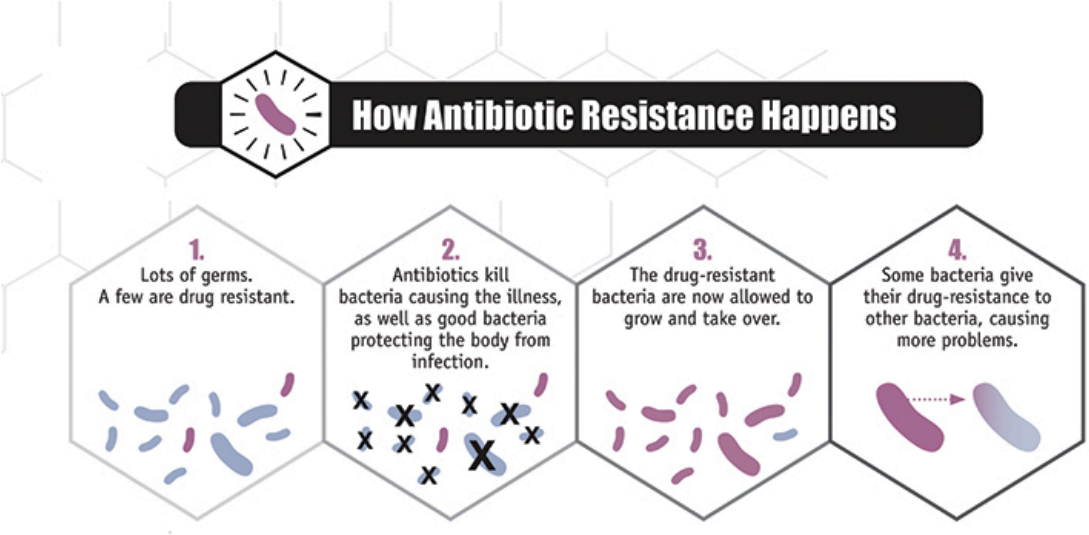
Transformation [1]



http://www.cdc.gov/drugresistance/about.html

Antibiotic resistance is a critical problem in healthcare today. How do bacteria become resistant to antibiotics? In this lab we will focus on #4 from the CDC infographic above. Some bacteria can transfer genes from one bacterium to another in a process called "conjugation" or may pick up genes from the surrounding environment (from dead bacteria), via "transformation". These genes, often on a plasmid, may code for antibiotic resistance mechanisms.

Learning Objectives:

After this lab you should be able to:
1. Define the vocabulary related to transformation, recombinant plasmid, selectable marker, vector, and competent cell, genetic engineering, gene regulation, etc.
2. Relate the mechanism of genetic recombination, specifically transformation, to the problem of antibiotic resistant bacterial infections.
3. Describe how gene regulation can be affected by a bacterium's environment.
4. Outline the process of transformation in bacteria.
5. Evaluate the outcomes of the bacterial transformation activity.

Introduction:

This lab will demonstrate principles of-
1. Bacterial reproduction
2. Genetic recombination in bacteria
3. Transformation as a method of genetic exchange and recombination
4. Transformation and genetic exchange as a vehicle of antibiotic resistance
5. Gene regulation in bacteria
6. Genetic engineering and recombinant organisms

Fred Griffith, a British microbiologist, discovered in 1928 that virulence could be transferred from killed pathogenic bacteria to live non-pathogenic bacteria.[2] He called this process "transformation". In 1944, Oswald Avery and his research group discovered that DNA was the factor being transferred.[3] These scientists had discovered and defined transformation, one of the mechanisms of bacterial recombination (Joshua Lederberg and others discovered the other two methods, conjugation and transduction). This was also the beginning of molecular biology and genetic engineering.

Today we know that genetic recombination in bacteria can be a source of antibiotic resistance. In addition, genes that code for antibiotic resistance mechanisms-like enzymes that break down the antibiotic in the bacterium-are carried on plasmids.

In this lab you will demonstrate the processes of transformation and gene regulation in bacteria, and genetic engineering, by forcing a bacterium to take up plasmid DNA from its environment (transformation) and express a gene (gene regulation) it would not normally have (genetic engineering). Typically, a very low percentage of bacterial cells will actually be transformed during this process. Some will successfully take up the plasmid; others may reject the plasmid in one of a few ways. But remember, most bacteria can grow so fast that, even if only one in a million are transformed with a plasmid that gives the cell resistance to an antibiotic, one cell can be millions in a matter of hours!

There are two sides to the process in the lab you will do. First of all, you will insert a plasmid (called pAMP) that contains a gene (*amp*^{R}) that confers resistance to the antibiotic Ampicillin into *E. coli*. Therefore, the *E. coli* is transformed and if subsequently exposed to Ampicillin it will not be affected and will grow as usual. Transformation happens in nature and is a vehicle for the spread of antibiotic resistance in bacteria. In a lab, antibiotic resistance genes can be paired with other genes on the same plasmid. These are called recombinant plasmids because they have been constructed from genes of different organisms. One could insert a plasmid that contains a specific gene for a desirable protein. But, it might be difficult to know which bacteria have successfully taken up the plasmid and the gene(s) it contains. To be efficient, one would want to select the bacteria that can make the protein (called transformants--the bacteria that have been successfully transformed), and not end up with bacteria that cannot. Therefore, if an antibiotic resistance gene is paired with the other gene (the gene of interest), then the acquisition of resistance, which is easily tested, indicates the acquisition of the gene of interest. The cells resistant to Ampicillin are then "selectable markers" for the other gene.

When I was a grad student at UCLA, I engineered a custom recombinant plasmid to express genes that I had mutated. The plasmid had the same amp^R *gene as pAMP and pGLO, so I was able to select for transformants by plating the cells on media that contained ampicillin. After inserting the mutated gene into the plasmid, I transformed E. coli cells and conducted growth experiments. My plasmid eventually became very popular with other researchers in the Microbiology, Immunology and Molecular Genetics Department, who nicknamed it 'pUC-Shane'!*

Dr. C. Shane Ramey

The second aspect of today's process, then, is that the plasmid with Ampicillin resistance also carries the gene (the gene of interest) to produce green fluorescent protein (called pGLO which has the *AMP*^{R} and *gfp* genes-see Fig. 1), which glows when exposed to UV light. Cells that have pGLO will fluoresce only when under the correct environmental conditions, specifically the presence of the sugar Arabinose. You will use the antibiotic resistance property to select for cells that, potentially, will fluoresce. Then, we'll feed the transformed bacteria Arabinose in order to "turn on" the genes for fluorescence. The presence of the Arabinose sugar is the environmental switch that helps regulate the expression/production of the green fluorescent protein. As you will observe in today's lab, bacteria plated onto media that contains Ampicillin will grow, and if the

media also contains Arabinose, they will glow! You will have successfully selected for a bacterium that you actually engineered to fluoresce.

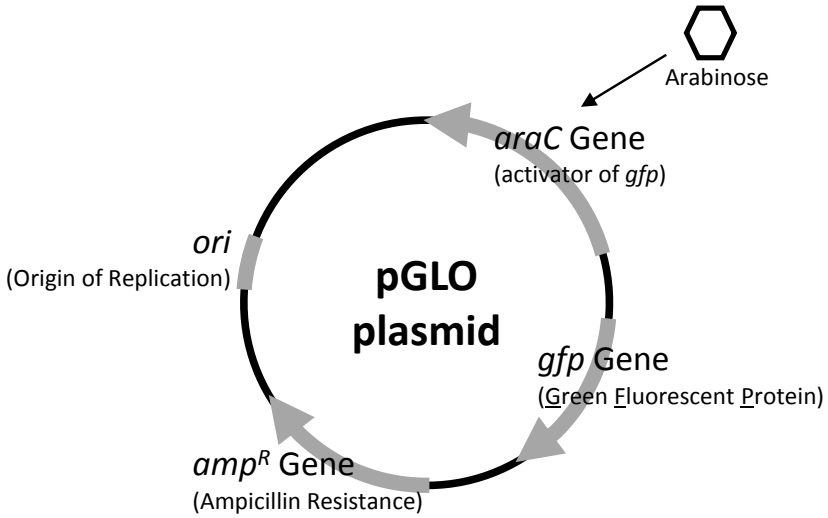


Figure 1. Components of the pGLO plasmid (C.S. Ramey)

In nature, not all bacterial cells can undergo transformation. Those that are able are called "competent" cells. In the laboratory, cells can be encouraged to undergo transformation by altering their environment and cells temporarily so that they will uptake DNA; they become competent due to lab manipulation. The method you will use is the calcium chloride/ heat shock procedure (see Figure 2). The positive Ca ions released in the solution will neutralize the negative charge on the DNA molecule (a plasmid in our case), which reduces the charge barrier for the DNA entering the cell (remember that cells have a net negative charge). Heating the cells increases the permeability of the bacterial membrane. Thus, DNA will more readily enter the bacterial cells and transformation may proceed. These steps must be done quickly in order not to damage or kill the cells. Later, you will calculate the "Transformation Efficiency" obtained by your bacterial culture. Not all cells will become competent, nor will all competent cells complete transformation.

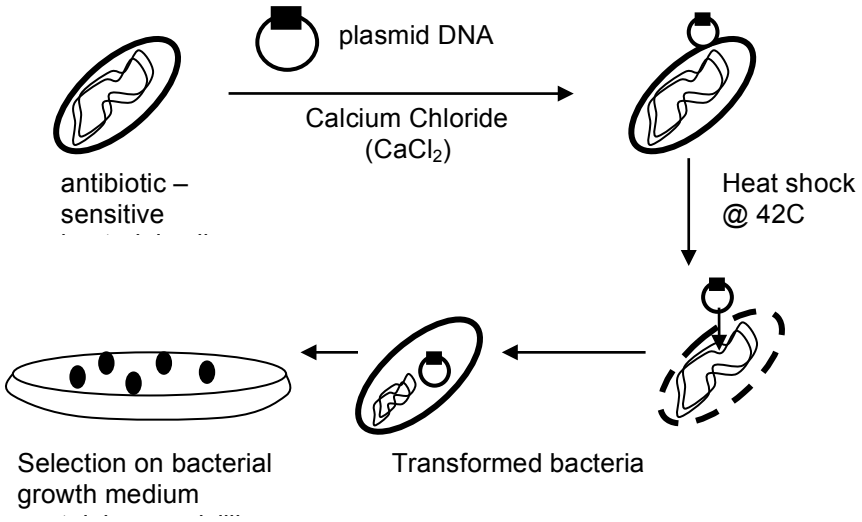


Figure 2: Bacterial Transformation. (K. Cude)

Pre-lab questions:

1. Define the terms transformation, recombinant plasmid, and competent cell.

2. Investigate green fluorescent protein, online, and describe what it means to "fluoresce". Did you find any unusual applications of genetically modifying organisms with this protein?

3. Explain the use of antibiotic resistance as a "selectable marker".

4. Predict what will happen if transformed cells are fed only:
a. Glucose

b. Glucose and arabinose

c. Glucose and sucrose

Materials:

Day 1-
200 ul micropipette (P200)
1000 ul micropipette (P1000)
Pipette tips
Microfuge tubes
E. coli culture (HB101 strain)
Ice bath
42C water bath
37C water bath
50mM $CaCl_2$ (keep ice-cold)
10 ul pGLO (plasmid concentration 0.2 µg/ µl)
Sterile LB broth
1 LB agar plates
2 LB+AMP
1 LB+AMP+Arabinose agar plates
2 Sterile Spreaders-small
Day 2-
Your inoculated plates from Day 1
UV lights

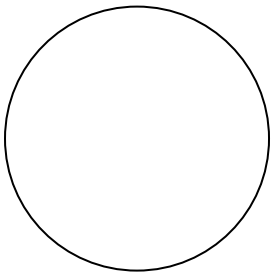
Procedures:

1. Label two microfuge tubes with the following: one as P+ (plasmid) and the other, P- (no plasmid, control).
2. Using a P1000 micropipette, add 500 ul of ice-cold 50mM $CaCl_2$ to one of the tubes, you will split this volume into both tubes later so it doesn't matter which tube you start with.
3. Using a sterile inoculating loop, transfer one to three isolated colonies of the starter *E.coli* colonies (a colony approximately the size of an o) into the tube.
4. Using a P200 micropipette and a clean pipette tip, suspend the cells by gently pipetting the solution in an out several times. Hold the tube up to the light to check for any cell clumps.
5. Now you will split this volume, half in each tube. Using a P1000 micropipette, remove 250 ul of the suspension and place it in the second tube so that you now have 250 ul of the suspension in both the P+ and P- tubes.
6. Replace the cap on the tubes and return them to the ice bath.
7. Incubate both tubes (P+ and P-) in ice for at least one minute.

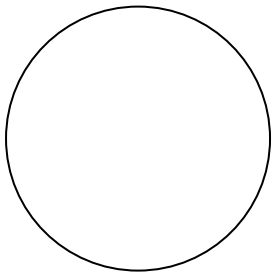
8. Add **10 ul of pGLO (plasmid concentration 0.2ug/ ul)** to the re-suspended cells in the **P+ tube only**. Mix the cells with the plasmid by gently and slowly pumping the solution in and out using a clean pipette tip. Return the P+ tube to the ice bath.
9. Incubate both tubes in ice for **20 minutes.**

10. While the tubes are incubating obtain 1 LB, 2 LB+AMP, and 1 LB+AMP+Arabinose agar plates and label them:
    a. Label the **bottom of the plates** of one set LB and LB+AMP with P-

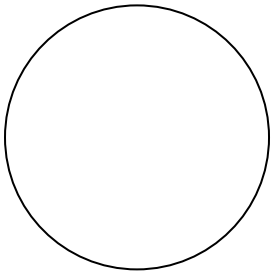
    b. Label the **bottom of the plates** of other set LB+AMP and LB+AMP+Arabinose with P+



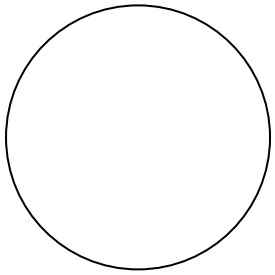
LB **(P-)**



LB +AMP **(P-)**



LB +AMP **(P+)**



LB +AMP+Arabinose **(P+)**

11. Following the 20-minute incubation in ice, carry your ice bath with the cells to the 42C water bath. Take the tubes directly from the ice bath and place them in the 42C water bath for 90 seconds to heat-shock the cells. Immediately afterward place the cells back into the ice bath for 2 more minutes.
12. Using a P1000 micropipette, and a clean pipette tip add 500 ul of sterile LB to the P+ and then with a new pipette tip, add 500ul of sterile LB to the P- tube. Gently, finger vortex (flick the tube with your finger) the tubes to mix the cells and broth together. Incubate the cells in the 37C water bath for 10 minutes.

13. After the 37C incubation period the cells are ready to be plated out onto agar.
    -Line up the two P- plates as shown in the above picture. Using a P200 micropipette, transfer 50 ul of cells from the P- tube to each of the two plates. Make sure to evenly spread the drops across the agar surface
    -Use a sterile cell spreader and rotate the plates to evenly distribute the culture across the agar. *Place the contaminated cell spreaders in the pipette disposal trays.*
14. Using a new micropipette tips and a sterile cell spreader, repeat the above procedure for the P+ plates by inoculating with the cells from the P+ tube.
15. Allow plates to dry, right side up, for about 5 minutes. Then place inverted plates in your incubator. Discard tubes in the biohazard containers.

Results:

1. After incubation, count and record the number of colonies on each plate.
2. While wearing safety goggles and in a dark place, illuminate the plates with the hand held UV light to determine which colonies are producing the Green Fluorescent Protein. Record results.

Table 1: Number of Transformed Colonies.

CELLS	PLATE	Growth? Y/N	Glowing? Y/N	AGAR PLATE Growth?	Growth? Y/N	Glowing? Y/N
Plasmid - (P-)	LB # of colonies:			LB +Amp # of colonies:		
Plasmid + (P+)	LB +AMP # of colonies:			LB +Amp + Arabinose # of colonies:		

3. Transformation Efficiency:

Show all your work. Include word equations, show substitutions, and include units!

a) What was the total mass (µg) of plasmid DNA added to the cell suspensions? Note in the experiment you used 10 µl of plasmid (step 8) that had a concentration of 0.2 µg /ml.

b) Using the information from a) and your colony count of transformed cells, calculate the number of colonies transformed per microgram of plasmid DNA. This is the **Transformation Efficiency** of your transformation event.

$$TE = \frac{\text{\# of transformed colonies}}{\text{ug DNA}}$$

Questions:

1. Explain how the bacterial cells, normally not competent, were made competent during the procedure.

2. Differentiate between the purposes of each of the 4 plates used in the exercise.

3. Contrast the uses of the antibiotic and arabinose in the exercise.

4. Why did the P- bacteria grow on the LB agar without ampicillin and not in the Petri dish with the LB agar with the ampicillin?

5. For the P+ plates, why did colonies "glow" on one plate and not the other?

6. How is the use of Arabinose a means of gene regulation?

7. *E. coli* cells can double about every 20 minutes with the proper environmental conditions. How long would it take a single transformed cell to become 100 million cells, all with resistance to ampicillin?

Conclusion:

Reflect on why transformation is potential method of increasing antibiotic resistance in populations of bacteria and on how this, and the lab exercise, reinforces the principles of aseptic technique in a hospital, home, lab, etc.

Resources:

1. Cude, K., Golbert, M. (2015). Transformation. In *Bio 107: Molecular and Cellular Biology Laboratory Manual*. 14th Ed.: (89-99). College of the Canyons. Adapted and used with permission.

2. Griffith, F. "The Significance of Pneumococcal Types." *The Journal of Hygiene* 27.2 (1928): 113–159. Print. Web. 12 November 2015 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2167760/

3. Avery, O. T., et al. "Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolation from pneumococcus type III." *The Journal of Experimental Medicine* 79.2 (1944): 137–158. Print. Web. 12 November 2015 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2135445/