

GROWING BACTERIA

INTRODUCTION

E. coli is a normal part of the bacterial flora of the human gut. It is not generally considered pathogenic, although some strains are highly toxic (recent food poisonings at Jack-In-The-Box for example). The strains commonly used for teaching laboratory work, such as MM294 that you will use throughout this semester, are ineffective at colonizing the human gut. Adherence to simple guidelines for handling and disposal makes work with *E. coli* a non-threatening experience.

Bacteria require a nutrient medium to grow. You will be using the Luria-Bertani (LB) recipe which provides the necessary carbohydrates, amino acids, nucleotide phosphates, salts and vitamins commonly used to propagate *E. coli*. The LB nutrients can be made up in water to give a liquid called **LB broth**. They can also be made up in an agar solution that is liquid at 60°C or above, and solidifies into a solid nutrient medium at cooler temperatures. This type is commonly poured into petri plates and called **LB agar**. Antibiotics may be added to either LB medium to select for desired characteristics in the bacteria.

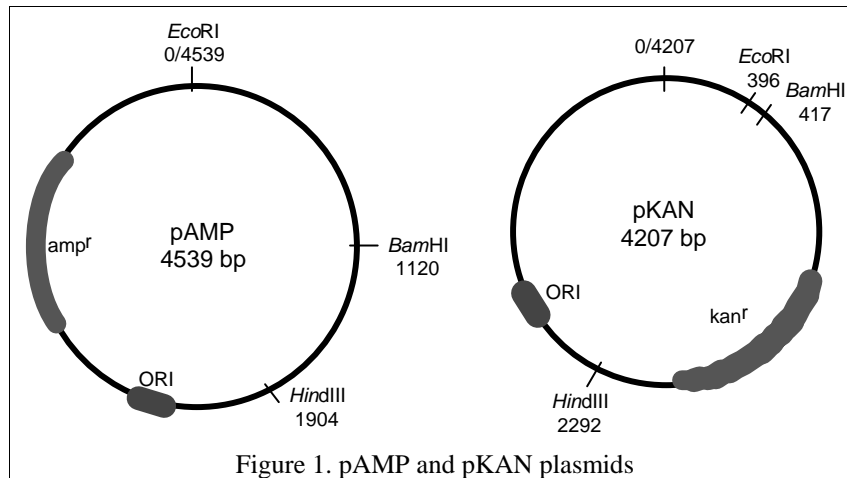
ANTIBIOTIC RESISTANCE AND PLASMIDS

Plasmids are small circular DNA molecules that occur in bacterial cells. They reproduce independently from the chromosome, but are sometimes controlled by chromosomal genes. In some cases, plasmids can even become integrated into the host's chromosome. Plasmids can carry a variety of different genes, for example, genes that provide the host with resistance to antibiotics, heavy metals, and other inhibitory compounds.

A very large number of plasmids are known; approximately 300 different naturally occurring ones have been isolated from strains of *E. coli* alone. In some cases, plasmids encode for properties which are thought to be fundamental to the bacterium in question. For example, the ability of leguminous plants to form root nodules that fix atmospheric Nitrogen into organic molecules (Nitrogen fixation), depends upon infection by the symbiotic *Rizobium* bacterium. The ability of *Rizobium* to induce root nodule formation depends on them carrying a *Sym* plasmid.

Among the most widespread and well-studied groups of plasmids are the *resistance plasmids* (R plasmids) which confer resistance to antibiotics and various other inhibitors of growth. A variety of antibiotic-resistance genes can be carried by an R plasmid. In general, these genes encode proteins that either inactivate the antibiotic, or affect its uptake into the cell. For example, plasmid R100 is a plasmid of 89,300 base pairs that carries resistance genes for sulfonamides, streptomycin, fusidic acid, chloramphenicol, and tetracycline. R100 also carries several genes conferring resistance to mercury, and a gene that allows it to transfer itself between enteric bacteria of the genera *Escherichia*, *Klebsiella*, *Proeus*, *Salmonella*, and *Shigella*. R plasmids with genes for resistance to kanamycin, penicillin, tetracycline, and neomycin are also known.

Plasmids are routinely used by molecular biologists to clone and transfer pieces of DNA that are of interest. They have been carefully designed and usually carry one antibiotic resistant gene to serve as a marker for the plasmids detection. These "engineered" plasmids do not carry genes that give them the ability to transfer themselves to other bacterial cells. They are passed on, however, to all the descendants of an infected cell (a clone).



Two examples of plasmids you will be using in this and future labs are shown in Figure 1: **pAMP** confers resistance to ampicillin (a member of the penicillin family); and **pKAN** confers resistance to kanamycin. pAMP is the larger of the two at 4,539 base pairs. Maps of both these plasmids are shown below, including cutting sites for *EcoRI*, *BamHI*, and *HindIII* restriction enzymes. Location of the antibiotic resistant genes and the origins of replication (ORI) are also shown. A plasmid must have at least one ORI in order to replicate within a cell.

HOW ANTIBIOTIC RESISTANCE WORKS

Antibiotics in the penicillin family (including ampicillin) interfere with cell wall synthesis and thereby prevent reproduction of the bacteria. Since human cells don't have to make cell walls, the drug does not inhibit or harm human cells. The ampicillin resistance gene (*amp^r*) carried on the pAMP plasmid produces an enzyme that specifically deactivates the ampicillin molecule. The kanamycin molecule irreversibly binds to the smaller ribosomal subunit, and prevents protein synthesis in the host cell. Without protein synthesis, the cell cannot reproduce. Kanamycin only combines with the smaller ribosomal subunit of prokaryotic cells and not those of eukaryotes. This of course makes it a suitable drug for use in humans. The kanamycin resistance gene (*kan^r*) codes for a protein that specifically adds phosphate groups to the kanamycin molecule, thereby blocking its ability to bind with the ribosome.

PREPARATION AND CLEAN-UP

1. Before starting lab work with bacterial cultures, remove all materials from the top of your lab bench, and wash with lab disinfectant. Allow to air dry. The instructor will demonstrate this.
2. Set-up a bio-hazard bag at your workspace.
3. Repeat the process when you are finished working with bacteria.
4. Properly dispose of bio-hazard bag.
5. Always wear gloves when handling bacteria

PREPARING AN "OVERNIGHT" CULTURE IN LB BROTH

Often it is necessary to grow large numbers of bacteria from a single colony. This is most easily done in an "overnight" culture in liquid broth. Bacteria divide so rapidly that just a few cells introduced into a container of broth will multiply into trillions and have used up all of their nutrients when incubated overnight.

EQUIPMENT

- MM294 *E. coli* culture in LB broth in 1.5 ml microfuge tube
- 1 test tube containing sterile LB broth
- Inoculating loop

PROCEDURE

1. Label the LB broth tube with your name.
2. Flame the loop and cool it by dipping it in the *E. coli* culture.
3. After the loop has cooled, swirl it around once or twice and carefully remove the loop and insert it into the LB broth and gently shake it.
4. Incubate inoculated tube for at 37°C for 24 h.

STREAK PLATE

Growing bacteria on agar plates is especially useful in isolating clones of cells. A colony visible to the naked eye will develop within 24-48 hours wherever a single cell lands on the agar surface. Each colony consists of millions of cells, all derived from and genetically identical to the original one (a clone).

EQUIPMENT

- Permanent marker
- Inoculating loop
- Bunsen burner
- MM294 *E. coli* culture in LB broth (in microfuge tube)
- MM294/pAMP *E. coli* with pAMP plasmid in LB broth (in microfuge tube)
- Two LB agar plates
- Two LB/amp (LB agar with ampicillin added) agar plates

PROCEDURE

1. Carefully observe the instructor's demonstration of sterile technique and preparing a streak plate.
2. Lay out the four plates so that the two LB plates are side by side, and the two LB/amp plates are directly under them. See the grid below.
3. Turn the plates over and mark the bottom of each plate with your name and date.
4. Label each plate according to Table 1.
5. Hold the inoculating loop like a pencil, and sterilize loop in burner flame until it glows red hot. Then continue to pass lower half of shaft through flame.
6. Cool for 5 seconds.
7. If the loop is laid on lab bench, it must be reflamed.
8. Remove cap or foam stopper from MM294 *E. coli* culture vial according to the instructor's demonstration.
9. Carefully dip the inoculating loop into the culture broth and remove.
10. Lift the lid of your "-pAMP" plate only enough to perform the streaking as shown below. Make sure you glide the loop back and forth across the surface of the agar, **do not gouge or scratch the agar**.

Table 1. Labeling agar plates		
	MM294	MM294/pAMP
LB agar	- pAMP	+ pAMP
LB/amp agar	- pAMP	+ pAMP

11. After the 1st streak flame the loop and cool it for 5 seconds, (before doing your next streak, check that your loop is cool by touching it on a clean part of the agar.) then make the 2nd streak as shown in Figure 2.
12. Make 3rd and 4th streaks sure to **flame and cool loop in between streaks** (Fig. 2).

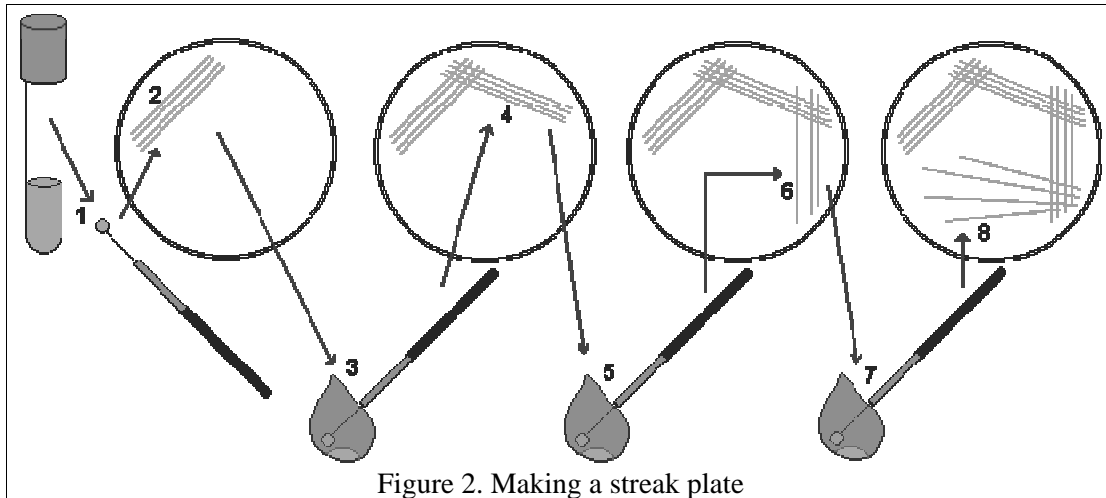
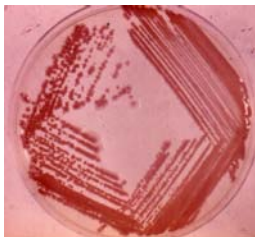


Figure 2. Making a streak plate

13. Make similar streak plates for the remaining three agar plates.
14. Flame the loop one last time before placing it on the lab bench.
15. Tape your agar plates together and place upside-down in the 37°C incubator. They will be incubated about 24 hours and placed in refrigerator until your next lab period. Below is an example of well-made streak plate.



SPREAD PLATE AND SERIAL DILUTIONS

Spread plates are similar to streak plates except that the cells are delivered to the agar surface in a measured volume of liquid (usually 100 μ l), instead of with the inoculating loop. A spreader is used to spread the liquid evenly around the dish before it is absorbed into the agar. This technique is most commonly used to determine the concentration of cells in a culture; e.g. if 50 colonies appear on a plate that was inoculated with 100 μ l of culture, you know that each colony came from one cell, so:

$$\frac{50 \text{ cells}}{100 \mu\text{l}} \times \frac{1,000 \mu\text{l}}{1.0 \text{ ml}} = 500 \text{ cells/ml}$$

Therefore concentration of cells in the original culture was 500 cells/ml.

Often times, the original culture is too concentrated to perform a direct spread plate with 100 μ l. In such cases, serial dilutions need to be performed first. The culture is diluted by 10 fold sequentially several times. Each dilution is then spread onto plates. The goal is to find one plate that has a countable number of colonies. The method for serial dilutions is described below and shown in Figure 3. After colonies have grown, plates with countable number of cells can be used to calculate the original culture's concentration. The general formula for the calculation is:

colonies/volume plated X dilution factor

The dilution factor is amount you diluted the culture. Therefore if you spread 100 μ l of a culture you diluted by 1/1000 onto a plate, and 25 colonies appeared, you would calculate concentration in ml as follows:

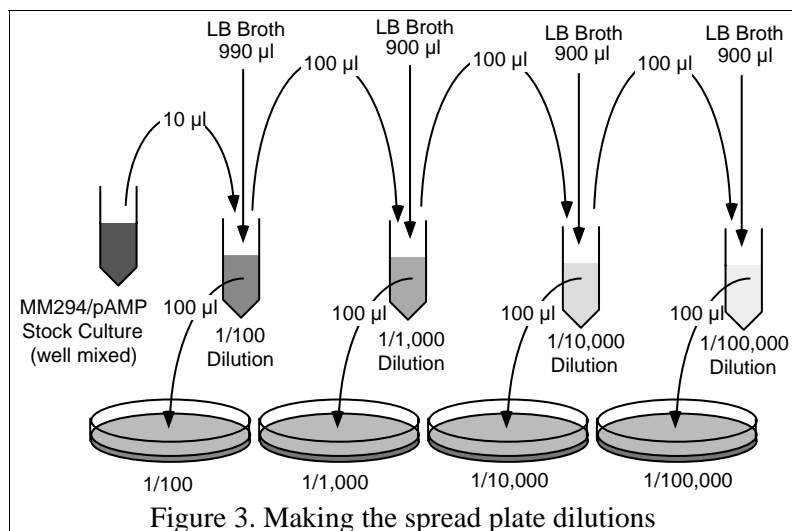
$$25 \text{ colonies}/100 \mu\text{l} \times 1,000 \mu\text{l}/1 \text{ ml} \times 1000 = 250,000 \text{ cells/ml}$$

EQUIPMENT

- Permanent marker
- MM294/pAMP *E. coli* culture in LB broth in 1.5 ml microfuge tube
- Four LB/amp agar plates
- Spreader and beaker of ethyl alcohol
- Four microfuge tubes
- 20-200 μ l micropipet, set to 100 μ l
- Sterile yellow micropipet tips
- 200-1,000 μ l micropipet, set to 900 μ l
- Sterile blue micropipet tips
- LB Broth
- Bunsen burner

PROCEDURE

1. Lay out the 4 LB/amp plates in a row across your desk.
2. Label all the plates with your name and date (bottom of plates).
3. Starting with the leftmost plate, label them: 1/100; 1/1,000; 1/10,000 and; 1/100,000
4. Set the microfuge tubes in a rack and label them the same.
5. Transfer 10 μ l of the MM294/pAMP cells from the culture stock to the microfuge tube labeled "1/100" as in Figure 3.
6. Add 990 μ l of LB Broth to the microfuge tube labeled "1/100" as in Figure 3. If you are careful not to touch the sides of the tube, you can use the same tip for all these dilutions. Use a fresh tip if the one you are using gets contaminated
7. Put a fresh tip on the 20-200 μ l micropipet and mix the 1/100 dilution by suctioning in and out several times, then transfer 100 μ l of the mixed 1/100 into the tube labeled "1/1,000."
8. Add 900 μ l of LB Broth to the 1/1,000 tube.
9. Put a fresh tip on the 20-200 μ l micropipet and mix the 1/1,000 dilution by suctioning in and out several times, then transfer 100 μ l of the mixed 1/1,000 into the tube labeled "1/10,000."



10. Add 900 μ l of LB Broth to the 1/10,000 tube.
11. Put a fresh tip on the 20-200 μ l micropipet and mix the 1/10,000 dilution by suctioning in and out several times, then transfer 100 μ l of the mixed 1/10,000 into the tube labeled "1/100,000."
12. Add 900 μ l of LB Broth to the 1/100,000 tube. Mix by suctioning in and out a few times.
13. Beginning with the 1/100,000 dilution and a fresh tip on the micropipet, transfer 100 μ l of dilution to the appropriate plate.
14. Immediately flame sterilize the spreader by lifting it out of the alcohol beaker and touching it to the Bunsen flame. Do not hold in flame.
15. Allow alcohol to completely burn off spreader. CAUTION: DO NOT RETURN FLAMING SPREADER TO ALCOHOL BEAKER.
16. Ten seconds after the flame is out, touch the spreader to the agar surface, away from the cells, to cool it.
17. Touch the spreader to the cell suspension and gently drag it back and forth, turning the plate slightly each time, to evenly distribute the cells over the agar surface.
18. Close the dish lid and return the spreader to the alcohol beaker.
19. Repeat adding 100 μ l of cells and spreading them for the remaining dilutions. Always going from dilute to more concentrated (so you don't have to use a new tip)
20. Keep it mind that all of the above steps must be done sterilely.
21. Tape your agar plates together and place upside-down in the 37°C incubator. They will be incubated about 24 hours and placed in refrigerator until your next lab period.

RESULTS

1. Record the results of your streak plate experiment in the Table below. Indicate whether there was *GROWTH* or *NO GROWTH*.

Table 2. Streak plate results		
	MM294	MM294/pAMP
LB agar		
LB/amp agar		

2. Record the results of your spread plate experiment in the Table below. If the colonies overlap and are to numerous to count, record these as "lawn."

Table 3. Spread plate results				
	1/100	1/1,000	1/10,000	1/100,000
Number of colonies				

3. Record the appearance of your LB broth overnight culture.

Tube:

QUESTIONS FOR YOUR REPORT

1. Present an abstract of your work. Your abstract should include a brief description (1/2 page) of 1) what you set out to learn, 2) what procedures you performed, and 3) a summary of your data or discoveries.
2. Turn in the results from the previous page.
3. Describe what an "overnight" culture is and how it is prepared using sterile technique. How do overnight cultures appear after incubation?
4. When do you use a streak plate versus a spread plate?
5. Explain why the terms "positive control," "negative control," and "experiment" could be inserted where they are in the streak plate table shown below.

Streak Plate Table		
	MM294	MM294/pAMP
LB agar	positive control	positive control
LB/amp agar	negative control	experiment

6. Describe the mechanism of ampicillin's ability to inhibit bacterial growth and the mechanism of ampicillin resistance imparted by the pAMP plasmid.
7. Sometimes students forget to flame their loops in between streaks on a streak plate. Draw a picture of a streak plate that would result from such an error.
8. Calculate the concentration of cells in ml of the stock MM294/pAMP culture used in the spread plate exercise. Show how you arrived at this figure.
9. (2 pts) Imagine that during the serial dilutions/spread plate procedure that your lab partner chose the wrong micropipet to transfer samples during the dilution. As a result, he/she ended up transferring 10 μ l instead of 100 μ l of culture in the last three steps. Therefore, the 1/1,000, 1/10,000, and 1/100,000 dilutions were actually much more diluted than expected. (Don't yell at your lab partner.) Based on your partner's mistake a) recalculate the actual dilutions and b) recalculate the concentration of cells from your stock culture.