NATIONAL CYSTIC FIBROSIS RESEARCH FOUNDATION
202 East 44th Street
New York, New York 10017

APPLICATION FOR RESEARCH SUPPORT

SECTION 1

TO BE COMPLETED BY PRINCIPAL INVESTIGATOR:

1. Abbreviated Title of Research Proposal: Incorporation of heterologous DNA into bacterial genomes

2. Type of Application: X New Project or ____ Reapplication

3. Dates of Entire Proposed Project Period: From: 7/1/69 Through: 6/30/72

4. Total Amount Requested for Entire Period: $45,000

   a) Amount Requested for First 12 month period: $15,000

5. Name of Principal Investigator: Joshua Lederberg

   a) Degree: Ph. D., Yale University

   b) Title of Position: Professor and Chairman

   c) Department, Service, Laboratory or Equivalent: Department of Genetics

   d) Major Subdivision: School of Medicine

   e) Mailing Address of Principal Investigator: Department of Genetics, Stanford University School of Medicine, Stanford, California 94305

   f) Telephone Data: Area Code: 415 & Telephone #321-1200 Ext. 5801

6. Identify Organizational Component Responsible for Conduct of Scientific Aspects of Project: School of Medicine

   a) Department: Genetics

   b) Address Where Research Will be Conducted: Stanford University School of Medicine, Stanford, California 94305

Abbreviated Title of Research Proposal: Incorporation of heterologous DNA into bacterial genomes.

* *

TO BE COMPLETED BY RESPONSIBLE ADMINISTRATIVE AUTHORITY

7) Applicant Institution: Stanford University (NAME)

a) (Address) (Street) Stanford, California 94305 (City) (State) (Zip Code)

8) Type of Organization:
a) Individual: ____
b) Public Institution: State: ____ Local: ____ Other: ____
c) Private Institution: Nonprofit: xx

9) Name, Title and Address of Official to Whom Checks should be Mailed:

Mr. Kenneth D. Creighton, Controller

Stanford University

Stanford, California 94305

10) Name and Title of Official Signing for Applicant Organization:

TERMS AND CONDITIONS

The undersigned agree to conform to the "Policies and Rules Governing National Cystic Fibrosis Research Foundation Research Grants".

11) Signature of Principal Investigator: Joshua Lederberg

12) Signature of Official Signing for Applicant Organization:

13) Name, Official Title and Department of All Professional Personnel Engaged on Project

Joshua Lederberg, Professor and Chairman, Department of Genetics

Research Associate (to be appointed)

Name, Official Title and Department of All Professional Personnel Engaged on Project (Continued)

14) Name and Address of Applicant Institution: Stanford University
Stanford, California 94305

15) Use This Space to Make a Broad Statement of your Research Objectives.
(Use Continuation Pages if Necessary)

See attached material, which was part of the application to the National Institutes of Health describing the project to which the present application is related.

SUPPORTING DATA

Summary Page

The DNA transfer system ("transformation") in Bacillus subtilis offers a particularly favorable opportunity to study the physical and chemical properties of DNA molecules in relation to their biological activity. Over the past several years my principal co-investigators have been Professor Walter F. Bodmer and Professor A. T. Ganesan. Their work now has become well differentiated and receives independent support, but the studies summarized in the attached publications were initiated during the current term of the grant.

The main questions we have examined have concerned:

The linkage system, as manifested in DNA transfer;

The fractionation of biologically active DNA, assigning different genetic activities to different average base compositions;

The effects of fragmentation of DNA molecules by physical shearing, and by nuclease attack;

The mechanism of integration of DNA in the course of the transformation process;

The association of the DNA replication process with a cell-membrane bound polymerase.

In collaboration with Professor Arthur Kornberg and other members of the Department of Biochemistry we had also attempted to demonstrate the replication of biologically active DNA by E. coli DNA polymerase. Those experiments were, however, unsuccessful--in considerable part, as we now know, because of our ignorance of polynucleotide ligase.

The main impact of these studies has been to verify the correspondence of the properties of genetic activity of DNA with distinctive sequences of nucleotides, and to provide some further technical facilities for the ultimate description of genetic information in chemical terms. During the period covered by this report many workers have entered similar fields and the literature now contains many examples of the building of new edifices on previously established foundations.

RESEARCH PLAN

SPECIFIC AIMS AND SIGNIFICANCE OF RESEARCH

As summarized in the Progress Report and List Publications, we have been committed for many years to study of the genetic functions of DNA in Bacillus subtilis.

When I moved to Stanford in 1959, I decided to shift my line of work to this direction from the sexual mechanisms in Escherichia coli. This was partly out of a sense of frustration about the opportunities for direct chemical analysis and intervention with "conjugal" as compared to "transforming" DNA.

During these years the specific research programs directed by Professors Walter Bodmer and A. T. Ganesan have become viable independent lines of work with their own support, and with which I now have a friendly connection rather than personal responsibility. For my own part, I have stressed some longer range gambles, most of which don't work. In addition, I must realistically admit to having progressively less time in which to concentrate on laboratory work under my own immediate direction. While I have administrative responsibility for some rather large programs of department- or school-wide scope, the present renewal application represents the only support for investigating my own ideas for research in chemical genetics. The budget remains at previous levels, which in view of cost increases over the years, reflects some retrenchment in its scope.

I propose now to concentrate on the natural or experimentally induced occurrence of insertions and inversions in B. subtilis. In enteric bacteria, mapping studies have shown a remarkable congruence of gene sequences among bacterial species which have already diverged significantly in DNA homology as tested by hybridization-reannealing experiments. Conservation of gene order is also evident in the organization and persistence of operons, showing coordinate repression; these are frequent in bacteria but rare in higher forms. Most or all of the examples of inversions found experimentally in bacteria can be explained either as methodological artifacts (1) or as consequences by crossing over of episome-bound segments with redundantly homologous segments of the whole DNA (2).

These observations were vague forerunners of the now accepted understanding that bacteria differ from eukaryons in 1) The simple organization of the chromosome as a single polynucleotide sequence, and 2) The absence of any common mechanism for "holo-ligation repair" of the chromosome. The recent clarification of the dark repair of single-strand breaks in DNA (reviewed in 3) has pointed up mechanisms of template-directed repair (hemiligation) in bacteria. These are fundamental to recovery from UV-damage, to recombination mechanisms, and probably to the post-editing of newly replicated DNA. There is, on the other hand, no good evidence for any normal mechanisms for joining or rejoining broken DNA strands except for hemiligation, i.e., when the broken strand is entwined with a complementary template strand that spans the broken ends.

It may be previous to define a process that may not exist, but we are then looking for holo-ligation, i.e., some way to join broken strands, or double helices,

without the help of a homologous template. The potential extent and limitations of holo-ligation relate to many current problems in chemical genetics and cell biology. These include, for example:

1) Genetic variation in recovery from X-ray damage and the mechanism of recovery from double-strand scissions.

2) A sharper confrontation with chromosome structure of eukaryons in which broken chromosomes can indeed be rejoined (but is this a covalent nucleotide assembly?)

3) Transcription problems: framing, the reading of inverted sequences, and strand selection.

4) The barrier to promiscuous recombination of DNA from different sources.

Point 4 may be the most far-reaching for every level of genetic manipulation, both investigative and applied. The rejection of foreign DNA by competent Bacillus subtilis cells may illustrate one evolved mechanism whereby a cell has been able to protect itself against unlimited intrusion by foreign genes, arising by chemical scrambling or by virus infection. The research utility of freely moving genes from another species into bacteria needs no elaboration, e.g. for the study of transcription-control, and to facilitate analyzing the genetic competence of DNA from differentiated tissues of higher organisms.

Important practical utilities would follow from the incorporation of human genes into suitable cryptic virus DNA for kind of transductional therapy for human genetic disease (4).

(Our work does not, however, involve the use of human subjects in the sense of PHS regulations on such research. For experimental convenience we will concentrate on the B. subtilis transforming system and DNA from the same, related and distant bacterial species. Eukaryotic DNA may be tested, probably only incidentally, unless very promising leads turn up in the bacterial work.)

METHOD OF PROCEDURE

The general experimental procedures will follow those used and developed in our previous work on B. subtilis, which are common to most workers in DNA biology today. They are detailed in the publications listed in the progress report.

Our research aim can be narrowly expressed as an effort to detect or contrive the holo-ligation of DNA molecules, i.e., effectively joining DNA strands with the direction of an existing complementary strand. Approaches to this aim may be further classified by some orthogonal labels like:

I. Direct Attacks:

Ligation of single or double stranded segments

Natural or contrived occurrence

DNA from same or different species (we will concentrate on B. subtilis at last for the acceptor chromosome)

Mediated by enzymes (in intact cells or purified extracts) or by organic chemical synthetic linkers

Enzymes of related or from other species, e.g. fertilized eggs and somatic cells which are known to be competent to repair chromosome breaks

II. Indirect Contrivances - Exploiting existing principles of recombination:

Rare coincidental homologies, possible with low efficiency of pairing among existing DNA's. Select on a large scale for rare events like gene inversion, introgression and study the progeny. Mistreat the cells in various ways designed to break DNA.

Nuclease deficient mutants - slower to edit or reject unpaired foreign DNA or the use of nuclease inhibitors.

Use input DNA previously cycled through a replication step with B. subtilis polymerase to exclude other labels that may mark DNA as "foreign", such as methylation.

Attach homopolymer ends to input DNA with terminal deoxyribonucleotidotransferase (4). Then consider various tricks to incorporate the homopolymer extensions as insertions into the bacterial DNA. For an example, see Fig. 1.

Once artificial sequences have been inserted it should be possible to introduce arbitrary genes by crossing over if these DNA segments are previous extended. Even more fanciful tricks can be speculated about.

These experiments raise the question of "fooling" the cell into inserting homopolyomer segments at the growing point of its own DNA, e.g., the attachment of $(ATAT)_n$ strains under guanine deprivation. The formation of poly-AT by GC-deprived DNA polymerase is well known. Small ATA segments in the DNA may allow poly-AT to be attached by polymerase in vivo under special conditions.

III. Ancillary Information Needed

1. Is the rejection of foreign DNA based on nuclease attack on unpaired DNA strands? We need a closer study of the fate of such material. (cf. 5)

2. Can short DNA segments be hololigated by chemical cross-linking agents? If so, will polymerase skip across such unnatural links with no more damage than a short deletion? The only basis for such a speculation is the supposition that mutagenesis by acridines involves a deletion implying some skip mechanism, not necessarily useful here. It should, however, be easy to test for biological activity of cross-ligated DNA.

3. What controls the spontaneous formation of poly (AT) in guanine-starved cells as compared to free polymerase? Preliminary experiments suggest that feedback inhibition of the triphosphate pool under G-deprivation may keep dAPPP and dTPPP from accumulating in intact cells. Nearest neighbor studies

are under way to determine the product into which exogenous, labelled dTP*PP is incorporated by toluene-treated bacteria under G-deprivation.

These forays are all rather opportunistic. It is not realistic to pursue all of these lines simultaneously, but the strategic outlook changes with each day's laboratory work, and it is not easy to predict nine months in advance which experiment will be done the following day. This concern for flexible strategy must also take into account the intense flow of new literature* and the highly relevant biochemical work being done in sister departments. By my present view, however, we should think of concentrating on:

1. Search for altered linkage arrangements in B. subtilis as a spontaneous occurrence, after mutagenic treatment of cells, and after chemical ligation of purified DNA.

   These new linkages should be fairly easy to select for by the examination of progeny of exceptional co-transfers of formerly unlinked markers. In addition. physical separation of DNA fractions by basic composition (melting point, buoyant density) can prefilter the heterogeneous DNA being tested.

2. Test the biological activity of DNA to which homopolymer ends have been added by a terminal transferase (b).

3. Long-shot trials of introgression of markers from distant species.

4. Optimizing the now low, but definite, rates of transfer from DNA of related bacilli. To do this may take special conditions, pre- or post-uptake, for example, annealing the foreign with recipient DNA, or finding mutant recipient bacteria deficient in "rejecting enzymes".

FACILITIES AVAILABLE

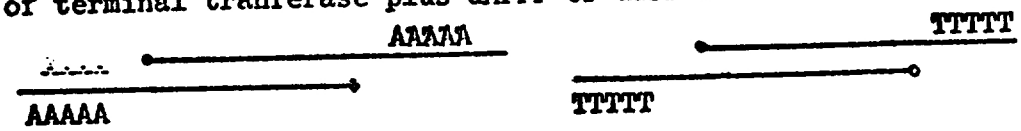
We were severely burdened by space limitations until the funding and construction of the Joseph P. Kennedy, Jr. Laboratories for Molecular Medicine, which were completed in April, 1966. Here we are fortunate to be compactly and efficiently housed in new laboratories in a way that encourages the sharing of equipment and the interchange of ideas among students and staff having a wide range of day-to-day technical preoccupations and broader interests.

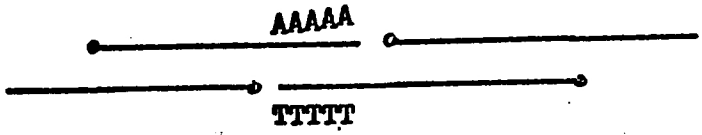
Major items of equipment available include:

Preparative and analytical ultracentrifuges: Beckman Model L and Model E, 3 International B-60's, Servall
Packard Tricarb Scintillation Counter; Packard Liquid Spectrometer System
Fraction Collectors
Zeiss Spectrophotometer; Fluorometer
B.& L. Recording Spectrophotometer
Gas chromatographic columns
LINC computer
Terminal access to computer facility in the Medical School.

Fig. 1. Hypothetical scheme for inserting artificial homopolymer sequences into DNA with terminal transferase and (hemi-)ligase.

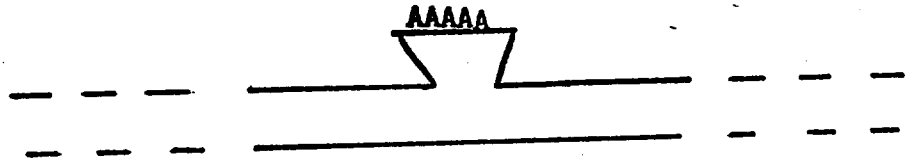
In two separate batches, prepare the following extended DNA's with the use of terminal tranferase plus dAPPP or dTPPP:



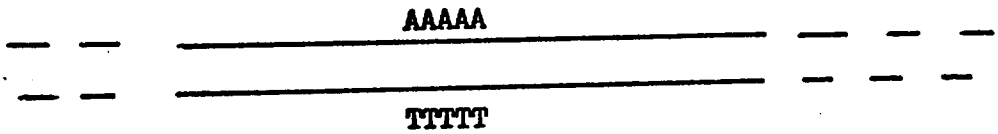


3. repair gaps with ligase (this may or may not be necessary as an explicit step in vitro, depending on the efficiency of such repair after uptake. However, it may be desirable nevertheless to fractionate the repaired material to concentrate it before putting it into competent cells.)

4. uptake by competent bacteria; integration in vivo.



5. further replication cycles, with inserted -AAAA- or -TTTT-



References (limited to those which clarify the discussion):

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2. Berg, Claire M. and Roy Curtiss III, 1967. Transposition derivatives of an Hfr strain of Escherichia coli K-12. Genetics 56:503-525.

3. Hanawalt, Philip C., 1968. Cellular recovery from photochemical damage. Ch. 11, Photophysiology, Vol. III (A. C. Giese, ed.) Academic Press, New York.

4. Rogers, Stanfield, 1966. Shope papilloma virus: A passenger in man and its significance to the potential control of the host genome. Nature 212:1220-1222.

5. Bodmer, W.F. and A. T. Ganesan, 1964. Biochemical and genetic studies of integration and recombination in Bacillus subtilis transformation. Genetics 50:717-738.

6. Krakow, J.S., C. Coutsogeorgopoulos and E. S. Canellakis, 1962. Studies on the incorporation of deoxyribonucleotides and ribonucleotides into deoxyribonucleic acid. Biochim. et Biophys. Acta 55:639-650.

Bollum, F. J., Eleanor Groeniger and Masahiko Yoneda, 1964. Polydeoxyadenylic acid. Proc. Nat. Acad. Sci. 51:853-859.

Relevant publications from Stanford Genetics Department laboratory:

Bodmer, Walter F. and A. T. Ganesan 1964. "Biochemical and Genetic Studies of Integration and Recombination in Bacillus subtilis Transformation" Genetics 50, 4: pp 717-738.

Nester, Eugene W., M. Schafer, J. Lederberg 1963. "Gene linkage in DNA transfer: A cluster of genes concerned with aromatic biosynthesis in Bacillus subtilis". Genetics 48, 4: pp 529-551.

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