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Project No. 742. The nature and action of the gene in bacteria. Lederberg (Mrs. Esther Lederberg, National Cancer Institute, Junior Research Fellow, Merton D. Zinder, Research Assistant, Donald A. Gordon, Research Assistant). State funds, W.A.R.F., Rockefeller Foundation, National Institute of Health.

The research undertaken in this field can be reviewed under the following headings:

1. Genetic Aspects of the life cycle of Escherichia coli.
2. Genetic control of fermentation enzymes in E. coli.
3. Genetic control of gene mutability. (Mrs. Lederberg)
4. Gene recombination in Salmonella. (Mr. Zinder)

1. Genetic aspects of the life cycle of Escherichia coli.

The discovery by Tatum and Lederberg (1) of a sexual cycle in strains of the common bacterium Escherichia coli opens up the field of bacteria to genetic analysis in a way that was not formerly possible. In this earlier work, conducted at Yale University in 1946-47, genetic recombination was demonstrated by the use of nutritional mutants, obtained with X-ray or ultraviolet light. These mutants have nutritional deficiencies which prevent them from growing on synthetic medium unless their particular growth factors are supplied. It was found that when different mutants were mixed together and inoculated into synthetic agar that a very small proportion of colonies did grow, and that these consisted of cells which could grow on the synthetic medium (i.e. prototrophs). By using mutants which differed in several other characters, it could be shown that these characters were redistributed to the prototrophs in all possible combinations, suggesting that the proper explanation of the occurrence of prototrophs is gene recombination. If one nutritional mutant is represented as A-B<sup>+</sup> and the other as A<sup>+</sup>B<sup>-</sup>, one would get by recombination A-B<sup>+</sup> x A<sup>+</sup>B<sup>-</sup> → A<sup>+</sup>B<sup>+</sup> and A-B<sup>-</sup>. In this case, only the A<sup>+</sup>B<sup>+</sup> would be recovered, as it alone could grow on the synthetic medium which is used to sieve it out from the two parents, whose cells preponderate. However, other markers can segregate freely into the prototrophs. From a study of the relative proportions of different recombination types, it was concluded that the genes of Escherichia coli were linked in linear order, as in higher forms, and there was probably but a single linkage group.

From this earlier work, it was concluded that a) cell fusion occurs only very rarely, about one zygote appearing per million cells; and b) the zygote does not proliferate, but undergoes reduction division immediately. This is based on the finding that individual prototrophs were uniform, each colony consisting usually of but a single recombination type which would be different from colony to colony. If the diploid zygote were to multiply, one would

expect to find several recombination types in a single prototroph colony, each derived from the segregation of a different zygote cell. These facts were discouraging to any hopes of seeing the zygote cytologically, or of determining such questions as dominance for which the diploid is necessary. We could put in a certain mixture of types, and get them out again in various recombinations, but the intermediate process was not directly accessible to analysis and had to be inferred. For the further development of genetical work on bacteria, it would be very important, if not essential, to clarify this intermediate process.

In the course of other work, and quite accidentally, an exception was noted to conclusion b); that is to say, a heterozygotic culture was found which could be maintained in the diploid condition, although it has a marked propensity to undergo reduction. The exceptional culture was a prototroph obtained in a cross between two parents which differed in nutritional factors (B, M, T, L, B<sub>1</sub>.) and in lactose fermentation (Lac-/Lac+). The indicator medium, Eosin-Methylene Blue agar is used to test fermentative characteristics (also see 5). When this culture originally thought to be Lac+ was streaked out and incubated for two or three days on the EMB medium, the colonies were observed to be mosaics of Lac- and Lac+ cells. Furthermore, when these segregants were tested, they proved to show all possible recombinations of the nutritional and other factors, including certain recombinations, corresponding to the A-B- above, which could not otherwise be secured. Evidently, this culture was prototrophic not because it was a recombination of the type A+B+, but because it was a diploid heterozygote ( $\frac{A+}{A-} \frac{B-}{B+}$ ). Further study of this culture and of its segregants suggests that its exceptional behavior may be the result of a mutation which occurred spontaneously in one of the parents, for outcrossing segregants to standard stocks results in prototrophs a substantial proportion of which are diploid heterozygotes.

Although it was formerly believed that there were no exceptions to rule

b) in crosses of standard stocks, current studies using better selective means now indicate that persistent heterozygotes may occur here too, although at a lower rate than among outcrosses of the above-mentioned exception. In this test, parents are used which carry different, closely linked, recessive Lac- in the "repulsion" phase, i.e.  $A^- \text{Lac}_1^- \text{Lac}_4^+ B^+$  x  $A^+ \text{Lac}_1^+ \text{Lac}_4^- B^-$ . When such a pair is crossed, most of the  $A+B^+$  recombinant prototrophs are either  $\text{Lac}_1^- \text{Lac}_4^+$  or  $\text{Lac}_1^+ \text{Lac}_4^-$ , because of the very close linkage of the two Lac loci. These are all lactose-negative; much less than 1% of the prototrophs obtained here are lactose-positive, and these can be detected visually by conducting the cross on a synthetic EMB medium. Even when "normal" stocks are used, about half the lactose-positive prototrophs are not  $A+B^+ \text{Lac}_1^+ \text{Lac}_4^+$ , but are heterozygous diploids  $\frac{A^- \text{Lac}_1^- \text{Lac}_4^+ B^+}{A^+ \text{Lac}_1^+ \text{Lac}_4^- B^-}$  as shown by their subsequent segregation. These diploids may differ from those previously obtained in being more stable, but this is not definitely established.

Detailed studies of the segregations from the exceptional heterozygote showed deviations from random distribution that can be best accounted for by assuming that one or both chromosomes of this diploid carry several deficient regions. These deficiencies, which would make inviable a segregant carrying them alone, may be the inciting factor for the persistence of this heterozygote, and detract somewhat from the usefulness of this type in genetic analysis. Whether the same situation prevails in the "normal" heterozygotes remains to be determined.

Studies are underway to determine the dominance relationships of a number of genes. The normal alleles of the nutritional mutations all seem to be completely dominant, and the functioning, "+", alleles of the several fermentation factors studied are nearly completely so. In addition, sensitivity to bacteriophage T1 is dominant to resistance, a fact of considerable importance

in interpreting studies on the induction of this mutation by radiations and chemicals (See 2). We have under way further experiments to determine the dominance of mutations affecting bacterial resistance to antibiotic and antibacterial agents.

2. Genetic control of fermentation enzymes in *E. coli*.

Investigations on *Neurospora* have led many workers to the conclusion that "single genes determine the specificity of single enzymes." (See 3.) Since bacteria are very favorable material for enzyme research, and we have now the capacity for genetical work on them, it has seemed desirable to study the problem of gene-enzyme relationships in *Escherichia coli*, to determine in the first instance whether the "one-to-one" theory quoted above could be verified.

The enzyme selected for study first is the lactase (beta-galactosidase) of *E. coli*. It is relatively easy to isolate mutants which have lost the capacity to produce this enzyme, by the use of the EMB indicator medium referred to earlier. By the examination of some millions of colonies of ultra-violet treated bacteria on this medium, several hundred, independently produced, lactose-negative mutants have been isolated. These mutants have been analysed by crossing them with each other to determine whether they are genetically identical. If two lactose-negative mutants are crossed, carrying the same Lac- mutation, then obviously there will be no lactose-positive recombinants. On the other hand, if two mutants carry mutations at different genetic loci, then they can be expected to give occasional lactose-positive recombinants with a frequency depending upon their linkage relationships. That is,  $Lac_1^- Lac_2^+ \times Lac_1^+ Lac_2^-$  can give  $Lac_1^+ Lac_2^+$ . With these tests, the mutants so far crossed can be placed into seven distinct groups, each carrying a distinct mutant gene which interferes with the production of lactase. Two of these mutant types also show enzymatic effects in addition to lactase. One of them,  $Lac_3^-$  is unable to ferment glucose or maltose;

another, *Laag-* is unable to ferment gluconate or maltose. These observations are not in accord with the simple "one-to-one" theory, but imply that the relationships between gene and enzyme are much more complex.

In order to support this conclusion, the enzyme has been extracted from the cells and studied in solution with the aid of an artificial substrate, *o*-nitrophenol  $\beta$ -galactoside, which releases a colored substance, *o*-nitrophenol when it is split by the enzyme lactase. This permits the reaction to be studied conveniently with a spectrophotometer. The enzyme has been partially purified with ammonium sulfate precipitation. These preparations are active in the absence of phosphate, pointing to a simple hydrolysis for the enzyme action. An interesting effect of alkali metals has been noted: the enzyme is strongly stimulated by sodium ions in fairly high concentration ( $M/50$ ), and is inhibited by rubidium. The inhibition by rubidium can be competitively reversed either with sodium, or with potassium, suggesting that all of the alkali metals compete for a position on the enzyme, certain combinations of metal-enzyme having a higher efficiency than others. It is presumed that  $H^+$  is also displaced by higher salt concentrations, but that while  $Na$ -enzyme is more active than  $H$ -enzyme,  $K$ -enzyme has the same activity. It has been observed, further, that most of the difference can be expressed in terms of the dissociation constant for the enzyme and its substrate so that these ions may be regarded as facilitating the absorption of the substrate to the enzyme. Ethylenediammonium and other substituted ammonium ions behave in much the same way as rubidium. These observations are strikingly parallel to the effects which have been noted by Snell on the growth of bacteria.

The enzyme lactase is strictly adaptive, i.e., it cannot be demonstrated in cells which have not been exposed to lactose for at least 2-3 hours. It is believed that the various mutations affecting lactase production do so via the

adaptation mechanism, which is now being studied in detail. While the all or none effect of most of the mutants makes it difficult to analyse their effects, an allele of  $Lac_3^-$  has been found which is responsive to temperature, and may be of great help. This mutant is wild type at  $30^{\circ}C.$ , but like  $Lac_3^-$  at  $40^{\circ}$ . At different intermediate temperatures, different enzymes can be formed. It has been possible to show that the temperature-sensitivity is not a reflection of this property of the enzymes themselves, once formed, but on the activity of the adaptation mechanisms.

### 3. Genetic control of gene mutability.

Rhoades has described "dotted" stocks of corn in which the status of one gene affects the mutability of another. In the presence of the  $\underline{dt}$  allele, the gene  $\underline{a}$  is quite stable and rarely if ever mutates to  $\underline{A}$  (shown as color in the aleurone). However, with increasing numbers of  $\underline{Dt}$  alleles,  $\underline{a}$  shows rapidly increasing numbers of such mutations. Although this phenomenon is of the greatest genetical interest, it is very difficult to study the possible chemical pathways because of obvious anatomical limitations.

The observation that various  $Lac^-$  mutants of E. coli, secured as already described, mutate at different rates back to  $Lac^+$ , suggested that here might be excellent material for a parallel type of study. The mutability of a  $Lac^-$  stock is readily determined by incubating its colonies on EMB agar for 2-3 days. At this time,  $Lac^+$  mutants are seen as papillate, dark outgrowths in the white or pink colonies.

Mutants at the  $Lac_1$  locus only were studied so far. As obtained by irradiation of the wild type, some of these mutants are quite stable, whereas others show many papillae in each colony. Genetic tests on such stocks have shown that the differences are due to different allelic states of the same gene, showing again that mutations which may be indistinguishable in all other respects can be distinguished in this way (i.e. are iso-alleles). The mutations

to Lac<sup>+</sup> in the mutable strains have also been studied, and proven to be true reverse mutations, restoring the Lac<sup>-</sup> gene which was originally impaired by ultra-violet light. Occasional Lac<sup>+</sup> mutations in the more stable strains have proven, however, to be due to mutations at other loci, such mutations having the effect of bypassing or "suppressing" the phenotypic manifestations of the original Lac<sup>-</sup> mutation. Such "suppressor" mutations have also been observed in the other Lac<sup>-</sup> mutants mentioned under Heading 2.

Attempts were then made to pick up effects of mutations of other loci on the mutability of Lac<sup>-</sup>. Stable strains were irradiated, and mutables looked for, without success so far. Conversely, mutable strains were irradiated, and derived stable strains have been recovered from them. Also, although the degree of mutability is quite a constant feature of a particular stock, a very few spontaneously occurring stable derivatives have been noticed.

Some of these derived stables, when tested genetically, proved to be more stable alleles of the original Lac<sup>-</sup>. At least one such stable, however, when crossed with wild type gave rise in addition to the parental types: Lac<sup>+</sup> and Lac<sup>-</sup>stable, the recombination class Lac<sup>-</sup>mutable. This shows that the apparent stability of this derived stock is due to a mutation at another locus. This new mutation also turned out to be associated with a nutritional deficiency, which is at least partially relieved by (autoclaved) cozymase (diphosphopyridine nucleotide). It is possible that this is not a true case of gene control of mutability, but that the second mutation interferes with the fermentation of lactose even when Lac<sub>1</sub><sup>-</sup> reverts to +. This point is now under study. A number of additional stable stocks remain to be studied.

#### 4. Genetics of Salmonella.

Escherichia coli, strain K-12, is, so far, the only bacterium in which a sexual phase has been demonstrated by the genetic methods detailed above.

It is naturally of interest to determine whether other bacteria will behave in the same way. Previous experiments on two other strains of E. coli have given negative results, but rather than pursue further strains of this species, we have been examining various Salmonella strains. Salmonella is much better understood serologically, and, furthermore, is an advantageous group with which, eventually, to pursue studies on pathogenicity.

Three strains of Salmonella typhimurium have been examined to date: SY-20, SY-21 and SY-23. Biochemical mutants have been isolated in each of these with the help of a new method using penicillin which we have just developed (4). Penicillin has a permanent bactericidal effect only on growing cells. If a mixture of wild type and mutant cells is inoculated into a synthetic medium to which penicillin is added, the wild type cells are killed at a much higher rate than the mutants. This property can be used to advantage to facilitate the isolation of biochemical mutants from irradiated populations in which they are far outnumbered by the original, non-mutated wild type cells.

No evidence of genetic recombination has been found so far in mixtures of mutants in the following combinations: SY-20 x SY-20; SY-20 x SY-21; SY-21 x SY-21; SY-23 x SY-23; SY-20 x SY-23. In several experiments, however, prototrophs have appeared in mixtures of mutants derived from SY-21 and SY-23. The situation has been complicated by the fact that SY-23 is lysogenic, i.e., carries without detriment to itself a bacteriophage which is active on SY-21. In addition, SY-21 is lysogenic, one of its phages is inactive on S. typhimurium strains, and is revealed only when S. gallinarum is used as a sensitive indicator; the other can attack SY-23. Lysogenicity is itself an imperfectly understood phenomenon, and in the present instance, it has not yet been possible to clarify its relationship to the recombination which may be occurring in SY-21 x SY-23. The prototrophs which can be recovered from such "crosses" are usually

ridden with phage, and it is likely that the very low yield with which they have been recovered may be accounted for by the destruction of many such prototrophs by these phages.

For this reason, we have tried to determine whether a lysogenic bacterium can be "disinfected" of its phage. Experiments with the ST-21 - S. gallinarum system have given rather discouraging results. All of the bacteria in such cultures seem to carry the phage, as determined by plating them out and testing individual colonies, and it has not been possible to eliminate the phage with such chemicals as Phosphine GRN and potassium arsenite which are reported to inhibit phage multiplication. Other agents will have to be tested.

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