M. & I.
May 13, 1950

SUMMARY SHEET

RG 1445(C2)
D

UNIVERSITY OF WISCONSIN

Joshua Lederberg
Assistant Professor of Genetics

"Genetics of Salmonella."

Year	Requested	Recommended	Previous Commitment	Application Period
3rd (C2)	$4,320	$4,320	$4,320	8/1/50-7/31/51

RECOMMENDATION: Approval for 1 year.

COMMENTS: The Study Section considers some progress has been made in this study and approval is recommended in view of the existing commitment for further support.

It is understood that the investigator has been unable to demonstrate genetic recombination in the genus Salmonella.

Priority Score: 191

Council Action:

S.G.Action:

Previous and Current Support, this Project

Grant No.	Requested	Granted	Application Period
RG 1445	$3,780	$3,780	7/1/48-6/30/49
1445(C)	3,780	3,780	7/1/49-7/31/50

Microbiology &
Immunology
May 5-6, 1951

SUMMARY SHEET

E 72(C3)
~~RG 1445(C3)~~

UNIVERSITY OF WISCONSIN

Joshua Lederberg
Associate Professor of Genetics

"Genetics of Bacteria."

Year	Requested	Recommended	Previous Commitment	Application Period
4th (C3)	$9,936	$4,320	-	9-1-51/8-31-52
5th (C4)	10,800	4,320		9-1-52/8-31-53
6th (C5)	10,800	-		
7th (C6)	11,880			
8th (C7)	11,880			

RECOMMENDATION: Approval

PRIORITY SCORE: 192

COMMENTS: Discussion revealed that the investigator's early work was done with E. coli. He then went to Wisconsin and applied similar technics to Salmonella species and did not have success. He has now gone off on a tangent but the new approach is extremely interesting. The need for competent investigation in the field of bacterial genetics was expressed. However, it is felt that he should continue at his former financial level until the new approach is tried and found productive.

FINAL ACTION:
June 15-16, 1951

PHS Support, this Project

	Requested	Granted	Application Period
RG 1445 thru (C)	$7,560	$7,560	7-1-48/7-31-50
1445(C2)	4,320	4,320	8-1-50/8-31-51

M & I
April 26-27, 1952 SUMMARY SHEET E 72(C4S)

THE UNIVERSITY OF WISCONSIN
Madison 6, Wisconsin

Joshua Lederberg
Associate Professor of Genetics

"Genetics of Bacteria."

Year	Requested	Recommended	Previous Commitment	Application Period
(C4)	~~10,800~~ 4,320	4,320	4,320	9-1-52/8-31-53
(C4S)	4,860	4,860	-	"

Budget with E-72(C4)

RECOMMENDATION: Approval

PRIORITY SCORE: 162

COMMENTS: This professor is a recognized authority in the field of genetics, who has demonstrated his originality and productivity. The supplemental fund requested is amply justified in accordance with the terms of the project and the competence of the investigator. Indeed, the funds requested represent almost the sum by which his previous request was reduced "until the new approach is tried and found productive."

FINAL ACTION:
June, 1952

PHS Support

Grant No.	Requested	Granted	Grant Period
E 72 thru (C2)	11,880	11,880	7-1-48/8-31-51
(C3)	9,936	4,320	9-1-51/8-31-52

THE GENETICS OF SALMONELLA RG-1445(c2)

National Institute of Health, U.S. Public Health Service, Bethesda, Md.

Third Annual Progress Report, submitted April 20, 1951.

Joshua Lederberg
Associate Professor of Genetics
University of Wisconsin, Madison.

SUMMARY

Current work suggests that, under certain conditions, Salmonella cultures form reduced cells which a) readily pass filters retaining the usual bacteria; b) are more resistant to disinfection (heat, alcohol, chloroform); and c) remain dormant in the absence of a stimulus from living cells. If verified, these findings might require a reexamination of concepts of bacteriological sterility.

This result was unexpectedly encountered in connection with work on the mechanism of genetic recombination. Such a process, analogous to that established in Escherichia coli K-12, seems to occur in some strains of S. typhimurium. Whether there is a specific connection between the filtrable agent and genetic recombination, as appears possible, is the subject of current experiments.

THE GENETICS OF SALMONELLA RG-1445(c2)

Third Annual Progress Report, submitted April 20, 1951.

Recapitulation and Introduction

This research project on genetic aspects of the biology of Salmonella is directly related to previous and concurrent studies on genetic recombination in the related group of coliform bacteria. It will help to outline progress and aims of the Salmonella project if the results from Escherichia coli are briefly recapitulated.

About five years ago, E. L. Tatum and the undersigned (at Yale University), discovered that genetic recombination occurs among cells of E. coli strain K-12 (1,2). This was demonstrated by mixing cells from different nutritionally exacting or auxotrophic mutant cultures on a synthetic agar medium. By suppressing the auxotrophic parents, this medium selects for a small proportion of cells which have become prototrophic, i.e., able to form colonies in the absence of supplementary growth factors. With appropriately developed mutant stocks it was possible to rule out the possibility that these prototrophs arise from intrinsic instability of the mutant parents; they could arise only from the interaction of the distinct mutants: e.g., Ab with aB give rise to AB. This type of recombination process immediately suggested the possibility of a sexual process in this bacterium. This possibility has been reinforced by a number of experiments which showed the following:

1) Recombination was not confined to nutritional factors. Many other genetic differences introduced with the parents (e.g., fermentation characters, virus resistance, drug resistance) reassort in all possible combinations among the prototrophs.

2) Recombination occurs pairwise. In mixtures of three kinds of mutants, only those recombinants occur which could arise from pairwise exchanges, whereas uniquely tri-partite exchanges are not found.

3) In certain crosses, cultures ("heterozygous diploids") have been isolated which carry the genetic factors from both parents, which may later segregate during the further proliferation of the culture (3). Single cell studies showed conclusively that this segregation involves the separation of intra-cellular units, not entire single cells (4).

4) Numberous attempts to effect genetic exchanges by means of culture filtrates, cell extracts, or other preparations not containing normal viable cells of both parents have failed completely. This requirement for intact cells from both parents supports the concept that the fusion of ordinary vegetative bacterial cells is the basis of genic conjunction (as in many other microbes) and that no special gametic forms need be invoked. However, the actual fusion of cells has not been observed in this material, so that our conclusions on this detail of the "sexual" process are entirely inferential.

Until recently, evidence for gene recombination in bacteria was confined to strain K-12 of E. coli. Cavalli, working at Cambridge, England, has discovered a strain there which can be crossed with K-12 (5) and more recently a considerable number of strains have been isolated (about 3% of a series of 500 tested) which also show this phenomenon (6). Many of these new "crossable" strains are quite different from K-12, some being classifiable as paracolon or as coliform intermediates. This result makes it all the more necessary and hopeful to scrutinize other bacteria for similar genetic processes. Two objectives are preeminent: a) to provide the basis for genetic analysis of problems unique to other bacterial groups, e.g.- antigenic and pathogenic variation in Salmonella, and b) to find better material for the determination of the mechanisms, scope, and ecological role of recombination.

Experimental Results with Salmonella

Considerable time was spent during the first two years in collecting suitable cultures, developing techniques for producing biochemical mutations in Salmonella (7), and in perfecting the training of the research assistant assigned to this problem (Mr. Norton D. Zinder). After an interval during which mutants were induced in a diversity of types, including S. typhimurium, poona, madelia, "coli", and others, it was decided to concentrate on a coherent set of cultures of S. typhimurium.

Such a set was provided by Dr. S. Lilleeungen (Stockholm) who had worked out a procedure for bacteriophage typing of this species. He kindly placed at our disposal representative cultures of each of his 22 types of world-wide origin. In this way we could be fairly sure of covering a comprehensive sample of S. typhimurium cultures without unnecessarily reduplicating our work.

Auxotrophic mutants have been induced in 20 of these types. The present material has allowed crossing tests (like those in E. coli K-12) to be made in about half of the 200 possible combinations (inter- and intra-strain crosses); the mutants required to complete all of the possible combinations are being produced from day to day. It should be pointed out that, despite technical advances, the production and characterization of at least two double auxotrophic mutants in each of 20 strains represents a considerable multiplication of effort and has occupied the larger part of the time spent on this project to date.

Of the 99 combinations so far tested, 9 have more or less consistently given prototrophs on minimal agar medium, while the parents separately do not form colonies under these conditions. This is preliminary presumptive evidence for recombination in Salmonella typhimurium. Some of the combinations have given very low yields of prototrophs, a result which hinders the further study of the mechanisms of the apparent recombination. Our effort has been focussed on one particular combination which proved to be exceptionally fertile. Culture "A" is a mutant derived in two steps from Lilleeungen's type 2, "B" from type 22. "A" requires histidine and methionine "B" phenylalanine "plus" tyrosine, and tryptophane. Cultures of A or of B by themselves have never been found to produce prototrophs, even when very dense suspensions were plated on minimal agar. However, mixtures of A with

B have given yields of prototrophs of at least 10^{-5} of the parental inoculum, (considerably higher than has been found in E. coli). Since this combination gives the highest yield of presumably recombinant prototrophs, it was selected for further study. Two directions are being followed a) the genetic rules of recombination, and b) its biological mechanism.

With respect to a) certain peculiarities have been noted already. Attempts to induce fermentation mutants in A have been mostly unsuccessful; a number of mutants have been induced in B, and such stocks as "B" Gal- Xyl- (galactose-, xylose-negative) developed; in distinction to the Gal + Xyl + characteristic of the original A and B. "Gal" and "Xyl" are here used as unselected markers, i.e., the distribution of + and - qualities among prototrophs of A Gal+ Xyl+ x B Gal- Xyl- is followed. So far, the prototrophs have been almost all Gal- Xyl- like the "B" parent, and a like result has been obtained with other markers. However, a very small proportion of Gal+ Xyl- and Gal- Xyl+ have been formed. The disproportion of types might be due to genetic linkage, but more work will be needed to clear this up.

It has also been noticed that many fermentation mutants lose the "fertility" characteristic of the original culture. This probably represents an inherent instability in the capacity to react with other strains which must be given close attention in our survey.

The most unexpected results deal with the mechanism of genetic interaction, and have been purposely left to the last. In view of their rather heterodox character, they will have to be subjected to more than usual scrutiny, and tested in other laboratories before they can be entirely acceptable to any large body of workers, including ourselves.

These experiments began with one modelled after a report by B. D. Davis (8). A U-tube was constructed with an ultra-fine sintered Pyrex filter in the horizontal arm. The tube was sterilized and filled with broth. "A" was inoculated in one arm, "B" in the other. By alternating suction on the two sides, the medium was flushed from one compartment to the other until the cells had become so dense as to clog the filter. Several experiments in which one side only was inoculated with A or B confirmed the integrity of the filter, and the stability of the parent cultures.

The cells in each compartment were harvested and washed separately. Neither A nor B cells from control experiments gave any prototrophs on minimal agar. However, the B cells from the U-tubes in which the opposite arm contained A, repeatedly gave numberous prototrophs; the A cells did not. As in the experiments in which the cells were mixed directly, most of the prototrophs carried the unselected markers of B, but other types have also been noticed. Evidently, the interaction of A with B involves an "agent" produced by A which can pass a filter that retains the typical cells of A and B. The agent has been studied further in culture filtrates and other preparations. It is not produced (except to a very limited extent in aged cultures) by A or B cultures separately. Mixed cultures of A and B grown for several hours were sedimented and the supernatants passed through two Mandler filters, one medium, one extra fine, (the first of these usually

suffices for sterile filtration). 0.1 ml. of such a filtrate plated with 10^8 cells of B usually yielded ca. 100-200 prototrophs. The filtrates themselves were sterile by the usual criteria (no colonies on synthetic or complete agar medium; no turbidity in yeast extract broth).

Two filtrable factors are demonstrated by these experiments: 1) from B which stimulates A to form the agent 2) which reacts with B to form prototrophs. The first factor is probably a latent lysogenic bacteriophage secreted by B, as it can be propagated on A concomitantly with phage lysis. It can be replaced by sublethal concentrations of crystal violet; other deleterious treatments are being studied.

The agent is readily assayed by plating test samples with washed B cells on minimal agar. The number of prototrophs formed is proportional to the volume of a given filtrate tested, i.e., the assay is linear. The agent is more resistant than ordinary cells to inactivation by heat, chloroform, benzene, or alcohol, and is relatively unaffected by exposures which effectively sterilize the cells of B. It is apparently nondialyzable. It is precipitated by 60-70% ethanol or 60% saturated ammonium sulfate; the sediment redisperses readily in water. The agent has also been sedimented directly from filtrates by ultra-centrifugation in the Spinco centrifuge. These findings simplify the concentration and preparation of the agent.

We have not succeeded in extracting the agent from cells of B killed by heat, or subjected to autolysis under conditions proven harmless to the agent itself. This and later findings suggest that the agent is a biological product rather than an intracellular component of B.

The most obvious interpretations of the agents are:

1. as a "transforming agent" similar to those of pneumococci or Hemophilus influenzae.

2. as a minute cell product, a "gamete", or a form similar to the L-forms reported by several other authors (see 9).

The following results are especially tentative but incline to the latter hypothesis. Microscopic examination of active sediments shows barely visible granules and rods resembling ordinary bacteria except for their greatly diminished size. Probably more important, platings of apparently sterile filtrates with various kinds of cells, including Escherichia coli, have resulted in colonies with the same cultural characteristics and mutant markers as the original B. This suggests that the agent consists of reduced cells which are a) "filtrable" b) are more resistant to antiseptic treatments, and c) will remain dormant except in the presence of living bacteria. The possible implications of this tentative result for broad problems of antisepsis and "sterility" are obvious. In addition, it should be taken into account in reviewing the mechanisms of "transformations" reported for various bacteria. Whether they have a unique sexual or gametic function is problematical. In preliminary experiments, however, the filtrates may have evoked prototrophs more readily and from a wider range of mutant cultures than did intact cells of B.

References

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5. Cavalli, L. L. and Heslot, H. 1949 Recombination in Bacteria: Out-Crossing E. coli K-12. Nature 164:1057-58.

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7. Lederberg, J. Isolation and Characterization of Biochemical Mutants of Bacteria. Meth. in Med. Res. 3:5-22.

8. Davis, B. D. 1950 Nonfiltrability of the Agents of Genetic Recombination in Escherichia coli. J. Bact., 60:507-508.

9. Stempen, Henry and Hutchinson, W. G. 1951 The Formation and Development of Large Bodies in Proteus Vulgaris OX-19. I. Bright Phase Contrast Observations of Living Bacteria. J. Bact. 61:321-335.

February 11, 1952

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The Genetics of Bacteria (E72-03)

Annual Progress Report submitted to the

Microbiological Institute, National Institutes of Health
Public Health Service, Bethesda 14, Maryland

by Joshua Lederberg, Associate Professor of Genetics
Department of Genetics, University of Wisconsin

This report covers the period from April 20, 1951 to February 1, 1952

A. Summary

In Salmonella typhimurium, a new mechanism of genetic exchange has been discovered: transduction. Many cultures engender a filtrable agent (FA) characterized by its genetic activity. FA transmits individual characteristics to individual cells, in contrast to the linkage complexes exchanged in sexual recombination. All of the traits studied (nutritional requirements; fermentations of sugars; resistance to streptomycin; and flagellar serotype) were transducible. For the most part, laboratory mutations of S. typhimurium were studied. In addition, the natural differences of S. typhi and S. typhimurium were exchanged resulting, for example, in the serological "hybrid" of the antigenic formula: IX, XII, i;--, (somatic antigen of typhi; flagella of typhimurium). Detailed studies of the genetic and kinetic properties of the FA have been initiated. It may be related to particles about .1 micron in diameter visualized with the electron microscope in partially purified active preparations.

In Escherichia coli, the sexual process underlying genetic recombination has been studied for some time. Many new interfertile strains have been found. For the first time, evidence of specific compatibility relationships, i.e., of sexual or heterothallic differentiation, has been uncovered.

Direct evidence of the spontaneity of drug-resistance mutation has been secured by a new method: replica plating.

B. Full Statement of Progress

1. Genetics of Salmonella.

The last report presented first evidence for a process of genetic exchange in Salmonella typhimurium. This report also gave a brief review of previous work [illegible] to these studies. At this time, it can be seen that certain of the conclusions of the 1951 report were incorrect, particularly those which attempted to rationalize genetic exchange in Salmonella in terms of "reduced cells" or gametes. The mechanism of recombination in Salmonella appears to be fundamentally different from the sexual processes of E. coli. To emphasize this distinction, the term "transduction" will be applied to the Salmonella system.

Most, if not all, strains of S. typhimurium are lysogenic, carrying one or more latent phages, acting on other typhimurium or other serotypes. Under appropriate conditions involving either attack by a latent phage from another strain, or the (rather obscure) activation of its own latent phage, almost all strains have been found to liberate a filtrable agent "FA" with remarkable genetic properties. Sterile, cell-free preparations of FA are capable of transferring individual traits from the donor strain to other susceptible strains.

For the most part, we have relied upon nutritional requirements (obtained as ultraviolet-light induced mutants, and isolated with the help of the penicillin method) as the genetic "markers" for these experiments. We have also used fermentative differences (both natural and mutational), resistance and susceptibility to streptomycin and the natural serological differences between serotypes (S. typhi and S. typhimurium). Since the frequency of transduction for a particular character is only about 10^{-6} to 10^{-5} of the treated cells, selective media must be used to detect the

changes. For nutritional changes this simply involves platings on synthetic agar medium. Fermentative or resistance transductions were detected by platings on sugar- or streptomycin-containing agar. Flagellar antigenic changes were selected by Gard's technique (inoculation of semisolid agar containing specific antiserum). For technical reasons, the genetic changes involving nutritional requirements have been emphasized for quantitative studies. Several of the auxotrophic mutants are sufficiently stable that spontaneous variations could not be detected; for some, the spontaneous reversions did occur frequently enough to require corrections for their rates. Perhaps the most important distinctive feature of transduction (aside from the filtrability of the agent) has to do with the uncorrelated behavior of different markers. In several different detailed experiments in which the FA-donor differed from the FA-recipient strain in several respects (e.g., nutrition; fermentation of xylose; fermentation of galactose; resistance to streptomycin), each of the individual traits was subject to transduction, but independently of all the others. That is, e.g., all of the streptomycin-resistant transduced cells remained like the parent cells in respect to fermentation and nutrition, and so on. This is in marked contrast to the unrestricted exchange of several markers in the non-filtrable E. coli system. We have therefore defined "transduction as a genetically unilateral exchange, in contrast to the union of equivalent elements in fertilization". In the last report, we were uncertain whether the Salmonella FA might not consist of some sort of "reduced cell" or "gamete", but the transfer of single elements is inconsistent with such an interpretation. We were also concerned about the persistence of occasional, possibly dormant, cells of the donor strain in the FA filtrates. We have since found that filtration through UF sintered Pyrex discs consistently removes all contaminating cells without impairing transductive

activity. The greater filtrability (through Mandler diatomaceous earth candles) of bacteria in FA preparations compared to ordinary cultures is of questionable relevance to the present problem, although it may still be significant in other connections (cf. "L-forms"). Most of the FA preparations used in our present work show no evidence of the persistence of the donor cells in any organized form. Since individual traits only are transferred, transduced types are usually readily distinguishable from both the donor and the recipient strain. Therefore, the sterility of the FA preparations (although well verified) is not critical for the validity of their effects. Some thirty different markers, in several different strains, have been tested for their transmission by FA, and gave comparable results in each case. There can be no doubt that the transductive system applies to most or all of the genetic material of Salmonella.

Reproducible, linear assays for FA have been developed, based upon the yield of prototrophs from suspensions of auxotrophic cells plated with the test samples on minimal agar. This has allowed studies of the stability and purification of FA, and of its adsorption on to susceptible cells. Concentrated preparations assaying close to 10^6 units/ml have been obtained by fractional centrifugation and precipitation with alcohol and ammonium sulfate. Such preparations are visibly opalescent, and show, under dark-field and electron microscopy numerous granules uniformly about .1 micron in diameter. The association of transductive activity with these granules is only a working hypothesis, but the size estimate is consistent with that secured by centrifugation and filtration experiments with gradocol membranes. The preparations are not yet, in our judgment, sufficiently pure to warrant direct chemical analysis. FA has proved to be resistant to several enzymes, including trypsin and desoxyribonuclease. The last point is the chief difference between the Salmonella transduction and the "transforming"

systems of the pneumococcus and Hemophilus, but may be a reflection of greater structural organization.

The adsorptive properties of FA are related to the "XII" somatic antigen of the salmonellae of groups B and D. Most of the serotypes tested from these groups adsorbed FA with high efficiency; rough S. typhimurium, or bacteria from other groups lacking the XII antigen fail to bind FA. Whether the adsorption of S. typhimurium FA by other serotypes is followed by genetic transduction has been tested only for S. typhi. The differential traits most readily available were streptomycin resistance (from a S^r S. typhimurium), fermentation of rhamnose and of arabinose, and the serological differences. The resistance and fermentative differences were individually transducible along the same pattern as between S. typhimurium strains. Monospecific anti-IX sera could not be obtained in sufficiently high titer for selective experiments involving the somatic antigens (owing to the complexity of the XII antigen common to typhi and typhimurium).

The differences in the flagellar antigens afforded an excellent opportunity to test the possibility that transduction would yield significant, new, "hybrid" types. S. typhi is diagnosed as IX, XII; d;—. S. typhimurium is IV, V, XII; i-1,2,3 . . . Barring the exceptional occurrence of artificial "j" phases, S. typhi cells are immobilized in semisolid agar containing d-antiserum. In about half of a score of trials, however, inocula of S. typhi cells exposed to S. typhimurium FA have resulted in a motile (non-d) phase. Following purification, these have been proven to be a new "hybrid" serotype: IX, XII; i-———. (We are indebted to Dr. P. R. Edwards of the P.H.S. Communicable Disease Center for confirming this diagnosis, and for providing many experimental materials). This type has not yet been encountered outside the laboratory. It is amply clear,

however, that transduction may well be an active mechanism for the continued evolution of new Salmonella types by the recombination of existing antigens.

Quantitative adsorption studies have been conducted only within S. typhimurium. Adsorption of FA occurs promptly: suspensions of 10^9 cells/ml become saturated within fifteen minutes at 37° C. No cofactors or pretreatments of the cells have been found necessary for adsorption (contra certain phages, or the pneumococcus transforming principle). The efficiency of transduction appears to be limited by the saturation of the susceptible cells. The experiments do not permit us to evaluate how many particles are adsorbed per cell. It is not necessary to conclude that one adsorbed particle excludes another, but this is a possible explanation for the single-ness of transduced effects. The saturation of cells exposed to excess FA has been verified by their refracteriness to transduction by a second FA preparation capable of mediating additional effects. The saturation level corresponds to the conversion of about one per 500,000 cells with respect to a single trait. The proportion of cells transduced for all other traits is certainly much higher, but no effects are discernible unless the donor and recipient cells are different. The most consistent picture is that each FA particle is associated with a single genetic potentiality. If a cell adsorbs a particle of a type relevant to later experimental tests, it will have a certain (so far undetermined) chance of being counted as a transduction. It is tempting to identify the FA particles as "naked genes", but the fate of comparable speculations on filtrable viruses serves as a warning. In any event, the particles appear to be too large to correspond to individual genetic units: either they are more complex than our experiments have so far revealed, or the particles are vehicles for a much smaller active unit.

This report is abstracted from a manuscript by N.D. Zinder and J. Lederberg "Genetic Exchange in Salmonella" already submitted for publication.

2. Mechanism of Development of Bacterial Resistance to Antibiotics.

Despite considerable evidence favoring the spontaneous mutation theory of "drug" resistance of bacteria, the question has continued to be mooted whether an antibiotic might not actually induce directed mutations for resistance. So long as resistant mutants could not readily be isolated except by exposing bacteria directly to the selective agents, only rather indirect and abstruse (biometric) evidence was available, and judging from many informal discussions, this has not been entirely convincing to many bacteriologists. A recent advance in methods--replica plating and indirect selection--has resolved this problem to some extent by making possible the isolation of resistant mutants without direct exposure of the selected bacteria to the drug.

Replica-plating is a method for "copying" or "printing" the pattern of microbial growth from an initial agar plate to a series of other media. A sheet of velveteen (previously steam-sterilized) is fastened to a support with circular cross-section slightly smaller than a Petri plate. The initial plate carrying the bacterial growth (colonies or otherwise) is pressed down on the velveteen, transferring an imprint of each colonly to the fabric. Plates of various agar media can then be pressed on the same fabric, and each of them will be imprinted with a replica of the original growth. Upwards of 200 colonies on a plate can be transferred in one operation to a series of other plates with accurate registration of their position. The applications, for example, to the more efficient detection of nutritional variants, or determination of antibiotic spectra, are nearly self-evident.

For the present application, the initial plate carries a uniform film of growth from a large, spread inoculum, rather than single colonies. If, for example, mutations of E. coli resistant to streptomycin have

occurred spontaneously during growth on this plain medium, the resistant cells should be distributed as families or clones of varying size. This prediction was readily verified, for replicas to a series of streptomycin-agar plates resulted in the development of resistant colonies at superimposable positions, corresponding to the locations of the mutant clones on the initial plate. The clonal occurrence of resistant cells is not consistent with the idea that resistance is directly induced by the streptomycin.

The experiment can be extended to give an even more rigorous conclusion. The initial plate has not been exposed to streptomycin, but the sites of many of the resistant clones have been revealed on the replica plates. Inocula taken from the indicated sites are considerably enriched in the proportion of mutant cells (from 100 to 1000-fold). The enriched inocula were replated at a higher dilution so that a few resistant cells were included. After growth on the second initial plate, replicas were again made on streptomycin agar, and the resistant sites again located. The repeated enrichment by 100-fold resulted after a few cycles in inocula of which a considerable fraction was resistant, so that pure resistant cultures were isolated from single colonies. At no point has the indirect selection line been exposed to the streptomycin; the replicas alone were so exposed, and these were used only to locate the mutant clones.

The pure resistant cultures were stable, resembling in every respect the mutants obtained by direct selection. The method was also applied to phage-resistance in E. coli, and should be of general utility.

These data are embodied in "Replic Plating and Indirect Selection of Bacterial Mutants", by J. Lederberg and E.M. Lederberg, 1952, J. Bact., In Press (March 1952).

Note on physiological mechanism of streptomycin-resistance: Other workers have reported (Smith, Oginsky, and Umbreit, 1949, J. Bact. 58: 761-767) that mutants of E. coli selected for streptomycin resistance show profound changes in their aerobic metabolism, so that the resistant forms are unable to be benefited in their growth by aeration. It might be thought that this modification was a direct effect of exposure to streptomycin, similar to the acriflavine-induced non-aerobic mutants of yeast discussed by Ephrussi, rather than a consequence of the resistance-mutation. This possibility would be of great genetic interest, and would be accessible to study be means of indirect selection. We soon found however that we could not reproduce the published findings: each of several resistant selections in several strains of E. coli was benefited by aeration to the same marked degree as its sensitive parent. The same held for strains provided by the workers cited. We have since learned that other investigators likewise failed to confirm the reported findings.

3. Genetic Recombination in Escherichia coli.

The methods, experiments, and reasoning that have led to the conclusion that a sexual phase operates in E. coli have been presented at length in several publications (cited in bibliography in support of Research Plan) as well as in previous progress reports, and will not be repeated here.

This process is under intensive study from many aspects: two new developments are of sufficient maturity and general interest to be recounted in this report.

a. Strain crosses. A screening method previously developed has been applied on a large scale in a search for additional sexually fertile strains of E. coli. The screening test identifies only those cultures capable of crossing with the initial K-12 strain. About 35 interfertile strains have been isolated in tests of about 1500 isolates from various sources. Most of the fertile cultures were obtained from human feces, urine, or infected lesions, although other sources were well represented. Nevertheless, no obvious cultural characteristic serves to identify the fertile cultures as distinct from the overall population. Most of them are fairly typical E. coli with individual differences in sugar reactions. A wide range of colicin and phage responses, colonial morphology and serological types is represented: the major somatic antigens are distinct in most or all of the strains tested. A program on the immunogenetics of E. coli has been initiated to determine the genetic complexity and mode of hereditary transmission of the somatic antigens. To date, it would appear that these antigens are inherited and recombinable as unit factors in the same sense as other mutative or natural differences.

b. Genetic and environmental control of fertility in E. coli K-12. Until recently, no experimental modification of the frequency of recombination had been discovered. All mutant derivatives of K-12 crossed among themselves

with about the same rate (equivalent to 1 recombinant per ca. 10^6 parental cells inoculated in the selective test system), and no experimental conditions were known to modify this rate. In particular, there has been no evidence of a mating-type differentiation: a search for this was a prime objective of the strain-crossing program. A "mutation" F- (as contrasted with the wild type F+) has been found with the following definitive property: two F- strains will not cross with each other. F+ x F+ and F+ x F- are fertile. Although quantitative studies have not yet been carried out, the latter crosses appear to be more fertile than the former, so that an incipient mating type differentiation is indicated. Certain F+ stocks show this preference for F- and incompatibility with F+ partners much more than others. The most surprising development concerns the inheritance of the F+/F- characters. The progeny of all crosses of F+ x F- were uniformly F+. After other genetic tests also raised the possibility that the F+ character could be directly transmitted to F- cells, the following type of experiment was done: F+ Lac- S^s cells were inoculated into broth together with F- Lac- S^r. After varying periods of incubation, the mixtures were streaked out on EMB or streptomycin agar to reisolate the Lac- S^r, presumably F- cells. Upon retesting, however, most of these colonies proved to be F+. This transmission of F+ is extraordinarily efficient. When 10^8 ml cells each of marked F+ and F- cells were incubated in broth for one hour, over 10% of the originally F- cells had become F+; over longer periods, almost all the F- became F+. No other genetic changes were noted. The transmission did not occur in non-nutrient media, in the cold, or via sterile-filtrates. Some preliminary experiments have been done on the possibility of transmission via cell-free extracts. The F+ agent is readily distinguished from lysogenic virus. This system is remarkable in providing the only example of a

non-sexual "transduction" in E. coli K-12, while the characteristic involved is the ability to undergo sexual recombination.

The F+ factor is of wide occurrence in E. coli, as tested by its transmission to F- testers. About half of the interfertile strains carry such an agent, and, in a limited number of tests, a few non-fertile isolates also show it. The non-F+ but interfertile strains form a new category, since some of them were shown to cross with F- K-12 although neither parent seemingly carried the standard F+.

It has also been found that some F+ cultures respond to aeration to simulate the F- behavior. This F- "phenocopy" is reversible, disappearing when the cells are recultured without aeration. Whether this and other differences of various F+ stocks are due to differences in the F+ agent itself is under investigation.

In addition to these studies, work has continued on the nuclear cytology of haploid and diploid E. coli; on formal and physiological genetics, especially of the loci controlling lactose-fermentation; and on the effects of radiations on the genetic behavior of E. coli. The present state of these problems is such that a formal statement of progress would not be very meaningful. Some details are included, however, in a summary analysis of this laboratory's work: "Recombination analysis of bacterial heredity", Lederberg, J., Lederberg, E., Zinder, N.D., and Lively, E.R., 1951, Cold Spring Harbor Symposia Quant. Biol., Vol. 16, in press (80 manuscript pages) of which reprints will be forwarded as soon as they are available.

C. Significant Accomplishments to Date.

The most significant accomplishment of this research program is to lay the groundwork for the genetic study of bacteria by recombination techniques. This has led to the discovery in Escherichia coli of a recombination mechanism that is almost certainly based on a sexual process, a mechanism previously excluded from bacterial biology. The larger part of our work since has concerned the details of this sexual process, its natural distribution, and its application to the genetic investigation of drug resistance, enzyme formation and antigens. It has been verified that resistance to streptomycin is a result of gene mutation. This mutation resembles genetic variations in higher forms in several detailed respects: it occurs spontaneously; it can be localized on a "chromosome" by means of linkage tests; it shows simple dominance relationships in heterozygous diploid cells (sensitivity is dominant to resistance).

Investigations on Neurospora have led other investigators to the conclusion that gene-enzyme relationships are simple and direct. We have found no support for this conclusion in studies on bacterial lactase. This enzyme is subject to control by any of a large number of different genes, some of which also affect other enzymes. Studies on mutants have contributed to the elucidation of "direct" fermentative pathways of disaccharides (e.g., the amylomaltase of E. coli). In the course of these studies, various methods applicable to biochemical and genetic studies have been developed, e.g.: determination of lactase by a chromogenic substrate o-nitrophenyl-B-D-galactoside; the penicillin method for the selective isolation of biochemical mutants; replica-plating method for large scale characterization of cultures.

Similar studies on a second group of organisms, Salmonella typhimurium, have led to a very different result. Recombination occurs, but not as a

result of a sexual process. Instead, individual genetic factors are singly transduced from one cell to another. This process has been experimentally verified to produce evolutionary novelties, for example, a serological "hybrid" of S. typhi and S. typhimurium.

These studies are not at a level where concrete accomplishments in the form of a new vaccine or antibiotic can be cited. They are concerned with the understanding of the biology of microorganisms, the details of which are necessary to long-range development of technological, medical, or epidemiological control.

The following publications have presented the main accomplishments; in most cases the titles are self-explanatory.

Lederberg, J. 1947 Gene recombination and linked segregations in E. coli. Genetics 32: 505-525.

1947 The nutrition of Salmonella. Arch. Biochem. 13: 287-290.

1949 Aberrant heterozygotes in Escherichia coli. Proc. Nat. Acad. Sci. U.S., 35: 178-184.

1950 Isolation and characterization of biochemical mutants of bacteria. Methods in Medical Research 3: 5-22.

1950 The beta-D-galactosidase of E. coli strain K-12. J. Bact. 60: 381-392.

1950 The selection of genetic recombinations with bacterial growth inhibitors. J. Bact. 59: 211-215.

1951 Single cell isolations of diploid heterozygous E. coli. J. Bact. 61: 351-355. (with M.R. Zelle).

1951 Prevalence of E. coli strains exhibiting genetic recombination. Science 114: 68-69.

1951 Streptomycin resistance: a genetically recessive mutation. J. Bact. 61: 549.

1951 Genetic Studies with Bacteria. pp. 263-289 in Genetics in the 20th Century, edited by L.C. Dunn. MacMillan, N.Y.

1951 Recombination analysis of bacterial heredity. Cold Spr. Harb. Symp., 16: In Press. (with E. Lederberg, N. Zinder, E.R. Lively).

1952 Replica plating and indirect selection of bacterial mutants. J. Bact.: In Press (March 1952) (with E.M. Lederberg)

195- Genetic exchange in Salmonella. MS ready for submission (with N.D. Zinder, pp. 37).

D. Plans for Next Year

The general outline of the next year's work is already inherent in the current experiments summarized in Part B. In the Salmonella transuction system, the following aspects should be given special emphasis: 1) The purification of the transducing agent, following procedures worked out in preliminary fashion (especially differential centrifugation); 2) morphological and chemical characterization of the purified material; 3) further exploration of the conditions of formation of FA, and its relationship to phage; 4) the distribution of FA-production and response in Salmonella; 5) exploration of serological "hybrids" of other Salmonella types, and the pathogenic properties of such hybrids in experimental animals.

In Escherichia coli, the program of immunogenetic study is marked for special emphasis. Antiseral reagents are being prepared against interfertile strains; some of them have been fractionated by reciprocal absorption. The segregation of antigenic differences in strain crosses; the examination of recombinants for non-parental antigens; and the antigenic behavior of diploid hybrids are the main features. In addition

to somatic agglutinogens, flagellar antigens and extractable precipitinogens are to be studied, the latter including the enzyme lactase. When the groundwork has been laid, it is hoped to look for mutations affecting antigenic specificity. Selective methods should be feasible: immobilization by serum-agar for flagellar changes, and bacteriolysis with complement for somatic antigen mutations.

The heredity or control of fertility by the F+ system is also of present interest. The mode of transmission of the F+ agent, its separation from the donor cells and the physiological basis of the F- modification by aeration are under present investigation. We are also studying the distribution of the F+ agent among bacteria, and will look further into its correlation with the potentiality of genetic recombination. In strain K-12, there is already evidence (the greater fertility of F+ x F- as compared to F+ x F+ and the sterile F- x F-) that the F-system leads to an incipient heterothallism as is characteristic of many fungi. The F+ agents from various sources will be compared to determine whether particularly compatible combinations can be found that will lead to a simplification of the study of recombination by genetic methods. If the frequency of recombination can be upgraded, and there is now the first evidence of rational control, it may be possible to approach the problem of bacterial sexuality by more direct cytological study.

mutation. J. Bact. 61: 549.