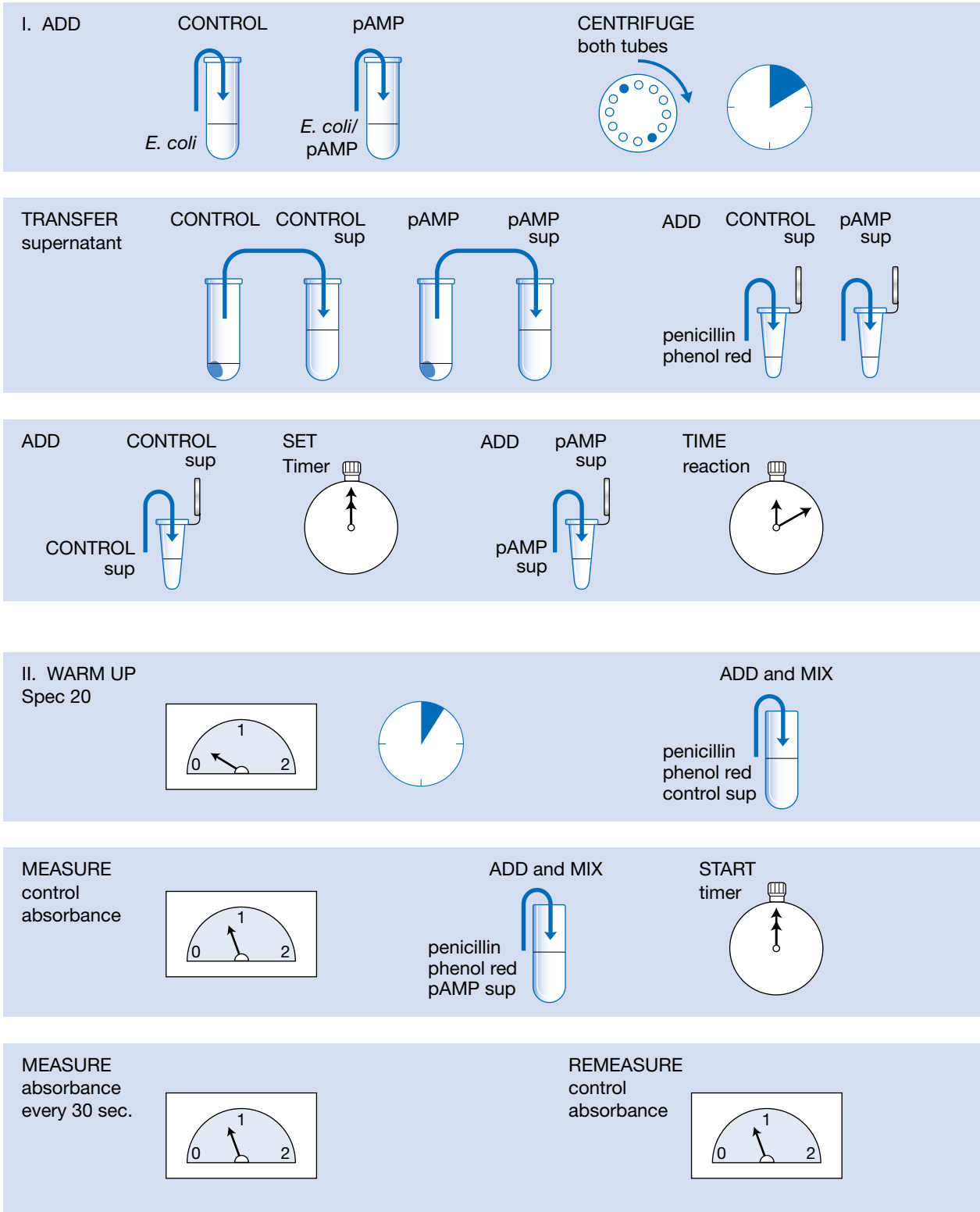
Assay for an Antibiotic Resistance Enzyme

LABORATORY 6 INTRODUCES WORKING WITH PROTEINS and performing an enzyme assay. In Laboratory 5, Rapid Colony Transformation of *E. coli* with Plasmid DNA, *E. coli* were transformed with a plasmid that rendered the cells resistant to the antibiotic ampicillin. The gene that confers ampicillin resistance codes for the enzyme β-lactamase, which breaks the β-lactam ring in ampicillin. In this laboratory, β-lactamase is isolated from ampicillin-resistant *E. coli*, and its enzyme activity is observed using an in vitro assay.

Ampicillin is a derivative of the antibiotic penicillin, and β-lactamase destroys penicillin even more efficiently than it destroys ampicillin. In this experiment, penicillin will therefore be used as the substrate in the β-lactamase assay. β-lactamase is isolated from overnight cultures of *E. coli* transformed with a plasmid that confers ampicillin resistance, such as pAMP. Because β-lactamase readily leaks out of the cells and into the culture medium, the media itself is used as a solution of β-lactamase. β-lactamase is then used to convert penicillin into penicilloic acid. The reaction is monitored using the pH indicator phenol red, which signals the formation of penicilloic acid by turning from red (basic pH) to yellow (acidic pH).

A kit based on this laboratory is available from the Carolina Biological Supply Company (Catalog no. 21-1137).

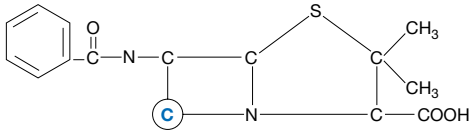

I. ADD
CONTROL
pAMP
E. coli
E. coli/
pAMP
CENTRIFUGE
both tubes
TRANSFER
supernatant
CONTROL
CONTROL
sup
pAMP
pAMP
sup
ADD
CONTROL
sup
pAMP
sup
penicillin
phenol red
ADD
CONTROL
sup
CONTROL
sup
SET
Timer
ADD
pAMP
sup
pAMP
sup
TIME
reaction
II. WARM UP
Spec 20
0
1
2
ADD and MIX
penicillin
phenol red
control sup
MEASURE
control
absorbance
ADD and MIX
penicillin
phenol red
pAMP sup
START
timer
MEASURE
absorbance
every 30 sec.
REMEASURE
control
absorbance

PRELAB NOTES

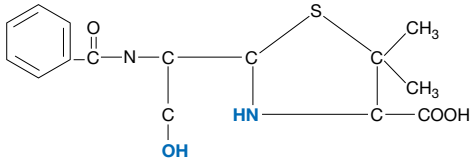
β-lactamase

β-lactamase is so-called because it acts to break a bond in a four-member ring in the ampicillin molecule called a β-lactam ring. The enzyme has a similar action on penicillin, which converts to penicilloic acid in the reaction. In fact, β-lactamase is commonly called penicillinase. Penicillin and penicilloic acid are shown below. The enzyme acts on the carbon marked with a circle.

Penicillin



Penicilloic Acid



β-lactamase is easy to isolate because it is stored in the *E. coli* periplasm, the space between the inner cell membrane and the cell wall, and leaks into the surrounding media. Thus, the growth media from an overnight culture of *E. coli* containing pAMP plasmid becomes a ready source of β-lactamase. Cells can be removed and the supernatant can be used as a β-lactamase solution. β-lactamase is fairly stable and can be frozen, thawed, and reused, but like any enzyme, it should not be left at room temperature for long periods of time. Keep β-lactamase on ice when not in use.

The β-lactamase solution may have a low pH, which can interfere with the assay. Test by mixing 100 µl of phenol red with 200 µl of β-lactamase solution. If the solution turns orange or yellow, the pH of the β-lactamase is too low. The pH of the β-lactamase can be adjusted to 7.0 using NaOH and a pH meter. Alternatively, increase the concentration of Tris (pH 7.9) in the phenol red from 10 mM to 20 mM or use Tris at a higher pH, such as 8.5 or 9.

Assay Conditions

The assay for β-lactamase is very simple. As the enzyme converts penicillin to penicilloic acid, the associated formation of hydrogen ions (H^+) can be monitored using a pH indicator. The pH indicator in this experiment is phenol red. At basic pH (>7.0), a solution of phenol red is red-pink. As the pH drops below 7.0 (i.e., becomes acidic), the solution changes first to orange and finally to yellow. Here, the reaction will be started at a pH of 7.9 (by adding 10 mM Tris [pH 7.9] to the phenol red solution). This colorimetric reaction can be quantitated using a spectrophotometer to measure the increase in absorbance at 430 nm, the absorption optimum for yellow. (Any spectrophotometer that can be used to read visible light, such as a Spectronic 20+, will suffice.) The rate of the reaction will vary, based on the quality of the β-lactamase. Generally, the reaction is complete within 10 minutes.

Plasmid Selection

Any plasmid carrying ampicillin resistance can be used in this experiment, although we recommend using pAMP. It is suggested that colonies obtained from Laboratory 5, Rapid Colony Transformation of *E. coli* with Plasmid DNA, be used as the source of ampicillin-resistant bacteria. Transformed colonies from Laboratory 5 can be stored at 4°C and used to make an overnight culture for up to 1 week. After 1 week, most or all of the cells on an LB-AMP plate will be dead.

Penicillin Solution

There are various forms of penicillin, but for this laboratory, we recommend using penicillin-G Procaine salt to make the penicillin solution. Do not worry if the salt does not completely dissolve; the solution is still usable. Note that penicillin-G Procaine salt is usually sold in units of activity. Generally, the number of units/gram is ~1000/mg. In addition, be aware that penicillin can break down into penicilloic acid spontaneously, especially if it is in solution for too long, if it is in solution at room temperature overnight or longer, or if the penicillin salt is old. Before use, check the penicillin solution by mixing 700 µl with 100 µl of phenol red solution. The phenol red may become pale red to pink, indicating the presence of a small amount of penicilloic acid. If the phenol red turns orange, we recommend that you make a fresh solution using a new stock of penicillin-G.

FOR FURTHER INFORMATION

The protocol presented here is based on the following published methods.

Skinner A. and Wise R. 1977. A comparison of three rapid methods for the detection of beta-lactamase activity in *Haemophilus influenzae*. *J. Clin. Pathol.* **30:** 1030–1032.

Wong K.W. and Soo-Hoo T.S. 1976. A rapid, simple agar-overlay method for the detection of pencillinase-producing *Staphylococcus aureus* in the clinical bacteriology laboratory. *Jpn. J. Microbiol.* **20:** 153–154.

PRELAB PREPARATION

1. Prepare a 0.04% solution of phenol red in 10 mM Tris (pH 7.9). This solution can be stored indefinitely at room temperature. Phenol red is typically sold as a 1% solution.
2. The day before the laboratory, prepare two *E. coli* cultures according to the protocol in Laboratory 2B, Overnight Suspension Culture. Inoculate the first culture with a cell mass scraped from one wild-type MM294 colony from an LB plate. You will need one 5-ml culture per experiment. Inoculate the second culture with colonies selected from the +LB/amp plate from Laboratory 5, Rapid Colony Transformation of *E. coli* with Plasmid DNA. You will need one 5-ml culture per experiment. Grow this culture in LB broth plus ampicillin. If possible, use a higher concentration of ampicillin (200 µg/ml) than previously suggested.

3. On the day of the laboratory, make up a 4 mg/ml solution of penicillin-G. Keep at 4°C until needed. If making the solution the day before, be aware that it will break down to penicilloic acid if left at room temperature. Store at 4°C overnight or at –20°C for long-term storage.
4. Test reagents on the day of the laboratory. Mix phenol red solution with the penicillin stock to check pH (see Prelab Notes).
5. Prepare aliquots for each experiment:

   1 *E. coli* overnight culture (5 ml) in 15-ml culture tube labeled "control"
   1 *E. coli*/pAMP overnight culture (5 ml) in 15-ml culture labeled "pAMP"
   6 ml of 4 mg/ml penicillin-G
   1 ml of 0.04% phenol red solution

MATERIALS

REAGENTS

E. coli overnight culture (1)
E. coli/pAMP overnight culture (1)
LB broth
LB broth/amp
Penicillin-G▼(4 mg/ml)
Phenol red▼solution (0.04%)

SUPPLIES AND EQUIPMENT

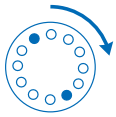
Beaker for waste/used tips
Bleach (10%)▼or disinfectant
Clinical centrifuge
Culture tubes (15 ml)
Micropipettor (100–1000 µl) + tips
Permanent marker
Spectrophotometer (optional)
Spectrophotometer tubes (clear)
Test tube rack
Timer or stopwatch
Tubes (1.5 ml)

▼ See Appendix 4 for Cautions list.

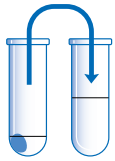
METHODS

I. Set Up β-lactamase Assay (30 minutes)

1. Collect the reagents and place them in a test tube rack on the lab bench.
2. Obtain two 15-ml tubes, one containing 5 ml of *E. coli*/pAMP overnight culture (labeled "pAMP") and a second containing 5 ml of wild-type *E. coli* culture (labeled "control"). Label the tubes with your name.
3. Place two tubes in a balanced configuration in a rotor of a clinical centrifuge. Centrifuge cells at 2500–4000 rpm for 10–15 minutes to pellet cells on the bottom-side of the culture tube.
4. While the tubes are in the centrifuge, label one fresh 15-ml culture tube "control sup." Label a second fresh 15-ml culture tube "pAMP sup." Label both tubes with your name.



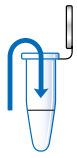
A tight pellet will form at the bottom of the tube after centrifugation and the supernatant should be clear. If the supernatant is cloudy, recentrifuge 5 minutes longer and if possible increase the speed.



You do not want any of the cell pellet and have plenty of β-lactamase, so leave a little of the supernatant behind to avoid disturbing the pellet.

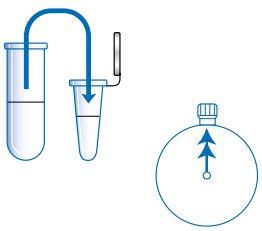
5. When the centrifuge stops, pour off the supernatant from each tube into the correspondingly labeled fresh 15-ml tube. *Do not to disturb cell pellet*. The "pAMP sup" tube contains β-lactamase that has leaked into the supernatant, and the "control sup" tube contains control supernatant. Bacterial pellets may be frozen for further analysis.
6. Obtain two 1.5-ml tubes. Label one tube "control sup" and the other "pAMP sup."
7. Use the matrix below as a checklist while adding reagents to each reaction. Read down each column, adding the same reagent to all appropriate tubes. *Use a fresh tip for each reagent*. Refer to detailed instructions that follow.

Tube	Penicillin-G	Phenol red	Control sup	pAMP sup
Control sup	700 µl	100 µl	200 µl	–
pAMP sup	700 µl	100 µl	–	200 µl



The phenol red solution may turn from dark red to a reddish orange in color. This is normal. If it turns orange or even yellow, the penicillin has already broken down to penicilloic acid. Get a fresh solution of penicillin.

8. Use a micropipettor to add 700 µl of penicillin solution to each 1.5-ml reaction tube.
9. Use a fresh tip to add 100 µl of phenol red to each reaction tube.
10. Use a fresh tip to add 200 µl of "control sup" solution to the 1.5-ml reaction tube labeled "control sup."
11. Note time, or set timer, and then use a fresh tip to add 200 µl of "pAMP sup" β-lactamase solution to the "pAMP sup" reaction tube. Invert tube to mix. Work quickly as the reaction will begin as soon as the β-lactamase is added. If working with a partner, one person adds the β-lactamase while the other sets the timer.
12. Begin timing the reaction. Place the tube in a rack to watch the color change. (Remember that temperature affects the rate of the reaction, so do not hold the tube in your hand as this will speed up the reaction.) Determine the time it takes for the reaction to turn yellow.

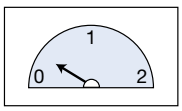


The red color should slowly fade to a rose color, then to orange, and finally, when the reaction is complete, completely to yellow. Try to be accurate in timing reaction. One variable may be your judgment of when the reaction is yellow. At this point, note the time again or stop the timer and record.

TIME FOR THE REACTION TO TURN FROM RED TO YELLOW _____

II. Quantitative β-lactamase Reaction (30 minutes)

Now follow the enzyme reaction quantitatively by monitoring in a spectrophotometer, such as the Spectronic 20+ (Spec 20). The increase in yellow absorbance is a measure of the formation of penicilloic acid.



1. Turn on the spectrophotometer to warm up and set wavelength to 430 nm.
   a. Zero the spectrophotometer following the manufacturer's instructions. A solution containing penicillin, phenol red, and fresh LB (mixed in the amounts shown in Step 2) can be used to "zero" the spectrophotometer. Peak absorbance of phenol red at basic pH (i.e., when red) is 560 nm, whereas peak absorbance at acidic pH (i.e., when yellow) is 430 nm.
   b. Test by measuring the absorbance of the phenol red diluted to 100 µl in 3 ml of water at several different wavelengths. Then add a little acid to the same phenol red solution so that it turns yellow and retake absorbance readings at different wavelengths.

The control tube will be used to measure the background level of 430 nm absorbing material in the reaction. It should have a very low absorbance.

2. When the spectrophotometer has warmed up for 5 minutes, make up a control tube to determine the background in the reaction. (If using a spectrophotometer with a 1-ml capacity cuvette, add volumes indicated in parentheses.)
   a. Add 2 ml of penicillin solution to the spectrophotometer tube (600 µl for a 1-ml cuvette).
   b. Add 100 µl of phenol red solution (50 µl for a 1-ml cuvette).
   c. Add 600 µl of "control sup" solution to the tube (200 µl for a 1-ml cuvette).
   d. Cover the tube with Parafilm and with a thumb over the top, invert it twice to mix the reagents.
3. Place the control tube in the spectrophotometer. Record the absorbance at 430 nm. This is the "control sup" background absorbance. Save this tube and read the absorbance again in Step 10.
4. Set up a table in a notebook as shown below to record the data.

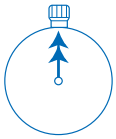
"CONTROL SUP" BACKGROUND INITIAL ABSORBANCE READING

Time (seconds)	pAMP sup
0	
30	
60	
90	
120	
150	
180	
210	
240	
270	
300	
330	
360	
390	
420	
480	

"CONTROL SUP" BACKGROUND FINAL ABSORBANCE READING

Using less pH indicator keeps spectrophotometer readings from going off scale. If reaction produces an absorbance value of 2, then repeat using half the amount of phenol red (50 µl for Spec 20 glass tube, or 25 µl if using 1-ml cuvette).

5. In a new spectrophotometer glass tube, add 2 ml of penicillin solution. (If using a regular spectrophotometer and a 1-ml capacity cuvette, add 0.6 ml of penicillin.)
6. To the same tube, add 100 µl of phenol red (50 µl for a 1-ml cuvette).
7. To the same tube, add 0.5 ml of "pAMP sup" β-lactamase solution (0.2 ml in a 1-ml cuvette).
8. Mix by pipetting up and down or by covering the top of the tube with Parafilm and inverting two or three times. Immediately transfer the tube to the Spec 20. The reaction starts as soon as the β-lactamase is added, so move quickly to establish the zero time reading.



If working with a partner, one person keeps track of the time and the other keeps track of the change in absorbance.

9. Simultaneously, start the timer and take an initial reading at 430 nm from the spectrophotometer. The initial reading of pAMP sup may not be as low as the control reading. Overnight *E. coli*/pAMP cultures contain cleaved ampicillin which, like penicilloic acid, is acidic and contributes to the color change from red to yellow. Record the readings every 30 seconds until no significant increase in absorbance is seen for 1 minute.
10. Place the control tube (from Step 3) in the spectrophotometer. Record this reading again to confirm that the absorbance of the control tube does not change over time.
11. Take time for responsible cleanup.
    a. Segregate for proper disposal culture tubes and micropipettor tips that have come in contact with *E. coli*.
    b. Disinfect cell suspensions, tubes, and tips with 10% bleach solution or disinfectant.
    c. Wipe down lab bench with soapy water, 10% bleach solution, or disinfectant (such as Lysol).
    d. Wash hands before leaving lab.

RESULTS AND DISCUSSION

In this laboratory, you have carried out an enzyme reaction using β-lactamase, also known as penicillinase. β-lactamase is the enzyme encoded by the gene *pAMP* that renders *E. coli* resistant to ampicillin, a form of penicillin. The enzyme converts penicillin into penicilloic acid. Although this reaction cannot be "seen," it can be monitored by following the associated accumulation of hydrogen ions. This experiment demonstrates the daily challenge of the molecular biologist: How to see what is not visible.

In this laboratory exercise, you should have observed that the "control sup" tube remained red throughout the incubation. On the other hand, you should have observed the "pAMP sup" tube change from pale red to orange to bright yellow. This color change represents the formation of hydrogen ions as a result of the formation of penicilloic acid. The color change in the enzyme reaction tubes should be fairly rapid. The rapidity of the change is a function of the activity and/or concentration of the enzyme.

1. Why is the phenol red solution made up in 10 mM Tris-HCl (pH 7.9)?
2. Write out the equation for the enzyme reaction that occurs in this experiment.
3. How would you predict the following factors to affect the rate of the reaction:
    a. Decreasing the temperature.
    b. Using a higher concentration penicillin-G.
    c. Heating the β-lactamase to 70°C for 10 minutes.
    d. Adding more β-lactamase.

e. Adding more phenol red.

f. Using 50 mM Tris (pH 7.9) to make up the phenol red.

g. Using 10 mM Tris (pH 9.0) to make up the phenol red.

4. Plot the spectrophotometer data for the "pAMP sup" β-lactamase reaction. Plot time in minutes on the *x* axis. Plot absorbance on the *y* axis.

5. Predict what the plots would look like if you took readings at 560 nm (the peak absorbance of phenol red at basic pH).

6. The rate of the reaction is defined as the amount of substrate formed over time. In this experiment, you indirectly measured substrate formation as an increase in $[H^+]$, which you measured as the increase in absorbance at 430 nm. Look at your plot and describe the point at which the rate of the reaction is greatest. Write the rate of the reaction as change in absorbance per minute.

FOR FURTHER RESEARCH

1. Enzymes are generally sensitive to heat. Heat-inactivate 1 ml of the β-lactamase solution by incubating at 70°C for 10 minutes and then repeat the assay, comparing the reaction of the heat-inactivated enzyme with untreated enzyme.

2. Use β-lactamase activity to destroy ampicillin on an agar plate. Do the following:

   a. Sterilize β-lactamase solution by filtering through a syringe filter into a sterile 15-ml tube.

   b. Obtain sterile cotton applicators, an agar plate containing ampicillin, and a tube of *E. coli* culture. Soak a cotton applicator in the sterile β-lactamase.

   c. Moving quickly, lift the plate lid and write your initials with the applicator on the surface of the plate. (You can redip into the β-lactamase.)

   d. Allow the plate to set for a few minutes, so that the β-lactamase soaks into the agar.

   e. Soak a fresh sterile applicator in the *E. coli* culture. Open the plate again and use the applicator to coat the entire surface with bacteria.

   f. Incubate the plate at 37°C (take time for responsible cleanup).

   g. After 24 hours, remove the plate; store at 4°C until it can be analyzed.

   h. Examine the plate and explain what has happened.

3. β-lactamase resides in the periplasm between the cell wall and the cell membrane. Normally, β-lactamase leaks into the media through the cell wall, but some remains trapped in the periplasm. Lysozyme is an enzyme from egg whites that destroys bacterial cell walls and releases the trapped β-lactamase into the media. Treat bacteria with lysozyme to release β-lactamase.

   a. Make up a stock of 20 mg/ml solution of lysozyme (pH 7.9) (store at –20°C).

   b. Thaw the "pAMP" and "control" bacterial pellets frozen in Part I, Step 5.

c. Add 5 ml of fresh LB broth to each tube containing the pellets. Close caps securely and vortex or shake the tubes vigorously for 1 minute.

d. Add 500 µl of lysozyme solution to each tube. Close caps securely and mix by inverting tubes several times.

e. Incubate for 10 minutes at 37°C or 15 minutes at room temperature. Close caps securely and vortex or shake tubes vigorously for 1 minute.

f. Place tubes in a *balanced* configuration in a clinical centrifuge. Centrifuge at 2000–4000 rpm for 10 minutes to pellet cells.

g. Label two fresh 15-ml tubes "pAMP + lys" and "control + lys."

h. When the centrifuge stops, pour the supernatant from "pAMP" and "control" tubes into "pAMP + lys" and "control + lys" tubes, respectively. These tubes contain β-lactamase released from the periplasmic space (pAMP + lys) and control supernatant (control + lys).

i. Proceed exactly as in Steps 6 through 12 from Part I of the protocol, replacing "control sup" with "control + lys" and "pAMP sup" with "pAMP + lys."

j. Compare the times the "pAMP sup" and "pAMP + lys" enzyme reactions take to reach completion.

4. Overnight *E. coli*/pAMP cultures contain cleaved ampicillin which, like penicilloic acid, is acidic. Compare the absorbance of your "control sup" and "pAMP sup" solutions by setting up two reactions as in Part II of this lab, but using water in place of penicillin-G. To each reaction, add 600 µl of water and 50 µl of phenol red. To one tube, add 200 µl of "control sup" and to the other tube add 200 µl of "pAMP sup." Measure the absorbances at 430 nm using a spectrophotometer. Do the values differ?

5. You can purify β-lactamase from a 100-ml overnight culture of *E. coli*/pAMP.

a. Add lysozyme to the culture to 2 mg/ml and incubate for 15 minutes at 37°C with shaking. Centrifuge to remove cells. Dissolve 15 g of ammonium sulfate in the supernatant. Place on ice for 10 minutes, and then centrifuge for 10 minutes. A precipitate will form at the bottom of the tube. Save this pellet and the supernatant. Resuspend the pellet in 2 ml of 10 mM Tris (pH 7.9) and set aside. Add another 25 g of ammonium sulfate to the supernatant and place on ice for 10 minutes. Centrifuge for 10 minutes and save the final supernatant. Resuspend the pellet in 2 ml of 10 mM Tris (pH 7.9).

b. Now determine which fractions contain β-lactamase activity by assaying the original supernatant, the resuspended ammonium sulfate precipitates, and the final supernatant with penicillin-G and phenol red. Determine the protein concentration of the original supernatant using a Bradford assay or Bio-Rad solution and compare with the other fraction containing β-lactamase activity. Finally, use a spectrophotometer as described in this laboratory to determine the relative β-lactamase activity of the original supernatant and the active fraction. Determine activity versus protein concentration.

6. You can visualize your β-lactamase protein by SDS-polyacrylamide gel electrophoresis. For details, see Laboratory 7, Purification and Identification of Recombinant GFP. β-lactamase samples are mixed with an equal volume of 2x denaturing buffer (100 mM Tris [pH 6.8], 200 mM dithiothreitol, 4% SDS, 0.1% bromophenol blue, and 10% glycerol) and boiled for 10 minutes prior to loading onto the gel. In addition, obtain 5-ml cultures of wild-type *E. coli* and *E. coli*/pAMP. Centrifuge and resuspend the cells in 1x denaturing buffer, boil 10 minutes, and load on gel. After running gel, determine the predicted size of β-lactamase and look for the corresponding band on the gel. It should be present in *E. coli*/pAMP but not in wild-type cells.