وراثة الاحياء المجهرية

العملي

Bacterial Genetics هي دراسة كل ما يتعلق بجينوم البكتريا كنموذج لفهم التضاعف والاستنساخ والتعبير الجيني وطرق انتقال المعلومات الوراثية بين الخلايا البكتيرية افقيا او عموديا وكيفية عزل الطفرات في المختبر

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Estimation the amount of DNA in *E.coli* by Burton method

The most important components of living cells are DNA , RNA and Ribosomes

DNA and RNA consist of nucleotides , the nucleotides have three major particles which include:

1- Base pairs (A,G,C)(T in DNA while U in RNA).
2- Deoxy Ribose in DNA and Ribose in RNA.
3- Phosphate group.

The nucleotides bind with each other by phospho diester bonds which represents the backbone of DNA and RNA.

DNA has a major role in carrying the genetic informations between organisms through generations and any change in the sequence of nucleotides leads to change in the phenotype of organisms .this change is called (mutation).

Procedure:

1- Prepare overnight culture of *E.coli* in Nutrient broth and incubate for 18 hrs.(the bacteria in log phase).
2- Take 10 ml of the growth and inoculate it in 50 ml of new Nutrient broth , this growth is considered the zero time of growth .the growth then incubated for different periods in shaker incubator in 37˚C.
3- Take 7.5 ml from the previously growth and add 2.5 ml from Perichloric acid (P.A) (1N) in a clean test tube
   Why do we add P.A. (1N) to the bacterial growth ?
4- Put the mixture in ice bath at 0 ˚C for 15 min this step is important because :-
   A. To reduce the heat generated from the addition of P.A. (1N).
   B. Freezing leads to precipitate the cell and DNA (after opening the cells).
5- Centrifuge the mixture for 15 min (3000 rpm) , the supernatant is discarded, while the DNA will be in the pellet in the tubes.
6- Add 2 ml of the P.A (0.5 N) to the pellet and mixed well , then put the mixture in water bath 70˚C for 15-30 min {P.A (o.5 N) breaks down the glycosidic bonds and the water bath speeds up the reaction and melts DNA}.
7- Centrifuge the mixture for 15 min (3000 rpm) , the DNA will be in the supernatant and other components of cell will settle down in the pellet .

8- Add Burton reagent "1 volume of the mixture from the previous step with 2 volumes of the reagent ''.
The blank is prepared from 4 ml of the reagent and 2 ml from P.A. (0.5 N).

9- Incubate the mixture and the blank for 18 hrs. at 37˚C, O.D. is measured at 600 nm, the colour of the mixture will change to greenish blue and the colour density depends on DNA concentration .

10- Draw a curve which represents the relationship between time and values of O.D.
O.D. will increase by time according to the increase of bacterial growth hour after hour, which leads to the increase of DNA concentration.

The Components of Burton:-

1- **Diphenyl amine** :- reacts with deoxy ribose sugar to produce greenish blue colour.
2- **Glycial acetic acid** :- break down hydrogen bonds between base pairs .
3- **H2SO4** :- breaks down the phosphor diester bonds in the nucleotides among the single strand of DNA (strong oxidant).
4- **Acetaldehyde** :- A co-factor that increases the reaction between deoxy ribose sugar and reagent and fixes the colour of the mixture .

The Principle of Reagent work:-

A. The main idea for this method depends on the reaction between deoxy ribose sugar "resulted from the broken DNAl'' and Burton reagent.
B. The principle of this reaction is that diphenyl amine in the reagent will react with deoxy ribose sugar which leads to greenish blue colour

The Preparation of Burton:-

1- Take 1.5 gm from Diphenyl amine and solve it in 100 ml of glycial acetic acid ,then 1.5 ml from the concentrated H2SO4 is added to the mixture .
2- Store the mixture in the dark (to prevent oxidation by the light)
3- 0.5 ml of acetaldehyde is added to 100 ml of the mixture to reach the final concentration of Burton.

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Standard curve of DNA

The aim of this curve is to estimate the amount of DNA which is extracted from *E.coli* through each hour from the previous experiment , this can be done by measuring the O.D. of standard gradient concentration of DNA.

The procedure:-

1- Prepare stock solution of DNA with 500 µg/ml by taking 25mg from powder DNA and solve with 50 ml of P.A. (0.5 N).
25mg/ml x 1000 = 25000 µg/ml
Stock (500 µg/ml) = 25000/50 = 500 µg/ml

2- Prepare gradient solution of DNA (25 ⟶ 200) from this original stock solution , P.A. is used with final volume up to 2 ml (apply this formula)

Concentration	C1V1 = C2V2	Take from stock	Complete it with
25	**500 x V1 = 25 x 2**	**0.1**	**1.9**
50	**500 x V1 = 50 x 2**	**o.2**	**1.8**
75	**500 x V1 = 75 x 2**	**0.3**	**1.7**
100	**500 x V1 = 100 x 2**	**0.4**	**1.6**
125	**500 x V1 = 125 x 2**	**0.5**	**1.5**
150	**500 x V1 = 150 x 2**	**0.6**	**1.4**
175	**500 x V1 = 175 x 2**	**0.7**	**1.3**
200	**500 x V1 = 200 x 2**	**0.8**	**1.2**

3- Add 4 ml of Burton reagent to each 2 ml of the previous concentration in the tubes.

4- Mix the tubes properly and put it in water bath in 100 ˚C for 10 min., cool tubes, then take O.D. by using spectrophotometer measuring 600 nm.

5- It is also important to use blank for measuring the O.D. in the spectrophotometer.

6- Draw the curve that visualize the relationship between O.D. and standard gradient concentration of DNA , then point the values of O.D. of previous experiment on the curve to obtain the unknown concentration for each O.D. after each hour .

Standard DNA		Test DNA		
Con. µg/ml	O.D.values	Hr.	O.D.	Con. µg/ml
25		0		
50		1		
75		2		
100		3		
125		4		
150		5		

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Estimation of RNA amount in E.coli

Types of RNA

1- **mRNA** :- The main function is to transfer the genetic information from DNA to the synthesis place of protein in living cell(ribosomes).

2- **tRNA** :- it is called transfer RNA because it has a role in arranging amino acids on the strand of mRNA , there are 20 essential amino acids in living cell.

3- **rRNA**:- it is called ribosomal RNA , because it builds the ribosomal structure with nucleoproteins and also has a role in protein synthesis in cytoplasm .

The Ribosome :- it is cytoplasm nucleoprotein molecules which has a role in protein synthesis by translating the transcript information from the DNA to protein particles.

Ethanol – Orcinol method

1- prepare the overnight growth of E.coli in nutrient broth at 37˚C for 18 hours.
2- Take 10 ml of growth and centrifuge it with cooling centrifugation at 4 ˚C for 15 min .with 3000 rpm.
3- Take the pellet and suspend it with 9 ml of saline citrate solution (0.15 M)
4- Add 1ml of P.A. (2.5 N) to the mixture.
5- Take 2 ml from the mixture above and add 3 ml of orcinol reagent (2:3 volume).
6- Prepare the blank (2 ml P.A. + 3 ml orcinol reagent)
7- Put the tubes in the water bath in 90 ˚C for 20 min. and leave it to cool then take O.D. with 660 nm.

Notes :-

1- **Why do use the cooling centrifuge instead of normal centrifuge ?**
To keep RNA particles far away from the effect of heat which will be produced in due to the centrifugation , since the RNA is unstable particle.

2- **What is the main benefit of P.A. (2.5 N)?**
Is to precipitate the proteins and other components of the living cells also contribute in breaking the bonds between nitrogen bases and phosphate groups that leads to increase the speed of reaction between the sugar and the reagent.

3- **What is the main benefit of SCS?**
SCS is a buffering reagent (PH=7).it keeps the primary structure of RNA, because the RNA particle is ionizable molecules.

4- **Why do you use the water bath in 90 ˚C?**
It helps to increase the reaction between ribose and orcinol reagent .the heat leads to increase the breaking of RNA bounds and release the ribose .

5- **What is the principle of orcinol ?**
It is indicator for the presence of ribose sugar which indicates also for the amount of RNA . the reaction between the sugar of RNA and the reagent produce green colored complex, the density of color reflect the amount of RNA.

As the following formula:

Ribose + acid-orcinol $\longrightarrow$ furfural

Furfural + FeCl3 $\longrightarrow$ Green complex at 600 nm

The components of orcinol reagent

1- **$FeCl3.6H_2O$ Solution** : prepared by solubilizing it in HCl
2- **Ethanol-Orcinol Solution** : prepared by solubilizing 6 gm from orcinol in 100 ml of absolute alcohol , save in dark because it is oxidized by the light so it loses its activity .
3- **The orcinol reagent** : prepared by adding 100 ml of the solution one and 35 ml of the solution two the solution color changes into the yellow and keep it far away from the light .
4- **SCS solution** : contain both NaCl and Tri sodium citrate.

Spontaneous Mutations

The mutation is divided as general into two main categories:

1- Spontaneous Mutations
2- Induced Mutations

Spontaneous Mutations

It is an auto mutation which occurs in the nature without the participating of the human and it occurs because of either physical and chemical reasons.

1- **physical reasons**: it represents by displaying to different types of rays or change in the temperature ,pH , pressure or other.
2- **Chemical reasons**:it represents by displaying to products of industry like pesticide , smocking, food preservatives …..etc.

Gradient Plate Method for isolation of the spontaneous mutation

This method is considered as simple and easy for the primary detection of Spontaneous Mutation

Procedure:-

1- Prepare nutrient agar ,sterile it and pour it into sterile plates as average 15 ml in each plate (half the plate) . after that plates are slopped by using any book edge ,leave it till it gets solidified.
2- Prepare another nutrient agar and sterile it then cool it to 45 ˚C .add the chosen antibiotic at concentration of 500 µg/ml to the media and move the flask to in a circle way to mix the antibiotic properly with the media .(foam should be avoided).
3- The (nutrient agar plus antibiotic) is poured over the previous solidified slopped nutrient agar until the plate is full and the media reaches to a straight line .
4- After the plates are solidified , they are inoculated with *E.coli* by taking 0.2 ml from the original growth and spread it by using a spreader over the whole surface of the media . incubate the plates in 37 ˚C for 24 hrs.

Discussion the results

1- If there is no growth , this means that the bacteria do not have the ability to resist the antibiotic in this concentration .

2- If there is full growth , this means that the bacteria have the ability to resist the antibiotic in this concentration.

3- If the growth only appears in the region of low concentration of antibiotic in the plate , this means that the bacteria can only resist the low concentrations of this antibiotic.

In this case loop is used to touch single colony and move it towards the high concentration region of the antibiotic.
Incubate the plate for 24 hrs. , then if there is any growth , the colony is transferred again to the higher concentration region , then watch the results.

A- If there is no further growth , this mutation is called (single step mutation).
single step mutation :- is a mutation in which the bacteria can resist limited concentration of the antibiotic but cannot grow on higher concentrations.

B- If there is any further growth, this mutation is called (multi steps mutation)
multi steps mutation :- is a mutation in which the bacteria can grow in concentration higher than the original concentration (it was isolated from in the first place)but in gradient way.

What are the main benefits of the gradient method?

1- The possibility to identify the approximate concentration for bacterial resistance.
2- The possibility to test more than one type of bacteria in the same plate and for the same antibiotic ,by growing the bacteria in parallel manner to insure that all types of bacteria will be exposed to the same concentration of antibiotic .
3- We can identify , the types the types of antibiotic and the bacterial resistance and the types of mutation whether is it a single or mulrti step mutation .

What are the disadvantages of the gradient plate method ?

1- There is no possibility to get fix the exact mutation , it is always an approximate manner , as an example the mutation grow till half the plate , we can say it resists half the concentration of the used antibiotic.
2- There is no possibility to account the mutation frequency in this method.

The Method of Preparation of Antibiotics Solutions

We should prepare a stock solution of antibiotic before adding it to the media . important notes should be taken in our mind during preparing the stock solution.

1- **Types of antibiotic in this case we have capsule antibiotic.**
2- **The final concentration of antibiotic**
3- **The volume of the used media**
4- **The amount of antibiotic solution that will be added to the media.**

Because the media used in this experiment is solid (nutrient agar) we must be careful not to add excess amount of antibiotic solution to the media because this will dilute it and it will not be solidified in a proper way ,therefore for each 50 ml of the media , the amount of antibiotic solution added should not be more than 0.6 ml .

Example 1:

Prepare (50 ml) of nutrient agar that contains Erythromycin 250 mg with final concentration is 500 µg/ml?

250 mg × 1000 = 250.000 µg

We suppose to dilute the powder in 5 ml of D.W. to prepare stock solution of that antibiotic

250.000/5 = 50.000 µg/ml the conc. Of the stock solution

C1 × V1 = C2 × V2

50.000 × V1 = 500 × 50

V1= 0.5 ml take this amount from the stock solution and add it to 50 ml of the media .

Ex .2:- prepare (50 ml) nutrient agar that contains Ampicillin 150 mg with final concentration is 400 µg/ml?

150 × **1000 = 150.000** µg

We suppose to dilute the powder in 5 ml of D.W. to prepare stock solution of that antibiotic

150.000 / 5 = 30.000 µg/ml the conc. Of the stock solution

C1 × V1 = C2 × V2

30.000 × **V1= 400 × 50**

V1= 0.66 ml we cannot add this volume because it is more than 0.6 ml so we will dilute the powder in 3 ml of D.W. to increase the concentration of the stock solution.

150.000 / 3 = 50.000 µg/ml the conc. Of the stock solution

C1 × V1 = C2 × V2

50.000 × **V1= 400 × 50**

V1= 0.4 ml take this amount from the stock solution and add it to 50 ml of the media .

Note :- if we want to prepare nutrient agar media with volume 100 ml for example then we calculate it according to the fact that for each 50 ml of media we need not more than 0.6 ml of antibiotic solution so for 100 ml we need not more than 1.2 ml of antibiotic solution and so on .

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Induced Mutations

Induced Mutations : the mutations which are occurred by human intervention using mutagens which have the ability to alter the genetic constitution of living cells including physical and chemical mutagens.

Physical mutagens : by using ultraviolet or X-ray , temperature or others.

Chemical mutagens : including many types of chemicals such as nitrous acid (HNO2) , mustard , Acridines ,ethyl methan and others.

Nitrous acid HNO2:- it is considered as one of the most widely used mutagens , used in research field for the isolation of the induced mutations for different purposes .this acid causes substitution mutations in nitrogen base with other .it is obvious that this acid can be produced by sodium nitrite salt(NaNO2) reacts with the water (H2O) .

Procedure:

1- Nitrous acid preparation by dissolving sodium nitrite in sodium acetate buffer and adjusting the pH value (pH=4.6) which is important for the reaction.

2- Collect 5 ml of *E.coli* growth in nutrient broth over night for 18 hrs. then centrifuged .the pellet will be taken and the supernatant will be neglected .

3- Resuspend the pellet by adding 5 ml of sodium acetate buffer then 0.3 ml of Nitrous acid (0.05M) for three treatment times which are zero time , 15 min , 30 min. in three different tubes while the forth tube will contain only the bacterial growth without addition of the acid and used as a control tube .

4- When the specified time is finished ,5ml of A- medium must be added to each tube to stop the activity of nitrous acid.

   A-medium: composed of the following salts (K2HPO4 , KH2PO4 , NH4PO4) with sodium citrate.

5- Centrifuge the tubes and take the pellet adding 10 ml of nutrient broth to it then incubated for 18 hr. to detect mutations

6- Culture 0.2 ml of two dilutions for the treated bacterial growth with nitrous acid and for the non treated original growth as a control on

Nutrient agar plates containing specific antibiotic with known concentration for isolation of mutations and incubated to the next day.

- **Mutations of the untreated cells considered as spontaneous Antibiotic – resistance mutations while the treated cells represents the induced mutations).**

Results

Results can be determined by calculating the viable cell count and the mutation frequency for both the treated and non-treated cells

We suppose that the V.C. for the untreated cells higher than that of the treated cells but the mutation frequency for the untreated cells will be less than the treated cells .

- **No. of dead cells = V.C. of the untreated cells - V.C. of the treated cells**
- **Killing rate = (No. of dead cells/ No. of the living cells of growth) *100**
- **No. of mutations (1ml) = no . of mutations * 5……(**if we take 0.2 ml inoculums)

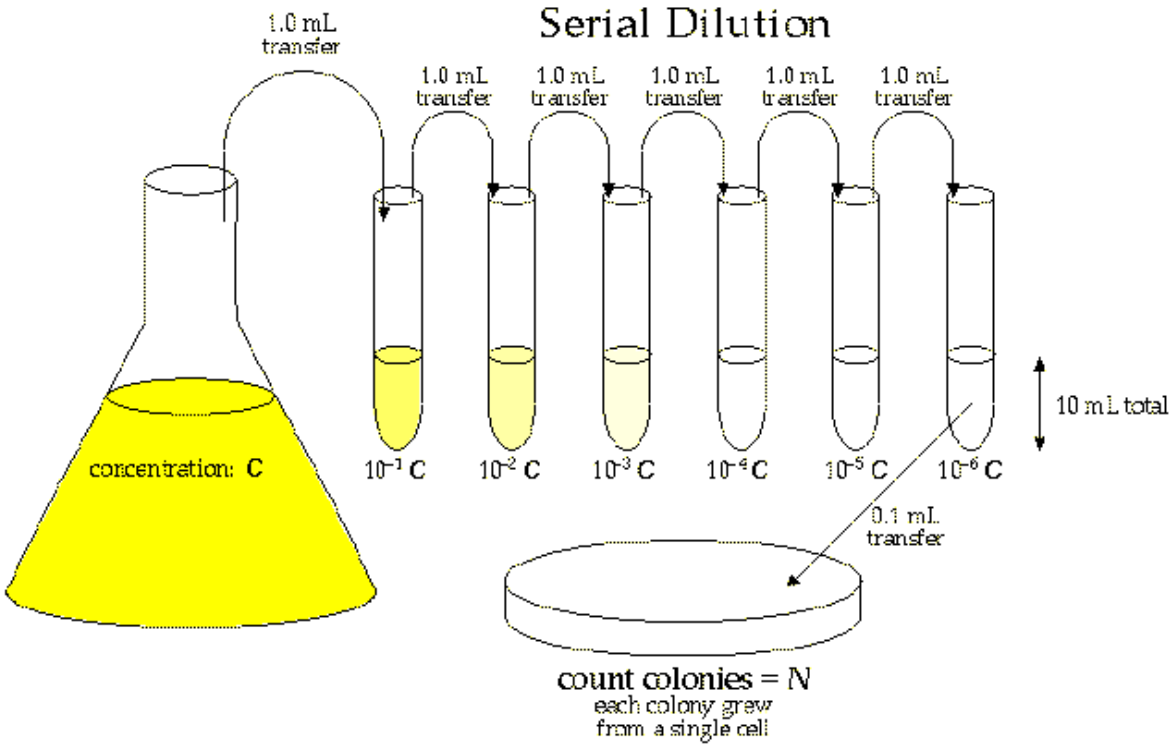
Calculation of mutation frequency by getting one single mutation for limited number of cells as in the following example:

1- 25 mutation for 190×10^9 cell/ml ……. 25/25 for 190×10^9/ 25 = **1 mutation for each 7.6×10^9 cells.**

2- 45 mutation for 190×10^9 cell/ml ……. 45/45 for 190×10^9/ 45 = **1 mutation for each 42×10^9 cells.**

3- 60 mutation for 190×10^9 cell/ml ……. 60/60 for 190×10^9/ 60 = **1 mutation for each 35×10^9 cells.**

So 1 mutation for each 7.6×10^9 cells is more frequently than the others for this antibiotic concentration because it happens in less number of living cells and vice versa



Serial Dilution preparation

Transformation

Is the genetic alteration of a cell resulting from the uptake , integration(incorporation) and expression of exogenous genetic material (Naked DNA) that is taken up through the cell wall.

Transformation occurs most commonly in bacteria and in some species it happened naturally during limited period of their growth.

Bacteria that are capable of being transformed are called competent cells

Artificial competence for bacteria that are unable to be transformed naturally in the environment such as E.coli .is induced by lab procedures and involves making the cells passively permeable to DNA by exposing it to specific conditions .

Transformation is also used to describe the insertion of new genetic material into non bacterial cells including animal and plant cells(genetic engineering)

Mechanism

Artificial competence

Bacterial transformation may be referred to as a stable genetic change brought by the uptake of **Naked DNA** (DNA without associated cells or proteins) and competence refers to " the state of being able to take up exogenous DNA from the environment"

Calcium Chloride transformation is a method of promoting competence .by chilling the cells in the presence of divalent cations such as Ca^{+2} (in Cacl2) prepares the cell membranes to become permeable to plasmid DNA ,the cells are incubated on ice with the DNA and then slightly heat shocked by exposing the cells to water bath (42 ˚C for 30-120 sec.) this allowing the DNA to enter the cells.

Frederick Griffith'S transformation experiment 1928

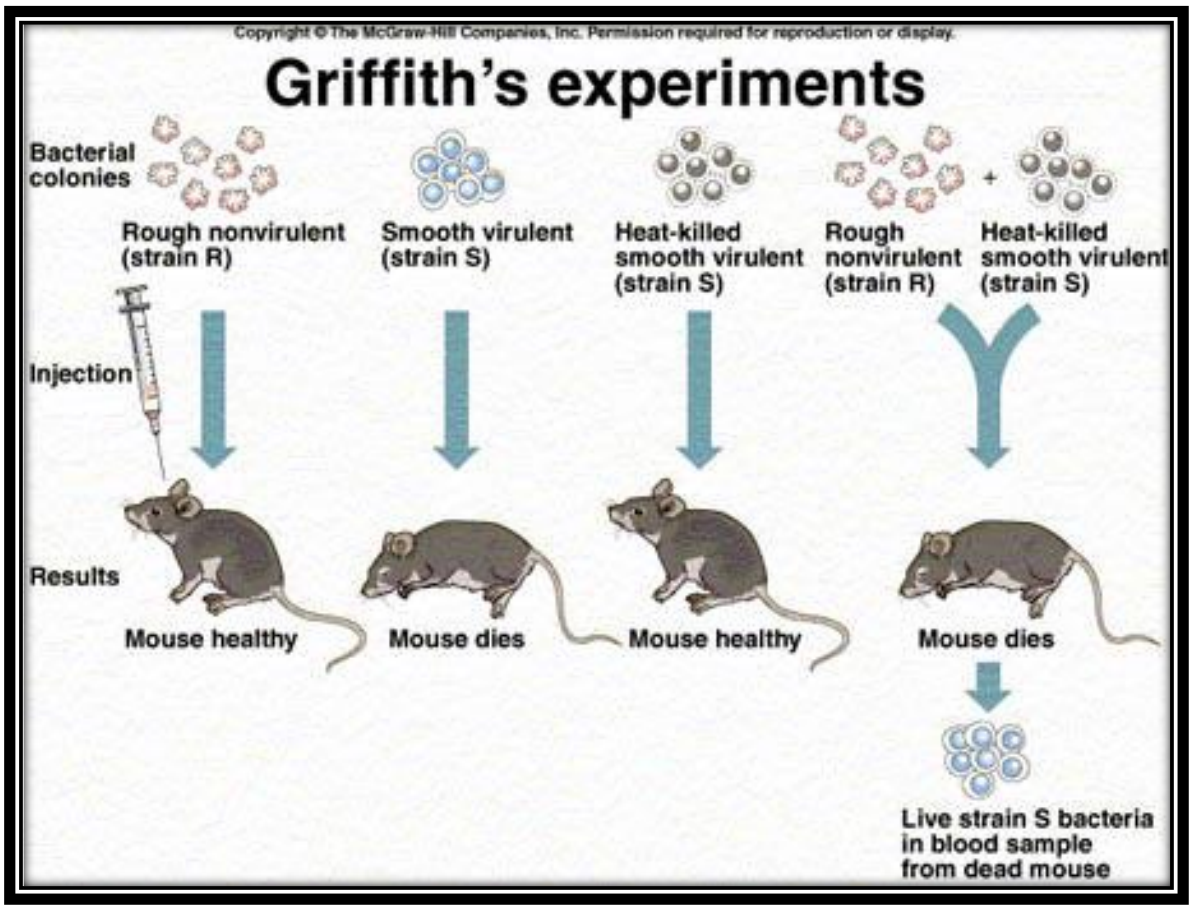
Two strains of Streptococcus pneumonia

1. Smooth

- Secrete a polysaccharide capsule
- Produce smooth colonies on solid media
- Virulent (caused a fatal bacteremia in mice.

2- Rough

- Unable to secrete a capsule
- Produce colonies with rough appearance
- A virulent (it did not have a pathological effect on mice)



Procedure

1- Prepare over night culture from
   a. E.coli sensitive to ampicillin (suspension I)
   b. E.coli resistant to ampicillin (suspension II)

2- lyse suspension 2 by incubation in water bath for 15 min 70 ˚C .

3- Take 3 ml of suspension 1 and centrifuge for 3000 rpm for 10 min. then add 2.5 ml of Cacl2 to pellet and keep it for 10 min. this step is required to prepare competent cells .

4- Mix (1 ml) from suspension 1 and 2 then put the mixture at 0 ˚C for 20 min.

5- Transfer the mixture to water bath at 42 ˚C for 90 sec. then again transfer it to ice bath at 0 ˚C for 2 min. to make shock in cell wall of suspension 1 (competent cells) and receive the DNA.

6- Incubate the mixture for 15 min. at 37˚C then add it to ampicillin agar plate with 300 µg/ml and incubate for 24 hrs. at 37 ˚C.

7- Discuss the results.

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Conjugation

Bacterial Conjugation : is the transfer of genetic material between bacterial cells by direct cell to cell contact or by a hair-like projection. bacterial conjugation is often regarded as the bacterial equivalent of mating since its involves the exchange of genetic material.

F- factor:

The conjugative plasmid is the F-plasmid ,Or F- factor .there can only be one copy of the F-plasmid in a given bacterium ,either free or integrated ,bacterial cells possess a copy are called F-positive (F^+) or

F-donor. Cells that lack F-plasmid are called F-negative (F^-) or recipient cells .

Hfr (High frequency of recombination):

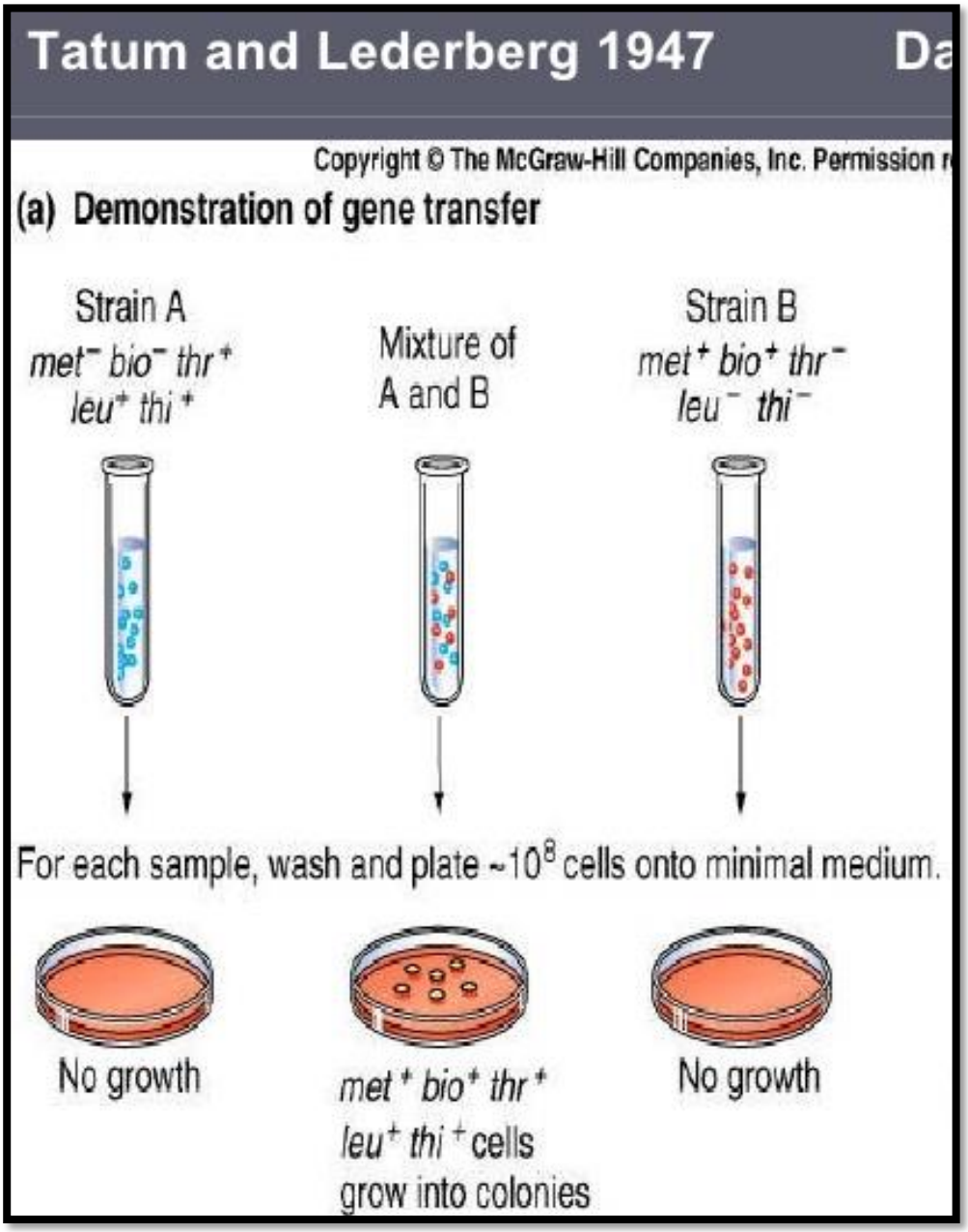
If the F-plasmid that is transferred has previously been integrated into the donor s genome .some of the donor s chromosomal DNA may also be transferred with the plasmid DNA .because such strains transfer chromosomal genes very efficiently they are called Hfr (High frequency of recombination).

Lederberg experiment:

This experiment was designed to demonstrate conjugation as follows

- Start with 3 cultures of bacteria :
  - Strain **A** (***thr*** $^-$ ***Leu*** $^-$) alone.
  - Strain **B** (***bio*** $^-$ ***met*** $^-$ ***thi*** $^-$) alone.
  - A mixture of strain **A** and strain **B**
- Grow all 3 cultures in minimal media supplemented with 5 nutrients (threonine , leucine , biotin, methionine and thiamine)
- Collect the cells , wash them in minimal media , and plate them on minimal media plates without any supplements.
- The results were as follows :-

- Plate 1 ------- Strain A alone --------no colonies were observed.
- Plate 2 ------- Strain B alone --------no colonies were observed.
- Plate 3 ------- mixture of Strain A and strain B -------- 1 colony per 10^7 cells plated was observed



Lederberg experiment in conjugation

PROCEDURE:-

1- Strain A : E coli strain (Sensitive to ampicillin and resistant to amoxicillin) was inoculated for 24 hrs. at 37 ˚C.
2- Strain B : E coli strain (Sensitive to amoxicillin and resistant to ampicillin) was inoculated for 24 hrs. at 37 ˚C.
3- Mix gently (0.1 ml from strain A and 0.1 ml of strain B).
4- Ampicillin and amoxicillin nutrient agar plate (with 100 µg/ ml for each antibiotics) is prepared by using previous calculation for preparation of stock solution of antibiotics.
5- Ampicillin and amoxicillin nutrient agar plate (with 100 µg/ ml for each antibiotic) is inoculated with 0.2 ml of mixture (strain A&B) and then is incubated for 24 hrs. at 37 ˚C.
6- Check the results ,if there is growth means that conjugation is happened between strain A & B because strain A alone or strain B alone cannot resist both ampicillin and amoxicillin with 100 µg/ ml.

Ex: In Conjugation experiment methodology , prepare 50 ml of media from ampicillin with 250 mg and amoxicillin with 300 mg with final concentration 500 µg/ ml? note: for amoxicillin stock dissolve in 3 ml D.W. and for ampicillin stock dissolve in 2 ml of D.W.

Ampicillin stock For

250 * 1000=250000 µg

C1 V1=C2 V2

250000 / 2 * V1 = 500 * 50 >>>>>>>>>>> **V1 = 0.2** ml take it and add it to 50 ml media

For Amoxicillin stock

300 * 1000 = 300000 µg

C1 V1=C2 V2

300000 / 3 * V1 = 500 * 50 >>>>>>>>>> **V1 = 0.25** ml take it and add it to 50 ml media

Note:- the sum of V1 for both antibiotics should not be more than 0.6 ml to 50 ml media