RESTRICTION ANALYSIS OF PLASMID DNA - Week 2

Last week you transformed bacteria by exposing them to one of three plasmids. Cells that were transformed acquired kanamycin resistance and formed colonies on plates containing kanamycin. Those that were not transformed were unable to grow. In addition, some of the colonies were able to synthesize β-galactosidase, because they were transformed by a plasmid that had an intact gene for the α-peptide. Other transformed bacteria were unable to synthesize β-galactosidase because they were transformed by plasmids containing *Rhodobacter sphaeroides* DNA inserted into the MCS, thus disrupting the gene for the α-peptide. Two of the plasmids had inserts that disrupted the gene for the α-peptide and the third did not. Colonies containing plasmids with *R. sphaeroides DNA* (white) were isolated from the agar plates, grown in LB and the plasmid DNA was isolated.

This week you will use restriction mapping to characterize the plasmid vectors containing *R. sphaeroides* inserts. This technique will allow you to distinguish between the two unknown plasmids carrying *R. sphaeroides* inserts as well as determine if your bacteria were infected with contaminating plasmids. Because we do not have adequate time for you to perform the plasmid isolation you will be provided with one of the two unknowns, either pKC106 or pKC107 (Figure 1).

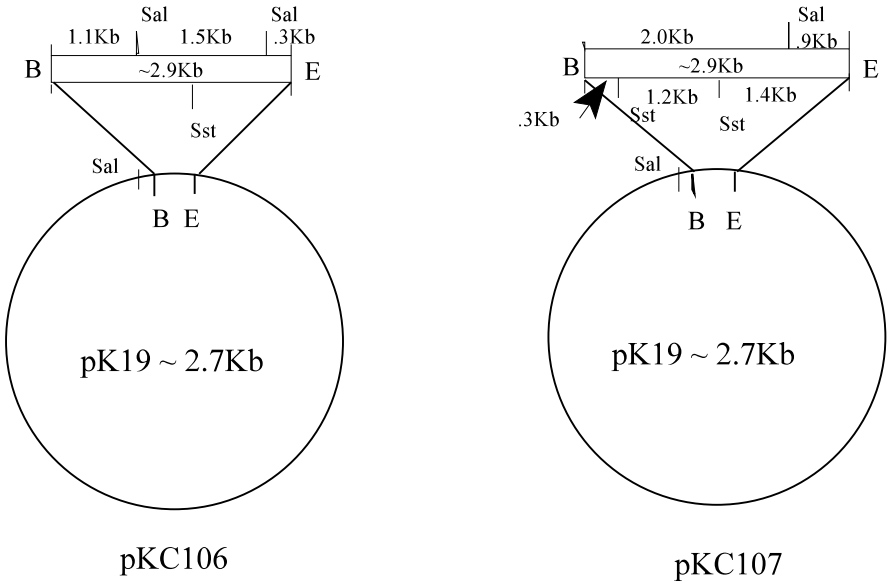

Figure 1 Restriction maps of pKC106 and pKC107. In the above diagram the ring represents the plasmid. The line above the plasmid is the 2.9 Kb *R. sphaeroides* insert. The unpaired ends of the *R. sphaeroides* DNA insert into the pK19 plasmid following the rules of base pairing. The BamHI (B) end inserts at the BamHI site on the plasmid and the EcoRI end inserts at the EcoRI (E) site on the plasmid. Restriction sites for SalI and Sst are also shown. The SalI site in the ring is very close to the EcoRI site. The lengths of the pieces are given in kilobase pairs (Kb).

Plasmids have many restriction sites. These sites provide a physical map of the plasmid and provide a means by which we can distinguish between plasmids of similar size. You are already familiar with the sites recognized by EcoRI and BamHI which were used to insert the *R. sphaeroides* DNA into the pK19 plasmid creating pKC106 and pKC107 (Figure 1). This week we will be utilizing two new restriction sites to help us distinguish between pKC106 and pKC107. One is cleaved by SalI and the other is cleaved by Sst (Figure 2). Restriction sites recognized by these two restriction enzymes are present in both pKC106 and pKC107. Although these plasmids are similar in size their restriction maps are quite different. Note the location of

Figure 2. SalI and SST recognize the base sequences shown above and cleave the DNA as indicated by the line.

the SalI and Sst sites on the plasmids in Figure 1. Cleaving the two plasmids with the same restricition enzymes will produce fragments of different sizes, providing a means to distinguish between bacteria carrying pKC106 and those carrying pKC107. If pKC106 and pKC107 are digested with SAL and SST the actual sizes of the unknown fragments can be determined by comparing the distance they migrate in an electrical field on an agarose gel to the distance DNA fragments of known sizes migrate. This process of separating DNA fragments in an electrical field is known as electrophoresis.

Gels for electrophoresis of DNA fragments are made from agarose which is derived from a compound isolated from a marine alga. A 0.8% agarose gel is made by heating 0.4 gm of powdered agarose in 50ml TAE buffer until it starts to boil. This causes the agarose to dissolve. This mixture is cooled to about 50°C at which time 2.5µl of ethidium bromide (a carcinogen) is added to the mixture and it is poured. As the gel cools a three-dimensional matrix is formed. DNA , which carries a negative charge in aqueous solutions is placed at the negative end of the gel and an electrical current is passed through the gel. The small linear DNA fragments orient in the gel and migrate through the gel toward the positive end. The rate of migration is inversely related to the size of the molecules. In this way molecules of different sizes can be separated.

When setting up a gel, mixtures of DNA with fragments of known sizes are generally placed in the outer and sometime one of the inner wells. For this lab we will use a 1Kb DNA ladder. The fragments of the 1Kb ladder separate in an identifiable pattern and it is possible to identify the various bands. By measuring the distance bands of known sizes migrate it is possible to set up a standard curve. If the $\log_{10}$ of the number of base pairs is graphed against the distance migrated, the result is a straight line. From this line it is possible to determine the size of an unknown fragment by interpolation. During this labyou will be creating a standard curve for the distance fragments of a 1Kb DNA ladder migrate.

Protocol

You will work in groups of four. Each pair will use a different enzyme system

You will be working with very small quantities. Make sure you pipette carefully. Place the drops along the side of the Eppendorf tube and allow them to run into the bottom.

1. Prepare the reaction mixture by adding each of the following in sequence. After each addition **spin, mix, spin**. When preparing these mixtures make sure that the buffer used is appropriate for the enzyme that is being added.

___ 2 µl plasmid DNA
___ 1µl 10x buffer
___ 6µl water
___ 1µl enzyme of choice
10µl total volume

2. Place mixture in the heat block at 37^{oC} for one hour.

During this incubation the instructor will review the principles of gel electrophoresis and the teaching assistant will demonstrate how to pour a gel.

3. Add 2 µl of 6x DNA loading buffer. **Spin, Mix, Spin.**
The loading buffer contains two dyes of different sizes and glycerol. Why is this important?

4. Load 12 µl of each sample into the wells. The gel will be submerged in TAE 1X buffer. Hold the tip of the pipette directly above the well of choice and gently depress the plunger. The sample should settle into the well. Remove the pipette before releasing the plunger.

5. Load 10 µl of 1Kb DNA standard into the designated wells.

6. Run a completely loaded gel at 125 volts for 45min-1 hour.

While your gel is running:

1. Determine the fragment sizes that will be produced by digesting pKC106 and pKC107;

2. Analyze the gel of pKC106 and pKC107 restriction digests;

3. Fill in course evaluation forms.

When the gel is finished:

The teaching assistant will take you to the third floor where you will examine and photograph your gel under UV transillumination. The ethidium bromide added when the gel was poured intercalates into the DNA molecule. Under UV light it flouresces bright orange making the bands of DNA visible. If time permits the teaching assistant will also demonstrate how to map your gel using the computer.