7.02 Fall 2001

RDM

Recombinant DNA methods

Agenda

DAY 1

General overview of module's two projects:

- Subcloning and expression of GFP
- PCR analysis of *ara* mutant from GEN module

Today's lab procedures:

- Restriction enzymes and digests
- Agarose gel electrophoresis and ethidium bromide
- Interpreting restriction digest results
- Restriction digest mapping: an example

Subcloning and expression of GFP

• Green Fluorescent Protein (GFP) from the Pacific Northwest jellyfish *Aequorea victoria* emits green light when irradiated with ultraviolet light. This fluorescence requires no other proteins or cofactors. GFP is active in a wide range of other creatures, including bacteria, as well as *in vitro*.
• The gene for GFP will be provided to us in a storage plasmid called pUC19. This plasmid has none of the DNA sequences that would be needed for expression of this gene.
• Using recombinant DNA methods, we will subclone the GFP gene out of the storage plasmid and move it into an expression plasmid, called pET, which contains a T7 promoter sequence. We will ligate the GFP gene into pET just downstream of this promoter, allowing GFP to be expressed in the presence of T7 RNA polymerase.
• The resultant plasmid, called pET-GFP, will be transformed into a strain of *E. coli* called AG1111. This strain has a high transformation efficiency, which is important because the ligation produces only a small amount of DNA. However, this strain lacks T7 RNA Pol, so it will not express GFP.
• We will use the "miniprep" technique to isolate plasmid DNA from these transformants and then perform analytical redigestions to verify that we have the desired plasmid construct.
• We will transform the confirmed plasmid into a second strain of *E. coli* called BL21. This strain has a lower transformation efficiency, but this is OK because the miniprep produced a large quantity of DNA. This strain expresses T7 RNA Pol, allowing expression of the plasmid-encoded GFP . Furthermore, this strain is deficient in protein-degrading enzymes (proteases) that would chew up the GFP.
• BL21 colonies resulting from successful transformation will fluoresce green under UV irradiation.

PCR analysis of *ara* mutant from GEN module

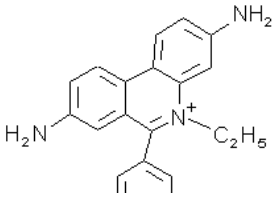
• In the genetics module, you isolated *E. coli* mutants with defects in the arabinose utilization pathway. We will revisit one of those ara^- mutants and compare it to an ara^+ strain, using PCR to amplify specific regions of the *araDABC* operon in both strains. The pattern of PCR products we obtain will allow us to conclude where in the mutant strain's *araDABC* operon the transposon inserted. We'll discuss this portion of the RDM module in more detail later on.

Restriction enzymes and digests

• Restriction enzymes are the immune systems of bacteria. They cut up foreign DNA such as invading viruses. The bacterium's own DNA is immune to the action of restriction enzymes because it is methylated by a modification enzyme.
• Restriction enzymes cut DNA very precisely at specific recognition sequences. Because of this, they are very useful tools for molecular biologists. They make recombinant DNA methodology possible.
• Restriction enzymes are fragile and must be handled with care. They must be kept in an ice bucket whenever they are outside the freezer.
• Always use a fresh pipet tip when removing enzyme from a tube, in order to avoid contamination.
• Always hold a tube of enzyme by the top, in order to avoid unnecessarily heating up the enzyme.
• Restriction enzymes need a special buffer in order to work. The buffer is usually supplied at 10X concentration and should be diluted to a final concentration of 1X in the restriction digest. The buffer has the following components:
    • an acid/base buffer such as Tris to maintain the proper pH.
    • magnesium, required as a cofactor in the DNA-cutting reaction.
    • a salt such as sodium chloride, needed for ionic strength.
    • a small amount of detergent such Triton, to prevent things from sticking to the tube.

Agarose gel electrophoresis and ethidium bromide

• A mixture of DNA fragments of different lengths (such as that resulting from a restriction digest) can be separated on the basis of their lengths using gel electrophoresis.
• An electric field is applied across an agarose gel. DNA, which has two negative charges for every base-pair, responds to this electric field by migrating away from the black (-) electrode and toward the red (+) electrode.
• The DNA has to travel through pores in the agarose. Smaller DNA fragments are able to maneuver better, and they get through the pores faster than larger ones. As the DNA progresses through the gel, the fragments sort themselves out by length, with the smallest ones moving to the front of the pack.
• DNA fragments separated by gel electrophoresis are visualized using ethidium bromide (EtBr), a planar molecule that intercalates between base-pairs in DNA and fluoresces red-orange under UV light. EtBr binds to DNA in a stoichiometric manner, i.e. the longer a DNA fragment, the more molecules of EtBr it binds.
• In our protocol, EtBr is added to the liquid agarose before pouring a gel.
• Ethidium bromide is a potent mutagen. (Why?) Always wear gloves when working with gels and gel boxes.



Structure of the ethidium cation

• Exposure to ultraviolet light can cause cancer. Always wear goggles or a face shield when working near UV light boxes.

Interpreting restriction digest results

• Electrophoretic behavior of DNA depends not only on fragment length, but on running time, voltage, buffer, and agarose percentage. Therefore it is crucial to include some DNA standards of known lengths on the gel. DNA fragments of unknown length can thus be compared to this "ladder" of standards.
• The distance traveled by a DNA fragment is proportional to the logarithm of that DNA fragment's length in base-pairs. Distance is measured from the wells where the DNA was loaded in the gel.

• Undigested plasmids are circular. A plasmid's true size can only be determined by measuring its mobility when linearized.
• Circular plasmids exist in multiple possible forms. Bacteria introduce twists into their DNA, rendering it supercoiled and more compact, and causing it to migrate faster in a gel than linear DNA with the same number of base-pairs. Nicked circular DNA, in which the supercoiling has been relaxed, is actually less compact than linear DNA of the same length, and so it migrates slower in the gel. Furthermore, plasmids can become concatenated inside the bacterium, linked to each other topologically like rings. These concatamers run much slower on the gel than unconcatenated plasmids.
• Faint secondary bands in a digest may represent partial digestion products such as uncut DNA (supercoiled, nicked, or concatenated plasmid) or singly cut DNA (in a two-enzyme digest). Some of these bands can be identified by comparing them to the bands in the undigested plasmid control lane.
• Very short DNA fragments (less than 500 base-pairs) may not be visible on the gel, either because they have run off the bottom of the gel, or because they fail to bind sufficient EtBr to generate a visible band. Because ethidium is positively charged, it migrates toward the top of the gel, exacerbating the difficulty of visualizing bands near the bottom of the gel.

Restriction digest mapping: an example

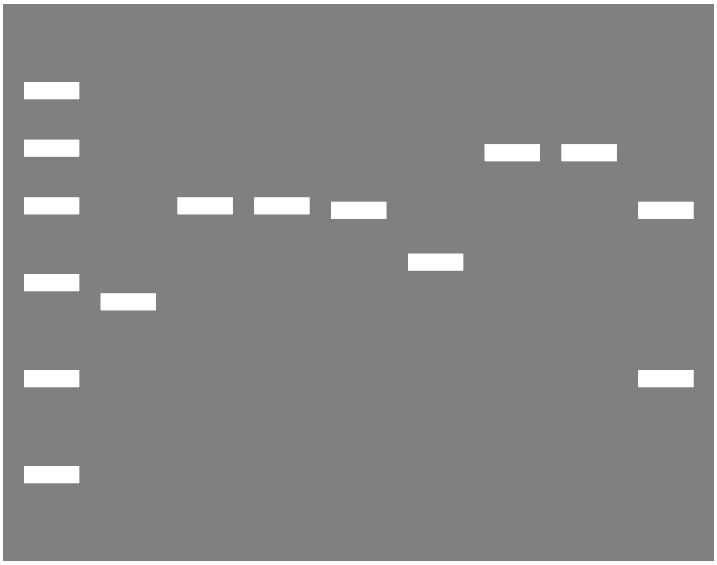
You are given the following materials:

- Two plasmids, named pA and pB
- Two restriction enzymes, named 1 and 2
- A ladder of DNA standards with the following lengths: 500, 1000, 2000, 3000, 4000, and 5000 base-pairs.

You are told that pB was constructed by ligating an insert into pA.

You perform restriction digests and run the following samples on a gel:

1. Ladder of DNA standards
2. pA undigested
3. pA digested with enzyme 1
4. pA digested with enzyme 2
5. pA digested with both enzymes
6. pB undigested
7. pB digested with enzyme 1
8. pB digested with enzyme 2
9. pB digested with both enzymes



The gel is shown at right.

Draw maps of the two plasmids, indicating where enzymes 1 and 2 cut and where the insert is in pB. Circle the band(s) on the gel which correspond to the insert DNA.