Transcript from filmed lecture
November 14, 1959

Lecture #43 - Bacterial Genetics: Clones
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Bacteria have become important subjects of experimental investigation for several reasons. First, as you all know, is their importance in the ecology of the terrestrial globe, their impact on agriculture, and their relationship to human disease. And this motivation has indeed underlain most work in bacteriology during the first 50 or 60 years of its existence. Second, bacteria proved to be extremely convenient objects of experimental investigation since very large populations can be manipulated with great ease in the laboratory. And third, the simplicity of their structure, which can be illustrated by this schematic diagram of a cell of Escherichia coli, which measures about 1 by 4 μ, making this among the smallest of all cells, and which contains within it a minimum of structural detail. We see the presence within each cell of two or sometimes four nuclei, which can be chemically defined as masses of deoxyribonucleic acid, containing approximately 6 million nucleotide units. This should be compared with the content of DNA in a mouse cell which is nearly one thousand times greater or 5 billion nucleotide units.

The slide which is now being projected will show you somewhat more realistically the appearance of a bacterial culture which has been stained so as to exhibit the nuclear material. We would like to know more than we do of the detailed morphological mechanism by which the nuclear material is divided at each cell division, since there is at the present time a continuing controversy as to the existence of a process displaying all of the features of mitosis as we see it in higher organisms. Still, there can be no doubt whatsoever as to the fundamental chemical similarity embodied in DNA between the

genetic material of bacteria and that of higher forms, and we likewise can have no doubt that there is a mechanism for the exact partition of the 5 million units or 6 million units of information which is contained in the DNA at each division of the bacterial cell.

An important feature of experimental treatment of bacteria is the reliance on methods of vegetative reproduction, since this, in all organisms, including those which have an auxiliary sexual mechanism, is by far the most important means for the increase of bacterial numbers. We, therefore, will recur again and again to the concept of the clone. A clone is defined as a population of individuals all derived from a single cell by vegetative reproduction. In consequence, barring some accident of genetic mutation, or some interesting event of genetic recombination with other cells, the clone should consist of a population of genetically equivalent individuals all of whom have identical copies of the same information which was present in the ancestor. Before dealing with the practical exploitation of clonal growth, we should consider the rapid rate at which bacteria multiply, and the large populations which can be obtained from them.

In a favorable medium a typical bacterium, like Escherichia coli, can divide vegetatively at intervals of about one half an hour. Consequently, if one plants into a tube of suitable medium, into nutrient broth, a single cell, we will have a geometrical increase in the number of cells along the lines indicated by this diagram. This doubling every half hour results in an accelerated increase in the population which can be defined by the expression that at any given time t, measured in hours, at which there will have been twice as many generations, small n, the population, large N, is given by the power 2 to the n, or 2 to the 2t. Consequently, after a period of 30 bacterial

generations, which would require just 15 hours, a tube of clear broth into which a single cell has been planted, will give rise to a population of 10 billion organisms, as indicated in this tube of turbid broth.

Now, in order to obtain single cells from which clones can be derived, which clones are then available for further biochemical and physiological and of course genetic study, several procedures can be used. The most tedious, although the most exact, is perhaps the direct isolation of a single cell by means of a micromanipulator. But this is a fairly slow and laborious process, and in practice is used only when special emphasis must be placed on the fact of the derivation of the entire clone from a single individual which had been seen under the microscope.

In practice many short cuts are available. And in general they are based on the fact that if one takes a suspension of bacteria in which there are large numbers of cells one can sufficiently dilute those suspensions by diluting samples into larger volumes of fluid, so that we will have a sample containing a few bacteria, which can then be spread on an agar plate. I have an example, of such an agar plate that had been spread 24 hours ago in my hand, and these are displayed further on this light box, in this example here. We have here about 200 colonies of bacteria, each of which has been derived from a single cell that had been planted on the surface of the nutrient agar medium, which fills this plate. As a result of the continued vegetative multiplication of each cell, the invisible cell, which had been randomly put at a particular spot on the agar, now gives rise during the course of 15, 20, or 25 generations to a visible colony. This colony is a clone, and, as I indicated before, unless there has been some interesting event to intervene, it will consist of individuals all having the same genetic constitution.

Another way in which one can conveniently separate cells in order to produce

clones is to use the well known, and very complicated instrument, the innoculating loop. One merely takes a sample from the broth culture, I'm omitting the sterile precautions which are necessary in practice, but a little hard to do at the lecture table, and simply spreads out the content of the loop onto a fresh agar plate, and then eventually there will be a point on the plate where single cells are deposited on the agar at some distance from one another. This gives rise to a plate that looks like this, and I've used the loop to streak out two different samples of different kinds of bacteria, and those are indicated by the different colors of the colonies on this half of the plate, on one hand, and the other half of the plate on the other.

This plate, that I've just shown, also illustrates how we must in practice rely on the average behavior of a large number of organisms for most of our genetic work. There are very few markers which can be accurately discerned in individuals cells. Now there are some kinds of experiments where one will rely on a cell marker, for example motility, which is based on the presence of flagella -- the organs of locomotion, but as a rule there simply is not enough material in a single cell to give us a convenient way of studying its phenotype, and we therefore rely on the fact of the genetic homogeneity of a clone, and the ability to recognize the physiological and biochemical behavior of a large number of identical organisms as a way of typing the original bacterium from which the clone had derived.

This plate, in fact, illustrates a physiological difference between a wild-type and a mutant strain of Escherichia coli which has been of some practical importance in the development of this subject. The medium on which these bacteria are plated is an eosin methylene blue agar medium containing the sugar lactose. And the dark colonies that appear at the top half of this plate represent organisms that are capable of fer-

menting lactose, and thereby generating the color that you see in the colony as a result of the acid produced from the fermentation. The bacteria that have been streaked on the lower part of this plate are a lactose-negative mutant, that had been derived from the original wild-type culture by exposing their cells to ultraviolet radiation. The comparison between the wild-type and the mutant strain has been carried to some degree of precision, and has in fact given us some insight into the genetic control of the formation of this enzyme responsible for lactose fermentation, beta galactosidase.

Before we touch further on physiological mutants of bacteria, it would be important to verify the fact that these are spontaneous, or ultraviolet, or X-ray induced mutations, in the same sense as applied to mutations of higher organisms. Since bacteria are so intimately exposed to their immediate chemical environment, one might inquire whether in these instances, for example, the production of lactose-positive reverse mutants from the lactose-negative strain, or in other cases, where one could insert some question of the inductive effect of the environment on the bacteria, whether these chemical environments have resulted in a direct way in the modification of the hereditary constitution of the cells.

This question,which may appear remote in the example I have just indicated, has been a very pressing one with respect to mutations of another form, mutations to resistance to antibiotics. It has been known, almost since the introduction of these agents in chemotherapy, that populations of bacteria which had been exposed to an antibiotic, for example t.b. organisms exposed to streptomycin during the course of therapy of tuberculosis, might on some occasions give rise to progeny which were resistant to the drug in question. At first sight the mechanism would appear to be quite simple, and has been so proposed by many workers. Namely, that some chemical reaction had taken place,

as between the streptomycin and the tubercle organisms to result in a change in hereditary constitution of these bacteria, and, consequently, the development of drug resistance. And this might seem to be the simplest statement of what, in gross, happens when one conducts this experiment either in the treated patient, or in the laboratory, when one adds a given amount of antibiotic to a large culture of bacteria.

However, the geneticist, accustomed as he is to the recognition of changes in populations on the basis of natural selection of spontaneously occurring mutants, has persistently raised an alternative hypothesis, namely that mutations to drug resistance, or mutations of other adaptive types, have in fact no relationship to the environment in which they occur, except that, once having occurred, these environments select those organisms that now have an adaptive value therein.

It has been a matter of some difficulty to establish in any precise way a criterion for deciding between these two interpretations since in most instances it would be necessary, in order to demonstrate the presence of one drug-resistant organism among a population of 10 billions of cells, to expose that population to the drug in question. How then can we determine whether the mutants were present in the population before the drug was added to it, that is to say, whether the mutants were pre-adaptive, or whether they had arisen as a result of a direct chemical interaction with the chemical -- whether they are post-adaptive?

One of the first approaches to this problem, that was at all convincing in its implications, was presented by Luria and Delbruck in 1943, the basis of their now well known fluctuation test. If we consider the clonal nature of bacterial growth we must keep in mind that when a mutation should occur during the course of development of a clone that the number of mutant cells which will be present in the final population will

depend not only on the mutation rate alone, on the frequency with which mutational events occur, but also on the extent to which individual mutants will have had an opportunity to multiply during the further growth of the clone. For example, a mutation which occurs late in the development of the clone, let us say at the last division before we examine it, will be represented by one mutant individual per mutational event. Much more infrequently, because there will have been many fewer cells at that time, we might have some mutations occurring say at the first or the second division. And these mutations will now have a very large mutant progeny, since the mutant character will be propagated to all of the descendants of those few cells that were present at that time. Consequently, the statistics of distribution of mutant cells in bacterial populations would be expected to be highly skewed, that is to say, there should be a very few cultures which have a very large number of mutants far in excess of the expectation from any normal distribution of the mutant individuals.

On the other hand, if the mutation were the result of an interaction of the final cells in the clone with the chemical used to demonstrate the mutants, we might then expect something much more nearly approaching a random or normal distribution.

The experimental finding which Luria and Delbruck found, and proposed as evidence that the mutations for resistance to viruses in bacteria and to drugs was based on spontaneous mutation, was that there was this highly skewed distribution that there were, so to speak, a few, and an unexpectedly large number, however, jackpots of cultures in which a very much larger number of mutants was present, than would have been predicted from the random occurrence of mutants in the final generations.

However, this argument is essentially a statistical one, and rigorous as it may be, it has not been understood as fully as might arguments of a more direct nature.

One of these is based on a new technique for handling the large numbers of clones which are necessary in analyzing problems of this kind. You will have recognized that we might have made a direct test of the pre-adaptive occurrence of resistant mutants in a very simple but tedious way. We might make a plating of a clone, so that there would be at our disposal some hundreds of thousands, or millions, or even billions if necessary, of isolated colonies. And we might then test those colonies in a way that I've indicated on this plate on the light box; namely, each colony might be put on a medium which lacks the drug, and then a sample of it stroked over to a line in which a certain amount of the drug had been added -- in this case the actual demonstration concerns streptomycin and certain mutants of E. coli which are resistant to streptomycin. If a sufficiently large number of randomly plated bacteria were used, and their clones tested against streptomycin, if these mutations can occur in the absence of the drug, one might in fact expect, without having subjected the bacteria to any prior exposure to the drug, that an occasional colony would be resistant. However, we now know that in order to do this experiment it would necessitate of the order of one hundred million, or one billion tests, and this is far beyond the range of any practical experiment that can be done by such a random procedure.

Fortunately, we have now at our disposal another method which makes it possible to handle large numbers of clones efficiently for this purpose. This is called replica plating, and it relies on the possibility of transferring the growth from one plate to another by means of a sheet of velvet which is placed on a hopp, as I have just done. If we start with an agar plate which has already had distributed on it a collection of colonies, whose types we are interested in determining, we can press the plate down on the velvet, leaving on the velvet surface an impression of the growth that was on the plate, and then use the velvet as a master to plant a corresponding pattern of growth on one or a series of additional plates, as I am now doing. I have on the light box an example

of this operation that was done about 24 hours ago, which indicates the identical patterns of growth which one can obtain, having started from this master, and here are two of the copies, and the similar distribution of colonies as between the two plates shows the extent to which one can obtain a faithful replica of the original pattern on one or several secondary plates.

The application to the present problem is the possibility of replica-plating a pattern growth from one master to a plate not containing the drug in question, to the standard medium, and then to additional plates which do contain the drug. On the plates containing say streptomycin, only those bacteria will be able to form colonies which are resistant to the drug. Consequently, by this method in a single procedure it is possible to test not just one colony for resistance to an antibiotic, but to test as many colonies as can be crowded onto a single plate. And this means that say a thousand clones can be tested in one operation.

In fact, even this is not sufficient for an efficient test of the pre-adaptation hypothesis. Since the frequency of occurrence of drug-resistant mutants in many cases is very much lower even than one in a thousand, and it may be one in a million or one in a billion.

Even so we can then use replica plating by starting with an original plate on which had been smeared, say one million or one billion organisms, which will give rise to small colonies so closely spaced together that there appears to be continuous growth. In that plate, however, there will be imbedded a few small clones, not discernible to the eye, which should, on the pre-adaptation hypothesis, be resistant to the drug. The position of these clones will be registered when replica plates are made from the original to subsidiary plates containing the drug. It is then possible to go back

to just the corresponding site on the original place corresponding to the resistant colony and from that site to obtain an innoculum which will be greatly enriched in the proportion of resistant as compared to sensitive cells. Because the colonies are so closely crowded together, the first return to the original plate will not give a pure culture of resistant organisms. However, it will have a higher proportion of them since one can go to the place where the resistant organisms are found, and ignore parts of the original plate which do not have these resistant mutants.

Using this as an innoculum it is possible to either repeat this enrichment procedure, or finally to do a plating at such a dilution that there will be discrete colonies and from such discrete colonies to detect the one which is resistant to the drug by making a replica plate. The point that should be stressed is that in these operations of transferring colonies from a master plate to the velvet, and then from the velvet to a subsidiary plate, when we save the master plate we do not expose any of its cells directly to streptomycin. We have taken only a sample of the cells from each colony in order to test the status of that colony or that site on the agar. Consequently, when, as has proven to be the case, it is experimentally possible in many instances to isolate drug resistant clones of bacteria by the repetitive use of the replica plating technique, we can be assured that the drug, say streptomycin, or penicillin or chloramphenicol, has not played a direct part in the production of the resistant mutants. These resistant mutants although present in very small numbers must have been present in the original clones before they were exposed to the drug.

Since we can so easily develop clones containing the 10 billions of organisms which I was indicating previously, and since it is such a simple operation to take the entire contents of such a culture and to plate into agar containing a drug like strepto-

mycin, we consequently have a very powerful method indeed for detecting the presence of even very small numbers of mutant cells in very large populations. We can, by this method, measure the smallest spontaneous mutation rate that has yet been possible to be measured, and this is in fact the mutation of Escherichia coli from its normal status of sensitivity to streptomycin to the status of streptomycin-resistance. This is a change which occurs approximately just once for one billion cell divisions. And we can visualize the difficulties that we would encounter if we set ourselves to the task of detecting, much less than enumerating the frequency, of this kind of mutant in populations of any other organism that we can use in the laboratory.

Having thus shown the spontaneous character of those mutants which arise and can be selected by the presence of deleterious agents, or similarly the spontaneous character of mutations that lead to improved adaptive quality of the organism in other stressful environments, for example the placing of nutritional mutants in media which lack the specific nutrients required by a given strain, we are then in a position to use this technique as a routine method for the specific isolation of those genotypes which may occur in a bacterial population and whose occurrence may give us important information as to genetic processes taking place in those populations.

In the next lecture we are going to discuss the application of such techniques for the discovery of genetic recombination. For the moment I think we can merely point to the fact that we now have available a method of precisely counting the incidence of mutants which may arise in bacterial cultures that had been exposed to specific treatments, particularly designed to induce the occurrence of various mutations. Historically, the most important of these is of course X-irradiation. And just as it does with every other organism we know, X-rays will cause an increased frequency of the pro-

duction of various kinds of mutants in bacterial populations. I would like very much to stress the various kinds of mutants, since neither with X-rays nor in fact with any other agent now available to us have we succeeded in producing any single unique type of mutant at will. This is presumably ultimately a reflection of the chemical structure of the hereditary material as represented in DNA. The fact that DNA has relatively simple units, the nucleotides of adenine, guanine, cytosine, and thymine, should tell us that we will face great difficulties in trying to obtain an agent which will specifically induce the mutation of any particular gene. Any gene in which we might be interested is certain to be composed of large numbers of these elementary units, differing from other genes only in their arrangement. In order to obtain a specific mutagen we should then require an agent not which merely separates its actions on thymine as compared to adenine, or on guanine as compared to cytosine, but which is able to recognize specific assemblages of these elementary nucleotides into larger units.

Such an agent, in other words, would have to have all of the necessary chemical discrimination which we now attribute to a gene. And we may reasonably expect that such an agent will have the same chemical complexity, and perhaps even structure, that the gene itself displays. This order of complexity is beyond our present powers of chemical synthesis, although it may perhaps eventually be within our grasp. Although specific mutagenesis is still for future investigation, this does not mean that we completely lack important information on the chemical environment concerned with mutation. For example, Novick and Szilard have found that purines, for example, caffeine, adenine, guanine, will greatly increase the mutation rate as observed in bacteria, and furthermore that the corresponding riboside derivatives will greatly de-

crease the mutation rates. These observations, and many others, have led us to the conclusion that spontaneous mutation, sporadic though it be with respect to the identity of its targets, is in many respects an incident of the normal metabolism of the cell.

Thank you.