

## ***Experiment 3***

# **Insertion of a Gene for Chloramphenicol Resistance from *Bacillus subtilis* into an *Escherichia coli* Plasmid**

With this experiment we begin a project that will run through the remainder of the quarter. The goal of the project is to subclone a gene for chloramphenicol resistance (CAP<sup>r</sup>) from the *B. subtilis* plasmid pPL608 into *E. coli*. In Experiment 3 we begin by creating chimeric plasmids. In subsequent experiments we will transfer the DNA into a host *E. coli* strain, identify the recombinant products, amplify the DNA, and characterize the chimeras by restriction mapping. Subsequently we will transfer the recombinant plasmids into *B. subtilis* and look at expression of foreign genes in *B. subtilis* and *E. coli*. In addition to CAP<sup>r</sup>, pPL608 carries a gene that confers resistance to both kanamycin and neomycin. Thus, the recombinant plasmids contain three antibiotic resistances; ampicillin (from pUC9) and chloramphenicol and kanamycin (from pPL608). Finally, we will perform a Southern blot to compare the recombinant plasmids with their parents. By the end of the quarter we will have worked through the basic steps of a cloning project.

In Experiment 3 we will cut the two parent plasmids, pUC9 and pPL608 with restriction enzymes and ligate them together. Joining of DNA's is most easily accomplished when both are cut with identical or compatible restriction enzymes. The two DNA's are then mixed and joined with DNA ligase under conditions that permit hydrogen bond formation. Upon annealing, the joint between the two DNA's will contain a pair of staggered nicks, one on each strand, and the ligase will form a phosphodiester linkage between adjacent nucleotides. Although the optimal temperature for ligase is 37<sup>o</sup>, incubation at this temperature would overcome the hydrogen bonding between the cohesive termini. Thus, ligation is usually performed at 4 - 15<sup>o</sup> for 12 - 24 hours. This reaction can be catalyzed by either T4 DNA ligase or *E. coli* DNA ligase. The former enzyme requires ATP as a cofactor and the latter requires NAD<sup>+</sup>. The primary difference between them is that the T4 enzyme will catalyze the ligation of blunt ends whereas the *E. coli* enzyme will not.

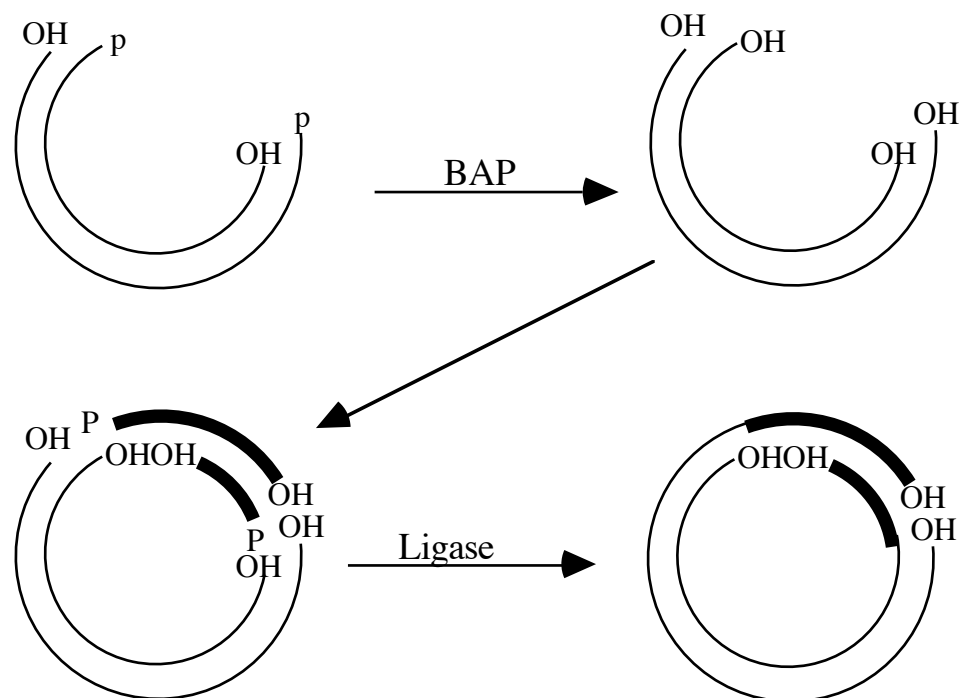
When performing a ligation reaction that involves a number of different fragments, any and all combinations of those fragments will occur, some of which may be undesirable. One such ligation is the rejoining of the cut ends of the vector. This will of course exclude the target fragment. Re-ligation can

## Cloning a Chloramphenicol Resistance Gene

---

be avoided by altering the vector such that its ends, when annealed together, do not provide a suitable substrate for ligase. This is accomplished by treating the cut vector with alkaline phosphatase.

In the alkaline phosphatase reaction, the 5'-phosphates are removed from the cut ends and replaced with 5'-hydroxyls. Upon self-annealing of the vector, a pair of staggered nicks will occur with both 3'- and 5'-prime hydroxyl ends, which cannot be joined by ligase. When a fragment containing 5'-phosphate and 3'-hydroxyls ends is inserted, one of the nicks at the joint will have 5'- and 3'-hydroxyl ends and the other will have normal 5'-phosphate and 3'-hydroxyl ends. The latter ends are joined by ligase and the former ends are not. The situation is the same at the other end of the fragment, except that the acceptable and unacceptable nicks are on the opposite strands. This ligated molecule, containing one ligated nick at each joint, can be introduced into *E. coli*. At the first round of replication, the cells will correct the unligated nicks.



### Preparation of Restriction Fragments

1. Cut pUC9 with *Bam*HI and pPL608 with *Bg*III. You will need to prepare two duplicate samples of pUC9, for a total of three digests. In addition you need to scale up the size of your digestions according to the table. Make sure to consult page 15 to determine the appropriate restriction buffers.

	Standard	4-fold scale-up
DNA	5 $\mu$ l	20 $\mu$ l
buffer	2 $\mu$ l	8 $\mu$ l
water	13 $\mu$ l	52 $\mu$ l
Total	20 $\mu$ l	80 $\mu$ l
Enzyme	1 $\mu$ l	2 $\mu$ l

2. Incubate the samples for at least 40 minutes at 37 $^{\circ}$ . We will do nothing further with these samples today, so you can actually let them go for an hour. The longer, the better.
3. It is important to inactivate the restriction enzymes before attempting a ligation so that you avoid re-cutting your ligated DNA. In this particular experiment, the two enzymes, *Bam*HI and *Bg*III are compatible, but do not recognize the same site. Thus when the ends are ligated, the fusion site is not recognized by either enzyme. However, it is still prudent to inactivate. This can be done simply by placing the DNA samples in a 65 $^{\circ}$  water bath for 20 minutes. Note, however, that the optimal temperature for some enzymes is 65 $^{\circ}$ . In these cases, inactivation must be carried out by phenol treatment.

### Bacterial Alkaline Phosphatase Treatment

1. Prepare a 10 mM Tris, pH8 solution.
2. Ethanol precipitate one of your pUC9 digests as follows: Adjust your DNA sample to 0.1 M NaCl using a 5 M NaCl stock solution. Add twice the volume of ice-cold ethanol (one volume is equal to the amount of your sample, in this case, 80  $\mu$ l, so you would add 160  $\mu$ l of ethanol). Place on ice for 15 minutes.
3. Centrifuge for 20 minutes at 19,000 rpm. Carefully decant and allow the residual ethanol to evaporate. Dissolve the DNA in an equal volume (i.e. the original volume, 80  $\mu$ l) of 10 mM Tris.

4. Add 5 - 10 units of BAP per  $\mu\text{g}$  of DNA. In this case, 1  $\mu\text{l}$  of BAP.
5. Incubate at  $65^{\circ}$  for 45 minutes.
6. Add an equal volume of phenol/chloroform. Mix well and allow to stand for 5 minutes. *Phenol/chloroform is extremely caustic!!*
7. Centrifuge briefly to separate the layers. The aqueous layer containing your DNA will be on top. Carefully pipette off the aqueous layer and transfer it to a fresh microfuge tube.
8. Ethanol precipitate your sample a second time to remove residual phenol/chloroform. Adjust your DNA sample to 0.1 M NaCl using a 5 M NaCl stock solution. Add twice the volume of ice-cold ethanol. Place on ice for 15 minutes.
9. Centrifuge for 20 minutes at 19,000 rpm. Carefully decant and allow the residual ethanol to evaporate. Dissolve the DNA in an equal volume of TE buffer.

**Preparation for  
Experiment 4**

1. Prepare 500 ml LB agar.
  2. Prepare 500 ml MacConkey + Ampicillin (50  $\mu\text{g/ml}$ ) agar.
- (Recipes for both are found in Experiment 4 on page 53)

**Ligation**

1. In general, you should prepare 30  $\mu\text{l}$  of reaction mixture (this may be scaled up or down as required):

20  $\mu\text{l}$  target + vector DNA's  
6  $\mu\text{l}$  5X ligation buffer  
3  $\mu\text{l}$  ATP (5 mg/ml in water, prepared fresh)  
1  $\mu\text{l}$  water  
1  $\mu\text{l}$  T4 DNA ligase

Notes:

- a. Target and vector DNA's may vary from being equal in proportion to being heavily weighted towards one or the other. The specific requirements of each ligation will indicate the proportions.

- b. When doing a blunt-end ligation use half the concentration of ATP
- c. Reasonable controls would be samples lacking either target, vector, or ligase. A volume of water equal to the missing item should be added.
2. Prepare 5 ligation mixes as follows:

	#1	#2	#3	#4	#5
pUC9 (-BAP)	5 $\mu$ l	0 $\mu$ l	15 $\mu$ l	0 $\mu$ l	0 $\mu$ l
pUC9 (+BAP)	0 $\mu$ l	5 $\mu$ l	0 $\mu$ l	15 $\mu$ l	0 $\mu$ l
pPL608	15 $\mu$ l	15 $\mu$ l	0 $\mu$ l	0 $\mu$ l	15 $\mu$ l
5X ligation buffer	6 $\mu$ l	6 $\mu$ l	6 $\mu$ l	6 $\mu$ l	6 $\mu$ l
ATP (5 mg/ml)	3 $\mu$ l	3 $\mu$ l	3 $\mu$ l	3 $\mu$ l	3 $\mu$ l
Water	1 $\mu$ l	1 $\mu$ l	6 $\mu$ l	6 $\mu$ l	6 $\mu$ l
Ligase	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l

3. Place the ligation mixes in refrigerator over night (or until the next period). Save the remainder of your cut plasmids.
4. After ligation, remove 10  $\mu$ l of each mix and 10  $\mu$ l of the original three cut plasmid samples and run them on a “baby gel” to check on ligation.
5. If the gel looks good, use 10  $\mu$ l of ligation mix for transformation into *E coli* in Experiment 4.

### TE Buffer

10 mM Tris, pH7.6  
1 mM EDTA

### 5X Ligation Buffer

0.25 M Tris pH7.6  
50 mM MgCl<sub>2</sub>  
5 mM dithiothreitol  
25% polyethylene glycol-8000