

Conjugation Lab
Hfr Mapping to Determine the Genetic
Distance Between Genes in *E. coli*

Report due: Oct. 13/14.

Readings: Bacterial Genetics:Hartwell Chapter 15 pp. 543-562.

Objectives: The experiment you will be doing today involves the 'mating' of two strains of *Escherichia coli* for the purpose of transferring genetic markers from one strain (the donor or male) to a second strain (the recipient or female). This process requires close cell-cell contact and involves the physical transfer of DNA from the donor strain to the recipient strain. Therefore, the two strains must be incubated together for conjugative DNA transfer to occur. With the discovery of bacterial conjugation, methods for genetic mapping of the *E. coli* genome developed quickly. A surprising result that arose from these efforts was the discovery that the *E. coli* chromosome was circular. Many years passed before the bacterial chromosome was actually observed in the electron microscope, confirming its circular topology.

Introduction: The donor strain for today's experiment contains the F plasmid integrated into the chromosome to form a high frequency recombination or *Hfr* strain. The *Hfr* being used is called *HfrH*, and the F plasmid is integrated at about 98 minutes on the *E. coli* genetic map. The integration of the F plasmid into the bacterial chromosome allows the transfer of chromosomal genes from the donor strain to the recipient strain. Notice that the donor strain is sensitive to the antibiotic streptomycin. This will be important in the design of today's experiment.

The recipient cell strain, *E. coli* AB1157, is resistant to streptomycin and contains many additional mutations, some of which are relevant to today's experiment. In particular, notice that the recipient strain requires both leucine and proline in the media in order to grow since it contains mutations in the biosynthetic pathways for both of these amino acids. The strain also requires several other amino acids that will be provided in the media. Each of these genes could also be used to select for recombinants provided they are transferred before the gene encoding streptomycin sensitivity/resistance.

The goal of today's experiment is to determine the time of entry of each of two genes on the *E. coli* chromosome. This is possible since an *Hfr* strain transfers genes linearly from a fixed point of origin. Thus, the experiment will provide information regarding the location of each gene relative to the origin of entry for the *HfrH* strain and relative to each other. Experiments like the one you are doing today were used to map the entire *E. coli* chromosome where, as a result of this approach to mapping the genome, the locations of genes are described in terms of *minutes* on the 100-minute *E. coli* map. Each lab group will do a single mating experiment and determine the frequency of recombinants for each of two genes as a function of the time the donor and recipient cells are in contact prior to "interruption" of the mating pair. The donor strain will donate the *leu* gene and the *pro* gene to allow a recipient, which acquires one or both of these genes by F-mediated transfer, to grow on an appropriate selective plate. Be sure to carefully label your plates. You should see different results depending on whether you are selecting for

leu+ recombinants or *pro+* recombinants.

Materials:

Minimal A Medium (1X)

Mix 15 g agar in 885 ml H₂O and autoclave

Autoclave separately and add 100 ml of 10X minimal salts

For 100 ml 10X salts:

K ₂ HPO ₄	10.5 g
KH ₂ PO ₄	4.5 g
(NH ₄) ₂ SO ₄	1 g
sodium citrate · H ₂ O	0.5 g

Autoclave

After autoclaving agar and salts mix and add the following: All stocks must be sterile. Vitamins and amino acids must be filter sterilized.

- 1 ml of 1 M MgSO₄·7H₂O
- 10 ml of 20% glucose
- 0.5 ml of 1% Vitamin B1 (thiamine)
- 4 ml of 10 mg/ml amino acid stock (Histidine, Arginine, Threonine, and Proline or Leucine as appropriate)
- Streptomycin to 50 µg/ml from a 50 mg/ml stock (filter sterilized)

The agar and minimal salt solution must be autoclaved separately and then combined to pour plates. It is generally convenient to make the salt solution in a more concentrated form (e.g. 10X) to add to an appropriate volume of a solution containing the agar.

'L' plates will select for *leu+* recombinants and will lack leucine but will contain arginine, proline, histidine and threonine as well as streptomycin to counter select the donor strain.

'P' plates will select for *pro+* recombinants and will lack proline but will contain arginine, leucine, histidine and threonine as well as streptomycin to counter select the donor strain.

2X YT media

per liter 16g tryptone
 10g yeast extract
 5 g NaCl

mix and autoclave (liquid media only)

Sterile saline

Per liter NaCl 9 g
Mix and autoclave

LB plates:

Per liter Tryptone 10 g
 Yeast extract 5 g

NaCl 5 g
Bactoagar 15 g

Mix first three, add agar and autoclave. Cool to 60 degrees in a water bath before pouring plates.

Donor Strain (relevant genotype/phenotype)

E. coli JC158 (HfrH *lacI22 serA6 spoT1 thi-1* Str^S)

Recipient Strain

E. coli AB1157 (F- *thr-1 leuB6 thi-1 lacY1 galK2 xyl5 mtl-1 proA2 his4 argE3 tsx-33 supE44 kdgK51* Str^r)

Materials required per lab group:

Sterile 250 ml Erlenmeyer flask containing 45 ml 2X YT media (1 per group)
Empty Sterile 125 ml Erlenmeyer flask (1 per group)
13 X 100 sterile test tubes (22)
sterile 1.5 ml eppendorf tubes in beakers (1 per group).
LB plates (8 per group)
'L' (lacking leucine) selective plates (24)
'P' (lacking proline) selective plates (24)
Pipetmen P200 and P1000
Sterile Blue tips
Sterile Yellow tips
Plastic cuvettes (4 per group)
Spectrophotometer (warmed up)
Sterile 10 ml pipets
Pipette pumps for 10 ml pipettes
Vortex mixer
Sterile spreader
Alcohol lamp
Sterile Saline (25 ml per group in Falcon tubes)
Waste container
2 test tube racks
Water bath at 37 degrees
Timer

Method:

Day 0. TA will set up a 5 ml overnight culture of AB1157, JC158 in 2X YT media and shake overnight at 37 degrees.

Day 1: Work in groups of 2.

Note for TA: In the morning, the TA will subculture the overnight cultures of the donor strain, and the recipient strain. For each strain, a 125 ml sterile flask containing 25 ml of 2XYT media will be inoculated with 0.5 ml of the overnight culture (Set up a separate flask

of each strain for each group in the shaking water bath). They will grow in a shaking water bath at 37°C to an OD₆₀₀ 0.5-1.0 (approximately 2-4 x 10⁸ cells/ml). This should take 1.5 hours. (TA: Start 45 minutes before the lab period begins).

Students: Before the lab period, read the protocol carefully. Make yourself a list of all the saline tubes you will need. They should be labeled with either the dilution they represent or the mating time point they represent. Make a list of all the bacterial plates you will need and how they must be labeled.

1 Prepare all the sterile click lock test tubes that you will need for vortexing the samples you remove from the mating mixture at each time point.

2. Prepare sterile eppendorf tubes with sterile saline solution for determining the original titer of the donor and recipient cell strains at the start of the mating (see details below).

3. Prepare all plates with labels. Label each plate with the type of media, the date, your initials, the dilution factor of the bacterial culture and the mating time point it will carry.

4. Prewarm flasks. Incubate an empty 125 ml Erlenmeyer flask at 37°C to use in step 6 below. You should also have a 250 ml flask with 45 ml medium in it prewarmed at 37°C.

5. The TA will check the OD₆₀₀ (optical density at wavelength = 600 angstroms) of your 25 ml cultures of AB1157 and JC158 and bring the cultures to you when they are in late log phase. We will try to have the cultures ready at 2:30.

Once the cultures have grown sufficiently, start the mating as described below. Once the mating is started, use the first 10 minutes waiting time to start diluting your two parental cultures in order to calculate the titre of bacteria in each starting culture. (finish the titrations as you have time).

6. Set up the mating and plate the 0 time point. To initiate mating, mix 10 mls of *E. coli* AB1157 with 0.5 ml of *E. coli* JC158 in the sterile 125 ml flask pre-warmed to 37°C you prepared in step 3. This establishes a 20:1 recipient:donor ratio and is time 0 for the mating experiment. Immediately remove a 0.1 ml aliquot using a P200 pipette with a sterile yellow tip and deposit it on to each of two labeled leucine (L) selective plates and two labeled (P) plates. This provides duplicate samples from this time point. In the interest of time, you may spread all four plates with the glass spreader without reflaming between each plate.

As soon as the four 0.1 ml samples have been removed, return the mating flask to a 37°C water bath and shake the mating mixture very gently.

Calculate the titer of bacteria in each parental culture as follows: Take 0.1 ml of each parental strain and transfer to the first tube for titration to determine the number of bacteria per ml of each original culture. Make serial dilutions, using sterile saline, of each of the two starting cultures to a final dilution of 10⁻⁶. This is accomplished as follows. Use a P20 pipette and a P200 pipette: With the P20 pipette, add 0.01 ml of cell culture to 0.99 ml sterile saline to make a 10⁻² dilution. Then add 0.01 ml of the 10⁻² dilution to 0.99 ml of sterile saline to make a 10⁻⁴ dilution. Use the P200 pipette to add 0.1 ml of the

10^{-4} dilution to 0.9 ml of sterile saline to make a 10^{-5} dilution. Finally, add 0.1 ml of the 10^{-5} dilution to 0.9 ml of sterile saline to make a 10^{-6} dilution. Mix each dilution thoroughly before making the next serial dilution. Use a new pipette tip for each dilution. Discard used pipette tips in your small waste can on your bench.

Using a P200 pipette deposit 0.1 ml of the 10^{-5} dilution of each strain onto each of two LB plates and spread as described below. Add 0.1 ml of the 10^{-6} dilution of each strain onto each of two more LB plates. Use the “flamed” glass spreader to sterily spread the bacterial suspension on the plates. Flame the spreader before spreading different dilution plates. These plates will be used to determine the titer (cell number) of each cell strain you used in the experiment. Label each plate with the type of media, your initials, the cell strain name, the dilution, the amount of the dilution plated, and the date.

7. Continue with the mating: At the 10 min time point dilute the mating mixture 1:10 by adding 5.0 ml of the mating mixture to 45 ml of pre-warmed 2X YT media in a 250 ml Erlenmeyer flask. This prevents the formation of additional mating pairs and thus minimizes the asynchrony in the initiation of chromosome transfer.

Immediately after making the 1:10 dilution at minute 10, withdraw a 1 ml aliquot from the diluted mating mixture into a 13 X 100 mm sterile test tube. Use either a 10 ml pipette or a P1000 pipette set at 1000 with a sterile tip. Vortex the sample vigorously for 15 seconds to interrupt mating pairs. Add 0.1 ml of this mixture to each of two labeled leucine (L) selective plates and two labeled (P) plates and spread the bacteria sterily.

It is important to keep the mating mixture aerated during the experiments. One partner should gently swirl the culture in your water bath set at 37 degrees while the other partner samples, vortexes and plates. Be sure to shake gently since hard shaking can disrupt the mating pairs.

8. At minute 15 withdraw a 1 ml aliquot from the diluted mating mixture. Vortex the sample vigorously for 15 seconds to interrupt mating pairs. Add 0.1 ml of this mixture onto each of two labeled proline (P) selective plates and two (L). This provides duplicate samples from this time point.

9. Continue this process of sample removal, dilution, mixing and plating in 5 minute intervals out to 60 min.

Note: It is sometimes very difficult to withdraw and plate samples every few minutes unless you are very well organized. Record the time the sample is actually withdrawn and don't be concerned if it takes a little more than a minute. It is more important to know the time that mating was actually interrupted.

10. Invert all plates after the bacterial suspension has soaked in and incubate at 37°C.

Day 2:

Count the colonies on the LB plates containing the donor strain and on the LB plates containing the recipient strain. Calculate the cell titer for each of the original cultures. There should be reasonable agreement between the dilutions for each culture. In other

words the 10^{-5} dilution should have 10 times more cells on the plate than the 10^{-6} dilution and the titer calculated from each dilution should be *approximately* the same. To arrive at a final titer for each cell strain take the average of the titers calculated using each dilution. Be sure to account for the dilution you made at the 10 minute time point and the volume of mating mixture that you put on each plate in your calculations.

Examine the selective L and P plates. (You may have to wait until day three to see the colonies). Count the number of Leu^+ and Pro^+ recombinants on each plate. There should be reasonable agreement between the duplicate samples that you prepared. To arrive at a final cell count, determine the average of the duplicate samples. If any colonies grew on the 0 time point L or P plates, subtract that number of colonies from the number on your other plates. Adjust your calculations to compensate for the change in dilution factor of the mating mixture between the 0 and 10 minute time points.

Calculate the number of *leu+* recombinants per 100 donor cells at each time point. Your titration of the original donor culture will tell you how many donor cells went into the original mating mixture. Calculate the concentration of donor cells at each dilution step in the protocol in order to determine how many donor cells were actually plated on the average selective plate. The transfer frequency (recombinants per 100 donors) is the average # of cells on the selective plates for a given time point divided by the number of donor cells plated X 100. Do the same for the average number of *pro+* recombinants per 100 donor cells at each time point.

Plot transfer frequency on the Y-axis against time of mating on the X-axis for transfer of each gene. Extrapolate from early time points back to the X-axis to determine the time of entry for the *leu* gene and the *pro* gene. In an interrupted mating experiment like the one performed here, the most accurate data is obtained at the early time points. Therefore, use early time points to draw a line that extends to the X-axis. This intersection represents the “time of entry” for that marker. The *leu+* and the *pro+* genes should have different times of entry.

Questions to be answered in the lab report

1. Neither the donor nor the recipient should grow on the L and P plates. Why is this true? Why is it important for the donor strain to be streptomycin sensitive instead of the recipient strain?
2. When you test the parental strains for growth on L and P plates, you may see a few colonies on each plate. This is normal since you plated about 10^7 cells. What could these colonies represent?
3. What is the time of entry for each gene?
4. Which of these two genes is closer to the origin of transfer?