into other Bacillus spp. and/or B. sphaericus will be carried out. Since

we have constructed a bifunctional plasmid that can replicate and code for endotoxin production in both B. subtilis and E. coli. This similar approach will be used in transferring delta-endotoxin gene of B. thuringiensis H-14 to other selected Bacillus spp.. It is more likely that B. sphaericus will be used as a host microorganism:

Another approach which dealing with transferring mosquito-toxin gene from B. thuringiensis HD-1 strain is also going on. We have domonstrated that protoplast fusion and conjugation technique can be used for transferring gene between B. thuringiensis and B. sphaericus. These two technique will be carried out in order to gain more experience and by the time when appropriate recombinant plasmids are available these two technique will be used.

Other technique that may be useful in identification and characterization of fusant and transcipient clones is enzyme analysis. The development of this technique is going on.

7. REFERENCES

Hanahan, D. J.Mol. Biol. 166, 557, 1983

Kado,C.I. and S.T. Liu. J. Bacteriol. 145,1365, 1981.

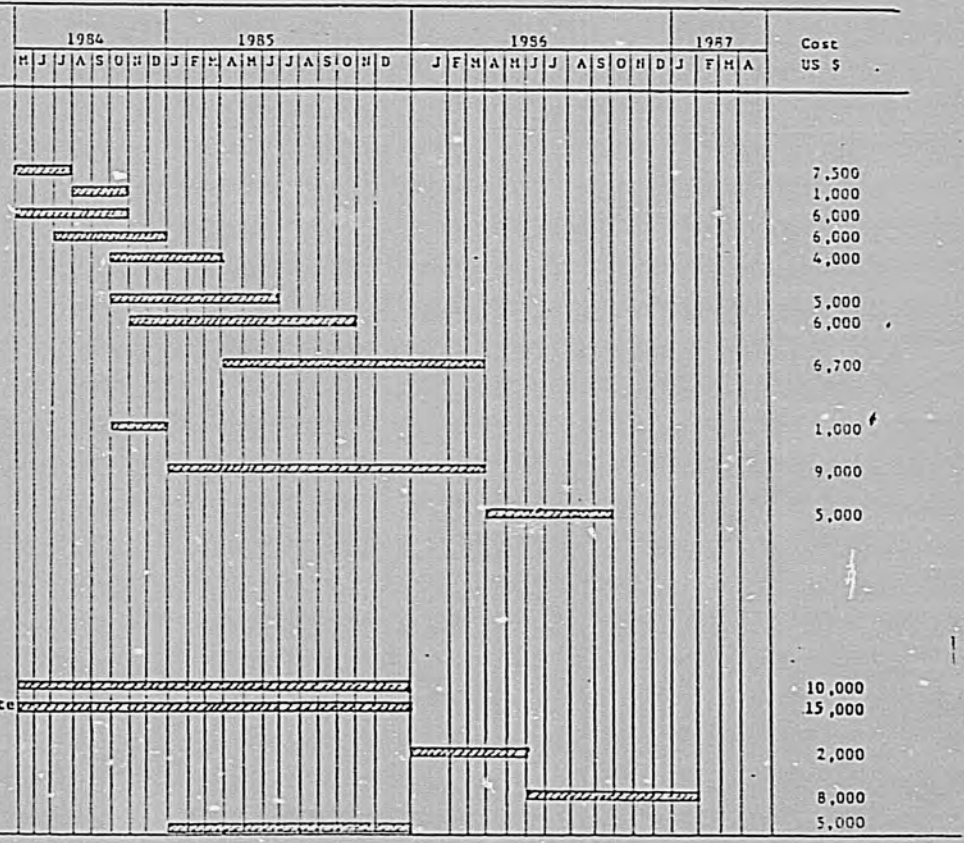
Kemp, D.J., et al. PNAS, USA. 80,3781, 1983

Lacey, L.A. AMCA. Bull. No.6, 132, 1985

Singer, S. AMCA. Bull. No.6, 123 1985

Revised activities and implementation schedule and financial plan (August 22, 1985)

Activities	Cost US $
1. Isolation and identification of the δ-endotoxin	
A. From B.T. H-14	
A1. isolation of δ-endotoxin	7,500
A2. development of antibodies to δ-endotoxin	1,000
A3. isolation of plasmid	6,000
A4. isolation of total DNA	6,000
A5. In vitro transcription-translation of δ-endotoxin gene	4,000
A6. Cloning of B.T.H-14 DNA into *E. coli*	5,000
A7. Identification of δ-endotoxin gene cloned into *E. coli* and characterization	6,000
A8. DNA sequencing of the δ-endotoxin gene	6,700
B. From B.T. HD-1	
B1. Development of antibodies to the mosquito toxin of strain HD-1	1,000
B2. Cloning of mosquito toxin gene into *B. subtilis*	9,000
B3. Characterization of the mosquito toxin gene	5,000
2. Introduction of the δ-endotxin gene into other *B. thuringiensis* strains and/or *B. sphaericus* strains	
2A. Development of transformation system in B.T. H-14	
2A.1. Construction of appropriate plasmid	10,000
2A.2. Search for and development of appropriate host	15,000
2A.3. Selection of mutant with defect(s) in δ-endotoxin	2,000
2A.4. Transformation of δ-endotoxin gene into B.T. H-14 mutants	8,000
2A.5. Conjugation	5,000



Revised activities and implementation schedule and financial plan (August 22, 1985)

Activities	1984 M	1984 J	1984 J	1984 A	1984 S	1984 O	1984 N	1984 D	1985 J	1985 F	1985 M	1985 A	1985 M	1985 J	1985 J	1985 A	1985 S	1985 O	1985 N	1985 D	1986 J	1986 F	1986 M	1986 A	1986 M	1986 J	1986 J	1986 A	1986 S	1986 O	1986 N	1986 D	1987 J	1987 F	1987 M	1987 A	Cost US $
2B. Development of spheroplast and mixed protoplast experiment using B.T. H-14 and *B. sphaericus* strains																																					
2B.1. Spheroplast production experiment																																					8,000
2B.2. Regeneration of spheroplast																																					1,000
2B.3. Mixed protoplast experiment																																					1,000
2B.4. Screening for fused zygotes																																					4,000
3. Strain selection																																					
3.1. Bioassay of larvicidal activity of genetically engineered strains																																					13,000
3.2. Production and maintenance of the selected strains																																					10,730
4. Scientific meeting																																					10,000
5. Report writing																																					5,000
Cost US$		41,720		5,750		6,450				12,912 .04		12,010				7,250			7,950			15,087 .06			12,400			8,100			9,700			10,600		149,930