PD-ABD-704

UNITED STATES OF AMERICA
AGENCY FOR INTERNATIONAL DEVELOPMENT
U.S.A.I.D. MISSION TO KENYA

UNITED STATES POSTAL ADDRESS
US AID MISSION TO KENYA
BOX 201
APO NEW YORK, NEW YORK 09675

INTERNATIONAL POSTAL ADDRESS
POST OFFICE BOX 30261
NAIROBI, KENYA

Dr. Ellie O. Osir
Senior Research Scientist
The International Centre of Insect Physiology and Ecology
P.O. Box 30772, Nairobi, Kenya

Subject: Grant No. 615-0261-G-SS-00-1068

Dear Dr. Osir,

Pursuant to the Authority contained in the Foreign Assistance Act of 1961, as amended, the Federal Grant and Cooperative Agreement Act of 1977, the Agency for International Development (hereinafter referred to as "A.I.D." or "Grantor" hereby grants to The International Centre of Insect Physiology and Ecology (hereinafter referred to as "Grantee" the sum of $149,020 to provide support for a research program entitled "The Molecular Basis of Selective Toxicity in B. thuringiensis" as described in the Schedule of this Grant, Attachment 2 entitled "Program Description", and the Grantee's proposal, as amended, which is made a part of the Grant and incorporated herein by reference.

This Grant is effective and obligation is made as of the date of this letter and shall apply to commitments made by the Grantee in furtherance of program objectives during the period beginning August 27, 1991 and ending August 26, 1995.

This Grant is made to the Grantee on condition that the funds will be administered in accordance with the terms and conditions as set forth in Attachment 1 entitled, the "Schedule", Attachment 2 entitled "Program Description", and Attachment 3 entitled "Standard Provisions", which have been agreed to by your organization.

Please sign all copies of this letter to acknowledge your receipt and acceptance of this Grant. Keep one copy for your files and return the original and all remaining copies to this office

Sincerely,

Stafford Baker for

John R. Westley
Grant Officer
USAID/Nairobi
Nairobi, Kenya

Attachments:

1. Schedule
2. Program Description
3. ~~Standard Provisions~~
4. Original Proposal with all amendments

ACKNOWLEDGED:

Grantee: The International Centre of Insect Physiology and Ecology

BY: EW Osir

TYPED NAME: Dr. Ellie O. Osir

TITLE: Senior Research Scientist

Date: _____

FISCAL DATA

PIO/T No.	:	N/A
Appropriation No.	:	72-1111021.6
Budget Plan Code	:	DDSA-91-29615-KG11
Allotment No.	:	146-51-615-00-19-11
Project No.	:	936-5600
Total Estimated Amount	:	$149,020
Total Estimated Amount	:	$149,020
Funding Source	:	AID/W
Project Office	:	AID/SCI
Voucher Paying Office	:	USAID/Nairobi Controller
Proposal No.	:	11.409

Funds Available
CONT/KENYA
Initials
Date 9/30/91

K210175

Grant No.615-0261-G-SS-00-1068

SCHEDULE

A. Purpose of Grant No. 11.409

The purpose of this Grant is to provide support for The International Centre of Insect Physiology and Ecology proposal entitled "The Molecular Basis of Selective Toxicity in B. thuringiensis" (11.409) which is hereby incorporated by reference.

B. Period of Grant

1. This is a four-year Grant and the effective date is August 27, 1991. The expiration date of this Grant is August 26, 1995.

2. No Cost Extensions

Subject to the ceiling amount of this Grant (C.1. below) and with the prior written approval of the Project Officer (F.1. below) the grantee is authorized to extend the completion date stated above, provided that such extension:

a. Does not cause the elapsed time for the completion of the work, including the furnishing of all deliverables except for the Final Performance Report (E.1.b. below) to extend beyond 180 calendar days from the original specified completion date, or beyond the overall Project Activity Completion Date (PACD) of December 31, 1999; or

b. Does not cause the elapsed time for completion of the work (all deliverable except the Final Performance Report - as in Paragraph B., above) to extend beyond 365 calendar days from the original specified completion date, or beyond the overall PACD (December 31, 1999), provided the cause of the delay in completion of the research work is due to research requirements or circumstances beyond the control of the Principal Investigator (PI); e.g., weather, civil uprising, change in the PI or co-PI, etc.

In either of the above cases, the Grantee shall attach a copy of the Project Officer's approval of any extension of the term of this grant to the final voucher submitted for payment.

C. Amount of Grant and Payment

1. AID hereby obligates the amount of $149,020 for the purposes of this Grant.

2. Payment shall be made to the Grantee in accordance with procedures set forth in Attachment 3 - Required as Applicable Standard Provision entitled "Payment - Cost Reimbursement".

3. All financial reports required by this provision shall be submitted to:

a. USAID/Kenya
Office of the Controller
Nairobi, Kenya

b. Voucher Identification
Grant No.615-0261-G-SS-00-1068
Project No. 936-5600
SCI Grant No. 11.409
Project Office: USAID/Nairobi
Budget Plan Code: DDSA-91-29615-KG11
Allotment Symbol: 146-51-615-00-19-11

D. Grant Budget

The following is the Budget for this Grant, including local cost items, if authorized. Revisions to this Budget shall be made in accordance with Mandatory Standard Provision of this Grant entitled "Revision of Grant Budget". Within the total estimated cost of the Grant, the Grantee may adjust the line items as reasonably as necessary for the performance of the grant program.

"Reasonably as necessary" has been interpreted as up to 15 percent. Any amount greater than 15 percent would require the approval of the Project Officer.

Line Item	Estimated Budget Amount
Personnel	$ 25,220
Equipment	49,200
Materials	26,000
Literature Searches	2,700
Professional Services/Consult.	2,400
International Travel	13,500
Outside Collaboration	30,000
Total	$149,020

Line item budget by fiscal year is attached as Attachment 2.A.

E. Reporting and Evaluation

1. Technical Reports

Reports must be sufficiently detailed to substantiate the findings and to permit a scientific evaluation of the research. Overseas collaborators shall be given fair credit for their participation in the research and a chance to review and comment on the Final Report before it is submitted. The principal investigator will share a draft of the Final Report with the A.I.D. Project Officer for comments prior to the formal submission. Publication of results in scientific journals is encouraged. Additional guidance on report preparation is given in the "Interim Guidelines on Performance Report Preparation for PSTC and CDR Projects," available from AID/SCI.

a. Performance Reports: Performance reports are required every six months. The principal investigator will submit reports stating what has been accomplished to date and detailing project management issues. A Financial Status Report will be attached to each report. Reports are due within sixty (60) days after the end of each six-month period. One copy of each report are to be submitted to the Office of the Science Advisor, Room 320 SA-18, Washington, D.C. 20523-1818; one copy to the USAID/Nairobi Project Officer; one copy to the A.I.D. Mission in Nairobi; one copy to the Technical Backstop Officer, Dr. Joel Cohen, S&T/AGR/AP, Room 412 - SA-18, Agency for International Development Washington, D.C. 20523-1809; and two copies to the National Academy of Sciences, BOSTID, 2101 Constitution Avenue, N.W., Washington, D.C. 20418.

b. Final Performance Report: Within sixty (60) days after the termination of the Grant, the principal investigator will submit one copy of the Final Performance Report to the Office of the Science Advisor, A.I.D., Room 320 SA-18, Washington, D.C., 20523-1818; one copy to the USAID/Nairobi Project Officer; one copy to the Technical Backstop Officer, Dr. Joel Cohen, S&T/AGR/AP, Room 412 - SA-18, Agency for International Development Washington, D.C. 20523-1809; one copy to the USAID/Nariobi; one copy to Bureau for Program and Policy Coordination, Center for Development Information and Evaluation (PPC/CDIE), Room 215C, SA 18, Washington, D.C., 20523-1802;and two copies to the National Academy of Sciences, BOSTID, 2101 Constitution Avenue, N.W., Washington, D.C. 20418.

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2. The Financial Status Report (SF-269) is required every six months as an attachment to the performance report and must be sent to the following Voucher Paying Office:

USAID/Nairobi
Office of the Controller
Agency for International Development
Nairobi, Kenya

F. Project Officer and Investigators

1. Project Officer:

Mr. James Dunn
USAID/Nairobi
Agency for International Development
Nairobi, Kenya

2. Project Backstop Officer

Dr. Joel Cohen
S&T/AGR/AP, Room 412 - SA-18
Agency for International Development
Washington, D.C. 20523-1809

3. Principal Investigator:

Dr. Maurice Odindo
The International Centre of Insect Physiology and Ecology
P.O. Box 30772
Nairobi, Kenya

4. Assistant
Mrs. Matilda Okech
The International Centre of Insect Physiology and Ecology
P.O. Box 30772
Nairobi, Kenya

5. Co-Investigator:

Dr. Douglas Bradley
Department of Botany, KB-15
University of Washington
Seattle, WA 98195

G. Special Provisions

1. Compliance with Federal Guidelines and Regulatory Procedure:

a. The recipient will implement this research activity in accordance with all relevant guidelines for U.S. Government funded research, such as:

(1) Guidelines for the handling of radioactive materials;

- (2) NIH and USDA guidelines for the handling of pathogenic microorganisms;

- (3) USDA-APHIS procedures for animal and plant health inspection;

- (4) The National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules;

- (5) Procedures issued by the USDA, EPA, or other appropriate Federal agency, regarding testing of genetically engineered organisms;

- (6) A.I.D.'s environmental procedures; and

- (7) Such other Federal guidelines and procedures as may apply during the course of research.

b. All existing comparable guidelines of the host country in which the research is actually located must be followed also.

c. Reports submitted under this activity to A.I.D. will address the cited regulatory issues. All modifications of protocols affecting these regulatory concerns must be reported. The investigators are responsible for reporting any difficulties encountered in implementing these protocols.

2. Laboratory Safety and Hazard Containment: Research will be conducted following the protocols described in attachment 4 (the original proposal or subsequent amendments, or letter from the Principal Investigator, which insure the safety of persons involved in the research). Notwithstanding, the research must be conducted following procedures issued by the U.S. Government and those issued by the government of the host country for the containment of these hazards.

If the protocols involving laboratory safety and hazard containment are revised, they must be re-reviewed by the investigator's institutional review committee(s) that approved the original protocol, and the Project Officer and Office of the Science Advisor must be informed in writing before the revised protocols are used. The revised procedures must be consonant with the guidelines of the country in which the laboratory is located, and of the United States. Copies of the approval of the revised protocols by the investigator's institutional review committee(s) should also be provided the Project Officer and the Office of the Science Advisor.

Similarly, the research will be conducted in the facilities described in attachment 4. If the research is moved to new facilities, or the facilities are modified in such a way to affect safety or hazard containment, a description of the new facilities must be provided to the Project Officer and to the Office of the Science Advisor before the research is affected. Any applicable institutional reviews of the facilities must be repeated, and the re-certification must be provided to the Project Officer and the Office of the Science Advisor.

3. Containment and Safe Disposal of Animal or Plant Pathogens or Pests: Research will be conducted following the protocols described in attachment 4, which insure the containment and safe disposal of animal or plant pathogens. Notwithstanding, the research must be conducted following procedures issued by the U.S. Government and those issued by the government of the host country for the containment of these pathogens or pests.

If any protocol is revised, it must be re-reviewed by the investigator's institutional review committee(s) that approved the original protocol, and the Project Officer and Office of the Science Advisor must be informed in writing before the revised protocols are used. The revised procedures must be consonant with the guidelines of the country in which the laboratory is located, and of the United States. Copies of the approval of the revised protocols by the investigator's institutional review committees should also be provided the Project Officer and the Office of the Science Advisor.

Similarly, the research will be conducted in the facilities described in Attachment 4. If the research is moved to new facilities, or the facilities are modified in such a way to affect safety or hazard containment, a description of the new facilities must be provided to the Project Officer and to the Office of the Science Advisor before the research is affected. Any applicable institutional reviews of the facilities should be repeated, and the re-certification should be provided to the Project Officer and the Office of the Science Advisor.

4. Recombinant DNA:

a. Research will be conducted following the protocols described in attachment 4 which ensure the containment of recombinant organisms. If any such protocol is revised, it must be re-reviewed by the investigator's institutional review committee(s) that approved the original protocol, and the Project Officer and Office of the Science Advisor must be informed in writing before the revised protocol(s) is used. Copies of the approval of the revised protocols by the investigator's institutional review committees should also be provided the Project Officer and the Office of the Science Advisor.

Similarly, the research will be conducted in the facilities described in attachment 4. If the research is moved to new facilities, or the facilities are modified in such a way to affect safety or containment, a description of the new facilities must be provided to the Project Officer and to the Office of the Science Advisor before the research is affected. Any applicable institutional reviews of the facilities should be repeated, and the re-certification should be provided to the Project Officer and the Office of the Science Advisor.

b. Notwithstanding the above:

(1) The cooperator cannot commence testing in any foreign location until written approval for such testing is obtained from A.I.D. and the Government of the country where testing is planned. Testing shall be conducted in accordance with all applicable regulations of that country.

(2) In addition, however, and prior to commencement of any such testing, the grantee shall make a judgment and communicate the same to A.I.D. as to whether the regulations, procedures or facilities of the country in question are adequate to ensure testing in an environmentally sound manner. In the event such judgment is that they are not, the grantee and A.I.D. will consult and agree on the conditions to be applied to the testing which will have such environmental effect.

(3) Reports submitted under this activity to A.I.D. will address regulatory issues as above related to the activity.

5. Humane Treatment of Experimental Animals:

a. Principals for the Treatment of Vertebrates: The Grantee will adhere to the following principals for the utilization, care and transportation of vertebrate animals used in testing, research and training. For guidance throughout these Principles, reference is made to the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council.

(1) Procedures involving animals should be designed and performed with due consideration of their relevance to human or animal health, the advancement of knowledge, or the good of society.

(2) The animals selected for a procedure should be an appropriate species and quality and the minimum number required to obtain valid results. Methods such as mathematical models, computer simulation and in vitro biological systems should be considered.

(3) Proper use of animals, including the avoidance or minimization of discomfort, d[illegible]ress, and pain when consistent with sou[illegible]tific practices, is imperative. Unless the [illegible]ary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain and distress in other animals.

(4) Procedures with animals that may cause more than momentary or slight pain or distress should be performed with appropriate sedation, analgesia, anesthesia. Surgical or other painful procedures should not be performed on unanesthetized animals paralyzed by chemical agents.

(5) Animals that would otherwise suffer severe or chronic pain or distress that cannot be relieved should be painlessly killed at the end of the procedure, or, if appropriate, during the procedure.

(6) The living conditions of animals should be appropriate for their species and contribute to their health and comfort. Normally the housing, feeding, and care of all animals used for biomedical purposes must be directed by a veterinarian or other scientist trained and experienced in the proper care, handling, and use of the species being maintained or studies. In any case, veterinary care shall be provided as indicated.

(7) Investigators and other personnel shall be appropriately qualified and experienced for conducting procedures on living animals. Adequate arrangements shall be made for their in-service training, including the proper and humane care and use of laboratory animals.

(8) Where exceptions are required in relation to the provisions of these principles, the decisions should not rest with the investigators directly concerned, but should be made, with due regard to U.S. and host country regulations, by an appropriate review group such as an institutional animal research committee. Such exception should not be made solely for the purpose of teaching or demonstration.

b. Applicable Regulations: The transportation, care and use of animals should be in accordance with the U.S. Animal Welfare Act (7 U.S.C. 2131 et. seq.) and other applicable U.S. Federal laws, guidelines, and policies. Notwithstanding, the research must be conducted following procedures issued by the government of the host country for the humane treatment of experimental animals.

c. Compliance with Reviewed Protocols: Research will be conducted following the protocols described in Attachment 2 (which is the original proposal or subsequent amendments, or letter from the Principal Investigator), which insure the humane treatment of experimental animals.

d. Revision of Protocols: If any protocol involving the experimental animals is revised, it must be re-reviewed by the investigator's institutional review committee(s) that approved the original protocol, and the Project Officer and the Office of the Science Advisor must be informed in writing before the revised protocol(s) is used. The revised procedures must be consonant with the guidelines of the country in which the animals are affected, and of the United States. Copies of the approval of the revised protocols by the investigator's institutional review committees should also be provided the Project Officer and the Office of the Science Advisor.

6. Environmental Hazards: Research will be conducted following the protocols described in attachment 4 which insure that there are no unacceptable environmental hazards incident to the research. Notwithstanding, the research must be conducted following A.I.D. environmental guidelines and those issued by the government of the host country.

If any protocol involving environmental hazards is revised, it must be re-reviewed by the investigator's institutional review committee(s) that approved the original protocol, and the Project Officer and Office of the Science Advisor must be informed in writing before the revised protocols are used. The revised procedures must be consonant with the guidelines of the country in which the research is located, and of the United States. Copies of the approval of the revised protocols by the investigator's institutional review committee(s) must also be provided the Project Officer and the Office of the Science Advisor.

In addition, however, and prior to commencement of any such activity which may create an environmental hazard, the grantee shall make a judgment and communicate the same to A.I.D. as to whether the regulations, procedures or facilities of the country in question are adequate to ensure the protection of the environment. In the event such judgment is that they are not, the grantee and A.I.D. will consult and agree on the conditions to be applied to the activity which will ensure environmental protection.

7. Intellectual Property Rights: Intellectual property rights stemming from the activities supported under this grant will be apportioned as described in attachment 4. If the participants chose to modify that apportionment, the revised apportionment must be submitted to the A.I.D. Project Officer for approval before it takes effect. Notwithstanding the intellectual property rights clauses provided in the following standard provisions apply.

H. STANDARD PROVISIONS

1. While in the field, the Grantee will keep the A.I.D. field mission generally appraised of the research, but will not request administrative support except for the usual in-country introductions as may be appropriate. The Grantee will abide by Mission and host government regulations and customs as they apply to AID-supported in-country activities.

2. Project Officer approval required by paragraph (a) of optional Standard Provision 3 of this grant entitled "Air Travel and Transportation," is hereby granted for travel between the United States and all other countries described in the project description.

3. The principal investigators and essential scientific staff of grantee and co-principal investigator of the collaborating institutions may not be changed during the life of the research without prior written approval of the Office of the Science Advisor.

4. The Standard Provisions applicable to this grant are entitled "Mandatory Standard Provisions for U.S., Non-governmental Grantees" and "Optional Standard Provisions for U.S., Non-governmental Grantees" and are included herein as Attachment 3.

I. Title to Property

The title to property acquired under this Grant will vest in the Grantee in accordance with Optional Standard Provision No. 18 entitled "Title to and Use of Property (Grantee Title)". When the principal Grantee makes a subagreement under this Grant Agreement, the principal Grantee will pass to the Subgrantee the provisions of the above cited reference, as appropriate. Equipment purchased on behalf of each collaborating institution shall remain the property of that institution.

J. Publications

Acknowledgment of A.I.D.'s contribution to any publication resulting from this Grant shall be made in accordance with procedures set forth in Attachment 3 - Optional Standard Provision No. 10 entitled "Publications".

K. Closeout Procedures

This section prescribes uniform closeout procedures for this Grant.

1. The following definitions shall apply for the purpose of this section:

   a. Closeout The closeout of a Grant is the process by which A.I.D. determines that all applicable administrative actions and all required work of the Grant have been completed by the Grantee and A.I.D.

   b. Date of Completion The date of completion is the date on which all work under the Grant is completed, or the date on the award document, or any supplement or amendment thereto, on which A.I.D. sponsorship ends.

c. Disallowed Costs Disallowed costs are those charged to a Grant that A.I.D. or its representative determines to be unallowable, in accordance with the applicable Federal cost principles or other conditions contained in the Grant.

2. A.I.D. closeout procedures include the following requirements:

a. Upon request, A.I.D. shall make prompt payments to a Grantee of allowable reimbursable costs under the Grant being closed out.

b. The Grantee shall immediately refund any balance of unobligated (unencumbered) cash that A.I.D. has advanced or paid and that is not authorized to be retained by the Grantee for use in other Grants.

c. A.I.D. shall obtain from the Grantee within 90 calendar days after the date of completion of the Grant all financial, performance, and other reports required as the condition of the Grant. A.I.D. may grant an extension when requested by the Grantee.

d. When authorized by the Grant, A.I.D. shall make a settlement for any upward or downward adjustments, to A.I.D.'s share of costs after these reports are received.

e. The Grantee shall account for any property acquired with A.I.D. funds, or received from the Government in accordance with the provisions of OMB Circular A-110, Attachment K.

f. In the event a final audit has not been performed prior to the closeout of the Grant, A.I.D. shall retain the right to recover an appropriate amount after fully considering the recommendations on questioned costs resulting from the final audit.

L. Source and Origin of Goods and Services

1. The country or countries where research or other scientific/technological cooperation takes place shall be deemed to be the cooperating country for the purpose of permitting local cost financing.

2. Goods and services, except for ocean shipping, financed by A.I.D. under the project shall have their source/origin in the Cooperating Country or the United States and Code 935 countries.

3. Ocean shipping financed by A.I.D. under the project shall, except as A.I.D. may otherwise agree in writing, be financed only on flag vessels of the United States.

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F. TECHNICAL WORKPLAN

1. Source of *B.t.* Strains

The *B.t.* strains that will be used in these studies were all isolated in Kenya and have a demonstrated toxicity against either *C. partellus* (Brownbridge and Onyango, 1990), *S. exempta* (Hamal *et al.*, 1990), or *G. morsitans morsitans* (Otieno and Kotengo, pers. comm.). Two strains, *B.t.*-43 and MF-4B-2, are highly toxic to *C. partellus* and *S. exempta*, respectively. The strains show very low levels of cross-toxicities. A third strain showing moderate levels of toxicity to both pest species will also be included for competition studies. The strain active against tsetse flies is totally innocuous to either of the lepidopteran species and will also be used in the studies.

2. Culture Techniques

The strains will be grown in a broth medium which will promote high levels of growth, sporulation and crystal endotoxin formation. Initially, starter cultures will be prepared in tryptone soy broth (TSB), using inocula taken from slant cultures of the respective strains to inoculate the medium. The starter flasks, 500 ml Erlenmeyer flasks containing 50 ml TSB, will be incubated (30° C, 250 r.p.m. for 12 h) in an orbital incubator. Second passage seed flasks will be similarly prepared and inoculated with 1 ml aliquots of the 12 h first passage seed. These will be incubated under the same conditions for 12 h. One ml aliquots will then be used to inoculate 70 ml of liquid culture medium carried in 500 ml Erlenmeyer flasks. The medium, pH 6.8, contains, in w/l: 5 g peptone; 5 g beef extract; 10 g glucose; 10 g glycerol; 2 g yeast extract; 0.1 g $CaCl_2$; 0.3 g $MgSO_4$ and 0.002 g each of $MnSO_4$, $FeSO_4$ and $ZnSO_4$. The flasks will be incubated (30° C, 350 r.p.m. for 72 h) to ensure full lysis of the cells. Cultures will be harvested by centrifugation (8000 x *g*, 4°C, 20 min) in a GSA rotor (Sorvall) and the supernatant portion discarded. The resulting pellet will be washed twice in ice-cold distilled water and stored at 4° C prior to the separation of the spores and crystals.

3. Crystal Toxin Preparation

The endotoxin crystals will be purified on 40-70 % (w/v) sodium bromide gradients by centrifugation (8000 x *g*, 4° C, 20 min) in an SS-34 rotor (Sorvall). Under these conditions, the crystals form a "cloud" about one-third of the way down the tube, with the spores forming a discrete band below them. The crystals will then be collected, washed twice with water and stored at 4° C before further processing.

4. Solubilization and Activation of Crystal Toxins

Lyophilized crystals will be suspended in a buffer of 50 mM Na_2CO_3/ HCl (pH 10.5) containing 10 mM dithiothreitol, and incubated (3 h, 37°C). Insoluble material will be removed by centrifugation (10,000 x *g*, 10 min, 4° C) in a Microfuge and the supernatant solution saved. The solubilized sample will be dialyzed against 0.1 M Tris-HCl buffer, pH 9.5 and incubated with trypsin (37°C, 3 h). Gel permeation (Superose 6 or 12) and ion-exchange (Mono Q) chromatography will be used to obtain pure preparations of the endotoxins. Protein estimations will be carried out by the BCA protein assay reagent (Pierce) with BSA as the protein standard.

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5. **Gel Electrophoresis, Isoelectric Focusing and Electroelution**

Electrophoresis on SDS-polyacrylamide gels will be carried out as described by Laemmli (1970). Gradients will be cast using a gradient mixer (BRL). Gels will be calibrated using low and high molecular weight standards (Bio-Rad). Staining for protein will utilize Coomassie brilliant blue R. Molecular masses will be estimated by the relation of log M_r to the relative mobilities of the standards. The isoelectric points of the endotoxins will be determined on a Pharmacia PhastSystem using commercial PhastGels according to the manufacturer's instructions.

Minor crystal subunits will be purified by elution from gels. Endotoxins will be separated by SDS-PAGE. After staining and destaining a vertical slice of the gel for protein, the gel will be cut in the parts corresponding to the subunits. Protein in the slices will be recovered by an electro-eluter (Bio-Rad) using the manufacturer's instructions. Elution will be facilitated in such cases by using N,N/-diallytartadiamide instead of N, N/-methylenebisacrylamide in the gel solutions.

6. **Bioassay of Protein Crystal Polypeptide Subunits**

All insects will be supplied by the Insect Mass Rearing Technology Unit of the ICIPE. Samples of the different polypeptide fractions, each diluted to achieve a range of concentrations, will be painted onto young maize leaves and third instar *C. partellus* or *S. exempta* larvae allowed to feed on them. In this way, the toxicity of the protein fractions from different *B.t.* strains to each insect species will be determined. Combinations of the fractions, at different concentrations, will similarly be administered to the insects to determine the interactions of the subunits and how this affects the toxicity of the parent *B.t.* strain against a given insect. This activity will later be studied at the molecular level. Similar methodologies will be adopted to determine the relative activities of the pro- and activated endotoxins derived from the *B.t.* crystal.

7. **Analysis of Carbohydrate Composition and Primary Structures**

The carbohydrate compositions of the endotoxins will be determined by the method of alditol acetates (Grimes and Greegor, 1976; Osir *et al.*, 1986 a). The analysis will be performed on a gas liquid chromatograph equipped with a flame ionization detector. A capillary column will be used according to the conditions previously described (Grimes and Greegor, 1976; Osir *et al.*, 1986 a). Carbohydrate standards of known retention times will be used.

Since *B.t.* endotoxins are glycosylated, NMR spectroscopy will be used to compare their primary structures. In this study, the endotoxins will be digested with pronase (Osir *et al.*, 1986 a) and the digests separated on a Bio-Gel P 6 gel permeation column. The amount of the carbohydrate in each column fraction will be determined by the phenol sulfuric assay (Dubois *et al.*, 1956). The major glycopeptide fractions will be prepared for analysis as previously described (Vliegenthart *et al.*, 1981; Osir *et al.*, 1986 a). Analysis will be carried out by a 500 MHz Proton Magnetic Resonance Spectroscopy at the Biological NMR Facility of the University of Arizona, Tucson, USA.

8. **Amino Acid Composition Analysis of the Toxins**

The amino acid compositions of endotoxins will be determined using an Applied Biosystems Model 420 A derivatizer/ 130 A separation/ 920 A data analyzer with automatic acid hydrolysis. This analysis will be carried out by the Macromolecular Structures Facility of the Department of Biochemistry, University of Arizona, Tucson.

9. **Antisera Production and Immunological Procedures**

Antisera against the endotoxins will be prepared in New Zealand white rabbits according to a previously described protocol (Osir *et al.*, 1986 b). The immunological relationships between the endotoxins will be analyzed by Western blotting. Protein samples will be separated by SDS-PAGE and then electrophoretically transferred onto Hybond N paper by the LKB NovaBlot transfer unit according to the manufacturer's instructions. The efficiency of the transfer will be ascertained by staining the blots with Rouge Ponnceau. After blocking non-specific binding sites with fat free milk, the blots will be incubated with antisera raised against the endotoxins. Bound antibodies will be visualized using Protein A conjugated to colloidal-gold particles (Bio-Rad). Alternatively, detection will

Milk-containing Bacillus

utilize horseradish peroxidase-labelled goat anti-rabbit IgG (Sigma) as specified by the manufacturer.

10. Radiolabeling of the Toxin Proteins

The endotoxins will be mixed with of carrier-free Na[^{125}I] (Amersham) and Iodobeads (Pierce) and incubated (30 min, 22° C) with gentle agitation (Osir and Law, 1986). The reactions will be stopped by pipetting the samples away from the Iodobeads. Unbound iodide will be removed using Bio-Gel P6 DG columns. Other radiolabelling methods (e.g Chloramine-T) will be used in the event that this method does not give satisfactory specific activities. The stability of labelled proteins will be assessed by SDS-PAGE and autoradiography. Labelled endotoxins will also be subjected to bioassays to assess whether iodination has any effect on insecticidal activity.

11. Preparation of Midgut Membranes and Analysis of Toxin Binding Characteristics

Midguts will be dissected from chilled third-instar larvae in ice-cold buffer (pH 6-8) containing 0.3 M mannitol, 5 mM EGTA, 17 mM Tris-HCl. After removing any other tissues, brush border membrane vesicles (BBMV) will be prepared as previously described (Wolferberger *et al.*, 1987). Alkaline phosphatase will be used as a marker for BBMV.

In order to establish the optimum binding conditions for the ^{125}I-labelled endotoxins, some initial experiments are planned. Since receptor-ligand interaction is a dynamic process, the time required for the binding reaction to reach equilibrium will be established by incubating BBMV (~50-100 μg protein in a buffer containing bovine serum albumin) with increasing concentrations of labelled endotoxins. Non-specific binding will be determined by carrying out the assays in the presence of an excess of radioinert endotoxins (~100-150 times the anticipated K_D). After incubation (0-60 min, 4° C or 22° C), the samples will be centrifuged (10,000 x *g*, 10 min, 4° C) in a Microfuge to separate bound from free endotoxin. The pellets will be washed rapidly of any residual non-receptor bound label and the radioactivity measured by a gamma counter. The pH dependency of toxin binding to the BBMV will be examined by varying the buffer pH between 6 and 8 using Tris-maleate (pH 6.0-7.5) or Tris-HCl (pH 7.2-8.0). The effect of various cations (e.g. Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}) on binding will be analyzed by varying their concentrations in the incubation buffer.

Once these experiments have been carried out, binding

studies will be repeated using the established optimum conditions. The equilibrium dissociation constant (K_D) and the binding site concentration (R_t) will be analyzed by the methods of Scatchard (1949) and Weder (1974). Alternatively, the LIGAND computer programme (Munson and Rodbard, 1980) will be used. In order to demonstrate binding specificity to BBMV, binding will be analyzed over a range of membrane concentrations. If the binding is to a biologically relevant site, it should increase with proportion to the increase in binding sites (receptors). Specificity will also be ascertained by using membranes derived from non-target insects (eg locust midgut or rat intestine BBMV).

In competition experiments, BBMV will be incubated with a fixed quantity of a given labelled endotoxin in the presence of increasing concentrations of the same (homologous) or different (heterologous) radioinert endotoxin. Similarly, the other crystal subunits (separated as described in F 5) will be tested. Dissociation will be tested by incubating labelled toxins with BBMV until equilibrium after which increasing amounts of radioinert toxins will be added. In all cases, bound endotoxin will be determined as described above and the data corrected for non-specific binding.

The three main criteria commonly required for a binding site to qualify as a receptor are: finite binding capacity, high affinity and reversible interaction with the ligand. The experiments outlined above should ascertain whether the BBMV binding sites are true endotoxin receptors.

12. Effect of Deglycosylation on Receptor Binding

In order to assess the possible role of the endotoxin carbohydrate moiety in receptor binding, the endotoxins will be deglycosylated with endo-β-glycosidase F or H (Boehringer) according to the manufacturer's instructions. Success of this treatment will be ascertained by periodate acid Schiff staining of the sample after separation by SDS-PAGE (Kapitany, 1973). The ability of the deglycosylated sample to compete with labelled endotoxin for membrane binding sites will be tested in the competition experiments described above.

13. **Solubilization of Membrane Toxin Receptors and Ligand Blotting**

Attempts will be made to solubilize the endotoxin receptors from homogenized brush border membranes using different detergents (Triton X-100/ 200, octyl-β-D-glucoside, Nonidet P 40 etc). In these experiments, the membranes will be mixed with various concentrations of detergents (0-50 mM). After incubation, insoluble material will be removed by centrifugation. Aliquots of the supernatant solution will be tested for binding to endotoxins as described above. Assuming that solubilization is achieved, then identification of the receptor will be carried out by ligand blotting. In case, solubilized membrane proteins will be separated by SDS-PAGE (in the presence or absence of sulfhydryl reducing agents), transferred onto nitrocellulose paper and then incubated with labelled endotoxins. Alternatively, the paper will be incubated with radioinert toxin and subsequently with ^{125}I-labelled anti-toxin antibodies. The control experiments will have excess radioinert toxins. Autoradiography will be carried out at -70° C using a Kodak XAR-5 film.

Once the receptor protein has been identified, purification will be carried out using various strategies: (i) electroelution from gel slices and (ii) lectin affinity chromatography, if the receptor is a glycoprotein. The purified receptor will then be characterized by the methods described above. The sensitivity of the toxin receptor to proteolytic cleavage will be assessed by incubating the receptor with pronase (37° C, 30 min) prior to the binding assays. Similarly, the effect of deglycosylation will also be studied.

14. **Cloning and Sequencing of the Genes That Code for the Crystal Proteins in the *B.t.* Isolates**

The determination of the genetic make up of the new *B.t.* isolates will be carried out in the laboratory of Professor Whiteley (University of Washington), since investigators in this laboratory have considerable experience with *B.t.* crystal proteins.

Professor Whiteley's laboratory has cloned the following Class I genes (lepidopteran-specific): *cryIA(a)* from two subspecies of *B.t.*; *cryA(b)* from two subspecies of *B.t.*, *cryIA(c)*, and *cryIB* (each isolated from a different subspecies of *B.t.*). This laboratory has also cloned and studied: *cryIIA*, a gene coding for lepidopteran-dipteran specific protein and a related variant, *cryIIB* coding for a lepidopteran-specific protein; *cryIIIA*, encoding a coleopteran-specific gene

and *cryIVD* and *crytA*, two genes that code for mosquitocidal proteins. Additional genes are currently under investigation.

The initial characterization of the new strains will involve a determination of the number and sizes of the proteins present in the crystals. Cultures will be grown in minimal media for 3-4 days to allow sporulation. Crystals will be separated from spores by centrifugation in renograffin or sodium bromide gradients and extensively washed to remove proteases. The crystals will be solubilized and proteins separated by SDS-PAGE and compared with similarly treated proteins from the above well-characterized *B.t.* strains. In addition, the new strains will be analyzed by immunoblotting using antibodies against proteins from all four of the major crystal protein classes, which are available in Professor Whiteley's laboratory. If none of the antibodies reacts with crystal proteins from the new isolate, the peptide bands from the new isolate will be cut from SDS-polyacrylamide gels and used to immunize rabbits.

It would be convenient to be able to monitor the toxicity of genes cloned from the newly isolated strains. Therefore, we plan to test the toxicity of the new isolates to larvae of *M. sexta* and *H. virescens*. These species are susceptible to many lepidopteran-specific *B.t.* strains and are readily available to us. If these insects are not susceptible to the new isolates, we will arrange for assays by the USDA laboratory in Yakima, Washington, which maintains colonies of additional lepidopteran species in the hope of finding suitable insects since we may not have access to *C. partellus*, *S. exempta* or *G. m. morsitans*.

Almost all crystal protein genes are borne on large plasmids. Plasmid DNA will be isolated from the new *B.t.* strains from Kenya and hybridized with the gene-specific probes described below. Varying the stringency of the hybridization conditions may indicate how closely related the crystal protein genes in the new isolates are compared to known genes. The plasmids that hybridize with one or more of the probes will be separated either by cutting out and eluting the band from the gel or will be separated by centrifugation in a sucrose gradient into "large" and "small" plasmid fractions for further experiments. The isolated plasmid DNA or enriched fraction will be digested with restriction enzymes (*Hind III*, *EcoRI* etc). The patterns will be compared with those produced from relevant known genes and hybridized with gene-specific probes. We would expect these strains to contain genes related to known Class I or Class II genes since the

strains will be selected for toxicity to specific lepidopteran species; it is likely that the new isolates may contain more than one crystal protein gene. If these preliminary experiments indicate the presence of a novel gene in the new *B.t.* strain, then the gene coding for the protein will be cloned.

15. Cloning of the *B.t.* Genes

A genomic library will be prepared from each strain by cloning 15-20 kb *SauIIIA* partial digest fragments of isolated plasmid or the enriched plasmid fraction (or total genomic DNA) into the lambda vector, EMBL3. The library will be screened by plaque hybridization with previously cloned gene probes at high and low stringencies to detect closely and more distantly related endotoxin genes, respectively. Positive clones will be transferred to the pUC118 plasmid vector for analysis.

Alternatively, a genomic library will be made using a partial digest (*SauIIIA*, or *BamHI*) of isolated plasmid or the enriched plasmid fraction and cloned into the *E. coli* plasmid pUC18. Transformants will be selected for ampicillin resistance and the loss of β-galactosidase activity and colonies will be screened by hybridization with a DNA probe. Positive colonies will be subjected to further analysis.

In screening for positive clones, we will use gene-specific segments of DNA from the genes cloned by the Whiteley laboratory as probes. In addition, we will use oligonucleotide probes (prepared by using a DNA synthesizer available in the Department of Microbiology, University of Washington) for *cryIC* and *cryID* or any other new Class I gene encoding a lepidopteran-specific endotoxin for which a DNA sequence has been published. To detect the presence of any crystal protein genes, we will use oligonucleotide probes to one or more of the five conserved regions (Hofte and Whiteley, 1989) that are found in all but one of the crystal protein genes whose DNA sequences have been published to date (the gene lacking the five conserved regions codes for a small mosquitocidal protein). If a positive clone is detected using a probe to a conserved sequence but not with a lepidopteran-specific probe, this would indicate the presence of a class II, III or IV gene which would react with a probe specific for one of these genes. If neither of the approaches outlined above yields positive results this may indicate the presence of a strong divergent novel gene. In that case, the crystal protein(s) from the new Kenyan isolates will be separated by SDS-PAGE and the N-terminal amino acid

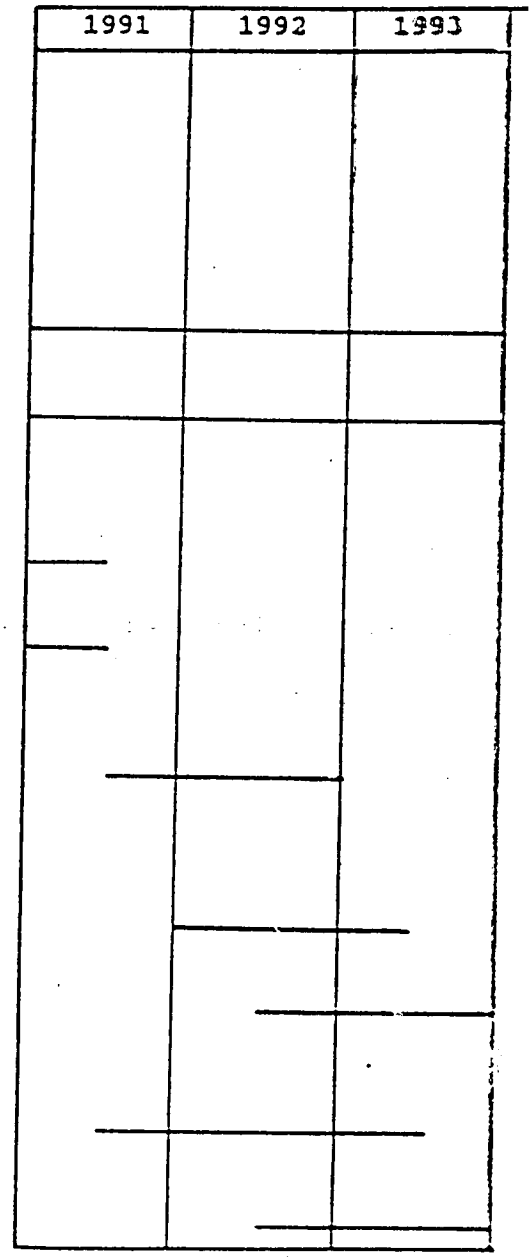
sequence of the major peptides will be determined (this service is available through the Department of Biochemistry, University of Washington). Knowing the N-terminal amino acid sequence, an oligonucleotide probe can be prepared and the gene can then be cloned.

The selected clones will be further subcloned and characterized by (i) hybridization of probes to restriction digests, (ii) immunoblotting to determine if the crystal protein of the expected size is produced and (iii) toxicity to locally available insects and to *C. partellus*, *S. exempta* and *G. m. morsitans*. Materials will be sent or carried to the ICIPE laboratory, if necessary for toxicity testing). Finally, if the properties of the cloned material indicate that a different gene has been isolated, the DNA sequence of the gene will be determined.

16. Sequencing of DNA

A set of deletions will be prepared from a subclone containing the gene of interest using *ExoIII* nuclease (using genes oriented in both directions) and the DNA sequence will be determined using the Sanger's dideoxy method. All of the methods needed for the proposed characterization of crystal protein genes are currently in use in the Whiteley laboratory. Most are well-known, published methods, many of which are described in standard laboratory manuals (e.g. Sambrook *et al.*, 1989).

CHART OF PROPOSED WORKPLAN (SUMMARY)



A. PATHOGENS

(a). Strain collection/ identification*

(d). Establishment of culture conditions*

(b). Bioassay development**

(c). Safety tests on non-target organisms**

B. PROTEIN BIOCHEMISTRY

(a). Toxin crystal preparation procedures**

(b). Protoxin/ Toxin preparation procedures**

(c). Physical/ chemical/ immunological studies (Protoxin/ Toxin)

C. MIDGUT TOXIN RECEPTORS

(a). Characterization of toxin binding sites

(b). Isolation/ characterization of midgut receptor proteins

D. ENDOTOXIN GENES

(a). Cloning of *B.t.* genes

(b). Sequencing and analysis of the genes

*Accomplished work
**Work in progress

V. BUDGET INFORMATION

I PERSONNEL	YEAR 1	YEAR 2	YEAR 3	TOTAL
1. Principal Investigator[a] (80%)	-	-	-	-
2. Principal Co-Investigator[a] (70%)	-	-	-	-
3. Scientific Officer[a] (40%)	-	-	-	-
4. Technician[b] (100%)	8,000	8,400	8,820	25,220
Total Personnel	8,000	8,400	8,820	25,220

II EQUIPMENT				
1. Gamma Counter and accessories[c] (Beckman)	30,000	-	-	30,000
2. Gamma vials (Beckman)	600	600	500	1,800
3. Culture bottles	400	300	300	1,000
4. Superose 6/Mono Q FPLC Columns[d] (Pharmacia)	1,200	-	-	1,200
5. Centrifuge tubes (Beckman)	500	500	500	1,800
6. Electro-eluter (Bio-Rad)	1,000	-	-	1,000
7. Fraction Collector (ISCO-WIZ)	1,000	-	-	1,000
8. Refrigerator/Freezer	1,000	-	-	1,000
9. Pipetman Set[e] (Gilson)	1,200	-	-	1,200

a = Salaries/benefits of personnel to be met from core funds as part of ICIPE's contribution to the project

b = Personnel to be hired on a full time basis. Includes salary and benefits

c = Equipment needed for gamma detection in receptor binding studies

d = Columns needed for toxin purification

e = Needed for measurement of various volumes

		YEAR 1	YEAR 2	YEAR 3	TOTAL
IV.	LITERATURE SEARCHES/ PUBLICATIONS/REPORTS	700	500	1,500	2,700
V.	PROFESSIONAL SERVICES/ CONSULTATION[h]	800	800	800	2,400
VI.	INTERNATIONAL PROFESSIONAL TRAVEL[i]	4,000	5,500	4,000	13,500
	Total (IV-VI)	6,000	5,800	6,800	18,600
VII.	OUTSIDE COLLABORATION	10,000	10,000	10,000	30,000

g = Cost of isotopes to be shared with other Centre users

h = Cost of amino acid composition (or sequence) and carbohydrate analyses

i = Cost of airfare (roundtrip) and per diem for one investigator per year.
In second year, a graduate student will spend a month at the University of Washington

		YEAR 1	YEAR 2	YEAR 3	TOTAL
10.	Microfuge (Beckman)	1,800	-	-	1,800
11.	Shaker	600	-	-	600
12.	Electrophoresis apparatus (BRL)	800	-	-	800
13.	Power Supply[f] (Bio-Rad)	3,000	-	-	3,000
14.	GLC Capillary Column	1,000	-	-	1,000
15.	Assorted glassware	2,000	-	-	2,000
	Total Equipment	46,200	1,500	1,500	49,200
III.	MATERIALS				
1.	Chemical and Reagents	9,000	6,000	5,000	20,000
2.	Radioisotope[g]	2,000	2,000	2,000	6,000
	Total Materials	11,000	8,000	7,000	26,000

f = Needed for electrophoretic separation of proteins and blotting

g = Cost of isotope to be shared with other Centre users.