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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

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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
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Budget Allocation for this period:	$12,400 (=฿ 322,400)

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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

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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 (1982) 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 H1 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.

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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 3.3 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.

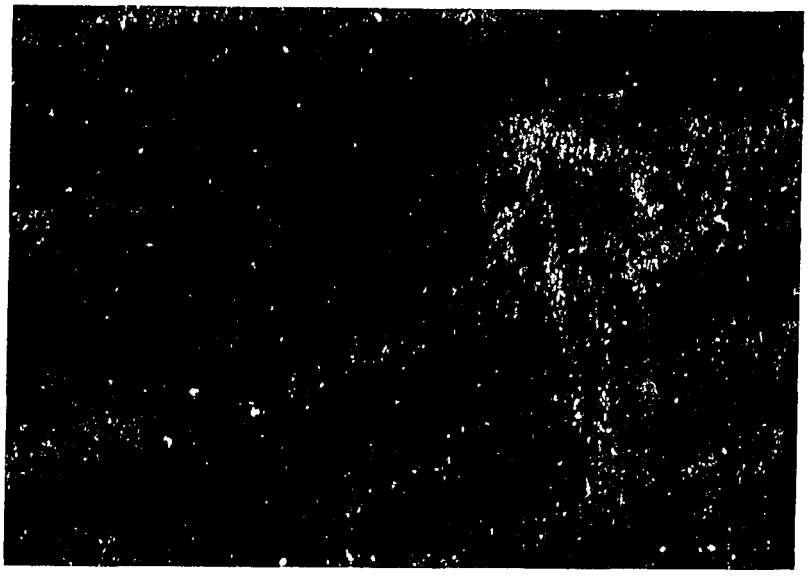

Figure 1. Characteristics of E. coli clones that contain endotoxin protein crystal.

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

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

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 $
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							1985											1986											1987			Cost US $
	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	
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