Experiment 6: Lysogeny and Induction of Phage λ

Soon after the discovery of bacterial viruses (bacteriophage), it was observed that certain phage were purely lytic, resulting in clear plaques of complete host cell lysis, while others caused cloudy or turbid plaques of incomplete lysis. These partially-lytic phage were referred to as "**temperate**" because they tempered (moderated) their destruction of the host population.

Within the population of surviving host cells following temperate phage infection one can generally find both wild-type cells that somehow simply avoided infection and mutants that are genetically resistant to infection. The majority of surviving cells, however, will have acquired a new phenotype as a result of successful phage infection. Although they are otherwise indistinguishable from the wild-type, when these cells are subjected to physical or chemical stress, they may subsequently lyse to release hundreds of new bacteriophage. Because they appear to induce their own lysis, these cells are referred to as **lysogens**. Subsequent work demonstrated that all lysogens carry a complete copy of the temperate phage genome, referred to as a **prophage**, incorporated within the chromosome. This prophage excises from the host chromosome and initiates its lytic growth cycle when the host cell is stressed. The process by which phage production is activated in a lysogen is known as **induction**.

The purpose of this experiment is to use the temperate bacteriophage λ to create and isolate lysogens of *E. coli*, then to induce lytic cycle replication within those cells by exposing them to uv light. In the process, we will experimentally distinguish between true lysogens , non-lysogens, and λ-resistant mutants of *E. coli*, as well as demonstrate the requirement for host-encoded RecA functions to induce the λ prophage.

BACKGROUND
Lysogeny and lysogen immunity. Temperate bacteriophage are characterized by their ability to replicate either by a lytic growth cycle at the expense of a host cell, or by a lysogenic cycle in which the phage genome is incorporated as a prophage into the host cell chromosome. In the case of bacteriophage λ, the "decision" of pursuing either a lytic or lysogenic growth cycle is regulated by a complex molecular regulatory mechanism that is itself influenced by the physiological state of the host cell. Entry into the lysogenic cycle is directly promoted by three regulatory genes called *c*I, *c*II, and *c*III. The "c" in each case stands for "clear", as mutants defective for any of these genes are incapable of lysogeny, and thus produce clear (fully lytic) plaques. The molecular mechanism by which λ activates lysogeny is discussed in pages 352-355 of the textbook.

The primary regulator involved in <u>maintaining</u> lysogeny once it's initiated is the product of the *c*I gene, commonly referred to as **CI protein** or, more simply, **λ repressor**. The function of CI is

to repress the expression of essentially all other λ genes, thus preventing the prophage from initiating lytic growth. The CI protein autoregulates its own synthesis as well, so lysogenized host cells maintain a near-constant level of free CI protein in the cytoplasm. This has the effect of rendering a lysogen effectively "immune" to subsequent infection by additional λ particles. New λ phage can attach and inject their DNA, but the free CI already present will quickly repress all lytic and lysogenic functions, so the new phage DNA is unable to replicate by either pathway and is ultimately degraded. This is why lysogens formed within a λ plaque are able to continue to grow and replicate in the presence of literally millions of free λ virions: the prophage is replicated as part of the host chromosome, it continues to produce free CI, and subsequent infections are inevitably non-productive. The maintenance of lysogeny and lysogen immunity is covered in pages 354-356 of the textbook.

One exception to the rule of lysogen immunity lies in a very rare class of "clear plaque" λ mutants referred to as $\lambda_{\mathbf{vir}}$, for "virulent". These phage are not mutant for any regulatory proteins, but instead have altered the operator sequences to which CI binds. As there are two major CI operator regions within the λ genome, the λ_{vir} phenotype actually requires multiple mutations— hence the rarity of this class of mutant. The effect of these operator mutations is to create a purely lytic phage that can't be repressed by CI because there are no binding sites for the repressor within the DNA. Even CI already produced by a resident prophage will have no effect, meaning λ_{vir} mutants will form clear plaques even on λ lysogens.

In addition to lysogens, a number of the cells surviving within the plaque of a temperate phage will be mutants that are simply genetically resistant to infection. Essentially all bacteriophage use normal host envelope proteins as specific receptors for their attachment and infection. (This absolute requirement for host structures forms the basis for the host specificity observed with most bacteriophage.) Resistant host strains are typically those cells that have altered or lost the receptor required for phage attachment as a result of spontaneous mutation. Such cells are no longer recognized as suitable hosts by the phage, and thus can't be infected. Consequently, λ-resistant mutants of *E. coli* won't be lysed even by virulent strains of λ $\lambda_{vir}.$

Induction. The action of the CI protein causes most λ lysogens to remain stable under a wide range of growth conditions. Ultimately, however, any prophage must be able to excise itself and initiate lytic growth at least under some conditions if it is ever to infect new hosts. This becomes especially important when the existing host cell itself is dying, since the strategy of a prophage is to rely entirely on the host lysogen for its replication. If the host is incapable of replicating further, it is entirely in the phage's best interests to abandon lysogeny and initiate lytic growth in order to find a new host better suited for replication. This is the process known as induction.

In the case of λ, the signal for prophage induction is the same signal used by the host cell for induction of the SOS response— that is, extensive DNA damage resulting in an abundance of

single-stranded DNA (ssDNA) within the cell. Interestingly, the λ prophage simply co-opts one of the host cell's own regulatory components for its induction. You previously learned about the activation of the SOS response: how RecA protein, activated by ssDNA, causes autocleavage of the LexA repressor, thus activating an entire array of SOS genes. In λ lysogens, activated RecA also catalyzes the autocleavage of all existing CI protein. With CI no longer present, the λ genes for prophage excision and lytic replication become activated, allowing the phage to escape its doomed host. One implication of this induction mechanism that will be tested in our experiment is the prediction that *recA*- *E. coli* lysogens should <u>not</u> be inducible for lytic growth. Induction of λ prophage is discussed in more detail on pages 356-359 of the textbook.

EXPERIMENTAL OVERVIEW
To isolate potential lysogens, we will first infect lawns of both wild-type and *recA*- mutant strains of *E. coli* with phage λ̃ . .   surviving cells from the resulting plaques and streak them to obtain 12 isolates from each parent strain. We'll then screen those isolates to distinguish the true lysogens from any non-lysogens or resistant cells by testing their sensitivity to both λ and λ$_{vir}$. Finally, we'll attempt to induce lytic phage growth in the isolates by treating the cells with uv light. In particular, we'll look for differences in prophage induction by the RecA+ and RecA- *E. coli* lysogens.

PROCEDURE
Day 1 (F, Feb 20)
> Broth cultures of a wild-type and an isogenic *recA*- strain of *E. coli* (Strains 12 and 13, respectively) will be provided.
>
> Add 0.1 ml of Strain 12 to a tube of molten LB soft agar, roll the tube between your palms to mix, then immediately pour it onto a plate of LB agar, tipping the plate so that you form an even top layer of soft agar. (<u>Do</u> <u>not</u> <u>remove</u> <u>the</u> <u>tube</u> <u>of</u> <u>molten</u> <u>soft</u> <u>agar</u> <u>from</u> <u>the</u> <u>water</u> <u>bath</u> <u>until</u> <u>immediately</u> <u>before</u> <u>use</u> <u>or</u> <u>it</u> <u>will</u> <u>solidify</u>!!!) Repeat this procedure with Strain 13 and allow the top agar lawns to solidify for 5 minutes.
>
> Use a mechanical pipette to apply a 5 μl drop of phage λ to the surface of each plate. (Use a separate pipette tip for each phage drop.)
>
> Allow the phage spots to dry for 10-15 minutes before incubating the plates <u>without</u> <u>inverting</u> at room temperature over the weekend.

Day 2 (M, Feb 23)

Your plates from Friday should each have a large plaque with a noticeably turbid center where the phage drops were placed. The turbidity is caused by the growth of cells that were not lysed by the bacteriophage.

Rub a sterile loop over the surface of each plaque and streak each sample for isolation on a separate plate of LB agar. Incubate the two streak plates at 37°C.

Day 3 (Tu, Feb 24)

Use sterile toothpicks to pick 12 isolated colonies from each of your streak plates, re-streaking each colony for isolation on LB agar. You should use a total of 4 new plates, streaking 6 isolates per plate.

Incubate the plates at 37°C. Store your original streak plates at 4°C until next period.

Day 4 (W, Feb 25)

Today you will screen your 24 isolates to distinguish the true lysogens from non-lysogens and λ-resistant mutants. To do this, you will cross-streak each strain across lines of wild-type phage λ and the lytic mutant strain λ_{vir}. Lysogens will be unaffected by wild-type λ, but will be lysed by λ_{vir}; non-lysogens will be lysed by both types of phage, and resistant cells will be unaffected by either phage. We use Green agar for this analysis because it enables us to distinguish the difference between unlysed and even partially lysed cell streaks fairly easily.

Use a sterile transfer pipette to dribble a thin, continuous line of phage λ across the surface of each of four Green plates. Use a second transfer pipette to repeat this procedure using a stock of λ_{vir}, as shown in Fig. 6-1. Be careful not to score the surface of the agar with the pipette in each case.

Allow the phage samples to dry completely (5-10 minutes). Use sterile toothpicks to pick isolated colonies of each of your possible λ lysogens and inoculate them as single streaks across the two phage stripes, as shown in Fig. 6-1. Cross-streak six of your potential lysogen strains per plate, as
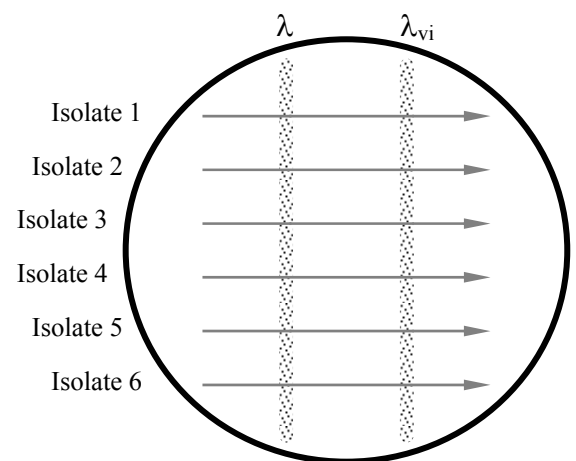

FIGURE 6-1. Screening of six putative λ lysogens by cross-streaking against λ and λ_{vir}.

shown. <u>Make</u> <u>sure</u> <u>that</u> <u>your</u> <u>streaks</u> <u>cross</u> <u>the</u> λ <u>stripe</u> <u>first,</u> <u>THEN</u> <u>the</u> <u>stripe</u> <u>of</u> λ_{vir}!!!
Again, try to avoid scoring the surface of the agar with the toothpicks.

Incubate your cross-streak plates at 37°C. Store your streak plates from Day 3 at 4°C, discarding the previous streaks from Day 2.

Day 5 (F, Feb 9)

Green agar is commonly used as an indicator medium for the detection of cell lysis. The medium contains a pH indicator that turns dark blue under acidic conditions. The cytoplasmic contents of most bacterial cells is slightly acidic, so areas of partial cell lysis can be detected as blue or mixed green and blue, rather than green (colorless) growth on the agar. Be aware that cells growing within grooves or scratches in the agar surface may well appear blue as a result of fermentation due to oxygen limitation; this is why it's important not to score the surface of the agar when inoculating the plates.

Score your strains for sensitivity or resistance to each of the two virus stocks and enter the results in Table 6-1. Even partial lysis should be scored as sensitivity to the phage. A control plate cross-streaked with known lysogen, non-lysogen, and resistant strains will be on display for purposes of comparison.

Day 6 (M, Mar 2)

A broth culture of a non-lysogen *E. coli* strain will be provided. Add 0.1 ml of this culture to a tube of molten LB soft agar, roll the tube between your palms to mix, then pour it onto a plate of LB agar to form an even lawn, as on Day 1. Repeat this procedure to produce a second plate containing an identical top agar lawn.

Once the top agar has solidified, place the two plates onto the patching grids shown on p. 33 of this manual. Inoculate each of your 24 potential lysogen strains onto <u>both</u> plates by picking an isolated colony from your refrigerated (Day 3) streak plate with a sterile toothpick and stabbing it into the agar of each plate. (You only need to pick a single colony once to stab both plates in turn.)

Place one plate under a uv lamp, remove its lid, then turn on the lamp for 10 seconds to induce phage excision. The second plate will serve as your uninduced (no uv) control. Incubate both plates at 37°C.

Return your streak plates to 4°C, as they may be required for Experiment 7.

Day 7 (Tu, Mar 3)

      Score your plates for the appearance of plaques around the stab inocula of each of your 24 possible lysogen strains. Record the results in Table 6-2.

EXPERIMENT 6 LAB REPORT (due Friday, Mar 6)
Your lab report should consist of Tables 6-1 and 6-2, and brief answers to the following questions.

1. Experiments demonstrate that the frequency of λ lysogeny (as opposed to lytic growth) is fairly low (~1%?) in healthy, growing host populations. This frequency actually increases in populations in which the potential host cells have largely stopped growing. Given that an integrated prophage is dependent on host cell growth for its own replication, this would seem to be a counter-productive response. Why is it still an evolutionarily viable strategy—indeed, a highly *successful* strategy—for λ survival?

2. In this experiment we used a *recA-* strain as a host for λ to demonstrate the relationship of host SOS induction with prophage induction. What results would you anticipate for lysogens obtained in our experiment if you had used a *recA- lexA-* double mutant as the host strain instead? Briefly describe and explain these results in terms of A) the cross-streak plates from Day 4, and B) the uv induction plates from Day 6.

3. As you learned in lecture, λ prophage integration is an example of site-specific recombination. How might your results have changed in this experiment if you had used a temperate phage that integrated by homologous recombination instead? (Assume that excision is still accomplished entirely by phage-encoded proteins.) Briefly describe and explain these changes in terms of A) the cross-streak plates from Day 4, and B) the uv induction plates from Day 6.

4. How might your results have changed in this experiment if you had used a λ mutant that synthesizes an unstable CI protein such that 0.1% of all lysogens will spontaneously induce excision and lytic replication? Briefly describe and explain the anticipated results in terms of A) the cross-streak plates from Day 4, and B) the uv induction plates from Day 6.

TABLE 6-1. Screening of Putative Lysogen Strains for λ Resistance or Sensitivity.

Parent Strain	Isolate Number	Resistance (R) or Sensitivity (S) to:		Strain Phenotype*
		λ	λ_{vir}	
Strain 12 (*recA*+)	1			
	2			
	3			
	4			
	5			
	6			
	7			
	8			
	9			
	10			
	11			
	12			
Strain 13 (*recA*-)	1			
	2			
	3			
	4			
	5			
	6			
	7			
	8			
	9			
	10			
	11			
	12			

* Classify each strain as either lysogen, non-lysogen, or resistant mutant, based on your results.

TABLE 6-2. Induction of the λ Lytic Cycle in Putative RecA+ and RecA- Lysogens.

Parent Strain	Isolate Number	Plaque Formation (+ or -) After:	
		UV Treatment	No Treatment
Strain 12 (*recA+*)	1		
	2		
	3		
	4		
	5		
	6		
	7		
	8		
	9		
	10		
	11		
	12		

Parent Strain	Isolate Number	UV Treatment	No Treatment
Strain 13 (*recA-*)	1		
	2		
	3		
	4		
	5		
	6		
	7		
	8		
	9		
	10		
	11		
	12		