SECTION I

Form Approved
O.M.B. 68-R0249

DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

GRANT APPLICATION

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TYPE	PROGRAM	NUMBER
2	R01	CA 16896-19
REVIEW GROUP		FORMERLY
MBC		GEN
COUNCIL (Month, Year)		DATE RECEIVED
Jan 77		5-28-76

TO BE COMPLETED BY PRINCIPAL INVESTIGATOR (Items 1 through 7 and 15A)

1. TITLE OF PROPOSAL (Do not exceed 53 typewriter spaces)

Genetics of Bacteria

2. PRINCIPAL INVESTIGATOR

2A. NAME (Last, First, Initial)
Lederberg, Joshua

2B. TITLE OF POSITION
Professor and Chairman

2C. MAILING ADDRESS (Street, City, State, Zip Code)
Stanford University School of Medicine
Department of Genetics
Stanford, California 94305

2D. DEGREE
Ph.D.

2E. SOCIAL SECURITY NO.
[redacted]

2F. TELEPHONE DATA — Area Code: 415 — TELEPHONE NUMBER AND EXTENSION: 497-5801

2G. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT (See Instructions)
Department of Genetics

2H. MAJOR SUBDIVISION (See Instructions)
School of Medicine

3. DATES OF ENTIRE PROPOSED PROJECT PERIOD (This application)
FROM: 05-01-77 THROUGH: 04-30-82

4. TOTAL DIRECT COSTS REQUESTED FOR PERIOD IN ITEM 3: $464,669

5. DIRECT COSTS REQUESTED FOR FIRST 12-MONTH PERIOD: $80,000

6. PERFORMANCE SITE(S) (See Instructions)
Department of Genetics
Stanford University School of Medicine
Stanford, California 94305

7. Research Involving Human Subjects (See Instructions)
A. [X] NO B. [ ] YES Approved: ________ Date
C. [ ] YES – Pending Review

8. Inventions (Renewal Applicants Only - See Instructions)
A. [X] NO B. [ ] YES – Not previously reported
C. [ ] YES – Previously reported

TO BE COMPLETED BY RESPONSIBLE ADMINISTRATIVE AUTHORITY (Items 8 through 13 and 15B)

9. APPLICANT ORGANIZATION(S) (See Instructions)
Stanford University
Stanford, California 94305
IRS. Number 94-1156365
Congressional District Number 12

10. NAME, TITLE, AND TELEPHONE NUMBER OF OFFICIAL(S) SIGNING FOR APPLICANT ORGANIZATION(S)
D'Ann B. Downey
Sponsored Projects Officer
Sponsored Projects Office

Telephone Number (s) (415) 497 2883

11. TYPE OF ORGANIZATION (Check applicable item)
[ ] FEDERAL [ ] STATE [ ] LOCAL [X] OTHER (Specify) Private Non-Profit University

12. NAME, TITLE, ADDRESS, AND TELEPHONE NUMBER OF OFFICIAL IN BUSINESS OFFICE WHO SHOULD ALSO BE NOTIFIED IF AN AWARD IS MADE
K.D. Creighton
Associate Vice President - Controller
Stanford University
Stanford, California 94305

Telephone Number (415) 497 2251

13. IDENTIFY ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR INSTITUTIONAL GRANT PURPOSES (See Instructions)
01 - School of Medicine

14. ENTITY NUMBER (Formerly PHS Account Number)
IRS Number - 94-1156365

15. CERTIFICATION AND ACCEPTANCE. We, the undersigned, certify that the statements herein are true and complete to the best of our knowledge and accept, as to any grant awarded, the obligation to comply with Public Health Service terms and conditions in effect at the time of the award.

SIGNATURES (Signatures required on original copy only. Use ink, "Per" signatures not acceptable)		DATE
	A. ~~SIGNATURE OF PERSON~~ NAMED IN ITEM 2A	
	B. SIGNATURE(S) OF PERSON(S) NAMED IN ITEM 10	

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DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

LEAVE BLANK

PROJECT NUMBER

RESEARCH OBJECTIVES

NAME AND ADDRESS OF APPLICANT ORGANIZATION

Department of Genetics
Stanford University School of Medicine, Stanford, California 94305

NAME, SOCIAL SECURITY NUMBER, OFFICIAL TITLE, AND DEPARTMENT OF ALL PROFESSIONAL PERSONNEL ENGAGED ON PROJECT, BEGINNING WITH PRINCIPAL INVESTIGATOR

Joshua LEDERBERG [redacted]	Professor and Chairman	Department of Genetics
Stanislav EHRLICH [redacted]	Research Associate	" " "
Hela Bursztyn [redacted]	Sr. Research Assistant	" " "

TITLE OF PROJECT

Genetics of Bacteria

USE THIS SPACE TO ABSTRACT YOUR PROPOSED RESEARCH. OUTLINE OBJECTIVES AND METHODS. UNDERSCORE THE KEY WORDS (NOT TO EXCEED 10) IN YOUR ABSTRACT.

We are studying the behavior and expression of DNA segments from Bacillus subtilis cloned in various vectors, in E. coli and back in B. subtilis. We will focus particularly on (a) the steps by which heterologous plasmids can deliver inserted segments for chromosomal integration in the course of transformation, and (b) the development of probes for t-RNA.

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DETAILED BUDGET FOR FIRST 12-MONTH PERIOD

FROM 05-01-77 THROUGH 04-30-78

DESCRIPTION (Itemize) PERSONNEL NAME	TITLE OF POSITION	TIME OR EFFORT %/HRS.	AMOUNT REQUESTED (Omit cents) SALARY	FRINGE BENEFITS	TOTAL
Lederberg, Joshua	PRINCIPAL INVESTIGATOR	15%	----	----	----
Ehrlich, Stanislav	Research Associate	100%	18,140	3,559	21,699
Bursztyn, Hela	Sr. Research Assistant	100%	16,316	3,201	19,517
Evans, Peter	Lab Technician	100%	12,592	2,471	15,063
Hollier, Sadie	Glassware Washer	100%	10,064	1,975	12,039
Staff Benefits @ 18.8% 5/1/77 - 8/31/77					
20 % 9/1/77 - 4/30/78					
			57,112	11,206	68,318

Item	TOTAL
CONSULTANT COSTS	------
EQUIPMENT Replacement centrifuge or similar parts	2,000 *
SUPPLIES Petri Dishes $1,700 Chemicals 3,700 Glassware & Pipetts 1,000 Isotopes 1,000 Media 1,300	8,700
TRAVEL — DOMESTIC	------
TRAVEL — FOREIGN	------
PATIENT COSTS (See instructions)	------
ALTERATIONS AND RENOVATIONS	------
OTHER EXPENSES (Itemize) Books, Reference $ 215 Equipment Maintenance and Repair 275 Postage 110 Communications 382	982
TOTAL DIRECT COST (Enter on Page 1, Item 5) →	$80,000

INDIRECT COST (See Instructions)

DATE OF DHEW AGREEMENT:

______ % S&W*

58% %NTDC* 19 September 1975

☐ WAIVED

☐ UNDER NEGOTIATION WITH:

*IF THIS IS A SPECIAL RATE (e.g. off-site), SO INDICATE.

BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED FROM PUBLIC HEALTH SERVICE
DIRECT COSTS ONLY (Omit Cents)

DESCRIPTION		1ST PERIOD (SAME AS DETAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED *(This application only)*					
			2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	7TH YEAR
PERSONNEL COSTS		68,318	73,442	78,950	84,871	91,236		
CONSULTANT COSTS *(Include fees, travel, etc.)*		------	------	------	------	------		
EQUIPMENT		2,000	2,150	2,310	2,483	2,670		
SUPPLIES		8,700	9,353	10,054	10,808	11,619		
TRAVEL	DOMESTIC	------	------	------	------	------		
	FOREIGN	------	------	------	------	------		
PATIENT COSTS		------	------	------	------	------		
ALTERATIONS AND RENOVATIONS		------	------	------	------	------		
OTHER EXPENSES		982	1,056	1,135	1,220	1,312		
TOTAL DIRECT COSTS		80,000	86,001	92,449	99,382	106,837		

TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD *(Enter on Page 1, Item 4)* ⟶ $ 464,669

REMARKS: ***Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)***

Years 02 - 05:

Equipment Costs - Replacement parts for existing equipment

Personnel - Annual increase = 7.5%

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
LEDERBERG, JOSHUA	Professor and Chairman Department of Genetics	5-23-25

PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX
Montclair, New Jersey	USA	[X] Male [ ] Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Columbia College, New York	B.A.	1944	
College of Physicians & Surgeons, Columbia Univ., New York (1944-46)			
Yale University	Ph.D.	1947	Microbiology

HONORS

1957 - National Academy of Sciences
1958 - Nobel Prize in Medicine

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Genetics, chemistry and evolution of unicellular organism and of man	Principal Investigator

RESEARCH SUPPORT (See instructions)

Please see attached list

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

1959-present	Professor and Chairman, Department of Genetics, Stanford University School of Medicine
1957-1959	Chairman, Department of Medical Genetics, University of Wisconsin
1947-1957	Professor of Genetics, University of Wisconsin

Publications:

1. Lederberg, J., 1959. A view of genetics, Les Prix Nobel en 1958, 170-89.
2. Nestar, E.W. and J. Lederberg, 1961. Linkage of genetic units of Bacillus subtilis in DNA transformation. PNAS 47:52-55.
3. Nestar, E.W., A.T. Ganesan and J. Lederberg, 1963. Effects of mechanical shear on genetic activity of Bacillus subtilis DNA. PNAS 49:61-68.
4. Ganesan, A.T. and J. Lederberg, 1964. Physical and biological studies on Bacillus subtilis transforming DNA. J. Mol. Biol. 9:683-695.
5. Ganesan, A.T. and J. Lederberg, 1965. A cell membrane bound fraction of bacterial DNA. Biochem. Biophys. Res. Com. 18:824-835.
6. Lederberg, J., 1966. Experimental genetics and human evolution. American Naturalist 100:519-531. Bull. Atom. Sci. 22:4-11.
7. Halpern, B., J.W. Westley, I. von Wredenhagen and J. Lederberg, 1965. Optical resolution of D,L amino acids by gas chromatography and mass spectrometry. Biochem. Biophys. Res. Com. 20:710-714.

(see continuation page)

Privileged Communication

Section II - Biographical Sketch
Lederberg, Joshua

Publications - continued:

8. Ciferri, O., S. Barlati and J. Lederberg, 1970. Uptake of synthetic polynucleotides by competent cells in Bacillus subtilis. J. Bact. 104:684-688.

9. Lederberg, J., 1972. Use of computer to identify unknown compounds: the automation of scientific inference in Biochemical Applications of Mass Spectrometry, ed. G.R. Walter, John Wiley & Sons, New York, p. 193-207.

10. Lederberg, J., 1972. Biological innovation and genetic intervention in Challenging Biological Problems - Directions Toward Their Solution (American Institute of Biological Sciences 25th Anniversary Volume, John A. Behnke, ed. Oxford University Press, New York, p. 7-27.

11. Patton, W., V. Bacon, A.M. Duffield, B. Halpern, Y. Hoyano, W. Pereira and J. Lederberg, 1972. Chlorination Studies. I. The reaction of aqueous hypochlorous acid with cytosine. Biochem. Biophys. Res. Commun. 48:880-884.

12. Lederberg, J., 1972. The control of chemical and biological weapons. Stanford J. International Studies 7:22-44.

13. Smith, D.H., B.G. Buchanan, R.S. Engelmore, A.M. Duffield, A. Yeo, E.A. Feigenbaum, J. Lederberg, C. Djerassi, 1972. Applications of artificial intelligence for chemical inference. VIII. An approach to the computer interpretation of the high resolution mass spectra of complex molecules. Structure elucidation of estrogenic steroids. J. Am. Chem. Soc. 94:5962-5971.

14. Masursky, Harold et al. (J. Lederberg included), 1972. Mariner 9 television reconnaissance of Mars and its satellites: preliminary results. Science 175:294-305.

15. Klein, H.P., J. Lederberg, A. Rich, 1972. Biological experiments: the Viking Mars Lander. Icarus 16:139-146.

16. Sagan, C. et al. (J. Lederberg included), 1972. Variable features on Mars: preliminary Mariner 9 television results. Icarus 17:346-372.

17. Lederberg, J., 1974. "A System-analytic Viewpoint" in How Safe is Safe? - The Design of Policy on Drugs and Food Additives. National Academy of Sciences, Washington, D.C., p. 66-94.

18. Masinter, L., N. Sridharan, J. Lederberg, D.H. Smith, 1974. Application of artificial intelligence for chemical inference. XII. Exhaustive generation of cyclic and acyclic isomers. J. Am. Chem. Soc. 96:7702-7714.

19. Sagan, C. J. Veverka, P. Fox, R. Dubisch, R. French, P. Gierasch, L. Quam, J. Lederberg, E. Levinthal, R. Tucker, B. Eross, 1973. Variable features on Mars, 2, Mariner 9 global results. J. Geophys. Research 78:4163-4196.

20. Harris-Warrick, R.M., Y. Elkana, S.D. Ehrlich and J. Lederberg, 1975. Electrophoretic separation of B. subtilis genes ($EcoR_I$/agarose gel electrophoresis). Proc. Nat. Acad. Sci. USA 72:2207-2211.

21. Bursztyn, H., V. Sgaramella, O. Ciferri and J. Lederberg, 1975. Transfectability of rough, gal^- strains of Salmonella typhimurium. J. Bact. 124:1630-1634.

Privileged Communication

Section II - Biographical Sketch
Lederberg, Joshua

Publications - continued:

22. Sgaramella, V., S.D. Ehrlich, H. Bursztyn and J. Lederberg, 1976. Biological activity and molecular structure of DNA. I. Enhancement of transfecting activity of bacteriophage P22 DNA upon exonucleolytic erosion. (Running title: Exonuclease and transfecting P22 DNA). J. Mol. Biol. (accepted for publication).

23. Ehrlich, S.D., V. Sgaramella and J. Lederberg, 1976. Biological activity and molecular structure of DNA. II. Bacteriophage T7 DNA transfects $CaCl_2$-treated E. coli cells and is activated by erosion with lambda exonuclease. (Running title: Exonuclease and transfecting T7 DNA). J. Mol. Biol. (accepted for publication).

24. Shih, K. and J. Lederberg, 1976. Effects of chloramine on Bacillus subtilis DNA. J. Bact. (accepted for publication).

25. Shih, K. and J. Lederberg, 1976. Chloramine mutagenesis in B. subtilis. Science (accepted for publication).

Privileged Communication - Section II [redacted]

Lederberg, Joshua

RESEARCH SUPPORT

GRANT NO.	TITLE OF PROJECT	CURRENT YEAR	PROJECT PERIOD	% OF EFFORT	GRANT AGENCY
Dr. Lederberg: personal research commitments					
5ROI CA16896-18	Genetics of Bacteria	$70,000 5/76-4/77	$195,000 5/74-4/77	15	NIH
NAS1-9692	Viking Mission Participation	$42,500 1/76-6/76	$62,572 1/75-3/77	5	NASA
Dr. Lederberg also functions as Principal Investigator ex officio on the following program-projects and training grants:					
NGR-05-020-632	Analytical Methodology for Biochemical Monitoring	$60,000 5/75-4/76	$180,000 5/73-4/76	2	NASA
NO1 CB 43902	Biomedical Markers that May Presage the Presence of Cancer	$95,000 6/75-6/76	$183,108 6/74-6/76	5	NIH
3TOI GM00295	Genetics Training Grant (graduate research training)	$121,000 7/75-6/76	$916,637 7/74-6/79	10	NIH
1T22 GM00198-02	Postdoctoral Training Medical Genetics	$48,133 7/75-6/76	$144,133 7/74-6/77	5	NIH
1P07 RR00785-03	Stanford University Medical Experimental Computer: National Computer Resource for Research on AI in Medicine	$358,000 8/75-7/76	$3,092,226 10/73-7/78	10	NIH
NGR-05-020-004	Instrumentation for Planetary Exploration	$110,000 9/75-8/76	$110,000 9/75-8/76	5	NASA
GM20832-02	Genetics Research Project	$241,432 5/76-4/77	$1,292,113 5/74-4/79	10	NIH

A personal note.

The Viking mission will have been consumated before this cycle begins and there is not likely to be any other avenue in the near future for study of extra-terrestrial biology (to speak to one source of distraction). While I continue to do some work for W.H.O. and similar agencies, I no longer take the active (and time-consuming) role in representing the ideals of science to the public that I did 5 years ago. In the previous renewal I expressed my hope and commitment to return again to an unqualified involvement in experimental molecular genetics. I believe this has been demonstrably successful and hope to continue in that way.

Joshua Lederberg

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Stanislav EHRLICH	Research Associate	12/6/43

PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX
Zagreb, Yugoslavia	Yugoslavia (J-1)	☒ Male ☐ Female

EDUCATION *(Begin with baccalaureate training and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
University of Zagreb, Yugoslavia	Lic. Sci.	1967	Organic Chemistry and Biochemistry
Institut de Biologie Moleculaire de la Faculte des Sciences de Paris, France	Ph.D.	1972	Organic Chemistry

HONORS

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
	Research Associate

RESEARCH SUPPORT *(See instructions)*

none

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)*

1975-present Research Associate, Department of Genetics, Stanford U. Medical School

1973-1975 Research Fellow, Department of Genetics, Stanford U. Medical School

1970-1972 Institut de Biologie Moleculaire, Paris, France

1968-1970 Laboratory of Dr. G. Bernardi (Centre de Recherches sur les Macromolecules), Strasbourg, France

1967-1968 Organic Chemistry and Stereochemistry Laboratory (Chief Dr. V. Skaric), Institut "R. Boskovic", Zagreb, Yugoslavia

<u>Publications:</u> see attached list

NIH 398 (FORMERLY PHS 398)
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☆ U. S. GOVERNMENT PRINTING OFFICE : 1974 584-253/3004

Section II - Biographical Sketch
Ehrlich, Stanislav

LIST OF REPRESENTATIVE PUBLICATIONS

Stanislav D. Ehrlich

S.D. Ehrlich, J.P. Thiery and G. Bernardi
Analysis of nucleosides on columns of fractionated Sephadex G 10,
Biochim. Biophys. Acta, 246:161 (1971).

S.D. Ehrlich, G. Torti and G. Bernardi
Studies on acid DNase. IX. 5'-hydroxy-terminal and penultimate
nucleotides of oligonucleotides obtained from calf thymus DNA,
Biochemistry, 10:2000 (1971).

U. Bertazzoni, S.D. Ehrlich and G. Bernardi
Analysis of labeled 3'-terminal nucleotides of DNA fragments,
Methods Enzymol., 29:355 (1974).

A. Devillers-Thiery, S.D. Ehrlich and G. Bernardi
Composition of the 3'-phosphate penultimate nucleotides released from
calf thymus DNA by spleen acid DNase,
Eur. J. Biochem., 38:416 (1973).

J.P. Thiery, S.D. Ehrlich, A. Devillers-Thiery and G. Bernardi
Specificity of spleen acid DNase,
Eur. J. Biochem., 38:434 (1973).

S.D. Ehrlich, U. Bertazzoni and G. Bernardi
Specificity of pancreatic DNase,
Eur. J. Biochem., 40:143 (1973).

S.D. Ehrlich, U. Bertazzoni and G. Bernardi
Specificity of *E. coli* endonuclease I,
Eur. J. Biochem., 40:149 (1973).

G. Bernardi, S.D. Ehrlich and J.P. Thiery
Deoxyribonucleases: Specificity and use in nucleotide sequence studies,
Nature N.B., 246:36 (1973).

S.D. Ehrlich, J.P. Thiery, A. Devillers-Thiery and G. Bernardi
A new approach to the study of nucleotide sequences in DNA,
Nuc. Ac. Res., 1:87 (1974).

S.D. Ehrlich, J.P. Thiery and G. Bernardi
The mitochondrial genome of wild-type yeast cells. III. The pyrimidine
tracts of mitochondrial DNA,
J. Mol. Biol. 65:207 (1972).

V. Sgaramella, H. Bursztyn, S.D. Ehrlich and J. Lederberg
Exonucleolytic degradations of the DNA of bacteriophage P22 increase
its transfecting activity on calcium chloride treated *Salmonella typhimurium* cells,
Genetics, 74:S249 (1973).

DO NOT TYPE IN THIS SPACE - BINDING MARGIN

Section II - Biographical Sketch
Ehrlich, Stanislav

List of Representative Publications - continued:

R.M. Harris-Warrick, Y. Elkana, S.D. Ehrlich and J. Lederberg.
Electrophoretic separation of B. subtilis genes ($EcoR_I$/agarose gel electrophoresis).
Proc. Nat. Acad. Sci. USA 72:2207-2211 (1975).

V. Sgaramella, S.D. Ehrlich, H. Bursztyn and J. Lederberg.
Biological activity and molecular structure of DNA. I. Enhancement of transfecting activity of bacteriophage P22 DNA upon exonucleolytic erosion. (Running title: Exonuclease and transfecting P22 DNA).
J. Mol. Biol. (in press) (1976).

S.D. Ehrlich, V. Sgaramella and J. Lederberg.
Biological activity and molecular structure of DNA. II. Bacteriophage T7 DNA transfects $CaCl_2$-treated E. coli cells and is activated by erosion with lambda exonuclease. (Running title: Exonuclease and transfecting T7 DNA).
J. Mol. Biol. (in press) (1976).

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
BURSZTYN, Hela	Senior Research Assistant	5/24/29
PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX
Warsaw, Poland	USA	☐ Male ☒ Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Javeriana University, Bogota, Colombia	"Licenciada en Bacteriologia"	1950	Bacteriology
Michigan State Univ., USA	Post grad. courses	1955-56	Immunology & Biochemist

HONORS

none

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Genetics	Senior Research Assistant

RESEARCH SUPPORT (See instructions)

N/A

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

1968-present Stanford University School of Medicine, Department of Genetics
1967-68 U.C.L.A., Department of Medical Microbiology
1965-1967 Weizmann Institute of Science, Biochemistry Section, Israel
1964 Suisse Institute of Cancer, Lausanne, Switzerland
1962-64 Columbia University, Biochemistry Department, Genetics Research
1960-62 Columbia University, New York, Cancer Research Institute
1959-60 Institute of Cardiology, Mexico, with scholarship for Research in Rheumatic Fever
1957-59 University of Andes, Bogota, as full-time professor of bacteriology and chief of the clinical lab
1953-55 Colombian Institute of Social Security, as chief of the bacteriology labs
1951-53 Columbia University, Department of Bacteriology

Publications

1. Bursztyn, H., V. Sgaramella, O. Ciferri and J. Lederberg, 1975. Transfectability of rough, gal⁻ strains of Salmonella typhimurium. J. Bact. 124:1630-1634.

2. Sgaramella, V., S.D. Ehrlich, H. Bursztyn and J. Lederberg, 1976. Biological activity and molecular structure of DNA. I. Enhancement of transfecting activity of bacteriophage P22 DNA upon exonucleolytic erosion. J. Mol. Biol. (submitted)

NIH 398 (FORMERLY PHS 398)
Rev. 1/73

Lederberg, Joshua

Privileged Communication

RESEARCH PLAN

Progress Report: March 1, 1973 to date.

Introduction

This summary of work since the previous cycle of renewal-application will be structured around a) brief citations to publications, and b) work in progress at the present time and the focus of further planning. (See below).

The continuity of our work was gravely impaired by the disruption of funding for a period of six months at the last round, when we received that all too familiar notice that renewal had been "approved but not yet funded" by the NIAID, when that institute was facing an unprecedented crisis in its own cash flow. Essentially all of my energies during that interval were spent on desperate measures to keep my laboratory going during the process of bureaucratic readjustment, hindered by some very bad luck on council deadlines. Fortunately, the National Cancer Institute [which was the original locus of support during the early history of this grant] was again able to take responsibility for funding, and just in the nick of time from the standpoint of keeping the laboratory intact.

Since December 1967, our laboratory's research has been oriented to what is now called "recombinant DNA". Our initial efforts to achive such molecular hybrids centered on trying to "deceive" cells of B. subtilis into accepting heterologous DNA aggregates coupled by unnatural chemical links, and on studying the barriers to interspecific transformation. We also indicated some possible enzymological approaches which were more promising in their conceptualizing than in what could be concretely realized at that time. Most of our work continues to deal mainly with these simple model systems in bacteria, (usually closely related species), but has had in mind that they would be extended to more disease-oriented applications like cancer biology once the basic principles could be worked out, which of course is precisely the follow-up history of my earlier research.

While we achieved some understanding of genetic heterology (and the not very suprising conclusion that sequence-heterology plays a larger role than DNA-restriction in inter-Bacillus transformations), none of these approaches gave much manifest reward towards the goals we had outlined. However, Dr. V. Sgaramella (1972) then discovered the flush-ended-duplex-joining activity of the DNA-ligase of phage T4; and at last this seemed to be the key to the controlled terminal joining of DNA segments from diverse sources. Unfortunately, or so it seemed it proved to be surprising difficult to obtain reliably flush-cut DNA with then-available methods, and his most important result (in this laboratory) was to demonstrate that phage P22- DNA could be oligomerized by this method, and that DNA segments obtained with other restriction endonucleases could not be joined to P22. This was one of the first hints that restricted DNA was NOT flush-ended, as many then believed. Our continued efforts at that time to exploit the T4-ligase method to make other recombinant DNA remained highly frustrating (we now realize we were suffering also from inefficient biological selection in our system, as well as from difficulties in the preparation of joinable segments.)

Then the splendid work of Cohen, Boyer, Berg, and their students and colleagues opened the floodgates with the elegant solutions to these problems based on 1) the special cohesive ends generated by EcoRI and other restriction endonucleases, 2) the use of E. coli plasmids as vehicles for the replication and expression of inserted DNA segments, and 3) the adoption of agarose-gel electrophoresis for the sizing of the segments obtained after endonuclease.

Within the last three years, these studies have indeed revolutionized the technology of handling DNA in the laboratory, and have inspired literally hundreds of able investigators to exploit them for studies of gene structure and to develop the technology even further. The literature is so rich as to make it hopeless to review it here— see any issue of the Proceedings of the National Academy of Sciences, or perhaps Curtiss' review in the current volume (1976) of the Annual Review of Microbiology.

Our own position is therefore necessarily in some transition: from that of playing some long-shot gambles looking for the breakthrough, to that of seeking the most effective role in a now intensely cultivated milieu. The opportunities

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for fruitful application are so vast; and are of course coupled with concerns about the socially responsible conduct of experiments that might be deemed to carry a risk of spawning serious new human or eco-pathogens, that it is difficult to lay out a long range program with high confidence that it is an optimum choice. We may again simply be scooped by others; we may face unforeseen difficulties (or equally challenging opportunities; we may well have to revise our research efforts in order to comply with official regulations, or with our own prudent judgment, for a cautious and responsible approach to this exploding field. Given this explanation, there now follows a more detailed outline of A] research progress during the past 3 years, including also some ancillary lines of work, and B] our research plan for the next interval.

A. Specific items for a progress report:

A1. Terminal joining of flush-ended DNA with T4 ligase.

As already mentioned, Sgaramella's work '72 in this laboratory gave the very encouraging result that P22 DNA could be oligomerized, with the retention of biological activity; but it proved to be surprisingly difficult to find other sources of flush-cut DNA to form recombinant molecules. The next section will review incidental findings on the biological activity of P22 DNA altered in different ways, noted incidentally to this central effort.

More recently, during another extended visit here by Dr. Sgaramella we have reexamined terminal joining, particularly in efforts to exploit the restriction endonuclease from B. subtilis, (Bsu) which attacks GGCC sites to produce flush-ended DNA segments.

With various DNAs, it was verified that Bsu segments were readily circularized by T4-ligase, giving an EM assay for the flush-ended activity of this enzyme (contra the sealing or sticky-end rejoining activity also shown by this ligase, and by others which do not show terminal joining.)

We have used the circularization assay to follow the purification of the T4 ligase, and have been able to show coincidence of chromatographic behavior on DE-cellulose columns of the three activities associated with the enzyme: ATP-PP exchange, cohesive joining (assayed by circularizing DNA molecules endowed with EcoRI-produced cohesive ends) and terminal joining. We have investigated the kinetics of the cohesive and terminal joining, and have found that the latter requires about 50 times more enzyme than the former, but that the extent of the joining reaction is similar in the two cases, close to 70%. Optimal joining temperature was found to be 25 degrees C for cohesive and terminal joinirg, 5 degrees C or 37 degrees C reducing the reaction by 15% and 25% respectively for the former, 25% and 50% respectively for the latter.

We are currently engaged in a search for a cloning vehicle for flush-ended segments. This requires identifying an enzyme-plasmid (or phage) system where the plasmid would be cut in a unique position, and a segment inserted in the site of the cut without impairing either the replication of the hybrid molecule or the expression of the genetic markers of the vector.

We have investigated the restriction pattern of small replicons (pSC101, ColE1) with the Bsu endonuclease, and have found that they are cleaved into a great many segments, which, in spite of their joinability, did not regain any biological activity upon ligation. After incomplete enzymatic digestion, however, ligation did restore some of the biological activity. However, the transformed clones sofar contained intact plasmids whose DNA was identical to the parent. We have not yet been able, thus, to create hybrid replicons from the pSC101 (or ColE1) DNA treated with the Bsu restriction enzyme.

We are investigating restriction patterns of various replicons with other enzymes believed to give rise fo flush-ended segments. HpaI and Sma cut pSC101 at a unique site; the same is true for Sma cutting of pMB9.

We were able to recircularize close to 20% of pSC101 and pMB9 cut by Sma, about 5% of pSC101 cut by HpaI (the extent to which a flush-ended segment can be circularized depends mostly on the purity of the restriction enzyme used to obtain the segment). Experiments designed to measure restoration of biological activity of pSC101 and pMB9 upon terminal joining and the effect of inserting segments at the site of the cut by the two restriction enzymes should be done shortly.

This work has not yet been published.

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Research Plan continued:

A2. Electrophoretic separation of Bacillus subtilis genes.

Many investigators have shown the utility of processing viral and plasmid DNAs with restriction endonucleases to produce ensembles of DNA segments which have a) specific termini [the endonuclease sites], and b) size distributions characteristic of the source DNA.

This paper shows that the same procedure is applicable to a bacterial DNA, albeit the resolution of DNA by size with gel-electrophoresis, does not afford clean separation of unique segments, with perhaps rare exceptions. However, the DNA does form bands in agarose gels which offer a 30- to 60-fold enrichment of biological activity. These separations are a prelude to the cloning of segments in plasmid vectors.

Remarkably, the tryptophan operon which is in many respects one of the most interesting and best mapped in B. subtilis is also remarkably large and can therefore be obtained in relatively high yield, even before cloning. [Ref. Warrick et al., 1975 in Bibliography].

Since that publication, we have been able to develop the methods initiated by Murray, Hogness and others for the NA-heteroduplex-staining of colonies and plaques, and extend them to DNA phoretogram bands in situ for the recognition of specific segments.

Extensive efforts were made to introduce the tryptophan operon into psc-101 with a view to cloning it. Although a variety of segments from B. subtilis could be cloned in this way, and recognized by the band-staining, it became evident that a) this system discriminates very strongly against the insertion of larger segments, and b) that the expression of such segments, in this vector, was highly problematical. At least, while this was the major research effort of our laboratory for the past 2 years, we did not succeed so far in obtaining the desired specific clones.

Similar studies were then undertaken (and are still in progress) with other potential vectors, including ColEl and lambda, each of which has also been used by several other workers to exploit particular technical advantages. While we have learned a great deal more about the idiosyncrasies of these vectors, the work [which had not to this point solve the problem at hand] is not at a stage where it can be readily summarized.

Besides the re-ligation of sticky-ended segments furnished by the EcoRI nuclease, we have also used terminal transferase to generate poly-A and poly-T sticky ends on sheared B. subtilis DNA, and ligated these to similarly treated vector segments. As already shown by others, this method can also be used to generate recombinant DNA clones; but we encountered a number of problems in its application in the present context.

However, when we turned to DNA segments from a better defined source, namely the subtilis phage phi-3-t, we were eventually more successful, as will be recounted in more detail.

A3. Studies with phi-3-t.

A number of interpretations could be offered for the negative results up to this point. They are hard to distinguish without first accomplishing one instance of insertion of a KNOWN segment from B. subtilis into the plasmid: one could then pursue the reasons why it might not be expressed, whether it underwent further changes in its new environment, whether it was restricted upon transfer back to B. subtilis, and so forth. We therefore turned our attention to another option where we could expect to isolate a given segment with still higher reliability than by the direct segmentation of the entire subtilis genome.

phi-3-t seemed to offer such an opportunity, and indeed it has given the most encouraging results to date: namely, the transfer and expression of a segment of DNA from a subtilis PHAGE in an E. coli plasmid, the amplification of this segment, and its transfer and expression back again in B. subtilis.

phi-3-t is a B. subtilis temperate phage, isolated by Tucker (1969), who has shown that it carries genetic information specifying thymidylate synthetase activity. The synthetase is produced in B. subtilis strains not only during virulent infection, but also in lysogens.

Young et al. (1976) have recently shown that the insertion site of phi-3-t lies in the terminus region of the B. subtilis chromosome, close to the ilv A gene. They have noticed that the transfection of thy- strains of B. subtilis

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with the phi-3-t DNA gives rise to thy+ prototrophs, which often do not contain the whole prophage. They have studied restriction patterns of the DNA with several endonucleases; 24 and 5 bands could be resolved by agarose gel electrophoresis subsequent to cleavage with EcoRI and Bam respectively (F. Young, personal communication; Wilson et al., 1974). They have observed that EcoRI cleavage but not Bam destroys the transforming activity of the gene coding for the thymidylate synthetase (thy P gene).

These data led to the following chain of reasoning:

1. Thy P, comprising an unusually large part of the vector in which it is embedded, should therefore be comparatively easy to isolate and insert in an E. coli plasmid.

2. Transforming activity of the gene is not lost even if it is not carried by the complete prophage; it should therefore be possible to identify the thy P gene cloned in E. coli, even if it were not expressed in that host, by transforming B. subtilis thystrains to prototrophy with the hybrid plasmid DNA.

3. Since the EcoRl-cloning vehicles are available more readily than the Bam ones, partially EcoRI degraded phi-3-t DNA should be amplified, as the complete degradation inactivates the thy P gene activity.

Cloning of thy P gene.

phi-3-t DNA was cleaved with EcoRI endonuclease so as to decrease the transforming activity of thy P gene about 10-fold. (Complete degradation led to a 1000-fold decrease, and resulted in a gel electrophoresis pattern similar to that observed by Wilson et al., 1974).

Fully EcoRI cleaved pSC101 DNA was added to the phi-3-t segments to a ratio of 1:10. Ligation was carried out at a DNA concentration of 20 micrograms/ml; T4-ligase catalyzed formation of close to 50% circular molecules in 1 hour at 20 degrees C. The ligated mixture was used to transform a E. coli thy- strain to either TcR (tetracycline-resistance, a marker carried by pSC101) or thymine prototrophy. TcR transformants were further screened by replica-plating for thy+ character and by hybridization using labeled RNA (C-RNA) synthesized on the phi-3-t DNA template by E. coli RNA polymerase (Grunsteign and Hogness, 1975). Table I summarizes our results.

Table I

selection	TcR	Thy+	Thy+ TcR
plated cells	10↑7	10↑8	10↑8
transformants	447	1	2
hybridize phi-3-t cRNA	31	-	2
TcR Thy+ phenotype	2	0	2

Selection for TcR, followed by replica-plating seems to yield Thy+ transformants at higher frequency (10^-7) than direct selection for Thy+ character (10^8). Colonies hybridizing cRNA were labeled 1-33; hybrid plasmids were named pFT followed by the clone number.

The thy gene is plasmid-borne.

Plasmid DNA was extracted by standard clear lysis procedures from the E. coli clones transformed to TcR phenotype. Less than 1% of linear molecules were seen in the preparations. That DNA was used to transform TcS thy- E. coli to thy+ TcR; pSC101 and lambda DNAs were used as standards for competence of cells. Results are summarized in Table II.

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Table II

Transformation frequency (colonies or plaques per DNA molecule)

DNA	TcR	TcR Thy+	plaques
lambdaDNA	-	-	10^{-5}
pSC101 DNA	1.5×10^{-5}	$<10^{-10}$	
pFT23 DNA	1.7×10^{-5}	1.7×10^{-5}	
pFT24 DNA	1.7×10^{-5}	1.7×10^{-5}	

TcR Thy markers are introduced in the E. coli at the same frequency as TcR markers when pFT23 or pFT24 DNA is used; efficiency of transformation with these two DNA's for the TcR is the same as that observed woth pSC101 DNA. This latter does not transform thy- strain to prototrophy.

Hybrid plasmid DNA was used to transform B. subtilis thystrains. Efficiency of transformation was measured with both the intact and the Bam cleaved DNA; intact and Bam-cut phi-3-t DNA was used as a standard. Results are summarized in Table III.

Table III

DNA	Transformation frequency (colonies/DNA molecules)
phi-3-t	1×10^{-5}
phi-3-t/Bam	1.5×10^{-6}
pFT23	6×10^{-8}
pFT23/Bam	5×10^{-8}
pFT24/Bam	5×10^{-8}
pFT24/Bam	5×10^{-8}

Plasmid DNA can transform B. subtilis to thymidine independence; this not only confirms that the thy gene is plasmid borne, but also indicates the phi-3-t origin of that gene.

The efficiency of transformation with the plasmid DNA is three orders of magnitude lower than that displayed by the phi-3-t DNA. This does not seem due to the circular structure of the plasmid: its cleavage with Bam, which cuts in the pSC101 part of the hybrid and yields linear molecules (see below), does not change the transforming activity of the DNA. An alternative explanation might be the existence of hitherto undetected restriction system of B. subtilis.

An additional evidence for the plasmid location of the thy character was obtained by curing the TcR thy+ E. coli transformants of the hybrid plasmid. This was achieved by coupling ethidium bromide treatment with the ampicilin selection in the presence of tetracycline. Loss of tetracycline resistance was accompanied by the loss of thymidine prototrophy; reintroduction of the hybrid plasmid restored both the TcR and the thy+ phenotype.

Characterization of hybrid plasmids.

The four hybrid plasmid DNas were cleaved with different restriction enzymes, and the segments characterized by gel electrophoresis, using SPP1 DNA cleaved with EcoRI as standard. Results are shown in Table IV.

Table IV

hybrid plasmid	pFT23	24	25	33
	---DNA MW x 10 -6 --			
EcoRI segments	6.2	6.2	6.2	6.2
	4.6	4.6	4.6	5.5
	0.67	-	0.67	0.67
	0.24	-	0.24	0.24
Bam segments	11.7	10.8	11.7	8.3 ; 4.4
Sma segments	11.7	10.8	11.7	8.0 ; 4.6
intact	11.7	10.8	11.7	12.6

The 6.2 megadalton segment is the pSC101 moiety of the plasmid. The 4.6 mD piece, released from pFT23, 24 and 25 comigrates with the band 5 of EcoRI cleaved phi-3-t DNA; the two smallest segments of pFT23 and 24 comigrate with bands 17 and 21. The 5.5 mD segment of pFT33 has a mobility identical to band 3 of the phi-3-t. Inserts in the pFT23, 24 and 25 do not contain either Bam or Sma sites: the plasmids are cleaved within the pSC101 part. To the contrary,the pFT33 insert has a Bam and Sma site, 1.4 mD apart.

EcoRI restriction patterns could be interpreted as follows: within the phi-3-t genome are contigous segments 3 (27,21), 5; thy P gene is contained within segments 17 and 21, which are cloned together with segment 3 in pFT33 and segment 5 in pFT23 and 25.

However, this simple interpretation seems to be contradicted by the fact that pFT24, does not contain segments 17 and 21. An alternative interpretation is that some rearrangements of inserted DNA have taken place in the hybrid plasmids, some of the observed coincident electrophoretic mobilities being accidental. This hypothesis takes support from the fact that the pFT23, introduced in an r- m+ E. coli host (W5445) is converted into a 10.8 mD molecule, identical to pFT24 in its EcoRI restriction pattern. Heteroduplex mapping of so modified pFT23 and pFT24 did not show any non-homologous region, which would indicate a very precise mechanism of rearrangement. We are presently investigating sequence relations between the four pFT's.

Evidence for phi-3-t sequences in the hybrid plasmids.

E. coli clones carrying pFT's hybridize phi-3-t cRNA (labelled transcript RNA), while the clones without a plasmid, or carrying pSC101 do not. This indicates that the sequences complementary to phi-3-t have been introduced into the former clones, coincidental with introduction of pFT's.

B. subtilis thy- strain SB591 does not hybridize phi-3-t cRNA, contrary to the SB168 (this latter does not carry a phi-3-t prophage, as judged by the lack of immunity to the phi-3-t phage). Thy+ transformants of SB591 with phi-3-t DNA or with pFT23 or 24 DNA do hybridize phi-3-t cRNA; all three treatments have led to introduction of phi-3-t sequences into the SB591. It can therefore be concluded that the pFT's encompass the phi-3-t sequences.

Additional evidence for the presence of phi-3-t sequences in pFT's 23 and 24 has been obtained by the electron microscopy inspection of heteroduplex molecules. Both plasmid DNA's, isolated after transfer into r- m+ host and cleaved with Bam, form a duplex with the phi-3-t DNA. The size of homologous region is 4.6 mD, identical to that determined for the insert by both gel electrophoresis (Table IV) and E.M. analysis of heteroduplex with pSC101 DNA. In spite of the rearrangement that the pFT23 has obviously undergone (see above), no single-stranded portions were detected interrupting the 4.6 mD heteroduplex. This indicates elimination of sequences from pFT23 proximally to the pSC101-phi-3-t joint.

Still further evidence for the presence of phi-3-t sequences in the pFT's is obtained by hybridizing cRNA synthesized on pFT's to EcoRI-produced segments of phi-3-t DNA, separated by gel electrophoresis (Southern, 1975). Exact localization of segments complementary to hybrid plasmids is presently under way; this should shed some light on both the position of thy P gene on the segments and the rearrangement pattern of the cloned sequences.

A4. Chlorine as a mutagen.
(abstracts from two brief papers now in press)

Effects of Chloramine on Bacillus Subtilis DNA, K. Shih and J. Lederberg, J. Bact.

The lesions induced in Bacillus subtilis DNA after treating bacterial cells (in vivo) and bacterial DNA (in vitro) with chloramine were studied biologically and physically. Single strand breaks and a few double strans scissions (at higher chloramine doses) accompanied loss of DNA transforming activity in both kinds of treatments. Chloramine was about three times more efficient in vitro than in vivo. DNA was slowly chlorinated; the subsequent efficiency of producing DNA breaks was high. Chlorination of cells also reduced activity of endonucleases in cells, however, chlorinated DNA of both treatments was sensitized to cleavage by endonucleases. The procedure of extracting DNA from chlorinated cells induced further DNA degredation. Both treatments introduced a small fraction of alkali-sensitive lesions in DNA. DNA chlorinated in vitro showed further reduction in transforming activity as well as further degradation after incubation at 50°C for 5 hours; whereas DNA extracted from chloramine treated cells did not show such a heat-sensitivity.

Chloramine Mutagenesis in Bacillus Subtilis, K. Shih and J. Lederberg, Nature

Chloramine (which occurs widely as a byproduct of sanitary chlorination of water supplies) is shown to be a weak mutagen, when reversion of trpC to trp^+ in Bacillus subtilis is used as an assay. Some DNA-repair mutants appear to be more sensitive to chloramine, suggesting the involvement of DNA targets in bactercide. The influence of plating media on survival of cells treated with chloramine suggests a bacterial repair system acting upon potentially lethal lesions induced by chloramine.

A5. Transfectability of Rough Strains of Salmonella typhimurium
H. Bursztyn, V. Sgaramella, O. Ciferri, and J. Lederberg,
Journal of Bacteriology, 124:1630-1634, 1975

Cells of rough (but not smooth) strains of Salmonella typhimurium become competent for transfection by phage P22 deoxyribonucleic acid after treatment with 0.1 M $CaCl_2$. The yield of infectious centers is about 10^{-s} per genome equivalent of deoxyribonucleic acid. However, different sorts of rough strains vary in their ability to become competent in a fashion that can be correlated with the level of the genetic block in cell wall lipopolysaccharide synthesis. The most amenable strains are blocked by defects in the addition of galactose units I and II of the lipopolysaccharide by the inability to synthesize uridine 5'-diphosphate-galactose (galE point mutants and gal deletion mutants). Strains blocked only in the addition of galactose I, glucose I, or heptose II have low levels of transfectability, whereas strains with either more complete or more deficient lipopolysaccharide core are not competent for transfection. When normal lipopolysaccharide synthesis is restored either genetically or by furnishing exogenous galactose (galE point mutants that can still use it), the cells are no longer competent for transfection.

A6. Studies on Phage DNA Eroded by Exonuclease.

Biological Activity and Molecular Structure of DNA. II Bacteriophage T7 DNA Transfects $CaCl_2$-Treated E. Coli Cells and Is Activated by Erosion with λ Exonuclease, S.D. Ehrlich[x], V. Sgaramella[+], J. Lederberg, J. Mol. Biol., In Press.

T7 bacteriophage infects, equally efficiently, restriction-proficient E. coli K12 cells and the restriction deficient mutants. To the contrary, the phage DNA transfects the latter at much higher efficiency ($2 \cdot 10^{-6}$ plaques/genome equivalent, pge*) than the former (10^{-9} pge). Transfecting activity of the DNA on restriction-deficient hosts increases another 20-fold (up to $4 \cdot 10^{-5}$ pge) by complete erosion of the redundant regions with λ-exonuclease, both in rec^+ and recB recC sbcB genotypes. Circles and linear oligomers arising from the annealing of eroded DNA show the same transfecting activity as the unannealed monomers. The terminal redundance of the genome, as measured by the annealability of eroded molecules, comprises 50-100 base pairs.

Enhancement of Transfecting Activity of Bacteriophage P22 DNA Upon Exonucleolytic Erosion, V. Sgaramella[+], S.D. Ehrlich[x], H. Bursztyn and J. Lederberg, J. Mol. Biol. accepted for publication.

The transfecting efficiency of P22 DNA on "rough" strains of S. typhimurium or non-restricting mutants of E. coli K12 approaches 5×10^{-8} plaques/genome equivalent (pge)*. It increases 20-fold upon complete erossion** of the terminally redundant regions of the DNA molecule with either λ exonuclease or exonuclease III. Eroded DNA molecules form circles and linear oligomers upon annealing. The circular monomers display transfecting activity about 10 timeshigher than that of eroded linear monomers or hydrogen-bonded oligomers. $recB^-$ $recC^-$ $sbcB^-$ strains of E. coli K12 are transfected with P22 DNA with an efficiency of 5×10^{-6} pge. The activity of DNA molecules on these strains is not augmented by erosion. This suggests that the activation observed on rec^+ genotypes following erosion is due to the formation of hydrogen-bonded circular molecules, which more readily escape degradation by the products of host genes recB recC and sbcB.

A7. Heterologous transformation between B. globigii (donor) and B. subtilis 168 strains (recipients), from: DNA Segmentation and Sequence Heterology in Transformation of Bacillus Subtilis, Ronald Morgan Harris-Warrick, Ph.D. dissertation (1975).

Very poorly transformable markers in the non-conserved regions of the chromosome were studied to detect any differences in transformation using these and conserved region markers. TrypC-aroB cotransformants were selected over 90% also cotransformed tyrA and aroE, an increase in linkage in marked contrast to the reduction of linkage seen in other systems (29). Mutations were introduced into the intergenote of one of the hybrid strains and transformed to prototrophy by B. globigii and B. subtilis DNA: both DNA's transfromed with the same efficiency, representing a 10^5-fold increase in the efficiency of the hetrologous donor's transforming ability. DNA from hybrids and B. subtilis homogenotes was compared for efficiency of transformation of hybrid and homogenote recipiets: in both directions, the homologous cross

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(hybrid -x hybrid or homogenote -x homogenote) was about ten times more efficient than the partially heterologous cross (hybrid -x homogenote or homogenote -x hybrid). The homogenote B. globigii donor transforms a homologous intergenote marker with 75-100% efficiency compared to a hybrid donor, and this ratio increases with decreasing DNA concentration. A model of intergenote transformation is presented where, in contrast to results in conserved regions of the chromosome, the heterologous intergenote in this non-conserved region must be cotransformed in its entirety with homologous sequences on both sides to aid in recognition and integration. This model is verified by the following facts: 1) heterologous intergenote transformation is unusally sensitive to shearing of the donor DNA; 2) all markers in the heterologous intergenote cross are cotransformed with 100% efficiency, even at very high levels of shear. Finally, a restriction activity was isolated from B. globigii that inactivates B. subtillis transforming DNA in vitro but is without effect on B. globigii DNA; this shows that the modification sites on the two DNAs are different. It is proposed that the major barrier to heterologous transformation between these strains is homology, and that restriction does not have a role.

(The B. globigii strains have been furnished to Dr. F. Young whose laboratory has found an interesting new exonuclease therein.)

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RESEARCH PLAN Parts A. and C.:
'Specific aims and significance of research'

As indicated in the introduction, it would be imprudent to take other than an opportunistic view of the prospects of further development of research with recombinant DNA. Our own orientation in making research plans is to concentrate on areas :

a) which take advantage of our historical experience with bacterial systems, like B. subtilis transformation

b) Which will continue to help in furnishing tools for others, as well as to illuminate theoretical issues

c) Which promise to be of some health-related practical value in the foreseeable future

d) Which despite my (publically stated) convictions that the potential hazards of research with recombinant DNA have generally been exaggerated in proportion to the benefits, conscientiously complies with all competent policy guidelines concerning safety, so as to leave neither real nor imputable hazard as an outcome of our work.

e) Which are as distinctive as is feasible from the well established lines of investigation that our numerous colleagues are pursuing.

For several of these reasons, we propose to continue to focus on the interface of the Bacillus transformation system and the insertion of its DNA segments into vectors either of Bacillus or of enterobacterial forms. We must admit that one of the major factors favoring the Bacillus system no longer applies, as there are now several approaches to DNA-transformation in enteric bacteria (unlike 5 years ago). However, there are several problems that are especially pertinent here:

1) The mechanism of integration of DNA segments into the chromosome
2) Comparing gene regulation and expression in a chromosomal (vs. a plasmid) context.
3) The likelihood that the separation of Bacillus from E. coli, though both are of course prokaryotic, will reveal interesting differences in basic mechanisms of gene action, membrane function, and other aspects of cell biology
4) The possibility (which others stress more than I would) that there may be safety advantages to an organism like Bacillus which is more remote than the commensals/pathogens of the coliform group from close ecological association with man.

We have summarized recent progress in our work in previous sections, and we expect our ongoing program to be an extension and elaboration of this, subject to all of the contingencies stressed throughout this application.

B. subtilis transformation has, of course, been one of the foundation stones of bacterial-genetic research since it was brought under experimental control by Spizizen (1958); see, for example Hotchkiss and Gabor(1970) for a review of unsettled questions.Regrettably, all of the work till now (including our own) has been faulted by the physical inhomogeneity of DNA isolated after physical fragmentation. Our new-found capabilities of cloning specific segments offer, for the first time, sources of large amounts of "reagent-grade DNA": well-defined, homogeneous material which will permit kinetic studies of unprecedented precision. As almost every step of the transformation process has been controversial, the opportunity for a fresh start appears sufficient compensation for what might otherwise appear like a dreary routine of "mapping the entire genome, segment by segment." But this is precisely the challenge ahead of us at this point, the first step having been Harris-Warrick et al's sizing of EcoRI nuclease segments of B. subtilis into resolvable bands in agarose gel electrophoresis. In my own experience such studies have never failed to give unexpected rewards from discrepancies that were totally unanticipated in prior planning. These studies require no more than a vector in which B. subtilis DNA can be amplified and retain its biological activity for transformation back into B. subtilis cells. Besides the systems already

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discussed, we will of course give special attention to standardized safety-disarmed agents as they become available. Insofar as these complexes are ALSO active in E coli cells, they offer the additional virtue of inter-species comparative study.

Access to large quantities of homogenous DNA also encourages us to reexamine the question of the extent to which "recombinant-DNA" might be formed by more nearly natural means, or indeed may play some evolutionary role for microorganisms. [I must confess to entertaining this prejudice about almost any "trick" that we play in the laboratory.] For example it will be possible to use strenuous selection for biological activity to determine whether even trace numbers of recombinant molecules are formed by intracellular processing of non-specific [chemically or physically coupled] aggregates of marked DNA from different sources. Depending on the circumstances, this may have an important bearing on whether or not recombinant-DNA formation continues to be viewed as a potentially hazardous laboratory artifact, or whether it is already pervasive in nature.

Correspondingly, this competence may be a special quality of particular strains of naturally occurring organisms, for which we would now have a powerful screening device. (Competition between inactive or irrelevant DNA and the active molecules in a heterogenous mixture may well dampen the detectability of the latter by even more than the 1000- or 10,000- fold factor of sheer specific activity.)

These systems also offer a reexamination of in-vitro mutagenesis, which has been only marginally interesting with the existing material. While there are objections to extrapolating from any simplified laboratory assay to the estimation of human toxicological hazard of specific chemicals, no one believes we have a surfeit of mechanistically reliable methods for exploring the basic aspects of mutagenesis (and by implication carcinogenesis.)

In seeking some aspects of these studies that might have tangible fallout for clinical utility, we have decided to focus on the genes for t-RNA as candidates for cloning and amplification. Initially, we will be working primarily in subtilis-subtilis and subtilis-coli systems, since the basic validation and preparation of reagents can be done readily here with minimal concerns about biohazards. In view of the evolutionary conservatism of t-RNA's, it is conceivable that these DNA-clones may already be useful as cytochemical probes for specific t-RNA in human tissue (and neoplastic) specimens — perhaps offering a new dimension in histopathological and pathogenetic study. [Other studies in this department already relate to disturbances of t-RNA metabolism in tumors that often result in the spilling of pseudo-uridine and beta-amino-isobutyric acid in the urine of bladder cancer patients.]

In any event, these pilot studies with bacterial t-DNA should be an important stepping stone to the isolation of analogous segments from human DNA [a project that we leave to entirely separate, future review when issues of biohazard policy, and facilities, as well as the technical problems pertinent thereto have been clarified. We look forward to even more audacious efforts at the preparation of probes for specific messenger RNA, and perhaps for chromosomal DNA, but in good time.]

Besides these efforts which center on subtilis-transformation, we have also already been obliged to learn a good deal about other vectors (like psc101, colE1, and lambda) as now standard vectors for cloning, and this of course has generated a number of optional topics. For example, pairs of replicons can be set up carrying markers that must recombine for the host cell to survive at a non-permissive temperature. These replicons may also be constructed to have no prior homology, so that recombination will now depend on the coincidence of homologous inserts in each of them. This is then a tool for a) selective characterization and isolation of homologous DNA, and b) to study the recombination process itself — e g. with synthetic homopolymers whose level of homology can be synthetically defined.

Finally, the T4-terminal ligase system is under new examination, as mentioned in the progress report. Dr. Sgaramella will have principal responsibility for this effort at his laboratory in Pavia; but as we are continuing in close communication, we expect him to make another extended visit to pursue these studies here during the term of the renewal application.

The availability of cloning vehicles for flush-ended segments is

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particularly interesting from the point of view of biological safety, mainly due to the fact that it affords cloning of very short DNA segments, containing little spurious potentially harmful genetic information, besides the one under investigation. With cohesive joining, both the vector and the inserted segments must have the same sticky ends. Thus, enzymes which produce short segments will as a rule abolish the biological activity of the vector (compare the example pSC101 cut with Bsu, preceding section). Terminal joining does not require that the ends to be ligated be produced by the same enzyme; one can thus clone very short segments, produced by the enzyme of low specificity, using a vector cut only once by a very specific enzyme. Potential conversion of single-strand ended segments into the fully base-paired ones via for example the repair action of DNA polymerase increases further the versatility of the flush-ended cloning vehicle, making it thus useful for amplifying segments resulting from action of any restriction enzyme. This versatility may also be helpful in the further development of secure vectors for research in this field.

Although we have remained well within the domain of projected efforts, almost none of the detailed experiments outlined in our progress report could have been predicted in writing research plans at the last cycle; and, if anything, the field is moving still more quickly, even turbulently today. It would be futile to offer a rigid framework of planned experiments, but the following two areas are emerging as those warranting our most focussed attention:

1. The Thy P segment of phi-3-t having been amplified now in psc-101 furnishes a powerful tool for studies of the mechanism of transformation. We already know that psc-101/Thy P hybrid DNA transforms B. subtilis, equally efficiently whether or not the psc-101 moiety is cleaved. Furthermore, studies of the transformed subtilis clones (so far) show DNA homologous to the Thy P moiety (presumably integrated in the chromosome) but none of the psc-101.
Further studies can show the precise boundary of the accepted segment. What is the cellular mechanism by which this discrimination is achieved?
This particular instance of transformation may be related more to viral functions than typical transformation, insofar as the recipient appears to be a thy- deletion mutant, which does not exhibit extensive homology with thy P segments of phi-3-t. (However shorter attachment loci would not be detected by physical hetero-duplex analysis.) We hope to do similar experiments with other segments from B. subtilis by amplifying them either in psc-101, or in the current psc-101/phi-3-t hybrids.
More generally, the availability of "reagent grade" amplified DNA opens the door to comprehensive reexamination of the fate of labelled segments throughout the entire process of transformation.
We can also look foward to the opportunity of studying the essential conditions for precise expression both in homologous and heterologous environments. At this point, the evidence concerning the Thy P segment is merely a) functional efficacy of thymidylate synthetase, and b) preliminary heteroduplex maps comparing the cloned segment (in psc-101) with phi-3-t. [These already show many unexpected complexities]. We have not yet decided whether Thy P, or some other B. subtilis marker, would be the ideal test situation for more precise studies of the fidelity of the continuity of the DNA and of its expression in m-RNA and in the sequence of the product enzyme. When this determination is made, such studies are urgently needed. If it can be managed, our preference would be either for the tryptophan operon, or for t-RNA segments.

2. With the help of these tools and insight, we also intend to focus on the genes for t-RNA as objects of amplification for the reasons outlined in the introduction. Clones of t-DNA can be recognized, and would be most interesting and useful even if they are not expressed by virtue of their ability to specifically hybridize with intracellular t-RNA. cRNA transcripts of t-RNA, even if not functional in amino-acid transfer, might also be prepared with enough specific radioactivity to warrant using them as cytochemical probes for the corresponding chromosomal DNA. And if the t-DNA is functional, it might be selected by by the use of super-suppressor genotypes with amber mutants.
A number of experiments are already in progress using DNA that is being pre-enriched for t-DNA components by the selection of phoretogram bands that stain with radiolabelled t-RNA.

Research Plan continued:

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* (For references to work from our laboratory, see the bibliographic section for Professor Lederberg)

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The MOLGEN project
(computer science)

[The following project depends on collaboration with the present laboratory research, and may be viewed as a spillover benefit of the experimental pursuits.]

For several years, I have been asking: "To what extent can we model scientific imagination and experiment-planning on computers?" Many times I had come to an exciting experimental insight as one more permutation of already established ideas, but one that suddenly illuminated the field, and clarified what had seemed to be observational discrepancies. Perhaps equally often it fell to someone else to do this, indeed sometimes even with my own data. Could one devise computer programs that even if — as would surely be true for a long time — they were unable to compute with human intelligence in its most creative tasks, could still function as mechanical proof-checkers, as aids to the design of experiments, as vigilant watchers for the missing control?

My initial work in this area has been embodied in the DENDRAL project, in collaboration with Professors E.A. Feigenbaum and Carl Djerassi here at Stanford. As outlined further, below, the scientific domain of DENDRAL has been structural organic chemistry, in part in connection with the use of massspectrometry as an analytical tool. This choice was motivated in part by other applications of this kind of analytical chemistry, e.g. for planetary biology and more recently for metabolic screening; in part by the ease with which HYPOTHESES -represented as structural formulas — could be handled in a mathematical formalism, and hence in computer programs. That work has been quite successful, and working programs are now actively being used in the proof-checking and similar modes through a national computer network centered at Stanford (the SUMEX-AIM system supported by the NIH Biotechnology Resources Branch).

Within the last year, the time has seemed ripe to plan for a long awaited convergence of these approaches with the molecular genetics research in my own laboratory. This is now an active program, in collaboration with several fellows and students led by Professors Feigenbaum and Bruce Buchanan in the Department of Computer Science, and Professor Nancy Martin of the University of New Mexico. This work is being funded primarily by the NSF through grants to the other principals. However, the close working relationships of my associates and students (principally Dr. S. Ehrlich and Mr. J. Feitelson) are indispensable as a way of grounding that research in the ongoing concrete problems of a running laboratory in the field.

Whereas the DENDRAL goal was a hypothesis (i.e., a chemical structure) to explain a set of experimental data, MOLGEN begins with a stipulated DNA structure and seeks suggested experiment plans that could either falsify or validate the asserted structure. At present, this entails a substantial effort in representing existing knowledge of experimental techniques (i.e., enzyme specificities, electron-microscopy, electrophoresis) and the physical biochemistry of DNA. We are finding it both painful and educational to examine even the most elementary procedures, and e.g. our own and published information on the specificity of enzymatic reagents, in the detail needed to represent them in the computer programs. For some time, the sheer task of communicating this material knowledge will ensure that the programs will be quite limited in the scope of their imagination compared to human intelligence, or to what can be accomplished a bit more easily in the context of organic molecular structure. However, we also have the side benefits of understanding our own thinking rather better as a result of these exercises; and we can anticipate that other developments in computer science will make more credible the idea of direct reading of published data by programs as the first crude, but most tedious, step in the acquisition of shared knowledge where it can then be refined by collegial criticism.

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To help clarify the mention of DENDRAL, the following abstract is copied from the SUMEX-AIM brochure:

The current emphasis in the field of AI (artificial intelligence) is to understand the underlying principles of a) efficient acquisition and utilization of material knowledge, and b) the programmed representation of conceptual abstractions in reasoning, deductive, and problem-solving activities. At present, these are far more specialized and inflexible than human intellectual functions; however, in special domains they may be of comparable or greater power, e.g., in the solution of formal problems in organic chemistry or in the integral calculus.

AI systems are characterized by complex computational processes that are primarily non-numeric, e.g., graph-searching and symbolic pattern analysis. They involve procedures whose execution is controlled by diverse types and forms of knowledge about a given task domain, such as models, fragments of "advice", and systems of constraints or heuristic rules. Unlike conventional algorithms commonly based on a well-tailored method for a given task, AI procedures typically use a multiplicity of methods in a highly conditional manner—depending on the specific data in the task and a variety of sources of relevant information. The tangible objective of this approach is the practical development of computer programs which, using formal and informal knowledge together with mechanized hypothesis formation and problem-solving procedures, will offer more general and effective consultative tools for the clinician and medical scientist. Contexts in which experimental data already are acquired by machine may offer even richer opportunities.

The DENDRAL project at Stanford, under the direction of Professor Lederberg, Genetics; Professor Edward Feigenbaum, Computer Science; and Professor Carl Djerassi, Chemistry, is aimed at assisting the biochemist in interpreting molecular structures from spectroscopic, physical and chemical information. In cases where the characteristic spectra of a compound are not catalogued in libraries, the DENDRAL programs carry out the rather laborious processes a chemist must go through to interpret the spectrum from "first principles". One of the DENDRAL programs, CONGEN (for CONstrained structure GENeration), is an interactive program designed to assist the chemist in the enumeration of structural isomers, based on inferences about structural features of an unknown compound. These inferences, whether obtained from physical, chemical or spectroscopic data, are supplied to CONGEN as structural fragments and related information, using a standard computer terminal connected to SUMEX-AIM. The program uses atoms and superatoms (non-overlapping structural fragments known to be present in the molecule) to construct structures; the procedure is restricted by a variety of constraints on desired and undesired substructures and ring systems. There is no direct algorithmic path available to determine such a molecular structure from the spectral data—only the inferential process of hypothesis generation and testing within the domain of reasonable solutions defined by a knowledge of organic and physical chemistry.

This process, as implemented in the computer, is a simplified example of the cycle of inductive hypothesis—deductive verification that is often taught as a model of the scientific method. (Whether this is a faithful description of contemporary science is arguable, and how it may be implemented in the human brain is unknown. Regardless, these are useful leads rather than absolute preconditions for the pragmatic improvement of mechanized intelligence for more efficient problem-solving.) The elaboration of these approaches with existing hardware and software technologies is the most promising approach to enhancing the application of computers to the vaguely structured problems that dominate our task domains.

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Comments on facilities and personnel.

We have been working since 1959 in these laboratories, which are well-equipped for molecular biology in respect to centrifuges, electron microscope and so on. With respect to biohazards, we have of course been in communication with the requisite branch of the NCI, and are awaiting the publication of the definite guidelines. Our experiments are planned to work at the lowest risk-levels commensurate with our scientific objectives. In any event, I believe we have always set a high standard of caution in dealing with our microbial specimens as if they were pathogens (and this is not just a supposition with Salmonella.) We believe we are working at the functional equivalent of P2 at the present time, and are prepared to upgrade our physical facilities formally to the p3 standard. If more stringent regulations still are adopted, we will probably respond by narrowing the frame of our experimental effort at Stanford, accordingly. We have access to the high-containment facilities at the NASA-AMES Research laboratory for intermittent experiments (e.g. retrospective selection of well-defined segments from "shotgun" trials of a total source DNA) that may be identified as of higher risk.

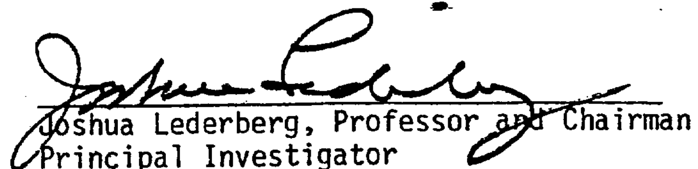
Certain aspects of the experimental program can be carried out strictly within B. subtilis, now that we understand the basic tricks and hAve some of the reagents —e.g., phi-3-t itself is a promising vector for amplification. Hence if there are still unforeseen difficulties from a regulatory standpoint, there is still some important work to be done that falls outside the context of interspecies recombinant DNA.

In any event, Stanford University is adopting a formal institutional framework to assure compliance with all requisite regulations, and we have the will and the intention to conduct our own work in accordance with such publically stated commitments. (If I seem to be overstating the obvious I might comment that I have been slandered by rumors that I was interested in biological warfare applications (sic!!!) of molecular genetics, notwithstanding my deep and strenuous involvement in the steps that led to the negotiation of the anti-BW convention which is now in force.)

The Department of Genetics at Stanford and at Pavia, Italy are formally affiliated as cooperating institutions under the Treaty for Technological Cooperation between Italy and the U.S. Under its terms, each department has received funds (from the U.S. NSF, Italy the CNR) to support the exchange of fellows and faculty. Dr. Vittorio Sgaramella and Orio Ciferri have been particularly important in the work of this laboratory, and we anticipate that they will again make extensive visits here during the course of the present project. We have indicated some of the lines of work that will be pursued independently at Pavia; however, the facilities there are not ideal for efficient work, and the effort can be refreshed to mutual advantage by these visits.

Dr. Sgaramella was also instrumental in recruiting Dr. S. Ehrlich as a senior fellow in my laboratory; the quality of his contribution permeates this report and the publications that are in press and in preparation.

The undersigned agrees to accept responsibility for the scientific and technical conduct of the project and for the provision of required progress reports if a grant is awarded as the result of this application.



25 May 1976
Date

Joshua Lederberg, Professor and Chairman
Principal Investigator