hment 1.

Date August 3, 1979

26 REVIEW BOARD ON THE USE OF HUMAN SUBJECTS, ICDDR,B.

ipal Investigator J.R. Murphy

Trainee Investigator (if any) _____

:ation No. 79-016

Supporting Agency (if Non-ICDDR,B) _____

of Study Isolation of Vibrio cholerae Transducing Phage

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

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

ource of Population: NA

) Ill subjects Yes No
) Non-ill subjects Yes No
) Minors or persons under guardianship Yes No

es the study involve: NA

) Physical risks to the subjects Yes No
) Social Risks Yes No
) Psychological risks to subjects Yes No
) Discomfort to subjects Yes No
) Invasion of privacy Yes No
) Disclosure of information damaging to subject or others Yes No

es the study involve: NA

) Use of records, (hospital, medical, death, birth or other) Yes No
) Use of fetal tissue or abortus Yes No
) Use of organs or body fluids Yes No

e subjects clearly informed about: NA

) Nature and purposes of study Yes No
) Procedures to be followed including alternatives used Yes No
) Physical risks Yes No
) Sensitive questions Yes No
) Benefits to be derived Yes No
) Right to refuse to participate or to withdraw from study Yes No
) Confidential handling of data Yes No
) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes No

5. Will signed consent form be required: NA
   (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 NA Yes No
7. Check documents being submitted herewith to Board: none
   ___ 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 Board for review.

ce to obtain approval of the Review Board on the Use of Human Subjects for any changes ing the rights and welfare of subjects before making such change.

Principal Investigator

Trainee

79-016
Recd 7/12/79

SECTION T - RESEARCH PROTOCOL

le: ISOLATION OF VIBRIO CHOLERAE TRANSDUCING PHAGE

ncipal Investigator: John R. Murphy, Ph.D.

rting Date: November 7, 1979

pletion Date: December 20, 1979

al Direct Costs:

ilability of Funds:

(a) Scientific Director's Remarks:

(b) Controller's Remarks:

tract Summary:

recent years there has been increased emphasis on the development of live
al vaccine strains of Vibrio cholerae. Mutant strains which produce extremely
w levels of cholera enterotoxin or defective toxin have been isolated in
veral laboratories. These strains have not been completely genetically characterized
cause of the lack of appropriate genetic methology. If successful, isolation of
cholerae transducing phage will allow for the development of the methodology
cessary for the genetic characterization and genetic construction of candidate
ve oral vaccine strains. To date transducing phage have not been described for
cholerae; however, such phage have been isolated in a number of other prokaryotic
nera. Transducing phage are detected by their ability to carry bacterial genes
to recipient cells in which the corresponding gene is inactive because of a
tation. Transducing phage then will complement the bacterial mutation and the
sogen will be prototrophic.

riews:

Research Involving Human Subjects: _____

Research Review Committee: _____

Director: _____

BMRC: _____

Controller/Administrator: _____

Isolation of Vibrio cholerae Transducing Phage

A. INTRODUCTION

1. Objective. The overall objective of this proposal is to isolate and characterize transducing phage(s) for Vibrio cholerae El Tor and Classical biotypes. Since transducing phage mobilize regions of the bacterial chromosome, these phage will provide a means for genetic characterization of potential live oral vaccine strains of V. cholerae that has not previously been available. In addition, transducing phage may also play an important role in the genetic construction of live oral vaccine strains.

2. Background. In general, the genetic characterization of prokaryotes has relied heavily upon results obtained by bacterial conjugation, transduction, and in some cases, transformation and/or transfection. In V. cholerae genetic mapping has been performed using the sex factor P to mobilize the bacterial chromosome. The frequency of mobilization of genetic markers from P^+ donor strains to P^- recipients is on the order of 10^{-6} to 10^{-8}. Unlike the case of Escherichia coli where high frequency recombinant (Hfr) donor strains (3) are readily available, Hfr strains of V. cholerae have not been isolated. The major limitation of P - factor mapping is the low frequency of marker transfer between donor and recipient strains. Recently this problem has been largely eliminated by the development of genetic mapping by transposon facilitated recombination (Tfr) (6). This method takes advantage of the homology of the transposon Tn-1 introduced into both the P^- factor and at random sites in the vibrio chromosome to stimulate recombination and as a result the frequency of marker transfer between donor and recipient strain is greatly enhanced. Johnson and Romig (6) have shown that the frequency of marker transfer is increased by 10^2 to 10^3. Genetic mapping in V. cholerae is now practical, and by using the Tfr system Johnson and Romig (6) have shown that the vibrio chromosome is, in fact, circular and many regions of the El Tor and Classical genomes appear to be markedly similar.

In studies of the molecular genetics of toxinogenesis in V. cholerae, Mekalanos et al. (10) have shown that the genetic locus conferring hyper-

production (htx) of cholera toxin maps between the streptomycin (str) and rifampin (rif) resistance loci. Mekalanos and Murphy (9) have further shown that mutations in this region of the chromosome result in a hypotoxinogenic (Ltx) phenotype. It is important to note that this locus, as well as the tox-1 locus described by Vasil et al. (27) and Holmes et al. (5) are clearly not the structural genes for cholera toxin.

Transducing phage for V. cholerae have, as yet, not been isolated. Transducing phage, in general, contain substitutions of bacterial DNA for phage DNA, and are detected by their ability to carry bacterial genes into a recipient cell in which the corresponding genes have been inactivated by mutation. Transducing phage has been isolated from Escherichia coli (11), Shigella sp. (7), Salmonella (7), Pseudomonas sp. (4), Staphylococcus (12), Bacillus subtilis (26), as well as many other bacterial genera. Therefore, the proposed isolation of transducing phages for V. cholerae is expected to be successful.

The isolation of vibrio transducing phage will, in theory, allow for the specific excision, and subsequent enrichment of any V. cholerae chromosomal gene. Subsequent analysis of the transducing phage DNA will allow for complete molecular genetic characterization. For example, in the development of live oral vaccine strains for cholera one would like to introduce a deletion mutation into the cholera toxin genes in order to insure genetic stability. The isolation of a transducing phage that carries the cholera toxin structural genes will allow us to characterize the nature of mutations down to the level of DNA sequence and allow the complete characterization of mutations in potential live oral vaccine strains.

Transformation and/or transfection with genetic information has not, as yet, been reported for V. cholerae. These methods of gene manipulation will be developed separately and will not require research support under this proposal.

Much of the prior work with vibrio phage has lead to the successful development of an effective vibrio phage typing scheme. This scheme has been used for epidemiologic characterization of outbreaks of cholera disease. In addition to the development of a vibrio phage typing scheme,

Mukerjee (13,14,15,16,17,18,19,20), Chatterjee and coworkers (1, 2, 8) and others have studied many of the properties of vibriophage. Takeya and coworkers (21,22,23,24,25) have studied the kappa-type phages of El Tor vibrios. Clearly, there is a rich literature on the isolation and characterization of vibriophage from the environment, from the stools of cholera patients (24), and from strains of V. cholerae.

3. Rationale. The rationale of this proposal is based upon the prior isolation of transducing phage in many other bacterial genera. There is every theoretical basis for the isolation of transducing pahge for V. cholerae. Funds are requested in this proposal to conduct the primary isolation of transducing phage from (*i*) the environment, (*ii*) the stool of cholera patients, (*iii*) the ultraviolet light induced lysates of V. cholerae strains, and (*iv*) high titre phage stocks in phage collections. The environmental sources and culture collections that will be available will provide the best opportunity for the isolation of V. cholerae transducing phages.

B. SPECIFIC AIMS

1. Isolation of specific and/or generalized transducing phage for Vibrio cholerae El Tor and Classical biotypes.

C. METHODS OF PROCEDURE

1. Background. As described above transducing phage are recognized by their ability to carry bacterial genes into recipient cells in which the corresponding genes have been inactivated by mutation. For example, V. cholerae has the ability to grow on a simple defined medium: glucose and minimal salts. Following mutagenesis strains that require specific nutrients (e.g., histidine) for growth can be readily isolated. A strain that requires histidine will not grow on glucose plus salts, but will grow on glucose, salts, plus histidine. Infection of this strain by a transducing phage that carries the histidine region of the vibrio chromosome will relieve the requirement for histidine in the growth medium and the lysogen will now have the ability to grow on glucose plus salts medium.

2. Bacterial strains. The principal investigators will bring a series of auxotrophic mutants carrying single requirements for purine and amino acids for growth. These strains will not grow on glucose salts medium and their rates of reversion to prototrophy have been determined.

3. Isolation of transducing phage. In most all prokaryotic systems that have been examined the frequency of transducing phage in lysates is on the order of 10^{-8}. Since the frequency of transduction is low, these lysates are called LFT (low frequency transducing). LFT lysates of V. cholerae will be prepared by ultraviolet light induction of strains isolated from patients, the environment, and culture collections that are available. It has been previously recognized that most all strains of V. cholerae carry temperate phage (23). Vibrio LFT lysates will also be prepared from universal indicator strain isolation of phage from environmental sources, as well as from .22μ filtrates of patients stools.

LFT lysates will be mixed with each of the auxotrophic strains and following incubation at 30°C for 20 minutes, cultures will be plated on minimal medium (glucose plus salts). Following incubation at 30°C for 36 hours, plates will be examined for colonies. Colonies which appear may be due to either (*i*) reversion of the existing mutation, or (*ii*) to infection with a transducing phage which complements the existing mutation. These two possibilities can be easily tested by ultraviolet irradiation of these colonies for induction of phage, followed by infection of the auxotrophic test strain, incubation, and replating. Revertants will not give rise to transducing phage; whereas, if transducing phage are present they will be amplified and the lysate will be called HFT (high frequency transducing). The titre of HFT lysates should be on the order of 10^6 - 10^{10} transducing phage per ml.

). SIGNIFICANCE

The currently available methods for the molecular genetic characterization of mutations in V. cholerae are insufficient. The development of potential live oral vaccine strains is therefore impeded. At present we can not make rational predictions about rates of reversion of candidate vaccine strains to wild type phenotype. The successful development of live oral vaccine strains and their introduction into cholera endemic

area is dependent upon (*i*) their efficacy, and (*ii*) their genetic stability. The understanding of the molecular genetic basis of all mutations introduced into candidate vaccine strains is essential. The isolation of V. cholerae transducing phage will provide another method for the genetic characterization and construction of candidate live oral vaccine strains.

E. FACILITIES REQUIRED

1. Office space: not required.
2. Laboratory space: The facilities required for the proposed work includes sufficient laboratory space for 2 persons, incubator (roller drum) or water bath for ~ 100 culture/day, and media preparation.
3. Hospital Resources: not required. Stool samples from patients with confirmed cholera will be obtained.
4. Animal Resources: not required.
5. Logistical Support: not required.
6. Major items of equipment: none required.
7. Special Requirements: none.

F. COLLABORATIVE ARRANGEMENTS

The study proposed is considered to be collaborative. Authorship on publications that may result will be mutually determined by the principal investigators and the ICDDR,B, Dacca supervisor.

Section III - BUDGET

1. Personnel Services:

	Nov. 7 - Dec. 20	
	% effort	
(a) Professional		
John R. Murphy	100%	
David Relman	100%	
Imdadul Huq	50%	$360.00
(b) Technical		
Q.S. Ahmed	100%	$225.00
(c) Secretarial		
None		
(d) Clerical		
None		

2. Supplies:

(a) Media

Glucose salts agar
Glucose salts broth
Casamino acid - Yeast Extract agar
Casamino acid - Yeast Extract broth
Petri dishes (700 - 1000/week) 4200 - 6000
Test tubes with caps
16 x 150mm - 2000

$ 925.00

Pipettes
10ml - 200
5ml - 200
1ml - 200
0.1ml - 800

$ 185.00

Filtration apparatus
Millipore Sweeney 10
Millipore .22μ filtrates 1000

$ 625.00

3. Equipment: none required
4. Patient Hospitalization: none required.
5. Outpatient Care: none required.
6. Transport:
7. Travel and Transportation of Persons $ 50.00

Local Travel - 2 trips for 4 persons

Overseas Travel

John R. Murphy - Calcutta, India - Dacca, Bangladesh (3 round trips) $ 108.00

David Relman - Boston, Massachusetts, U.S.A. - Dacca, Bangladesh (round trip) $1730.00

8. Transportation of Things: none
9. Rent, Communication and Utilities

David Relman - Guest house accommodations
Nov. 7, 1979 - Dec. 20, 1979 - 44 days X$15.00 $ 660.00

John R. Murphy - Guest house accommodations
total 2½ weeks - 17 days X $15.00 $ 255.00

10. Printing and Reproduction: none.
11. Other Contractual Services: none.
12. Construction, Renovation, Alterations: none.

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