TRANSFORMATION OF BACTERIA WITH DIFFERENT PLASMIDS

Objectives

- To understand the concept of DNA as genetic material through the process of transformation.
- To test the conditions that make cells competent for use in DNA-mediated transformation.
- To study the characteristics of plasmid vectors.

Introduction

Transformation

Modern molecular biology began with the experiments of Avery, MacLeod and McCarty (1944) on two strains of *Pneumococcus* bacteria. When grown on an agar plate, the wild type virulent strain had smooth glistening colonies designated as type S while an avirulent strain had colonies with irregular shape and rough surface, designated as type R. The change from type R to S could be mediated if a DNA extract from S was added to type R bacteria in a test tube. The term "transformation" was coined for such a change. Other contemporary scientists did not easily accept these experiments and what they implicated, mainly because the method of identifying DNA was not yet well established at the time. It took a decade before the validity of such experiments and their conclusions became fully appreciated as a result of rapidly increasing knowledge and understanding of the chemical and physical nature of DNA.

Normally grown *E. coli* cells can not take up the exogenously supplied DNA. However, if the cells are soaked in an ice cold calcium chloride solution for a short time before the addition of DNA and a brief (90 seconds) heat shock (42°C) is given, DNA uptake by the cells is facilitated (Hanahan, 1983). When bacteria have been prepared in this special manner to easily accept the foreign DNA, they are said to be "competent".

Although DNA-mediated transformation is universally applicable to all prokaryotes as well as a variety of eukaryotes such as fungi, mammalian cells, etc., not all species can be made competent by the same procedure. This suggests that different mechanisms may be at work for this phenomenon in different organisms. However, it seems that most species require Ca^{+2} ions prior to DNA uptake.

The theory proposed for the creation of competency is rather recent and is based on the charges present on the phospholipids in the cell membrane as well as on the exogenous DNA molecule. Thus foreign DNA is normally repelled by the cell membrane, although there are pores (called adhesion zones) large enough in the cell membrane to allow the DNA to pass through. The addition of Ca^{+2} neutralizes the negative charges on both the phospholipid layer as well as the DNA and cooling of the cells on ice congeals the lipid membrane. Sudden heat shock in the next step would increase the temperature

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outside the cell while it is still low on the inside. This temperature gradient would facilitate the movement of DNA into the cell (See animation of this process at www.dnalc.org/ddnalc/resources/transformation2.html).

Cloning and Cloning Vectors

The cloning process that opened the door to molecular bioengineering technology is a very common procedure now. In its simplest form, the ends of a gene and the circular DNA of a vector (discussed below) are both cut with a molecular scissor called a restriction enzyme (See Appendix: Restriction Enzymes) to obtain sticky ends. The sticky ends are then annealed (joined) together. The resultant recombinant DNA is then inserted into a competent bacterium and the bacterium is grown to produce millions of copies (or clones) of the vector and consequently of the desired gene.

When the genomic DNA is cut into small pieces and each piece is cloned in a vector, we have what is called a "genomic library". The cutting can be done by mechanical shearing of the DNA or by partial or complete digestion via restriction enzymes or other enzymes such as DNAses. The vector is actually a carrier DNA molecule that is capable of replicating in a host cell. Modern vectors are engineered to have specific characteristics.

Some of the main characteristics of a vector are: it is a small molecule; it contains an origin of replication called "Ori" and it carries one or more marker genes that enable one to select or counterselect for them. There are three main classes of vectors currently used in molecular experiments; plasmids, phages and cosmids. We will consider only the plasmid vectors in this discussion.

Plasmid vectors, or plasmids for short, are small, double-stranded, circular DNA molecules that reside inside some bacterial species and replicate independently of their host DNA. They range in size from 1 to 200 kb (1 kb = 1000 base pairs). Each plasmid contains an origin of replication (also called a replicon) and one or more genes that code for enzymes that confer resistance to one or more antibiotics. However, for its replication the plasmid relies completely on its host enzymes and proteins.

Plasmids are transferred to the cell progeny in a random manner; i.e., the daughter cells do not receive the same exact number of plasmids. Plasmids can also be transferred to cells via the conjugation or transformation process. In the lab, it is a rather routine process to transform cells with small plasmids. However, large plasmids are transformed very inefficiently.

Some plasmids can exist as single copies while others can have up to 700 copies in a host cell. Those which can multiply freely are called "relaxed plasmids" and are of more use in cloning than those with few-copies or "stringent plasmids". The control of copy number resides in the replicon. Plasmids are grouped into "compatibility groups" according to the type of their replicons. Usually there can only be one strain of a plasmid present in the same cell at one time.

The plasmids used in research now have been artificially manipulated to bring into a single plasmid all or most of the advantages of other plasmids or other vectors.

For example, there are now available plasmids that have an efficient replicon, replicate in a relaxed fashion, contain two antibiotic markers and a multiple cloning site (MCS; a short DNA sequence recognized by several important restriction enzymes; these restriction sites are absent in any other part of the plasmid) but have a deletion of all of their non-essential DNA sequences.

The most common antibiotic markers used in cloning are resistance genes to ampicillin (Amp^R), tetracycline (Tet^R), chloramphenicol (Cm^R) and kanamycin (Kan^R). The Amp^R gene encodes for an enzyme that moves to the periplasmic space and hydrolyzes the ampicillin molecules before they have the chance to bind and block the enzymes that synthesize the cell wall material. The Tet^R gene encodes for a membrane protein which does not allow the antibiotic to enter the cell and bind to the 30S ribosomal protein. The Cm^R gene codes for an enzyme that catalyzes the formation of hydroxyl acetoxy derivatives of chloramphenicol and these derivatives cannot bind the 50S ribosomal subunits. Finally, the Kan^R gene encodes for the enzyme needed to phosphorylate kanamycin or neomycin and render them harmless to the cell by not being able to inhibit protein synthesis.

The first plasmid that was extensively used in research in the beginning era of molecular biology for more than a decade was pBR322 (originally discovered and manipulated by Bolivar and Rodrigues (Bolivar, F., Rodriguez, R.L., et al. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2, 95) which is 4.36 kb. This plasmid had an efficient replicon, contained two antibiotic markers and a number of unique cloning sites.

All or most other plasmids are either direct descendants or derivatives of pBR322. The decrease in the size of the plasmid was an essential step. A small plasmid has the following advantages: 1) transformation efficiency is increased, 2) larger DNA fragments are accommodated in a smaller plasmid and 3) smaller plasmids usually replicate to a higher copy number.

Later, multiple cloning sites (MCS) were added. For example, pUC19 (2.69 kb), a very common and important plasmid, contains a part of the lac operon (see below) and within it 13 unique restriction enzyme sites. When a bacterium containing this plasmid is grown on a medium containing an inducer of the lac genes and a chromogenic substrate, a blue color precipitate is produced. The inducer used is isopropylthio-β-galactose called IPTG and the chromogenic substrate used is 5-bromo-4-chloro-3-indolyl-β-D-galactoside called Xgal. If the lac gene is interrupted (for example, by the insertion of a foreign piece of DNA into the MCs), no blue color is formed. This is a rapid and easy test to visually distinguish between the recombinant and non-recombinant plasmids. To understand the need for IPTG and Xgal, we need to learn about gene regulation and presence of operons in bacteria

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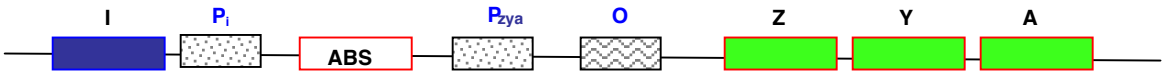
Gene Regulation and Operons

All genes that have transcriptional products (mRNA, tRNA, or rRNA) carry a stretch of nucleotide sequence called promoters. Such sequences are the binding sites for RNA polymerase (RNAp); promoters are the initiation signals for transcription. Many genes in bacteria are closely linked together into functional units called "operons". In other words, an operon is a group of contiguous genes under the control of a single operator. What is unusual in an operon is that several genes that are often functionally related have a single promoter so that the result of the transcription is one long RNA chain encoding several proteins. Promoters themselves encode no protein product.

The next unusual feature of operons is that transcription initiation at promoters can be entirely prevented. Thus the genes in an operon are simultaneously subject to an on-off control switch and we find that their protein products are either on (synthesis) or off (no synthesis).

The elements that have the most control of the operon are called the "operator" (O) and "repressor" (I) elements. Among the regulatory elements, only the repressor gene has a protein product (R protein), also called the repressor protein. R protein is a freely diffusible cytoplasmic protein, synthesized constitutively at a very low rate (so that there are only about 10 copies of it present in the cell at any time), and it is an allosteric protein: it has two binding sites and two three-dimensional shapes. It is this ability to change shape that confers regulatory function on the repressor. The operator is a DNA binding site for the repressor. So, one of the repressor's binding sites (its active site) recognizes the operator's nucleic acid sequence.

The lactose (*lac*) operon of the bacterium *Escherichia coli* is an example of such a system. It contains the structural genes specifying the proteins (enzymes) necessary for the utilization of lactose as a carbon source. It is diagrammed and notated as follows:



Regulatory genes:
I : the repressor gene
P_i: promoter of the I gene
ABS: activator binding site
P_{zya}: promoter of the genes Z, Y and A
O: the operator

Structural genes
Z: gene for β-galactosidase
Y: gene for galactoside permease
A: gene for galactosidase acetylase

Note: In this Lab Manual, we will show genes in italicized lower case letters and gene products in regular font with the first letter capitalized.

The R protein has a tendency to bind to the operator. When the repressor attaches to the operator, it blocks the movement of the RNAp that could bind at the promoter (P_{zya}). The repressor prevents RNAp from transcribing *lac* Z gene and accompanying structural genes (*lac*Y and *lac*A). This situation defines what is called a negative control system: synthesis is off when the control molecule is bound to the DNA.

In the case of *lac* operon, synthesis of mRNA and therefore synthesis of proteins corresponding to *lac*Z, *lac*Y and *lac*A genes do not occur as long as the repressor is bound to the operator. This off condition is clearly the usual state for the *lac* operon and, moreover, makes sense since lactose is not a carbon source frequently encountered by *E. coli*.

Please note that the *lac* operon does not completely shut off even when there is no lactose present. The reason has an evolutionary basis that the cell needs to be prepared in case lactose ever becomes available and so *lac* operon is expressed at very low levels at all times.

Now suppose lactose becomes *E. coli* 's sole carbon source. How is the *lac* operon turned on fully? Since the R protein is made constitutively and is active when synthesized, the cell must have some method for inactivating this repressor. The system is induced to activity by the lactose itself. Slowly, lactose molecules enter the cell and bind to repressor molecules, inactivating them. The inducer (lactose) binds to the repressor's allosteric site and induces a shape change in the repressor that makes it unable to bind to the operator; the repressor molecule actually bound to the operator is forced off the DNA. Now RNAp is able to transcribe *lac*Z, *lac*Y and *lac*A genes into a polycistronic translatable mRNA. And so, after a certain lag period, β-galactosidase, galactoside permease and galactoside acetylase enzymes appear in the cell and *E. coli* metabolizes lactose and grows. As long as the inducer (lactose) is present, synthesis continues, but when lactose is exhausted, the repressor is free to bind again to the operator and turn the system off.

There is a structural analog of lactose that is also capable of inducing the activity of the lac operon. It is isopropylthiogalactopyranoside (IPTG). IPTG is a better inducer than lactose for use in experiments because it gets into the cells readily without help from any permease (this eliminates most of the lag time before enzyme synthesis begins), and it is not a substrate for β-galactosidase so no breakdown occurs inside the cell.

Finally, we must be aware that there is one growth condition under which the *lac* operon cannot be induced by its regular inducer, namely, the presence of glucose in the growth medium. Under conditions of high glucose presence, most of AMP in the cell will be transformed to ATP. This causes the scarcity of AMP and cAMP. Usually cAMP makes a complex with a readily available protein in the cell called CAP or catabolic activator protein. The complex of cAMP and CAP bind to the ABS region of the lac operon and facilitate the attachment and functioning of RNAp. However, if cAMP is scarce causing the absence of the complex cAMP-CAP, RNAp is not bound to the promoter and no *lac* transcription can occur even when inducer is added. This phenomenon of "catabolite repression" by glucose is economical for the cell. The cell makes the numerous glucose-metabolizing enzymes constitutively and survives quite readily on glucose alone so that there is no need to waste energy in the production of enzymes to metabolize other sugars at the same time.

In the above instance, the *lac* operon behaves as a positive control system. There are many other systems similar to the *lac* operon for other sugars in which the

control is by a dual regulation: negative control by the repressor protein and positive control by the catabolite activator protein.

Laboratory Supplies

Freshly-grown *E. coli* NR62 (grown in the morning; very light growth)	6 ml/group
Tube of 100 mM $CaCl_2$	2 ml/group
Sterile distilled water	2 ml/group
Eppendorf tubes	10/group
Automatic pipetters, one set of P20 and P200	1/group
Pipet tips, large and small	1 box of each/group
pBR322 DNA, 5 ng/μl	20 μl/group
pUC19 DNA, 5 ng/μl	20 μl/group
L broth	12 ml/group
L agar plate*	1/group
L agar + Amp plate*	9/group
Spreader and alcohol beaker	1/group
Xgal, 20 mg/ml	500 μl/group
IPTG, 20%	120 μl/group
Sterile glucose solution, 25%	400 μl/group
Water bath at 37 ° C	1/lab
Heat block at 42° C	1/lab
Microfuge	2/lab
Liquid waste beaker	2/table
Discarded pipet tips beaker	2/table

*All plates should be dried at 37°C for 3-4 hours with lids half open.

Procedures

Students at one side of a table will do treatments 1, 3, 5, 7 and 9 and the students on the other side will do 2, 4, 6, 8 and 10.

Preparation of Competent Cells

1. You will be provided with a freshly grown culture of *E. coli* DH5α (NR 62) broth culture. Purposefully, a very light broth is prepared here to give us a chance to be able to count the number of colonies that are produce on the plates.

2. Before you start your exercise, do the following:

   -Obtain an ice bucket, fill it with ice and place the bacterial culture on it.
   -Place a tube of 100 mM $CaCl_2$ solution and a tube of distilled water on ice.
   -Check that the temperature of the heat block (42°C) and water bath (37°C).
   -Label 10 Eppendorf tubes 1 through 10 and place on ice.
   -Practice using micropipetters.

2. After 10 min, dispense 0.5 ml of the culture into each of the 10 ice-cold Eppendorf tubes and place them on ice for another 5 min.

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3. Microfuge all tubes for 1 min, discard the supernatants into the liquid waste beaker and invert the tubes over paper towel for complete drainage. It may not be possible to see pellets at the bottom of the tubes because of the light growth given you.

4. Add 300 μl of ice-cold sterile distilled water to tubes 2, 4, 6, 8 and 10 and 300 μl of $CaCl_2$ to the rest of the tubes. Re-suspend cells by vortexing, then place tubes on ice for 2 min.

5. Microfuge tubes for 1 min, discard the supernatants and add 60 μl of ice-cold water to tubes 2, 4, 6, 8 and 10 and 60 μl $CaCl_2$ to the other tubes. Re-suspend cells again by vortexing, then place tubes on ice for 5 minutes or longer. These are your competent cells.

Transformation of Competent Cells

1. Use the competent cell tubes as shown in the following table:

Tube	Add*	Remarks
1	5 μl of pBR322 DNA	High cation
2	5 μl of pBR322 DNA	No cation
3	5 μl of pBR322 DNA	High cation + glucose
4	5 μl of pBR322 DNA	No cation + glucose
5	5 μl of pUC19 DNA	High cation
6	5 μl of pUC19 DNA	No cation
7	5 μl of pUC19 DNA	High cation + glucose
8	5 μl of pUC19 DNA	No cation + glucose
9	5 μl of water	High cation (Negative Control)
10	5 μl of water	No cation (Positive Control)

*Each μl of pBR322 or pUC19 contains 5 ng of DNA.

2. Gently tap the prepared tubes to mix the competent cells and the plasmid DNA or water and store tubes on ice for 15 min.

3. Heat shock the tubes at 42°C for exactly 90 seconds and return to ice immediately.

4. After 2 min on ice, add 1 ml L broth to each tube, vortex and incubate at 37°C for 30 min or more for the antibiotic genes to express themselves.

5. As incubation of tubes is going on, perform the following:

   - Obtain one L agar plate and 9 L agar + Amp plates and label them.

   - Dip a spreader in alcohol, flame it and evenly spread 50 μl of Xgal on all 10 plates.

Note: **Important.** To sterilize the spreader, dip it in the 95% ethanol jar, touch the spreader to the inside of the jar above the alcohol level to get rid of the extra alcohol and then quickly take the spreader through a flame. Do not hold the spreader in the flame for more than a second. (Our purpose is to kill any bacterium present via the alcohol and not via the heat.) **Be extra careful** as flaming alcohol drops may fall on objects and cause a fire hazard. Also while the alcohol is burning off, keep the spreader head down so the dripping alcohol will not spread to your fingers and hand. Your TA will do a demonstration of this technique for you.

- When the surface of the agar is dry enough, spread 12 μl of IPTG in the same manner on the surface of all plates.

- Again, when the surface of the agar is dry enough, aseptically spread 300 μl of glucose solution on the surface of agar on plates number 3,4,7 and 8. (See above table)

6. Microfuge the Eppendorf tubes for 1 min and discard the supernatant.

7. Resuspend the cells of each tube in 1 ml of sterile, distilled water and spread 50 μl from each tube on the corresponding prepared plate. Note that the L plate (without ampicillin) is used for tube number 10.

8. As soon as the agar surface is dry, invert the plates and incubate them at 37°C for bout 24 hours (never longer). Your instructor will store your plates in the refrigerator for your observation.

   Note: Make sure plates are removed after 20-24 hours of incubation, otherwise satellite colonies will start growing on the plates and interfere with counting the actual colonies that had already grown.

9. Count the number of colonies on each plate and note their color.

Results of Transformation Lab Exercise

NAME _____ DATE _____ GROUP NAME _____

Name(s) of partner(s) _____

1. Draw the pBR322 and pUC19 plasmids and label the origin of replication and the genes present on each. Describe the differences between restriction site locations on the two plasmids.

2. Fill in the following table:

Plate #	Growth (Yes/No)	No. of white colonies*	No. of blue colonies*
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

*If too numerous to count, write TNTC.

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3. Describe why there is a color difference between colonies on plates #1 and #5.

4. Transformation efficiency is defined as the number of transformants per μg of plasmid DNA. You know exactly how much plasmid DNA you used and you have also counted the number of colonies on each plate. Adjust this number to 1 ml of cells spread over the plate. Then calculate the transformation efficiency for tubes 1 and 5. Show your calculations. (Note: If you obtain Transformation Efficiency of 10^3 to 10^5, you have done a great job!)

5. Why is it necessary to include both a positive and a negative control tube in the experiment?

6. Do you see a color difference for the colonies growing on plates 5 and 7? Explain the reason for your observations.

7. Do you see a difference between plates 3 and 7? Explain what this difference is due to.

8. We ordered pUC19 DNA from the Sigma Chemical Company and it arrived in a vial containing 10 μg DNA in a total volume of 22 μl. As you have seen, each group of students uses 20 μl of 5 ng/μl of pUC DNA in their experiment. If you were to prepare an exact amount of pUC to give to the class, assuming 8 groups per class, how would you prepare the material to be used by the class? Use water as your diluent and show all your calculations.