A FACTOR (OR MUTATOR GENE) INFLUENCING MUTATION RATES IN ESCHERICHIA COLI

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There is now considerable evidence, particularly in maize and in Drosophila, for the existence of factors which influence the rates of mutation of genes. The interpretations which have been offered are varied and involve, for example, mutator genes, heterochromatin, or even infective, virus-like particles. Although much still remains to be done, the study of these phenomena constitutes one approach to the general problem of the nature of gene action.

The present paper is concerned with the evidence for the existence of analogous effects in a microorganism, a variant of the well-known K12 strain of Escherichia coli. Our recognition of the special properties of this bacterial variant is not without its instructive, even amusing aspects: in the course of an investigation on possible changes in the nutritive requirements of bacteria after selection for drug resistance, we had observed that a number of streptomycin-resistant K12 stocks acquired a need for one or more amino acids before growth ensued. In one series a requirement for phenylalanine was indicated for a number of independent isolates, in a frequency somewhat beyond that expected by chance. To investigate further this apparent association, we then determined the resistance of a stock phenylalanine-requiring K12 auxotroph which had not previously been in contact with streptomycin. At the inoculum dilution used, the culture appeared completely resistant to streptomycin, inocula of 0.1 ml. of full-grown culture growing out in subcultures containing 100 μg. or more streptomycin per milliliter, while other K12 stocks did not grow in drug concentrations beyond 5–10 μg. This appeared to be a verification of our working hypothesis concerning phenylalanine requirement and increased resistance to streptomycin.

Further investigation, with greater dilutions of the culture, revealed, however, that we were dealing not with a high level of resistance for the average organism in the culture but rather with an unusually high rate of mutation to resistance, which resulted in an exceptional number of resistant organisms in an otherwise susceptible population. Of perhaps more general interest is our later observation that, as in the Drosophila material, this increased mutation rate obtains toward a considerable variety of agents, although the mutation rate is not increased uniformly to all states and in fact appears to be normal in at least one instance. Finally, clear evidence has been obtained that the maintenance of this high rate of mutation is in no way related to a nutritional requirement for phenylalanine and that our original hypothesis in this regard, useful as it was in leading to further observations of interest, must be regarded as only a tentative working hypothesis, based on too small a series of initial observations and to be replaced in the light of additional experiments.

Cultures.—The relationships of the principal K12 cultures used, which are of importance for the argument below, are best understood from the following chart.
K12 (Wild type, no amino acid requirement)  
58  (Biotin requirer)  
58-278 (Biotin + phenylalanine requirer, normal rate of mutation)  
UV-irradiated  
Y24 (Biotin + phenylalanine + cystine requirer, normal rate of mutation)  

Several routine stock culture passages  
(Requirements as for 58-278, but is high mutating)

All the above organisms, except stock 58-278, were kindly supplied to us by our colleague, Dr. David Bonner. In the initial stage of this investigation, the fact that the unique properties of stock 58-278 M* were apparently acquired (by mutation?) during the culture passages was not appreciated, since the full chart had not been plotted. After the observation that stock Y24, which we had assumed to have been derived directly from stock 58-278 M* by further irradiation, did not possess exceptional mutating properties, the above chart was reconstructed from the records, and an effort was made to locate a sample of stock 58-278 which had not been passaged and which should therefore be more directly in the true line of descent than stock 58-278 M*. We were fortunate in that an original lyophilized sample of stock 58-278 meeting these requirements could be contributed by Professor E. L. Tatum, of Stanford University, who had preserved it from the above irradiation experiments, which he had conducted while still at Yale University. On test (see below), this culture was found to have only the normal, wild-type rate of mutation to streptomycin resistance. While this finding threw no light on the mechanism of origin of the high-mutating properties of stock 58-278 M* (except to suggest that they arose during the maintenance passages of the latter subline), it did account satisfactorily for the negative findings for stock Y24. As will be noted further below, the high-mutating properties, once introduced into a culture, are quite stable and persist even after further irradiation. Their absence from stock Y24 therefore required explanation.

**Demonstration of Increased Numbers of Resistant Forms.**—Evidence of resistant forms in an otherwise susceptible population may be provided by one of two types of experiments: titrations with varying inocula or direct plate counts on plain and on drug media.

In the former type of experiment it is common experience that, even with inocula as high as 1 ml. of full-grown culture, any of a variety of K12 or other coliform strains do not exhibit, more often than once in three samples of this size, a resistant organism capable of growing in 100 μg. of streptomycin per milliliter. The chance that an organism of this degree of resistance will be present decreases, of course, tenfold with each decimal dilution of the inoculum used, so that, with the inocula ordinarily employed in resistance tests (0.1 ml. or less), most cultures will be scored as "susceptible." In contrast, cultures of stock 58-278 M* invariably contain at least one resistant organism per hundredth of a milliliter. With less than this amount of inoculum, positive scores for resistance are obtained with decreasing frequency. On the other hand, in cultures of truly resistant organisms, each indi-
individual microorganism possesses a considerable resistance, and practically the same dilution end-points are exhibited in plain and in drug-containing media. The behaviors of these three types of cultures, as estimated in part from other data given below, are illustrated in Table 1.

**TABLE 1**

**Probabilities of Positive Scores for Resistance, as Influenced by Inoculum Size, in Tube Titrations of K12 Strains**

(Screening Concentration = 100 μg. Streptomycin per Milliliter)

<table>
<thead>
<tr>
<th>Inoculum Size (ML.)</th>
<th>Wild or 58-278</th>
<th>58-278 M*</th>
<th>Selected from 58-278 M* or Non-M*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>+/3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.1</td>
<td>+/30</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.01</td>
<td>+/300</td>
<td>+/5</td>
<td>+</td>
</tr>
<tr>
<td>0.001</td>
<td>+/3,000</td>
<td>+/50</td>
<td>+</td>
</tr>
<tr>
<td>0.0001</td>
<td>+/30,000</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

Note: + = growth in drug, with consequence positive "score" for resistance of culture; +/3 = positive an average of only once in 3 trials; +/30 = positive an average of only once in 30 trials, etc. The fully resistant strains are derived in the usual manner, by an initial isolation on streptomycin agar.

More quantitative estimates are afforded by platings on solid media (Fig. 1). The various non-M* cultures, when plated directly, without preselection for resistance, give counts which decrease rapidly with increases in the drug concentration (curve A). A similar type of behavior has been recorded for other organisms and antibiotics.4 A fully resistant culture, on the other hand, gives a quantitative recovery over this range (curve B). The M* culture, although following the general course of curve A down to about 0.0001 per cent of the initial population, then diverges significantly from this distribution because of the appreciable number of more highly resistant organisms which are present (curve C).

**Random Origin of Resistant Forms.**—In a typical experiment a culture of stock 58-278 M* was diluted to give about one hundred organisms per inoculum, as checked by plate counts. Since the ratio of susceptible to resistant organisms is of the order of 10^6, an inoculum of this size is very unlikely to contain a "preformed" resistant organism. Portions of this inoculum were then distributed into each of nine broth tubes, which were incubated to full density, after which the total
number of organisms and the percentage of resistant forms in each tube were determined. Each value recorded in Table 2 is the average of two plate counts. The large variance for the counts on drug agar, in contrast to the small variance for the total count, in these determinations, for a series of cultures originating from common small inocula, may be taken as evidence for the random origin of the resistant forms.\textsuperscript{5, 6}

\begin{table}
\centering
\caption{Means and Variances for Counts of Total and Resistant Organisms in Series of Cultures from Common Small Inocula}
\begin{tabular}{|c|c|c|c|}
\hline
Tube No. & Total Count \((\times 10^6)\) & Resistant Count \((\times 10)\) & Total Count \(\times 10^6\) \\
\hline
1 & 94 & 150 & 0.63 \\
2 & 74 & 83 & 0.89 \\
3 & 108 & 100 & 1.08 \\
4 & 112 & 68 & 1.65 \\
5 & 99 & 204 & 0.49 \\
6 & 101 & 126 & 0.80 \\
7 & 98 & 290 & 0.34 \\
8 & 139 & 123 & 1.12 \\
9 & 87 & 77 & 1.52 \\
Mean & 101 & 133 & 0.95 \\
Variance & 323 & 5,465 & 19.7 \\
Var./mean & 3.2 & 41 & 21 \\
\hline
\end{tabular}
\end{table}

In a variation of this experiment, a culture of 58-278 M\textsuperscript{*} was plated on brain heart agar, and, after incubation, eight typical colonies were picked and each resuspended in separate 0.5-ml. portions of water. These inocula were then each diluted serially, by tenfold steps, through sets of broth tubes. After the latter had been incubated, the last tube in each set which showed growth was identified. It could be assumed, from the manner of preparation, that each represented a culture begun with ten, or less, organisms. These eight secondary cultures were then plated (in appropriate dilutions) on plain agar and on agar containing 100 \(\mu\)g streptomycin per milliliter. The statistical measures obtained were as follows: total count, mean \(= 4.7 \times 10^6\), with variance 1.7 times the mean; resistant count, \(2.8 \times 10^4\), with variance 20.1 times the mean. This difference in the variance ratios leads to the same conclusion as the previous experiment.

\textit{Mutation Rates to Streptomycin Resistance}.—These were determined from the number of tubes which contained no resistant variants.\textsuperscript{5} The inocula used varied from 10 to 500 organisms in the different experiments; they were checked by plate counts in each instance.

For the determination of the mutation rates of all the “normal” comparison cultures, 1.0 ml. of undiluted brain heart broth was used for each tube in the set. For the M\textsuperscript{*} cultures the broth was diluted 1:300 before inoculation, so as to reduce the final density; without this step, the density in each tube would be such as to result in a hundred or more resistant organisms, and the invariable positive scores would preclude quantitative evaluation of the rate.

Following incubation to final density, all tubes were diluted with 5–10 volumes of broth containing streptomycin; those failing to show secondary growth after reincubation were scored as negative. When difficulty was experienced in reading,
the contents of a tube were streaked on streptomycin agar for verification of the absence of resistant forms. The rates determined are tabulated in Table 3; that for the "high-mutating" or M* strain is seen to be about one hundred times the "normal" rate.

**TABLE 3**

**Mutation Rates to Streptomycin Resistance of "Normal" and "High-mutating" K12 Strains of E. coli, as Estimated by the Negative Tube Method**

<table>
<thead>
<tr>
<th>Culture (with Auxotrophic Requirement)</th>
<th>Final Screening Concentration of Streptomycin (µg/ml)</th>
<th>Individual Values Observed</th>
<th>Mean</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>58-278 M* (biotin-, phenylalanine-, large colony form)</td>
<td>100</td>
<td>5.0, 2.0, 1.5, 7.0, 3.8</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>58-278 B (small non-M* colonies derived from 58-278 M* above)</td>
<td>500</td>
<td>7.3, 6.3, 2.1, 2.7, 3.0, 2.5, 6.6, 4.1, 4.1, 2.2, 3.7, 8.6</td>
<td>4.4</td>
<td>2.2</td>
</tr>
<tr>
<td>58-278 (b'otin-, phenylalanine-)</td>
<td>100</td>
<td>3.6</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Biotin-, methionine-</td>
<td>500</td>
<td>3.2, 1.6, 2.3, 3.0, 5.5, 5.0, 2.2, 1.5</td>
<td>3.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Wild type K12</td>
<td>500</td>
<td>6.7, 3.1, 3.0, 1.4, 8.7, 2.6, 2.3</td>
<td>4.0</td>
<td>2.7</td>
</tr>
</tbody>
</table>

All determinations were made at 37° C., in brain heart broth of pH 7.4. Each individual value tabulated is from an experiment employing 50–100 tubes. The significance of the large and small colony forms is explained in the text.

**Streptomycin Dependency.**—It is general experience that there is a marked strain influence on the percentage of streptomycin-resistant forms isolated which are also streptomycin-dependent. For some coliform organisms it may run as high as 40–50 per cent; for K12 wild type we have found about 25 per cent of dependent forms. The low rate of isolation of dependent forms from the M* cultures is especially noteworthy; for 58-278 M* it is only 0.5 per cent. These latter dependent forms are atypical in that, once isolated, they exhibit an extraordinary tendency toward reversion to streptomycin independency, presumably due to the close association of the M* factor (or gene) with this locus. As an example, in one experiment 1 independent form was secured for each 420 streptomycin-dependent organisms plated, while for the wild type this ratio is increased to 1 in 10⁷ plated.

**Separation of High-mutational Property and Nutritional Requirement.**—The invalidity of our original suggestion concerning an association between streptomycin resistance and a requirement for phenylalanine was evident from the data for cultures Y24 and 58-278 (see chart above). This was confirmed by analysis of a series of auxographs derived from E. coli, strain W-NZ, kindly furnished us by Dr. Bernard Davis. Each of these cultures exhibited a requirement for phenylalanine (with or without additional amino acid requirements), but none had any unusual resistance or mutational characteristics.

Although the evidence appeared conclusive that a requirement for either biotin or phenylalanine was not invariably, or even frequently, associated with the initiation of a high mutational rate, there still remained the possibility that these particular requirements were in some way essential for its maintenance, once it had been established. The invalidity of this second hypothesis could readily be proved.
by recombination experiments, through which the auxotrophic requirements could be removed without disturbing the high-mutational characteristics.

For these, the original procedure of Tatum and Lederberg\(^4\) was followed. The first cross effected was as shown below:

\[
\text{Biotin-}, \text{phenylalanine-}, M^* \times \text{Threonine-, leucine-, non-M*} \\
(58-278 M^*) \downarrow \text{Recombination conditions, with} \\
\text{final screening on minimal agar} \\
24 \text{prototrophs, all M*}
\]

This cross has since been repeated in a number of experiments, with yields of hundreds of prototrophs of similar characteristics. In addition, it is possible by irradiation of the prototrophs to introduce new auxotrophic markers without disturbance of the M* characteristic. When some of these new auxotrophs are recombined with certain other non-M* cultures, not all the resulting recombinants are found to possess the M* characteristic. This behavior, which will be reported in more detail elsewhere, is not, however, necessarily in conflict with other data reported here on the stability of the M* characteristic but may merely reflect differences in segregation, such as have been reported for other crosses.\(^7\)\(^8\)

Mention should be made here of another observation made early which is of some interest. When the original culture of 58-278 M* was received, it was plated on agar and found to give rise to both small and large colonies. The high-mutational characteristics and the ability to recombine were limited to the latter colonies, which then constituted our stock 58-278 M* for subsequent experiments. The small colonies, designated 58-278 B (Table 3), not only possessed the normal mutational rate but, unlike culture 58-278 itself, gave no prototrophs in recombination experiments. This observation, which was anomalous at the time, may now be viewed in the light of recent work on strain polarity.\(^7\)\(^8\) Experiments to test this are now in progress.

**Specificity of the High-mutational Effect.**—A survey was made of seventy-eight cultures isolated as *E. coli* in the clinical laboratory of our hospital, utilizing as screening technique any unusual numbers of resistant organisms which appeared after plating on streptomycin agar (100 \(\mu\)g. per milliliter). Some twenty such cultures yielded significant numbers of colonies, although most yielded none at all. As might be expected from the procedure used, most of these high positive scores were due to sampling variations, in which there was by chance a resistant mutant initially present or a mutation had occurred early during growth, and on replication of these with larger numbers of independent culture tubes no unusual number of resistant organisms appeared. Two stocks did, however, give positive scores on this confirmatory test, and, although one gave variable results on further repetitions, it is likely that one or both of these represent cultures which have mutation rates to streptomycin resistance significantly above the average. Unfortunately, these cultures were discarded before the more general properties of stock 58-278 M* were ascertained, and it therefore cannot be stated whether or not the high mutational rate of these other coliforms is limited to streptomycin resistance.

Although the present report deals largely with the latter characteristic, as has just been suggested the unusual features of stock 58-278 M* embrace a wider range. Thus it appears from preliminary experiments involving platings on agar containing various agents that from ten to several hundred times as many colonies appear
from the M* stocks as arise from the normal comparison strains. Included in this
respect are resistances to diverse agents, including chloramphenicol, sodium fluoride,
or certain T phages, such as T1, T4, T5, or T3h. Mutations to abilities (or revers-
sals from abilities) to ferment certain sugars, particularly xylose, also appear to be
increased. Significantly, however, the rate of mutation to resistance to phage T2
appears to be entirely in the "normal" range, as judged by comparisons with stock
58-278 and the wild type. This is the sole exception so far encountered.

The further quantitation of most of these processes is entirely feasible, and ex-
periments to this end are now under way. The possibility of differential selective
effects\(^8\) may make simple colony comparisons unreliable, however, and the more
laborious mutation-rate determinations must ultimately be made, as has been done
above for streptomycin resistance. With proper design, such data should be inde-
dependent of any selective forces which might bias the rate determinations.

The fact that selective effects alone are not responsible for the larger number of
mutants observed in the M* stocks, as contrasted with the non-M* stocks, could
readily be demonstrated by specially designed reconstruction experiments. In
these, inocula of a resistant stock which contained either 0.5–2.0 or 5–20 organ-
isms were introduced into tubes containing \(10^6\) "susceptibles" of stock 58-278,
and streptomycin was then added. In all instances resistant organisms were
recovered in the same frequency as for inocula of the resistant stocks alone, in plain
broth. In its preparation this resistant stock, derived from culture 58-278, had
been given only one purification transfer in streptomycin, so as to militate against
any selective forces, except for resistance, which would differentiate it from its
parental stock. We may therefore conclude that in the present instance even a very
small number of resistant variants derived from stock 58-278 can grow out in the
presence of a large number of "susceptibles," and hence the lower mutation rate
for this non-M* culture is not due to a masking effect, or to false negatives. Al-
though care was taken to have the resistant organism as homologous to the parental
culture as possible, this experimental approach does not guarantee that there may
not be present other types of potentially resistant organisms which cannot compete
at all with the "susceptives" even to the degree of occasional growth necessary for
isolation. It may only be added that additional attempts to detect their presence
by marked dilution of the culture\(^6\) were unsuccessful.

Implications.—Although, as cited earlier, the properties of mutator genes in
higher forms have been described by numerous investigators, detailed descriptions
of their occurrence in bacteria appear to be lacking. Evidence for varying rates
of acquisition of drug resistance,\(^10\) or for reversions of alleles at a single locus (in
\textit{Neurospora}\(^11\)) has, however, been published.

Cultures of exceptional mutation rate require special recognition in the design
of clinical tests for resistance, which must score a culture as either "resistant" or
"susceptible," at some preassigned drug level. The danger of false scoring for resis-
tance would appear to be overcome by employment of two amounts of inoculum,
one low and one high, much as in the test for potential penicillinase producers.

Although the numbers of resistant forms produced may be sufficient to effect, with
some tests, a scoring as a "resistant" culture, they may not be numerous enough
to affect the clinical course under chemotherapy, in situations in which cellular or
humoral immunity can dispose of the majority of the organisms present. On the
other hand, where the clinical situation demands that all organisms be checked by chemotherapy alone, the high rate of production of resistant forms may have serious consequences, even though the laboratory test indicates that the bulk of the culture population is sensitive.

The potentialities for the use of the high-mutating factor, here tentatively considered to be a mutator gene, in the exploration of problems in microbial genetics have scarcely been realized. Fortunately for laboratory work, this characteristic appears to be quite stable and has been preserved during dozens of culture transfers over a period of three years; this is in marked contrast to the lability of some mutator genes in higher forms.1

The nonuniformity of the increases in mutation rates to different states, apparently the result of mediation by the mutator gene, is a matter of some interest. Further quantitation is, however, needed before valid comparisons with linkage data and map distances can be made. The zero effect recorded in the case of mutation to resistance to phage T2 is of especial interest here; in higher forms there is evidence that mutator genes may influence the mutation of genes located in different chromosomes.1, 2

The unequivocal interpretation of quantitative data for M* and non-M* comparison strains is not simple and often involves additional information which is not readily available. Thus, even if differences in the mutation rates appear for some phenotypic changes, it is not always readily ascertainable whether these are due to comparable genic changes or whether there may not be factors other than the mere presence or absence of the mutator gene which are quantitatively influencing the observed rates. Experiments recognizing this difficulty are now under way and, if successful, should permit a more exact delineation of the mutator gene properties in E. coli, strain K12.

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5 S. E. Luria and M. Delbrück, Genetics, 28, 491–511, 1943.