# Introduction to the Fluctuation test

ICB 5751 - The origins of Molecular Biology

Beny Spira
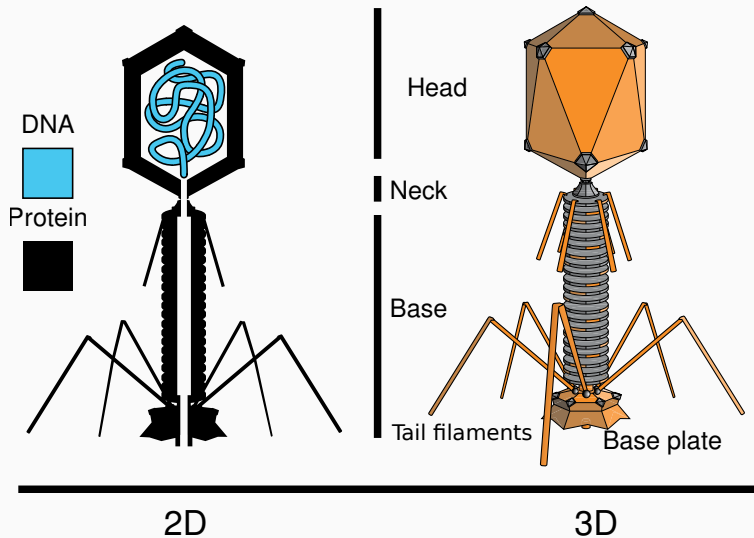
# MUTATIONS OF BACTERIA FROM VIRUS SENSITIVITY TO VIRUS RESISTANCE[1,2]
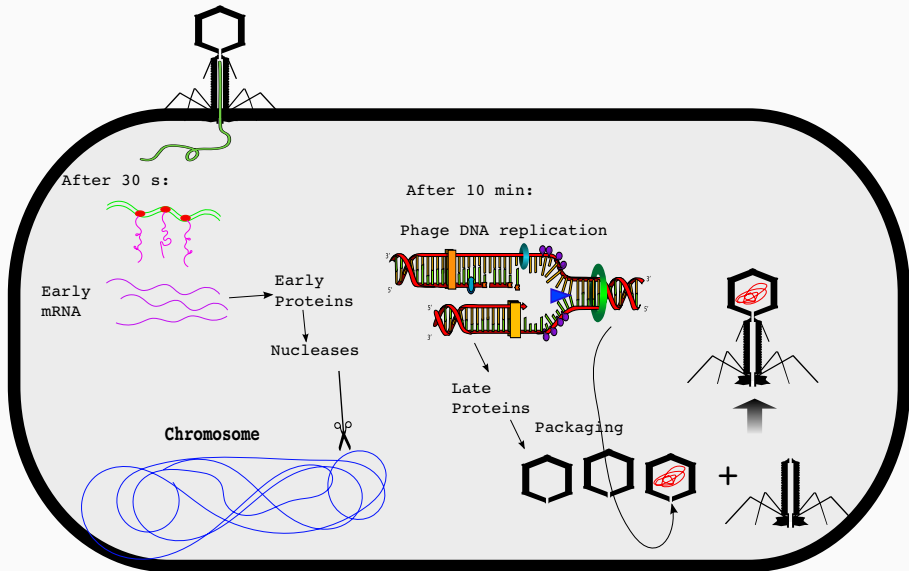
S. E. LURIA[3] AND M. DELBRÜCK

*Indiana University, Bloomington, Indiana, and*
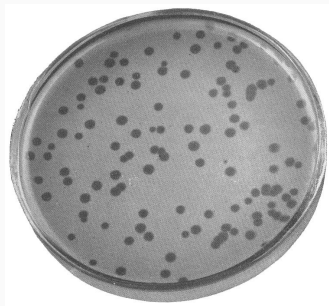*Vanderbilt University, Nashville, Tennessee*

# Bacteriophages or phages



DNA

Protein

Head

Neck

Base

Tail filaments

Base plate

2D

3D

# Phage T4: lytic cycle



After 30 s:

Early mRNA

Early Proteins

Nucleases

Chromosome

After 10 min:

Phage DNA replication

Late Proteins

Packaging

# Kinetics of infection



Plating after addition of $CHCl_3$

Phages per cell

Eclipse

Non-adsorbed phages

Time after infection
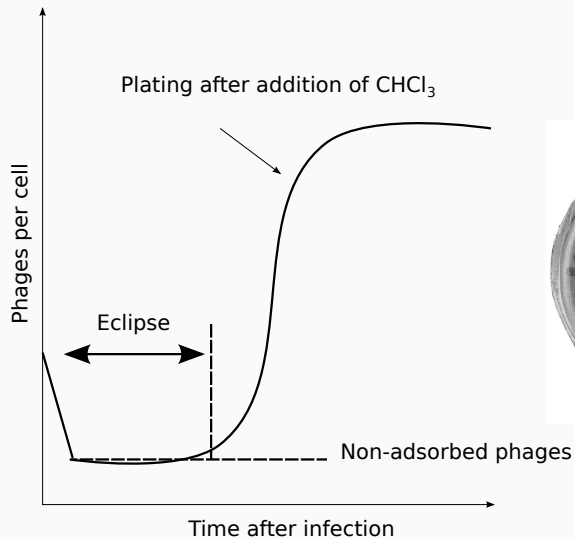
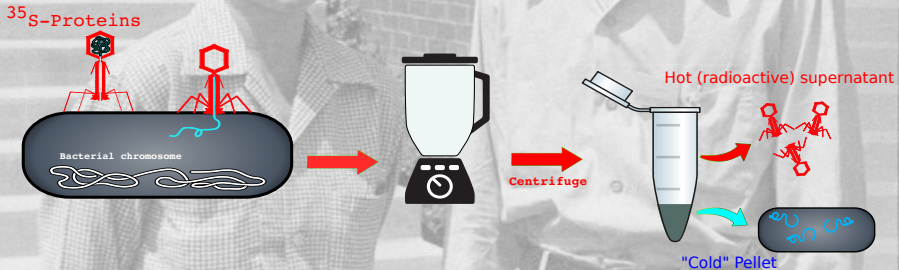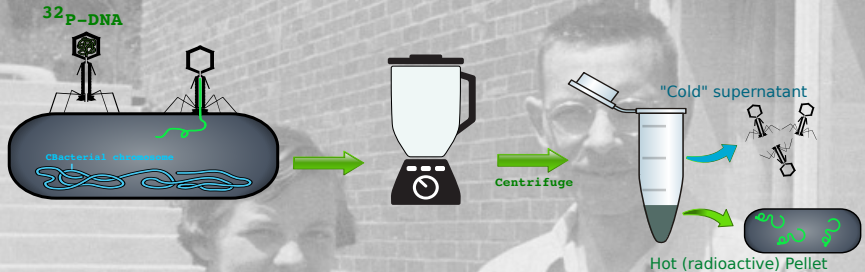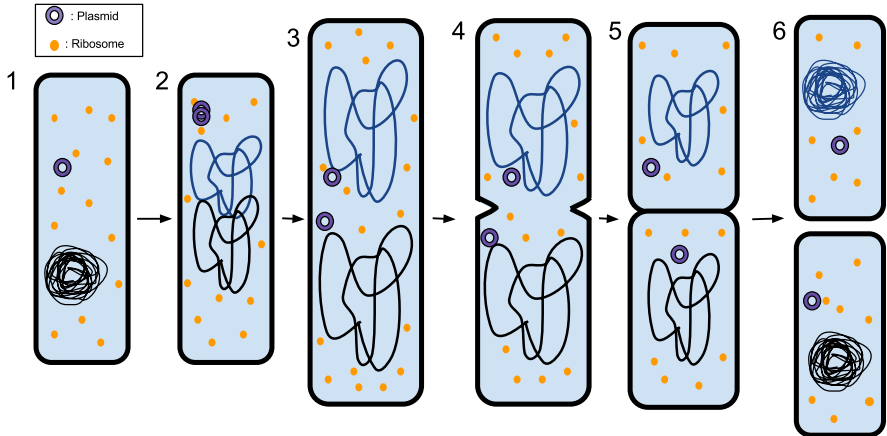# DNA injection

*The study of the growth of bacterial cultures does not constitute a specialised subject or a branch of research: it is the basic method of microbiology.*

*J. Monod*

*The dream of a bacterium is …*

$$2^0 \rightarrow 2^1 \rightarrow 2^2 \rightarrow 2^3 \rightarrow 2^4 \rightarrow 2^5 \rightarrow 2^6 \rightarrow 2^7 \rightarrow 2^8 \ldots$$

$$N = N_0 \times 2^n$$

$$\log N = \log N_0 \times \log(2^n)$$

$$\log N - \log N_0 = n \log 2$$

$$n = \frac{(\log N - \log N_0)}{0.301}$$

N = Final conc. of bacteria

$N_0$ = Inital conc. of bacteria

n = no. of generations

# Exponential growth

$$2^0 \rightarrow 2^1 \rightarrow 2^2 \rightarrow 2^3 \rightarrow 2^4 \rightarrow 2^5 \rightarrow 2^6 \rightarrow 2^7 \rightarrow 2^8 \ldots$$

$N = N_0 \times 2^n$

$\log N = \log N_0 \times \log(2^n)$

$\log N - \log N_0 = n \log 2$

$n = \frac{(\log N - \log N_0)}{0.301}$

N = Final conc. of bacteria

$N_0$ = Inital conc. of bacteria

n = no. of generations

# Exponential growth

$$2^0 \rightarrow 2^1 \rightarrow 2^2 \rightarrow 2^3 \rightarrow 2^4 \rightarrow 2^5 \rightarrow 2^6 \rightarrow 2^7 \rightarrow 2^8 \ldots$$

$$N = N_0 \times 2^n$$

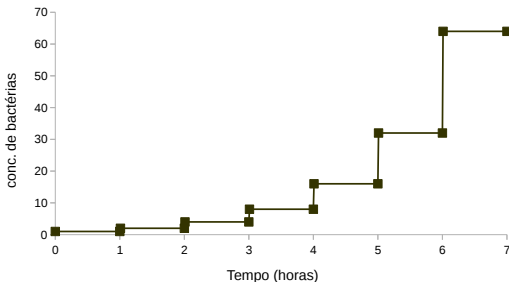$$\log N = \log N_0 \times \log(2^n)$$

$$\log N - \log N_0 = n \log 2$$

$$n = \frac{(\log N - \log N_0)}{0.301}$$

N = Final conc. of bacteria

$N_0$ = Inital conc. of bacteria

n = no. of generations

$$2^0 \rightarrow 2^1 \rightarrow 2^2 \rightarrow 2^3 \rightarrow 2^4 \rightarrow 2^5 \rightarrow 2^6 \rightarrow 2^7 \rightarrow 2^8 \ldots$$

$$N = N_0 \times 2^n$$

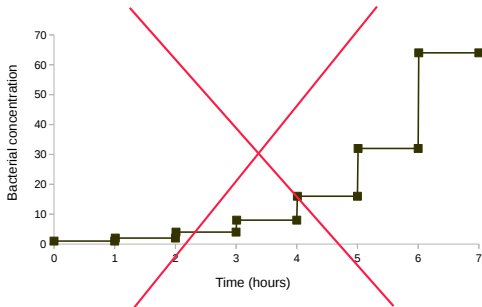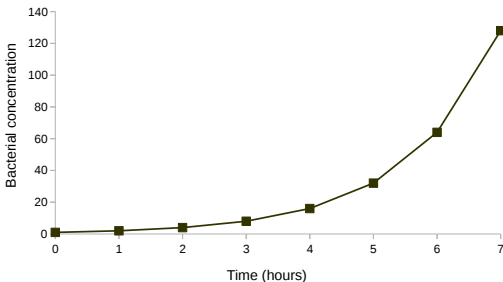$$\log N = \log N_0 \times \log(2^n)$$

$$\log N - \log N_0 = n \log 2$$

$$n = \frac{(\log N - \log N_0)}{0.301}$$

N = Final conc. of bacteria

$N_0$ = Inital conc. of bacteria

n = no. of generations

## Problems

1. Calculate the final concentration of bacteria in a culture in which $N_0=10^6$, $\mu$ =0.5 and T= 5 h.

2. Calculate the generation time of the bacterial cultures.

3. Calculate $\mu$ and g of a bacterial population which initial concentration is $5 \times 10^5$/ml and the final concentration is $5 \times 10^{10}$/ml in a 10 h period.

4. How many generations elapsed since the arrival of modern humans (200,000 years ago), assuming a generation time of 20 years?

# Problems

1. Calculate the final concentration of bacteria in a culture in which $N_0 = 10^6$, $\mu = 0.5$ and T= 5 h.

   N = 1,22 x $10^7$ bacteria

2. Calculate the generation time of the bacterial cultures.

   g = 1,39 h

3. Calculate $\mu$ and g of a bacterial population which initial concentration is $5 \times 10^5$/ml and the final concentration is $5 \times 10^{10}$/ml in a 10 h period.

   µ= 1,15 $h^{-1}$; g = 0,603 h

4. How many generations elapsed since the arrival of modern humans (200,000 years ago), assuming a generation time of 20 years?

   n = 200.000 years/20 years = 10.000 generations

# Graphical analysis of growth

Graphical analysis

## Detailed Definition

Mutations are alterations in the nucleotide sequence in the genome of an organism. Mutations are the result of unrecoverable lesions in the DNA (by radiation or chemical mutagens), or errors in the replication process, or the insertion or deletion of mobile genetic elements. Mutations may or may not cause phenotypical alterations.

## Nomenclature for genotypes and mutations (in prokaryotes)

- Gene names are always italicized or underlined. Example: *lacZ* or <u>lacZ</u>

- Δ= Deletion. Example: Δ*phoA*; Δ(*phoA-proC*)

- :: = Insertion. Example: *phoA*::Tn*10*

- Wild-type gene names are usually not written

- Wild-type strain: natural isolate of a species

- Auxotrophic strain: mutant with one or more nutritional deficiency

- Prototrophic strain: wild-type strain from which the auxotrophic mutant comes from
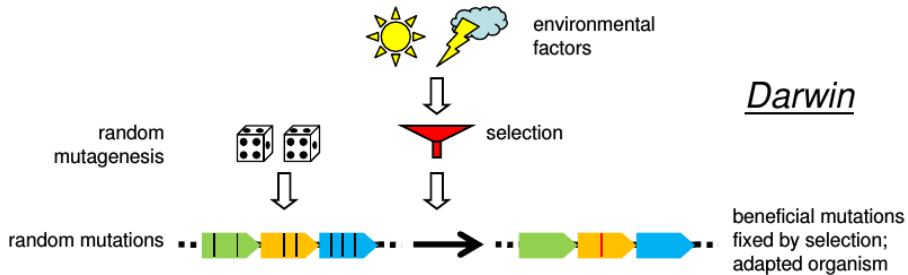
Adapted from Koonin and Wolf 2009

environmental factors

mutation-directing mechanism

beneficial mutations → adapted organism
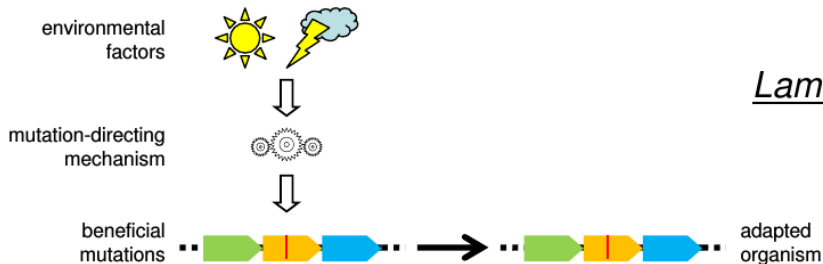
*Lamarck*

Adapted from Koonin and Wolf 2009

*Microbiology is the last bastion of Lamarckism.*

*Salvador Luria*

(A) Induced mutations
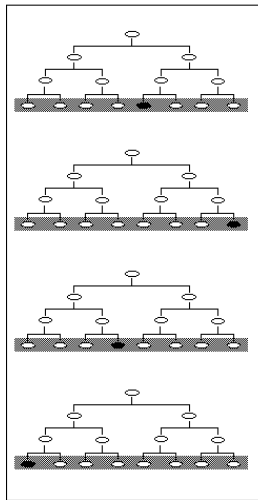
(B) Spontaneous mutations
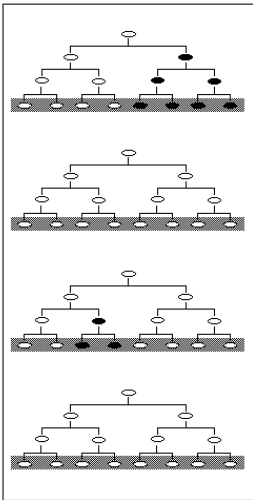
**Max Delbruck (1906-1981)**
**Salvador Luria (1912-1991)**

Luria S and Delbrück M. *Mutations of bacteria from virus sensitivity to virus resistance.* Genetics 28: 491, November, 1943

20 ml
*E. coli* T1$^S$

$2 \times 10^8$ bacteria/plate

2 x 10$^8$ bacteria/plate

0.5 ml   0.5 ml   0.5 ml   0.5 ml   0.5 ml   0.5 ml   0.5 ml   0.5 ml

$2 \times 10^8$ bacteria/plate

Jackpot
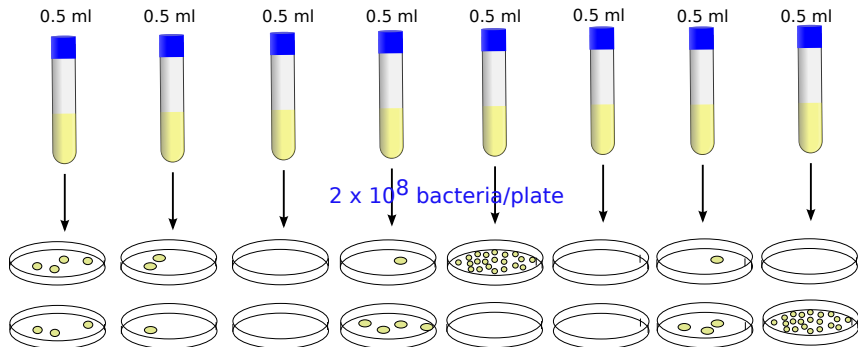
Jackpot
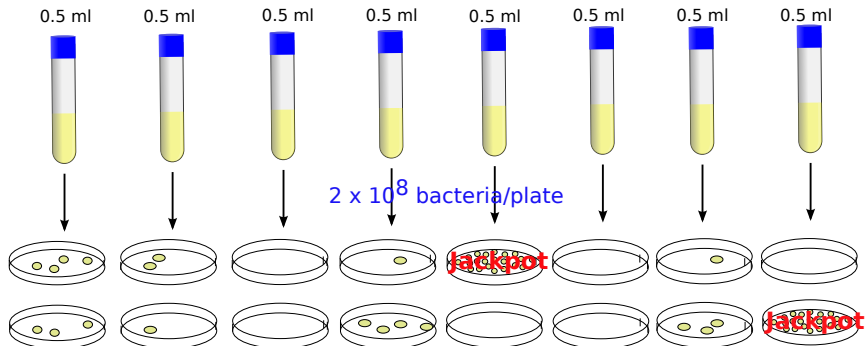
# Fluctuation test: Results

The number of phage T1 resistant *E. coli* mutants in a fluctuation test.

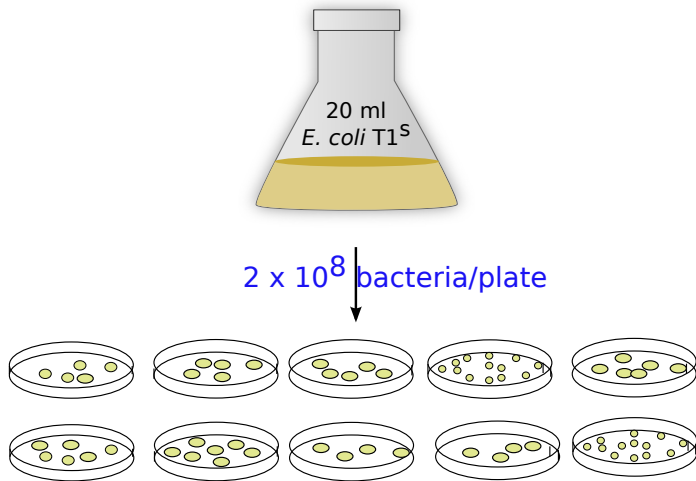| | Separate cultures | | Large culture | |
|---|---|---|---|---|
| | Culture number | # T1$^R$ mutants | Sample number | # T1$^R$ mutants |
| | 1 | 1 | 1 | 14 |
| | 2 | 0 | 2 | 15 |
| | 3 | 3 | 3 | 13 |
| | 4 | 0 | 4 | 21 |
| | 5 | 0 | 5 | 15 |
| | 6 | 5 | 6 | 14 |
| | 7 | 0 | 7 | 26 |
| | 8 | 5 | 8 | 16 |
| | 9 | 0 | 9 | 20 |
| | 10 | 6 | 10 | 13 |
| | 11 | 107 | | |
| | 12 | 0 | | |
| | 13 | 0 | | |
| | 14 | 0 | | |
| | 15 | 1 | | |
| | 16 | 0 | | |
| | 17 | 0 | | |
| | 18 | 64 | | |
| | 19 | 0 | | |
| | 20 | 35 | | |
| | | | | |
| mean | | 11.3 | | 16.7 |
| variance | | 694 | | 15 |
| variance / mean | | 60.8 | | 0.9 |

$$Mean = \frac{mut}{n}$$

mut = mutants per culture
n = no. of cultures

$$Variance = \frac{\Sigma(mut - mean)^2}{(n)}$$

Data from Luria, S. and M. Delbruck. 1943. Genetics 28: 491.

## Poisson binomial distribution

The Poisson distribution is a discrete probability distribution for the counts of events that occur randomly in a given interval of time (or space).
It applies when:

1. The event is something that can be counted in whole positive numbers;

2. Occurrences are independent, so that one occurrence neither diminishes nor increases the chance of another;

3. The average frequency of occurrence for the time period in question is known.

$$P(X|\lambda) = \frac{\lambda^x \cdot e^{-\lambda}}{X!}$$

e = 2,71828
X = number of events
λ = average number of events
P(X|λ) = probability of occurring X events knowing that the mean is λ

# Poisson distribution

1. A radioactivity monitor counts 4 particules per milisecond. What is the probability that 6 radioactive particles would enter the monitor at a given milisecond?

   R: $P(X = 6|\lambda = 4)\frac{4^6 \cdot e^{-4}}{6!} = 0.10422$

2. On average a mutation occurs at every 1000 replications of E. coli chromosome. What is the probability that not a single error would occur after 5000 replications?

   R: $P(X = 0|\lambda = 5)\frac{5^0 \cdot e^{-5}}{0!} = 0.00674$

## Quantifying mutations

### Mutant Frequency

$$f = \frac{no.\ de\ mutantes}{N}$$

N = Number of cells in the population

### Mutation rate (*Luria-Delbrück*)

$$a = \frac{m}{N}$$

$$m = \frac{no.\ de\ mutac\tilde{o}es}{cultura} = -\ln \frac{(no.\ de\ culturas\ sem\ mutac\tilde{a}o)}{(no.\ total\ de\ culturas)}$$

N = number of cell divisions/culture

The equation above is based on the Poisson distribution: $P_i = \frac{m^i \cdot e^{-m}}{i!}$

$Pi$ = probability of the culture having $i$ mutations

$m\ (\lambda)$ = mean of mutations per culture

The easiest way to find out $m$, is to determine the frequency of 0 events:

$$P_0 = \frac{m^0 \cdot e^{-m}}{0!} = \frac{1 \cdot e^{-m}}{1} = e^{-m} \rightarrow m = -\ln P_0$$

## Exercise

1. A scientist conducted a fluctuation test to find out whether mutations in the *rpoS* gene are spontaneous or directed by an external trigger. Forty out of 50 cultures containing each $10^7$ bacteria were found out to be sensitive to stresses. Assuming that those sensitive bacteria underwent mutations in rpoS calculate the mutation rate.

$m = -\ln(\frac{10}{50}) = 1.6$

$a = \frac{m}{N} = \frac{1.6}{10^7} = 1.6 \cdot 10^{-7}$ mutations/generation

## A transcendental formula for mutation rate (Eq. 8)

$$r = \mathbf{a} \cdot N_t \cdot ln(N_t \cdot C \cdot \mathbf{a})$$

$r$ = average of mutants (among all cultures)

$a$ = mutation rate

$N_t$ = number of bacteria in each single culture

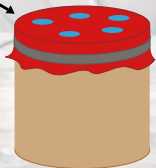$C$ = number of cultures

How to solve a transcendental equation?

# Replica plating



non-selective plate

Replica on selective plates
(suplemented with phages

Offset of all
colonies

Resistant colonies

incubation

## Mutations in *E. coli*

Knowing that:

- there are $10^{14}$ bacteria in the adult intestine; 0.1% of them belong to the species *E. coli*
- Rate of spontaneous mutations: $10^{-10}$ nucleotides/generation
- *E. coli* genome: $5x10^6$ bp = $10^7$ nucleotides
- ∴ 1 mutation for every 1000 genome replications

1. If each bacterium replicates once a day, how many new bacteria of the species *E. coli* would emerge each day?
   R: 100 billion bacteria/day
2. How many mutations occur in bacteria of the species *E. coli* per day per colon (person)?
   R: 100 milhões de mutações/dia

# Max Delbrück

- Born in Berlin in 1906, son of the famous historian *Hans Delbrück*
- Studied astronomy, but in 1926, after attending a seminar by *Erwin Schrödinger* (in which *Einstein* was present), and despite the fact that he did not understand a word, decided to study quantum physics
- His PhD supervisor was *Max Born*, Delbrück worked in Kopenhagen under *Niels Bohr*, in Zurich with *Wolfgang Pauli* and in Germany with Lise Meitner, but realized that all great discoveries in this field have already been done
- With the Russian geneticist *Nicolai Timoféeff-Ressovsky*, that have been studying radiation-induced mutations and the biophysicist *K. G. Zimmer*, wrote the first article (theoretical) about gene structure: "*About the nature of the gene mutation and the gene structure*" (1935). There he described the "Delbrück model of the gene"
- In 1937 moved to Caltech to work with *Thomas Morgan*, but soon realized that he will not find out the basic laws of genetic replication working with Drosophila. A simpler organism was required

## Max Delbrück

- Under crisis because he thought he has failed, met *Emory Ellis*, who introduced him to the study of phages. Delbrück immediately adopted phages as a study model. Published his first paper on the quantitation of phage reproduction (1939)
- In December 1940 he met *Salvador Luria*
- Together with Luria and Alfred Hershey, started the "Phage Group", and from 1945 onwards, the famous "Phage Course" at CSH.
- The central idea of the Phage group was to study the nature of the gene by using the most simple known organism as a model
- At that time the laws of genetics and evolution were applied only to sexual organisms. It was not known if bacteria and phages have genes
- With *Luria* devised the famous "Fluctuation test", which served two purposes: (1) to prove that mutations are spontaneous and random with respect to their outcome and (2) to create a method for measuring mutation rate
- Delbrückian philosophy:
    - *Max* thought (correctly) that the "gene" must be a molecule
    - Following his mentor's logic- *Niels Bohr*, he (wrongly) expected to uncover new physical laws that might explain biological phenomena
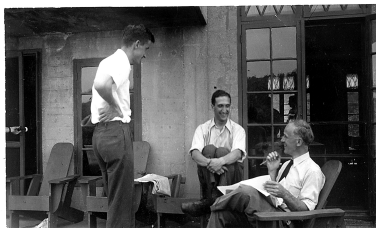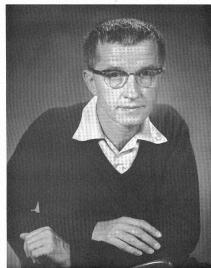
## Salvador Luria

- Born *Salvatore Luria* in 1912 in Turim into an Italian Jewish family
- Studied medicine, graduated in 1935. Specialized in radiology and moved to Rome
- Joined *Enrico Fermi*'s group, where he studied physics for a year
- Have come across radiobiology and the Delbrückian idea about gene structure (gene = molecule)
- In parallel Luria came across phages. Pioneered the idea that they would be the perfect organism for the study of *Delbrück*'s theory
- In 1938 ran away to Paris. When the Nazis invaded France, in 1940, escaped by bicycle to Marseille, and from there to the USA

## Salvador Luria

- Met Max in 1940
- In 1941 published, together with the biophysicist *Thomas Anderson*, the first phage micrographies
- In 1943 was hired by the University of Indiana. This same year also published the Fluctuation test with *Delbrück*
- His first PhD student was James Watson
- Besides the Fluctuation test, he made two more great contributions:
  - Luria was the first to observe the phage restriction phenomenon
  - He also found out that two phages killed by UV radiation can be reactivated upon entering the same bacterium and going through recombination
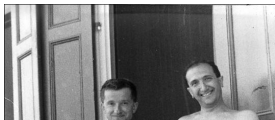
Max Delbrück
(1906 - 1981)

Alfred D. Hershey
(1908 - 1997)

Salvador E. Luria
(1921 - 1991)

## Quotes

*Suppose [an] imaginary physicist, the student of Niels Bohr, is shown an experiment in which a virus particle enters a bacterial cell and 20 minutes later the bacterial cell is lysed and 100 virus particles are liberated. He will say: "How come, one particle has become 100 particles of the same kind in 20 minutes? That is very interesting. Let us find out how it happens! How does the particle get in to the bacterium? How does it multiply? Does it multiply like a bacterium, growing and dividing, or does it multiply by an entirely different mechanism ? Does it have to be inside the bacterium to do this multiplying, or can we squash the bacterium and have the multiplication go on as before? Is this multiplying a trick of organic chemistry which the organic chemists have not yet discovered ? Let us find out. This is so simple a phenomenon that the answers cannot be hard to find. In a few months we will know. All we have to do is to study how conditions will influence the multiplication. We will do a few experiments at different temperatures, in different media, with different viruses, and we will know. Perhaps we may have to break into the bacteria at intermediate stages between infection and lysis. Anyhow, the experiments only take a few hours each, so the whole problem can not take long to solve."*

*[Eight years later] he has not got anywhere in solving the problem he set out to solve. But [he may say to you] "Well, I made a slight mistake. I could not do it in a few months. Perhaps it will take a few decades, and perhaps it will take the help of a few dozen other people. But listen to what I have found, perhaps you will be interested to join me."*

*Max Delbrück*

## Quotes

*I have often noticed in later years that biologists are readily intimidated by a bit of mathematics laid before them by chemists or physicists. It was one of the blessings of my too short stay among physicists to be immunized against mathematical humbug.*

*S.E. Luria*

*I have deeply regretted that I did not proceed far enough at least to understand something of the great leading principles of mathematics, for men thus endowed seem to have an extra sense.*

*Charles Darwin*