7.02, Fall 2001: Genetics Recitation Two

Agenda

I. Overview of *ara::lacZ* fusion generation and stabilization
II. Transposon Mutagenesis
III. Results of Viable Cell Counts and Correlation with OD data
IV. Results of Phenotypic Characterization (Streaking)

I. Overview of *ara::lacZ* fusion generation and stabilization
- A. Infect *E. coli* strain pNK/BW140 with bacteriophage λ1205 carrying the mini-Tn*10* transposon**(GENETICS DAY 2)**
  1. Similar strain to what we grew in Day One, except for pNK
     - a. pNK plasmid carries copy of gene encoding transposase that is under control of lac repressor
       - i. Prof. Amon will talk about the *lac* operon in her next lecture, what's important for today's purposes is that transcription of the transposase gene is induced by IPTG
  2. λ1205 is a bacteriophage carrying mutations that make it a good delivery vehicle for mini-Tn*10*
     - a. A bacteriophage is a virus that infects bacteria.
       - i. Once inside the bacteria, it takes over the host replication machinery turning the bacteria into a virus making machine.
       - ii. When viral DNA is replicated and packaged into mature phage particles, the bacterial cell is lysed and phage are released that can infect other bacteria.
       - iii Lysed bacterial cells within a bacterial lawn appear clear, and are called plaques.
     - b. λ1205 has a mutation deleting its attachment site - can't integrate into host genome
     - c. λ1205 has an amber mutation in gene required for DNA replication, so it can't replicate.
       - i. An amber mutation is a missense mutation (See Glossary)
     - d. Why does mini-Tn*10* need a delivery vehicle? (*E. coli* does not take up DNA from the environment under normal conditions, λ1205 injects DNA into cell.)
  3. mini-Tn*10* is a transposon
     - a. Transposon is a naturally occurring DNA element that is capable of inserting into DNA
     - b. Transposon insertion is random
     - c. Transposons can be stably inherited as part of bacterial genome unless acted upon by tranposase
     - d. Transposon insertion into a gene usually results in loss of protein function
     - e. Structure of mini-Tn*10*
       - i. Inverted repeats at end of tranposon
         - Recognized by tranpsosase, necessary for tranposition
       - ii. Kanamycin resistance gene
         - Has its own promoter, ribosome binding site and initiation codon

- Does Kanamycin resistance require insertion into a gene in the correct reading frame? (No, because it has its own promoter, ribosome binding site, and initiation codon. It has everything necessary for transcription and translation.)

iii. *'lacZ* gene
- Fragment of *lacZ* gene which has no promoter, ribosome binding site, or initiation codon
- Remember that *lacZ* encodes the enzyme β-galactosidase responsible for cleaving lactose
- Expression of β-galactosidase requires insertion of transposon in correct orientation and reading frame because *lacZ* fragment not capable of initiating transcription or translation
- What's the chance that β-galactosidase will be expressed? (1/6 = 1/2 (chance of right orientation)* 1/3 (chance of correct reading frame))

B. Select for transposon insertion into the genome and screen for insertion into the arabinose operon
1. Selection vs. screen

2. How will we select for tranpsoson insertion into the genome? (Kanamycin resistance)
3. How can we screen for mutations in the arabinose operon? Hint: Is there an indicator we can use for this screen? (MacConkey Arabinose– look for white colonies, those that aren't fermenting arabinose)
    a. Remember that MacConkey is a rich media, so cells that aren't fermenting arabinose are finding other carbon sources (such as amino acids)
4. Arabinose System - (Professor Amon will discuss this in lecture and this is also covered in the appendix to your manual)
    a. Arabinose is five carbon sugar found in plant cell walls
    b. Enzymes responsible for converting L-arabinose to D-xylulose-phosphate encoded by *araBAD*
        i. D-xylulose-phosphate can enter central metabolism and be fermented
        ii. Mutation in *araD* is lethal because toxic intermediate builds up
        iii. Expresion from *araBAD* is regulated by *araC*
            - Transcription is induced by *araC* in the presence of arabinose
            - *araC* also involved in repression – (see appendix)
    e. How are Ara- mutants generated? Into what genes must the Tn insert?
        i. Insertion into *araB, araA,* or *araC; araD* insertion = lethal

C. Pick and purify mutants **(GENETICS DAY 2.5 –** Done by staff)
1. Establish pure clone (descendant of a single cell with a specific mutation) for further phenotypic testing

D. Further characterize arabinose and *lacZ* phenotypes of mutants (**GENETICS DAY 3 & 3.5)**

1. Screen Ara- mutants on Minimal media containing arabinose
    a. Why can't they grow? (Minimal media doesn't contain a carbon source other than arabinose for the bacteria to use.)
2. Test whether *lacZ* is transcribed and translated and whether transcription is inducible by arabinose or constitutive
    a. What indicator would we need in these plates? (X-gal – turns blue when acted upon by β-galactosidase, the enzyme encoded by *lacZ*)
    b. Transcription of *araB* and *araA* is induced by *araC* in the presence of arabinose
    c. *araC* is transcribed in the absence of arabinose
    d. If *lacZ* has inserted into *araB* or *araA* in the correct orientation and reading frame, will its transcription be inducible by arabinose or constitutive? (Inducible) What about an insertion into *araC*? (Constitutive)

E. Based on results of D, you've now identified *ara::lacZ* translational fusions

F. Stabilizing the mutation
1. Why do we need to stabilize the mutation? (The transposon insertion is still in a strain that contains the transposase gene. Although we are not inducing the tranposase gene with IPTG, the *lac* repressor doesn't work perfectly, so we are still getting a little transcription and translation of the tranposase gene. We don't want the mutation we've worked so hard to get to "hop" out of our gene, so we are going to move the mutation into a strain that doesn't have the tranpsosase gene around.)
2. How do we do this? P1 transduction (**GENETICS DAY 4, 5 & 6)**

    b. Procedure

G. Mapping Mutation using P1 transduction (**Day 4, 5, 6 & 7)**

II. Transposon Mutagenesis

A. Provided with culture that has been pregrown overnight in LBMM + tetracycline. What are those components and why are they important?
1. LB is a rich media that supports rapid growth
2. Maltose is a sugar added to the media. The target of λ phage on the surface of *E. coli* is the LamB protein, which is involved in maltose transport
3. $MgSO_4$ – Mg++ ions stabilize adsorption of phage to surface of bacteria
4. Tetracycline is an antibiotic, it selects for the pNK plasmid which carries tetracycline resistance

B. The culture has been diluted in LBMI. What's I? Why is it important?
1. I = IPTG, necessary to induce transcription of transposase

C. You have 3 test tubes - One = cells alone, another = phage alone, third = cells + phage
1. What are your controls and why do you need them? (Controls = cells, phage alone;You want to make sure that there was nothing wrong with your transduction, such as a contaminant in your cells or phage, that could confound your results.)
2. Would you expect either control to grow when spread on Mac Ara Kan?
    a. Phage alone won't grow
    b. Cells alone won't grow because they're not Kan^R

D. Incubate all 3 test tubes at 37° for 30 minutes without shaking
1. Preadsorption of phage to cells

2. Avoid shaking because you don't want to disrupt attachment

E. Dilute your stock culture 1/50 with media and read OD_{550}. Use this to calculate the cell concentration based on your growth curve calculations..

F. Add sodium citrate to reaction
   1. Sodium citrate chelates Mg++ ions, stops phage adsorption
   2. Why did we add sodium citrate to control reactions? (Want to treat controls exactly the same way we treat our experiment, if we introduce a mistake anywhere in the protocol, we want to find out.)

G. Add LB to tubes and incubate with shaking at 37° C for 1 hr.
   1. What is going on during this time? *E. coli* are growing, those that contain transposon insertions are starting to produces the protein that phosphorylates kanamycin, making cells resistant to kanamycin.

H. Spread on Mac Ara Kan plates & LB X-gal Kan plates
   1. Why are we spreading so many plates? (We expect that a random insertion into the arabinose operon to be a rare event, so we need to screen many colonies.)
   2. Why do we spread on the LB X-gal Kan plate? (We can determine what proportion of transposon insertions were in the correct orientation and reading frame to be transcribed constitutively. We also know that there are no problems with our *lacZ* gene.)

III. Results of Viable Cell Counts and Correlation with OD data

A. Weighted Averages take into account error that was introduced with each subsequent transfer during serial dilution.
   1. Number of colonies on 10^{-4} plate has least error = 1, 10^{-5}=0.1, 10^{-6}=0.01
   2. To figure out the number of colonies at a given time point, we calculate the weighted average:

      $$300 = (295+32+6)/1.11$$

   3. Use this number to calculate the CFU/ml

      $$3*10^{7}\ \text{CFU/ml} = 300\ \text{colonies}/((0.1\ \text{ml})(10^{-4}))$$

B. Organize OD readings with viable count in a chart for your time points

C. Plot on a semi-log plot with CFU & OD on the log axes
   1. They should form a similar curve which will allow correlation of OD with a known CFU/ml
   2. You should also be able to determine the doubling time. How much time elapsed before your culture doubled?

IV. Results of Phenotypic Characterization (Streaking)

A. Do your results compare with what you expect given the phenotypes of the strains?
   1. Try to fill out a chart similar to the one presented during recitation one. Ask yourself what each indicator tells you.
   2. If you still had problems streaking for singles, make sure to ask for help.
   3. Save your LB-Kan plate, you need it for a control later on.