1956 Research Report - Bacterial Genetics - Wisconsin - J. Lederberg

Introduction and notes.

The focal interest of this laboratory remains the recombinational analysis of bacterial heredity. For the most part, this proceeding along the lines of our past work, with various collaborators and students concerning themselves with different aspects. The detailed work is better summarized in the scientific publications, which are listed separately. Attached is an account of two main lines of work, having to do, respectively, with bacterial protoplasts and a prophage-linked system of transduction. The protoplast work is a new line, and is being followed primarily in the hope that it may lead to a DNA-mediated transduction, or to new mechanisms of cell fusion. In addition, it is a basis of a biochemical-genetic study of bacterial cell-wall syntheis.

The scientific workers connected with this project are:

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Protoplasts.

In botanical usage a protoplast is the living content of a cell, lying within the rigid cell wall. Attention was focussed on bacterial protoplasts in 1953 by Weibull's demonstration that the protoplasts of Bacillus megaterium could be released when the outer wall was dissolved by lysozyme. The protoplasts appeared as spherical bodies which could be maintained only in a hypertonic protective medium, and promptly lysed when transferred to water. These two criteria, spherical shape and osmotic fragility, are the easiest to distinguish bacterial protoplasts from intact cells. In gram-positive (but not gram-negative) bacteria, moreover, the cell wall is readily demonstrated by simple staining procedures.

The protoplasts of B. megaterium have already been the subject of several important studies--directed on one hand to the missing functions of the cell wall, on the other to the use of protoplasts as easily disrupted and extracted biosynthetic units. These results (e.g. Spiegelman and Landman 1956) stimulated the hope of comparable experiments with E. coli a) for the use of already well-studied enzymatic mutants, phage, etc., in physiological studies, and b) to test for the uptake of DNA (as genetic fragments) by the wall-less protoplasts.

Early in 1956, several investigators became interested in this problem (Repaske and collaborators at Indiana; Zinder at Rockefeller Institute) and succeeded in finding special conditions under which lysozyme would dissolve the existing walls of E. coli cells. Simultaneously, in this laboratory, penicillin was found to inhibit wall formation in growing cells. It had long been known that penicillin lysed growing cells; by analogy with Weibull's results, it was found that in a growth medium containing M/3 sucrose and M/50 Mg^{++}, this lysis was averted, and the cells transformed instead into spherical, osmotically fragile 'protoplasts' (Lederberg, 1956). This observation also furnished clear evidence that the antibiotic action of penicillin depended on wall-inhibition: the protoplasts would remain viable only so long as they were suspended in the protective medium, and rapidly lysed (and of course died) when diluted in ordinary medium. On the other hand, if the penicillin was removed, the protoplasts gradually reverted to viable bacillary forms, presumably by resynthesis of the normal wall. This hypothesis on the mode of action of penicillin is in excellent accord with Park's finding of the accumulation of cell-wall precursors in penicillin-blocked staphylococci (cf. Lederberg 1957; Park and Strominger 1957).

The synthetic abilities of E. coli protoplasts were then studied: they were found to continue to grow (increase in mass and volume) many fold when incubated in penicillin-broth, but without increase in numbers, or microscopically detectable proliferation. More specifically, the protoplasts were also shown to be capable of synthesizing large amounts of an inducible enzyme, B-galactosidase (Lederberg 1956). Provocative biosynthetic studies on this system are being actively pursued by Professor Spiegelman at the University of Illinois.

Our interests have been concentrated on a) a search for genetic interactions between protoplasts of different genotypes, or between protoplasts and extracts, and b) the biology of protoplasts and L-forms. As to the former, while compatible genotypes have been found to mate with either parent a protoplast, no new forms of genetic interaction (such as recombination between F- x F-, or protoplasts and extracts) have been found so far.

It is difficult to be certain precisely what is new in these findings, except their application to E. coli. Many authors have described the formation of spherical 'large bodies' under the influence of penicillin (see a recent review by Liebermeister and Kellenberger, 1956 and the extensive reports by Dienes and Klieneberger quoted there) and there are many hints (at least) of the osmotic fragility of the L-forms to which the large bodies are related. The several studies on L-forms have, however, so far lacked a unifying principle that might transfer them from obscure morphological curiosities to generally interesting objects for quantitative, physiological study. The current studies on E. coli protoplasts suggest that the absence of a normal wall, whether by external inhibition or internal genetic defect, accounts for the L-form cycle.

As already mentioned, in broth the protoplasts E. coli fail to divide, and after extensive increase in mass, eventually burst. After some empirical experimentation, the conditions for proliferation into L-forms have been found. In addition to the protective medium already mentioned, these conditions are 1) a suitable strain, 2) at least 1,000 units of penicillin per ml. 3) One percent agar (Difco) and 4) One percent meat extract (Lemco). Some of these conditions are quite rigorous, and have no certain explanation at present. For example, while 100 units of penicillin is quite sufficient for the production of protoplasts in broth or agar, 10 times this concentration is required for L-forms. Perhaps the lower concentration does not completely abolish wall-synthesis, and this is necessary for L-form development.

The transformations of individual cells have been followed by direct phase microscopy of agar cultures. (In broth, a single blister appears which balloons out to form a spherical protoplast). One or several blisters appear, to give either a spherical or a lobulate 'protoplast'. Then, further blisters may appear, to generate new lobes, or thin processes develop, the tips of which then balloon out at some distance from the original body. The connection may later disappear. As a result of these processes, the cell develops into a cluster of granules and spheres of various sizes, the oft-described L-colony. In 2 or three days, the L-colonies may reach a diameter of 2-3 mm.

The L-colonies may be transferred in series by grinding blocks of agar in broth and plating the minces. When plated with penicillin, they regenerate new L-colonies; without penicillin, only normal bacillary colonies develop (even after 10 serial passages as L-forms). However, the viability of the L-colony is very low, averaging only about 10 plating units per colony which may contain 10^3 to 10^5 visible granules and spheres.

The requirement for agar is a physical one: L-colonies will develop in small blocks of agar, while protoplasts at the surface or immersed with the agar blocks in the same fluid medium will enlarge and explode. L-colonies which penetrated to an agar-glass interface formed a conglomerate of large and small droplets which adhered imperfectly to the glass.

These observations can be rationalized in the following working hypothesis: in the absence of the normal wall, the protoplast assumes a spherical shape in an isobaric medium by virtue of interfacial forces. There is no mechanism by which it can divide its mass. It has a fluid or semifluid consistency and, in contact with glass which it wets to some extent, the balance of interfacial forces breaks it up into droplets of varying size. In agar, however, the expanding protoplast is subject to local mechanical stresses, from the fibrils in which it is enmeshed, and can therefore flow into local crevices in the agar, to give blisters or processes. At these points, there

is a further weakening of the residual (lipid?) membrane and secondary or daughter protoplasts are ultimately budded off. In a sense, the agar mesh has taken over the functions of the missing wall. The low viability may be a reflection of the extent to which a daughter globule receives an intact genetic complement (nucleus), or simply to the mechanical fragility of these elements.

This interpretation of L-forms is at present a working-hypothesis. The basic observations are not greatly different from those of Dienes and others working on different species. It happens that in E. coli, the osmotic fragility of the protoplasts is especially pronounced, and therefore more readily analyzed. In other bacteria, notably Streptobacillus moniliformis whose L-forms can be cultivated in liquid medium, we may suppose a stronger residual wall or another layer is left. The liquid-culture L-forms of Proteus appear to be instances where the structural ghosts of exploded protoplasts remain as an outer shell to provide the framework in which still viable units can proliferate at the center. Reports on the filtrability of viable elements of L-forms require some circumspection: on the one hand, most of the elements are not dramatically smaller than ordinary bacteria; on the other, the fluidity or plasticity of the L-forms raises some questions as to the dimensions of the filtrable units. Insofar as these protoplasts do behave as naked globules of bacterial 'protoplasm' they may be expected to lead to new avenues of experimentation. However, the existing data give no basis to doubt that an essential organizational unit corresponding to the content of an intact cell is still required for the persistent viability of the protoplastic elements.

An interesting corroboration of these proposals comes from the behavior of a mutant of E. coli (B. Davis) which requires diaminopimelic acid (DAP). This substance had been found to be a unique constituent of the cell walls of a number of bacteria. The mutant cells, when deprived of DAP in a protective medium, undergo the same transformations (protoplasts and L-colonies) as described for the effect of penicillin. When DAP is restored to these forms, they then revert to the bacillary type. However, as DAP does not reverse the antibiotic effect of penicillin, compounds relate to different aspects of cell wall synthesis. A systematic search is underway for other biosynthetic mutants defective in wall formation.

It is still uncertain exactly what reaction is inhibited by penicillin. The penicillin-protoplasts appear to be limited only by a thin residual membrane; however, india-ink preparations show a prominent but transparent capsule: immunological tests are contemplated to determine whether this capsule represents the remains of the old wall, or the accentuated development of a carbohydrate (capsular, K) antigen. When a protoplast is lysed in water, it does not freely dissolve; instead, india-ink preparations show a large clear area, probably the cell-contents in a gelatinous lump, which may help account for the persistence of protein- synthetic preparations in partial lysates (Spiegelman).

(The foregoing observations are being coordinated into a manuscript for publication in the near future).

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Transduction of Gal factors. Prophage linked transduction (M. L. Morse, E. M. Lederberg and J. Lederberg)

The transduction of genetic markers for galactose fermentation has been recounted in previous reports and published in detail. Briefly, the phage, λ, can transmit the Gal markers of a lysogenic donor bacterium to a suitable recipient. This reaction can be symbolized donor--x recipient. In the simplest case, Gal^+ --x $Gal^- \rightarrow Gal^+$, the transduction is detected by the occurrence of Gal^+ (galactose-positive) clones among the Gal^- recipients. Further study has shown that an intermediate "heterogenote" often persists, a clone which carries the (exogenote) donor fragment together with the intact recipient chromosome. A later step may be an exchange between the exogenote and the chromosome, viz., Gal^+ --x $Gal^- \rightarrow$ Gal^+ ex/$Gal^- \rightarrow Gal^+$.

More intimate studies have involved combinations of different Gal^- mutants. Thus 1-2+ (an abbreviated symbol for $Gal_1^- \ Gal_2^+$) --x 1+2- $\rightarrow$ 1-2+ ex/1+2- $\rightarrow$ 1+2+, 1+2-, 1-2+, 1-2- and other more complex types. Further research has been devoted (1) to the physiological relations of different mutants (2) to the concomitant behavior of prophage in transduction and (3) the effects of irradiation of the phage.

a. Almost all galactose-negative mutants are closely linked to one another. Their linkage sequence has however not been determined as yet. No definite recurrences of mutations at the same locus have been identified. A series of at least 12 distinct loci has been established already.

b. Kalckar and Kurahashi (at N.I.H., Bethesda) have worked out the enzymatic steps in galactose fermentation, according to the following scheme:

1. Galactose + ATP ↔ Galactose -1-P + ADP (galactokinase)
2. Gal -1-P + UDP Glu ↔ UDP Gal + Glu -1-P. (transferase)
3. UDP Gal ↔ UDP Glu (epimerase)

Sum: Gal + ATP → Glu -1-P + ADP
Then Glu -1-P → CO_2, lactic acid/general glycolytic pathway. by the

They have also analyzed the enzymatic defects of a number of mutants. Group A (Gal 2, Gal 8) lack galactokinase. Group B (Gal 1, Gal 4, Gal 6, Gal 7) lacks the transferase, and thus corresponds to the genetic disease, congenital galactosemia, in man. No epimerase mutants have been found so far. (See Kurahashi, K. 1957 Enzyme formation in galactose-negative mutants of *Escherichia coli* Science 125, 114-116).

c. Certain heterogenotes, e.g., 1+2-/1-2+ give a normal wild type, galactose positive reaction. However, others, 1+4-/1-4+ are galactose-negative, in contrast to 1+4+/1-4-, galactose positive. That is, Gal 1/Gal 4 forms a cis-trans position-effect group. In sum, Gal 1, Gal 4, Gal 6, Gal 7 form one position effect group (or "cistron"-Benzer), any pair from the group giving a cis-trans position-effect. Gal 2 and Gal 8 form a second cistron. Combinations of a mutant from one cistron with one from the other are galactose positive. Thus, as far as these experiments go, each cistron corresponds to one enzyme.

However, one other mutant, $Gal\ 3^-$ shows position-effects with both cistrons. Its enzymology is not yet known.

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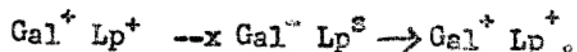
Current theories of gene-enzyme relationships are converging on a linear correspondence between the informational content of a protein product, and of a polynucleotide string in the chromosome. Many experiments are in accord with this simple correspondence, e.g., the relationship between the group A and B cistrons and the enzymes. For this reason, it is especially important to analyze apparent exceptions, like Gal_2^- to see whether they support, or contradict what may be an oversimplified scheme.

Prophage relations in transduction.

The only markers known to be transduced by lambda are the Gal complex. A concerted search for additional transducible markers is underway, so far without success. The phage obtained by lytic growth is ineffective, and it must be obtained by the ultra-violet light induction of lysogenic donors. Since Gal is the only marker closely linked to lambda (Lp) in sexual crosses, its specificity in transduction is connected with this linkage to the prophage. In fact, when lambda infects a sensitive cell, and lysogenic survivors are obtained, this may be considered a transduction of the lambda prophage. When this is coupled with Gal, we may write:



$$Gal^+ \; Lp^+ \; \text{--x} \; Gal^- \; Lp^s \longrightarrow Gal^+ \; Lp^+.$$

As the phage from heterogenotes has a very high efficiency of transduction, we have been able to study individual transformed clones. These are invariably mixed with unchanged $Gal^- Lp^s$ cells, which probably are the uninfected segregants from the multinucleate initial cell. The Gal^+ components of these transductional clones have usually been Lp^+, a minority (about 1/3) give the Lp^r (immune) reaction. The Lp, or prophage is therefore directly associated with Gal^+ transduction. We will now deal with experiments designed to look for the segregation of prophage from heterogenotic cells, where Gal is segregating.

1. $Gal^+ \; Lp^+$ --x $Gal^- \; Lp^+$. When both parents are lysogenic, the heterogenotic progeny are pure lysogenic, as expected on almost any hypothesis.

2. $Gal^+ \; Lp^+$ --x $Gal^- \; Lp^s$. Two types are found, a) Gal^+/Gal^- which are pure lysogenic, that is either homogenotic (Lp^+/Lp^+) or hemogenotic (Lp^+) for the prophage. This would seem to contradict the expectation that prophage is coupled with Gal, and therefore should segregate with it. b) Gal^+/Gal^- which give the immune (Lp^r) reaction. However, these types are invariably unstable: all haploid segregants (with respect to Gal) are Lp^s, and vice versa, while all Lp^r derivatives are still homo- or hetero-genotic for Gal, and subject to further segregation. Our first hypothesis was that this type was segregating for a defective prophage, Lp^r/Lp^s. However as no Lp^s homogenotes have been found, it seems more likely that the immune phenotype is either an Lp^s/Lp^s, homogenotic for the Lp^s-mutant-prophage, or an Lp^s on which the exogenote confers immunity. Since this would imply that the exogenote carries at least part of the prophage, the two hypotheses are more or less equivalent. For further discussion, we will postulate that this phenotype is $Gal^+ \; Lp^s/Gal^- \; Lp^s$, and abbreviate the prophage condition as Lp^{rs}. The immunizing effect of the exogenotic Lp^s would then be either a dosage effect of Lp^s (with dosage compensation, cf. the sensitive phenotype of Lp^s/Lp^s homozygotes) or a special effect of Lp^s in the exogenotic location. On this formulation, the result a) is also homogenotic, Lp^+/Lp^+.

This hypothesis does not explain why no segregating prophage heterogenotes, Lp^+/Lp^s are found, and we simply have to resort to ad hoc proscriptions against this type. This type of segregation would be the only tangible evidence for our hypothesis that the prophage is coupled with Gal in the exogenote. The deeper significance of this hypothesis is the homology between prophage and other segments of the bacterial chromosome.

3. $Gal^+ Lp^+$ --x $Gal^- Lp^s / Gal^- Lp^s$ (that is, homogenotic recipients). This transduction has not been sufficiently studied. However, they have been found to give lysogenic heterogenotes, which then segregate to give Lp^+, Lp^{rs}, and Lp^s progeny, as if they were $Gal^+ Lp^+/Gal^- Lp^s/Gal^- Lp^s$. No effort has been made to characterize the simple $Gal^+ Lp^+ / Gal^- Lp^s$ which might issue. The result does, however, give the most direct support so far for the representation of Lp^+ on the exogenote.

4. $Gal^+ Lp^h$ --x $Gal^- Lp^+$. To circumvent the possible pecularities of Lp^+/Lp^s (i.e. prophage/sensitive) confrontations, we have used the h (host-range) mutant of lambda furnished by Appleyard, here designated simply as Lp^h. The usual results are, however, pure Lp^+ or, less often, pure Lp^h. Again, we would have to find an ad hoc rule against prophage segregation. Perhaps the first event in establishment, and a necessary one for the survival of a heterogenote, is a crossover which ensures homogenosis for the Lp locus. In favor of this is the coincidence of Lp^+ and Lp^{rs} heterogenotes in the same primary transductional clone, as has been observed once or twice, i.e., these would be the complementary Lp^+/Lp^+ and Lp^s/Lp^s homogenotes from the initial, forbidden Lp^+/Lp^s.

5. $Gal^+ Lp^+$ --x $Gal^- Lp^r$. The recipient here is a haploid, immune (defective prophage) type, not to be confused with Lp^{rs}. The results are mostly Lp^r, with rare Lp^+ heterogenotes. This is, therefore, analogous to experiment 4. One or two types which behave as if Lp^+/Lp^r were also isolated, but the segregational coupling of Lp to Gal was not maintained. This is not understood.

6. $T4_{II}$. Benzer (1955) has reported that Lp^+ is killed by certain r mutants of phage T4, but does not support their growth, while Lp^s supports normal growth of T4r. This stock is therefore a further test of prophage action. All lambda-lysogenic and immune stocks, including Lp^{rs}, and Lp^r as well as Lp^+/Lp^s diploids were found to be 'immune' to $T4r_{II}$.

7. $Gal^+ Lp^+ P434^s$ --x $Gal^- Lp^s P434^+$. P434 is a new temperate phage, partly homologous with lambda, described by Jacob. It was used here as an additional means of marking the exogenote in transduction. A wide variety of lysogeny-types has issued from this transduction, including Lp^+, Lp^r and Lp^s in various combinations with $P434^+$ and $P434^s$. The most pertinent one, behaves as a $Gal^+ Lp^+ P434^s/ Gal^- Lp^s P434^+$, i.e., it is segregating both prophages, in coupling with Gal. This result together with experiment 3, gives strong, but tentative, support to the exogenotic prophage hypothesis. The special immunity relationships between lambda and P434 may have something to do with the contrast between this result and the simpler experiment 2.

The complexity of these results awaits a unifying generalization. Experiments 3 and 6 support the working hypothesis of prophage segregation; the others are equivocal. At issue is the conclusion that the prophage is an integral part of the bacterial chromosome. Its behavior must be analysed further to see whether its peculiarities are incidental to a fundamental homology of prophage with other genes, or whether entirely new principles must be postulated.

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