Outline

1. Zen of Screen vs Selection
2. Mutation Rate

Question:

"*In this post-genomic era, why should we have to learn about classical bacterial genetics, classical phage biology, or the historical papers describing principles we can now get in a kit?*"

Finding and Analyzing Mutants.

"A selection is worth a thousand screens." David Botstein

"You get what you select for, but you don't know what you selecting for until you get it." Tom Silhavy."

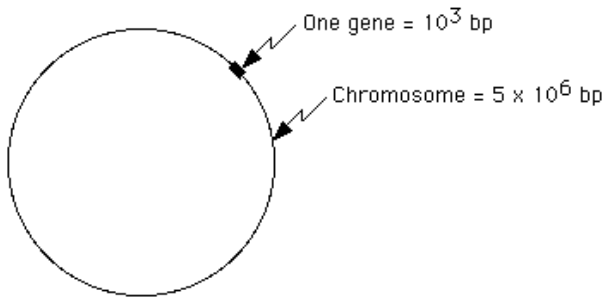
"It is better to be a mutant than to be dead."
Colin Manoil

Difference between selection and screen?

A Selection is a growth condition that allows for the selective propagation of genetically marked cells.

A Screen is a growth condition where both mutant and wild type are able to grow but can be distinguished phenotypically

What is the probability of a isolating a mutant in one gene on the chromosome?



How many mutants would you need to look at to find the one you want?
-if the average gene is 10^3bp and the genome is $5x10^6$, your chances would be 1/5000 or you need to look at 5000 colonies.

However, not all mutations result in a phenotype (what would these be?).
About 90% of base changes are silent. Therefore:
1/5000 x 1/10: $1/5x10^4$
-1 in 50,000 would be the one you want!
Divide this by 250 colonies per plate: 200 plates to find one mutant!

Nature Genetics June 2003, p420

Table 1 | **Common selections and phenotypic screens in bacteria**

Method	Result	Example
Selection		
Growth on various carbon sources (such as lactose, maltose and galactose)	Expression of wild-type genes that encode catabolic pathways	Isolation of Mel$^+$ revertants of lacO^o (see main text)
Prototrophy, growth in the absence of further biosynthetic intermediates (such as amino acids, nucleotide precursors and vitamins)	Expression of wild-type genes that encode biosynthetic pathways	Reversion of *his* auxotroph
Resistance to phage	Loss of function or decreased expression of gene that encodes phage receptor	Identification of receptor for bacteriophage-λ
Resistance to antibiotic	Loss-of-function mutation in gene that encodes transporter; missense mutation in gene that encodes antibiotic target; up regulation of efflux system; gene that encodes inactivating enzyme	Decreased outer-membrane permeability for β-lactams; resistance to streptomycin alters ribosomal protein S12; plasmid encoded tetracycline exporter; plasmid encoded aminoglycoside phosphotransferases
Resistance to metabolic analogues	Loss of function in gene that encodes transporter; missense mutation in gene that encodes target enzyme	(See main text)
Phenotypic screen		
Ability to ferment sugars, (such as lactose, maltose and galactose), MacConkey, tetrazolium and so on;colour changes indicate the amount of acid production from catabolism	Various colour changes indicate the overall degree of fermentation, correlated to levels of gene expression	(See main text)
Chromogenic substrate (such as Xgal)	Formation of coloured product correlated to levels of hydrolytic enzyme (such as β-galactosidase)	(See main text)
Light production from luciferase	Formation of light correlated to levels of luciferase enzyme reporter	Identification of quorum-sensing systems using *lux* genes
Fluorescence from green fluorescent protein (GFP)	Intensity indicates level of *gfp* gene expression; intracellular location can indicate specific localization signals	Identification of promoters that are activated during intracellular growth of bacterial pathogens

Why do selections?

A brute force approach to genetics would be to assay individual colonies for a particular phenotype. Suppose each assay could be done in one second. How long would it take to assay a culture of overnight bacteria for a mutant?

$$10^9\ assays \times \frac{1\ \text{sec}}{assay} \times \frac{1\ hr}{360\ \text{sec}} \times \frac{1\ day}{24\ hr} = 11574\ days = 31.7\ yr$$

$$10^8\ assays \times \frac{1\ \text{sec}}{assay} \times \frac{1\ hr}{360\ \text{sec}} \times \frac{1\ day}{24\ hr} = 1157.4\ days = 3.17\ yr$$

$$10^7\ assays \times \frac{1\ \text{sec}}{assay} \times \frac{1\ hr}{360\ \text{sec}} \times \frac{1\ day}{24\ hr} = 115.74\ days$$

$$10^6\ assays \times \frac{1\ \text{sec}}{assay} \times \frac{1\ hr}{360\ \text{sec}} \times \frac{1\ day}{24\ hr} = 11.57\ days$$

Selections are a good thing!

Why use bacteria and phage and yeast to study molecular genetics?

- **They grow fast! *E.coli* and *Salmonella* will double every 20-25 minutes in rich media with good aeration.**
- **Phage will grow just about as fast**
- **Qualitative methods can be employed to measure population size, phage particle number, and morphology of individual colonies/plaques**
- **Relatively small genomes**
- **Haploid genomes (not yeast)**
- **Growth on defined media possible, thus allowing isolation of mutants unable to grow-defines metabolic/catabolic pathways**

Another reason to use bacteria to study genetics in bacteria:

Processes described in bacteria often have close homologues in eukaryotes such as DNA replication and repair are good examples. Many other examples, such as basic transcription, translation, etc.

The biotechnology field stands on the shoulders (if they had them) of the bacterial factory.

C. Why is it an advantage to work with bacteria in culture to study complex genetic systesm?

1. -Bacterial cultures reach large homologous populations, greater than 10^9 cells per ml (E.coli and Salmonella).

2. Large populations sizes are needed to measure changes in the appearance of mutants. Do you think a flask of E.coli growing on shaker remains genetically static? Roberto Kolter.

3. Large populations let you identify rare events, like mutation rate.

D. Bacteria and phage offer genetically tractable systems to study complex processes. Can you think of a bacteria that has no genetic system? Hint: pathogens-which one?

E. Finally, understanding how bacterial pathogens respond to the host environment, regulate virulence genes, and evade host defenses is important to combat disease.

1. The tools developed in the early days of bacterial and phage genetics have allowed the near complete genetic dissection of some very important pathogens.
2. Many of the more clever genetic techniques to identify genes utilized by pathogenic bacteria were developed using old fashioned techniques.
3. Some bacteria cannot be culture in vitro and have not genetic systems. However some surrogate model systems have been developed. <u>Can you think of one?</u>

Type of Mutants we will be discussing and you are familiar with in the laboratory section.

1. **Conditional lethal mutants**. These are the class of mutations in genes that are needed for normal, vegetative growth. Examples: DNA and RNA polymerase, ribosomal RNA, etc. The cell cannot function without these genes.

2. **Catabolic mutants.** These mutants are unable to break down more complex energy sources to grow. Examples would those mutants unable to grow on arabinose, galactose, maltose. What is special about these sugars?

3. **Metabolic mutants.** This class of mutants are unable to synthesize compounds needed to grow. Examples: aroA-a mutation in the first step to make aromatic amino acids such as tryptophan and histidine. This class of mutants are also known as auxotrophic mutants. How could you tell the difference between these classes of mutants?

4. **Temperature sensitive mutants**. This type of mutation is generally refers to a change in the amino acid composition of a protein. This change alters or abolishes its function at a particular temperature. The secondary and tertiary structure of the protein is altered by the mutation such that at lower temperatures, the protein is functional. At higher temperatures, the structure of the protein changes with accompanying loss of function. Examples, Hsp60-heat shock protein. A particular point mutation in Hsp60 allows for cellular growth only at 25°C, but not at 37°C. How would you go about making such mutants? What would a leaky mutation mean in this context?

What is the difference between mutation rate and mutation frequency?

Mutation Rate: the number of mutations per cell division. Because the number of cells in the population is so large, the number of cell divisions is approximately equal to the number of cells in the population (N). Therefore, the value of “h” can be determined by a fluctuation test.

Mutation Frequency: is the ratio of mutants/ total cells in the population. This is measured by plating out the cell on selective and non-selective media and counting the number of mutants per culture. This method is easier, but may show large fluctuations depending on when the first mutation arose in the population.

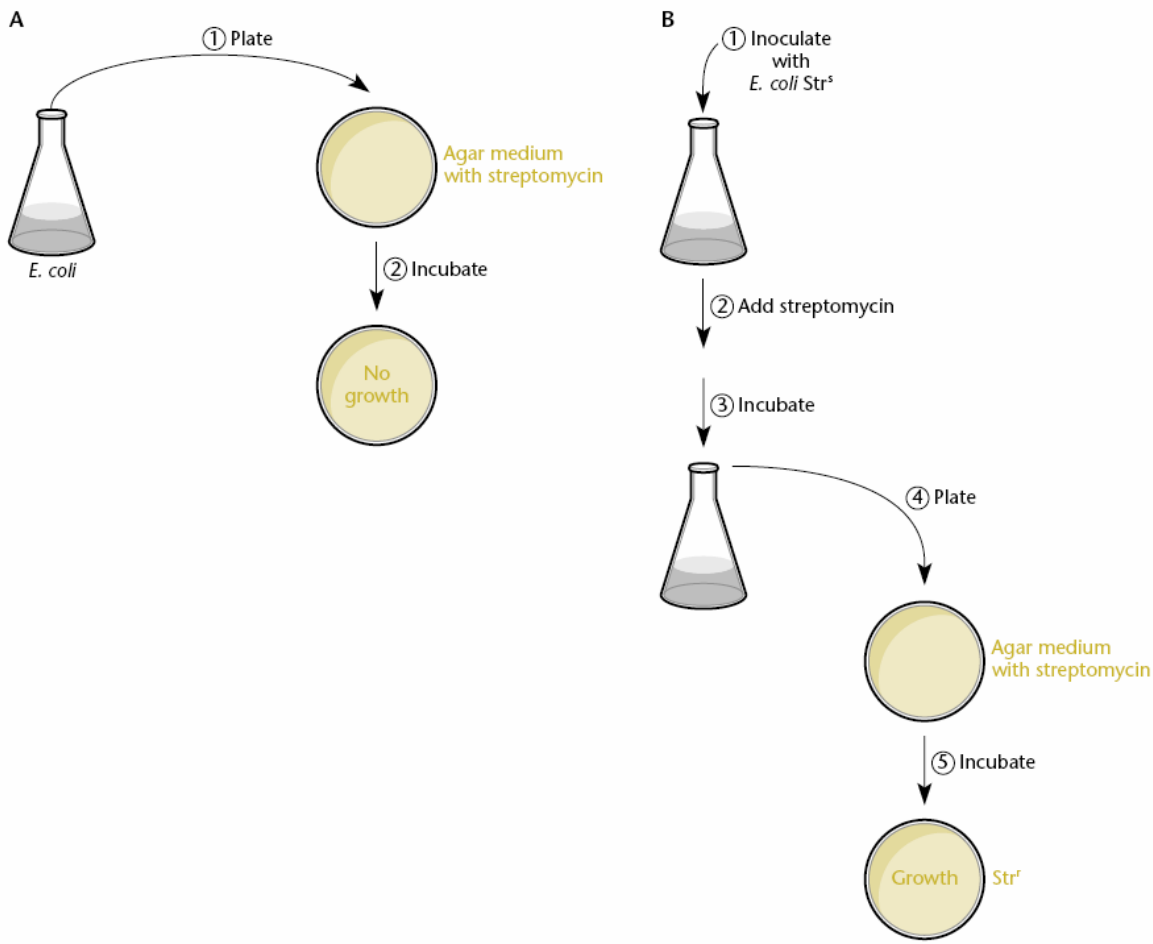
Two theories of mutation in 1943: how did mutants arise in a culture of bacteria

1. Adaptation theory-resistant mutants occurred only as a specific physiological adaptation to a selection. Resistant mutants were not present prior to the application of the selection. The is Lamarckian Inheritance.

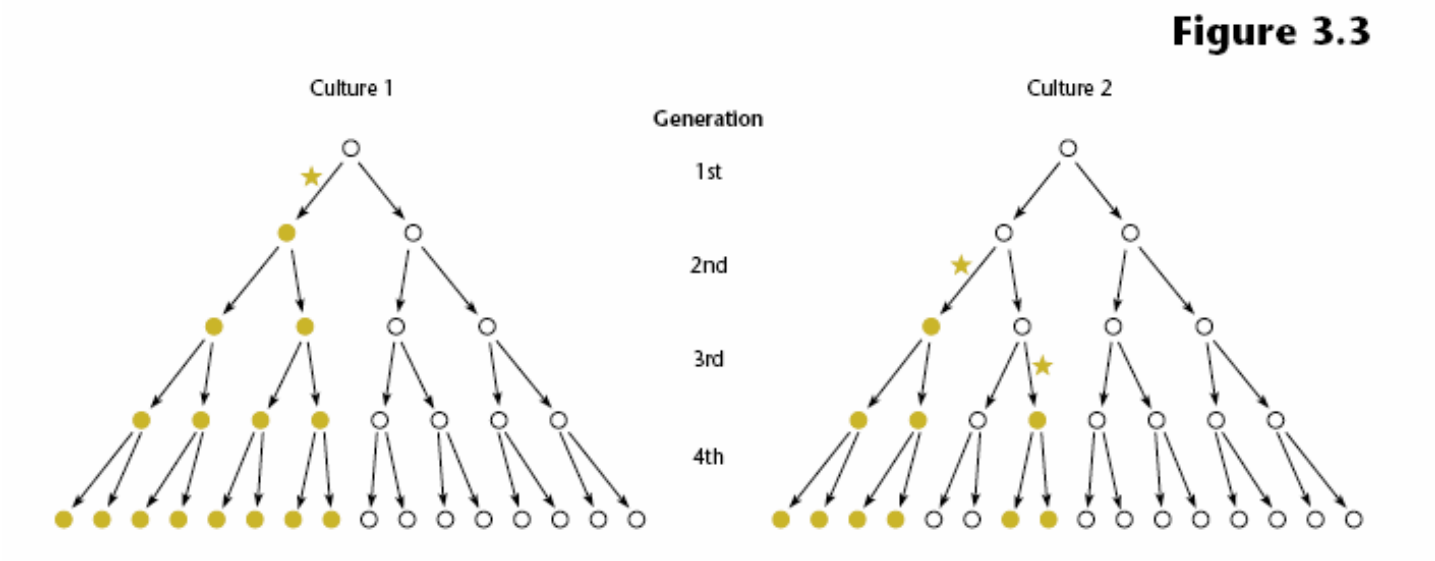
2. Mutation theory-resistance is due to random genetic mutation and resistant mutants were present before the selection The is “neo-Darwinian” Inheritance

Luira Delbruck experiment Genetics 28(1943), p491

Figure 3.2

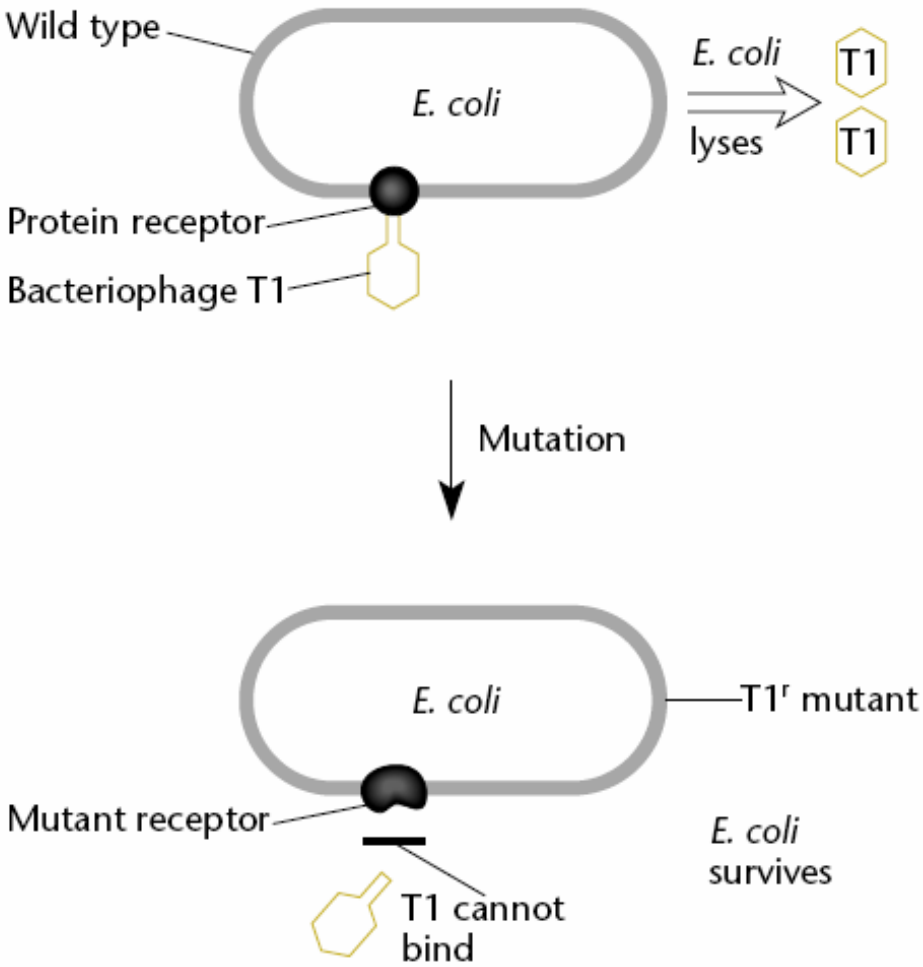


Directed change: the compound directly causes the resistance to strep. Strep resistant bacteria would only occur in the presence of the antibiotic.



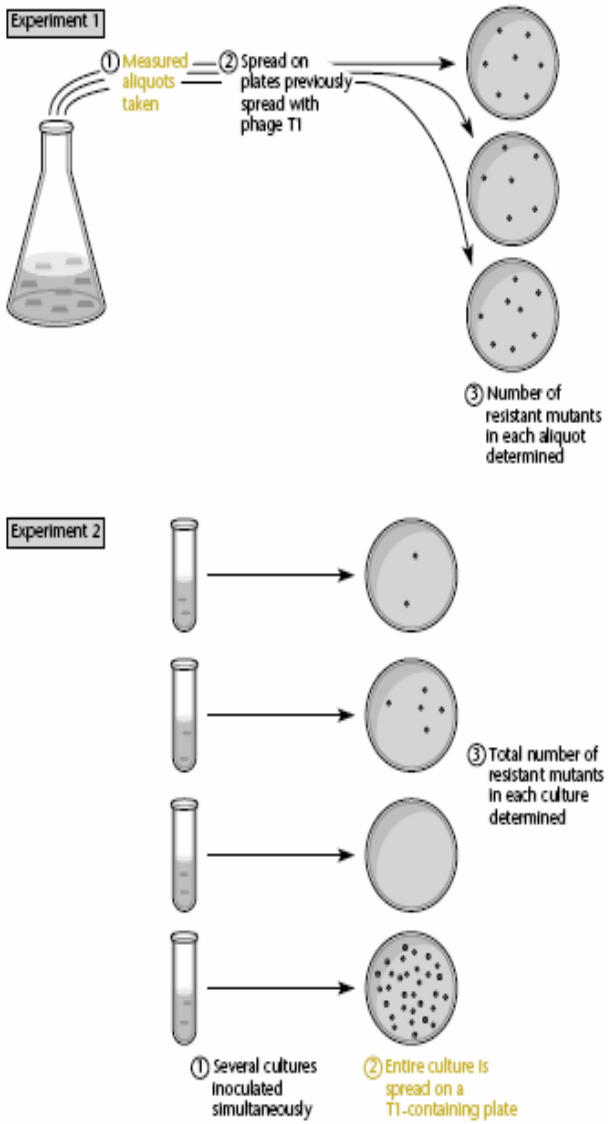
Random Mutation: the mutation can occur early or late in the growth cycle of the culture. Depending on when the mutation occurred would determine the number of mutants in the culture. Here is the problem, how do you tell when the mutation occurred and at what frequency?

Figure 3.4


Wild type
E. coli
E. coli
lyses
T1
T1
Protein receptor
Bacteriophage T1
Mutation
E. coli
T1r mutant
Mutant receptor
E. coli
survives
T1 cannot
bind

Fluctuation Test: Luria and Delbruck

Figure 3.5



If the ton^r mutation arose by exposure to T1, then there would be an equal number of mutants on all plates. In other words, there is equal probability of mutation to ton^r in all culutres. Differences in numbers of mutants isolated could be attributed to sampling error.

If mutation to ton^r is spontaneous and occurred before exposure to T1, then the number of ton^r mutants isolated from each culture should be different from tube to tube.

Table 3.2

TABLE 3.2 The Luria and Delbrück experiment

Experiment 1		Experiment 2	
Aliquot no.	No. of resistant bacteria	Culture no.	No. of resistant bacteria
1	14	1	1
2	15	2	0
3	13	3	3
4	21	4	0
5	15	5	0
6	14	6	5
7	26	7	0
8	16	8	5
9	20	9	0
10	13	10	6
		11	107
		12	0
		13	0
		14	0
		15	1
		16	0
		17	0
		18	64
		19	0
		20	35

Mean: 16.7
Variance: 15
Variance/Mean: 0.9

Mean: 113
Variance: 694
Variance/Mean: 60.8

In either of these two cases, if multiple samples from a single culture of bacteria were plated on phage T1, each of the resulting plates should yield approximately the same number of colonies. However, the two possibilities can be distinguished mathematically by comparing the mean and variance of the number of the number of mutants in each culture:

$$Mean = \frac{(m)}{(n)} \quad \text{and} \quad Variance = \sum_{x=1}^{n} \frac{(m - Mean)^2}{(n-1)}$$

where m = Number of mutants per culture and n = number of cultures. If approximately the same number of resistant mutants are obtained on each plate as with multiple samples from a single culture or as predicted by the directed-mutagenesis hypothesis, the mean should be approximately equal to the variance. In contrast, if there is large variation in the number of mutants per plate, the mean will be much less than the variance.

Mutation Rate Calculations:

To calcuate the mutation rate, you need to know the number of cell divisions.

-generally, one starts with an known number of cells and dilutes them into a known volume of media giving a known number of cells/ml

-at the end of the experiment, the number of cells are determined by plating on nutrient media, no selection.

$A=m_2-m_1/N_2-N_1$

$M_{2:}$ =mutant number at the end, M_1-mutant number at the beginning

N_2=number of cells at the end, N_1= number of cells at the beginning

M= -ln(number of tubes with 0-mutations/total number of tube)

Luria-Delbruck results:

M= 11/20 11 tubes had no mutations

A=m/n =0.59/10^7cell divisions/tube=

6x10^{-8} mutations/tube

Sample problems

Measuring resistance to streptomycin is a classic way to determine mutation rate. To determine the frequency of StrR mutants a fluctuation test was done with 50 tubes each containing 10^8 cells and 42 of the tubes contained no mutants. Use the Luria-Delbruck calculation to determine the mutation rate to StrR.

ANSWER:

First calculate the average number of hits per cell

$m = -\ln (42/50) = -\ln(0.84) = 0.17$

Then divide the average number of hits per cell by the number of cells in the population

$a = m / N = 0.17 / 10^8 = 1.7 \times 10\text{-}9$

Real life experiment: Stanley Maloy's laboratory. The Maloy lab studies the function of the *put* genes, genes needed to transport proline needed for carbon and nitrogen utilization and osmotic balance. To determine the frequency of putP mutants a fluctuation test was done using 20 tubes with a final concentration of 10^7 bacteria each. From each tube 0.1 ml of culture was plated on medium that selects for putP mutants. Seventeen of the tubes yielded putP mutants but 3 of the tubes yielded no mutants. Based upon these results, use the Luria-Delbruck calculation to determine the mutation rate to putP-.

ANSWER:

First calculate the average number of hits per cell

m = -ln (3/20) = 1.9

Then divide the average number of hits per cell by the number of cells in the population

a = m / N = 1.9 / 107 = 1.9 x 10-7

Suggest two reasons why the rate of mutation to StrR is so much less than the rate of mutation to Pro-.

ANSWER: One reasonable explanation is that any mutation that disrupts any of the proline biosynthetic genes would result in a Pro- phenotype, but only very specific base substitutions in ribosomal genes result in streptomycin resistance (i.e., Str resistance is a smaller target size for mutations) -- this is the actual reason. A second potential reason could be that there are redundant genes that encode the wild-type Str sensitive phenotype and the Str resistant mutant phenotype is recessive to the wild-type.

Fitness of Mutations/Mutants. Can some mutations be deleterious?

By definition, if you can isolate a mutant, it is not lethal. However, some mutations render the cell less fit than wild type. **How would you determine this?**

Competitive Index. This is a measure of how well the mutant can compete with wild type in the same culture condition mixed at a ratio of 1:1.

1. Two strains, wild type and mutant, are grown to the same density, diluted and inoculated into fresh media at a ratio of 1:1. The mutant is marked with an antibiotic marker so it can be identified.
2. At various times, an aliquot is removed from the culture, diluted, and plated onto media with and without antibiotics.
3. Count the total number of bacteria growing on non-seletive media
4. Count the total number of bacteria growing on selective media only-this is the number of mutant bacteria in the culture

$$\text{CI:} \quad \frac{\text{Ratio of mutant/wild type recovered}}{\text{Ratio of mutant/wild type input}}$$

What would a ratio of 1 mean?

What would a ratio of .00001?

Ames Test for Mutagens

Bruce Ames used a *his* and a *trp* mutant of Salmonella Typhimurium as a bioassay for DNA damage.
-treat bacteria with compound
-plates on minimal plates and look for frequency of colonies able to grow
-the revertants are the result of DNA damage/repair

Several different types of his mutants are used to test for different classes of mutagens -- for example, frameshift mutagens will revert a frameshift mutation in *his* or *trp*. The Salmonella his mutants used have three additional properties that make them more sensitive to mutagens.

1. They have a rfa mutation that makes the outer membrane more permeable to large molecules.
2. They have a mutation that deletes the uvrB gene, to eliminate excision repair of DNA damage.
3. They carry the plasmid pKM101 which increases error-prone repair of DNA damage. Thus, reversion of the his mutations in these strains provides a sensitive test for a broad spectrum of mutagens.

These strain sets are commercially available and used widely today.