Experiment 7: Genetic Mapping of λ-Resistance Mutations by P1 Transduction

You may have isolated λ-resistant (λ^r) mutants as part of Experiment 6. As explained below, the majority of spontaneous mutations conferring resistance to λ will occur in one of two specific genes, *lamb* or *malT*. For this experiment you will perform generalized transduction on an uncharacterized λ^r *E. coli* strain and attempt to determine by cotransduction mapping which, if either, of these genetic loci mutated to confer λ resistance to your particular mutant.

BACKGROUND

Cellular resistance to phage λ. Bacteriophage typically recognize and attach to their host cells by using normal cell envelope molecules as their specific receptors. A common evolutionary mechanism for resistance to phage infection is thus the cell's loss or alteration of the surface molecule used by the bacteriophage. Phage λ uses the outer membrane maltose transport protein LamB as its receptor for infection. Although LamB is the only protein required for λ infection, it is one of seven different proteins required for the transport and catabolism of maltose in *E. coli*. The genes for these seven proteins are all under the control of a single transcriptional activator protein called MalT. Resistance to phage λ can thus occur as a result of either of two different mutations: loss of the receptor itself (*lamB*-) or loss of the activator protein required for expression of the receptor (*malT*-). A more detailed description of the *E. coli* maltose regulon is given in the textbook on pages 525-528.

Transduction of chromosomal mutations. Transduction refers to the transfer of foreign (non-viral) DNA into another cell by means of a viral vector. Certain types of bacteriophage will occasionally package random segments of the host cell chromosome into viral capsids (protein coats) instead of the normal viral genome. The result is not a normal bacteriophage, but rather a **transducing particle**, consisting of a phage capsid containing bacterial DNA. Transducing particles are still capable of introducing their DNA into new host cells by the same mechanism as wild type phage. Because the introduced bacterial DNA carries none of the viral sequences required for replication, however, it will generally be degraded unless it first recombines with a homologous region of the chromosome in the new host cell. The result of such an event is a recombinant cell called a **transductant** that has replaced part of its chromosome with the corresponding DNA acquired from another cell. In most cases, the specific portion of the bacterial genome that is packaged and transduced is completely random, meaning that essentially any part of the bacterial chromosome can be transferred in this way. The transfer of chromosomal sequences by such bacteriophage is therefore referred to as **generalized transduction** to reflect its non-specific nature. See pages 336-337 of the textbook for a more detailed description of generalized transduction.

Transduction can also be used to map the relative locations of different bacterial genes on the chromosome. The principle is simply that the amount of DNA carried by any one transducing particle represents a very small portion of the total DNA of the bacterial cell. Any two genes that can be contained within a single transducing particle (meaning they can be "cotransduced") must, therefore, be relatively close to each other on the chromosome. By measuring the **cotransduction frequency** of two or more genes, one can estimate the relative proximity of those genes. The simplest analysis is to select for the transfer of one gene (for example, a gene encoding antibiotic resistance), then to screen the population of transductant cells for the inheritance of a second gene. The cotransduction frequency is measured as the percentage of cells that have inherited <u>both</u> genes, rather than the selected first gene by itself. The higher their cotransduction frequency, the closer two genes must be to each other. See pages 181-183 of the textbook for more discussion of gene mapping by cotransduction.

EXPERIMENTAL OVERVIEW

To map the possible location of an uncharacterized λ^r mutation, we'll use two strains of *E. coli*, each containing a unique kanamycin resistance (Kan^r) marker in a gene located near *lamB* or *malT*. Strain 14 contains a Kan^r marker inserted into the *aceA* gene, located adjacent to *lamB*. Strain 15 contains the same Kan^r marker inserted into the *glpR* gene, adjacent to *malT*.

These two strains will be infected with the transducing phage P1 to create two transducing lysates. These transducing lysates will consist of cell-free mixtures of both wild-type P1 phage and transducing particles that contain random pieces of the bacterial chromosome. Some of these transducing particles will contain the specific chromosomal fragment that includes the Kan^r marker from each strain.

We'll then use these transducing lysates to infect an uncharacterized λ^r strain of *E. coli*. Following infection with the appropriate transducing particles, some of these *E. coli* cells will become kanamycin-resistant as a result of recombining the donated DNA fragment that contains the Kan^r marker. We'll identify these transductants simply by selecting for growth on kanamycin agar.

After selecting for acquisition of the Kan^r marker, we'll screen the transductant cells for <u>loss</u> of their original λ^r phenotype. As already explained, our expectation is that most λ^r strains of *E. coli* will have mutations in either the *lamB* or *malT* gene. Wild-type alleles of *lamB* or *malT* that are cotransduced with the selected Kan^r markers could potentially replace these mutant (λ^r) alleles by recombination, resulting in the loss of λ resistance in the transductant cells. As shown in Figure 7-1, the *lamB* gene at 91.5' and the *malT* locus at 76.6' on the chromosome are much too far apart to be cotransduced together, so any loss of the λ^r phenotype should strictly correlate with the selection of one or the other of the adjacent Kan^r-marked genes (*aceA* or *glpR*). By this method you should be able to deduce the probable identity of your strain's original λ^r mutation.

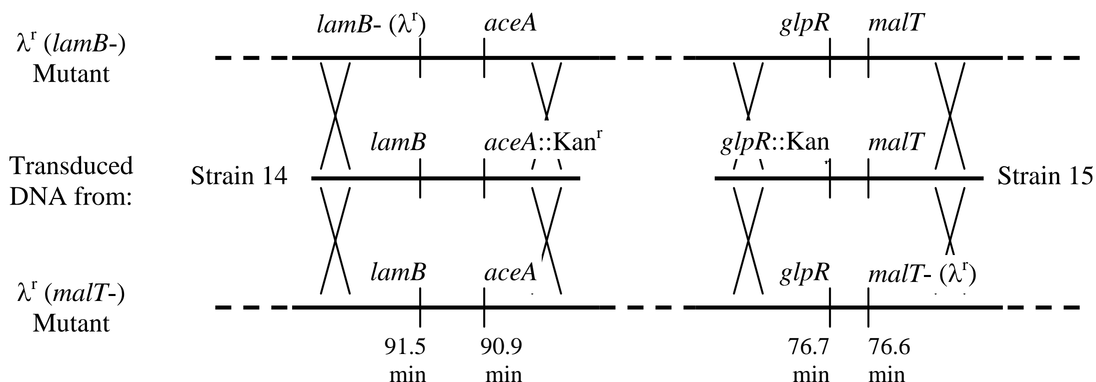

FIGURE 7-1. Mutational mapping by cotransduction of wild-type alleles flanking selectable (Kan^r) markers. Transduction of the *aceA*::Kan^r marker of Strain 14 (middle left) into a *lamB*- λ^r *E. coli* mutant (top) could potentially cause the loss of λ resistance due to cotransduction of the wild-type *lamB* allele. Transduction of the *glpR*::Kan^r marker from Strain 15 (middle right) would have no such effect on the strain's λ^r phenotype. The opposite result would be expected for a *malT*- λ^r *E. coli* recipient (bottom): transduction of *glpR*::Kan^r marker from Strain 15 could potentially result in the cotransduction of a wild-type *malT* gene (thus replacing the chromosomal *malT*- allele), while transduction of the *aceA*::Kan^r marker from Strain 14 would have no effect on the λ resistance of the strain.

PROCEDURE

Day 1 (Tu, Mar 3)

Broth cultures of two different strains of *E. coli* (Strains 14 and 15, below) will be provided. Strain 14 contains a kanamycin resistance (Kan^r) marker inserted in the *aceA* gene, located at 90.9 minutes on the *E. coli* chromosome. Strain 15 contains the same marker inserted into the *glpR* gene, located at 76.7 minutes. Since *lamB* and *malT* are located at 91.5 and 76.6 minutes, respectively, this puts *aceA*::kan adjacent to *lamB* in Strain 14 and *glpR*::kan adjacent to *malT* in Strain 15 (see Fig. 7-1).

Strain 14 (JW3975.2): *aceA*782::Kan^r
Strain 15 (JW3386.1): *glpR*756:: Kan^r

YOU WILL PREPARE A TRANSDUCING LYSATE FROM ONLY ONE OF THESE TWO STRAINS. If your group number is even, you will work with Strain 14; if your group number is odd, you'll work with strain 15. Even- and odd-numbered groups will share lysates on Day 4.

Use the mechanical pipettor to add 50 µl of the provided transducing phage $P1_{vir}$ to your broth culture and place the tube in the 37°C water bath for 20 minutes to allow infection.

Following this incubation, use a 1 ml pipette to add the entire culture volume to a tube of molten H top agar. Roll the tube between your palms to mix, then immediately pour it onto a plate of LB agar, tipping the plate as you pour to form an even top layer of soft agar. (You should perform this task at your bench, but do not remove the tube of molten top agar from the water bath until immediately before use or it will solidify! Once the top agar lawn begins to solidify, you should STOP attempting to spread it further—it is far preferable to have "gaps" in the lawn than to have wrinkles and folds in the surface.)

Allow the agar to solidify completely (~5 minutes) and incubate the plates without inverting at 37°C.

Day 2 (W, Mar 4)

Check the soft agar lawn of your plate for evidence of cell lysis. This indicates that the P1 phage are producing new viral particles. A small percentage of these will contain bacterial DNA in place of the viral chromosome—that is, they will be transducing particles.

Pour the entire volume of a tube containing 5 ml of sterile LB + 5 mM $CaCl_2$ onto the surface of your agar plate and carefully place the plate in the refrigerator to incubate overnight. During this time, phage particles will elute from the top agar into the broth.

Day 3 (Th, Mar 5)

There are two things to do today to prepare for tomorrow's transduction procedure.

First, take your lysis plate from the refrigerator and carefully collect 1 ml of the broth supernatant using a mechanical pipettor (P1000 Pipetteman). Be careful not to disturb the soft top agar when collecting the sample. Dispense the 1 ml sample into a sterile Eppendorf (microcentrifuge) tube and centrifuge for 5 minutes at high speed, using one of the provided 1 ml blanks as a balance. (It is ESSENTIAL that your tube is balanced!) The centrifugation will pellet any agar fragments, cellular debris, and surviving or resistant *E. coli* cells.

Use a P1000 Pipetteman to transfer the supernatant to a new sterile Eppendorf tube. In the hood in the inner lab (T-378) add 3-4 drops of chloroform to the tube. (There should be a small but visible bubble of chloroform at the bottom of the tube.) Cap the tube and rock it gently several times to mix the chloroform before placing it upright in the refrigerator for the weekend. The chloroform should kill any remaining *E. coli* cells, leaving the P1 phage unaffected.

CAUTION: Avoid any contact with the chloroform! If you get any on your skin, flush immediately with copious amounts of water.

Second, if you have isolated an *E. coli* mutant that is clearly λ^r based on your results from Experiment 6 (i.e., by both cross-streaking and prophage induction tests), you should use an isolated colony of that mutant from your refrigerated Day 3 streak plate to inoculate a new culture. (It doesn't matter if the parent strain of this mutant is RecA+ or RecA-.) If you did not obtain any λ^r mutants in Experiment 6, agar plates containing isolated colonies of uncharacterized λ^r mutants will be provided.

Inoculate one isolated colony of an uncharacterized λ^r mutant strain of *E. coli* into a tube of LB broth and turn it in to be incubated in a rotator at 37°C overnight.

Day 4 (F, Mar 6)

Use a sterile loop to streak a sample of your overnight λ^r *E. coli* culture onto a plate of LB agar and incubate the plate at 37°C. (Colonies from this plate will serve as an "untransduced control" inoculum on Day 7.)

Use the P1000 Pipetteman to dispense 1 ml of your overnight culture into a sterile Eppendorf tube and centrifuge it at high speed for 5 minutes to harvest the cells. (Again, please be sure that the tube is properly balanced before starting the microcentrifuge!)

Use the P1000 Pipetteman to collect and discard the supernatant. Using a new tip, resuspend the cell pellet in 1 ml of MC buffer (100 mM $MgSO_4$, 5 mM $CaCl_2$). Transfer the cell suspension back into the glass test tube that originally contained the buffer and leave it at room temperature for at least 15 minutes while you prepare the next part of the procedure.

Label seven sterile tubes #1-#7. Collect your P1 transducing lysate from the refrigerator. (Be careful not to disturb the chloroform in the bottom of the tube—if you transfer any part of it, you'll sterilize your transduction cultures. That would be bad.)

The additions to be made to each tube are summarized in the table on the next page. Use a P20 Pipetteman to dispense 10 µl of your transducing lysate into tube #1. Use a P200 Pipetteman to dispense 100 µl of your lysate into tubes #2 and #3. Borrow a transducing lysate that was made with the other *E. coli* strain from Day 1 (i.e., either Strain 14 or 15) and dispense 10 µl of that lysate into tube # 4, and 100 µl into tubes #5 and #6. Finally, dispense 100 µl samples of your λ^r *E. coli* suspension into tubes #1, #2, #4, #5, and #7. (Use a separate pipette tip for each *E. coli* culture addition.)

Tube 1	10 µl <u>your</u> transducing lysate	100 µl λ^r *E. coli*
Tube 2	100 µl <u>your</u> transducing lysate	100 µl λ^r *E. coli*
Tube 3	100 µl <u>your</u> transducing lysate	—
Tube 4	10 µl <u>borrowed</u> transducing lysate	100 µl λ^r *E. coli*
Tube 5	100 µl <u>borrowed</u> transducing lysate	100 µl λ^r *E. coli*
Tube 6	100 µl <u>borrowed</u> transducing lysate	—
Tube 7	—	100 µl λ^r *E. coli*

Incubate all 5 tubes in the 37°C water bath for 20 minutes to allow infection to occur.

After the 20 minute incubation, use the P200 Pipetteman to add 200 µl of 1 M Na-Citrate (pH 5.5) to each of your 7 transduction tubes. The function of the Na-Citrate is to chelate all available Ca^{+2} ions. Since Ca^{+2} is required for P1 adsorption, this treatment will prevent re-infection and lysis of any transduced *E. coli* cells by wild-type P1. (We hope.)

Spread 200 µl of each transduction culture onto a separate plate of LB + Na-Citrate + kanamycin. Only those λ^r *E. coli* cells that have recombined the Kan^r marker from Strain 14 or 15 should be able to grow. The "lysate only" controls (tubes #3 and #6) will identify any Kan^r donor cells that may have survived during the construction of your transducing lysates. The "*E. coli* only" control (tube #7) ensures that none of the λ^r recipient cells can grow in the presence of kanamycin by themselves.

Allow the plates to dry and incubate them inverted at 37°C overnight.

Day 5 (M, Mar 9)

Remove the streak plate of your λ^r *E. coli* strain from the 37°C incubator and store it at 4°C until tomorrow

Examine the transduction plates for the appearance of colonies. Such colonies represent individual transductants that now contain the same Kan^r insertions transduced from Strains 14 and 15. Record the approximate numbers of transductants observed for each plate in Table 7-1.

Carefully pick 24 isolated colonies produced from <u>each</u> <u>transducing</u> <u>lysate</u> (i.e., 24 colonies transduced with the Strain 14 lysate and 24 colonies transduced with the Strain 15 lysate) and streak each for isolation on LB + Na-Citrate + kanamycin agar. It doesn't matter which of the two plates (10 µl or 100 µl additions) each of the transductants are picked from. You should streak 6 isolates per plate, making a total of 8 plates.

Incubate your streak plates inverted at 37°C overnight. (You may discard the transduction plates.)

Day 6 (Tu, Mar 10)

Suspensions of the lytic mutant phage λ_{vir} will be provided. Use the P200 Pipetteman and an ethanol-sterilized glass rod to spread each of two Green plates with 150 µl of the λ_{vir} suspension. Allow the plates to dry fully (~15 minutes) before continuing.

For each of your two sets of transductants, pick and patch isolated colonies from all 24 of your streaks onto a single plate of Green agar spread with λ_{vir}. (Use the same grids from Experiment 4, p. 33, as templates.)

Agar plates containing isolated colonies of Strains 14 and 15 will be provided. In the margins of one of your Green plates make additional patches of each of these organisms, as well as a third patch of an isolated colony of your λ^r strain (obtained from the streak plate you refrigerated on Day 5.)

Incubate the two patch plates inverted at 37°C.

Day 7 (W, Mar 11)

Examine your plates for the appearance of blue color within the patch of growing cells. This is evidence of cell lysis due to the infection of cells by λ_{vir}, so only those transductants that have lost their original λ^r mutation should appear blue. Score each of your two sets of 24 transductants for the loss of λ-resistance and record the results in Tables 7-2 and 7-3.

Based on these results you should now be able to deduce whether your λ^r *E. coli* strain carried a mutation in *lamB*, *malT*, or some other locus. (Examples of other *E. coli* mutations that can confer resistance to λ include the *nus* genes, discussed on pp. 348-349 and p. 373 of the textbook.) For a synopsis of our transduction mapping strategy, please review the Experimental Overview section of this lab.

WE'RE DONE!!! Please take this opportunity to discard ALL remaining plates and tubes from all five of the incubators (4°, room temp., 30°, 37°, and 42°). Remember to remove any labeling tape from glass tubes before discarding. (Thanks!)

EXPERIMENT 7 LAB REPORT (due by 4:00 Monday, Mar 16)
Your lab report should consist of Tables 7-1, 7-2, and 7-3 and brief answers to the following questions.

1. Why was it necessary for you to streak out already-isolated colonies of your transductants on Day 5? What result might you anticipate if we patched them directly onto green agar to screen for cotransduction of a λ-sensitive phenotype? (HINT: Note the composition of the medium you used for re-streaking!)

2. The *E. coli* chromosome is approximately 4.5 Mb (4.5×10^6 bp) in size and requires about 100 minutes to be transferred in its entirety by conjugation. How close in base pairs must two genes be in order to be cotransducible by P1 phage?

3. Imagine you mistakenly chose an *E. coli* isolate from Experiment 6 that was a λ lysogen instead of a λ-resistant mutant. What results would you expect to observe in terms of the cotransduction of a λ-sensitive phenotype (Table 7-2) if this strain was used for our transduction experiment? Briefly explain your reasoning.

4A. Like the *lac* operon, the maltose regulon of *E. coli* is subject to catabolite repression, meaning that expression of its genes (including *lamB*) requires the action of Catabolite Activator Protein (CAP—also known as CRP) and its effector molecule cyclic-AMP (cAMP). As a result, cells that are mutant for either CAP or adenyate cyclase (the enzyme that synthesizes cAMP), would also be expected to be resistant to phage λ, since they will not express the LamB receptor. The *cap* gene is located at 75.1' on the *E. coli* chromosome, while adenyate cyclase (*cya*) maps to 86.0' on the chromosome. Using our procedure, what would be the expected cotransduction results (Table 7-2) for λ^r strains carrying either of these two mutations? Briefly explain your answers.

B. Assuming you might, in fact, have isolated a *cap*- or *cya*- λ^r mutant, what is one additional, easily testable prediction you could make about its phenotype that would distinguish it from a *malT*- or *lamB*- mutant?

TABLE 7-1. Transduction of Kan^r gene insertions

Transducing lysate used:	Volume of lysate added:	Number of Kan^r transductant colonies from:	
		λ^r *E. coli*	Lysate alone
Strain 14	10 µl		—
	100 µl		
Strain 15	10 µl		—
	100 µl		
No lysate	—		—

TABLE 7-2. Cotransduction of λ-Sensitivity and Selected Kan^r Markers.

Transducing lysate used:	Number of Kan^r transductants showing:		Probable λ^r mutation of original *E. coli* strain*
	λ resistance	λ sensitivity	
Strain 14			
Strain 15			

* Indicate "*lamB*-", "*malT*-", or "other".

TABLE 7-3. Response of Control *E. coli* Strains to λ_{vir} Infection.

λ resistance or sensitivity ("R" or "S") of:		
Strain 14	Strain 15	λ^r *E. coli*