Chem 405 Biochemistry Lab I
Experiment 4
Bacterial Transformation of the MDH-GFP-6X His (MGH) Plasmid

Introduction: Last wek you purified plasmid DNA. The goal for this week is to place the DNA into bacteria cells (transformation) so that we can later grow the bacteria with your DNA and induce the cells to produce your fusion protein MGH.

Fusion Protien Background: A fusion protein is formed by combining two (or more) parts of whole genes of proteins as one larger gene. This fusion or chimeric gene is then placed in either a bactera cell (to make protein for purification) or placed into orther cells for some biochemical/cell biological study.

Many fusion proteins use either an affinity or an epitope tag. These are short sequences of amino acids that are either recognized by specific antibodies (for detection) or amino acids with special affinities for chromatographic resins (for purification). His tagged proteins (6X His) include six histadines at end of a protein. There, these histadines can then bind nickel coated beads for purification. The His tag can also be used for detection when using the antibiodies commercially available for His tags. A second common gene used in fusion proteins is the Green fluorescent protein (GFP) from the jellyfish *Aequorea Victoria*. GFP is a revolutionary reporter molecule for monitoring gene expression and protein localization *in vivo*, *in situ*, and in real time.

The GFP fluoresces bright green upon mere exposure to UV or blue light—unlike other bioluminescent reporters which require additional proteins, substrates, or cofactors to emit light. Upon mechanical stimulation A. victoria emits a green light.

Fig 1. *The source of our fun.*

However, when the calcium-triggered photoprotein aequorin was puriefied from A. victoria photocytes, it was found to generate blue, rather than green light. GFP acts as a secondary fluorescent protein, receiving energy from activated aequorin.

Plasmids. *E. coli* is a small rod-shaped bacterium that contains two different kinds of DNA, the large genomic DNA (4500 kb) that is found in most cells and small circular DNA (0.03 - 0.3 kb) called plasmids. Plasmids are double stranded closed circular DNA molecules that grow within the host *E. coli*. They are self-replicating and do not integrate into the genomic DNA. When plasmids are used in molecular biology, they are used as vectors to insert foreign DNA into host cells. For these plasmids to be useful as vectors they must have three features: a selectable marker, an origin of replication and a cloning site.

Transformation. The uptake of exogenous DNA by cells that alters the phenotype or genetic trait of a cell is called transformation. For cells to uptake exogenous DNA they must first be made permeable so the DNA can enter the cells. This state is referred to as competency. In nature some bacteria become competent due to environmental stresses. We can purposely cause cells to be competent by treatment with chloride salts of metal cations such as calcium, rubidium or magnesium and cold treatment. These changes affect the structure and permeability of the cell wall and membrane so that DNA can pass through. However, this renders the cells very fragile and they must be treated carefully while in this state.

The amount of cells transformed per 1 µg of DNA is called the transformation efficiency. Too little DNA can result in low transformation efficiencies, but too much DNA also inhibits the transformation process. Transformation efficiencies generally range form 1×10^4 to 1×10^7 cells per µg of added DNA. We will determine the transformation efficiency of the cells we transform during this lab. We will do this by counting the number of colonies of bacteria that form on LB plates containing antibiotic.

What are we going to do? Our six-week project is to study MGH. For this week's part of this project we want to transform the *E. coli* cells with plasmid containing the gene for MGH so these cells will grow and in doing so make lots more plasmid. But, we need to be sure that the *E. coli* we culture actually contain the plasmid of interest. Therefore, we must have a method to screen for bacteria containing the MGH plasmid. We will use antibiotic resistance to ampicillin to screen for bacteria containing the MGH containing plasmid.

E. coli bacteria are normally poisoned by the antibiotic ampicillin. Ampicillin acts to damage the membranes of *E. coli* by inhibiting the crosslinking of the bacterial membrane. This results in bacteria which are structurally very weak. In the hypotonic media in which these cells grow, the cells exposed to ampicillin will swell and burst. For cells to survive, they must include a means to break down the ampicillin. *The plasmid has an additional gene that codes for a protein, ß-lactamase, that is excreted by the cells and in a local area will hydrolyze the ampicillin. Therefore by adding ampicillin, only bacteria that contain the plasmid will survive*. We also need to be sure not to allow our transformed *E. coli* to become overgrown. If the colonies on the LB plates are large they will break down enough ampicillin so that surrounding bacteria (satellite colonies) will form that may not have the plasmid insert due to the lack of remaining antibiotic.

See Chapter 11 in At the Bench for background in handeling and using bacteria.

References

1. Cody, C. W. et al., Biochemistry (1993) 32, 1212-1218
2. Heim, R., Prasher, D. C., Tsien, R. Y., Proc. Natl. Acad. Sci. USA (1994) 91, 12501-12504
3. Chalfie, M., Photochem. Photobiol. (1995) 62, 651-656
4. Chalfie, M. et al., Science (1994) 263, 802-805

Today's Experiment: *E. coli* Transformation.

Procedure - We will identify *E. coli* containing plasmid by selection on ampicillin plates. Culture plates without ampicillin will be used for controls. You will prepare the plates, calculate what you need to make the Luria Broth (LB) plates and use the ampicillin (Amp).

First, you MUST read the protocol below and determine how many plates you must prepare with and or without Amp. It will be up to you to do the proper calculations. Ensure this goes into your notebook. The protocols linked on the web on bacterial culturing and transformation are a general description if you need to do this in the future. When in doubt, this protocol overrides this during class. Outside of class use the general protocol on the web.

<u>I. Preparation of LB agar plates</u>.

1. Calculate how many plates with and without Amp you will need. Plan to use 10 ml of LB per plate. It is usually good to prepare at least 5-10 ml extra.
2. Melt the agar by re-heating in the microwave and allow cooling to 55^{O}C. (Note: you should be able to comfortably hold your hand on the bottom of the flask. Adding antibiotic when the agar is too hot will destroy the ampicillin).
3. As needed, add enough stock ampicillin to make 1X (100 μg/ml). Prepare your Amp + plates separately from the Amp – plates. MIX THE PLATES AND WORK QUICKLY TO MOVE TO THE NEXT STEP.
4. Pour liquid agar (8 to 10 ml) into 60 mm petri dishes and allow to solidify. The exact volume isn't as important as the LB should be about ½ up the plate. Work quickly, you can not re-heat the Amp containing LB for reuse.
5. Leave the lids ajar on the plates until the condensation disappears. This usually takes 30 minutes to an hour.
6. Be sure to label plates as + Amp or – Amp on the bottom of the plate.
7. Store unused plates in the cold room covered in tin foil.

<u>II. Transformation of *E. coli* cells.</u>

When using frozen competent bacterial cells, it is extreamly important that you make all calculations ahead of time and that the cells go from frozen to thawed on ice as quickly as possible. Thawing should be done by rolling the tube between your fingers just until the cells are barely thawed. After this thawing, be sure to keep the cells on ice at all times. As the cells become warm they "seal up" and will no longer take in plasmid DNA. Even on ice, the cells will lose the ability to take up the DNA. <u>***Speed is an important factor here***</u>.

1. Determine the concentration of the DNA in the elution in last week's experiment. Calculate how many μl you need to use to add 1-5 μg of plasmid DNA. You may need to create a small amount of dilute plasmid DNA to do this…

2. Into each of two sterile microfuge tubes, pipet 50 μL of competent cells. Label the tops of the tubes as #1 control (-DNA) and the second tube #2 plasmid (+DNA).

3. To tube #1 of competent cells add 5 μL of TE. This will be your control. To tube #2 add 1 - 5 ng of DNA as calculated in step 1.

4. Incubate both tubes on ice for 30 min. During this time DNA is associating with the cell membranes.

5. Heat shock cells by transferring the cells to a 42^{O}C water bath for EXACTLY 2 min. Heat shocking is necessary to increase the transformation efficiency. However, prolonged exposure to heat in rubidium/glycerol buffer kills competent cells. Carefully monitor heat shock times.

6. Add 0.2 ml SOC medium (without ampicillin) to each sample, and incubate at 37^{O}C for 45 min with gentle orbital shaking. During this period, competent cells recover and transformed cells express the antibiotic resistance gene needed to replicate on selective plates.

7. Aliquots of the transformation reactions need to be plated on appropriate plates with and without ampicillin (see figure below).

   However, suggested plating volumes may be the following:
   Control: place 100 μL on a +Amp plate and 100 μL on a - Amp plate.
   Plasmid: Place three different volumes of cells on the center of the + Amp plates.

8. Use the hockey stick to spread the cells. Flame the stick after diping the stick in the ethanol between uses. Ensure the stick is cool before moving on to the next plate.

9. Incubate the plates with the top off and agar facing up in the 37^{O}C incubator for several minutes, until the inoculum of cells is completely absorbed into the agar.

10. Cover the plates and incubate in the 37^{o}C incubator over night. Be sure the plates are placed upside down in the incubator.

11. The next morning, use parafilm to seal the plate and cover in tin foil in the cold room. Sometime before the next class you need to determine the transformation efficiency for your transformation procedure.

12. Count the colonies on your +AMP plates and carry out the following calculation:

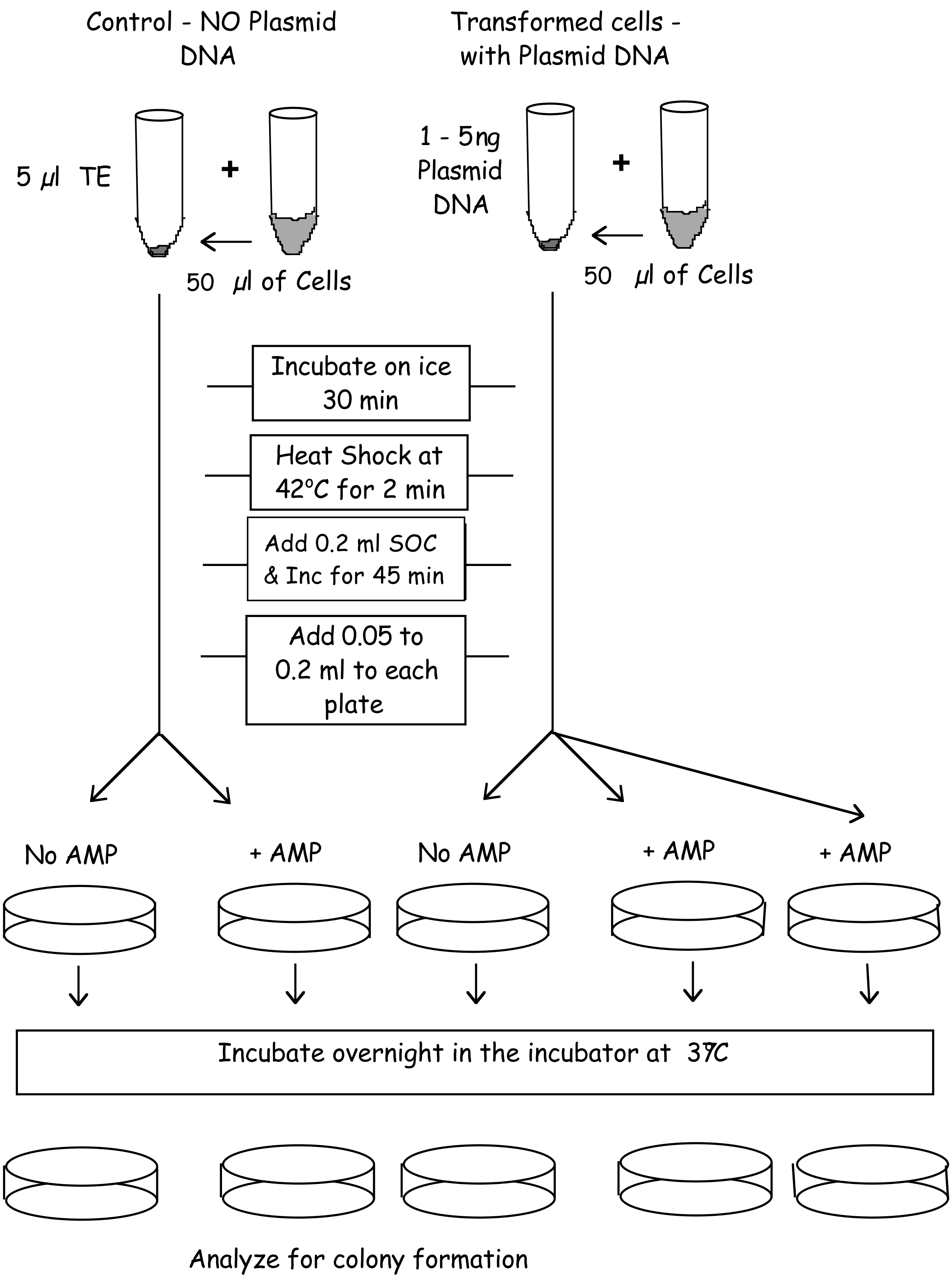
$$\frac{\#\text{ of Transformants}}{\mu\text{g DNA}} \text{ X } \frac{\text{Recovery Volume (mL)}}{\text{Volume Plated (mL)}} = \#\text{ of Transformants}/\mu\text{g DNA}$$

The recovery volume = the amount of DNA, buffer, compitent cells and SOCR

The colonies are now ready to grow for MGH expression and purification.

The instructors will be looking at your plates and picking one or two colonies for culture, protein expression and lysing. You will be purifying the MGH protein from this lysate during your next lab.

Transformation of E.coli



Lab Notebook:

Accurately record the procedures conducted during lab. This will include:

- Observations of the experiment
- Data and calculations for quantifying your transformation efficiencies.

Your final report (paper not the lab notebook) should include some of the following points

- The introduction to the MGH fusion protein as well as the background info on transformation.
- Final results for each of the plates in transformation
- Why some plates grew many colonies other plates only a few and yet other plates had no colonies?
- What is the role of the plasmid in the transformation?
- Why will transformed cells grow in the presence of amp?