Joshua Lederberg's Legacy to Bacterial Genetics

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Joshua Lederberg (1925-2008) was an extraordinarily gifted person. Starting his professional career at the age of 17 as a dish washer in Francis Ryan's laboratory in Columbia University, he rose to be the President and later University Professor Emeritus at Rockefeller University, occcupying chairs of Genetics at Wisconsin and Stanford Universities. He was only thirty three when he received the Nobel Prize, along with George W Beadle and Edward L Tatum in 1958. He also received the Presidential Medal of Freedom and the National Medal of Science. His scientific work encompassed not only bacterial genetics but also astrobiology (exobiology, as he called it) and artificial intelligence. He was part of the Stanford team which developed the artificial intelligence software program DENDRAL. With his passing away in February 2008, the last of the founding fathers of bacterial genetics is gone. It is an honour for me to write this small article in his memory. In this article, I will focus on just two of his outstanding contributions to bacterial genetics, namely, the spontaneous, selection-independent origin of bacterial mutations and the discovery of genetic recombination and sexuality in *Escherichia coli.*

Introduction

The history of bacterial genetics can be divided into two eras: the pre-double helix and the post-double helix. While the latter is strewn with brilliant discoveries and conceptual advances, the former is an age of uncertainty and chaos. It is also an era when the genetics of higher organisms (Mendelian genetics) advanced by leaps and bounds giving rise to offshoots like population genetics and eugenics.

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are several reasons for this sorry state of affairs. In plants and animals it is possible to spot and pick out 'variants' which differ in some property (such as flower colour, eye colour, height of individuals, texture of seeds) from the bulk of the population. The variant properties are stable and can be passed on to the progeny. Therefore they are heritable traits and hence are mutations and the individuals carrying them are mutants. In bacteria it is simply not possible to spot out a mutant without resorting to what is known as 'selection', i.e., exposing millions of bacteria to a condition which will allow the growth of only the mutant(s) but not the bulk of the population. It is impossible to say unambiguously whether the selection picked out a mutant that existed in the population or created one as Jean Baptiste Lamarck had proposed in the 18th century. (We will return to this vital question later on). Moreover, in plants and animals, it is possible to 'cross' two variants, each displaying one or more unique variant characters and look at their distribution in the progeny. It could be recalled this is the way Gregor Mendel discovered the basic laws of heredity in the mid 19th century. In the early days of bacterial genetics, such crossing was simply impossible to do with bacteria. Other difficulties like the apparent absence of cytologically observable nucleus and chromosomes compounded the problem. No wonder none spoke of genetics of bacteria. Even some of the reputed textbooks of bacteriology in the 1930s simply avoided the term 'mutants' of bacteria. In spite of all these difficulties, microbiologists like Martinus Beijerinck believed that microbes could mutate much the same way that plants and animals do. Some early pioneers like I M Lewis even showed that variants exist in a population and the variant characters are indeed heritable. Such reports were ignored by and large.

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Whether the origin of variants was by mutational or non-mutational adaptive mechanisms could not be settled one way or the other. It was left to the wisdom of three extraordinarily gifted people – Salvador Luria, Max Delbrück and Joshua Lederberg – to clear up all the uncertainties and lay the firm foundations of modern bacterial genetics. In order to understand and appreciate

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Lederberg's contribution in this area, it is first necessary to know something about the origin of variants in bacteria.

The Fluctuation Test of Luria and Delbrück

The single vexing question which eluded a definitive answer in the early years of bacterial genetics concerns the origin of bacterial variants. Do they arise spontaneously in the course of a single cell multiplying exponentially into a large population (mutation) or *after* a large number of non-variant cells are subjected to selection (adaptation)? Adherents of each view interpreted experimental data to suit their own whims and fancies. A landmark experiment to settle this question was done in 1943 by Luria and Delbrück. They approached the question as a problem in population genetics, amenable to statistical analysis.

Let us imagine that we spread a large number, say 100 million, of bacterial cells on the surface of a solidified nutrient medium in a Petri dish and spray the plate with a bacterial virus (bacteriophage) to which the bacteria are sensitive. The virus will infect and kill all the cells except a very few variants which are resistant to the virus. In this case the virus acts as a selective agent. The resistant cells which survive the virus attack will grow into macroscopic colonies the next day. The question is, how do the resistant variants arise? Do they exist before exposure to the selective agent and show up after the virus-sensitive cells are killed or do they arise after exposure to the virus? If we do the experiment with a single culture, of course we will get some virus resistant colonies which we can count. But we cannot answer the question we set out to resolve. Let us say we do the experiment with a large number (25 or 50) of parallel cultures each started up with a small number of cells and grown to a large sized population. The experimental conditions being identical with each culture flask, we can reasonably assume that the population size will be approximately the same in all of them. (If we want to be very fastidious we can even determine the number of cells in each culture tube). If an aliquot from each tube is spread on solid nutrient medium and exposed to the virus, we will have 25 or 50 parallel plates of

approximately the same number of virus-infected cells. What would be the result expected? This depends on how the variants arise in the population. If they arise by an adaptive mechanism where each cell is assumed to have a small and equal probability to develop resistance *after exposure* to the virus, the number of resistant colonies showing up the next day would be more or less equal on all the plates, following a Poisson distribution. On the other hand, if they arise at a small and equal probability *at every generation* as the culture grows but *before exposure to the virus*, the number of resistant colonies will fluctuate widely among the plates (*Figure* 1). The mutation to virus resistance could have

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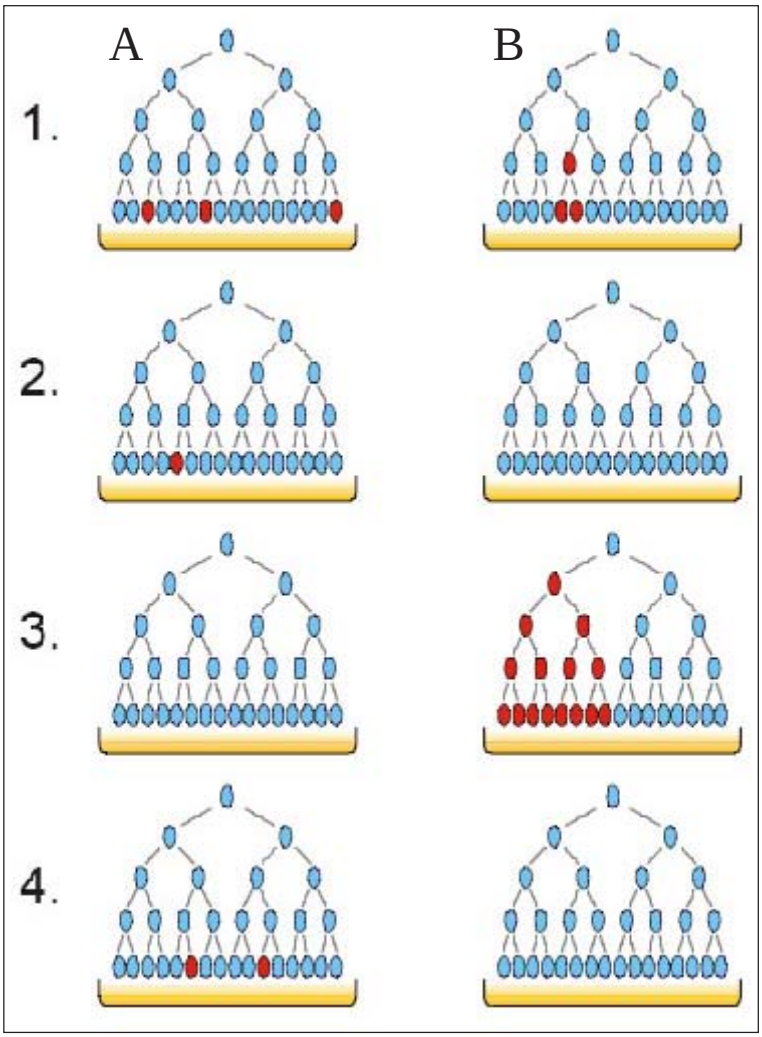

Figure 1. Experimental strategy to differentiate between mutations that are induced by selection (A) and mutations that occur spontaneously before selection (B). The Petri dish represents selection (Adapted from [3]).

occurred early in one tube or sometime in the middle of the growth cycle in another tube or late in some tube. There may even be a tube in which it has not occurred at all. Since there was no restriction to the growth of virus-resistant cells relative to sensitive ones, the size of the 'clone' of resistant cells will fluctuate from 0 (or a small number) to a large number among the parallel cultures. This is exactly what Luria and Delbrück found using *Escherichia coli* and bacteriophage T1. This landmark experiment settled the question of the origin of bacterial variants in favour of the spontaneous mutation hypothesis (For a description of the statistical analysis of the data and another simpler and related experiment called the Newcombe experiment, the reader is referred to standard text books on bacterial genetics).The Luria–Delbrück fluctuation experiment is generally considered to mark the starting point of bacterial genetics. Other workers, notably Demerec, Latarjet and Witkin, used the fluctuation test to investigate radiation resistance, resistance to antimicrobials, etc.

Joshua Lederberg and Indirect Selection of Mutants

1. *Replica Plating*

In spite of its elegance and scientific rigour, the Luria–Delbrück experiment had a thorn in the flesh. It did show that mutation to phage resistance, and by extrapolation, mutations in general, arise independent of selection. Nevertheless selection was still necessary to reveal the mutants. It could be argued, for the sake of argument that variation to virus resistance really occurs adaptively after exposure to selection but the fluctuation is due to non-uniform probability of its occurrence among the virus-infected populations. Therefore the cause of the fluctuation is not pre-selection events (mutations) but post-selection events (adaptation). The ideal experiment would be to show the presence of mutants in the population in the *absence of exposure* to the selective agent.

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mutation and selection, using a laughably simple experimental tool. This was done by a method which has come to be called 'replica plating'.

Let us imagine a population of *E. coli* cells in which one out of a billion cells is resistant to an antibiotic. If we spread one billion cells on solidified nutrient medium supplemented with the antibiotic, the single resistant cell alone will grow out to form a colony. If we want to isolate the resistant mutant without exposure to the antibiotic, we have to screen one billion colonies (obtained without antibiotic exposure), one by one, to see whether they are sensitive or resistant to the antibiotic. This would be an enormously laborious task. If we could test one billion colonies for antibiotic sensitivity/resistance in a single step, the job will be finished in one day! This is what the Lederbergs accomplished using the 'replicator'. It is nothing but a cylindrical wooden block, slightly smaller in diameter than the standard Petri dish, fitted with a vertical handle on the top. (Imagine the commonly used circular rubber stamp). A piece of sterile velvet cloth is tied to the bottom of the cylindrical block. By gently pressing the replicator on the surface of a Petri dish containing solidified nutrient (non-selective) medium with a large number of colonies on it (the 'master' plate) and then on the surface of a fresh plate, keeping the orientation of the replicator unchanged, a replica of the master plate could be obtained. The bristles on the velvet cloth act as tiny inoculation needles. For the sake of simplicity let us imagine a master plate containing 200 colonies on it, of which one is resistant to antibiotic A and another to antibiotic B. If we replicate the master plate on two other plates, one supplemented with A and the other with B, only A-resistant cells will form a colony on the first plate and B-resistant on the other; all the other (200–1) will not grow at all. Since the orientation of the replicator with respect to the master plate is not changed on the second set of plates, it is easy to spot out A-resistant and B-resistant colonies on the master plate. They could be picked up, purified and tested further to see if every cell in them is A or B resistant, as the case may be. What is important to realize in this simple experiment is

that the final pick up of the mutants is from a source which has *not encountered* the selective agent at all. Therefore selection only enables the recognition of mutants against a background of non-mutants. This technique could be used to isolate any type of mutants; drug resistant, metabolic, phage resistant, temperature sensitive, etc. All that is needed is to devise appropriate methods for selection.

2. Sib Selection in Liquid Cultures

In 1956 Cavalli-Sforza and Lederberg devised another method for indirect selection of mutants in liquid cultures. This is called the 'sib selection' technique. Suppose we spread 10^8 cells of an antibiotic sensitive *E. coli* culture on solid nutrient medium supplemented with the antibiotic and obtain 1 resistant colony the next day. The frequency of antibiotic resistance in the culture is $1/10^8$ (10^{-8}). Can we get the resistant cell in pure culture without exposure of the cells to the antibiotic? Of course one can resort to the replica plating technique described earlier. The sib selection method accomplishes the same in liquid culture.

Suppose we dilute the above culture of 10^8 cells 10-fold by making the final volume 10ml, the cell density will now be 10^7cells/ml. The original frequency of antibiotic resistance being $1/10^8$ (which is equal to $0.1/10^7$), the frequency in the diluted culture containing 10^7/ml cells will be 0.1 resistant cell/ml, or one resistant cell/10ml. If we distribute 10 ml of the diluted culture into 10 tubes of 1 ml each, one of the tubes (the 'lucky' one) will receive the lone resistant cell while the other nine will not. Now the frequency in the lucky tube is $1/10^7 = 10^{-7}$, a 10-fold enrichment with respect to the original culture. This can be verified after growing all the 10 cultures to saturation and determining the total and resistant cells in each. The 'best' tube is the one where the single resistant cell landed (the initial ratio being 1 resistant cell/ 10^7 total cells), which could grow along with the sensitive cells generating resistant siblings. Now if this culture is diluted to 10^6 cells/ml in nutrient broth and dispensed in 10 tubes of 1 ml each, one of them (the lucky one) will receive the single resistant cell

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and 10^6 total cells, the frequency now being $1/10^6 = 10^{-6}$. This is a 10-fold enrichment with respect to the immediate predecessor and 100-fold with respect to the parent cultures ($10^{-8} \rightarrow 10^{-7} \rightarrow 10^{-6}$). If this enrichment procedure is repeated again and again, the frequency keeps increasing 10-fold at every step such that ultimately one will obtain a culture in which the resistant to total cells will be 1/1, that is, all the cells will be resistant. Note that plating on selective medium was only to identify the best tube at each step and not for picking up of the desired mutant.

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What has been presented above is an idealized description of the method. In practice, however, there may be difficulties arising due to differing growth rates of mutants relative to non-mutants. For example, streptomycin-resistant mutants grow slower than sensitive cells. Therefore, appropriate corrections have to be made to take care of the difference in growth rates. Moreover, it was assumed that none of the cultures yield additional mutants during the course of the experiment; only the ones present initially kept growing and got enriched relative to the non-mutants at successive steps. This need not be true (even if some cells in any of the tubes in any of the steps mutated, the proportion of mutants will be less than the best tubes which started off with a mutant). In their experiments Cavalli-Sforza and Lederberg observed that only the best tube contained large number of mutants while the others had none or very few. They isolated streptomycin and chloramphenicol-resistant mutants using the sib selection technique. At that time a few people, notably Sir Cyril Hinshelwood (a very distinguished physical chemist and Nobel Laureate) and his group maintained that antibiotic-resistance is the result of physiological adaptation which in turn is caused by changes in the kinetics of chemical reactions in cells when exposed to antibiotics. The replica plating method (see above) convincingly showed that antibiotic resistant mutants exist in cultures in the absence of exposure to the antibiotic. The sib selection method not only confirmed it but also showed that the resistance property was heritable; cells from a best tube always yielded a best tube in the next step. These observations are not in conformity with the

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adaptation postulates of Hinshelwood but can only be explained on the basis of spontaneous mutation hypothesis. Although not designed to refute Hinshelwood, the sib selection technique did so indirectly, besides being another method for the indirect isolation of mutants. (In all fairness it must be admitted that Hinshelwood did not refute the mutation-selection hypothesis of the origin of bacterial variation or assert that physiological adaptations are heritable. However, he held views radically different from the majority and was often accused of "uncritical application of mathematics to biology" (see [1], [2]).

The Luria–Delbrück fluctuation test and Lederberg's indirect selection of mutants established an important paradigm in genetics, namely, the independence of the occurrence of mutations (mutagenesis) and selection. In other words mutagenesis does not depend on the usefulness or harmfulness that might ensue consequently, either immediately or in the future. Selection on the other hand does not influence mutagenesis but only exposes the intrinsic fitness differences of the mutant *vis-à-vis* the non-mutant. This is clearly a Darwinian idea. It should be mentioned in passing that four and a half decades after the Luria–Delbrück and Lederberg experiments were published, a few reports of apparently selection-dependent, Lamarckian type of origin of at least some types of mutations appeared. However, more recent studies have brought these results under the purview of natural selection, revealing that even non-dividing cells can mutate.

Joshua Lederberg and the Discovery of Mating, Sex and Genetic Recombination in *Escherichia coli*

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Bacterial genetics in the pre-double helix era suffered from a major lacuna in that there was no concrete evidence for the existence of chromosomes in bacteria. The occurrence and nature of mutations, evidence of (genetic) linkage revealed by techniques such as DNA-mediated transformation and phage-mediated transduction, were fragmentary and did not provide a picture of the overall genetic organization of bacteria. William Hayes who made outstanding and path breaking contributions on the

sexuality of *E. coli* in subsequent years compares the state of affairs to "viewing a football match through a telescope". One could appreciate the skills of one individual player but not the overall game strategy.

Some of the initial groundwork to gain insights into the genetic organization of bacteria (*E. coli*) was done by Lederberg and Tatum during 1947–52. Both of them had independently isolated nutritionally deficient mutant strains of *E. coli*. Such mutants are called auxotrophs. Unlike the wild type strains which are called prototrophs, the mutants cannot synthesize growth factors like amino acids or vitamins or purine/pyrimidine bases from simple precursors like glucose and ammonium sulphate. They grow well in rich media (nutrient broth) but cannot grow on minimal glucose + salts media unless the required nutrient is supplied from the medium. They are generally designated as Met^- (methionine requirer), Leu^- (leucine requirer), Trp^- (tryptophan requirer) and so on. Lederberg and Tatum also isolated mutants designated as Ton^R (resistant to phage T1), Str^R (resistant to streptomycin), etc. They had also isolated double mutants such as (Thr^- Leu^-), (Pro^- Thr^-), which were extremely useful not only to Lederberg and Tatum, but to other investigators in subsequent years. With these mutants they set out to see whether it is possible to 'mate' or 'cross' any two to know the possible existence of mating types, sterility factors, etc., in *E. coli*.

The basic idea behind the mating experiment is to mix two strains each with a different auxotrophic mutation and look for prototrophic 'recombinants'.

$$A^-B^+ + A^+ B^- \rightarrow A^+ B^+$$

In the above example one strain is auxotrophic for amino acid A (designated as A^-) but prototrophic for amino acid B (designated as B^+). In the other strain the markers are reversed. Neither of them will grow on glucose + salts minimal medium. If some genetic exchange and recombination occurs when they are 'mated' so as to yield cells which are A^+ B^+, the latter will grow on

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minimal medium since they will not require supplementation of amino acids A or B. Note that the auxotrophic mutations are used not only as genetic markers but also as selective agents. A likely technical difficulty in such an experiment is either of the two strains could suffer a 'back mutation' (reversion) to give A^+ B^+cells. There is no way to distinguish between revertants and recombinants. To avoid this difficulty Lederberg and Tatum used double mutants like A^- B^- and C^- D^- as mating partners. Since the frequency of reversion of double mutants will be the product of the single reversion frequencies, double mutants will revert at extremely low and negligible frequencies, of the order of $1/10^{14}$ or $1/10^{16}$. With such sound reasoning, Lederberg and Tatum set up crosses between various auxotrophic mutants. As it always happens, the initial trials ended in failure. After sustained efforts they finally did get a pair of strains in which the mating succeeded. This cross, given below is hailed as Lederberg's "classical cross" in the literature.

Strain 58-161: $Thr^+Leu^+Bio^-Met^-$ ↘

$\rightarrow Thr^+Leu^+Bio^+Met^+$ (prototroph)

Strain Y10: $Thr^-Leu^-Bio^+Met^+$ ↗

Lederberg presented his paper in the highly prestigious Cold Spring Harbor Symposium of 1946. Like all significant discoveries, the initial reaction to his paper was lukewarm, if not hostile. Many doubts and alternative explanations poured in. Perhaps in anticipation, Lederberg had taken care to do a number of control experiments to rule out possibilities like transformation by DNA released from lysed cells, persistent mating partners, and cross-feeding (syntrophic feeding) between the cells. Eventually truth prevailed and within a year their initial observations were substantiated. When more mutants were available many more successful matings were done. By introducing a third marker like Ton^R in one of the mating partners, the inheritance of that marker (not selected) among the prototrophic recombinants could be scored. The results showed that some of the recombinants inherited the non-selected Ton^R marker and some did not. The frequency depended on which partner had the Ton^R marker. It tended

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to go along with Thr^+Leu^+ more often than other markers indicating some linkage between $Thr^+Leu^+$and Ton^R. By 1947–48 Lederberg was able to construct a partial linkage map of *E. coli*, based on the results of mating experiments.

A major technical advancement that occurred during this period was the demonstration by Bernard Davis that successful mating between *E. coli* strains required cell-cell contact. Mating failed to occur if the two partners were kept in the two arms of a U-tube, separated by a filter that allowed the mixing of the medium but prevented cell-cell interaction. This device was later used by Lederberg to show that another mode of gene transfer he originally discovered in *Salmonella typhimurium*, called transduction, did not require cell-cell contact but was mediated by a filterable agent, a bacteriophage. Lederberg viewed the mating between *E. coli* cells as being analogous to that occurring in higher organisms. He proposed that two haploid cells form a transient diploid (zygote?) followed by meiosis (without cell division) to regenerate haploid cells. Genetic exchange probably occurs during the transient diploid state. After the pathbreaking work of William Hayes on conjugation which followed (1952–57), Lederberg's views on the mechanism of mating were abandoned. Although Lederberg could construct linkage maps of *E. coli* with limited number of markers, all of them could not be arranged into a single linear array. What resulted was a four-armed branched linkage map. These knotty issues were resolved by the work of Hayes in the following years. George Beadle, Edward Tatum and Joshua Lederberg received the Nobel Prize in 1958; Lederberg was only thirty three!

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Significance of the Work of Joshua Lederberg on Bacterial Sexuality

It is amazing that a very simple experiment of mixing two auxotrophic mutants and plating out for recovering prototrophic recombinants had such an impact on bacterial genetics. Lederberg's experiment pointed the way to many future lines of investigation on the genetic organization of *E. coli* and, by extrapolation,

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bacteria in general. Just a year earlier, in 1945, Beadle had remarked "in bacteria where cell reproduction is vegetative, there are presumably units functionally homologous with genes of higher organisms, but there is no means by which these can be identified by the techniques of classical genetics". The very next year Lederberg and Tatum's work opened up a grand new vista. Many people had earlier looked for sexual phenomena in bacteria, but without success. Crosses could not be done in bacteria; the techniques used in earlier bacterial "crosses" were not sensitive enough to reveal rare recombinants. The use of nutritional auxotrophs, especially double mutants to avoid reversion artifacts, not only as genetic markers but also as selective agents was a stroke of brilliance. Moreover there was a feeling at that time that genes in bacteria, even if they existed, were analogous to extrachromosomal factors in higher organisms. This view is based on the knowledge of cytoplasmic inheritance in algae, protozoa, etc. The phenomenon of bacterial transformation was known since its discovery by Griffith in 1928. Avery, McLeod and McCarty had shown just a couple of years earlier (1944) that Griffith's "transforming principle" was really DNA (see *Resonance*, September 2007). This remarkable finding had not had its full impact in 1946 when Lederberg's mating experiments were published. The double helical structure of DNA proposed by Watson and Crick lay seven years ahead. More significant is the fact that while Lederberg looked at the genetic outcome of the cross, William Hayes who followed, looked at the mechanisms of the cross and came up with remarkable findings. It led to the discovery of extrachromosomal genetic elements (plasmids) in *E. coli*. Hayes' work also led to the development of a powerful technique of genetic mapping and construction of a linkage map of *E. coli*, assigning a unique position for every gene on the linkage map. Surprisingly the *E. coli* linkage map was circular unlike those of higher organisms in which the linkage maps were linear. Thus a whole new branch of molecular genetics was born. Little wonder that Luria, writing about the state of affairs in bacterial genetics in 1947, eulogized the mating experiments of

Lederberg and Tatum as one "among the most fundamental advances in the whole history of bacteriological science".

Suggested Reading

[1] **William Hayes, *The Genetics of Bacteria and their Viruses*, Editions I and II. John Wiley & Sons Inc, New York, USA. Although the second (and last) edition of this treatise is 40 years old, it is still an outstanding source of classical information on bacterial genetics, generally not found in contemporary textbooks, 1964, 1968.**

[2] **T D Brock, *The Emergence of Bacterial Genetics*, Cold Spring Harbor Press, Cold Spring Harbor, New York, USA. An excellent source of historical information on bacterial genetics, 1990.**

[3] **G S Stent and R Calendar, *Molecular Genetics: an Introductory Narrative*, W H Freeman and Company, San Francisco, USA, 1978.**

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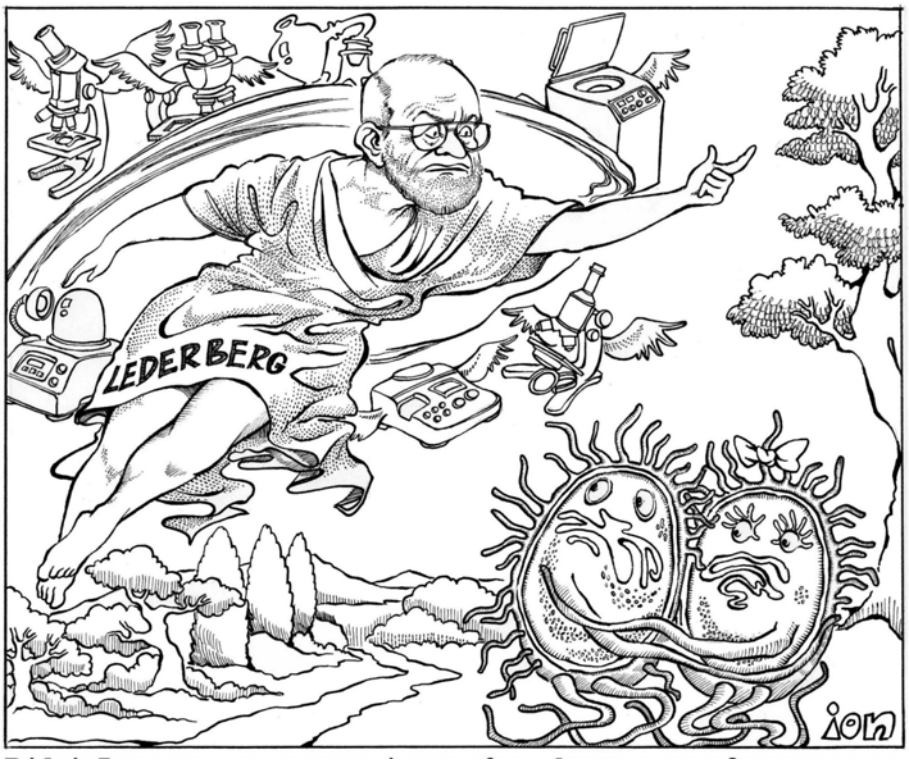

Didn't I warn you not to conjugate & exchange genes?