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

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A. Summary

Studies on the mechanism of genetic transduction and its application to immunogenetic analysis are detailed. A wide variety of serotypes can now be artificially synthesized.

In *E. coli*, single cell studies and the analysis of persistent diploids have confirmed the picture of sexual recombination in this species as including the interaction of two intact parental cells and the migration of an intact nucleus from one parent to the other.

This is not a publication, but may be informally quoted as a private communication.

Genetics of Bacteria

SALMONELLA

During the period covered by this report, our program has enjoyed the close collaboration of Dr. C. C. Spicer and Dr. A. Bernstein, both on leave from the British Public Health Laboratory Service, and especially of Dr. P. R. Edwards of the Enteric Bacteriology Laboratory, Communicable Diseases Center (P.H.S.), Chamblee, Ga. Some of these observations are also directly related to previous work of Dr. Bruce Stocker (presently at the Lister Institute, London) and the dissertation of Mr. (now Dr.) N. D. Zinder, presently at the Rockefeller Institute.

In previous reports and publications (1,9,10,11) an account has been given of the discovery of genetic transduction by means of temperate bacteriophage, which forms the basis of most of our work in Salmonella genetics at this time. Briefly, it has been established that temperate bacteriophage particles may carry small hereditary fragments from the cells on which they have been grown to new host cells. Provided that the new hosts are not killed outright by the phage, they may then acquire the genetic traits thus transduced. Since each of a wide variety of genetic markers is subject to transduction, it is inferred that they are typical of the entire heredity of the bacterium. However, the fragmentation of the genotype in transduction does not necessarily imply a similar disorganization in the normal vegetative history of the bacterium. More likely, the hereditary content is normally organized in chromosomes or nuclei, and is only broken up (and transferred to other cells) by the lytic action of the growing phage. The most perplexing problem is the means by which such fragments can be implanted in the stable genetic structure of the new host. However, this enigma is shared by students of related processes of genetic exchange among bacteria, viz. the "type transformations" of the pneumococcus and the influenza bacillus. These phenomena betray a fundamental similarity that justifies their being subsumed as comparable examples of genetic transduction. The unique feature of transduction in Salmonella is the intervention of bacteriophage; in transduction in the pneumococcus, nuclear fragmentation is achieved by chemical means, but the manner in which the fragments are able to penetrate the bacterium without the help of a biological vector is not understood at all.

BACTERIOPHAGE AS THE VECTOR OF TRANSDUCTION

Previous reports (1,10; see also 12) have cited the evidence for the gross morphological and surface (i.e. size, reactions with antisera and bacterial receptors, behavior in chemical purification and response to inactivating chemicals and heat) similarities of the Salmonella transducing agent (FA) with phage. However, these findings would be reconcilable with the release of two categories of particles by bacteria in which phage has grown: phage, strictly speaking, and superficially similar particles carrying FA, to the exclusion of phage nuclei as inhabitants of phage skins (13). The only contribution to this problem has been the finding of a statistical association of the incidence of induced lysogenicity among transductions and their unaltered neighbors under conditions where not all the surviving progeny of infected bacteria are lysogenized. As in *E. coli* (4,14), infected Salmonella cells generally give rise to mixed clones with sensitive and lysogenic survivors, and the observed correlations presumably refer to the coordinate distribution of lysogenicity and the transduced trait among the progeny of infected individuals. This point has been much more definitely established with an *E. coli* system (see below). The relationship of phage to FA has also been examined with the help of a virulent mutant (22V) of the temperate phage 22. As bacteria lysogenic for 22

are immune to 22V, selective survival of 22-infected cells is achieved by exposing them to 22V. With a low ratio of phage 22 to bacteria, 22V was found (as expected) to destroy most of the bacteria, but the incidence of transduction was not concurrently reduced, i.e., transduction is associated with protection against 22V. As the surviving bacteria are all lysogenic for 22V, it is concluded that the FA particle contains both a phage 22 nucleus and a bacterial fragment. That this association is fortuitous is suggested by the virtual absence of specificity of transduction, i.e., any single trait is subject to transfer at a rate within a 50-fold range in the neighborhood of one per million phages. In addition, the phage nucleus may be functionally separated from FA in either of two ways: the use of a recipient host immune to the phage, which does not impede transduction so long as the phage is adsorbed by the bacterium, or by treating the phage with ultra-violet light, which destroys the lytic and lysogenizing function much more rapidly than transducing competence. Since very large doses of UV are known to interfere with the adsorbing power of phage, it is questionable whether any direct action on the enclosed fragment has yet been demonstrated, however interesting this might be in anticipation of mutagenesis studies with "free genes".

The phages employed in previous transduction work were all obtained from lysogenic *S. typhimurium*, and are probably related to Boyd's A1 series (6,15). The "virulent mutant" 22V has a somewhat reduced transducing competence, provided, of course, immune (lysogenic or otherwise) recipients are used. Baron (16) has reported that the *S. typhi* typing phage Vi II, in several of its adapted states is also competent. Through the courtesy of Dr. A. Felix and Dr. E. S. Anderson it has been possible to test a number of typing phages for *S. paratyphi* B and type-determinant phages of *S. typhi* (9); of the former, "B.A.O.R." and of the latter, k, proved to be capable of transduction. Thus at least four, serologically unrelated, phages of the *Salmonella* group will function in transduction, pointing to the possible generality of the phenomenon. Unfortunately, the effective host range of the other phages is generally rather less than that of 22, though the Vi II might be expected to function with Vi+ strains of *S. paratyphi* C (8). To date, however, transductions have been limited to groups A, B and D. Although these groups share the "XII" somatic antigen complex, it has not been possible to inculcate any specific component as the unique receptor.

ANALYSIS OF NON-MOTILE VARIANTS

The solidification of the principles of transduction analysis now permits their application to specific genetic problems. The first of these to be considered was the determination of the flagellar apparatus (6). Previous conclusions on the existence of three levels of determination--the formation of flagella, their antigenic content, and their locomotor function--by distinct sets of non-allelic genes have been confirmed in further studies. The restoration of motility has proven to be a useful artifice for the typing of non-motile *Salmonella* variants which could not otherwise be diagnosed as to serotype for epidemiological control.

LINKAGE

A few exceptions have been noted to the otherwise well-founded rule that only a single factor can be transduced at a time (the basic evidence for fragmentation as the first step of the process). The most clear cut of these exceptions involves one of the factors for motility (Fla_1) and the factor controlling the flagellar antigen of phase 1 (H_1). Transductions of motility to a non-motile strain of *S. paratyphi* B, SW-666, whose constitution may be given as $Fla_1 - H_1^b$, will result in Fla^+ , i.e., motile

selections whose flagellar antigen may be either H_1^b or that characteristic of the donor strain. That is, if this is *S. typhimurium*, $Fla_1^+ H_1^i$ \times $Fla_1^- H_1^b$ may engender either $Fla^+ H_1^b$, as required by the single transduction of the Fla marker, but also in some $Fla^+ H_1^i$, as would indicate the concurrent transduction of the H_1 marker as well. An extensive series of backcrosses and tests with a variety of distinct donors (6,7) have helped to verify that this is indeed a dual transduction, occurring much too frequently to be explained by coincidence, and therefore to justify the invocation of linkage. It is not unlikely that linkage is as characteristic of the genetic makeup of *Salmonella* as of *E. coli*, but that it will be obscured by the mechanics of transduction except for markers unusually closely related to one another. Similar observations have been made for the transduction systems of other organisms (reviewed by Zinder, 12).

As there is no means of selecting for the other combination, $Fla_1^- H_1^i$, this is not an ideal system for linkage studies. However, other Fla^- markers linked to H_1 have been found, e.g. in an *S. dublin* (recognizable as such after the transduction of motility!) from Guatemala. Attempts were made to map these two Fla factors and H_1 to determine if they would fit a unique linear sequence, but the results were ambiguous owing to the looseness of one of the linkages. (This program is being extended by Dr. Stocker with more comprehensive materials). However, significant progress in mapping studies will probably ensue only after additional linked pairs are discovered. The negative results of previous efforts portend that the projected search will be tedious.

ABORTIVE TRANSDUCTIONS

With most markers, it is possible to score only those genetic transfers that result in stable, hereditary changes insofar as the necessary selection and identification procedures require the handling of very large clones. However, motility can be scored directly or indirectly on single cells, so that it has been possible to detect abortive transductions of motility to non-motile variants. These were first observed (6) as trails of small colonies that have worked themselves deep into soft agar, but do not swarm out as motile clones. The trails were concluded to represent abortive transductions, i.e., the consequence of the introduction of a genetic factor (Fla^+ for motility) into a non-motile recipient in such a fashion that the factor can function but can not reproduce. The motile cell thus deposits a spoor of non-motile progeny at each fission. This interpretation has been verified by direct micromanipulatory studies on the motile transductions (Stocker, private communication, and confirmed, Lederberg, unpublished) which also promise to give more intimate knowledge of the early steps of the transduction process. The possibility that abortive transductions result from the transmission of fragments of a size or configuration unsuitable for their incorporation in the stable chromosome set is a leading hypothesis that has, as yet, no particular evidence. It would be difficult to imagine that the transduced fragment is directly incorporated so as to displace actively the previous homologue; instead, it may be incorporated (either directly, or as a copy) at a subsequent replication of the genetic material.

An alternative hypothesis for the trails would have assumed that they reflect the persistence of an unincorporated residue including Fla^+ after part of a fragment had been incorporated by crossing over. However, tests of trails from transductions with linked markers have failed to uncover crossover types such as the $Fla_1^- H_1^i$ referred to under Linkage. The antigenic character of the motile bacterium in this case would be of interest, but has not yet been settled. As trails are inhibited by either serum it may be b+i, i.e., reflect a "double heterozygous" condition for the segment, $Fla^+ H_1^i / \text{---}Fla_1^- H_1^b\text{---}$. If this is generally the case, it would suggest that linked factors are separated by some sort of cross-over process in addition to the primary fragmentation (as has been shown in the *E. coli* system).

IMMUNOGENETICS

The Salmonella group is especially appropriate for immunological studies, both from the point of view of the fund of consolidated information, and the importance of systematic findings on the serological and immunogenetic structure of a medically important bacterial group. The flagellar, or H, antigens are especially handy. Previous accounts (7,10) relate the results of systematic serotypic recombinations. This report will emphasize otherwise unrecorded details. They support the thesis that the mosaic structure of the group can be most readily explained by recombination processes. But it must be reemphasized that the genetic possibilities are only the initial raw material for evolutionary forces. The typhoid fever syndrome is still associated with a bacterium with certain biochemical peculiarities and, i.a., the d flagellar antigen, though it is dubious whether these are all directly concerned with pathogenetic properties.

Although S. gallinarum and "S. pullorum" are serotypically indistinguishable the former can be shown to carry a covert H₁ antigenic factor gm, identical with that of S. enteritidis (reciprocal absorptions by P. R. Edwards, priv. comm.), as well as certain Fla⁺ factors. On the other hand, S. pullorum has given completely negative results in similar experiments, supporting the differentiation of these species (though identical serotypes) which cause distinct diseases in the fowl. No criticism is implied of the serological scheme nor of the application of binomial epithets to serotypes! To be consistent, however, a serotype should include all serologically similar taxons (e.g. S. paratyphi C and S. cholerae-suis) and nothing else, and not be confused with species, the delineation of which has wisely been held in abeyance by international agreement.

Experiments like those reported previously on the mechanism of diphasic flagellar variation (7,10) have been continued and amplified, supporting but not proving the same tentative hypothesis: that the two alternative phases reflect the allelic states of two distinct loci, resembling e.g., the Rh:CDE and the ABO systems of human blood groups. In Salmonella, however, only one phase of a cell is expressed at a time, and almost all of the progeny of any cell remain in the same phase. The chief uncertainty concerns the mechanism of this alternation and its heredity, but the data still suggest a local fluctuation from active to inactive states, and vice versa, at the two loci. More decisive experimental material and methods are being sought.

A POSSIBLE GENETIC DUPLICATION

In a systematic examination of genetic homologies, the "specific" or phase-1 factors of a wide variety of serotypes were found to be mutually homologous (i.e., would displace one another in transduction experiments), and the same for the "group" or phase-2 factors. However, a strain of S. paratyphi B, var. java (CDC-157) (7) was found to be exceptional. This was first examined as an apparent "monophasic" 1,2... type, but its 1,2... factor was found to be homologous with typical phase-1 (rather than phase-2 as expected for this antigen). The parent strain, showing b:1,2 (as expected for this serotype) was then studied. It showed a quite sluggish phase variation from b to 1,2, whose reversal was progressively more difficult. Nevertheless, (by transductions in both directions) it was possible to show that both the b and the 1,2 factors of this strain are H₁ homologues. I.E., the immunogenetic constitution of this strain must be represented as H₁^b H₁^{1,2}, though as in typical diphasics, H₁^b H₂^{1,2}, only one locus is patent at any time. This constitution is required to explain not only the homology of 1,2 with specific phases in transductions from this strain, but the synthesis of such types as b:a and then c:a (otherwise unheard of) from transductions to the strain from typical stocks. Though both H₁ factors are

linked to Fla₁, attempts to demonstrate their linked transduction were frustrated by the progressive diminution of phase variability required to demonstrate the full antigenic potentiality. Monophasic variants of *Salmonella* species are well known for their usefulness as antigenic reagents. Three types have been distinguished by genetic analysis. 1) *S. typhi*: no locus homologous to H₂ can be demonstrated; if ever present it has presumably been deleted; 2) Some monophasic *S. paratyphi* B strains: an H₂ locus is demonstrable by transductions to the strain leading to the diphasic condition, but not otherwise. These can be understood as carrying a "null" allele at the H₂ locus. 3) *S. abortus-equi*: an allele is present at the unexpressed locus. The genetic background is presumably unfavorable to phasic variation in any direction, but has not been satisfactorily dissected. When transduced to other stocks, the components show normal behavior in at least some cases.

For technical reasons, the somatic antigens (O, Vi) have not proved so amenable to genetic study. Some antigenic variations have been noticed incidentally, e.g., the acquisition of the V factor (by a *S. abortus-equi* in the course of transductions involving its monophasic character. However, neither this example, nor alterations in the I component of *S. paratyphi* A are convincing examples (compare 18), as they have not been consistently related to the occurrence of a comparable factor in the donor cell. In fact, just these antigens are known to undergo "Form Variation" in at least some stocks, and it is possible that circumstances of the experiments apart from the transduction itself played a part in the selection of the antigenic variants. These experiments are being extended in several other laboratories (Washington, London, Copenhagen).

IMMUNOCHEMICAL DIFFERENTIATION OF FLAGELLAR PHASES

An earlier, and generally overlooked report (19) on the differentiation of phase-1 and phase-2 has been confirmed and amplified. In tube tests, after 2-4 hours at 37 C., acriflavine will agglutinate smooth, flagellar phase-2 cultures, but not phase-1. The differentiation has been found to be general throughout the group (except that H₁^{1,2} is agglutinated. However, H₂^{1W}, *S. wien*, is agglutinated; H₁^{1W}, *S. dar-es-salaam* is not.) The same reaction holds for the separated flagella. The agglutination is remarkably similar in appearance, and in timing and reaction to heat to that of a serum H agglutination. O-forms are unresponsive. The reaction is specific from pH 6-8; salt is not required. Several basic amino-acridines are equally effective, but 1,2,3,4-tetra-hydro-5-aminoacridine (generously furnished by Prof. A. Albert) was not, in surprising agreement with the bactericidal specificities of these compounds. Experiments with purified flagella have suggested that both phases will combine with the dye. Thus there may be a subtle difference in the physical structure of the flagella in the two phases, corresponding to the determination by two loci.

ESCHERICHIA COLI

These studies enjoy the active participation of Dr. E. M. Lederberg, Dr. T. C. Nelson and Mr. M. L. Morse. The sexual recombination mechanism of *E. coli* (5,20,21) is to be sharply distinguished from transduction as it operates in *Salmonella*, e.g. the interaction occurs exclusively between intact cells of the two parents, and the interchange of genetic factors occurs in large blocs approaching or equivalent to the entire genotype. In *Salmonella*, interchange generally involves just one marker at a time (very exceptionally two) and is mediated by filtrable bacteriophage particles. More recent observations (22) have indicated a formerly unsuspected polarity in the sexual behavior which is best explained by the assumption that one parent transmits only a nucleus, the other both nucleus and cytoplasm to the zygote.

But despite contrary speculations, (23), there remains no evidence to justify the implication of an extracellular, virus-like agent as the vector of the migratory nucleus, all experiments designed to detect recombination via extracellular elements having been categorically negative. The working hypothesis that still fits all present factual knowledge is that the zygote proceeds from a conjugation (contra copulation) of two cells in the course of which nuclear migration takes place. However, while this hypothesis has the support of the negative evidence just quoted, it has not yet been affirmatively substantiated by direct morphological study, for which there are considerable technical obstacles.

The discovery of the differentiation of F⁺/F⁻ states has been recounted previously, (2,3,10). F⁻ cells appear to be able to function only as the stationary parent; F⁺ as either migratory or stationary, possibly on the basis of a physiological heterogeneity of the cells in a culture. Thus F⁻ x F⁻ crosses are sterile, while other combinations are fertile. The remarkable, and least understood fact is that, i.a., the state of a culture depends on the presence of a hypothetical "F⁺" agent. That is, F⁻ cells can be converted to the F⁺ state (which persists as a stable hereditary potentiality) by mixed culture with other F⁺ cells. The simplest interpretation of this finding is to assume an infective, hereditary particle, the "F⁺ agent". But despite an extraordinary efficiency of transfer of the F⁺ state in mixed cultures, all experiments with cell-free preparations have been entirely negative. For example, actively exchanging populations have been separated through membrane filters directly into a competent recipient, in a matter of a few seconds. The contagious properties of the F⁺ state would make this a very sensitive test, but it gave a negative result. It can only be concluded that F transfer requires the direct, superficial contact of two intact cells: it is not necessary to assume cytoplasmic connections. Experiments to duplicate the experiments under microscopic control are in process. If the intact cell is the normal biological vector of the F status in transmission experiments, there may thus be a sense in which one could refer to the F agent as the vector of recombination, i.e., this is the cell itself. This reconciliation of apparent antitheses makes it likely that any resolution will have to follow the physical separation of the F agent as an extracellular entity; any functional separation, such as already recorded, will be equivocal. However, insofar as the presence of an infective F agent has been only one of several genetic and physiological factors controlling incompatibility, and in particular several other lines of *E. coli* seem to function in recombination as well with as without a transmissible F agent, it would seem premature to place the primary burden of genetic exchange on this obscure principle.

The analysis of crossing data in *E. coli* K-12 has been obscured by discrepancies from mendelian segregation for which no rationale was in sight prior to the discovery of F polarity. While, in 1947, one had no basis to doubt the single linear linkage grouping of the then available markers (20; see also 25) the discovery of aberrant heterozygotes in 1948 indicated that "the linkage map of *E. coli* K-12 will have to be systematically reexamined, with the use of several unrelated sets of stocks" (24), a warning that has since been reechoed (5,11,21). The qualitative anomaly of these stocks was hemizyosity (i.e. deficiency in one of two homologous chromosomes) for certain markers, particularly Mal and S (controlling maltose fermentation and streptomycin-resistance, respectively). The possibility was considered, at that time, that the deficiencies reflected defects already in the parental gametes, but had to be rejected for reasons similar to the argument below.

Not the least difficulty in rationalizing the segmental elimination that was postulated to explain the hemizygous defect was its non-random involvement of the contributions from the two parents. It was later realized that these parents were polarized in regard to F, and that it was the contribution of the F⁺ parent that was usually, but not always eliminated. In all these studies, heterozygosity for Mal

had never been observed (barring a single primary nondisjunction of different origin), including tests of nearly a thousand diploids in whose parentage Mal⁺ was either an "unselected" marker, or in which Mal⁺ (having come from the F⁺ parent) was selected on synthetic maltose medium. The Mal⁻S region thus clearly follows a different rule from other factors such as Lac and Mtl which are regularly heterozygous whether selected or not.

An explicit study of the role of F polarity on the character of diploid exceptions has therefore been undertaken, and recently concluded for the Mal⁻S region (26). To simplify the account, markers of the F⁻ parent will be referred to as orthotypic, from the F⁺ as paratypic; when both are represented in part, we have metatypic. It was first verified that regardless of parental couplings of F⁺/F⁻ elimination in the Mal⁻S region was usually paratypic, i.e., about 85% of the diploids (selected as Lac +/- prototrophs) retained the orthotype Mal⁻S markers. However, 13% were orthotype eliminations, retaining the paratype segment, and 2% were metatype, retaining one of the markers from the F⁺, one from the F⁻. The latter two classes, and the complete absence of heterozygotes for this region are inconsistent with a prezygotic defect of the F⁺ gamete, as proposed elsewhere (23). The metatypic hemizygotes exculpate possible reversals of polarity, by means of transmission of F on the cross plates, a possible error also excluded by other evidence. It is therefore concluded that segmental elimination occurs after a complete zygote has formed from the union of two intact genetic nuclei. This elimination will usually involve the paratypic segment. Where it does not, one may assume that there has been a preceding cross-over between the markers and specific site of chromosome separation (see also 30). A superimposed pre-zygotic elimination in some cases is not excluded, but supererogatory. For most analyses, it is immaterial whether the elimination is genetic or zygotic; as a rule only experiments permitting the observation of the unreduced diploid will allow a distinction.

Microscopic studies of E. coli recombination have been initiated to define more closely the cellular events of the process. "Hfr" strains are available which allow of up to about 10% recombination after about 4 hours growth in broth and tests of colonies from dilute platings on unselective media. (Other criteria of the incidence of recombination are inadmissible without detailed kinetic analysis owing to residual growth of heavier inocula even on selective media.) These crosses show a polarization favoring orthotypic markers of the Mal and Gal regions to an extreme degree, while Lac, V₁ and Ara are much less distorted. At first sight, it would be tempting to ascribe such a polarization to a failure of paratypic markers to enter the zygote, but single cell analyses have proven otherwise.

Under conditions permitting microscopic control, the maximum incidence of recombinants is somewhat reduced. 301 single cells have been separated from crossing cultures and allowed to form clones, later examined for their content of genotypes. 284 of these clones were pure cultures of one of the two parents. Of the remaining 17 single-cell clones, all engendered progeny typical of the F⁻ parent, but in addition, 7 also contained cells carrying the full genetic complement of the F⁺ parent. These clones also contained numerous recombinants, but these were generally orthotypic. Thus in 7 single cells, the full genetic content of the two parents was represented transiently, but the recombinant output (some possibly from subsequent crossing) was still restricted to the orthotypes. The remaining 10 clones contained only orthotypic recombinants and parents. Thus, the frequent exclusion of paratypic markers from the recombinant progeny cannot be ascribed to their exclusion from the zygote, but is assigned to a later elimination process as already suggested by the study of more persistent diploids.

These experiments are designed ultimately to define the formation as well as the fate and content of the zygote. The efficiency of mating is still too low to make the solution of the problem as direct as may seem. There has been a possible correlation of zygote formation with clumps of the two parent cells, but the aggregates have been difficult to analyse. The zygote cells appear to be slightly larger than the parents. No evidence of copulatory fusion or of specialized sex structures or spores has been found; on account of trivial clumping and transient associations, a passing conjugation would not be so readily delineated. These studies are continuing coordinately with cytological studies of fixed and stained preparations.¹

LYSOGENIZATION AND TRANSDUCTION

Studies are continuing of the genetic role of bacteriophage in *E. coli* K-12. It has been possible to confirm previous findings on the localization of the determinant of lysogenicity, Lp^+ , (prophage?) on the bacterial chromosome in the vicinity of a set of determinants for galactose fermentation, (Gal). These findings have also been confirmed, extended, and subjected to attempted reinterpretation by various other workers (27,28,29). While crosses of lysogenic x sensitive give progeny generally orthotypic for this trait and for Gal, para- and meta-typics are also found, so that Lp^+ behaves like any other marker in its transmissibility by either parent. But the most decisive evidence for chromosomal localization stems from diploids, obtained by rather involved sequences of crosses, that are heterozygous for $Lp^+ Gal^+ / Lp^S Gal^-$. Such diploids, which segregate these markers usually, but not always, in the parental couplings, are also obtained by the infection of Lp^S / Lp^S , homozygous diploids, giving rise to segregation for Lp^+ / Lp^S such as has not been observed from the infection of haploids.

A nuclear basis for the fixation of symbiotic bacteriophage was also suggested by the segregation of lysogenicity among the progeny of presumed single infected cells (4; also the subject of a more extensive study, 14). This point remains to be decisively verified by single cell methods, whose application is currently planned.

A limited system of transduction by this phage, confined to the markers closely linked to Lp , is the subject of the dissertation work of Mr. M. L. Morse.

¹ Many of the foregoing problems have been the subject of a collaborative correspondence with Dr. L. L. Cavalli of Milan, Italy. We are anticipating a visit from Dr. Cavalli during the term of the present grant which will allow an even more direct association.

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