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FINAL REPORT

IMPROVEMENT OF BACTERIAL AGENTS FOR CONTROL OF MOSQUITO VECTORS

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SUBMITTED BY

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INTRODUCTION

Since the discovery of *Bacillus thuringiensis* var. *israelensis* (serotype H-14 or *B.t.i.*)in 1977, *(B.t.i)* has become one of the most promising potential bioinsecticide against larvae of mosquitoes. A number of field trial studies are in progress to utilize this bacterium for biologically control of mosquito vectors. There are three properties which are foreseen as limitation to the effectiveness of *B. thuringiensis* var. *israelensis* for mosquito control. The first is its genetic instability from the point of view of toxin production, the second is its limited persistence in natural environments and finally its narrow host range in which the dosage required for control malaria-carrying mosquitoes (*Anopheles* spp.) is rather high. On the other hand, *B. sphaericus*, another famous biological control agent, demonstrates a good potential on recycling and persistence in certain conditions[1-4]. However, recycling does not always occur, but seems to be favored by organic pollution. In general, *Anopheles* are more susceptible to *B. sphaericus* than they are to *B.t.i.* but this varies according to species and even strains of the same species. Particulate matter, strong ultraviolet radiation, low temperatures and alkalinity decrease larvicidal activity[5-8]. 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. Thus, improvement in larvicidal activity and broadening of the host

range is a more appropriate alternative against malaria-carrying mosquitoes.

Gene manipulation techniques have brought a new dimension to applied genetics. The initial step in developing a biotechnological process is generally a search for suitable organisms. Such organisms will be expected to create a product or service that will generate a financial return to that industry. Selection of improved organisms is tedious and time demanding and most of the methods available to the geneticists up until recently have involved trial and error. However, new genetic technologies, i.e. protoplast fusion, conjugation and the use of recombinant DNA techniques, are allowing new approaches by which useful genetic traits can be inserted directly into the chosen organisms. Thus, totally new capabilities can be engineered and microorganisms in particular may be made to produce substances beyond their naturally endowed genetic capabilities.

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 or 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 at 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 microorganisms. It has been shown that the activity of *B.t.i.* and *B. sphaerieus* 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.

The objective of this study was to increase the toxicity of bacterial pathogens of malaria-carrying mosquitoes through genetic engineering.

MATERIALS AND METHODS

Chemicals

All chemicals used throughout this study were of analytical grade or the purest grade available.

Microorganisms

Bacillus thuringiensis var. *israelensis* (serotype H-14) was obtained from Institut Pasteur and used as a source of delta-endotoxin gene. *Escherichia coli* strain JM 107 and *B. subtilis* were used as recipients in gene cloning. *B. thuringiensis* var *kurstaki* (HD-1) and *B. sphaericus* 1593 were used in the protoplast fusion experiment.

Plasmid vectors used for the construction of recombinant DNA plasmids were pUC12, pUR290, pUR291, pUR292, pUN121 and pUB110.

Restriction enzymes used for the construction of recombinant DNA plasmids, restriction enzyme mapping and BNA restriction analysis were Acc I, Acy I, Bam HI, Bel I, Bst NI, Cal I, Dde I, EcoRI, Hae III, Hind III, Hpa II, Msp I, Pst I, Pvu II, Sau 3AI, Sau 961, Xba I, and Xho II.

B.t.i wild-type strain (Department of Microbiology, Faculty of Science, Mahidol University, Bangkok) was used to prepare total RNA. *B.t.i* strain 4Q272 carrying only the 110-kb plasmid (Dr. D.H. Dean, Bacillus Genetic Stock Center, Columbus, Ohio) was used as the source of the 110-kb plasmid. *E. coli* JM107[7] and plasmid vector pUC12[8] have been previously described.

Mosquitoes:

The mosquitoes used in the assay for toxicity were *Aedes aegypti*, *Culex quinquefasciatus*, *Anopheles minimus* and *An. maculatus*. All mosquitoes was from stocks reared for several generations at the Department of Microbiology. The colonies of *Ae. aegypti* and *Cx. quinquefasciatus* were started from field-collected larvae from Bangkhen, Bangkok. *A. minimus* and *A. maculatus* were from stocks reared from eggs obtained from the Department of Biology. The colonies were maintained using a standard mosquito rearing techniques in a temperature and humidity-controlled rearing room to assure maximum vigor of the progeny selected for testing.

Preparation of B. thuringiensis H-14 protein crystals

Bacillus thuringiensis H-14 cells were grown in a nutrient broth supplemented with mineral salts (NBS) at 30°C. in a rotary shaker at 200 rpm for 5 days or until fully sporulated cells were obtained. The fully sporulated cultures were harvested by centrifugation at 10,000 rpm for 10 min in sorvall RC-5 super speed refrigerated centrifuge. The pellets which contained spores and crystals were resuspended in a solution containing 1 M NaCl and 0.01% Trit n X-100, and the suspension was dispersed by sonication. The sonicates were centrifuged on continuous renografin gradients of 40-75% at 4,800 rpm for 30 min using a HB-4 rotor. The band containing crystals was located by sampling aliquots of 0.5 ml and examining of each fraction with phase-contrast microscope. The fractions containing crystals were collected and pooled together. The renografin was

eliminated from the purified crystals by diluting the suspension and centrifugation.

Identification of delta-endotoxin gene

The purified delta-endotoxin 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 hr 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 kDa protein purified from Sephadex G-100. The mixtures of individual protein components that reacted with the antibodies were analyzed by SDS-PAGE followed by Western blot transfer, probing with the antibody and subsequent ^{125}I-protein A coupled with autoradiography.

Isolation of plasmid DNA. The 110-kb plasmid DNA was isolated from *B.t.i.* strain 4Q272 according to the method of Lereclus et al.[7]. The plasmid vector pUC12 was prepared from *E. coli* JM107 by a lysozyme detergent-lysis method[8]. The plasmid preparations were purified by centrifugation in a CsCl-ethidium bromide (EtBr) gradient.

Cloning of DNA. The 110 kb *B.t.i.* plasmid DNA and pUC12 vector DNA were digested separately in high-salt buffer[8] with XbaI (BRL). The plasmid DNA digests were

mixed, heated at 65°C for 10 min and precipitated with ethanol. The pellet was dissolved in ligation buffer[8] containing T_4-DNA ligase (BRL) and incubated at 16°C for 20 h. Portions of the ligation mixture were used to transform *E. coli* JM107[9], and the recombinants were selected on X-gal plates containing 100 μg/ml ampicillin.

Radioactive RNA probes. Total RNA was extracted from *B.t.i.* wild-type strain harvested at midlog phase where there was not detectable amount of delta-endotoxin and at delta-endotoxin-mRNA-enriched stage (5 h after onset of stationary phase) [1] the method of Wong et al.[10] RNA was further purified by CsCl centrifugation. The purified RNA was labeled in vitro with [-^{32}P]ATP (Amersham, 3000 Ci/mmol) and T_4-polynucleotide kinase (BRL) as described by Donis-Keller et al.[11]

Colony hybridization. *E. coli* clones containing the *B.t.i.* delta-endotoxin gene were detected by colony hybridization[12].The prehybridization step was carried out with an excess of non-labeled *E. coli* JM107 RNA plus 500 μg/ml of non-labeled *B.t.i.* RNA extracted at midlog phase. The filters were then hybridized with in vitro-labeled delta-endotoxin-mRNA-enriched RNA ($1x10^7$ cpm/ml).

Radioimmune screening and Western blot analysis. The method was based on those described by Burnette[13] and Johnson et al.[14]. The colony immunoassay was accomplished by in situ lysis of colonies transferred to nitrocellulose filters. The filters were soaked in BLOTTO solution (5% skim milk, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 120 mM NaCl, pH 7.4) and

incubated with rabbit anti-*B.t.i.* crystal antibody diluted 100 fold. ^{125}I-Protein A (Amersham, 0.5-1.0x10^{8} cpm/ml) was added after removal of unbound antibody. The colonies containing materials capable of reacting with the antibody were detected by autoradiography. Protein samples were solubilized in sample buffer (30 mM Tris-HCl pH 8.3, 5% glycerol, 1% SDS and 2.5% B-mercaptoethanol) and boiled for 5 min, then electrophoresed on 10% polyacrylamide gel, transfered electrophoretically to nitrocellulose, and probed with antibody followed by detection with ^{125}I-protein A.

Restriction endonuclease analysis. The recombinant plasmid DNA (0.3-0.5 μg) was treated at 37°C for 2 h with at least 1 unit of the following enzymes: XbaI (BRL) and EcoRI (BRL), in high-salt buffer[8] and PstI (BRL), HindIII (BRL) and PvuII (Amersham) in medium salt buffer. Electrophoresis of DNA was carried out in a horizontal 0.7% agarose gel in TB buffer[8] for 2-3 h at 80 V. Gels were stained for 30 min in 0.5 μg/ml EtBr solution before being photographed under UV light.

Mosquito-larvicidal assay. The assays were modified from Tyrell et al.[15]. The cells were grown for 12 h at 37°C in LB-broth supplemented with 100 μg/ml ampicillin to O.D.$_{600}$ of approximately 1.2. Pellets of 3 ml culture were resuspended in 1 ml distilled water and fed in triplicate to ten 2nd instar mosquito (*Ae. aegypti*) larvae. Dead larvae were scored at room temperature at 12 and 24 h after feeding.

Protein synthesis in minicells and immunoprecipitation. The *E. coli* minicell-producing strain P678-54 was transformed

with purified pMU388 and selected on LB plate containing ampicillin[16]. Minicells were purified and incubated in 500 µl of M9 medium plus amino acid mixture, at 37°C for 45 min. The minicells were pelleted and resuspended in SDS sample buffer, boiled for 5 min and subjected to 10% SDS-polyacrylamide gel. The protein bands were stained with Coomassie brilliant blue. For immunoprecipitation of the protein product, minicell pretein was incubated with the rabbit antiserum at 4°C for 1 h. The antigen-antibody (Ag-Ab) complex was incubated with Protein A-Sepharose at 4°C for 1 h and the Ag-Ab-Protein A-Sepharose complex was separated by centrifugation. The pellet was washed twice and finally resuspended in SDS sample buffer and boiled for 3 min. After centrifugation, the supernatant was loaded onto 10% SDS-polyacrylamide gel.

DNA sequencing. DNA sequence was determined by the chain termination method of Sanger et al.[17], following subcloning of the XbaI fragment containing the EcoRI and PstI sites into M13mp18 and M13mp19 vectors, followed by introduction of appropriate deletions. analysis of sequence homology used the DNASIS Program from Hitachi Software Engineering Co. Ltd.

Transfer of delta-endotoxin gene into other Bacillus.

Attempts to introduce the delta-endotoxin gene into *Bacillus subtilis* were also done by conjugation. The 130 kDa delta-endotoxin gene was inserted into pUB110 by joining pUB110 plasmid vector with the recombinant *E. coli* plasmid containing the 3.8 kb gene encoding 130 kDa

delta-endotoxin. the joining was done at Bam HI site. This recombinant plasmid was transferred into *B. subtilis*. 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.

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.

Bacterial strains. All mutant strains of *B.t.i.* used in this study were derived from the wild type, serotype H-14, isolated from a powder preparation provided by WHO. The *B.t.i.* strain A 084 was selected from the wild-type strain by plating an overnight culture on nutrient agar containing 200 μg/ml streptomycin. *B. thuringiensis* strain O 016 was obtained from curing experiment designed to obtain strains which do not harbor any plasmid using the method of acriflavin at 1.25 μg/ml as curing agent. For treatment with acriflavin, 1% of overnight culture of *B.t.i.* was transferred

into nutrient broth containing acriflavin and the culture was incubated with shaking at 30°C for 4-6 hr. *B. cereus* GP 7 which harbored pBC 16 plasmid was a gift from Dr. W. Goebel. *B. subtilis* strains HVS 62 and HVS 104 which harbored pC 194 and pHV 33, respectively, were gifts from Dr. S. D. Ehrlich. All stock cultures were grown on nutrient agar slants at 30°C for 2 days and subsequently kept at 4° until used. Subcultures were made on the same media once every month.

Unless stated otherwise, nutrient broth supplemented with mineral salts (NBS) as described by Delafield et al.[18] was used in this study.

Routinely sporulating cultures were prepared by transferring a loopful of the bacteria from a stock culture into a 500-ml Erlenmeyer flask containing 100 ml NBS medium. The culture was then incubated with shaking at 200 rpm for 24 hr at 30°C. It was used to provide 1% inoculum for further cultures in NBS for 3 days at 30°C on rotatory shaker at 200 rpm.

Mosquitoes and toxicity assays. A strain of *Aedes aegypti* originally obtained in the form of eggs from the Department of Medical Science, Thailand and referred to as the Bangken strain was used for all toxicity tests. The toxicity assay was carried out as described by Panbangred et al[19].

Preparation of plasmids. Plasmid DNA was extracted by using a rapid alkaline extraction procedure described by Birnboim and Doly[20] with the following modification. Before

treatment with lysozyme, the pellet was washed once with buffer G (50 mM Tris, pH 8.0, 1 mM EDTA, 10% glycerol). The final concentration of lysozyme was increased to 10 mg/ml in the presence of 1 mM DTT.

Restriction of plasmid DNA with restriction endonuclease. Digestion of plasmid DNA was performed in the general buffer described by Maniatis et al.[9] to 14 µl of plasmid DNA, 5 ml of deionized distilled water, 5 ml of 5X buffer and 1-2 unit of restriction endonuclease were added. Incubation was carried out for 2 hr at the temperature recommended by the manufacturer and the reaction was terminated by heating at 65°C for 10 min.

Characterization of plasmid DNA. Agarose gel electrophoresis was used to characterize plasmid DNA fragments. Before loading, 15 µl of plasmid DNA was mixed with 5 µl BJ II solution (550% sucrose, 50 mM EDTA , 0.05% Bromphenol blue). The mixture was loaded into previously prepared slots of a horizontal 0.7% agarose gel. Tris-borate buffer (89 mM Tris, 89 mM boric acid, 25 mM EDTA, pH 8.3) was used throughout. Gels were usually run at a constant 80 V at room temperature, stained in ethydium bromide (1 µl/ml) for 30 min and destained in water for 30-60 min. Fluorescence was visualized with a UV light transilluminator and photographed on Kodak film.

Estimation of molecular weights of DNA fragments was performed using HindIII fragments of DNA as the standard. The published values for these fragments in megadaltons were averaged (15, 6.1, 4.3, 2.8, 1.5, 1.3) and used to determine

unknown fragment size graphically, assuming a logarithmic relationship between molecular weight and electrophoretic mobility.

Protoplast transformation in B. thuringiensis O 016. The method used for protoplast formation in *B. thuringiensis* O 016 was similar to that described for *B. subtilis* ML 1II by Chang and Cohen[22] with some modifications. The Penassay broth that was used in formation of *B. subtilis* protoplast was replaced by L-broth medium (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, pH 7.4). The protoplast suspension was used in transformation experiments with plasmid DNA by following the procedure described for protoplast transformation in *B. subtilis*[21] with following modification. The SMMP used for *B. subtilis* was substituted by SMML (equal volume of 2X SMM and 4X L-broth). The transformed protoplasts were allowed for phenotypic expression by incubation at 30°C for 2 hr. Subsequently, the protoplasts were plated on modified DM-3 medium (per liter: 200 ml 4% agar, 250 ml 1 M sucrose, 100 ml 5% Difco casamino acid, 50 ml 10% yeast extract, 100 ml 3.5% K_2HPO_4 and 1.5% KH_2PO_4, 25 ml 20% glucose, 20 ml 1 M $MgCl_2$, and 5 ml filter-sterile bovine serum albumin) containing appropriate concentration of antibiotics for direct selection of transformants. Transformation frequency was scored after incubation of protoplast on regeneration plates at 30°C for 2 days.

Conjugation-like mating. To prepare for mating experiments, donor and recipient cells were separately cultured by inoculating a loop of cells from a stock culture

into 5 ml L-broth medium subsequently shaken at 200 rpm at 30 °C. After 12-14 hr, 50 µl from each of these cultures was used as inoculum for further subcultures in 5 ml L-broth medium shaken under the same conditions for another 2.5 hr until the absorbance at 600 nm reached 1.0. For mating, 1 ml each of the donor and recipient cultures were mixed in 5 ml L-broth which was immediately filtered through a Millipore filter (0.45 µm). The filter was then placed on L-agar medium and incubated at 30°C. After 24 hr the filter was removed and agitated in 5 ml normal saline solution to prepare a suspension that was then appropriately diluted and plated on L-agar containing antibiotics. The plates were incubated at 30°C for 48 hr and the number of apparent transcipients was scored. The total number of donors and recipients was also determined by plate counts on agar with addition of appropriate antibiotics.

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-nitrosoguanidine) 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.

Drug resistant mutants of *B. sphaericus* strain 1593 were also selected from the standard strain using the same

technique described above.

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 of the clones were analysed.

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 considera. le 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 w s 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. After purification, the tentative fusant was confirmed for the presence to plasmid(s) using agarose gel electrophoresis technique. After plasmid extraction, electrophoretic patterns of the transcipients were compared with those of the parent strans.

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. The plasmids were isolated from *B. thuringiensis* strain HD-14 and purified by zonal centrifugation through a CsCl density gaadient. Recombinant plasmids were constructed by ligating fragments of *B. thuringiensis* HD-1 plasmids which had been restricted with etiher Bam HI, HindIII or EcoRI into either one of pUN 121 and pBR 322 vectors. The ligated product was used to transform *E. coli* C600 and the transformants were selected and screened for delta-endotoxin production using enzyme-linked.

Immunosorbant Assay (ELISA). The positive clones were also tested for larvicidal activity against *Ae. aegypti* larvae.

RESULTS AND DISCUSSION

Cloning in E. coli of DNA fragments responsible for larvicidal activity

CsCl-purified 110 kb plasmid DNA of *B.t.i.* strain 4Q272 was digested with XbaI and cloned into pUC12 vector. Eight hundred recombinant clones were obtained, 32 of which were found to hybridize to delta-endotoxin-mRNA-enriched RNA:17 of these 32 clones gave positive signals when probed with antiserum raised against the delta-endotoxin; 5 of the immunopositive clones (pMU14, 258, 303, 388 and 678) exhibited the strongest signal. All the immunopositive clones and 3 negative clones were tested for *Ae. aegypti* larvicidal activity as shown in Table 1. Only the five strongly immunopositive clones showed toxicity to the larvae. *E. coli* clones containing other recombinant plasmids or pUC12 alone were not toxic to the larvae, while *B.t.i.* wild type and *B.t.i.* strain 4Q272 were highly tixic.

Table 1. Larvicidal activity of B.t.i. and recombinant E. coli

Sample	Dead larvae (dead/total)	
	12 h	24 h
B.t.i.		
Wild type	10/10	10/10
4Q272	10/10	10/10
E. coli		
pUC12	0/10	0/10
pMU14	2/10	10/10
pMU42	0/10	0/10
pMU90	0/10	0/10
pMU126	0/10	0/10
pMU188	0/10	0/10
pMU221	0/10	0/10
pMU258	3/10	10/10
pMU303	2/10	10/10
pMU319	0/10	0/10
pMU360	0/10	0/10
pMU380	0/10	0/10
pMU388	3/10	10/10
pMU402	0/10	0/10
pMU500	0/10	0/10
pMU544	0/10	0/10
pMU671	0/10	0/10
pMU679	1/10	10/10
pMU694	0/10	0/10
pMU695	0/10	0/10
pMU701	0/10	0/10

The cell pellets from 3 ml culture were suspended in 1 ml distilled water and fed to ten 2nd instar mosquito (Ae. aegypti) larvae. The results are averages of triplicate assays

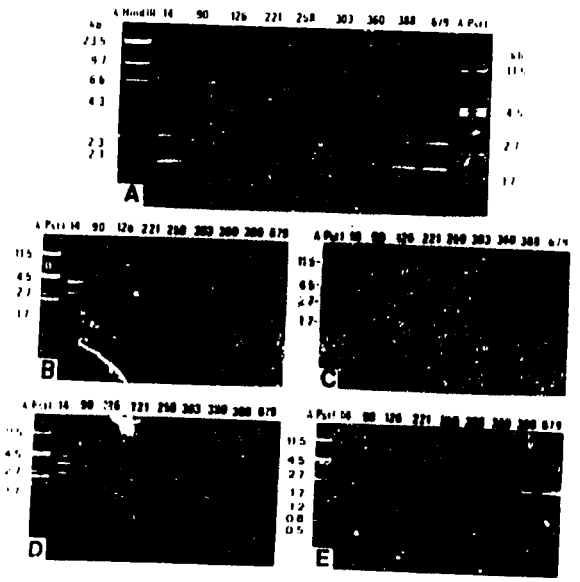

Fig.1A-E. Ethidium bromide staining patterns of restrictio endonuclease fragments of recombinant plasmids. Plasmid pMU14, pMU90, pMU126, pMU221, pMU258, pMU303, pMU360, pMU38 and pMU679 were each digested with XbaI (A), EcoRI (B), Pst (C), HindIII (D), and PvuII (E) and the DNA fragments analyzed on 0.7% agarose gel. Lambda DNA digested with either HindIII or PstI was used as molecular weight marker

Restriction endonuclease mapping

Restriction enzyme analysis of the plasmids from *E. coli* clones which exhibited strong (pMU14, 258, 303, 388 and 679), weak (pMU90, 126, 221) or negative (pMU360) immunoreactivities are shown in Fig.1. The banding patterns from XbaI, EcoRI, PstI, HindIII and PvuII digests revealed that pMU14 was identical to pMU679, pMU90 to pMU221, and pMU258 to pMU303 and pMU388, whereas pMU360 was different. Construction of restriction maps of these plasmids (Fig.2)

revealed that pMU258/303/388 and pMU14/679 had identical inserts of 3.8 kb but in opposite orientations. Similarly, pMU90/221 and pMU360 has identical inserts of 1.9 kb but in opposite orientations. The 1.9-kb insert was one half of the 3.8 kb insert. pMU126 was the same as pMU90/221 but had an extra insert of 2.4 kb.

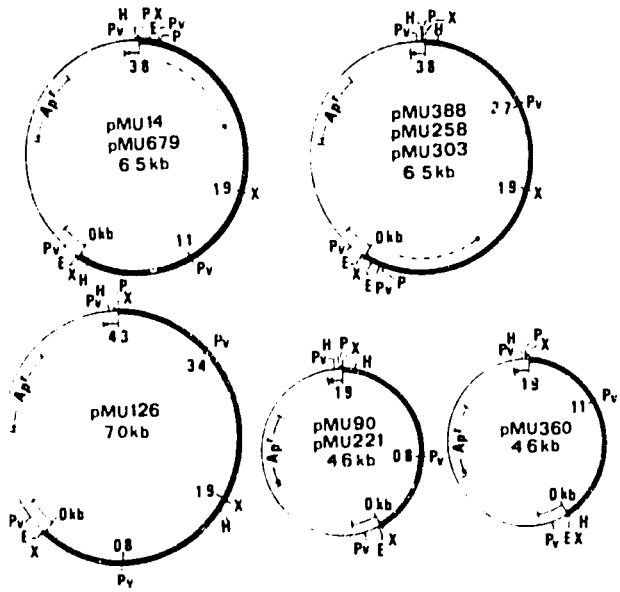

Fig.2. Restriction endonuclease maps of the recombinant plasmids. The maps were constructed from known map of pUC12 and from the patterns in Fig.1. The *thin line* represents pUC12 and the thick line DNA insert. *Solid arrow* represents transcriptional direction of pUC12 promotors, and *dashed arrow* indicates transcriptional direction of insert. *Pv, H, P, X, E;* PvuII, HindIII, PstI, XbaI and EcoRI, respectively.

Western blot analysis of the immunoreactive products

The results thus far showed that, irrespective of orientation, the 3.8 kb insert produced a strong

immunoreactivity to the delta-endotoxin antibody. Clones pMU90/221 with the 1.9 kb insert oriented in the same direction as that of pMU258/303/388 exhibited immunoreactivity, whereas pMU360 with the insert in the opposite orientation did not. Protein extracts of these immunoreactive clones were subjected to SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose paper and then probed with the delta-endotoxin antibody coupled with ^{125}I-protein A (Fig.3). Clones containing pMU14 (or pMU679) and pMU388 (or pMU258/303) gave several immunoreactive bands with that of 130 kDa predominating. Clones harboring pMU90 showed a major band of 65 kDa, whereas those harboring pMU360 or pUC12 gave no immunoreactive bands. IPTG induction of clones containing pMU90 resulted in a large increase of the 65 kDa protein. The IPTG induction produced a slight increase in 130 kDa protein in the clone containing pMU388 but a slight decrease in the clone harboring pMU14. The results indicated that the 3.8 kb insert encoded 130 kDa protein irrespective of orientation, and the 1.9 kb half encoded 65 kDa protein if oriented in the right direction. The results from IPTG induction suggested that the transcriptional direction of the 3.8 kb insert of clones pMU258/303/388 was the same as that of *lac* Z promoter, whilst that of clones pMU14/679 was opposite to that of the *lac* Z. It would thus appear that the 3.8 kb insert contained a necessary regulatory region for expression of the delta-endotoxin gene. The restriction endonuclease map (Fig.2) would indicate that the regulatory region was in the XbaI fragment bearing the EcoRI site, and

that the transcriptional direction would be from EcoRI towards XbaI, PuvII and HindIII sites. Consistent with this suggestion were the findings that pMU90/221, but not pMU360 (insert in the opposite orientation), produced a 65 kDa protein by employing the *lac Z* promotor. The clone harboring pMU126 produced a 65 kDa protein in a similar manner to pMU90/221, indicating that the terminating codon for delta-endotoxin synthesis resided in the XbaI fragment containing PuvII and HindIII sites (Fig.2).

Fig.3. Autoradiogram of Western blot analysis of protein products. Extracts from about 10^7 cells of *E. coli* haboring recombinant plasmids (pMU14, pMU90, pMU360, pMU388, pMB3.45, pMB3.6) and vectors (pUC12, pBR322) were subjected to 10% SDS-PAGE, transferred onto nitrocellulose paper and then probed with antiserum raised against *B.t.i.* crystal proteins, a ^{125}I-Protein A. (') indicates induction with 1 mM IPTG;(S) is solubilized *B.t.i.* crystal proteins.

Further analyses of the protein encoded by the 3.8 kb fragment

The plasmid pMU388 was used to transform *E. coli* P67854 from which minicells were produced. Proteins synthesized in minicells were analyzed by SDS-polyacrylamide gel electrophoresis as shown in Fig.4. It was evident from the Coomassie blue staining patterns that the minicells with pMU388 produced 130 kDa protein which was not observed in the minicells alone or that containing pUC12. When the minicell proteins were immunoprecipitated with the delta-endotoxin antibody, a 130 kDa protein was precipitated (Fig.4, lane E). When the minicells (10^7/ml) were fed to *Ae. aegypti* larvae, only the minicells producing 130 kDa protein killed the larvae within 24 h.

The HindIII fragment containing 3.6 kb of the 3.8 kb insert from plasmid pMU14 (see Fig.2) was transposed from pUC12 to pBR322, a non-expression vector, at the single HindIII site. Proteins synthesized by the *E. coli* harboring the new recombinant (pMB3.6) plasmid were analyzed by Western blot as shown in Fig.3 (track pMB3.6). The result clearly illustrated that 130 kDa protein was the major immunoreactive product. When fed to 2nd instar larvae of *Ae. aegypti*, cells harboring the pMB3.6 were as toxic as those harboring pMU14 or pMU388.

Observation by phase-contrast microscope of *E. coli* containing pMU388 showed a small bright particle located at one end of the cell. These particles could be extracted by solubilization under alkaline conditions in thioglycolate or

dithiothreitol. The SDS-polycrylamide gel pattern of the extract is shown in Fig.4 track F; note the major protein band of 130 kDa protein extract was highly toxic, with LC_{50} of 200 ng/ml.

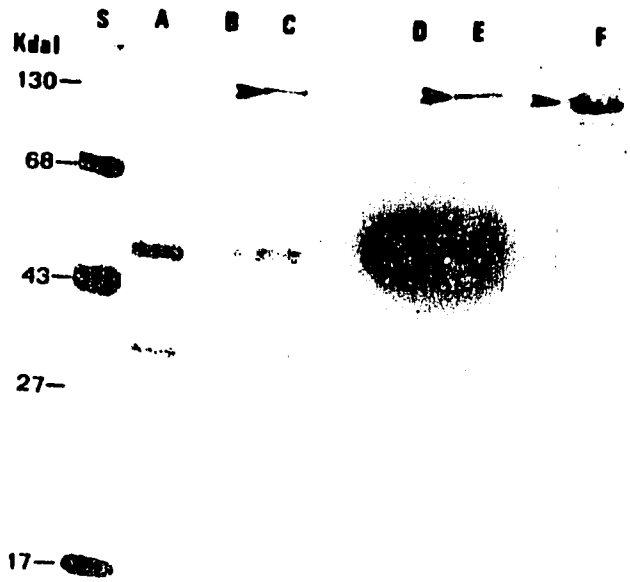

Fig.4. Synthesis of 130 kDa protein in minicells. The minicells harboring pMU388 were prepared as described in Materials and methods. The protein synthesized was extracted and analyzed in 10% SDS polyacrylamide gel, and stained with Coomassie brilliant blue. A, B, C, protein patterns from minicells alone, minicells harboring pUC12, and minicells harboring pMU388, respectively. D, E, protein patterns from minicells harboring pUC12 and pMU388 after immunoprecipitation with the delta-endotoxin antibody. F, Coomassie blue staining pattern of alkaline-thioglycolate-solubilized product of the bright particles from *E. coli* cell harboring pMU388. Arrow indicates 130 kDa protein. The thick band is IgG. S, standard molecular weight marker.

DNA sequence of the 5'-terminal coding strand

A partial DNA sequence of the 3.8 kb insert corresponding to 5'-terminal coding strand is shown in Fig.5. Of the ATG codons yielding an open reading frame, only the ATG at base 151 was preceded by the Shine-Dalgarno sequence, GGAGG, starting at base 139. The implication that this ATG was the translational start site of the 130 kDa protein is further supported by the observations that subcloning of the HindIII fragment of pMU14 insert into pBR322 vector gave an *E. coli* clone producing 130 kDa larvicidal protein (Fig.3, track pMB3.6). The recombinant (pMB3.45) containing the EcoRI-HindIII fragment of the pMU14 insert from which the first 153 bases had been removed, did not code for the 130 kDa protein (Fig.3, track pMB3.45)

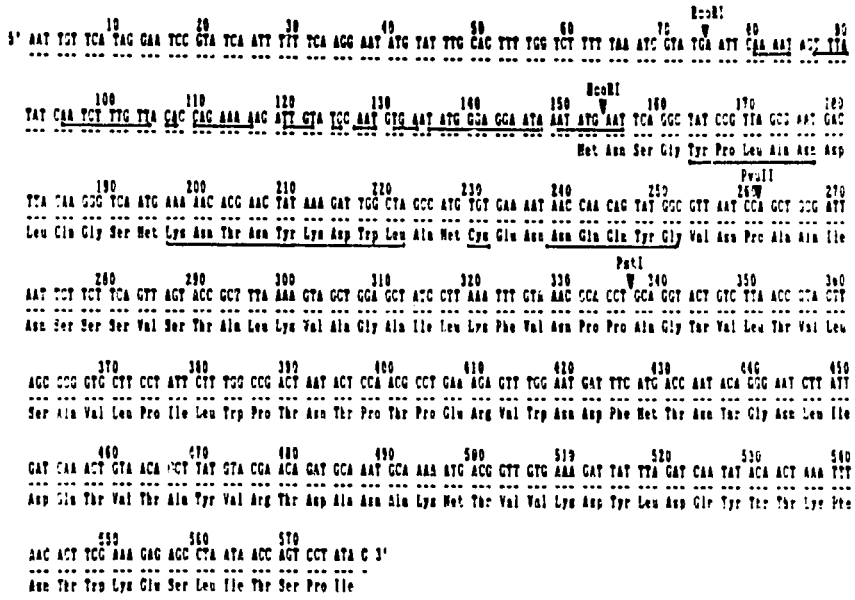

Fig.5. DNA sequence and deduced amino acids sequence of the 5'-terminal coding strand of the 3.8 kb insert. The SD sequence GGAGG is at bases 138-143. EcoRI, PuvII, and PstI sites are shown by *arrows*. The *underlines* are sequences identical to 72-kDa mosquito-larvicidal gene of Thorne et al.[22]

Transfer of a Tetracycline Resistance Plasmid from B. cereus into B.t.i.

The difficulty in carrying out transformation in *B.t.i.* using protoplast transformation or competent cell transformation lead to an investigation of the possibility of using a conjugation-like gene transfer process on Millipore filters (see Materials and Methods). The mating experiment was carried out between *B. cereus* GP7 harboring pBC16 which carried a tetracycline resistance (Te^R) marker and *B.t.i.* strain A 084, a streptomycin resistant (Str^R) mutant. After

mating, tentative transcipient cells with pBC16 were selected on plates containing 5 μg/ml tetracycline and 20 μg/ml streptomycin. Under these conditions, the donor and recipient strains could not multiply even at very high concentrations of cells. Data in Table 2 shows that the frequency of apparent transfer of pBC16 into *B.t.i.* by this conjugation-like process was 1.0×10^{4}.

To confirm that the Tc^{R} trait in the tentative transcipients came from the presence of plasmid pBC16, 4 clones (Str^{R} Tc^{R}) were randomly chosen. After plasmid extraction, electrophoretic patterns of the four clones were compared. Plasmid bands which comigrated with the plasmid pBC16 could be detected in the plasmid prep rations from all four of the clones. All these transcipients were stable after many cycles of subculture in media with or without antibiotics.

To further characterize the plasmid from a transcipient clone, one Str^{R} clone was randomly selected, purified and designated as *B.t.i.* A 084-16. Plasmids were extracted from strain A 084-16 and digested with six different restriction enzymes. The restriction patterns of the plasmids from the transcipient clone were compared with the patterns obtained from plasmid pBC16 of *B. cereus* GP 7 (Fig.6). They had practically identical restriction patterns. The pBC16 plasmids in both hosts had two restriction sites for EcoRI, a single restriction site for BamHI and no restriction site for SmaI, PstI, KpnI, and BglII (Fig.6 and data not shown). Clearly, the plasmid pBC16 from *B. cereus* could be

TABLE 2

Frequency of Plasmid Transfer in *Bacillus thuringiensis* and *Bacillus cereus*

Mating pair No.	Donor	Recipient	Number of potential recipients[a]	Number of transcipients[b]	Transfer frequency
1.	*B. cereus* GP 7 (pBC 16)	*B. thuringiensis* A 084	6.0×10^{7}	6.2×10^{3}	1.0×10^{-4}
2.	*B. thuringiensis* O 016-194 (pC 194)	*B. thuringiensis* A 084	1.9×10^{8}	1.3×10^{3}	7.0×10^{-8}
3.	*B. thuringiensis* A 084-16 (pBC 16)	*B. thuringiensis* A 084-194	8.6×10^{7}	7.0×10^{3}	8.1×10^{-5}

[a]The number of each cell type per milliliter of mating mixtures was determined by plating on media containing appropriate antibiotics.

[b]The numbers of transcipients were determined on media containing streptomycin and tetracycline for mating pair No.1, streptomycin and chloramphenicol for mating pair No.2, and streptomycin, tetracycline, and chloramphenicol for mating pair No.3.

[c]Rate of transfer is expressed as the number of transcipients per one potential recipient cell.

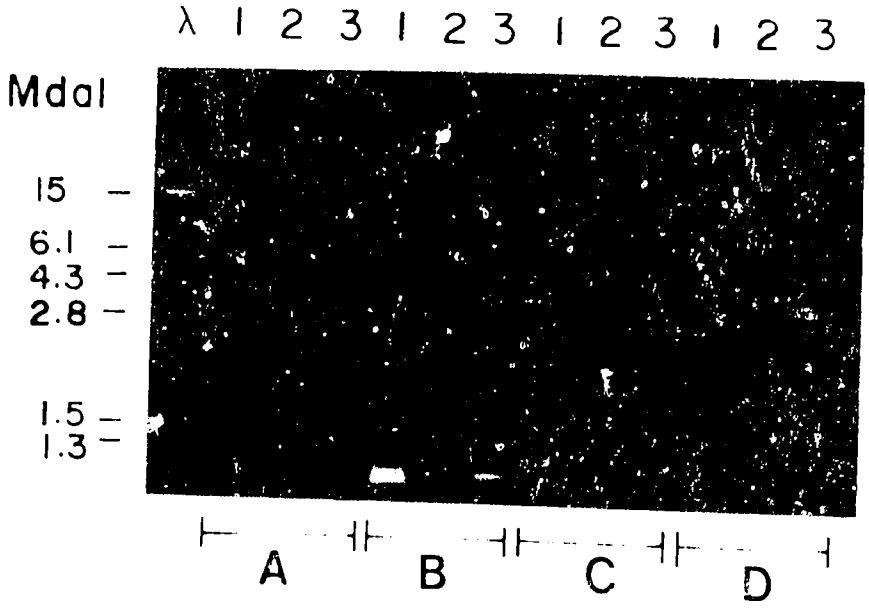

Fig.6. Agarose electrophoresis gels of restriction enzyme digested plasmid DNA. (A) Undigested plasmid DNA; (B) EcoRI digested plasmids; (C) BamHI digested plasmids; (D) SmaI digested plasmids. Lanes numbered 1, *B. cereus* harboring pBC 16; lanes numbered 2, *B.t.i.* wild type; lanes numbered 3, transcipient *B.t.i.* A 084-16; lane marked , HindIII digested DNA.

transferred and maintained in strain A 084-16. Furthermore, strain A 084-16 still maintained the other small plasmids commonly found in wildtype *B.t.i.*. It is of interest to note that the pattern of plasmid of the wild-type strain shown here differed considerably from that reported by Clark et al.[22] which indicated the presence of nine plasmids. The discrepancy may lie in the different methods of extraction or in the alteration of the bacterial strains.

Some Physiological Properties of B.t.i. strain A 084-16

Cultures of the donor (*B. cereus* GP 7), recipient (strain A 084) and transcipient (strain A 084-16) were compared using phase contrast microscopy. After 48 hr of growth, no parasporal bodies were detected in sporulating cells from *B. cereus* GP 7 harboring pBC16, whereas they were present in all cells of strain A 084-16 and had a size and shape similar to those found in strain A 084.

To further characterize transcipient strain A 084-16, it was grown in NBS medium for 48 h and tested toxicity to second instar *Ae. aegypti* larvae according to the method described under Materials and Methods. As shown in Table 4, the sporulating culture of the transcipient strain showed high toxicity to *Ae. aegypti* with an LC_{50} of 3.2×10^3 cells/ml. This LC_{50} is similar to that of strain A084, and the wild type. Larvae exposed to a culture of *B. cereus* GP 7 at a concentration of 10^7 cells/ml were unaffected (Table 4).

Transformation of B. thuringiensis O 016 Protoplasts with Various Plasmids

When PEG induced protoplast transformation was performed in *B. thuringiensis* strain O 016, it was found that plasmid pC 194 and pHV33 could be transformed into it with frequencies of 2.6×10^{-2} and 1.3×10^{-3}, respectively (Table 3).

To ascertain the successful introduction of both plasmids, five transformants with the chloramphenicol resistant (Cm^R) phenotype were randomly selected from two separate transformation experiments, purified and analysed for plasmid patterns using 0.7% agarose gel electrophoresis. All clones showed the presence of plasmids which comigrated with plasmids pC 194 or pHV33 from the original *B. subtilis* host. The Cm^R phenotype of all the transformants was stable after many subcultures in media without the drug. These results showed that pC 194 and pHV33 could be successfully introduced and maintain in *B. thuringiensis* O 016. One transformant harboring the pC 194 plasmid, *B. thuringiensis* O 016-194, was used in further experiments.

Transfer of the Chloramphenicol resistance plasmid from B. thuringiensis strain O 016-194 into B.t.i. strain A 084

B. thuringiensis strain O 016-194 described in the last paragraph was used as the donor in mating experiments with a streptomycin resistant strain of *B.t.i.* strain A 084. After mating on Millipore filters (see Materials and Methods), tentative transcipients were selected on L-medium containing 10 μg/ml chloramphenicol and 30 μg/ml streptomycin. Under

TABLE 3

Frequency of Protoplast Transformation in *B. thuringiensis* Strain O 016[a]

Treatment	Number of potential recipients[b]	Number of transformants	Frequency of transformation[c]
Without plasmid addition	9.5×10^5	0	0
Added pC 194	4.3×10^5	1.1×10^4	2.6×10^{-2}
Added pHV 33	8.1×10^5	1.1×10^3	1.3×10^{-3}

[a] The procedure used for this strain was the same as that for protoplast transformation in *B. thuringiensis* subsp. *israelensis* except that the washing step and the addition of 1 mM DTT were omitted. The concentration of lysozyme was 2 mg/ml and the amount of DNA added was 1 ug/ml for both pC 194 and pHV 33.

[b] DM-3 medium was modified as described under Materials and Methods section.

[c] Frequency of transformation is expressed as the number of transformants per one potential recipient cell.

these conditions, the donor and the recipient strains could not grow. Data in Table 2 shows that the rate of transfer was very low; only 13 transcipients per 1.9×10^8 cells.

To confirm the presence of plasmid pC 194 in the transcipients, plasmid DNA from randomly picked clones was extracted and analyzed by agarose gel electrophoresis. Plasmid patterns from two of the tentative transcipients showed clearly that pC 194 had been successfully transferred to *B.t.i.* A 084.

Additionally, the putative pC 194 plasmid in strain A 084-194 was further characterized by digestion with the restriction enzyme HindIII. The result is shown in Fig. 7. The two Mdal plasmid in strain A 084-194 showed a single site for HindIII (lane 1, Fig.7), as reported for plasmid pC 194. Clearly, plasmid pC 194 from *B. thuringiensis* O 016-194 was transferred and maintained in *B.t.i.* A 084-194.

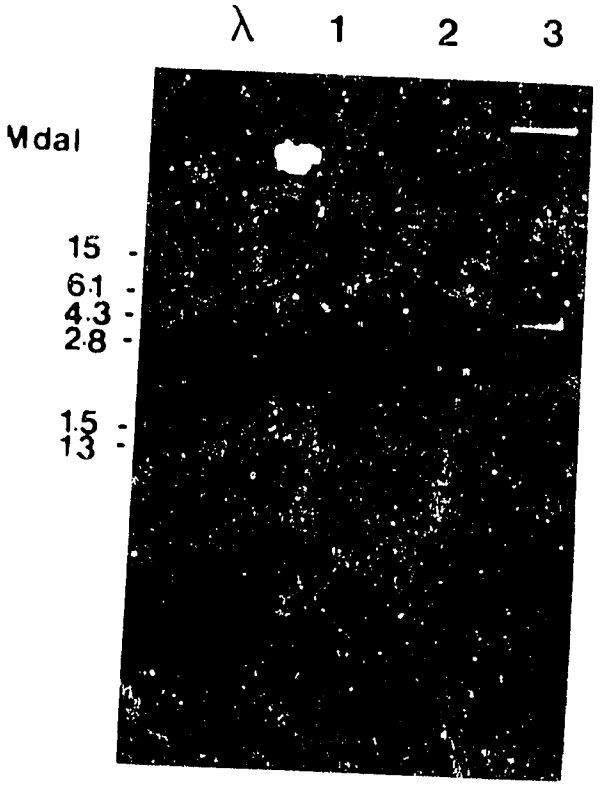

Fig 7. Agarose electrophoresis gel of HindIII digests of plasmid DNA from various strains of *B. thuringiensis*. Lane 1, *B. thuringiensis* O 016-194; lane 2, *B. thuringiensis* subsp. *israelensis* wild type; lane 3, *B. thuringiensis* subsp. *israelensis* strain A 084-194; lane marked HindIII digested DNA.

Physiological Properties of the B. thuringiensis subsp. israelensis A 084-194

Toxicity of the transcipient A 084-194 to *Ae. aegypti* larvae was found to be similar to that of wild-type *B.t.i.*. As shown in Table 4, two samples of sporulating cultures of transcipient strains A 084 194a and A 084-194b showed high toxicity to second instar of *Ae. aegypti* larvae with LC_{50} 1 x 10^3 cells/ml. This LC_{50} was identical to that of strain A 084. *Ae. aegypti* larvae exposed to a 48 h culture *B.*

TABLE 4

Toxicity of the Various Strains of *B. thuringiensis* subsp. *israelensis* and *B. cereus* GP 7 Toward *Ae. aegypti*

Bacterial strains	LC_{50} (cells/ml)
B. cereus GP 7 (Tc^R)	$> 10^7$
B. thuringiensis O 016-194 (Cm^R)	$> 10^7$
B. thuringiensis subsp. *israelensis* (wild type)	1.0×10^4
B. thuringiensis subsp. *israelensis* A 084 (Str^R)	1.0×10^3
B. thuringiensis subsp. *israelensis* A 084-16 (Str^R Tc^R)	3.2×10^3
B. thuringiensis subsp. *israelensis* A 084-194a (Str^R Cm^R)	1.0×10^3
B. thuringiensis subsp. *israelensis* A 084-194b (Str^R Cm^R)	1.0×10^3

thuringiensis O 016-194 (harboring pC 194) at a concentration of 10^7 cells/ml did not show any toxicity effects (Table 4).

Transfer of Plasmids between Strains of B.t.i.

In this experiment, intraspecific transfer of plasmids pC 194 and pBC 16 in *B.t.i.* was examined by mating strain A 084-194 (Str^R Cm^R harboring pC 194), and strain 084-16 (Str^R Tc^R harboring pC 16) by the filter procedure (see Materials and Methods). Transcipients were selected on L-agar containing both Tc and Cm. As shown in Table 2, 7.0 x 10^3 transcipients which possessed the $Str^RTc^RCm^R$ phenotype were obtained (frequency 8.1 x 10^{-5}). To confirm the transfer of plasmids, one of the putative transcipients was purified and cultured. Plasmids were extracted and analyzed by agarose gel electrophoresis. The result was shown in Fig.8 where lane 4 indicates the presence of both pC 194 and pBC 16 in the transcipient strain A 084-194-16 which possessed the $Str^RTc^RCm^R$ phenotype. The four small plasmid bands commonly found in *B.t.i.* were also still present in this transcipient strain. This evidence verified the transfer of both pBC 16 and pC 194 between strains of *B.t.i.*. the transcipients retained the ability to form parasporal bodies and maintained a high level of toxicity toward *Ae. aegypti* larvae.

Fig.8. Agarose electrophoresis gel of plasmid DNA from various strains of *B. sphaericus* subsp. *israelensis*. Lane 1, *B. thuringiensis* subsp. *israelensis* wild type; lane 2, *B. thuringiensis* subsp. *israelensis* strain A 084-16; lane 3, *B. thuringiensis* strain A 084-194; lane 4, *B. thuringiensis* subsp. *israelensis* strain A 084-194-16.

Transfer of plasmids from B. thuringiensis HD-1

Fourteen *E. coli* C-600 ELISA positive clones were obtained from the ligated product of BamHI cut mosquito-toxin gene joined with BamHI digested pBR322 plasmid vector, 10787 *E. coli* C-600 ELISA positive clones were obtained from the

ligated product of HindIII cut mosquito-toxin gene joined with HindIII digested plasmid vector pUN121, and 205 *E. coli* JM 107 ELISA positive clones were obtained from the ligated product of XbaI cut mosquito-toxin gene joined with XbaI digested plasmid vector pUC 12. Only those ELISA positive clones were used in larvicidal activity screening test against *Ae. aegypti* larvae. One out of 205 *E. coli* clones obtained from the transformation by recombinant plasmid demonstrated a good larvididal 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.

Introduction of the delta-endotoxin gene into B. subtilis

In an attempt to introduce the delta-endotoxin gene into *Bacillus* spp., the 130 kDa delta-endotoxin gene was used to construct the recombinant plasmid and introduced into *B. subtilis*, and *E. coli*. Both clones exhibited toxicity towards *Ae. aegypti* larvae. The protein crystal was observed only in *E. coli* cells under the phase-contrast microscope. The bifunctional plasmid was found to be stably maintained in *B. subtilis* as well as in *E. coli*.

It is now believed that the successful in transferring of mosquito-toxin gene from *B. thuringiensis* H-14 into other organisms can be done and is relying on the selection of an appropriate plasmid vector that can be expressed in the recipient cell. The development of methods and gene transfer systems will lead to successful transfer of an individual

gene coding for the mosquito-toxin into a host microorganisms 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 of mutants. The most difficult part of it is the matter of selection of an appropriate plasmid vector for the construction of recombinant 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.

The discovery by Gonzalez and Carlton[23] of plasmid transfers in the insect pathogen, *B. thuringiensis*, opened up new prospects for genetic investigations on parasporal body synthesis and for creation of more toxic strains with a wider host range among harmful insects. Such genetic engineering work requires knowledge about the genetic determinants carried by the cryptic plasmids in *B. thuringiensis* and effective transformation systems. This study focused mainly on development of genetic transformation systems in *B.t.i.*. Recently three laboratories, using the protoplast transformation technique, have published reports on transformation in two derivatives of *B. thuringiensis*, *B. thuringiensis* strain HD-1 and *B. thuringiensis* subsp. *galleriae*. The plasmids used were pC 194[24]pBC 16[25] and pUB

110[26]. Unfortunately, the transformation frequencies were extremely low when compared with those achieved using a similar technique with *B. subtilis*. Furthermore, plasmid pC 194 could not be detected in the transformants and the authors suggested that it had been integrated into the chromosome. One of the reasons for inefficient transformation with *B. thuringiensis* protoplasts was probably the high resistance of the cell wall of *B. thuringiensis* to lysozyme[27].

In this study, attempts to establish a transformation system in *B.t.i.* by using PEG induced protoplast transformation were also not successful. Attempts to use the "competent cell" transformation techniques also failed.

In 1984, Fisher et al.[28] reported the transfer of plasmid pC 194 into various subspecies of *B. thuringiensis*. However, in that report, pC 194 could not be transferred into *B.t.i.*, the strain of most interest in our study. Of the two mating techniques used in our study the filter technique was superior in obtaining higher and more consistent numbers of *B.t.i.* as the donor, a closely related bacterium, *B. cereus* which harbored pBC 16 (Tc^R), was chosen. Under our conditions, pBC 16 from this source could be transferred to *B.t.i.* A 084, a Str^R strain, with relatively high frequencies and it was stably maintained. It was proved to be pBC 16 by electrophoretic mobility and restriction pattern analysis in comparison with pBC 16 from *B. cereus*. Additionally, the Tc^R transcipients contained typical parasporal bodies and showed toxicity to *Ae. aegypti* equal to

that of *B.t.i.*, the wild-type strain. The rate of plasmid transfer between *B. cereus* and *B.t.i.* was comparable to that obtained in mating between *S. faecalis* and *B.t.i.* by Lereclus et al.[28].

After successful transfer of plasmid pBC 16, we further attempted to transfer plasmids such as pC 194 (Cm^R) in *B. subtilis* to *B.t.i.* Using the same mating technique, no transcipient cells were observed. A similar result was reported by H.M. Fisher et al.[28] and was not entirely unexpected since the two organisms are not closely related. However, the plasmids pC 194 as well as pHV33 could be introduced into a strain of *B.t.i.* using conjugation-like process if the plasmids were first introduced into another strain of *B. thuringiensis*. The method for introduction of plasmid pC 194 into *B.t.i.* began first with the introduction of plasmid pC 194 into a strain of *B. thuringiensis*, i.e., strain O 016 by means of protoplast transformation. The transformant obtained, *B. thuringiensis* O 016-194 was mated with *B.t.i.* A 084 to obtain Str^RCm^R transcipients.

The *B. thuringiensis* O 016 which was employed here as an intermediate strain for introducing plasmid pC 194 into *B.t.i.* was of special interest. This strain gave somewhat distinct colony morphology from that of other *B. thuringiensis* and was sensitive to lysozyme. After treatment with 2 mg/ml lysozyme for 30 min, 100% protoplasts could be obtained and a high regeneration frequency of 2.13% was achieved after regeneration in modified DM-3 medium. The transformation frequency for *B. thuringiensis* O 016

protoplasts by pC 194 and pHV33 was relatively high (2.6×10^{-2} and 1.3×10^{-3}) when compared with similar experiments that have been reported by other investigators[24,28]. Morphological and biochemical examination confirmed that this strain was *B. thuringiensis*.

Into a specific plasmid transfers between strains of *B.t.i.* were attempted to determine whether a transcipient harboring both pBC 16 and pC 194 could be obtained from the mating pair and whether the two plasmids of different origin could be maintained in *B.t.i.* The results of mating between strain A 084-16 (Tc^R) and strain A 084-194 (Cm^R) revealed that a high frequency of plasmid transfer could be achieved and that the transcipients were stable. The existence of other plasmids in the transcipients did not interfere with the maintenance of the two newly aequired plasmids.

The stable maintenance of plasmid pC 194, as judged by the presence of plasmid as well as drug resistant marker, in *B.t.i.* suggested that the limiting factor in earlier protoplast transformation experiments was the difficulty in introducing plasmid DNA into the cell. If a lysozyme sensitive mutant of *B.t.i.* could be obtained, successful protoplast transformation might be possible with high frequency. This could be of some advantages since the protoplast transformation first reported by Chang and Cohen[21] was a very efficient means of plasmid DNA transformation without a requirement for oligomeric molecules. Furthermore, plasmid DNA was preferred to chromosomal DNA, and in contrast to competent cell

transformation, restriction and modification of the transforming DNA did not occur. Such a protoplast transformation system for *B.t.i.* would constitute a very significant achievement in its genetic manipulation program.

The conjugation-like process used successfully in this study was first discovered by Gonzalez et al.[29]. By using this technique, Klier et al.[30] transferred the plasmid carried crystal gene from strain berliner 1715 into an acrystalliferous *B. thuringiensis* strain where it promoted the synthesis of crystal protein. Also this method, the crystal gene of strain berliner was introduced into a wild-type strain of *B.t.i.* The resulting transcipient strain produced two types of delta-endotoxin which were active on both lepidopteran and dipteran larvae. In addition, Gonzales and Carlton[31] used a conjugation-like process to show that a large 75 Mdal plasmid was required for crystal toxin production in *B.t.i.*

One of the major factors that has hindered successful development of a gene transfer system in *B. thuringiensis* has been the absence of selective markers. This study shows that two plasmids specified Tc^R and Cm^R can be introduced and stably maintained in *B.t.i.* This opens the possibility of constructing an ideal two-marker plasmid vector, a high frequency protoplast transformation system in *B. thuringiensis* O 016 and the conjugation-like process between *B. thuringiensis* O 016 and *B.t.i.*, new possibilities are opened for genetic manipulation in *B. thuringiensis*.

Five recombinant *E. coli* clones exhibiting mosquito

larvicidal activity contained a 3.8 kb DNA fragment of 110 kb *B.t.i.* plasmid. Three clones (pMU258/303/388) had the 3.8 kb insert with the same transcriptional direction to the *lac* Z promoter of pUC12 vector, whereas two clones (pMU14/679) had the 3.8 kb insert in an opposite transcriptional direction. All five clones produced a major delta-endotoxin of 130 kDa, which was highly toxic to *Ae. aegypti* larvae. This study clearly demonstrated that among the three major *B.t.i.* delta-endotoxin proteins, the 130 kDa protein alone could exhibit mosquito larvicidal activity. The 25-28 kDa protein had been reported to be the protein responsible for mosquito larvicidal activity[32,33,34]. However, antibody raised against the 25-28 kDa protein did not cross-react with the 130 kDa protein produced by the *E. coli*; nor did the antibody raise against the 130-kDa protein produced by the pMU388 clone cross-react with the 25-28 kDa protein. It would thus appear that the 130 kDa protein is not a protoxin form of the 25-to 28-kDa and that the toxicity exhibited by *B.t.i.* delta-endotoxin crystal is derived from both 130-kDa and 25-28 kDa proteins.

The observation that the 3.8 kb insert produced 130 kDa protein regardless of the vectors (pBR322, pUC12) or its orientation in the pUC12 vector suggested that the insert contained both the regulatory region and the structural gene. As the coding region for 130 kDa protein alone requires at least the 3.8 kb DNA fragment, this would indicate that the 3.8 kb insert was a minimal sequence necessary for expression of the 130 kDa delta-endotoxin gene in *E. coli*.

DNA sequence of the 5'-terminal of the coding strand revealed that there were about 150 5'-non-translating nucleotides in the 3.8-kb insert, sufficient to contain the necessary regulation sequences which normally require some 80 base pairs. Comparing the sequence obtained with those of genes encoding lepidoptera toxin from *B. thuringiensis kurstaki*[10,22], *B. thuringiensis berliner* 1715[35], *B. thuringiensis sotto*[36] and the 28-kDa *B.t.i.* gene[37], we did not find any significant homology. However, some homologies in both regulatory and coding sequences (Fig. 5, underlined sequence) were observed with the 72-kDa *B.t.i.* gene[22]. The Shine-Dalgarno region, TATGGGAGGAATA, was identical. The coding regions from bases 163-177 and 196-252 coded for 20 identical amino acids, suggesting that this protein region was highly conserved. There were no significant homologies in other regions, suggesting that the 72 kDa gene was not part of the 130 kDa gene reported here.

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