

BACTERIAL TRANSFORMATION WITH RECOMBINANT DNA

For information on the plasmids used in this lab contact Ted Lee: Theodore.Lee@fredonia.edu

In this lab we are going to learn some basic techniques and concepts used to clone DNA molecules. A DNA molecule (or gene) is said to be cloned if it is contained in a vector DNA molecule from which the cloned DNA can be readily isolated. There are different types of cloning vectors such as plasmids, phage, and cosmids. In this lab we will use plasmid vectors.

Plasmids

Plasmids are small circular DNA molecules found in bacteria. They replicate independently of the bacterial chromosome and depending on the plasmid there may be from 1 to over a hundred copies per cell. If a given plasmid used for cloning is present in a hundred copies, then one can isolate a large amount of cloned DNA from a small number of cells by isolating the plasmid.

Plasmids have been developed which contain several features advantageous for cloning. Nearly all plasmids used for cloning have a gene encoding antibiotic resistance. When such a plasmid infects a bacterial cell the antibiotic resistance is transferred. Only bacteria containing this resistance gene will grow on a media containing the antibiotic. This makes it possible to isolate bacteria that contain the plasmid. Plasmids also contain restriction enzyme sites in specific locations. These sites make it possible to open the circular plasmid and insert foreign DNA. In addition, many plasmids also have differential markers to allow for the identification of recombinant plasmids containing a DNA insert.

During the next two weeks you will be working with plasmids derived from the pK19 plasmid (Figure 1). The pK19 plasmid contains a resistance gene for the antibiotic kanamycin. It also contains several **restriction enzyme sites** where the DNA molecule can be cut and foreign DNA can be inserted. In the pK19 plasmid these restriction sites are located very close to each other forming a **multiple cloning site (MCS or polylinker)**. The MCS lies within the Lac Z gene. This gene encodes a fragment of the β -galactosidase enzyme called the β -peptide. Adjacent to the multiple cloning site is a copy of the promoter for the lac operon. When the promoter is turned on, the DNA in the MCS and beyond is transcribed into mRNA, which is then translated. This results in the synthesis of the β -peptide. If a foreign DNA molecule is inserted into the MCS the gene encoding the β -peptide is disrupted and the β -peptide is not synthesized. Since the β -peptide is an essential component of β -galactosidase, bacteria lacking the ability to produce the β -peptide will not produce functional β -galactosidase unless they are transformed by plasmids lacking inserts.

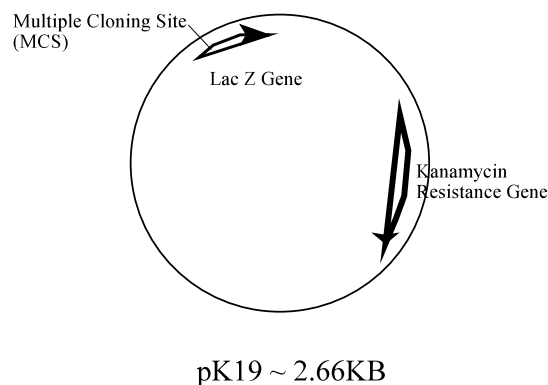


Figure 1 pK19 plasmid showing the relative position of the multiple cloning site, the Lac Z gene and the kanamycin resistance gene.

Transformation

Transformation is the uptake of DNA by bacterial cells. The pK19 plasmid can replicate its DNA using the bacterium *Escherichia coli* as a host organism. In order for *E. coli* to take up pK19 they must be made competent. Any bacterial cell that is competent can take up DNA. *E. coli* can be made competent by incubating them in the presence of specific salts such as CaCl_2 . We will transform *E. coli* using a method called **electroporation**. In this protocol cells are made competent by washing them in 10% glycerol. Competent cells can be stored at -80°C until they are needed. When DNA is added to competent cells, some of the cells will take up the DNA and are transformed. Selecting the appropriate *E. coli* strain to replicate

the plasmid is a critical step in the cloning process. The strain you will be using is J107. This strain contains a deletion in the gene encoding β -galactosidase. It will synthesize most of the β -galactosidase protein, but it will not be active because it does not make the β -peptide. If the β -peptide is supplied to the *E. coli* by inserting the pK19 plasmid, it complements the polypeptide encoded by the bacterial chromosome and the cell will have β -galactosidase activity.

How do you know if the β -galactosidase produced by the bacterial cell is active? Recall that β -galactosidase cleaves the disaccharide lactose into glucose and galactose. X-gal is a lactose analogue similar to the ONPG which was used in earlier labs. X-gal turns blue when cleaved by β -galactosidase. Bacterial colonies containing cells with functional β -galactosidase are blue and contain a pK19 plasmid lacking an insert. When foreign DNA is inserted at the MCS the gene for the β -peptide is disrupted, β -galactosidase is inactive and the bacteria colonies are white. By growing the bacteria on solid media it will be possible to determine which cells have been transformed by a plasmid containing an insert (the DNA you eventually want to clone) and which have been transformed by plasmids lacking an insert.

Cloning of DNA molecules

Our ability to clone DNA depends on the availability of **restriction enzymes**. These enzymes are proteins that recognize specific base sequences and cleave the DNA molecule. Each restriction enzyme recognizes and cuts the DNA at a unique sequence. These sequences are usually 4-6 base pairs in length. When the enzyme cleaves the molecule the strands separate at the point of cleavage and tails with unpaired bases result. These unpaired tails make it possible to isolate DNA from one organism and insert it into a cloning vector.

When one desires to clone a DNA fragment, they need to put it into a cloning vector such as a plasmid. Prior to this laboratory two DNA fragments were isolated from *Rhodobacter sphaeroides* and inserted into the pK19 plasmid. The DNA fragments were isolated by cutting the DNA of *R. sphaeroides* with the restriction enzymes EcoRI and BamHI (Figure 2). The pK19 plasmid DNA was also cut with these two enzymes. By adding DNA ligase to a mixture of cut plasmid DNA and the cut bacterial DNA it is possible to create plasmids that contain portions of the bacterial DNA. Isolation of genes from the pK19 plasmid was

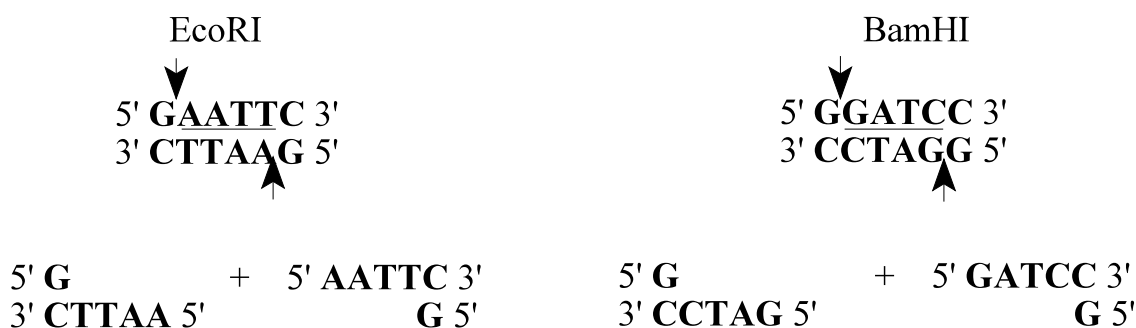


Figure 2. The restriction enzymes EcoRI and BamHI recognize the specific base sequences shown above and cleave the sugar-phosphate backbone at the points shown by the arrows. The hydrogen bonds connecting the two DNA strands break and the molecule separates into two fragments. Each fragment contains unpaired bases at the 5' end. When DNA *ligase* is added these unpaired bases can anneal with complementary strands. DNA ligase is an enzyme that assists in the reconnection of the sugar phosphate backbone.

the research focus of Dr. Ted Lee and his Kenyon Honors students.

Creation of Recombinant Plasmid

A 9 Kb DNA fragment was generated from the bacterium *Rhodobacter sphaeroides* by cutting the bacterial chromosome with EcoRI (Figure 3). At each end of the fragment there are unpaired bases.

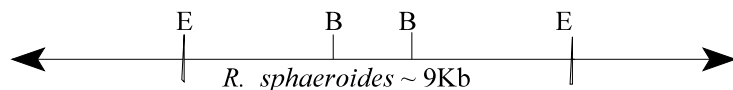


Figure 3. A 9Kb fragment of *R. sphaeroides* DNA located between two EcoRI (E) restriction sites. In addition to the two EcoRI sites there are two BamHI sites. The DNA molecule continues in the direction shown by the arrows.

The multiple cloning site of plasmid pK19 contains both EcoRI and BamHI sites (Figure 4). When pK19 plasmid rings opened at the EcoRI site are mixed with EcoRI digested fragments of *R. sphaeroides* and a DNA ligase is added, recombinant plasmids containing the bacterial fragment can form (Figure 4). The recombinant plasmid is named pKC105. We know the base pair sequence of the region of the *R. sphaeroides* insert lying between the two BamHI sites. It contains two genes. The second of these the *dnaJ* gene has only been partially sequenced since it extends beyond the BamHI site. Students in Dr. Lee's lab have isolated the regions of the insert between EcoRI and BamHI and inserted them into pK19 plasmids. This was done by simultaneously digesting pKC105 with both EcoRI and BamHI. This results in DNA fragments with an EcoRI site on one end and BamHI on the other. These fragments are then mixed with pK19 plasmids that have also been digested with both enzymes creating pKC106 and pKC107 (Figure 5).

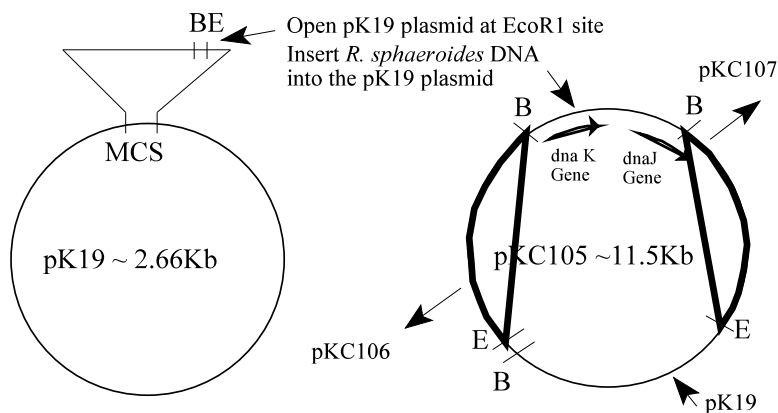


Figure 4. The pK19 plasmid showing the MCS site and the pKC105 plasmid which is created by adding a 9Kb *R. sphaeroides* insert. The insert is at the top of the ring and runs from one EcoRI site to the other. The plasmid DNA runs between EcoRI sites at the bottom of the ring. Also note that both the insert and plasmid have BamHI (B) sites. The fragments of *R. sphaeroides* of interest are indicated by the bold lines.

This week you will be transforming bacteria with either pK19, pKC106, or pKC107. Next week you will use restriction mapping to distinguish between bacteria transformed with pKC106 and pKC107. The

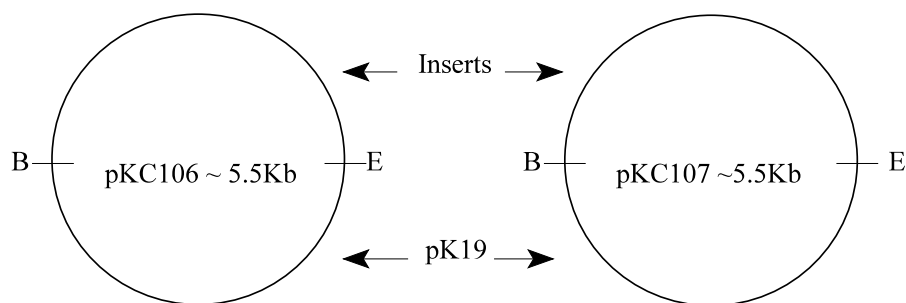


Figure 5 Plasmids pKC106 and pKC107 contain DNA from *R. sphaeroides* that was isolated from the pKC105 plasmid. The *R. sphaeroides* DNA is at the top of the plasmid ring and the DNA derived from pK19 is at the bottom of the ring.

challenge is that pKC106 and pKC107 are the same size. This technique was used to isolate the unknown region of the *dnaJ* gene.

Protocol: You will work in pairs. Each pair will have either pK19, pKC106 or pKC107. The plasmids will be identified as either A, B, or C.

1. Thaw competent cells on ice. You will use the *E. coli* strain J107 Δ (Lac Z)M15 because it lacks a kanamycin resistance gene and in addition cannot make β -galactosidase unless the β -peptide is supplied by an intact pK19 plasmid. (*Spin down*)
2. Add 1 μ l of unknown plasmid DNA to competent cells. To do this successfully expel the air from the micropipetter before drawing up the DNA. Check to make sure you have DNA in the pipette. When adding DNA to the competent cells expel the DNA directly into the cells, then work the cell mixture up and down in the pipette. (*Spin down*)
3. Remove electroporation chamber from ice bucket. Place 25 μ l of cells between the protruding “bosses” in the electroporation chamber to make a bridge.
4. Place the chamber in the electroporator.
 - Make sure the white knob is pointing at the cell you put your chamber in.
 - Close and lock the lid.
 - Connect Electrodes
 - Set to **Charge** and use **Up** to charge the unit to 405-408 volts
 - Switch to ARM
 - Press **Trigger** when 400 volts are shown. You should hear a blip this means current is passing through the cells. A current of 2.25-2.5 is ideal for transformation.
 - Unplug the electrodes, open the lid, and remove the chamber
5. Rinse the cells with 1ml of Lauria Broth (LB). Close the chamber.
6. Incubate the cells in a shaking water bath at 37°C for one hour. During this time the cells will be getting ready to express their genes but there will be no cell division.

During the incubation your instructor will demonstrate how to spread the cells on an agar plate containing kanamycin, IPTG and X-gal. Only cells containing plasmids with the kanamycin resistance gene will grow on these plates. Cells with β -galactosidase activity will be blue and all others will be white.
7. Label the bottom of two plates and place one on the spreader. Holding the lid at an angle over the plate place 100 μ l of “transformed” bacteria in the center of the plate. Replace the lid.
8. Wash the hockey stick in 95% ethanol and quickly flame it.
9. Spread the bacteria to assure that cells are widely spaced on the plate. This way colonies that result from the division of single cells will be less likely to grow together.
10. Concentrate the bacteria. Use a 1000 μ l followed by a 200 μ l pipette to transfer the remaining fluid to an Eppendorf tube. Pellet the remaining bacteria - 1 min at maximum speed. **Gently** pour off the fluid. Use the 200 μ l pipette to resuspend the pellet. Then spread a second plate.
11. Incubate your plates in an inverted position at 37°C overnight.
12. Return the following day to count the number of blue and white colonies. Then place your plates in the refrigerator on the shelf designated for your section.