Bio 210A

Bacterial Transformation-Gene Cloning

Purpose
- To understand the relevance of gene cloning to the biotechnology industry.
- To understand the definition of transformation, clone, and cloning vector.
- To differentiate between cloning and expression plasmid vectors.
- To understand the plasmid vector elements necessary for gene cloning: origin of replication, selectable marker, control elements.
- To successfully carry out a transformation/gene cloning procedure.

Introduction
This cloning exercise demonstrates the cloning of a gene into a bacterium using a plasmid. Plasmids are small circular DNA molecules that exist apart from the chromosomes in most bacterial species. Under normal circumstances, plasmids are not essential for survival of the host bacteria. However, many plasmids contain genes that enable bacteria to survive and to prosper in certain environments. For example, some plasmids carry one or more genes that confer resistance to antibiotics. A bacterial cell containing such a plasmid can live and multiply in the presence of the antibiotic. This can occur in nature or by the genetic engineering performed by a molecular biologist.

Ampicillin is an antibiotic that inhibits the growth of bacteria by interfering with the formation of the bacterial cell wall. It is an organic molecule with a Beta-lactam ring at the center of its structure (square ring). The ampicillin resistance gene (amp') encodes for a protein called beta-lactamase which opens the lactam ring deactivating the ampicillin antibacterial properties.

The Chemical Structure of Ampicillin

In this lab, you will perform a procedure known as genetic transformation. Remember that a gene is a piece of DNA which provides the instructions for making (codes for) a protein. This protein gives an organism a particular trait. Genetic transformation literally means change caused by genes, and involves the insertion of a gene into an organism in order to change the organism’s trait. Genetic transformation is used in many areas of biotechnology. In agriculture, genes coding for traits such as frost, pest, or spoilage resistance can be genetically transformed into plants. In bioremediation, bacteria can be genetically transformed with genes enabling them to digest oil spills. In medicine, diseases caused by defective genes are beginning to be treated by gene therapy; that is, by genetically transforming a sick person’s cells with healthy copies of the defective gene that causes the disease.

You will use a procedure to transform the bacteria Escherichia coli (E. coli) with either a gene that codes for Red Fluorescent Protein (RFP), “tomato protein”. The real-life source of the RFP is a Green Fluorescent Protein (GFP) gene from a bioluminescent jellyfish Aequorea victoria. Green Fluorescent Protein causes the jellyfish to fluoresce and glow in the dark. Following the transformation procedure, the bacteria express their newly acquired jellyfish gene and produce the fluorescent protein, which causes them to glow a brilliant green color under ultraviolet light. The RFP, tomato gene, is a modified GFP that is also red in bright light.
In this activity, you will learn about the process of moving genes from one organism to another with the aid of a plasmid. In addition to one large chromosome, bacteria naturally contain one or more small circular pieces of DNA called plasmids. Plasmid DNA usually contains genes for one or more traits that may be beneficial to bacterial survival. In nature, bacteria can transfer plasmids back and forth allowing them to share these beneficial genes. This natural mechanism allows bacteria to adapt to new environments. The recent occurrence of bacterial resistance to antibiotics is due to the transmission of plasmids.

In this activity, you will use the Amgen’s plasmid rpARA as the foreign DNA which carries two genes the tomato gene and the amp’ gene. The rpARA also contains a specific gene regulation system, which can be used to control expression of the tomato gene in transformed cells. The tomato gene can be switched “on” in transformed cells by adding the sugar arabinose to the cells’ nutrient medium to induce expression of the tomato gene for the cells to produce the Tomato protein. To identify cells that have been transformed with rpARA DNA, selection is accomplished by growing bacterial cells on plates containing nutrient agar and the antibiotic ampicillin. Transformed cells will appear white (wild-type phenotype) on plates containing ampicillin and no arabinose, and red when arabinose is included in the nutrient agar medium with ampicillin.

You will be provided with the tools and a protocol for performing genetic transformation. During Lab 1, your task will be to:
1. Take competent cells which are able to take up plasmid and carry out the genetic transformation. Note bacteria can pick up DNA from their surrounding only under certain conditions.
2. Follow the transformation protocol, below.
3. Plate out the cells according to the protocol provided and incubate the plates at 37ºC overnight to allow bacterial cells to grow.

During Lab 2, you will:
4. Collect the data and observe the use of a selection marker in gene cloning
5. Determine the degree of success in your efforts to genetically alter an organism.

Materials
- E. coli starter plate 1
- Microfuge tubes
- Poured agar plates (4 total)
  - 1 LB plates
  - 2 LB+ Ampicillin /Amp plates
  - 1 LB+Amp + Arabinose/Ara plates
- Transformation solution, 50mM CaCl₂ solution 1
- LB nutrient broth (recovery broth) 1
- Sterile inoculation loops 7 (1 pk of 10)
- Micropipettetrs (P-20, P-200, P-1000)
- Foam microtube holder/float 1
- Ice bucket full of crushed ice (foam cup) 1
- Marker pen 1
- Water bath at 42ºC, Incubator, Ice bath
- Sterile supplies: pipettes, microfuge tubes, tips, blue tips, hockey sticks (bent glass rods or Pasteur pipettes)
Procedure

1. Take 2 microcentrifuge tubes and label them ‘+’ and ‘-’. (This ‘-’ tube will act as a control because it will not be exposed to the plasmid; therefore, it will NOT take up the gene).
2. Place 250uL of ice cold transformation solution into each tube, and place the tubes in ice.
3. Pick one colony of *E.coli* from the culture plate with a sterile loop and mix it into the ‘+’ tube. Be sure that all the colony material on the inoculating loop comes off into the transformation solution. Repeat for the ‘-’ tube with another sterile loop. Place both microfuge tubes on ice.
4. Now using separate sterile pipette tips, add 10uL of rpARA plasmid to ‘+’ tube and 10uL sterile water to ‘-’ tube. Mix them well and keep them on ice for 10 mins. This treatment stresses the bacterium, rendering its cell membrane and cell wall permeable to the plasmid. This process will make the recipient *E.coli* "competent" to uptake the plasmid.
5. Place both the tubes from the ice container into the water bath at 42°C for 50 sec. The transfer from ice to the water bath should be immediate. This step is called the "Heat-Shock" step, and it will maximize the uptake of the plasmid through the wall and membrane of the cells.
   When incubating the tubes in the water bath, be sure that the tube bottoms are all the way into the water below the rack.

Carry the tubes over to the water bath in the ice and replace them when done.

6. After 50 sec., place these tubes immediately on ice for 2 min.
7. At room temperature, add 250uL of recovery media (LB broth) to the both tubes, mix gently, and leave at room temperature for 15mins.
   Transfer 200 uL of the mixture in the ‘-’ tube to the following plates and spread it with the help of sterile loop.
   Plate 1:  ‘-’  LB Plate  
   Plate 2:  ‘-’  LB+Amp Plate

Gently spread the content over the media surface in the plate with the help of a sterile loop. Use different sterile loop for each plate.
8. Now transfer 200 uL of the mixture in the ‘+’ labeled tube content to the following plates.
   Plate 3:  ‘+’  LB+Amp Plate  
   Plate 4:  ‘+’  LB+Amp+Arabinose Plate

9. After 30 min., invert these plates and incubate at 37°C for 24hrs.
Next Lab Period
10. After incubation, observe these plates using visible light.

<table>
<thead>
<tr>
<th>Agar Type</th>
<th>E. coli</th>
<th>Number of Colonies</th>
<th>Color of colony using bright light for rpARA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB Plate</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LB+Amp</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LB+Amp+Arabinose</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>LB+Amp+Arabinose</td>
<td>+</td>
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</tbody>
</table>
A. In-class Questions (Lab 1)

Before collecting data and analyzing your results, answer the following questions.

1. On which of the plates do you expect to find bacteria most like the original non-transformed *E. coli* colonies you initially observed? Explain your predictions.

2. If there are any genetically transformed bacterial cells, on which plate(s) would they most likely be located? Explain your predictions.

3. Which plates should be compared to determine if any genetic transformation has occurred? Why?

4. What is meant by a control plate? What purpose does a control serve?
B. In-class Analysis of Results (Lab 2)
The goal of data analysis for this investigation is to determine if genetic transformation has occurred.
1. Which of the traits that you originally observed for *E. coli* did not seem to become altered? In the space below list these untransformed traits and how you arrived at this analysis for each trait listed.

<table>
<thead>
<tr>
<th>Original trait</th>
<th>Analysis of observations</th>
</tr>
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</table>

2. Of the *E. coli* traits you originally noted, which seem now to be significantly different after performing the transformation procedure? List those traits below and describe the changes that you observed.

<table>
<thead>
<tr>
<th>New trait</th>
<th>Observed change</th>
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</table>

3. If the genetically transformed cells have acquired the ability to live in the presence of the antibiotic ampicillin, then what might be inferred about the other genes on the plasmid that you used in your transformation procedure?

4. From the results that you obtained, how could you prove that the changes that occurred were due to the procedure that you performed?