

The Effects of Plasmid on Genotype and Phenotype

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Introduction

Plasmids are small circular DNA molecules that often found in bacteria in addition to the large circular DNA molecule of the bacterial chromosome. Plasmid DNAs replicate independently of the bacterial chromosome, and many plasmids can also be transferred naturally among their bacterial hosts. Generally, plasmid genes code for proteins that are not essential for the survival of their host in its normal environment. However, some plasmid DNAs encode genes that confer on their host the ability to live in conditions that would ordinarily kill the host. For example, plasmids can encode proteins that can break down antibiotics that would otherwise prevent the bacterial cell from growing.

One such protein is an enzyme called beta-lactamase which can break down penicillin and certain modified penicillins such as ampicillin. If a bacterial cell contains a plasmid carrying this gene, then the bacteria can grow in the presence of ampicillin and are said to have the phenotype "ampicillin-resistant". The plasmid you will be using in your experiments is a plasmid constructed by scientists for use in recombinant DNA experiments. The beta-lactamase gene was included in this plasmid in order to be able to easily test for its presence in bacteria by determining whether they can grow on media containing ampicillin. A gene used this way is commonly referred to as a "selectable marker". Since having this gene allows bacteria to grow in the presence of the antibiotic, you can readily appreciate how this type of gene can cause serious medical problems when it occurs in pathogenic bacteria. For this reason, the plasmids such as pUC 18 which are used in recombinant DNA experiments were designed so that they cannot be exchanged with other bacteria except by special techniques used in the laboratory.

In this laboratory, you will be incorporating plasmids into bacteria (*E. coli*) and this process is called transformation. It involves treating the bacteria in such a way that their cell wall becomes permeable to DNA for a period of time. The transformed bacterial cells will be cultured on agar broth containing Luria broth, ampicillin, and a chemical X-gal (5-bromo-4-chloro-3-indolyl-betaD-galactoside). Luria broth provides essential nutrients for growth and ampicillin will act as a selection agent. When X-gal is broken down by an enzyme called beta-galactosidase, it forms a blue color. Therefore bacterial cells containing a gene coding for the enzyme will form blue colonies on this agar. pUC18 is a plasmid that includes in its DNA a gene for a portion of beta-galactosidase. The bacterial hosts used with pUC 18 contain in their chromosome genetic information coding for the remainder of the complete enzyme.

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Any bacterial cells which have taken up pUC 18 and contain a functional beta-galactosidase will form blue colonies on this medium. If the gene in pUC 18 has been inactivated, the colonies will be white. Scientists using pUC 18 as a gene cloning vector take advantage of this by inserting foreign DNA into a site within the beta-gal gene and inactivate it in order to distinguish those plasmid DNAs containing the new DNA from the parental unmodified pUC 18 plasmids.

During this laboratory exercise you will be examining the effects of plasmids on the phenotype of E. coli. In addition, you will use gel electrophoresis to examine some aspects of the genotype of the plasmids you are using in these experiments. In the third part of the laboratory you will screen the colonies of transformed E. coli from the first part of the laboratory for the presence of plasmid. You will do this by extracting and recovering plasmid DNA from the cultures.

- I. The Effects of Plasmids On Phenotype
- II. Plasmid Genotype
- III. Screening for Plasmids

Please note:

As you can probably tell, there are many new terms that you will learn by doing these labs. The complexity of the lab procedure, the expense of the lab supplies, and the need to use time wisely REQUIRES that you read and study the lab procedures and introductions prior to coming to class. This lab requires that you get the maximum use of time out of the limited time available.

Common Sense Safety

Prior to performing any of the following procedures, make sure you remember to review the following guidelines:

- A. Remember to wash your hands with soap before and after doing any of the following exercises. Also wash your working area before and after each exercise with disinfectant. If you think you have spilled any bacteria, clean the area with disinfectant and consult your instructor.
- B. Remember not to touch any bacteria. Keep cultures out of the open air as much as possible to avoid contamination.
- C. Read all lab procedures prior to lab. Always ask questions if you are unsure about any procedure. Handle the cultures carefully to avoid any kind of contamination.
- D. Place all contaminated instruments and used cultures in the appropriate receptacles so that they may be autoclaved or treated for disposal.

I. The Effects Of Plasmids On Phenotypes

In this first section, you will be inserting 2 types of pUC18 into *E. coli*. One type of the plasmid will be normal pUC18 (see figure 1a). The other plasmid will be a modified form of pUC18. The modified plasmid will have DNA inserted into one of its genes. The DNA insert is in the middle of the *lac Z gene* (see figure 1b.)

Inserting DNA into the *lac Z* gene affects the function of the *lac Z* gene. The *lac Z* gene is necessary for beta-galactosidase activity. Therefore, the *E. coli* that contain the insert will not manufacture beta-galactosidase. These *E. coli* cell colonies will appear white. The colonies with the normal pUC18 plasmid (without the DNA insert) will break down the X-gal and will appear blue when grown on the plates. Of course, the bacteria without ANY plasmid will not grow; for, they do not contain the genes necessary to break down ampicillin.

Questions:

1. In your own words what is a plasmid?
2. On what kind of media will you grow your *E. coli*?
3. Why is X-gal necessary for this lab?
4. What is beta-galactosidase?
5. After inserting the plasmids into the *E. coli* and culturing them, how will you know if the *lac Z* gene is active or inactive in a particular colony?

Preparation of Competent Cells

Calcium chloride is used to make the cells competent (ready for the uptake of plasmid). It is unclear exactly what the calcium chloride does to the cells to get them ready. But, it does have some kind of effect on the cell wall of the E. coli. The calcium chloride probably breaks open pores in the cell membrane and also helps to carry the plasmid DNA near the openings (see figure 2). After adding the calcium chloride, the mixture is put on ice to cool everything down. Then, the mixture is “heat shocked” by immediately placing it in a 42°C water bath, it is placed back on ice, and is then incubated so that new cells containing the plasmid are generated.

In most cells, plasmids replicate at the same time as the host cell; so, when the cells divide, the new cell will have the plasmid. That form of plasmid replication is called “stringent control.” In plasmids under “relaxed control,” replication of plasmid DNA is not coupled to replication of the host.

Procedure:

1. Use a sterile pipet to add 250 μ L of ice-cold 0.1 M calcium chloride solution to each of the four 1.5 mL tubes. Label the four tubes: A, B, C, and X (X is the control.) Place all tubes on ice.
2. Flame an inoculating loop, then cool it by sticking it into an agar plate in an area where no bacteria are growing. Use the loop to transfer a large cell mass into tube A.
 - Be careful not to transfer agar from the plate along with the cell mass.
 - Vigorously tap the loop against the wall of the tube to dislodge the cell mass.
 - Suspend the cells *immediately* by repeated pipeting using a sterile blue micropipet tip. Examine the tube by holding it up to the light and continue until no visible clumps remain.
3. Return tube A back to the ice and transfer a second cell mass into tube B, and a third and fourth cell mass into tubes C and D by repeating step 2.
4. All tubes should now be on ice.

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5. Use a micropipet to add 10 μL of unknown solution A into the tube you have labeled A. (Solutions A, B, and C are unknown--they could be pUC18, pUC18 with DNA insert, or distilled water.)
 - Repeat Step 5 using solutions B and C.
6. Incubate all of the tubes on ICE for 15 minutes.
7. While you are waiting, obtain one Luria Broth plate (LB) and 3 LB/Amp/X-gal plates.
 - Label the LB plate "LB control"
 - Label the 3 LB/Amp/X-gal plates "A Amp/X-gal, B Amp/X-gal, and C Amp/X-gal"
8. After the 15 minutes for Step 6 has expired, carry the tubes ON ICE to the 42°C water bath and heat shock the cells immediately by placing them into the bath for 60 seconds. (*It is essential that the cells be given a sharp and distinct shock: you must remove the tubes directly from the ice into the water bath.*) After the 60 seconds, immediately place the cells back ON ICE for one or more minutes.
9. Use a sterile blue micropipet tip to add 250 μL of Luria broth (LB) to each tube. Mix all the tubes by tapping the tubes with your finger. Let the tubes reach room temperature. While the tubes are cooling, flame an inoculating loop in preparation for Step 11.
10. Select the X tube. Use a micropipet to add 100 μL of cell suspension from tube X onto the center of the LB only plate. Immediately proceed to Step 11.
11. Immediately spread the cells over the surface of the plate with a flamed inoculating loop.
12. Repeat Step 11 to spread suspension A on plate A, suspension B on plate B, and suspension C on plate C. Remember to flame the inoculating loop between each suspension.

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13. Allow the plates to sit for a minute, then wrap them together with tape, and label your name(s) and the date. Invert the plates and incubate for 8-24 hours at 37°C.

Results:

<u>Plasmid</u>	<u>Number of Colonies</u>	<u>Color of Colonies</u>
A		
B		
C		

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II. The Effects Of Plasmids On Genotypes

In this part of the lab, you will use gel electrophoresis to analyze and compare the genotypes of the two different plasmids (the pUC18 and the pUC18 with DNA insert). You will be given three unknown samples. One of the samples will be pUC18 and another sample will be pUC18 with the DNA insert. The last of the three samples will contain no plasmid at all.

You will cut the circular plasmids using a restriction enzyme. The particular enzyme that you will use is called EcoR1 endonuclease. EcoR1 cuts the plasmid in the middle of the lac Z gene (the EcoR1 site). The lac Z gene is also the site where the DNA insert is located in the plasmid with the DNA insert. The EcoR1 endonuclease will cut on both sides of the insert, leaving several different sizes of DNA fragments. Using gel electrophoresis, you will determine the lengths of the DNA fragments and which plasmid is which, based on genotype.

Questions:

1. What is a restriction enzyme?
2. Where does EcoR1 cut *E. coli*?
3. Why should you expect to see differences between the two plasmids after running them on a gel?

Procedure:

1. Label three small microtubes A, B, and C.
2. To each tube, add:
8 μ L of appropriate plasmid
1 μ L of EcoR1 buffer
1 μ L of EcoR1 enzyme
3. Incubate the tubes for 60 minutes at 37°C.
4. During the incubation, prepare 1.2% agarose gels. (1.2 g of agarose per 100 mL of buffer and 3-4 drops of Carolina Blu stain)
5. At the end of the 60 minute incubation period, add 2 μ L of electrophoresis loading dye ("blue juice") to each tube.

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6. Load 15 μL of each sample into the sample wells as follows:

<u>Sample Well</u>	<u>Sample</u>
1	A
2	B
3	C
4	DNA markers
5	A
6	B
7	C
8	DNA markers

Note: The DNA marker sizes are: 784, 1120, 2040, and 3621 base pairs.

7. Electrophorese until the bromophenol blue in the samples has migrated to within 1mm of the positive electrode end of the gel.
8. At the end of the electrophoretic run, remove the gels from the unit and stain them with Carolina Blu. The Carolina Blu stain should take about 20 minutes. After 20 minutes, SAVE the stain and soak the gel in distilled water.

Analysis:

1. Place your gel on a light box and measure the distances of all DNA bands (in mm) from the sample origin. (*Measure from the FRONT of the well to the FRONT of the band you are measuring.*) Record the values below.

<u>Lanes</u>	<u>Distances (mm)</u>
1. A	
2. B	
3. C	
4. markers	
5. A	
6. B	
7. C	
8. markers	

2. On semilog paper, plot the distance migrated by the DNA bands in the DNA marker sample. The lengths (in base pairs) are 784, 1120, 2040, and 3621.

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3. After extrapolating, use the graph to estimate the lengths of the fragments in samples A, B, and C.

Length (in base pairs)

Sample A

Sample B

Sample C

4. Examine all of your results based on phenotype and genotype. Make a data table and record your observations of all three of the samples. Finally, identify the three unknown samples.

III. Plasmid Screening: How to Extract a Plasmid

During this final part of the lab, you will be extracting the plasmids out of the colonies that you cultured. The plasmid can be taken out of the blue and white colonies of *E. coli* and analyzed by gel electrophoresis. The results you obtain can be compared to your results from section II.

First, you will grow a fresh, new culture of *E. coli* containing the plasmids. Then, you will concentrate the cells and add lysozyme solution which removes the cell's outer lipid layer and breaks down the peptidoglycan layer of *E. coli*. Alkaline SDS will be added next to disrupt the lipids of the cell membranes and the NaOH in the SDS denatures the chromosomal and plasmid DNA by breaking the hydrogen bonds. Finally, acidic sodium acetate (NaOAC) will be added to allow the plasmid DNA to renature. The chromosomal DNA is too complex to renature; so, it is precipitated out along with the SDS and various proteins. Plasmid DNA is about all that is left. (See figure 3)

After isolating the plasmid DNA, gel electrophoresis is used to examine the genotypes of the plasmids. This is an important step in helping to confirm that the plasmids were indeed taken in by the bacteria.

Questions:

1. Explain what the following substances do to help isolate a plasmid from *E. coli*:
 - a. Lysozyme
 - b. SDS
 - c. NaOAC
2. What is a peptidoglycan layer?

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Procedure:

1. Obtain the plates containing the colonies with the two types of plasmid (the blue and white colonies). (By now you should know which type of plasmid is in each colony.) Also obtain two sterile small test tubes and label them according to the two plasmids you have.
2. Add about 10 mL of luria broth (containing ampicillin) to each test tube. Be careful not to contaminate the broth or test tubes.
3. Inoculate the two labeled test tubes with the appropriate plasmid-containing *E. coli* and grow them overnight.
4. Transfer 1.5 mL of each culture into an eppendorf tube. Spin for 10 seconds in a microfuge to form a pellet of cells. Pour off the supernatant. Continue to do this until you have a good-sized pellet. If questionable, ask your instructor to confirm the pellet size is large enough (50 μL +).

Do the following for each tube:

5. Add 100 μL of lysozyme solution. Vortex the mixture vigorously until the pellet is resuspended.
6. Let the mixture sit at room temperature for 10 minutes.
7. Add 200 μL of alkaline SDS and shake the mixture semi-vigorously until the mixture is clear. Invert the tube about 25 times.
8. Add 150 μL of 3 M NaOAC (pH 4.7) and mix gently by inverting five times.
9. Spin 5 minutes in a microfuge.
10. Pour the supernatants (plasmids) into a new tube.
11. Discard the pellet (called "E. coli brains").
12. Add 1 mL of cold ethanol to the supernatants. Invert the tube 5-6 times.
13. Spin for 2 minutes in a microfuge.
14. Pour off the ethanol supernatant.

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15. Dry the pellet by “wick drying.” To do this, twist a Kim-wipe into a “wick” without touching the end of the Kim-wipe. Dry off the ethanol very carefully.
16. Add 10 μ L of TE buffer (Tris-EDTA).
17. Add 5 μ L of 6X loading buffer (“blue juice”).
18. Run the plasmids on a gel. The results will be discussed in class.