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Attachment 1.
(FACE SHEET)

Date December 10, 1991

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr. Zia Uddin Ahmed Trainee Investigator (if any) ______

Application No. 91-016 Supporting Agency (if Non-ICDDR,B) ______

Title of Study Cloning of gene(s) that code for toxin production by enterotoxigenic Bacteroides fragilis and development of diagnostic tests based on the toxin.

Project status:
( ) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

Circle the appropriate answer to each of the following (If Not Applicable write NA).

1. Source of Population:
   (a) Ill subjects Yes No
   (b) Non-ill subjects Yes No
   (c) Minors or persons under guardianship Yes No
2. Does the study involve:
   (a) Physical risks to the subjects Yes No
   (b) Social Risks Yes No
   (c) Psychological risks to subjects Yes No
   (d) Discomfort to subjects Yes No
   (e) Invasion of privacy Yes No
   (f) Disclosure of information damaging to subject or others Yes No
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) Yes No
   (b) Use of fetal tissue or abortus Yes No
   (c) Use of organs or body fluids Yes No
4. Are subjects clearly informed about:
   (a) Nature and purposes of study Yes No
   (b) Procedures to be followed including alternatives used Yes No
   (c) Physical risks Yes No
   (d) Sensitive questions Yes No
   (e) Benefits to be derived Yes No
   (f) Right to refuse to participate or to withdraw from study Yes No
   (g) Confidential handling of data Yes No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes No
5. Will signed consent form be required:
   (a) From subjects Yes No
   (b) From parent or guardian (if subjects are minors) Yes No
6. Will precautions be taken to protect anonymity of subjects Yes No
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ___ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

   * If the final instrument is not completed prior to review, the following information should be included in the abstract summary:
   1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
   2. Examples of the type of specific questions to be asked in the sensitive areas.
   3. An indication as to when the questionnaire will be presented to the Cttee. for review.

We agree to obtain approval of the Ethical Review Committee for any changes involving the rights and welfare of subjects before making such change.

Zia U. Ahmed
Principal Investigator

Trainee

A- 032065

APPLICATION FOR PROJECT GRANT

1.	Principal investigator	:	Zia Uddin Ahmed, Ph.D.
2.	Other investigators	:	R.B. Sack, M.D One Senior Research Officer One Research Officer
3.	Title of project	:	Cloning of gene(s) that code for toxin production by enterotoxigenic *Bacteroides fragilis* and development of diagnostic tests based on the toxin
4.	Starting date	:	As soon as possible
5.	Date of completion	:	Two years from the starting date
6.	Funding source	:	Not known
7.	Total budget requested	:	$ 56,330
8.	Head of programme	:	

M. Islam
Acting Head, LSD

9. Aim of the project

a) *General aim*

To contribute towards an understanding of the possible virulence mechanism of *B. fragilis* and to development of diagnostic tests based on the toxin gene and its product.

b) *Specific aims*

i) to study whether toxin-production in *B. fragilis* is plasmid-mediated

ii) to make a cosmid-based gene bank of total cellular DNA of *B. fragilis*

iii) to select, by using a tissue culture assay and DNA probes, clone(s) producing toxin of *B. fragilis*

iv) to subclone the toxin gene in a small DNA fragment to facilitate further studies towards identification of the toxin, its mode of action and studies aimed at developing diagnostic tests

c) *Significance*

Same as general aim

10. Ethical implications : None

11. ABSTRACT SUMMARY

Attempts will be made to test whether any plasmid is involved in the expression of toxigenicity in *Bacteroides fragilis*. Total cellular DNA from a toxigenic strain of *B. fragilis* will be isolated and partially digested with the restriction endonuclease *BamH1*. Using this digest, a gene bank will be made in the cosmid pHC79. Clones from the gene bank will be screened for toxin production using a tissue culture assay. Positive clones will be isolated and the coding region will be subcloned using standard procedures. Attempts will be made to produce DNA probes using sequences of the toxin gene and to obtain pure toxin using an *in vitro* DNA-dependent translation system.

12. BACKGROUND, RESEARCH PLAN, BIBLIOGRAPHY

[A] BACKGROUND

It is recognized that a large proportion of diarrhoeal episodes has no known etiology (Guerrant *et. al.*, 1983). In recent years, the possibility of some strains of *Bacteroides fragilis*, a Gram-negative, strictly anaerobic bacteria, being associated with diarrhoeal illness has drawn attention and interesting results have been reported. Some isolates of *B. fragilis* have been demonstrated to elaborate a type of toxin which is functionally similar to the known toxins produced by other enteric bacteria. The toxin has a demonstrable secretogenic activity in certain assays, that is,in lamb and calf ligated ileal loops (Myers *et al.*, 1984, 1985). Such strains of *B. fragilis* have been designated as enterotoxigenic *Bacteroides fragilis* (ETBF). There are other animal models also where entero-virulence of such strains can be readily demonstrated. For example, adult rabbits with ligated ceca and

healthy infant rabbits can be infected with ETBF strains which cause lethal diarrhoeal illness (Myers *et al.*, 1987a; Myers *et al.*, 1998).

Although very clear data are at present lacking, association of ETBF with diarrhoea in humans is becoming increasingly significant as a result of careful studies. The first report on the isolation of ETBF from cases of acute and chronic diarrhoea (with no other detectable etiology) was based on a population in an Indian Reservation in Arizona (Myers *et al.*, 1987). In a recent, more extensive case-control study in this same population, it was found that ETBF isolation was significantly associated with cases of acute diarrhoea, as compared to control cases (Sack *et al.*, US-Japan meeting, 1990).

Toxin produced by ETBF strains is an extracellular product, released by the cells into the growth medium. Standard assays which are employed to detect enteric bacterial enterotoxins such as Y-1 adrenal cells, Chinese Hamster Ovary (CHO) cells, suckling mice, hamster, pig and rabbit ligated ileal loop assays, have failed to demonstrate enterotoxigenic effects of ETBF strains that can be identified by lamb and calf ileal loop assays. Nor has it been possible to detect DNA sequences in such strains coresponding to the sequences of the cholera toxin (CT) or heat-labile enterotoxin of *E. coli* (Myers *et al.*, 1985). Although enterotoxigenic activity can be demonstrated in several whole animal models, such as adult rabbits with ligated ceca and in infant rabbits, the secretogenic activity of the toxic principle in ETBF strains can also be demonstrated clearly in lamb and calf ligated ileal loops. These rather costly and cumbersome assays make studies on the epidemiology of ETBF strains and on the biology of the toxin considerably difficult.

Recently, a tissue culture assay using the cloned human colonic epithelial cell line HT29/C_1 has been developed (R.B. Sack, US-Japan meeting, 1990).

This assay which is highly sensitive (87%) and specific (100%) for ETBF strains thus provides an opportunity for more extensive laboratory-based experimental work on the toxin and its mode of action. In the present protocol, we propose to study whether or not the gene coding for toxin production is plasmid-borne and to clone the gene using the standard gene cloning techniques and the above HT29/C_1 tissue culture assay. Genes of *Bacteroides* sp. have been successfully cloned and expressed in *E. coli* K-12. For example, Anderson *et al.* (1984) cloned the gene coding for fimbriae using a positive selection plasmid vector, pTR262. Matsushito *et al.* (1990) cloned endoglucanase gene from *B. ruminicola* in a bacteriophage -based genomic library and achieved successful over-production of the enzyme by placing the gene under the control of the *tac* promoter.

[B] RESEARCH PLAN

MATERIALS AND METHODS

Bacterial strains and plasmids

A strain of well-characterized ETBF of human origin strain, 077225-2, will be used as a reference ETBF strain and as the source of DNA for use in constructing a gene bank. The gene bank will be made in the cosmid pHC79 and maintained in *E. coli* K-12 strain DH1. For subcloning purposes, we propose to use the insert-selection plasmid vectors pUC18 or pUC19.

Reagents

Restriction endonucleases, cosmid pHC79 and the packaging kit, plasmid pUC18 and pUC19 and restriction buffers will be obtained from Boerhinger Mannheim,

Germany. The *in vitro* transcription/translation kit will be purchased from Amersham.

Cultivation of ETBF strain

The ETBF strain will be grown on brain-heart infusion slants and stored in sterile defibrinated bovine blood at -70°C. For large volume cultivation, brain heart infusion broth (BHIB) will be used. A seed culture will first be made by streaking frozen material on a blood agar plate and incubating the plate anaerobically at 37°C for 48 hours. Five or more colonies from the plate will be inoculated into 100 ml of BHIB in a conical flask and incubated anaerobically for 48 hours.

For the cultivation of *E. coli* strains, standard media such as trypticase soya broth or agar, and Luria-Bertain medium will be used. X-gal plates will be used for insert selection in plasmid pUC18.

Isolation of DNA

Plasmid DNA will be isolated by the method of Birnboim and Doly (1979). For the isolation of genomic DNA, one or more of the several procedures commonly used in Gram-negative bacteria will be employed. The DNA will be of sufficiently high molecular weight, pure enough so as to allow its digestion by restriction endonucleases. We do not foresee the need for purification of DNA using CsCl-ethidium bromide density gradient centrifugation. The procedure with which we will start is the following.

Cells from 100-500 ml BHIB cultures will be collected by centrifugation and suspended in 3 ml of a solution of 8% sucrose, 0.5 MEDTA and 20 mM Tris.Cl, pH

8.0 followed by the addition of 0.6 ml of lysis solution (lysozyme 10mg/ml in 10 mM Tris.Cl, pH 8.0). After mixing, the cell suspension will be left at room temperature or at 37°C for 10-30 min. Then, 9 ml of a solution containg 0.5 MEDTA, 0.5% SDS and 100 µg/ml proteinase K will be added and the resultant viscous lysate will be incubated at 56°C for 2 h. The lysate will be extracted once with an equal volume of phenol saturated with 10mM Tris-HCl, pH 8.0 followed by one extraction with phenol/chloroform and one with chloroform.

The aqueous phase will be dialyzed against 5 L of 1mM Tris, pH 8.0 overnight. After making the solution 0.3 M by adding 1/10 th volume of a solution of 3M NaCl, the DNA will be precipiated with two volumes of ethanol. The fibrous precipitate will be collected, washed with 70% ethanol and vacuum-dried. The DNA will be dissolved in 1mM Tris.HCl, pH 8.0 and stored at 4°C.

A procedure has been recently described to obtain DNA from *Bacteroides ruminicola* (Matsushita *et al.*, 1990). If pure DNA is not obtained by the above procedure, the latter method will be used.

Correlation of plasmid with toxicity

Toxigenic and non-toxigenic isolates of *B. fragilis* will be examined for the presence of easily detectable plasmids. Reports on the occurrence of plasmids in *B. fragilis* are scanty. Stiffler *et al.* (1974) detected the presence of plasmids in clinical isolates by labelling the DNA with ^{3}H-thymidine and subjecting to dye-buoyant density-equilibrium centrifugation. These relatively small and low-copy plasmids were found to have no obvious association with drug resistance. The isolates examined by these authors were clinical in origin but it is not known whether these were toxigenic.

If a correlation is detected by studying a sample of known toxigenic and non-toxigenic isolates, using routine rapid plasmid isolation techniques, then it will be worthwhile to study the plasmid further. Study of the biology of a plasmid which does not carry a selectable marker is not be easy. Such studies, in a major way, are dependent on obtaining plasmidless variants. If the plasmid is highly unstable, such variants could be obtained easily by actually examining a small number of colonies (about 200 or so). Otherwise, it will be necessary to mark the plasmid of interest with a selectable marker -- a drug-resistance transposon -- which could then be used to follow plasmid-loss or plasmid-transfer. It is difficult to predict how difficult will these manipulations be in *B. fragilis*.

Restriction endonuclease digestion

Total DNA will be partially digested with *Sam*3A. That is, the DNA solution will be digested with a certain amount of enzyme for different lengths of time (2-10 min) and the different digests will be mixed. This is expected to result in a partially digested preparation.

Fragment size selection

The digest will be electrophoresed in a preparative agarose gel. The region of the gel corresponding to 35 kb will be removed and the DNA fragments electro-eluted. After extraction with phenol, phenol-chloroform and chloroform, the DNA fragments will be precipitated with ethanol. This population of DNA fragments will be used to make the gene bank.

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Gene bank

The cosmid pHC79 will be digested with *BamHI* to linearize the molecule, and then bacterial alkaline phosphatase will be added. The solution will be incubated at 37°C to dephosphorylate the 5'-ends of the DNA molecules. After usual phenol-chloroform and chloroform treatments, the DNA molecules will be precipitated with ethanol.

DNA fragments of the *B. fragilis* genome, prepared as described above will then be ligated with the linearized, dephosphorylated cosmid molecules using T4 DNA ligase.

The ligation mixture will be packaged in bacteriophage heads using the packing kit obtained from Boerhinger Mannheim.

The packaged heads will be used to transfect competent cells of *E.coli* strain DH1 by using standard procedures (Maniatis *et al.*, 1982). The transfected cells will be stored in 1 ml aliquots at -70°C after making the cell suspension 15% with respect to glycerol.

Screening the gene bank

Transfected *E. coli* cells will be plated onto LA medium containing 50 µg/ml ampicillin (to select the cosmid-containing cells). Ampicillin-resistant colonies will be tested for the production of toxin using the tissue culture assay.

Each Amp^r clone will be grown in 10 ml BHIB overnight at 37°C under ampicillin selection. The culture suspernatant obtained after centrifugation will be concentrated approximately 20-fold using Amicon Centriprep Concentrators or

Amicon Stirred cell apparatus (Myers *et al.*, 1984). The concentrate will be filter-sterilized and tested for toxic activity in the tissue culture assay.

Sub-cloning

Toxin-positive clones (of which attempt will be made to isolate more than one) will be identified. Plasmid DNA from the clone will be isolated by using the alkaline lysis procedure of Birnboim and Doly (1979).

The plasmid DNA will be digested with one of several different restriction enzymes so as to obtain a small number of fragments. These fragments will be isolated from preparative agarose gel, purified and cloned into pUC18 by following standard procedures (Maniatis *et al.*, 1982). The sub-clones will be tested for cytotoxicity using the cell culture assay. The sub-cloning strategy depends entirely on expression of the toxin gene in *E. coli* which may not be the case. Alternative approach to screening the library is to develop a DNA sequence using the N-terminal sequence of the toxin, affinity-purified by using antisera and use the synthetic DNA sequence as a probe.

Validation of the Tox$^+$ clones

Each Tox$^+$ clone identified by the cell culture assay will be tested in lamb and calf ligated ileal loops and compared with a reference ETBF strain with respect to the level of toxin-activity.

Projected directions for further studies

After a subclone containing the toxin gene in a relatively small fragment (2-3 kb) is obtained, further studies could follow two major directions, both

aimed at developing diagnostic tests. One approach could be directed to developing DNA probes for the detection of toxigenic *B. fragilis*. The other approach could be based on the toxin molecule. The toxin could be purified to homogeneity and used to raise monoclonal antibodies for a diagnostic ELISA.

DNA probes and external collaboration

Probes can be made using restriction fragments of the cloned DNA or by determining the sequence of the gene one can design synthetic oligonucleotide probes. For these studies we propose to identify in the near future an external laboratory where facilities for such experiments are in routine operation.

This phase of the project is expected to commence during the second year -- that is, after the cloning and subcloning part has been successfully completed.

Identification of gene product

The cloned product could be identified by recourse to *in vitro* transcription/translation assays. Such systems are commercially available (Amersham). These experiments will be planned in more details after the toxin gene has been cloned. Our immunology laboratory will be involved in these studies. Details of these experiments are, therefore, omitted from this protocol.

FACILITIES AT ICDDR,B AND COLLABORATION

Facilities available at ICDDR,B are adequate for the gene cloning work proposed in this protocol. Fine structure analysis of the Tox^+ insert leading to sequencing the gene will best be done at an external laboratory. For this, a collaborating centre needs to be identified which we intend to do in the near future. Physical facilities available are adequate for the proposed work and no major capital investments are anticipated.

BIBLIOGRAPHY

Anderson, B.J., M.M. Bills, J.R. Egerton, and J.S. Mattick. 1984. Cloning and expression in *Escherichia coli* of the gene encoding the structural subunit of *Bacteroides nodosus* fimbriae. J. Bacteriol. 160, 748-754.

Birnboim H.C., and J. Doly. 1979. A rapid alkaline procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7, 1513-1523.

Guerrant, R.L., Kirchhoff, D.S. Shields, M. K. Leslie *et al.* 1983. Prospective study of diarrhoeal illness in Northeastern Brazil : Patterns of disease, nutritional impact, etiologies and risk factors, J. Infect. Dis. 184, 986-997.

Maniatis, T., E.F Fritsch and J. Sambrook. 1982. Molecular cloning : a laboratory manual. Cold Spring Harbor, New York.

Matsushita, O.J.R. Russell and D.B. Wilson. 1990. Cloning and sequencing of *Bacteroides ruminicola* B_14 endoglucanase gene. J. Bacteriol. 172, 3620-3630.

Myers, L.L., B.B. Firehammer D.S. Shoop and M.M. Border. 1984. *Bacteroides fragilis* : a possible cause of acute diarrhoeal disease in newborn lambs. Infect. Immun. 44, 241-244.

Myers, L.L., D.S. Shoop ., B.D. Firehammer, and M.M. Border, 1985. Association of enterotoxigenic *Bacteroides fragilis* with diarrhoeal disease in calves. J. Infect. Dis. 152, 1344-1347.

Myers, L.L., D.S. Shoop., and T.D. Byars. 1987a. Diarrhoea associated with enterotoxigenic *Bacteroides frafilis* in foals. Am. J. Vet. Res. 48, 1565-1567.

Myers, L.L., D.S. Shoop, L.L. Stackhouse, F.S. Newman, R.J. Flaherty, G.W. Letson and R.B. Sack. 1987b. Isolation of enterotoxigenic *Bacteroides fragilis* from humans with diarrhoea. J. Clin. Microbiol. 25, 2330-2333.

Myers, L.L., D.S. Shoop, J.E. Collins and W.C. Bradbury. 1989. Diarrhoeal disease caused by enterotoxigenic *Bacteroides fragilis* in infant rabbits J. Clin. Microbiol. 27, 2025-2030.

Myers, L.L., D.S. Shoop and J.E. Collins. 1990. Rabbit model to evaluate enterovirulence of *Bacteroides fragilis*. J. Clin. Microbiol. 28, 1658-1660.

Stiffler, P.W., R. Keller, and N. Traub. 1974. Isolation and characterization of several cryptic plasmids from clinical isolates of *bacteroides fragilis*. J. Infect. Dis. 130, 544-548.

WORK PLAN

Year 1 :

- Setting up the tissue culture assay
- Preparation of gene bank
- Subcloning of the toxin-containing sequence using cell culture assay and by DNA probes

Year 2 :

- Toxin purification using a transcription-translation system *in vitro*
- Restriction endonuclease analysis of the toxin gene and sequencing

BUDGET

YEAR 1

Personnel

Research Officer	GS-5	...	US$	6,000
Lab Attendant	GS-1	...	"	2,000

Operating Cost

(a) Materials, supplies, interdepartmental	...	"	10,000
(b) Miscellaneous (contractual services)	...	"	2,000
		US$	20,000

YEAR 2

Year 1 + 15% increase	...	US$	23,000
		US$	43,000
Overhead	...		13,330
TOTAL		US$	56,330

Z4:CLONING.PRO

Dr. Zia. U. Ahmed

THE UNIVERSITY OF ADELAIDE

Department of Microbiology and Immunology

FACSIMILE MESSAGE

To: Dr Môyenul Islam
Acting Head
Laboratory Sciences Division
ICDDR, B
Dhaka Bangladesh

Fax No.: 0011 880 2 883 116

No. of pages: 4.

From: Prof Paul A Manning

Date: 16 October 1991

Dear Dr Islam

Enclosed is my report on the Grant application which you sent to me for assessment. If I can provide any further comment, please do not hesitate to contact me.

Yours sincerely

Paul A Manning
Professor

Project title: Cloning of gene(s) that code for toxin production by enterotoxigenic Bacteroides fragilis and development of diagnostic tests based on the toxin.

Principal Investigator(s): ....

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project		←→	←→
Adequacy of Project Design		←→	←→
Suitability of Methodology			✓
Feasibility within time period		✓	
Appropriateness of Budget		✓	
Potential value to field of knowledge	✓		

CONCLUSIONS

I support the application:

a) without qualification ☐

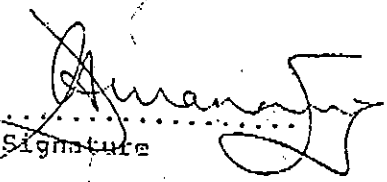
b) with qualification:
- on technical grounds ☑
- on level of financial support ☑

I do not support the application ☐

Name of Referee: .....Prof Paul A Manning.....

Position: ....Professor..........

Institution: ....University of Adelaide........



Signature

16.10.91.
Date

DETAILED COMMENTS

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel they are justified.

(Use additional pages if necessary)

This project is a seemingly straight-forward proposal to identify the genetic location of the gene for *Bacteroides fragilis* enterotoxin, to clone the gene using a suitable detection system and to develop a gene probe. Most of the methods to be used are routine and there is no particularly novelty in the project design. However, there are several flaws in the project as it stands.

1. The analysis of plasmids and correlation with toxicity relies on the fact that if the toxin(s) is plasmid encoded then it is stable in this form. However, many toxins are associated with mobile genetic elements and it is not unreasonable to assume that the same could apply in *Bacteroides* and consequently it may not be possible to find a correlation.

   The methods for plasmid analysis are not the most appropriate and alkaline-lysis without radioactively labelling is preferred.

2. The digestion of the DNA for constructing of the library is inappropriate. The cosmid vector pHC79 requires at least 31kb and a maximum of 45kb of DNA to be cloned to be able to be packaged. Thus, fragments of 20-25kb are only likely to produce mixed cosmids which may provide confusion sorting out genomic organization. I suggest that the DNA should be sized to around 35-40kb. Also, a partial *Bam*HI digest is not ideal since it has a 6bp recognition sequence and there is the possibility of getting non-clonable fragments. This can be overcome by using partial *Sau*3A digests which also help to randomize the cloning better so that a truly representative selection of cosmid clones covering the genome is obtained.

3. Dephosphorylation of the vector should be done at about 37°C. 68°C will inactivate the enzyme.

4. The cloning strategy relies on expression of the toxin in *E.coli*. However this could be a serious flaw, especially since we know that expression of a variety of toxins are regulated via species specific transactivating elements. Thus, it is possible to clone the toxin gene(s) and not get any expression. Thus, looking for the biological activity would fail. An alternative approach is to work up an antiserum (or even a MAb, if

reasonably concentrated and partially pure preparations of toxin can be obtained) and use that to affinity purify material for N-terminal sequence analysis. This could be used to predict an oligonucleotide for probing the genomic library.

5. Expression of the toxin clone in minicells is not a feasible approach to get pure toxin.

The time scale of the project seems appropriate, however, I am not convinced that the number of personnel requested is justified. Certainly the operating costs seem excessive and need to be justified. The project should be able to be done on about half the maintenance budget requested.

What is the $48,000 overhead?

Is a 15% increase for year 2 excessive?

Project title: Cloning of gene(s) that code for toxin production by enterotoxigenic Bacteroides fragilis and development of diagnostic tests based on the toxin.

Principal Investigator(s): ....

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project	✓		
Adequacy of Project Design	✓		
Suitability of Methodology	✓		
Feasibility within time period	✓		
Appropriateness of Budget	✓		
Potential value to field of knowledge	✓		

CONCLUSIONS

I support the application:

a) without qualification [✓]

b) with qualification:
- on technical grounds [ ]
- on level of financial support [ ]

I do not support the application [ ]

Name of Referee: Dennis J. Kopecko, Ph.D.

Position: Asst. Chief, Dept. Bacterial Immunology

Institution: Walter Reed Army Inst. Research

[signature]

Signature

6 Nov. 91

Date

DETAILED COMMENTS

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel they are justified.

(Use additional pages if necessary)

This represents a well-defined project and utilizes the appropriate technology to achieve the objectives. Purifying the toxin and cloning the toxin genes from ETBF has very significant practical consequences and will be an important advancement in diarrheal disease research. I strongly support this application.

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RESPONSE TO REVIEWERS' COMMENTS

Dr. D. Kopecko has given suuport without qualification.

Following are the response to Dr. P. Manning's comments

1. We will resort to alikaline lysis for plasmid analysis. It is true that a correlation may not be abvious between the presence of plasmid and toxin production by a strain. However, it is not the most significant part of the project. If a correlation however does appear as significant, furtehr studies as to the stability of the association and possible transposon involvement will be examined.

2. We will now use Sau3A instead of BamH1. This is a very good suggestion, and fortunately we do have this enzyme at hand for ready use.

3. BAP should indeed be used at 37°C — 68°C was a typographic error.

4. We will test for both biological activity and use probes which will have to be disigned. We have incorporated the lead given by Dr. Manning towards the development of a useful probe.

5. The minicell portion is withdrawn as it indeed is a highly technical area and the results may not be easy to interpret.

All of the comments of the reviewer have been most enlightening which I accept with gratitude.