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Bacterial, Phage and Molecular Genetics

An Experimental Course

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Preface

During the mid-forties bacteria and phages were discovered to be suitable objects for the study of genetics. Genetic phenomena such as mutation and recombination, which had already been known in eukaryotes for a long time, were now shown to exist in bacteria and phages as well. New phenomena as lysogeny and transduction were discovered, which gained great importance beyond the field of microbial genetics.

Bacteria and phages are of small size, multiply rapidly, and have chemically defined growth requirements. Many selective procedures can be applied to screen for rarely occurring mutants or recombinants. Therefore, they offered ideal conditions to investigate genetic processes and to interpret them in molecular terms. Many new methods were developed (e.g. CsCl density gradient centrifugation) and old techniques were improved and modified for new purposes (e.g. chemical mutagenesis). Hypotheses, such as the semiconservative replication of DNA, mutation by transition and transversion and operon regulation, have had an extraordinarily stimulating effect on the research in general genetics. Thus, in the past two decades, from the genetics of microbes (including fungi) the field of molecular genetics developed. Many text books compete in presenting the latest knowledge on this subject. But to date there are only a few laboratory manuals which introduce the student of biology to the manifold experimental techniques of microbial and molecular genetics. This laboratory manual is an attempt to redress the balance.

The experiments were selected for this book such that each part of microbial and molecular genetics is about equally represented. Care was taken to ensure that most of the experiments could be performed with standard laboratory equipment. Among the experimental objects used here, are *Escherichia coli* and some of its phages and also *Serratia marcescens* and phage κ. At present, certain interesting experiments (extracellular UV mutagenesis) can be carried out more conveniently with *Serratia* phages than with *E. coli* phages; in other experiments, such as phage crosses and complementation, it is less important which particular phage is used. Obviously a study of phage crosses could be extended to circular linkage maps, different types of heterozygotes and the use of deletion mutants for mapping, in which case phage T4 would be more suitable.

The experiments described were tested in several laboratory courses lasting from 3 to 6 weeks. Each time approximately 20 students of biology and biochemistry participated. The students were in about their third year of study, and up to this time had had little practice in experimentation. In order to facilitate experimentation by the beginner and to help him to find his way through the multitude of test tubes on his bench, we have taken care to give detailed descriptions of the experimental procedures. We have also included extensive data sheets for the execution and evaluation of the experiments. On the one hand, these data sheets are to serve as example for the recording of experimental results; and on the other hand, they are to aid the instructor to criticize the work of 20 or more students in as short a time as possible. The students can compare their own records and results with the data obtained in our laboratory while working out the experiments for this course (Section IV). Our results of the experiments should not be considered as absolute. Changes in laboratory conditions (chemicals, equipment) naturally influence the results to a certain degree.

At the beginning of each experiment, we have briefly summarized the theory on which it is based. These introductions can not substitute for a text book; but, with the literature quoted at the end of each experiment, they should help the student to brush up on his theoretical knowledge and, thus, to gain insight into the experiment.

We have written this book to stimulate the student's interest in genetics by giving him detailed instructions to "rediscover" experimentally genetic phenomena which he already knows. Besides this we wanted to make the experiments quantitatively evaluable and to demonstrate a series of methods of experimental and theoretical-statistical nature.

Requests for the necessary bacteria and phage strains (Section III/B) used are welcome. Specimens will be sent at a minimal charge.

Acknowledgements. At this point we would like to thank all technicians of the institute who have helped us in working out the experiments. We are indepted to Mrs. Sigrid Mickley who has typewritten the English manuscript and to Mrs. Rosi Winkler who prepared the graphs. We are also grateful to Professor R.W. Kaplan for his valuable advice on the statistics section. We wish to express our thanks to all our colleagues who placed strains of bacteria and phages at our disposal.

Bochum, January 1976 The Authors

Contents

I. General

A. Abbreviations and Expressions Frequently Used

1. Nucleic Acids and Bases

A	adenine	DNA	deoxyribonucleic acid
T	thymine	RNA	ribonucleic acid
G	guanine	mRNA	messenger RNA
C	cytosine	tRNA	transfer RNA
U	uracil	rRNA	ribosomal RNA

2. Units

length	1 m = 10^3 mm = 10^6 µm = 10^9 nm = 10^{10} Å
weight	1 g = 10^3 mg = 10^6 µg
volume	1 l = 10^3 ml = 10^6 µl
radioactivity	1Ci = 10^3mCi = 10^6µCi (Ci = Curie)

1 Ci: 3.7×10^{10} radioactive decays per sec or
2.2×10^{12} radioactive decays per min

3. Other Abbreviations and Symbols

Abbreviations for the characterization of the genotype of bacteria and phages are listed in Section IIIb. For the abbreviations of nutrient media and solutions see Section IIIa.

cpm	counts per min
dpm	decays per min
ε	molar extinction coefficient
EDTA	ethyldiaminetetraacetic acid
η	refractive index
x g	gravitational force
k, -k	growth constant or inactivation constant
log cells	bacteria in the logarithmic (exponential) growth phase
M	molar
m	multiplicity of infection = phages/bacterium
µ	mutation rate, induced
N	total number of cells, viable cells or plaque formers

O.D.	optical density, synonymous with extinction
Φ	quantum yield
P-buffer	phosphate buffer
ρ	density (g/ml)
S	Svedberg unit
σ	effective cross section
SSC	standard saline citrate
stat cells	bacteria in the stationary phase
t	time
T_m	melting point
rpm	revolutions per min
UV	ultraviolet light

We have tried to be consistent with the lettering most frequently used in other textbooks. Thus, it could not always be avoided that one symbol was used for two different meanings. Deviations from the definitions listed above are given in the text of the respective experiment.

4. Explanation for Some Expressions Used in the Text

- Denaturation of DNA. The conformational change of native DNA by physical or chemical treatment, i.e. the melting of double stranded DNA (helix-coil transition).

- Fractioning a gradient. The emptying of a centrifuge tube, fraction by fraction, at the end of a CsCl or sucrose gradient centrifugation. Usually the bottom of the tube is pierced with a hypodermic needle and a constant number of drops per fraction is collected.

- Soft-agar layer plates. Agar plates, layered with 3 ml of soft agar, which contains 0.1 ml of a bacterial suspension and, when needed, the addition of 0.1 ml of a phage suspension. Often used for the determination of viable counts or plaque titers.

- Indicator bacteria. When determining phage titers by the plaque method, indicator bacteria form the bacterial "lawn" on which the plaques are formed.

- Culture, logarithmic. A culture of growing bacteria, such as one obtains by a 2-4 hrs incubation of a 1:50 dilution of a stationary culture. Log cells are freshly grown prior to the beginning of the experiment and then kept in an ice bath until they are used.

- Culture, stationary. A bacterial culture which has been grown over night (approx. 15 hrs) and whose cells are in the stationary phase.

- Culture, rolling. A bacterial culture which is aerated by rolling in a slanting position during incubation. This procedure prevents the formation of foam!

- Lysate. A suspension of phages which have been released by lysis of infected bacteria.

- Marker, genetic. A mutation, by which a gene is "marked" for genetic experiments, e.g., for crosses. Point mutations must make the mutants phenotypically distinguishable from the wild type, if they are to serve as genetic markers.

- Marker, selective. A mutation, which under suitable conditions, provides the mutated organism with a selective survival or growth advantage.

- Multiplicity of infection. The ratio of infecting phages to bacteria in the infection mixture.

- Scintillation counting. A method which allows a quantitative measurement of the decay of radioactive substances by means of the transfer of the energy of radioactive decay to scintillators (organic ring compounds). The scintillations emitted by the scintillators upon excitation by radioactive decay are then registered electronically.

- Streaked plates. Agar plates upon whose surface 0.1 or 0.2 ml of a bacterial suspension has been evenly spread with a sterile glass rod. The method is used to determine the number of colony forming bacteria (viable counts).

- Centrifugation, low speed. Centrifugal forces up to 5,000 times the gravitational force are employed. Practicable at room temperature.

- Centrifugation, high speed. Centrifugal forces up to 50,000 times gravitational force are employed. The rotor must be cooled to compensate for heating due to friction.

- Centrifugation, ultra. Centrifugal forces higher than 50,000 times gravitational force are employed. The rotor must spin under vacuum and must be cooled in order to avoid aerodynamic buoyancy and frictional heating.

B. Basic Equipment for the Experiments

The experiments were planned for groups of two students each. The following items of laboratory equipment are used in all the experiments. Hence they are not listed again in the "Material" section of each experiment.

1. Material for each Group

- 1 plastic bucket for used pipettes
- 1 plastic tub for used test tubes
- 1 stop watch for laboratory use
- 1 hand tally for counting colonies or plaques
- 1 bunsen burner with tubing and lighter
- 1 pipette aid, e.g., propipette
- 2 pairs of goggles
- 1 wooden block used as a stand for 2 inoculation loops
- 1 pair of pointed forceps
- 1 large-holed test tube rack, empty
- 1 can each with sterile 10, 1 and 0.1 ml pipettes
- 1 styrofoam container for ice
- 1 beaker (250 ml) to hold two glass rods, suitable for streaking
- 1 glass rod with fine polished ends, 4-5 mm diameter
- 1 crayon
- 1 pocket lens, approx. 6x magnification
- 1 screw-cap bottle with approx. 200 ml sterile phosphate buffer
- 1 wash-bottle with distilled water

2. Material for two Groups

- 1 counter for the enumeration of colonies and plaque plates
- 1 large-holed test tube rack, 2/3 filled with large sterile test tubes, and to 1/3 filled with small sterile test tubes (a total of 60-90)
- 1 box of soft absorbent paper for the cleaning of cuvettes, etc., e.g., Kleenex

1 dissecting microscope (magnification 5 to 10x)

1 water bath at 47°C with small-holed test tube rack (for soft agar layer tubes)

1 water bath at 30° or 37°C

1 spectrophotometer (e.g. the Bausch and Lomb (SPECTRONIC 20) with cuvette holder and 2 round cuvettes (diameter 1 cm)

1 microscope with phase contrast optics (magnification 10 × 40)

1 Petroff-Hauser counting chamber

C. Calculation of Titers and Some Statistical Methods

All studies in quantitative biology are made with the goal of obtaining results which are fairly representative. However, in most experiments one studies only a small sample. In order to make this procedure valid, samples should be taken randomly.

1. The concentration (titer) of colony-forming cells in a given bacterial suspension can be obtained by taking a random sample from the suspension, diluting it appropriately and spreading 0.1 ml of an appropriate dilution on nutrient agar. After overnight incubation the titer can be calculated from the colony-count by dividing the number of colonies found by the factor of dilution. Details of the calculation may be taken from the example given below.

Dilution	Sample	Colonies/0.1 ml	Statistical weight (arbitrary)
1×10^{-4}	1	560	1
	2	625	1
3×10^{-5}	1	138	0.3
	2	171	0.3
1×10^{-5}	1	45	0.1
		1,539	2.7

Titer: $1{,}539/2.7 \times (1 \times 10^{-4}) = 5.70 \times 10^{6}/0.1$ ml
5.70×10^{7}/ml

Bacterial titers calculated in this way become increasingly reliable by enlarging the size of the sample. The size of the sample is taken into account by the square root of the total number of colonies considered ($\pm \sqrt{N}$). The ratio $100/\sqrt{N}$ (%) = v expresses the error as percentage of the titer calculated. For more information see e.g. CAVALLI-SFORZA, 1969, p. 49 ff.

$\sqrt{1{,}539}/2.7 \times (1 \times 10^{-4}) = 39/2.7 \times 10^{-4} = 0.15 \times 10^{7}$/ml
Titer = $5.70 \pm 0.15 \times 10^{7}$/ml

$v = 100/\sqrt{1{,}539} = 2.6\% = 0.15/5.70$

The same procedure is also applicable to the determination
- of phage titers based on plaque counts on indicator plates.
- of cell titers based on microscopic cell counts.

2. Significance tests. If two or more random samples are taken from a larger group, the composition of the group from which each sample was taken can be predicted by the use of statistical methods. In order to determine whether two such groups are composed identically (e.g. whether two bacterial cultures contain an identical number of a certain mutant) the samples can be compared by a "significance test". By a similar statistical procedure one can decide whether the experimental results match the results predicted by a hypothesis.

a) Using the t- or STUDENT-test, one can calculate whether the difference between two measurements (x_1; x_2) is significant or not. The t-test can only be applied if the values to be compared represent random samples from two normal distributions of nearly the same variance. Instead of absolute values one may also use the arithmetic means ($\bar{x}_1$; $\bar{x}_2$) from each of a series of continuously or discontinuously varying values. (After CAVALLI-SFORZA, 1969.)

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s_D} \times \sqrt{\frac{z_1 \times z_2}{z_1 + z_2}} \qquad (1)$$

$\bar{x}$ = the arithmetic mean from several measurements (absolute frequencies), e.g. average number of colonies per plate

z = number of samples taken, e.g. number of plates

$z - 1$ = number of degrees of freedom

s_D = standard deviation of the difference $(\bar{x}_1 - \bar{x}_2)$

$$s_D = \sqrt{\frac{\Sigma(x_{i1} - \bar{x}_1)^2 + \Sigma(x_{i2} - \bar{x}_2)^2}{z_1 + z_2 - 2}} \qquad (2)$$

For t-values calculated according to Eq. (1), the corresponding P-value (level of significance) can then be taken from Figs. 2 and 3 (PÄTAU, 1943). For details see (2d).

b) The Chi-test can be used to investigate whether the difference between two empirically found percentage frequencies is significant or not. This test is only for discontinuous variables. Chi is equal to the difference between two single frequencies (p_1; p_2), divided by the square root of the sum of the squares of the standard deviations (s_1^2; s_2^2):

$$\text{Chi} = \frac{p_1 - p_2}{\sqrt{s_1^2 + s_2^2}} \qquad (3)$$

$p = M/N \pm s$

M = sum of elements of interest in a random sample, e.g., recombinants, mutants, etc.

N = sum of all elements in the same sample, e.g., mutants and non mutants.

s = standard deviation = $\sqrt{p\,(1 - p)/N}$ (4)

Standard deviations can be read directly from Fig. 1 (KOLLER, 1940). The procedure is as follows:

- If a point on the p-scale is joined by a straight line to a point on the N-scale, the middle scale will be intersected at the corresponding value s or 3s, resp. If this intersecting line rises less than the next neighboring "licence line", when observed from the N-scale, then the sample size N is too small for the frequency p. For example, when p = 3.0% then N must be ⩾500.

- If p is larger than 50% then the reciprocal value must be inserted.

For all Chi-values the corresponding P-values (level of significance) can be taken from Figs. 4 and 5 (PÄTAU, 1942).

c) With the Chi^2-test one can investigate whether absolute or relative frequencies of several events found in random samples (observed frequencies) deviate significantly from "theoretical" frequencies which are expected on the basis of a given hypothesis. The experiments No. 12 and 13 of this book include Chi^2-tests. P-values can be taken from Figs. 4, 5 and 6.

d) The level of significance, P, is a measure of the significance of the difference between an observation and a hypothesis. Consequently, only small P-values (⩽0.01) are really valuable, i.e. those indicating that the observed data do not agree with a given hypothesis. If P-values, however, are greater than 0.05 the hypothesis under consideration is neither excluded nor proven. The meaning of P can be best illustrated by an example: Let us say a certain experiment was performed once and a P-value of 0.003 was found. This means that the chance of again finding the same or a greater deviation between observation and hypothesis is 0.003 : 1 or 1 : 333.

Generally the following limits of significance are accepted:

P ⩽ 0.01	the difference is significant
P = 0.01 to 0.05	the difference is probable
P > 0.05	the difference is uncertain.

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