TBT4145 Molecular Genetics

Experiment 1: Transfering a gene that specifies resistance against kanamycin from the plasmid pUC128Kan to plasmid pBR322.

Group 4

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Summary

Restriction digestion of the plasmids pUC128Kan and pBR322 was performed using the restriction enzyme BamHI. DNA fragments in the two mixtures were separated using gel electrophoresis and DNA from the desired bands was extracted from the gel. DNA extracted from the band presumed to contain the *kan* gene, encoding kanamycin resistance, from plasmid pUC128Kan was ligated with DNA from the band likely containing the linearized pBR322 plasmid. Religations were also performed. After overnight ligation, the ligation mixtures were transformed into competent *E. coli* cells that were plated out at different dilutions on media containing different antibiotics. After overnight incubation, colonies from these plates were diluted once more and plated out on a series of media containing different antibiotics. The growth observed on the two sets of plates was assessed.

The cells transformed with the *kan* gene without a plasmid did not grow on any antibiotic. The cells presumed to contain religated pBR322 grew on ampicillin and tetracyclin, and the cells presumed to contain the *kan* gene within pBR322 grew on ampicillin and kanamycin. These results were as expected based on the plasmid sequence and show that the *kan* gene was successfully inserted into the pBR322 backbone.

1. Introduction

The information in this section was found in the lab manual [1] and the course textbook [2].

Gene cloning is the laboratory process used to create recombinant DNA, a DNA molecule constructed *in vitro* by joining all or parts of two different DNA molecules. It is one of two widely-used methods (along with PCR) used to direct the replication of any specific DNA sequence chosen by the experimentalist. The gene-cloning procedure involves two essential steps:

1. The incorporation of the gene of interest into a small self-replicating chromosome (*in vitro*).
2. The amplification of the recombinant minichromosome by its replication in an appropriate host cell *in vivo*, producing recombinant DNA molecules.

In a typical laboratory procedure, the DNA sources to be used are first digested with restriction endonucleases, enzymes that target specific DNA sequences called restriction sites and cleave both strands within these sequences. A wide variety of restriction enzymes are available commercially and as long as the sequence of the DNA that is to be cut is known, detection of restriction sites within the DNA is easily done using available software. When the restriction sites are known, appropriate enzymes can be selected for cleaving DNA in the desired locations. After digesting vector (backbone) and the source, typically a plasmid containing the insert, the resulting fragments can be separated by gel electrophoresis and insert and backbone can be obtained by extraction of DNA from the appropriate bands on the gel. These fragments are then ligated together using a DNA ligase, utilising the complementary overhang sequences generated by the restriction endonucleases, and transformed into an appropriate bacerial host. Selection of colonies that are likely to contain the correct plasmid can be done by resistance testing, achieve by growing the bacteria on a medium containing one or several antibiotics.

In this experiment, the objective is to transfer the *kan* gene, encoding kanamycin resistance, from the plasmid pUC128Kan to another plasmid, pBR322. Maps of these plasmids are shown in Figure 1.1 and Figure 1.2, respectively. As can be seen from these figures, pUC128Kan contains two restriction sites for the restriction enzyme BamHI, wheras pBR322 contains one. Thus, three DNA fragments should be obtained when these plasmids are cut wih BamHI: two from pUC128Kan, one of which should contain the *kan* gene, and the linearized pBR322 plasmid. The fragment containing *kan* and the linearized pBR322 now have complementary overhang sequences, and therefore the result of ligating these two fragments should be a circular plasmid with kanamycin resistance in which pBR322 is the backbone. Insertion of the *kan* gene will disrupt the tetracyclin resistance gene of pBR322 (*tetA*), leaving the plasmids that are likely to contain an inserted *kan* gene with resistance to ampicillin (from backbone) and kanamycin (from insert). Thus, insertion of *kan* into pBR322 can easily be detected by selection of transformants on media containing different combinations of antibiotics.

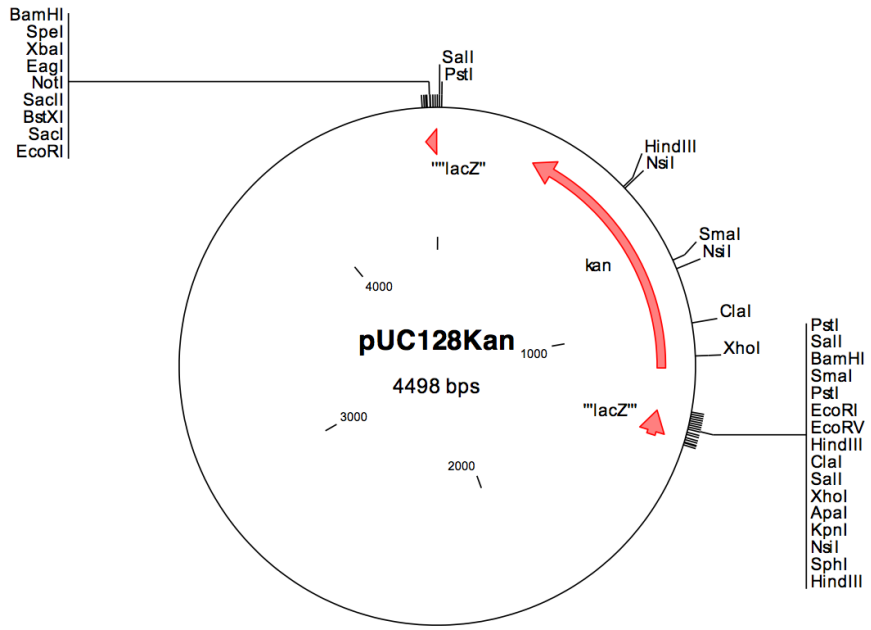


Figure 1.1: Plasmid map of pUC128Kan [1] with the *kan* gene inserted as a BamHI fragment. Some restriction sites are indicaed.

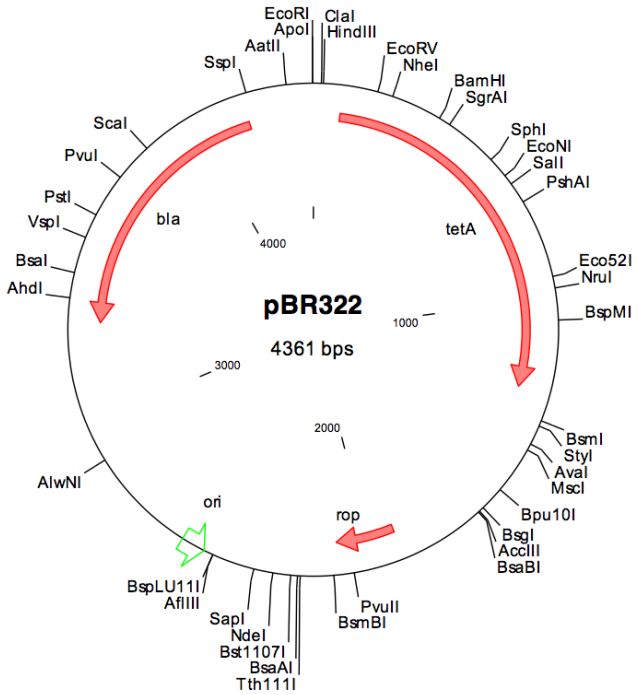


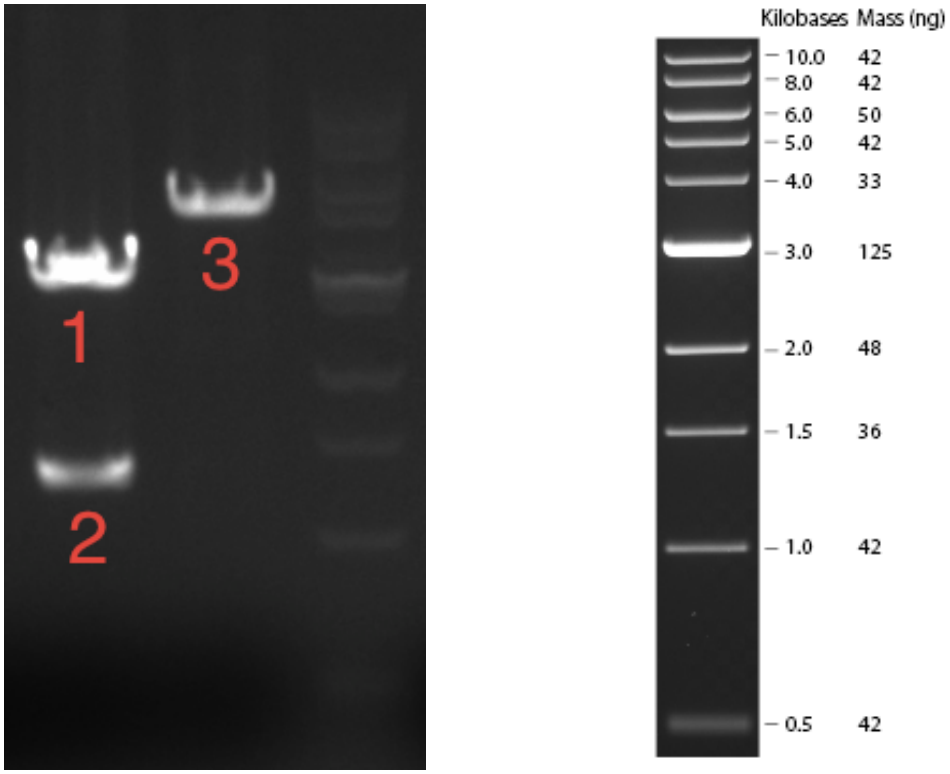
Figure 1.2: Plasmid map of pBR322 with genes and some restriction sites indicated [1]. *tetA* conferrs tetracycline resistance, *bla* encodes β lactonase, conferring ampicillin resistance, and *rop* is the gene responsible for copy number regulation.

2. Materials and methods

The experiment was performed as described in the lab manual [1] wih the following exceptions:

- Digestion mixture A was prepared using 11.5 rather 16.5 µL sterile H_2O and 10.0 rather than 5.0 µL pUC128Kan.
- 1 mL LB medium was added instead of the same volume of SOC medium before the 60 minute incubation at 37 °C during tranformation (step 4).

3. Results



(a) (b)

Figure 3.1: (a) Visualization of gel from gel electrophoresis performed on cutting mixtures A and B containing the plasmids pUC128Kan and pBR322, respectively. Both plasmids were cut with BamHI. Mixture A was added to the well on the left, mixture B to the middle well and a 1 kb ladder to the right well. The 1 kb ladder is shown in (b). The bands numbered 1 and 2 contain DNA from mixture A and the band numbered 3 contains DNA from mixture B. The bands 1 and 2 correspond to approximately 3.2 and 1.2 kb, respectively. Band 3 corresponds to about 4.4 kb.

Figure 3.1 shows the gel obtained by gel electrophoresis of the cutting mixtures in which pUC128Kan and pBR322 were digested with BamHI. Three distinct bands were obtained, as indicated in the figure. The bands 1, 2 and 3 correspond to approximately 3.2, 1.2, and 4.4 kb, respectively. DNA from band 2 and band 3 was ligated together and transformed into *E. coli*. Religations of these bands were also performed. The growth of the resulting transformants on media containing different antibiotics is summarized in Table 3.1. As can be seen from this table, the cells containing DNA from ligation mixture C grew in the presence of ampicillin and kanamycin, whereas the transformants from ligation mixture B grew on the plates containing ampicillin. The A transformants did not grow on any of the antibiotics.

Table 3.1: Growth of cells transformed with ligation mixtures A, B and C on LA media containing ampicillin (amp), kanamycin (kan) and tetracyclin (tet). + indicates growth and - indicates no growth.

Ligation mixture	Growth?		
	LA+amp	LA+kan	LA+tet
A	-	-	-
B	+	-	-
C	+	+	-

Table 3.2: Growth of the colonies that grew on the first set of plates on a new set of plates with LA media containing ampicillin (amp), kanamycin (kan) and tetracyclin (tet). The origin of the colony is denoted by one letter indicating the ligation mixture (A, B or C) along with three letters indicating the antibiotic on which the colony was selected (amp, kan or tet). The numbers 1 to 5 denote the five different colonies that were picked from the original plates. + indicates growth and - indicates no growth.

Medium	Origin of colonies	Growth?				
		1	2	3	4	5
LA+amp	Bamp	+	+	+	+	+
	Camp	+	+	+	+	+
	Ckan	+	+	+	+	+
LA+kan	Bamp	-	-	-	-	-
	Camp	-	-	-	-	-
	Ckan	+	+	+	+	+
LA+tet	Bamp	+	+	+	+	+
	Camp	+	+	+	+	+
	Ckan	-	-	-	-	-

Table 3.2 summarizes the growth on the second series of plates containing different antibiotics. All the B colonies that grew on ampicillin also grew on the second ampicillin

plate as well the plate containing tetracyclin. All the C colonies that grew on ampicillin once again grew on ampicillin and also on tetracyclin. All the other C colonies, those first selected on kanamycin, now grew on kanamycin as well as ampicillin.

To summarize the results from Table 3.1 and Table 3.2, the B and C transformants that first grew on ampicillin also grew on tetracyclin, and the C cells that first grew on kanamycin also grew on ampicillin.

4. Discussion

Information about the structure of the plasmids present in the different transformants can be deduced from their antibiotic resistance and knowledge of the structure of the original plasmids. The insert, the *kan* gene, confers resistance against kanamycin and, when inserted into pBR322 at the restriction site of BamHI, interferes wih resistance against tetracyclin. This can clearly be seen from the plasmid map of pBR322 in Figure 1.2, as the restriction site for BamHI is located a few hundred basepairs downstream of the start of the *tetA* gene that confers tetracyclin resistance. The pBR322 plasmid also provides ampicillin resistance via the *bla* gene. Thus, a plasmid in which the *kan* gene is properly inserted, is expected to confer resistance against ampicillin via the backbone and against kanamycin via the insert. Cells containing the religated pBR322 plasmid are expected to be resistant against ampicillin and tetracyclin. The *kan* gene alone does not have the structure nor contain the apparatus necessary to facilitate transfer into daughter cells and will therefore not allow host cells to propagate on media containing antibiotics.

The cells that were transformed with ligation mixture A, expected to contain only the *kan* gene excised from pUC128Kan, did not grow on media containing any of the three anibiotics. This result is as expected and shows that no DNA material conferring antibiotic resistance was taken up by the cells. The cells that were transformed with DNA from ligation mixture B, likely to contain the religated pBR322 plasmid, also behaved as expected, growing on ampicillin and tetracyclin but not on kanamycin. The observed tetracyclin resistance shows that the *tetA* gene was intact, and thereby that pBR322 was successfully religated in these cells. The C transformants that first grew on kanamycin grew on ampicillin as well, but not on tetracyclin, confirming that these cells contained the *bla* gene of the pBR322 backbone as well as the *kan* gene inserted within *tetA*.

A noteworthy observation is the growth on tetracyclin of all C cells that were first selected on ampicillin combined with no growth of these cells on kanamycin. This shows that all the colonies that were selected from the original ampicillin plate contained the religated pBR322 plasmid and that none of them contained the inserted *kan* gene. By first selecting on ampicillin, cells containing both these plasmids were expected, as both will possess ampicillin resistance. A sample size larger than the five colonies that were tested could have reflected this. It should be noted that this observation does not interfere with the conclusion, that the *kan* gene was successfully inserted into the pBR322 backbone; it merely shows that religations of pBR322 also did occur in ligation mixture C.

5. Conclusion

The *kan* gene was successfully excised from the pUC128Kan plasmid and inserted into the pBR322 plasmid.

Sources

[1] Lab Manual, 2012, *Lab Manual Molecular Genetics 2012*. NTNU, Trondheim, pp. 5-11.

[2] Snustad D. P., Simmons, M. J., 2010, *Principles of Genetics*. 5th ed., John Wiley & Sons, Inc., pp. 419-421.

A. Dilution of cell cultures

The transformation suspensions A, B and C were diluted 1:10 with LB medium in a series down to a dilution of $10^{-}6$. Each dilution was prepared from the one preceding it, i.e. the $10^{-}1$ dilution was prepared from the original cell culture, the $10^{-}2$ dilution was prepared from the $10^{-}1$ dilution and so on. In each case, the total volume prepared was 500 µL, meaning that 450 µL LB medium was mixed with 50 µL of cell culture. The concentration of dilution n is thus given by

$$C_n = C_{n-1} \cdot \frac{V_{cells}}{V_{LB} + V_{cells}} = C_{n-1} \cdot \frac{50}{450 + 50} = \frac{C_{n-1}}{10}$$

where C_n and C_{n-1} are the concentrations of dilution n and dilution $n - 1$, respectively, V_{cells} is the cell culture volume, and V_{LB} is the LB volume.