GENETIC ENGINEERING

RECOMBINANT DNA
GENETIC ENGINEERING
PART I: PRODUCING A STRAIN OF *E. coli* THAT GLOWS IN THE DARK

Introduction – Plasmids

Penicillin is one of the most important anti-infective agents used in clinical medicine because it is inexpensive, a bactericidal, and its toxicity for human cells is almost nonexistent. Penicillin interferes with the synthesis of the bacterial cell wall and will thus cause osmotic lysis of susceptible microbes. Penicillin is not a single compound but a group of compounds with related structures and activities. Many of these compounds are semi-synthetic in that part of each molecule is made by a mold to which the chemist adds another chemical group. Over 500 semi-synthetic penicillins have been made during the past 30 years. Ampicillin is a broad-spectrum semi-synthetic penicillin that will kill a number of bacterial species including *Escherichia coli*. Occasionally, *E. coli* cells are found in nature that are resistant to the toxic effects of ampicillin. In this lab, you will create such an ampicillin-resistant population of *E. coli*.

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 drug. Indeed, antibiotic-resistant *E. coli* isolated in many parts of the world contain plasmids that carry the genetic information for protein products that interfere with the action of many different antibiotics. In this laboratory, you will introduce a plasmid that contains an ampicillin resistance gene into *E. coli*.

One plasmid that you will use in this lab is called pUC18. A map of the basic structure of pUC18 is shown below.

![Partial Map of Plasmid pUC18](image-url)
Plasmid pUC18 is a circular DNA molecule that contains only 2,686 nucleotide pairs. The small size of this plasmid makes it less susceptible to physical damage during handling. In addition, smaller plasmids generally replicate more efficiently in bacteria and produce larger numbers of plasmids per cell. As many as 500 copies of this plasmid may be present in a single *E. coli* cell. Plasmid pUC18 contains an ampicillin-resistance gene that enables *E. coli* to grow in the presence of the antibiotic. Bacteria lacking this plasmid, or bacteria that lose the plasmid, generally will not grow in the presence of this antibiotic. The ampicillin resistance gene of pUC18 codes for the enzyme β-lactamase (penicillinase) which inactivates ampicillin and other penicillins.

In the laboratory, plasmids can be introduced into living bacterial cells by a process known as transformation. When bacteria are placed in a solution of calcium chloride, they acquire the ability to take in plasmid DNA molecules. As illustrated below, this procedure provides a means for preparing large amounts of specific plasmid DNA since one transformed cell gives rise to a clone of cells that contains exact replicas of the parent plasmid DNA molecule. Following growth of the bacteria in the presence of the antibiotic, the plasmid DNA can readily be isolated from the bacterial culture.
Plasmids are very useful tools for the molecular biologist because they serve as gene-carrier molecules. A basic procedure of recombinant DNA technology consists of joining a gene of interest to plasmid DNA to form a hybrid, or recombinant molecule that is able to replicate in bacteria. This is illustrated in the following diagram.

**Formation and Amplification of a Recombinant DNA Molecule**

<table>
<thead>
<tr>
<th>Cell DNA</th>
</tr>
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<tbody>
<tr>
<td>AATT----TTAA--</td>
</tr>
<tr>
<td>EcoR1</td>
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<table>
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<tr>
<th>Linear Plasmid DNA Molecule</th>
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<tbody>
<tr>
<td>AATT----TTAA--</td>
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<tr>
<td>Anneal and seal nicks with DNA ligase</td>
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<table>
<thead>
<tr>
<th>A recombinant DNA molecule composed of plasmid DNA &amp; cell DNA insert</th>
</tr>
</thead>
<tbody>
<tr>
<td>AATT----TTAA--</td>
</tr>
<tr>
<td>AATT----TTAA--</td>
</tr>
<tr>
<td>AATT----TTAA--</td>
</tr>
</tbody>
</table>
In order to prepare a recombinant molecule, the plasmid and gene of interest are cut at precise positions by specific deoxyribonucleases (restriction endonucleases) and then the molecules are spliced together. After the hybrid plasmid molecule has been prepared, it is introduced into *E. coli* cells by transformation. The hybrid plasmid replicates in the dividing bacterial cells to produce an enormous number of copies of the original gene. At the end of the growth period, the hybrid molecules are purified from the bacteria and the original gene of interest is recovered. This method has enabled scientists to obtain large quantities of more than 1,000 specific genes including the genes for human interferon, insulin, and growth hormone.

B. The *lux* Operon

The emission of light by living organisms is a fascinating process. Luminescent bacteria are the most abundant and widespread of the luminescent organisms found in nature. The genes responsible for light emission in a few of these organisms have been well characterized. The genetic system required for luminescence in the bacterium *Photobacterium (Vibrio) fischeri* is the *lux* operon. This operon contains two genes that code for luciferase (the enzyme that catalyzes the light-emitting reaction) and several genes that code for enzymes which produce the luciferins (which are the substrates for the light-emitting reaction). A genetic map of this operon is shown below.

**The *lux* Operon**

<table>
<thead>
<tr>
<th>R</th>
<th>T</th>
<th>C</th>
<th>D</th>
<th>A</th>
<th>B</th>
<th>E</th>
<th>G</th>
<th>Rib</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1</strong></td>
<td>Kb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The *lux* operon contains two genes for the luciferase enzyme (A and B). This enzyme is composed of two different polypeptide chains. The operon also contains several other genes (R, T, C, D, E, G, and Rib) that are thought to code for enzymes which produce the substrates for the light-emitting reaction. These substrates are called luciferins and are long chain fatty aldehydes.

C. The Lab

**Objective:** In this lab, you will create a luminescent population of bacteria by introducing into *E. coli* a plasmid that contains the *lux* operon. The success of the transformation is readily apparent since the *E. coli* colonies that take up this plasmid glow in the dark. You will also introduce a control plasmid into *E. coli* that does not contain the *lux* operon and these cells will not glow in the dark. Both plasmids contain an ampicillin resistant gene and therefore both cell types will grow in the presence of the antibiotic.
Materials
Control plasmid (plasmid pUC18)  Sterile tubes
Plasmid Lux  CaCl₂ solution
Ampicillin-nutrient agar plates  E. coli
Nutrient broth  37°C water bath
Inoculating loops  Ice water bath
Sterile transfer pipets

Procedure:

I. Preparation of Competent Cells (performed by instructor)
   1. Place the vial of CaCl₂ and the tube of E. coli in the ice bath.
   2. Using a sterile pipet, transfer about ½ mL of the CaCl₂ solution to the tube containing the bacteria.
   3. Using the same pipet, transfer the contents of this tube back into the vial that contains most of the CaCl₂ solution.
   4. Tap the vial with the tip of your index finger to mix the solution.
   5. Incubate the cells for about 10 minutes on ice. The cells are then called competent because they can take up DNA from the medium. If desired, the cells can be stored in CaCl₂ for 12 to 24 hours on ice before use.

II. Uptake of DNA by Competent Cells
   6. Label one small tube “C DNA” (for control) and one tube “L DNA” (for plasmid lux).
   7. Place the two tubes in an ice bath.
   8. Using a sterile micropipet, add 10 μL of the control plasmid to the tube labeled “C DNA” and 10 μL of plasmid lux to the tube labeled “L DNA”.
   9. Gently tap the tube of competent cells with the tip of your index finger to ensure that the cells are in suspension. Then, using a sterile transfer pipet, add 120 μL of the competent cells to each of the two tubes. Tap each of these tubes with the tip of your index finger to mix these solutions and store both tubes on ice. The competent cells, which are suspended in CaCl₂, will now begin to take up the plasmid DNA.
   10. To prepare a control with no plasmids, 120 μL of competent cells need to be added to each of two tubes labeled “NP” for no plasmid.

III. Recovery
   11. Transfer the tubes to the 37°C water bath for 5 minutes.
   12. Add 0.7 mL of nutrient broth to each tube and incubate at 37°C for 30 minutes. The nutrient broth should be dispensed with a sterile pipet. This incubation period allows the bacteria time to recover from the CaCl₂ treatment and to begin to express the ampicillin-resistance gene on the plasmid.
IV. Plating the Bacteria for Selection of Cells that have taken up the Plasmid

13. Obtain two ampicillin-nutrient agar plates and label one plate “C DNA” and the other plate “L DNA”.
14. Using a sterile pipet, remove 0.25 mL of the mixed bacterial suspension from the “C DNA” tube, remove the lid from the “C DNA” plate and dispense the bacteria onto the agar. Use an inoculating loop to spread the bacteria evenly onto the agar surface. Be careful not to break the surface of the agar.
15. Transfer 0.25 mL of the bacterial suspension from the “L DNA” tube to the “L DNA” plate and spread these cells onto the agar surface as described above.
16. Cells from the two tubes labeled “NP” should also be plated on two plates labeled “NP”.
17. Replace the lids on the plates and leave the plates at room temperature until the liquid has been absorbed (about 10 to 15 minutes).
18. Invert the plates and incubate them in the dark at room temperature.

Analysis and Questions

1. Colonies should appear in about 2 or 3 days at room temperature. Plates must be viewed at that time since bioluminescence decreases with time after colony formation. Allow at least 3 minutes for the eyes to adjust to the dark in a light-free room. View your plates and the plates of your classmates in the dark and then in the light and record your results below.

<table>
<thead>
<tr>
<th>Plate</th>
<th># of Colonies</th>
<th>Bioluminescent colonies (+ or -)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP: No Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C DNA: Control Plasmid (pUC18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L DNA Plasmid lux</td>
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</tr>
</tbody>
</table>

2. Why do the cells transformed with pUC18 and plasmid lux grow in the presence of ampicillin?
3. Name one enzyme that is produced by cells transformed with plasmid lux that is not produced by the cells transformed with pUC18.

_________________________________________________________________

4. A student has forgotten which antibiotic plasmid she used in her E. coli transformation. It could have been kanamycin, ampicillin, or tetracycline. She decides to make up a special set of plates to determine the type of antibiotic used. The plates below show the results of the test.

Plate I. LB agar with kanamycin  Plate II. LB agar with ampicillin  Plate III. LB agar with tetracycline  Plate IV. LB agar

Which antibiotic plasmid has been used? ______________________________________

How do you know? ____________________________________________________________

_________________________________________________________________

5. In a molecular biology laboratory, a student obtained competent E. coli cells and used a common transformation procedure to induce the uptake of plasmid DNA with a gene for resistance to the antibiotic kanamycin. The following results were obtained:

Plate I. LB agar + kan plasmid  Plate II. LB agar with kanamycin + kan plasmid  Plate III. LB agar no plasmid added  Plate IV. LB agar with kanamycin no plasmid added

On which petri dish do only transformed cells grow? _____________________________

Which of the plates is used as a control to show that nontransformed E. coli will not grow in the presence of kanamycin?

_________________________________________________________________
6. Match the definition or description with the correct term.

A. DNA ligase  
B. Restriction enzyme  
C. Restriction fragments  
D. Restriction site  
E. Sticky end

_____ Enzymes that protect bacteria against the invasion of foreign DNA by cutting foreign DNA at specific sites
_____ A symmetrical sequence of 4 to 8 nucleotides recognized by restriction enzyme
_____ Pieces of DNA produced when the DNA is subjected to restriction enzymes
_____ The single-stranded end found on DNA fragments
_____ The enzyme that fuses pieces of DNA

7. Match the definition or description with the correct term or process.

A. DNA fingerprint  
B. DNA sequencing  
C. Gel electrophoresis  
D. Polymerase chain reaction  
E. Restriction fragment length polymorphisms  
F. Southern blotting  
G. Transgenic organisms

_____ The separation of nucleic acids or proteins, on the basis of their size and electrical charge, by measuring their rate of movement through an electrical field in a gel
_____ Differences in DNA sequence on homologous chromosomes that result in different patterns of restriction fragment lengths; useful as genetic markers for making linkage maps
_____ A technique for amplifying DNA in vitro by incubating with special primers, DNA polymerase molecules and nucleotides
_____ The process of determining the sequence of bases in a section of DNA
_____ A hybridization technique that enables researchers to determine the presence of certain nucleotide sequences in a sample of DNA
_____ Organisms that contain genes from another species
_____ A specific pattern of bands produced through electrophoresis that is of forensic use because the probability that two people would have the exact same set of RFLP markers is very small
8. The gel at the right was produced from four samples of single-stranded DNA fragments that were incubated with radioactively labeled primer, DNA polymerase, the four nucleotides, and a different one of the four dideoxy nucleotides.

What is the sequence of nucleotides shown in this gel?

__________________________________________________________

What is the sequence of nucleotides in the original single-stranded DNA fragment?

__________________________________________________________

9. What is satellite DNA and how is it used as markers for DNA fingerprinting?

__________________________________________________________

__________________________________________________________

__________________________________________________________

__________________________________________________________