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PROGRESS REPORT NO. 6

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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Project Duration:	3½ years (May 1984 to November 1987)
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Budget Allocation for this period:	$20,161.53 (=฿ 524,200)

1. BACKGROUND/INTRODUCTION

Several strains of *Bacillus thuringiensis* have been discovered and studied over the past two decades as microbial insecticides. One strain, *B. thuringiensis* H-14, discovered in Israel in 1976 by Goldberg and Margalit had been found to be an extremely promising agent for the control of mosquitoes and blackflies. After the extensive study of this bacterium, it is now clear that *B. thuringiensis* H-14 is effective in the field against some of the major mosquito and blackfly vector species. However, data on the degree and extent of its efficacy against the different vector species were meagre.

One problem with *B. thuringiensis* H-14 is that its spores do not usually multiply in the environment to an extent sufficient for long-term larvicidal purposes, so that its residual activity is of short duration. Different formulations of *B. thuringiensis* H-14 produced commercially have been tested, including powders, liquids, prolonged-release granules, pellets and briquettes, but so far none has increased the duration of residual efficacy by more than two weeks (Goettel et al., 1982; Molloy et al., 1984; Rettich, 198? Preliminary data from Thailand suggest that under certain conditions residual activity can persist for up to fifteen weeks. (Silapanuntakul et al., 1983), but the organism's residual efficacy is still far from optimal for vector control in tropical countries.

Second, with all *B. thuringiensis* H-14 formulations so far tested, large quantities of the product are required for control purposes, which creates problems of logistics and cost.

Third, despite the widely confirmed safety of *B. thuringiensis* H-14, reservations have been expressed by some health officials about the exten-

sive use in rivers and other sources of drinking water of an organism whose spores are naturally designed to last under the most adverse conditions.

Finally, a problem with sporogenic B. thuringiensis H-14 is that the larvicidal toxin is present only within the spores. The fermentation process involved in the large-scale-production of this agent may well be more complex than it would be with a non-spore producing organism.

B. sphaericus, another larvicidal bacterium, is toxic to several genera of mosquito but not to the blackfly species against which it has so far been tested. Unlike B. thuringiensis H-14, though, it persists or recycles ,even in heavily polluted waters. However, the spectrum of activity of this organism or its residual efficacy under different ecological conditions is not fully known. Moreover, strains then available were not suitable for-large-scale fermentations.

The pathogenicity of B. sphaericus to vector mosquitoes has been assessed mainly in relation to Culex quinquefasciatus, an annoyance and a common filariasis vector in many countries. It has been shown that its effectiveness against C. quinquefasciatus is very high and last for several months. In addition, evidence was obtained of its effectiveness against Anopheles larvae. In particular, two strains (strains 2362 and 2297) were found to be slightly more active than Bacillus thuringiensis H-14 against Culex mosquito larvae. However, the residual efficacy of organism depends on its ability to survive in the environment and multiply in the host. Efforts have been made to determine the residual efficacy of B. sphaericus under a wide variety of ecological conditions. It has been demonstrated that the duration of its efficacy varies widely

according to the degree of water pollution which ranging from three weeks in clean water to six months in highly polluted water (Silapanuntakul et al., 1983). As with B. thuringiensis H-14, the greater the degree of water pollution, the higher the dosage required, but with greater residual efficacy than for B. thuringiensis H-14.

Further studies have shown that the activity of B. sphaericus against the different vector mosquito species depends on larval gut enzyme activity, which varies from species to species and prevents the B. sphaericus toxin from entering the vector's hemolymph. Attempts have been explored to find ways of neutratizing these enzymes in order to widening the bacterial spectrum of activity. One approach being explored is genetic manipulation. As with B. thuringiensis H-14, it is hoped that recombinant DNA methods could be used to ensure toxin production by more easily handled organisms, like E. coli of by other organisms which can multiply freely in environmental conditions.

2. OBJECTIVE

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

3. MATERIALS AND METHODS

3.1 Isolation and identification of the delta-endotoxin

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 than 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 that can kill wider host range. Two bacterial species, B. thuringiensis strains H-14 & HD-1 and B. sphaericus 1593, were used as models in the study.

A. FROM B. THURINGIENSIS H-14

Attempts to clone delta-endotoxin gene from 72 md. plasmid derived from B. thuringiensis H-14 into E. coli were done with an aid of restriction enzymes, EcoRI, Sau3AI and Bal31. Recombinant clones that carried a portion of B. thuringiensis H-14 DNA were selected from those colonies which produced proteins that could react with rabbit antibody prepared to crystal proteins of B. thuringiensis H-14 as demonstrated by colonies immunoassay.

Colonies immunoassay was done by transferring suspected recombinant colonies onto Nitrocellulose papers. Nitrocellulose papers were placed onto towel papers previously saturated with 1% SDS for 15 minutes to lyse bacterial colonies. The nitrocellulose papers were transferred into a plastic box in which its atmosphere was suturated with chloroform. The papers were left in the chloroform box for 15 minutes before soaking in 150 ml Blotto solution which contains 16 mM $Na_2HPO_4.2H_2O$, 4 mM $NaH_2PO_4.2H_2O$, 120 mM NaCl, and 5% skim milk to make

the final pH at 7.4. The residues of colonies were scrapped out of the nitrocellulose papers until no visible colony was envisaged by naked eye. Then, the nitrocellulose papers were transferred into a plastic box containing 200 ml Blotto solution. The nitrocellulose papers were gently shaken in the Blotto solution for 2 hours to prevent the papers from non-specific antibodies binding. The nitrocellulose papers were incubated with antibody solution in a plastic bag in a flip-flop manner with an aid of mixer at room temperature for one hour. The antibody solution was consisting of 10 ml of 10 µl/ml rabbit antiserum prepared to B. thuringiensis H-14 crystal proteins (25 KDa delta-endotoxin polypeptide) in Blotto solution. The plastic bag was voided of antibody solution and the excess antibody was washed for 15 minutes 4 times by gently shaking the nitrocellulose papers in 200 ml Blotto solution. The nitrocellulose papers were then incubated with 100 ml 20 µci ^{125}I-protein A in Blotto solution for 1 hour with occasionally shaking. The excess ^{125}I-protein A was removed by washing with 200 ml Blotto solution. The nitrocellulose papers were dried on towel papers and exposed to X-ray films by autoradiography.

Recombinant clones which produced proteins detectable by antibody to protein crystal were selected for characterization using DNA restriction analysis, Southern blot and Western blot.

B. FROM B. THURINGIENSIS HD-1

From our previous reports, attempts were further investigated to construct recombinant plasmid from plasmids derived from B. thuringiensis HD-1. Recombinant plasmids containing mosquito toxin gene were constructed by igating fragments of B. thuringiensis HD-1 plasmid which had been restricted with either Hind III, Bam RI, Xba I or EcoRI into

expression vectors, pUN121, pUC12 and pUB110.

The ligated products derived from plasmid vector pUN121 following the restriction of HD-1 plasmids with either Hind III or Eco RI were used to transform E. coli C-600 and the transformants were selected on the medium containing tetracycline which was used as a selective marker. The ligated products derived from plasmid vector pUC12 following the restriction of HD-1 plasmids with either XbaI or Hind III were used to transform E. coli JM107 and the transformants were selected on the medium containing ampicillin and substrate for galactosidase. The ligated products derived from plasmid vector pUB110 following the restriction of HD-1 plasmids with XbaI were used to transform B. subtilis and the transformants were selected on the medium containing Kanamycin.

All of transformants were screened for the presence of mosquito toxin gene by using enzyme-linked immunosorbant assay (ELISA). Those clones which yielded ELISA positive reaction were then screened for larvicidal activity towards Ae. aegypti larvae.

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

The introduction of the delta-endotoxin gene from B. thuringiensis H-14 into B. sphaericus 1593 was done by using two techniques, protoplast fusion and conjugation-like process. Several clones of transcipient and fusant were obtained. The zymogram technique, a means of visualizing isoenzymes and comparing differences between related species, was developed. The zymogram technique was done by electrophoresis of culture samples in polyacrylamide slab gels, the separated enzymes were stained with coomassie blue dye. The protein profiles and

isoenzyme profiles were examined in comparison with those profiles demonstrated by parent strains.

4. RESULTS AND DISCUSSION

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

Evidence of the 72 md plasmid involved in the production of mosquito toxin of B. thuringiensis H-14 was recently demonstrated by several groups of investigators (Ward and Ellar, 1983; Gonzalez and Calton, 1984). A recombinant clone containing a portion of delta-endotoxin gene from B. thuringiensis H-14 was obtained from the digestion of 72 md plasmid with restriction enzyme EcoRI and was designated as clone 1.5. This recombinant clone carried a portion of B. thuringiensis H-14 DNA of about 5,000 basepairs which responsible for the production of proteins reacted to rabbit antibody prepared against crystal proteins of B. thuringiensis H-14 as demonstrated by colonies immunoassay. However, the ability to produce proteins of this clone was very poor, thus proteins and toxicity of this clone were difficult to characterize. Another recombinant which expressed B. thuringiensis H-14 crystal gene was also obtained and was designated as clone O_3. This recombinant clone was found to habour a 500 basepairs sau 3AI fragment of B. thuringiensis H-14 DNA and it could produce proteins detectable by specific antibody prepared to 25 kd polypeptide derived from B. thuringiensis H-14 protein crystal. Further investigation revealed that clone O_3 could not produce detectable larvicidal activity against Ae. aegypti mosquito eventhough it was shown that clone O_3 carried a part 25 kd delta-endotoxin gene. It was believed that the non-toxin phenotype of clone O_3 was probably due to the ability of the in-

serted DNA of 500 basepairs could not encode full length of delta-endotoxin polypeptide that ~ufficient to exert toxic activity against mosquito larvae. This incomplete polypeptide may loss its biological activity via the absence of the active residue. When the relationship between DNA of clone 1.5 and O_3 was determined, it was found that they contained homologous sequence of inserted DNA. Thus, it was believed that clone 1.5 should carry the delta-endotoxin gene as of clone O_3. Therefore, attempt was made to clone inserted DNA of clone 1.5 into E. coli by using partial Sau 3AI and Bal 31 digestion. After all recombinants were screened for DNA that had homologous sequence to DNA of clone O_3 by using ^{32}P labeled of inserted DNA prepared from clone O_3 as the probe on DNA hybridization precess.

Three recombinants were obtained and designated as recombinants F 052, H 043 and A 136. Recombinants F 052 and H043 were derived from partial Sau 3AI digestion of the precursor DNA whereas a recombinant A 136 was derived from Bal 31 digestion.

Recombinants F 052 and H 043 were found to carry a part of the delta-endotoxin gene, and their inserted DNA restriction endonuclease mapping were similar to each other. This evidence suggested that DNA of these two recombinants came from the same region of the inserted DNA precursor. Unfortunately, protein products from this inserted DNA were not toxic to Aedes aegypti larvae. Only recombinant clone F 052 could produce proteins immunological related to proteins coded by 25 kd delta-endotoxin gene of B. thuringiensis H-14. The reason for the recombinant clone F 052 could express the delta-endotoxin gene was that the inserted DNA of clone F 052 was in the correct reading frame of lacZ promotor of pUC12. However, once the molecular weitht of the protein was analyzed, it was found that the protein had

molecular size of only 18 kd.. This indicated that the product of the inserted gene of recombinant clone F 052 was incomplete delta-endotoxin proteins.

A recombinant clone A 136 produced protein products of molecular size about 106 kd and this protein had immunological relationship to the 130 kd delta-endotoxin of B. thuringiensis H-14. Its inserted DNA had no relationship to DNAs of recombinant clones F 052 and O_3, however, it inserted DNA could hybridize to pMU388 DNA eventhough the restriction map of both DNAs were different. The recombinant clone A 136 expressed mosquito-larvicidal activity but less than those of pMU388 and the original B. thuringiensis -14.

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

As reported previously, we could successfully introduced the 130 kd delta-endotoxin gene prepared from B. thuringiensis H-14 into Bacillus subtilis following the joining of pUB110, the Bacillus plasmid, with 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 and as a result of the joining, a bifunctional plasmid was obtained. It was shown that this bifunctional plasmid could replicate and code for endotoxin in both E. coli and B. subtilis. Both recipients exhibited toxicity towards Ae. aegypti larvae but only E. coli could produce protein crystal that could demonstrate under the phase-contrast microscope. However, this bifunctional plasmid could not express in B. sphaericus. Further investigation is being done to introduce delta-endotoxin gene into B. sphaericus.

Attempts of cloning of mosquito-toxin gene derived from B. thuringiensis HD-1 were also carried out in parallel to those from B. thurirgiensis H-14. Twelve out of 4,515 transformants resulting from the ligated products of Hind III cut mosquito-toxin gene derived from B. thuringiensis HD-1 joined with Hind III digested vector plasmid pUN121 that transformed E. coli C-600 gave ELISA positive reation.

Seven out of 4,091 transformants resulting from the ligated products of EcoRI cut mosquito-toxin gene derived from B. thuringiensis HD-1 joined with EcoRI digested vector plasmid pUN121 that trans formed E. coli C-600 gave ELISA positive reaction. Thirty-five out of 2,356 transformants resulting from the ligated products of XbaI cut mosquito-toxin gene derived from B. thuringiensis HD-1 joined with XbaI digested vector plasmid pUC12 that transformed E. coli JM107 gave ELISA positive reaction whereas no transformant was obtained from the ligated products of XbaI cut mosquito-toxin gene derived from B. thuringiensis HD-1 joined with XbaI digested vector plasmid pUB110 and used to transform B. subtilis. Only ELISA positive clones will be used in larvicidal activity test against Ae. aegypti larvae. The larvicidal activity test is being done during the period of preparing this report.

In the meantime, attempts of cloning of mosquito-toxin gene derived from B. sphaericus 1593 were also carried out in parallel to those from B. thuringiensis HD-1 using the same approach. Eleven transformants that demonstrated larvicidal activity against Culex quinquefasciatus larvae were obtained as a result of the ligated products of EcoRI cut mosquito-toxin gene derived from B. sphaericus 1593 joined with EcoRI digested vector plasmid pUB110 and used to transform B. subtilis. In addition, 122 transformants that demonstrated larvicidal activity against Culex quinquefasciatus larvae were obtained as a re-

sult of the ligated products of EcoRI cut mosquito-toxin gene derived from B. sphaericus 1593 joined with EcoRI digested vector plasmid pUN 121 and used to transform E. coli C-600. Further investigation on DNA of these transformants is being done.

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

Attempts to identify and characterize transcipient clones resulting from conjugation-like process between B. thuringiensis H-14 and B. sphaericus 1593 were done after the development of zymogram had been completed. It was found that the antibiotic resistant gene could be transferred from either B. thuringiensis H-14 or B. sphaericus 1593 to one another following conjugation-like process. After the protein patterns were analyzed, the ratio between B. thuringiensis H-14 and B. sphaericus 1593 to serve as recipients of gene transferring was about 3 : 1.

From these data, it is believed that any plasmid or toxin gene can be transferred back and forth between these two species of bacteria. However, it is more likely that B. thuringiensis H-14 will serve as a better recipient than B. sphaericus. This conclusion is made from the ratio obtained between the enzyme-phenotype of the transcipients following the conjugation-like precess between B. thuringiensis H-14 and B. sphaericus 1593.

5. DISCUSSION/REMARKS

It is now believed that the successful in transferring of mosquito-toxin gene from either B. thuringiensis H-14 or HD-1 or B.

sphaericus 1593 into other organisms can be done and is relying on the selection of an appropriate vector plasmid that can be expressed in the recipient cell. We believe that with our developed methods and gene transfer systems, an individual gene coding for the mosquito-toxin can be successfully transferred into a host microorganism and the organism can be grown in volume to yield the product. However, this objective seems to be very difficult to obtain. This is due to the fact that different organisms have different properties even within the same species that consisting of several strains or mutants. Once one or another strain is selected we have to determine the properties of that particular organism and we usually get stuck with another problem. The most difficult part of it is the matter of selection of an appropriate vector plasmid. This is based on the fact 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 or organism. Moreover, the yield and purity of cloned genes and the efficacy of ligation and transformation are also greater with smaller DNA molecules. Thus, further investigation is being done.

6. WORKPLAN FOR THE NEXT PERIOD

A successful transfer of mosquito-toxin gene from either B. thuringiensis H-14 or HD-1 or B. sphaericus 1593 into E. coli in-which the recombinant clones exhibited the larvicidal activity towards either Ae. aegypti or Culex quinquefasciatus was obtained. Works in progress are attempting to characterize the DNA insert and the protein product to find out if this endotoxin gene is the right one. Individual appropriate vector plasmids will be selected for the introduction of mosquito-toxin gene from either B. thuringiensis H-14 or HD-1 or B. sphaericus

1593 into selected recipients.

7. REFERENCES

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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 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																																					
2B.2. Regeneration of spheroplast																																					8,000
2B.3. Mixed protoplast experiment																																					1,000
2B.4. Screening for fused zygotes																																					1,000
																																					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