LABORATORY

Molecular Genetics: Recombinant DNA

18

OVERVIEW

During the last decade there has been a technological revolution in the field of molecular genetics. Scientists can now explore and "engineer" changes in the genomes of a variety of organisms by obtaining pieces of DNA molecules and **recombining** them in different ways.

One of the key developments in **recombinant DNA technology** was the discovery of special enzymes called **restriction endonucleases.** These "restriction enzymes" have been isolated from a variety of prokaryotic organisms, especially bacteria. They protect bacteria by restricting foreign DNA, particularly viral DNA, from entering and functioning within cells. Restriction enzymes cut the foreign DNA at specific base sequences (restriction sites). The small pieces are then easily destroyed by other bacterial enzymes. Scientists have learned to use the same restriction enzymes as "molecular scissors" to cut all types of DNA molecules into smaller segments at specific locations.

The small pieces of DNA snipped from bacterial cells, fruit flies, frogs, or even humans can be recombined with other DNA. Often, the pieces are inserted into viruses that have been disabled or into bacterial **plasmids** (small, double-stranded DNA molecules located outside the bacterial chromosome). The plasmids or viruses act as **vectors** or carriers to transfer the DNA into the cell of a host—perhaps another bacterial cell or a eukaryotic cell. Bacterial host cells and some eukaryotic cells can multiply to form **clones** (a collection of copies of themselves) that can express the new genetic information and make new gene products. The cells have been **transformed** and may even express a new phenotype as a result of the added gene products. Commercially we can produce large quantities of rare proteins or other specific gene products, such as insulin or growth hormone, using recombinant DNA techniques. **Gene therapy,** the transfer of beneficial genes into the human body is also possible.

In this laboratory, you will investigate some of the basic principles of genetic engineering. Plasmids containing specific fragments of foreign DNA will be used to transform *Escherichia coli* cells, conferring both antibiotic (ampicillin) resistance and lac^+ phenotype (ability to metabolize lactose) to recipient cells.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

EXERCISE A Bacterial Transformation: Constructing Recombinant Plasmids

The bacterium *Escherichia coli (E. coli)* is an ideal organism for genetic manipulation and has been used extensively in recombinant DNA research. It is a common inhabitant of the human colon and can easily be grown in standard nutrient mediums.

The single circular chromosome of *E. coli* contains 5 million DNA base pairs (1/600th the total amount of DNA in a human cell). In addition, the cell contains small, circular, *extrachromosomal* (outside the chromosome) DNA molecules called **plasmids.** These fragments of DNA, 1,000 to 200,000 base pairs in length, also carry genetic information. Some plasmids replicate only when the bacterial chromosome replicates and usually exist only as single copies within the bacterial cell. Others replicate autonomously and often occur in as many as 10 to 200 copies within a single bacterial cell. Certain plasmids, called R plasmids, carry genes for resistance to antibiotics such as ampicillin, kanamycin, or tetracycline.

In nature, genes can be transferred between bacteria in three ways: conjugation, transduction, or transformation. **Conjugation** is a mating process during which genetic material is transferred from one bacterium to another "sexually" different type. (See Laboratory 14, Exercise C.) **Transduction** requires the presence of a virus to act as a **vector** (carrier) to transfer small pieces of DNA from one bacterium to another. **Bacterial transformation** involves transfer of genetic information into a cell by direct absorption of the DNA from a donor cell.

Through the process of bacterial transformation, a bacterium can acquire a new trait by incorporating and expressing foreign DNA. In the laboratory, the DNA used most commonly for transformation experiments is bacterial plasmid DNA. These plasmids often carry a gene for antibiotic resistance. The presence of the antibiotic-resistance gene makes it possible to **select** bacteria containing the plasmid of interest; the bacteria that contain the plasmid will grow on a medium that contains the antibiotic, whereas bacteria lacking the plasmid will not be resistant to the antibiotic and will die.

Transformation can occur naturally, but the incidence is extremely low and is limited to a relatively few bacterial strains. During the growth cycle of these strains, there exists a short period of time when the bacteria are most receptive to uptake of foreign DNA. At this stage the cells are said to be **competent.** (Competence to absorb DNA usually develops toward the end of the logarithmic growth phase, just before cells enter the stationary phase in culture.) The mechanism by which competence is acquired is not completely understood, but in the laboratory, the competent state can be induced by treating bacterial cells with divalent cations such as Ca^{2+} and Mg^{2+}.

In this exercise, you will simulate the construction of a recombinant plasmid. Plasmids can transfer genes such as those for antibiotic resistance which are already a part of the plasmid, or plasmids can act as carriers for introducing foreign DNA from other bacteria, plasmids, or even eukaryotes into bacterial cells. Restriction endonucleases are used to cut and insert pieces of foreign DNA into the plasmid vectors (Figure 18A-1).

Each restriction endonuclease "recognizes" a specific DNA sequence (usually a 4- to 6-base-pair sequence of nucleotides) in double-stranded DNA and digests phosphodiester bonds at specific sites in the sequence (recall that phosphodiester bonds link one nucleotide to the next in a DNA polynucleotide chain). If circular DNA is cut at only one site, an open circle results. If the restriction endonuclease recognizes two or more sites on the DNA molecule, two or more fragments will result. The length of each DNA fragment corresponds to the distance between restriction sites (restriction sites flank the fragment at its ends). Some restriction endonucleases cut cleanly through the DNA helix at the same position on both strands to produce fragments with blunt ends. Other endonucleases cut specific nucleotides on each strand to produce fragments with overhangs or "sticky ends" (Figure 18A-2). Using the same restriction endonuclease to cut DNA from two different organisms produces complementary sticky ends, which can be realigned in a "template–complement" manner, thus recombining the DNA from the two sources (Figure 18A-2).

In bacteria, restriction enzymes provide protection by breaking and destroying the DNA of invaders, such as that of bacteriophage viruses. However, since the recognition sites for restriction endonucleases also occur within the bacterial DNA itself, bacteria have a mechanism for preventing their own restriction enzymes from digesting their own DNA. For each restriction endonuclease produced by a bacterium, there is a corresponding enzyme that methylates the bacterial DNA at that enzyme's specific recognition

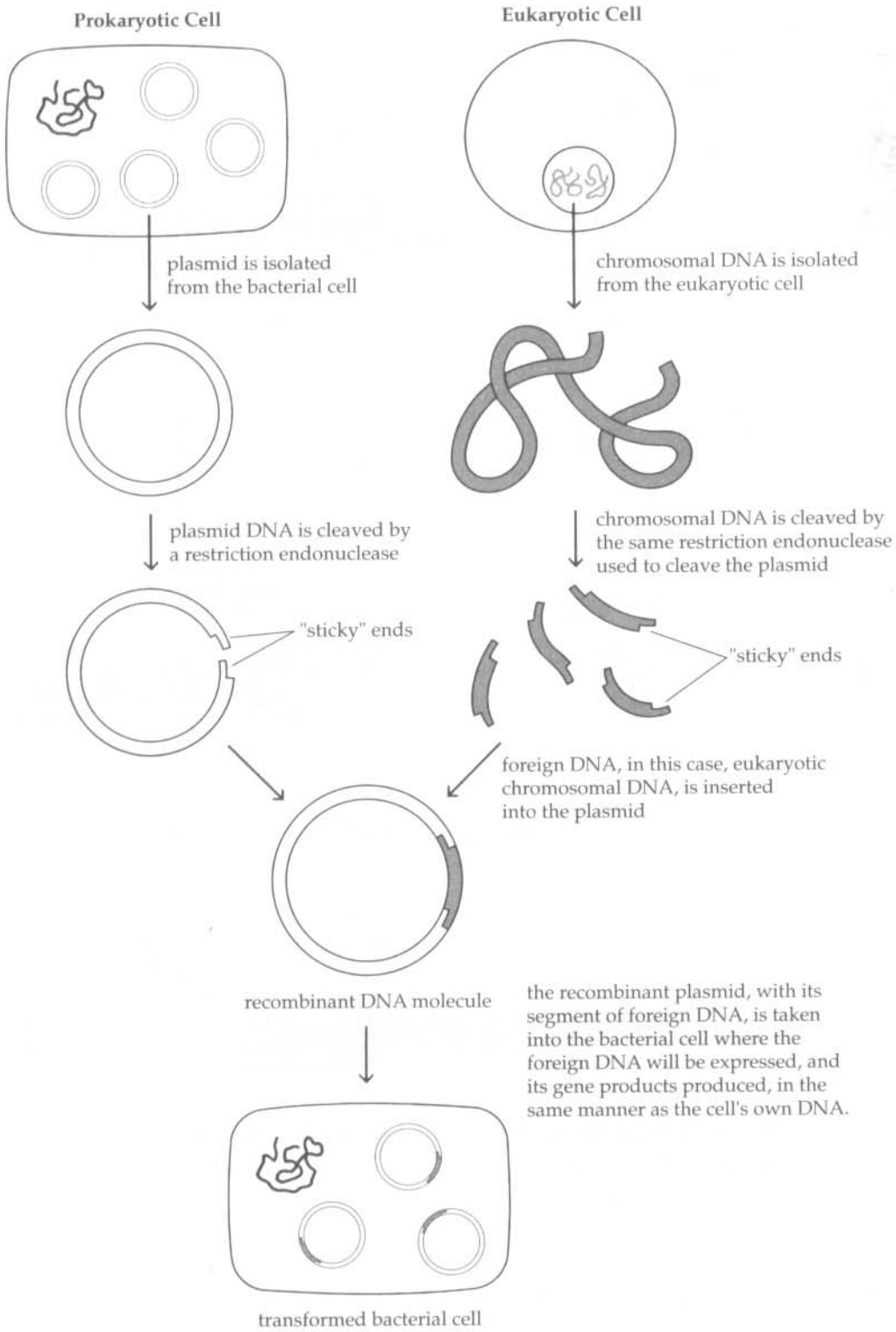


Figure 18A-1 *A typical recombinant DNA experiment.*

site: addition of a methyl group to the nucleic acid base prevents a close association from forming between the restriction endonuclease and the recognition site. In this way, bacteria can break down the DNA of invaders while protecting their own genetic material from destruction.

Objectives

- ☐ Describe how to construct a recombinant DNA plasmid.

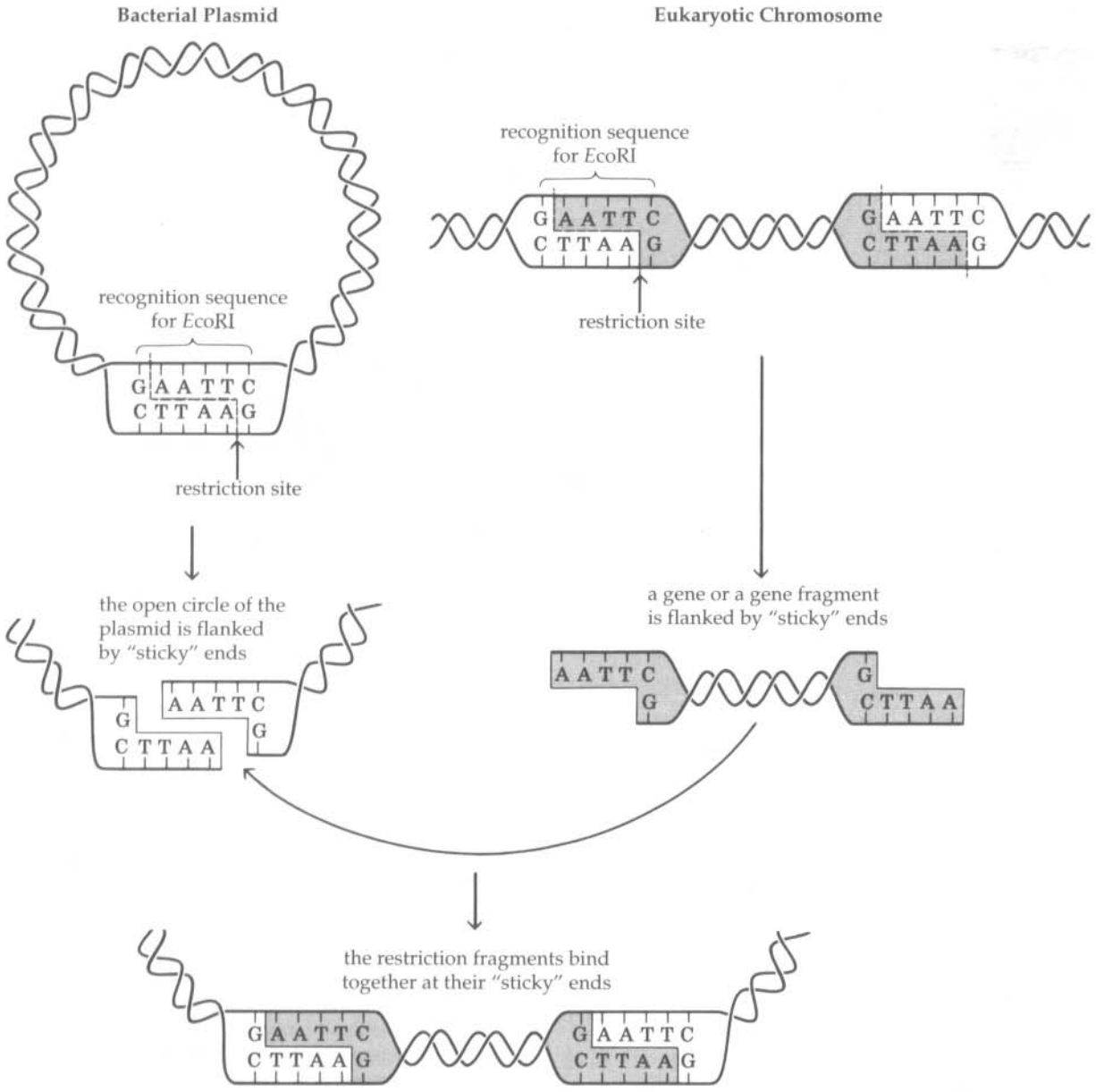


Figure 18A-2 *An example of how a bacterial plasmid and a fragment of eukaryotic chromosomal DNA are cleaved by the* EcoRI *endonuclease and then recombined.*

Procedure

The restriction endonuclease *Hin*dIII, isolated from *Haemophilus influenza,* recognizes the following restriction site and produces fragments with "sticky ends."

cut
↓
5′ –A–A–G–C–T–T– 3′
3′ –T–T–C–G–A–A– 5′
↑
cut

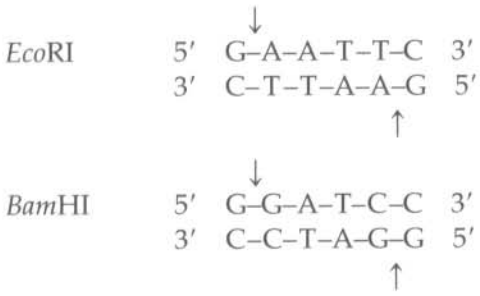
1. The shaded pieces of DNA in Figure 18A-3 (page 18-15) represent a segment of DNA from a human chromosome. The white piece of DNA represents a circular *E. coli* plasmid. Tape the

nucleotide strands together as indicated in order to form a circular plasmid of bacterial DNA and a long linear strand of eukaryotic DNA.

2. Use a pair of scissors to cut the DNA of both the *E. coli* plasmid and the human DNA sequence as they would be cleaved by *Hin*dIII.
3. Insert the human DNA into the plasmid and tape the fragments together.

   a. *DNA ligase is used to join the fragments. What do you notice about the 3′ and 5′ ends of the restriction fragments as they are recombined?* _____

4. Suppose you wish to insert a second gene into the plasmid. You have *Eco*RI and *Bam*HI restriction endonucleases available. They cleave the DNA as follows:



   Design a method for recombining the second gene with your plasmid.

   b. *Which restriction endonuclease would you use?* _____ *Why?* _____

   _____

   c. *What would be the required characteristics of the human DNA sequence?*

   _____

   _____

5. Use the "empty" DNA fragment in Figure 18A-3 to construct an appropriate human DNA fragment and insert it into the plasmid.

EXERCISE B Rapid Colony Transformation with pAMP: Ampicillin Resistance*

Normally, *E. coli* cells are destroyed by the antibiotic ampicillin. In this exercise, you will induce competent *E. coli* cells to take up the plasmid pAMP, which contains a gene for ampicillin resistance. Only *E. coli* cells that have been transformed will be able to grow on agar plates containing ampicillin. Thus we can **select** for transformants: those cells that are not transformed will be killed by ampicillin; those that have been transformed will survive.

Objectives

- ☐ Discuss the principles of bacterial transformation.
- ☐ Describe how to prepare competent *E. coli* cells.
- ☐ Outline the general procedure for gene transfer using plasmid vectors.
- ☐ Carry out the transfer of the antibiotic gene Amp^r and describe how to select for transformed cells that contain the Amp^r gene.

**Exercise B was developed by Dr. David Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, and Dr. Greg Freyer, Columbia University College of Physicians and Surgeons.*

Procedure

Formulate a hypothesis on which to base an investigation of how *E. coli* cells can be transformed by the pAMP plasmid.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** will happen when ampicillin-sensitive E. coli cells are transformed by pAMP?

What is the **independent variable** in this investigation?

What is the **dependent variable** in this investigation?

Use the following procedure to test your hypothesis.

1. Use a sterile micropipette to add 250 μl of ice-cold 0.05 M $CaCl_2$ to two Eppendorf microcentrifuge tubes.
2. Sterilize an inoculating loop by flaming it and then cool it by sticking it into the agar plate in an area where no bacteria are growing. Use the sterile inoculating loop to transfer a large (3-mm) colony of *E. coli* to one of the tubes. Be careful not to transfer any agar.
3. Vigorously tap the loop against the wall of the tube to dislodge the cell mass.
4. Suspend the cells immediately by vigorous pipetting using a 100-μl micropipette with a sterile tip or a sterile plastic transfer pipette.
5. Mark this first tube "(+)" and return it to the ice.
6. Repeat steps 2 to 5 for the second tube. Mark the tube "(−)."
7. Use a sterile inoculating loop to transfer 1 loopful (10 μl) of pAMP plasmid directly into the cell suspension in tube (+). At the correct angle, you will be able to see the plasmid solution form a film across the loop (much like what happens on a toy bubble-maker loop). Immerse the loop in the (+) cell suspension and mix well. Be sure to introduce the plasmid solution directly into the cell suspension—do not touch the wall of the tube as you insert the inoculating loop. Mix by tapping the tube with your finger.
8. Return the tube to ice for 15 minutes.
9. While the tubes are incubating, obtain two LB agar and two LB/Amp agar (LB agar containing ampicillin) plates. Label one LB agar plate "LB+" and the other "LB−." Label one LB/Amp plate "LB/Amp+" and the other "LB/Amp−." Mark your name on the lids.
10. A brief pulse of heat facilitates entry of foreign DNA into the *E. coli* cells. Heat-shock cells in both the (+) and (−) tubes by placing the tubes in a 42°C water bath for 90 seconds. (Tubes can be floated on the water by making an appropriate-sized hole in the center of a thin piece of Styrofoam to suspend the sample tube.) It is essential that cells be given a sharp and distinct shock, so work quickly.
11. Immediately return cells to ice for 2 minutes.
12. Use a sterile micropipette to add 250 μl of Luria broth to each tube. Mix by tapping with your finger and set at room temperature for recovery. Let sit for 10 minutes. During this period, the *Amp*^{r} gene, newly introduced into the transformed cells, codes for the synthesis of β-lactamase (an enzyme that destroys the antibiotic properties of ampicillin by cleaving its

β-lactam ring). The transformed cells are now resistant to ampicillin: they possess the gene whose product renders the antibiotic ineffective.

13. Place 100 μl of (+) cells onto the "LB+" plate and 100 μl of (+) cells onto the "LB/Amp+" plate. Place 100 μl of (−) cells onto the "LB−" plate and 100 μL of (−) cells onto the remaining "LB/Amp−" plate.
14. Immediately spread the cells using a sterile spreading rod. (Remove the spreading rod from ethanol and briefly pass it through a flame. Cool by touching it to the agar on a part of the dish away from the bacteria. Spread the cells and once again immerse the rod in alcohol and flame it.) Repeat the procedure for each plate.
15. Allow plates to set for 5 minutes. Tape your plates together and incubate *inverted* overnight at 37°C.
16. After 12 to 24 hours, indicate on which plates you observe growth.

    LB− _____ LB/Amp− _____

    LB+ _____ LB/Amp+ _____

    a. *What is the purpose of the (−) plates?* _____

    b. *Why was no growth observed on the LB/Amp− plates?* _____

Do your results support your hypothesis? _____ *Your null hypothesis?* _____

What do you ***conclude*** *about the ability of ampicillin resistance to be transferred from one bacterium to another?*

_____

17. Transformation efficiency is expressed as the number of antibiotic-resistant colonies per microgram of pAMP.
    a. Determine the total amount of pAMP used: _____ μg. [You used 10 μl of pAMP (0.005 μg/μl); see step 7.]
    b. Determine the concentration of pAMP (in μg/μl) in the total suspension of cells plus Luria broth used for recovery (250 μl $CaCl_2$ + 10 μl pAMP + 250 μl Luria broth; see steps 1, 7, 12): _____ μg/μl
    c. Determine the total amount of pAMP in the 100-μl spread on the plate (see step 13): _____ μg pAMP/100 μl.
    d. Count the number of colonies on the plate: _____ colonies. (If there are too many, divide the plate into quarters, count one quarter, and multiply by 4.)
    e. Divide the number of colonies by the amount of pAMP in the 100 μl of cell suspension spread on the plate (step c) to give colonies/μg pAMP (use scientific notation): _____ colonies/μg pAMP. This is the transformation efficiency.

EXERCISE C Transformation of *E. coli* with pBLU: The *lac*$^+$ Phenotype

In this exercise, you will work with the plasmid pBLU.™* In addition to the gene for ampicillin resistance (*Amp*^{r}), this plasmid carries a gene for production of the enzyme β-galactosidase (Figure 18C-1). This is one of the enzymes necessary for the complete breakdown of lactose, a carbohydrate that can be used in place of glucose as a source of nutrition for bacteria if glucose is unavailable. Restriction enzymes were used to insert the β-galactosidase gene into the pBLU plasmid.

**The pBLU™ plasmid was developed by Dr. Greg Freyer, Columbia University, College of Physicians and Surgeons, expressly for Carolina Biological Supply Company. Exercise C is adapted from the work of Dr. David Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, and Dr. Greg Freyer.*

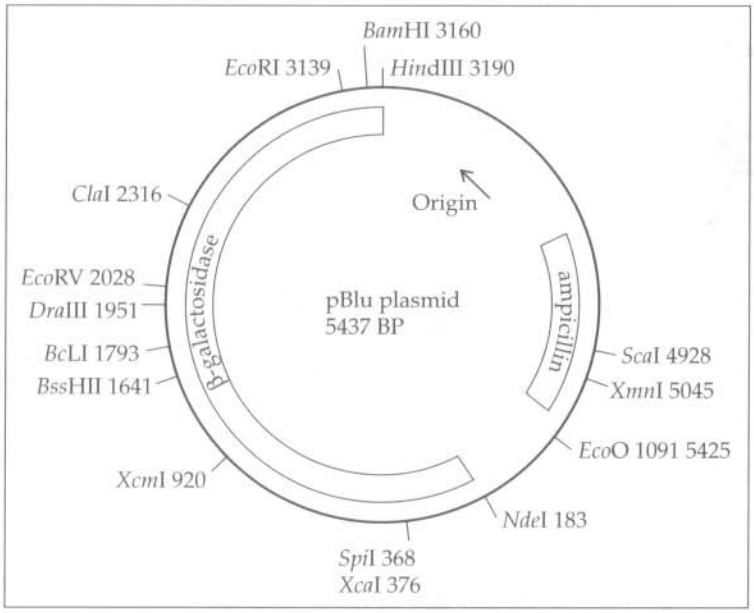


Figure 18C-1 *The pBLU plasmid.™ A restriction map for the pBLU plasmid shows sites for cleavage by restriction enzymes used to insert the β-galactosidase gene into a plasmid carrying the* Amp^r *gene.*

The host cells used for the study are bacterial cells, *E. coli* strain JM101. These bacteria are *not* resistant to ampicillin. They are also *unable* to metabolize lactose (they are lac^-), due to a mutation in the *lac z* gene—the gene in the *lac* operon (Figure 18C-2) that produces β-galactosidase. JM101 cells, however, can acquire resistance to ampicillin (be transformed) by insertion of the pBLU plasmid carrying the Amp^r gene. And, at the same time, JM101 cells can acquire an undamaged copy of the β-galactosidase gene from the pBLU plasmid, transforming the cells to lac^+. Transformed (lac^+) cells can use lactose if it, instead of glucose, is supplied in the agar medium. Lac^+ cells can also use X-gal, a substitute for lactose that, when broken down by β-galactosidase, turns blue. As the transformed cells multiply to form **colonies,** the colonies will appear blue. The expression of the blue phenotype verifies that the gene for β-galactosidase (the *lac z* gene), contained in the pBLU plasmid, is being expressed.

Note that *selection* of transformed cells is actually based on their acquired ampicillin resistance and not on their ability to digest lactose. The agar used to select transformed cells contains ampicillin (in addition to X-gal), and only transformed cells (cells that now contain the Amp^r gene also carried on the plasmid) are able to grow. Selection for the lac^+ phenotype would require that the bacteria be grown on a medium containing *only* lactose as a food source, so that only those cells that are transformed to lac^+ could survive. This is not the case in this experiment, since some glucose is present in the agar medium.

Objectives

- ☐ Describe how to "engineer" a plasmid to include a piece of foreign DNA that confers the lac^+ phenotype to transformed cells.
- ☐ Carry out the transformation of *E. coli* using a plasmid that confers both ampicillin resistance and the lac^+ phenotype.

Procedure

Work in pairs. Formulate a hypothesis on which to base an investigation of how *E. coli* cells can be transformed by the pBLU plasmid.

HYPOTHESIS:

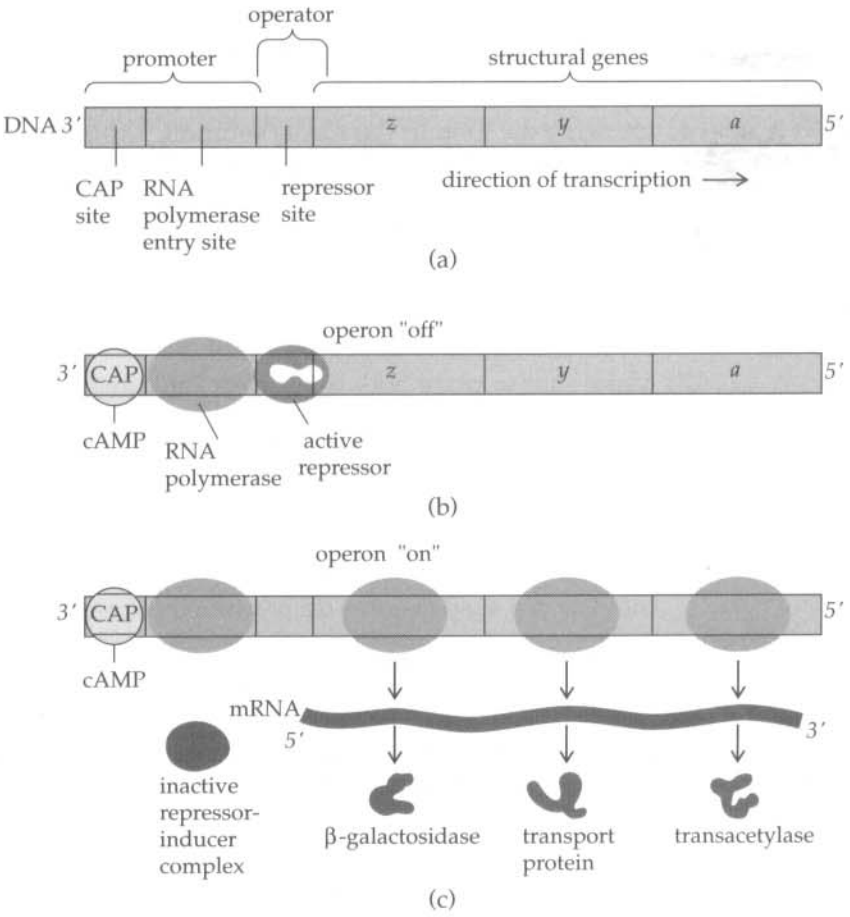


Figure 18C-2 *The* lac *operon. (a) The gene for production of β-galactosidase is the* lac z *gene. This is one of three structural genes in the* lac *operon. In combination with the* lac y *and* lac a *genes, the* lac *operon produces the enzymes necessary for the breakdown of lactose. The operon consists of a* promoter *region for the binding of RNA polymerse (as well as a binding site for catabolite activator protein/cyclic AMP, or CAP-cAMP, which enhances transcriptional activity) and an* operator *site where repressors can bind. The* lac *operon is under negative control by an active repressor that binds to the operator region where it blocks the movement of RNA polymerase. (b) The operon will remain "off" when glucose is readily available and lactose concentrations are low. (c) The* lac *operon turns "on" when allolactose (a derivative of lactose) acts as an inducer to inactivate the repressor (causing the repressor to fall off the operator region). This occurs when lactose concentration in the medium is high and glucose concentration is low. RNA polymerase can then transcribe a polycistronic messenger RNA that codes for the three gene products of the* lac *operon.*

NULL HYPOTHESIS:

What do you **predict** will happen when JM101 E. coli cells that are not able to use lactose as a food source are transformed by pBLU?

What is the **independent variable** in this investigation?

What is the **dependent variable** in this investigation?

Use the following procedure to test your hypothesis.

1. Obtain two sterile plastic test tubes (with caps) to serve as "transformation tubes." Mark one "(+)pLBU" and the other "(−)pBLU."
2. Pipette 250 μl of ice-cold 0.05 M $CaCl_2$ into each of the two plastic tubes (this will be used to make the cells competent) and place both tubes on ice. (Everything must be kept cold!)

3. Using a sterile transfer (inoculating) loop, transfer a large colony of *E. coli* JM101 cells from the agar plate supplied by your instructor into one of the tubes containing $CaCl_2$. (If colonies are small, use two colonies.) Be careful not to dig the loop into the agar! If you do, start over.
4. Immerse the loop in the $CaCl_2$ solution. Tap the loop vigorously against the sides or bottom of the tube—in the $CaCl_2$—until the cells have been dislodged.
5. Immediately, use a sterile transfer pipette to break up the lump of cells. Work the cells in and out of the *tip* of the pipette until, when held up to the light, no clumps of cells are visible.
6. Repeat steps 3, 4, and 5, adding cells to the second "transformation tube." Be sure to disaggregate cell clumps by vigorous pipetting.
7. Make sure to place both tubes back into the ice.
8. Use a sterile inoculating loop to transfer one loopful (approximately 10 μl) of pBLU plasmid solution (0.005 μg/μl as supplied by Carolina Biological Supply Company) into the (+)pBLU tube only. At the correct angle, you will be able to see the plasmid solution form a film across the loop (much like what happens on a toy bubble-maker loop). Immerse the loop in the (+)pBLU cell suspension and mix well. Be sure to introduce the plasmid solution directly into the cell suspension—do not touch the wall of the tube as you insert the inoculating loop.
9. Place on ice for 15 minutes.
10. While the cells are incubating, obtain the following set of agar plates:
    a. Two LB (Luria broth) agar plates. Mark the *bottom* of one plate "(+)" and the bottom of the second plate "(−)".
    b. Two LB/Amp agar plates. Mark one plate "(+)" and the other "(−)" as above.
    c. Two LB/Amp/X-gal plates. Mark one plate "(+)" and the other "(−)" as above.
11. After 15 minutes on ice, you must heat-shock the cells to assist with plasmid uptake. Remove both tubes and immediately place the tubes into 42°C water for 90 seconds. Immediately return the tubes to ice for *at least* 1 minute before proceeding.
    a. Why do you think you must also heat-shock the cells in the (−)pBLU tube when no plasmid was added to the tube? _____
12. Use a sterile transfer pipette to add 250 μl (0.25 ml) of sterile Luria broth to each of the two tubes. Tap with your finger, gently, to mix and let the tubes stand at room temperature (place in a test tube rack) for 10 minutes. This is the recovery period—it will give cells, if transformed, a chance to start producing β-lactamase (see Exercise B) so that when exposed to ampicillin they will be able to degrade the antibiotic.
13. Use a sterile transfer pipette to add 100 μl of (+)pBLU cells to each of the three plates marked (+). Be careful *not* to touch the tip of the pipette to the agar—if you do, discard the pipette and obtain a clean one. Be sure to use an aseptic technique: only lift the lid above the plate—do not take it off or lay it down. You do not want air-borne bacteria and fungal spores to settle on your plates.
14. Use a second sterile transfer pipette to add 100 μl of the cell suspension from (−)pBLU to each of the three plates marked (−). Follow the same procedures and cautions given in step 13.
15. Use a sterile glass "spreader" to spread the cells across the surface of the agar plates. Dip the spreader in alcohol, and briefly pass it through the flame from a Bunsen burner or alcohol lamp. Always allow the alcohol to "burn off." Lift the lid on one of your Petri dishes and cool the spreader by placing it on the surface of the agar away from the cells—don't be surprised if it sizzles. When cool (but do *not* touch it with your fingers), use the spreader to

distribute the cells over the surface of the plate by gently rubbing back and forth at various angles. Lower the lid gently and return the spreader to the alcohol. (Do not flame the spreader before placing it back into the alcohol.) Repeat this procedure for the remaining dishes. If two spreaders are available, one partner should spread the cells on the (+) dishes, while the other partner spreads cells on the (−) dishes. Always put the spreader back into alcohol, and reflame it between using it on different Petri dishes.

16. Allow plates to stand for 5 minutes and then bundle the six plates into a stack. Tape the plates together and place *inverted* (top side downward) in a 37°C incubator. Incubate for 12 to 24 hours. If an incubator is not available, incubate at room temperature—it will simply take longer for the cells to grow and reproduce and for you to get your results!

b. Which plates will serve as experimental control plates? _____ *Do you expect to see cells growing on these plates?* _____ *Why or why not?* _____

c. Why did you put both (+)pBLU and (−)pBLU cells on LB agar plates?

_____

d. If growth occurs on both LB agar plates, what does this tell you? _____

_____

e. If growth does not occur on either LB agar plate, what might you conclude?

_____

17. Indicate in Table 18C-1 what you expect to see, using G for growth and NG for no growth, on your plates after they have been incubated.

Table 18C-1 pBLU Transformation

Plate	Cells	Growth (G) or No Growth (NG)
LB	(+)pBLU	
LB	(−)pBLU	
LB/Amp	(+)pBLU	
LB/Amp	(−)pBLU	
LB/Amp/X-gal	(+)pBLU	
LB/Amp/X-gal	(−)pBLU	

Next Day

18. After incubation, record the number of colonies growing on the experimental LB/Amp/X-gal plate. If there are too many colonies to count, divide the plate into quarters using a marking pen. Count the number of colonies in one quarter and multiply by 4. Number of colonies: _____. Color development indicates that the β-galactosidase gene is functioning and the cells have been transformed to *lac*$^+$.

f. What color are the colonies? _____

If colonies are large, only their centers may be blue. X-gal is rapidly depleted from the medium as the colony grows.

g. Did you see any small white colonies at the edges of the blue colonies? _____

These white colonies are feeder colonies. Often, the destruction of ampicillin by the transformed bacteria forms an area around the colony where nontransformed cells can grow. The nontransformed cells, however, will appear white. *h. Why?* _____

_____

19. Note the results from the control plates.

i. Did cells grow on the LB agar plates? _____ *Why or why not?*

_____

j. Did cells grow on the LB/Amp plates? _____ *Why or why not?*

_____

k. Explain the reasons for growth of the transformed cells on the LB/Amp/X-gal plates. Why are the cells resistant to ampicillin? _____

Do your results support your hypothesis? _____ *Your null hypothesis?* _____

What do you ***conclude*** *about the ability of transformed JM101.* E. coli *cells to use lactose and X-gal?*

_____

20. Transformation efficiency is expressed as the number of antibiotic-resistant colonies per microgram of pBLU.

a. Determine the total amount of pBLU used: _____ μg. (You used 10 μl of pBLU (0.005 μg/μl); see step 8.)

b. Determine the concentration of pBLU (in μg/μl) in the total suspension of cells plus Luria broth used for recovery (250 μl $CaCl_2$ + 10 μl pBLU + 250 μl Luria broth; see steps 2, 8, 12):

_____ μg/μl

c. Determine the total amount of pBLU in the 100-μl spread on the plate (see step 13):

_____ μg pBLU/100 μl

d. Count the number of colonies on the plate: _____ colonies. (If there are too many, divide the plate into quarters, count one quarter, and multiply by 4.)

e. Divide the number of colonies by the amount of pBLU in the 100 μl of cell suspension spread on the plate (step c) to give colonies/μg pBLU (use scientific notation): _____ colonies/μg pBLU. This is the transformation efficiency.

Because transformation is limited to those cells that are competent, increasing the amount of plasmid used does not necessarily increase the probability that a cell will be transformed. A sample of competent cells can usually be saturated with small amounts of plasmid, and excess DNA may actually interfere with the transformation process.

l. How does the transformation efficiency of pBLU compare with that of pAMP (Exercise B)?

_____

Laboratory Review Questions and Problems

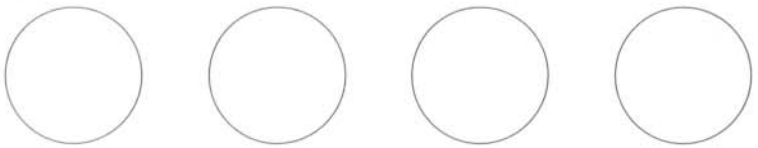
1. You are given the following assignment by a biotechnology firm. Transfer gene *A* from a mouse chromosome to plasmid pBR322 of *E. coli*. What would be your first consideration in choosing one or more restriction endonucleases to cut the plasmid and the chromosome? (*Hint:* Various genes on the eukaryotic chromosome are flanked by various nucleotide

sequences.) Gene *A*, by the way, is responsible for production of a protein that could be economically important if it could be produced in bulk.

2. You have successfully completed a transformation experiment. There are 800 colonies on your plate. You used 50 μl of a solution containing *E. coli* cells mixed with 1×10^{-3} μg/ml of plasmid DNA to inoculate the test plate. What was the transformation efficiency in this experiment?

3. Assume that a bacterial plasmid carries the gene for resistance to the antibiotic kanamycin. Using restriction enzyme A, you open the plasmid and insert a segment of a biologically important gene isolated from a mouse. The gene was excised from the chromosome as part of a fragment cut from whole DNA by using the same restriction enzyme A. After conducting the appropriate steps in a typical bacterial transformation, you plate the transformed cells (+) and control cells (−) on LB agar containing kanamycin and on LB agar alone.

   a. What do you *expect* to see? Indicate this on the plates below:



   b. What you actually observe is no growth on either LB/Kan plate, but growth on both LB plates. You try the experiment again using a different restriction enzyme B. This time you get growth of transformed (+) cells on LB/Kan but no growth of control (−) cells on LB/Kan. You get growth of both transformed (+) and control (−) cells on LB plates. How might you explain these observations? Propose a map for the bacterial plasmid and the restriction sites for restriction endonucleases A and B.

4. You are working in a recombinant DNA laboratory and are asked to clone a gene from a very rare strain of bacteria. The gene produces an important protein used in the oil industry to clean up oil spills. You need large amounts of this product. Outline the steps you would take to get an ordinary bacterium such as *E. coli* (which does *not* normally make the protein) to produce large amounts of this protein.

Figure 18A-3 *Directions: Cut out the top white strand (the "plasmid") and position tab Y under X to form a circle; tape ends together. To form the eukaryotic "chromosome," cut out the two long shaded strands and tape tab B over tab A; cut out the short shaded strand and tape tab D over tab C.*