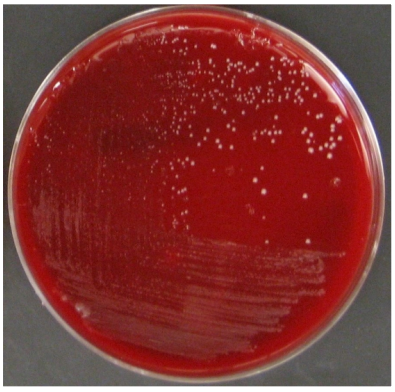
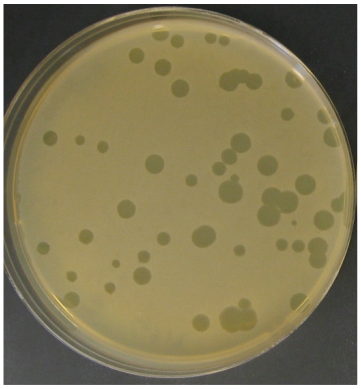
The Skin Microbiome

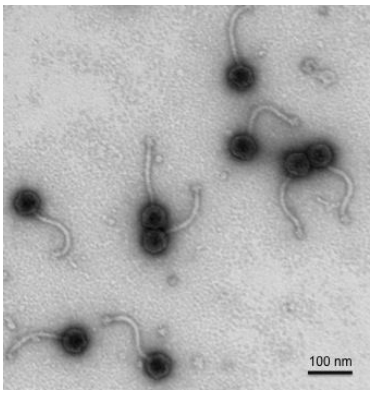
Propionibacterium acnes and Their Bacteriophage



Acne bacteria
Colonies of P. acnes bacteria grown in culture.
(Image credit: UCLA/Modlin lab)



Killer virus
Dark circles reveal where *P. acnes* virus has killed off acne bacteria.
(Image credit: UCLA/Modlin lab)



Magnified virus
A view of P. acnes virus particles under the electron microscope.
(Image credit: Charles Bowman/University of Pittsburgh)

PROJECT OVERVIEW

In conducting the procedures associated with this project, we will investigate the skin microbiome by isolating the bacterium *Propionibacterium acnes* (*P. acnes*) and bacteriophage that infect it. In the course of the experiment, you will discover and characterize a new phage.

You will design **two experiments** using your *P. acnes* and phage. In the first experiment, you will explore the **antibiotic resistance of your bacterial isolate**. In the second experiment you will explore **host-pathogen specificity** using *P. acnes* and phage isolated in the class.

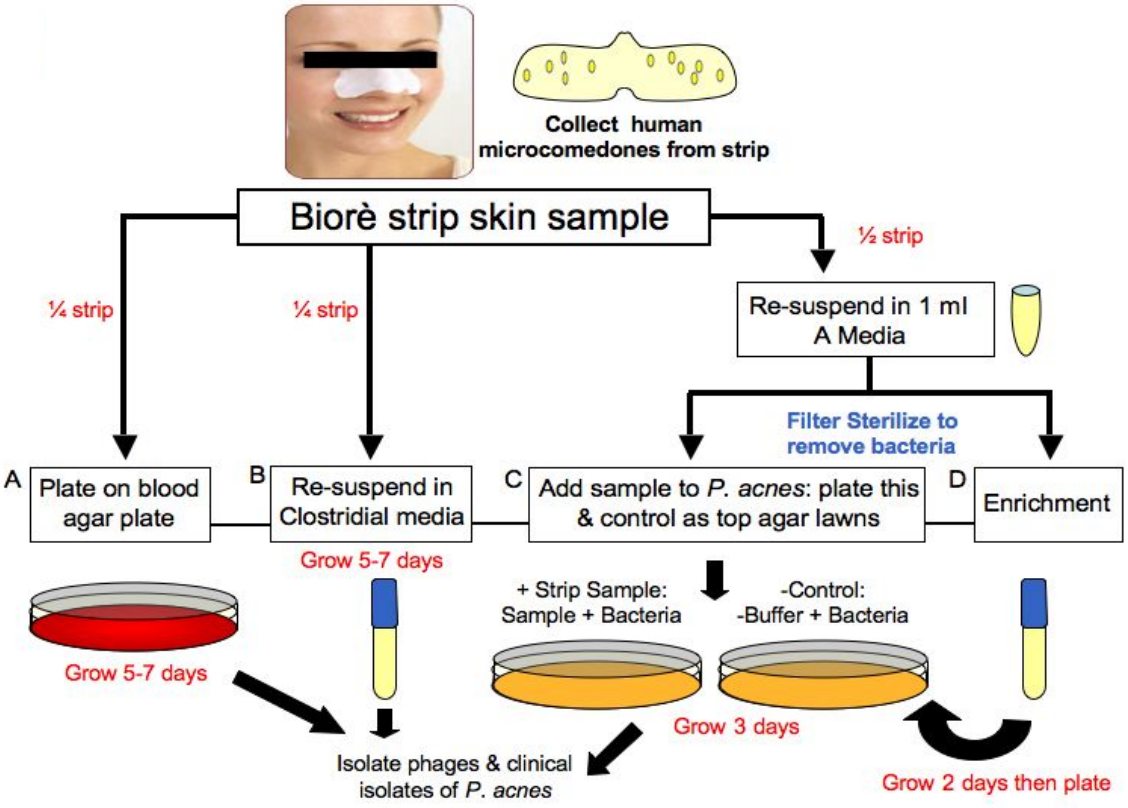
Unlike the other bacteria that we have used this semester, *P. acnes* is an aerotolerant anaerobe. This means that we can work with the bacterium on the benchtop, but need to incubate it under anaerobic conditions to grow.

For this lab module, you will work in groups of two. One lab partner will graciously donate a sample of his or her skin microbiome.

The *P. acnes* project protocols were provided by Laura Marinelli in Robert Modlin's laboratory at UCLA. The lab manual was adapted that used by the Life Science Instructional Labs at UCLA, edited by Erin Sanders.

DAY 1: ISOLATION OF *PROPIONIBACTERIUM ACNES* AND BACTERIOPHAGE FROM THE SKIN MICROBIOME

The purpose of the first part of module is to isolate the material – or microcomedones – from the pilosebaceous follicles (commonly referred to as 'pores') on your nose. For this you will use a commercially available pore strip, which adheres to the skin on your face similar to a band-aid and facilitates microcomedone removal. You will isolate facial bacteria (predominantly *Propionibacterium acnes*) by streaking the material directly from the strip onto a blood agar plate, which is selective for propionibacteria. You also will inoculate material from another part of the strip directly into liquid media. Once grown, you will attempt to isolate both phages and bacteria from this culture. You will inoculate material from the rest of the strip into growth media. This will be filtered to remove all the bacteria; however, any phages that are present will pass through the filter and are not removed. The filtered material – which includes any phages – will then be added to cells from a *P. acnes* culture, and plated as a soft-agar overlay. Phages present in the filtrate that can infect *P. acnes,* will be visualized as a clear area (plaque) in the lawn of bacterial cells.



***P. acnes* and Phage isolation protocol**. Note there are four approaches (A, B, C and D). A is for isolation of *P. acnes*, B is to isolate phage from a culture of the donor's *P. acnes* if needed, C & D are two different approaches to isolate phage using a stock strain of *P. acnes*.

Materials (*per group*)

P. acnes isolation:

Bioré Pore Strip
Brucella Blood Agar plate
Reinforced Clostridial Medium (RCM)
Sterile empty petri dish

Phage Isolation:

A Media plates (2)
1 ml plastic syringe
0.22 μm syringe filter
Phage buffer
0.5% A Media Top Agar
Fresh *P. acnes* culture
Sterile microcentrifuge tubes
P1000 and P200
micropipettors

Isolate Microcomedones:

1. Choose one lab partner to graciously donate bacteria and phage from his or her nose for the lab module.

2. Wash your hands thoroughly with antibacterial soap and warm water, and put on a fresh pair of laboratory gloves. The donor should wet his or her nose with tap water.

3. Remove Bioré Strip from package; twist strip to loosen from plastic backing and separate slits (see illustration on package).

   Peel the strip off of the plastic liner. Apply to the nose (re-wet if necessary), smooth side (the side that was in contact with the plastic backing) down. Press down to ensure good contact with the skin. Let the strip dry for 15 minutes until it feels stiff or cast-like. Avoid wrinkling your nose while strip is drying to prevent it from becoming dislodged

4. Once the strip is dry, put on a fresh pair of gloves and grasping the very edges of the strip, slowly and carefully peel it off starting at the edges and pulling toward the center. Immediately place strip in a sterile petri dish and close the lid.

5. Any residual glue remaining on your nose can be rinsed off with tap water.

Isolate *P. acnes*:

- Streak onto Brucella blood agar plate - Part A in the figure.
- Inoculate broth culture - Part B in the figure.

Caution should be used when handling the Bioré Strip to avoid contamination as much as possible. Always make sure your hands are clean and wear gloves when handling the pore strip.

1. Label the bottom of a Brucella Blood Agar plate and a test tube with your group's name and date.

2. Turn on your Bunsen burner and make sure your metal inoculating loop is close at hand.

3. Put on a fresh pair of gloves making sure you are careful only to touch the very edge of the strip.

4. Flame your loop until it glows orange and cool for about 5 seconds. Then scrape the microcomedones from ¼ of the strip – making sure to rub the strip hard enough so the material from the pore strip sticks to the loop – and inoculate the Brucella Blood Agar plate. Use the three-streak method to streak the sample on the plate. This step is part A in the figure.

5. Flame your loop again and cool. Scrape microcomedones from another ¼ of the strip and inoculate the material into the culture tube containing 3 ml of RCM. This step is part B in the figure.

6. Put the plate and the culture tube in the airtight box

Isolate Phage from the Bioré Pore Strip

- Phage isolation by direct plating on *P. acnes* in soft agar - Part C in the figure.
- Amplification of phage by growth with *P. acnes* in liquid medium - Part D in the figure.

1. Label two A Media plates with your group's name and the date; label one '+ Strip Sample' and the other 'Negative Control'.

2. Using a P1000 micropipettor, aliquot 1 ml of phage buffer into a sterile microcentrifuge tube; close the lid.

3. Flame your loop and cool. Then scrape the microcomedones from the remaining unscraped half of the strip, making sure that the material from the pore strip is sticking to the loop – it may help to wet the sterilized loop with phage buffer from

your tube in order to solubilize the microcomedones; inoculate your tube of phage buffer with material scraped from the strip.

- Remember to use caution when handling the Bioré Strip to avoid contamination as much as possible.

4. Obtain a fresh microcentrifuge tube and place in a rack on your bench. Attach a 0.22 μm syringe filter to a 1 ml syringe, and filter the contents of the first microcentrifuge tube into the second microcentrifuge tube; label with your group's name and date.

5. Obtain two clean test tubes, and label these: '+ Strip Sample' and 'Negative Control'. Using your P1000 and aseptic technique, add 500 μl of *P. acnes** culture to each tube.

   * The *P. acnes* we will use is strain 6919 from the American Type Culture Collection (ATCC). Because phage must invade a host cell to reproduce and you won't have a culture of your own *P. acnes* yet, we will use the 6919 strain to amplify the phage.

6. To the tube labeled '+ Strip Sample', add <u>one-half</u> of the filter sterilized contents of your microcentrifuge tube (should be about 300 μl; <u>save the rest</u> for enrichment step); add an <u>equal amount</u> of sterile phage buffer (PB) to the tube labeled 'Negative Control'.

7. Allow the tubes to sit on your bench at room temperature for 30 minutes. This incubation provides time for phage to bind to the *P. acnes* cells and start infecting the bacteria.

8. When the incubation time is over, get a tube of 0.5% A Media Top Agar from the water bath. Working <u>quickly</u> but <u>carefully</u>, add the contents of the tube labeled '+ Strip Sample' (~800 μl) to the tube of molten A Media Top Agar. Then rotate it rapidly between the palms of your hands to mix the contents (do not shake so that air bubbles are introduced). Immediately pour the entire contents of the tube onto the surface of an A Media Hard Agar plate. While rocking the plate gently but rapidly, spread the melted A Media Top Agar over the entire surface of the plate before it has time to solidify. Avoid splashing it onto the sides of the Petri dish.

9. Place the plate on a level surface and allow it to stand undisturbed until the soft agar is solidified.

10. Next add the contents of the tube labeled 'Negative Control' (~800 μl) to a second tube of molten A Media Top Agar. Rotate it rapidly between the palms of your hands to mix the contents, and immediately pour the entire contents of the

tube onto the surface of an A Media Hard Agar plate. While rocking the plate gently but rapidly, spread the melted A Media Top Agar over the entire surface of the plate before it has time to solidify. Avoid splashing it onto the sides of the Petri dish.

11. Once both plates have had time to solidify, they may be stacked, taped together, and inverted in preparation for incubation at 37°C.

12. While your plates are solidifying, set up the enrichment culture – Part D in the figure. Obtain a tube with 3 ml RCM. Using your P1000, you will then add 1 ml *P. acnes* culture and your remaining filter sterilized sample (should be about 300 μl). Use a fresh tip for both the *P. acnes* culture and your sample.

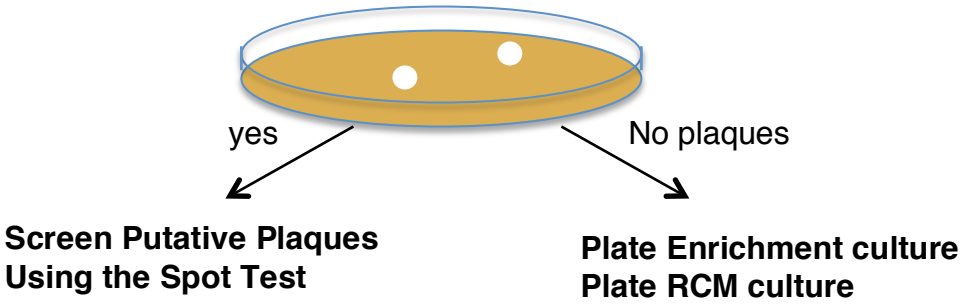
13. Put both plates (inverted), along with your enrichment culture, into the sealable pouch. Open the packet holding the GasPak and put it in the pouch too. Seal the pouch and incubate at 37°C.

14. Record the following information in your notebook:
    - ✓ Area of the face sampled (i.e., your nose)
    - ✓ Describe the appearance of the pore strip after it is removed. Can you see the individual microcomedones?
    - ✓ Other: problems with the protocol, additional observations, etc.

DAY 2: PHAGE ISOLATION and RESTREAK *P. ACNES*

1. Examine all your plates for the presence of plaques, which will be visualized as circular clear areas in the bacterial lawn on your '+ Strip Sample' plates. If plaques are present, proceed to the **Screen Putative Plaques Using the Spot Test** procedure (pages 12-15); if there are no plaques, proceed with the **Phage Isolation from Enrichment Culture and RCM Liquid Culture** procedure (pages 9-11).

    - Your 'Negative Control' plates should not have any plaques. If plaques are observed it may indicate contamination. See the instructor to discuss options.



2. **Restreak candidate colonies** of your *P. acnes* isolates from Day 1 (procedure on page 16).

PHAGE ISOLATION FROM ENRICHMENT CULTURE and FROM RCM LIQUID CULTURE

The purpose of this procedure is to plate the supernatant from your enrichment culture and RCM culture in order to isolate are any phage present in these samples, in case you did not obtain any plaques from the direct plating of your pore strip sample.

Enrichment culture: *P. acnes* ATCC 6919 was inoculated with the filtered sample from the pore strip to make the Enrichment culture. The supernatant from this culture should be enriched for phage that were able to infect and lyse the *P. acnes*.

RCM culture: This culture was inoculated directly from the material on your pore strip. If there were phages present on the strip that were missed by the other isolation procedures using the ATCC strain of *P. acnes*, they may be in this culture. It is also possible that some of the bacteria isolated by the strip were lysogens, which were induced in the RCM culture.

Materials (*per group*)
A Media plates (3)
1 ml plastic syringe (2)
0.22 μm syringe filter (2)
Fresh *P. acnes* culture
0.5% A Media Top Agar
phage buffer
Sterile microcentrifuge tubes
P1000 and P100 micropipettors

Procedure

1. Wash your hands thoroughly with antibacterial soap and warm water, and put on a fresh pair of laboratory gloves.

2. Label three A Media plates with your name and the date. Label them '+ Enrichment', '+ RCM', and 'Negative Control'.

3. Obtain a fresh sterile microcentrifuge tube, and label with your group name and EC (for enrichment culture). Using your P1000, aseptically remove 1 ml from your **enrichment culture** and transfer into the microcentrifuge tube.

4. Obtain a fresh sterile microcentrifuge tube, and label with your group name and RCM (for RCM culture). Using your P1000, sterilely remove 1 ml from your **RCM culture** that was inoculated directly from the pore strip and pipette into the microcentrifuge tube.

5. Place your tubes in the microcentrifuge across from each other so that the rotor is balanced. You can also balance your tube with another student's tube or a balance tube. Make sure your tubes are labeled clearly.

6. Spin at max speed (~14K rpm) for 5 minutes.

7. For each sample, attach a 0.22 μm syringe filter to a 1 ml syringe. If you are preparing both the RCM and enrichment cultures, you will need two filters and two syringes (one set for each sample).

8. Promptly remove your tubes at the end of the spin, being careful not to disturb the pellet.

9. Filter the supernatant from each sample into fresh sterile microcentrifuge tubes (labeled with your groups name, EC or RCM, and the date). Remember to use a separate filter and syringe for each sample.

10. Obtain 3 sterile test tubes; label these '+ Enrichment', '+ RCM', and 'Negative Control'. Using your P1000, sterilely aliquot 500 μl of *P. acnes* into each of the tubes.

11. Add 100 μl of your filter-sterilized enrichment culture sample to the tube labeled '+ Enrichment'.
    Add 100 μl of your filter-sterilized RCM sample to the tube labeled '+ RCM'.
    Add 100 μl of phage buffer to the tube labeled 'Negative Control'.
    Allow these to sit, undisturbed, at room temperature for 30 minutes.

12. When the incubation time is over, get a tube of 0.5% A Media Top Agar from the water bath. Working quickly but carefully, add the contents of the tube labeled '+Enrichment' (~600 μl) to the first tube of molten A Media Top Agar. Rotate it rapidly between the palms of your hands to mix the contents (do not shake so that air bubbles are introduced). Immediately pour the entire contents of the tube onto the surface of an A Media Hard Agar plate. While rocking the plate gently but rapidly, spread the melted A Media Top Agar over the entire surface of the plate before it has time to solidify. Avoid splashing it onto the sides of the Petri dish.

13. Add the contents of the tube labeled '+ RCM' (~600 μl) to a second tube of molten A Media Top Agar. Rotate it rapidly between the palms of your hands to mix the contents, and immediately pour the entire contents of the tube onto the surface of an A Media Hard Agar plate. While rocking the plate gently but rapidly, spread the melted A Media Top Agar over the entire surface of the plate before it has time to solidify. Avoid splashing it onto the sides of the Petri dish.

14. Next add the contents of the tube labeled 'Negative Control' (~600 μl) to a third tube of molten A Media Top Agar. Rotate it rapidly between the palms of your hands to mix the contents, and immediately pour the entire contents of the tube onto the surface of an A Media Hard Agar plate. While rocking the plate gently but rapidly, spread the melted A Media Top Agar over the entire surface of the plate before it has time to solidify. Avoid splashing it onto the sides of the Petri dish.

15. Place all plates on a level surface and let them stand undisturbed until the soft agar is solidified (at least 20 minutes).

16. Put all plates (inverted) into the sealable pouch. Open the packet holding the GasPak and put it in the pouch too. Seal the pouch and incubate at 37°C.

17. Record the following information in your notebook:
    - ✓ Appearance of enrichment culture
    - ✓ Appearance of the RCM culture
    - ✓ Other: problems with the protocol, additional observations, etc.

SCREEN PUTATIVE PLAQUES USING THE SPOT TEST

The purpose of this procedure is to purify any *P. acnes* phages that you isolated and to confirm that they are phages (and not plate bubbles).

Materials (*per group*)
Phage Buffer
Sterile test tube (1 or 2)
Fresh *P. acnes* culture
A Media plates (1 or 2)
0.5% A Media Top Agar
Sterile microcentrifuge tubes
P100 & P20 micropipettors

Procedure

Pick Plaques

1. Wash your hands thoroughly with antibacterial soap and warm water, and put on a fresh pair of laboratory gloves.

2. Determine how many different types of plaques (plaque morphologies) you can observe on your plate(s). For example, plaques may be large or small, turbid (cloudy) or clear, they may have rings (halos) around them or they may have sharp, defined edges. You will record these data in your notebook.

3. Obtain a sterile microcentrifuge tube for EACH different type of plaque you observe. Number your tubes, and legibly label with your group name. Using your P100, aliquot 100 μl phage buffer into each tube.

4. Pick one representative of each plaque type, and number these on the back of the plate; these will be the ones you pick.

5. Place your plate on your bench, agar side down, with the lid facing up. Put a fresh tip on your P100, depress the plunger to the first stop, and hold it there. Remove the lid from your plate and carefully push the tip into the center of your first plaque (labeled #1 on back of the plate). Slowly pull the tip out of the agar, and as you do so, slowly release the plunger of the micropipettor. This should pull an 'agar plug' into the tip.

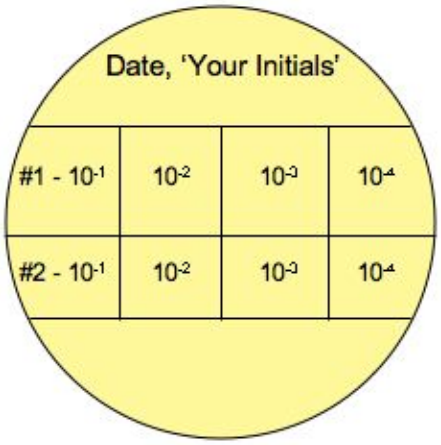
6. Open the microcentrifuge tube labeled '#1' and pipette the agar plug into the phage buffer. You should see the plug coming out of the tip, but you may have to pipette up and down a few times to get it out. It will look like a tiny, clear worm in your tube. Close the tube.

7. Repeat steps 5-6 for your remaining plaques (#2 – n). You may choose up to 10-12 plaques as long as they appear to exhibit unique plaque morphologies, although you will only proceed with 4 plaques today. All will be saved and stored at 4°C in case you need to go back to them at a later time.

Prepare plates with P. acnes in Top Agar

8. Obtain fresh A Media plates; one for every TWO phages that you picked and diluted (e.g. if you have 4 picked plaques/sets of dilutions, you will need 2 plates).

9. Label the back of these plates as illustrated below. If you only have one phage, you will only need to label one row.



A-agar plate

Diagram of plate with dilution scheme for spot test.

10. Retrieve a tube of 0.5% A Media Top Agar from the water bath.

11. Working quickly but carefully, add 500 μl of *P. acnes* culture to the tube. Then rotate it rapidly between the palms of your hands to mix the contents (do not shake so that air bubbles are introduced). Immediately pour the entire contents of the tube onto the surface of an A Media Hard Agar plate. While rocking the plate gently but rapidly, spread the melted A Media Top Agar over the entire surface of the plate before it has time to solidify. Avoid splashing it onto the sides of the Petri dish.

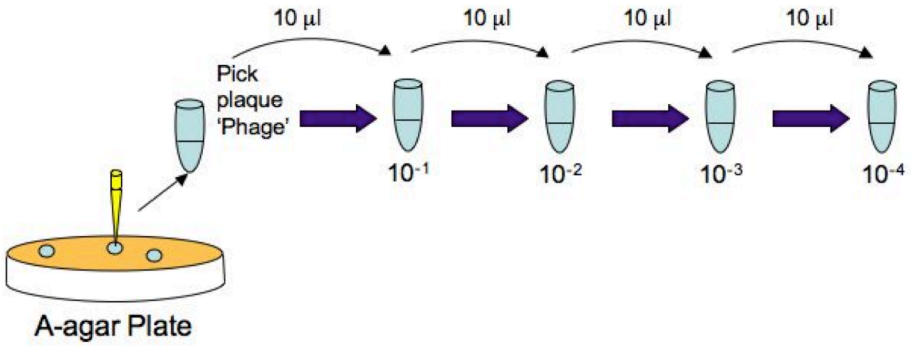
12. Place the plate on a level surface and allow it to stand undisturbed until the soft agar is solidified (at least 20 minutes).

13. Repeat steps 10-12 with a second tube of Top Agar, *P. acnes*, and the second A Media Hard Agar plate.

Prepare serial dilutions of picked plaques

14. Obtain a set of 4 sterile microcentrifuge tubes for EACH plaque you choose (16 total). Label <u>each set</u> with the plaque number and the following: '-1', '-2', '-3', '-4' (e.g. #1-1, #1-2, #1-3, #1-4). Using your P100 micropipettor, aliquot 90 μl of phage buffer into each tube.

15. As shown below, you now will make serial 10-fold dilutions of the plaques that you picked in step 8. Flick the tube containing your first picked plaque (#1) a few times to mix, and remove 10 μl using your P20 micropipettor. Pipette this into a tube labeled '#1-1'. Flick this tube to mix; using a fresh tip, remove 10 μl, and pipette this into the tube labeled '#1-2'. Repeat and continue (using a fresh tip each time) until you reach the tube labeled '#1-4'.



16. Repeat **Step #9** to make serial dilutions of each four plaques you picked. Make sure each set is <u>labeled clearly</u> so they do not get mixed up.

Spot plaque dilutions on P. acnes top agar

17. Using your P20, carefully spot 5 μl of each dilution onto the correspondingly labeled spots on the surface of your plates (e.g. spot 5 μl of the 10^{-1} dilution of plaque #1 into the spot labeled "#1-10^{-1}"). Allow spots to dry <u>completely</u> before moving your plates.

18. Once spots are dry, invert your plates and place the sealable pouch. Open the packet holding the GasPak and put it in the pouch too. Seal the pouch and incubate at 37°C.

19. Record the following information in your notebook:
    a. Whether or not you obtained plaques from the direct plating and/or the enrichment culture
    b. Description of all of the different plaques observed on your plates <u>and</u> which plate they came from

c. Note which plaques you picked, where they came from, and what they looked like (e.g. Plaque #1 was large with a clear center; Plaque #2 was small and turbid….)
d. Other: problems with the protocol, additional observations, etc.

ISOLATING CANDIDATE COLONIES OF *P. ACNES*

Check Blood agar streak plates for colonies

Today you will check for bacterial growth on the Brucella Blood Agar plates that you streaked with material from your pore strip. You will record your observations, and chose 3 individual colonies to re-streak on blood agar.

Materials

Brucella Blood Agar plates (3)
Metal inoculating loop

Procedure

1. Wash your hands thoroughly with antibacterial soap and warm water, and put on a fresh pair of laboratory gloves.

2. Examine your blood agar streak plate, which was inoculated with material taken directly from your pore strip.

3. Record the following information in your notebook:
   - ✓ Overall appearance of plate (e.g., Can you observed clear areas or any changes in color? Were you able to streak for single colonies?)
   - ✓ Describe the appearance of the colonies (size, color, shape). How many different types (morphologies) do you see?
   - ✓ Other: any additional observations, problems that may have occurred.
   - ✓ *P. acnes* forms round gray/silver colonies and usually do not cause clearing in the blood agar (non-hemolytic). Choose these colonies to restreak. You can compare your plate to a plate with *P. acnes* 6919

4. Chose 3 isolated colonies to pick and re-streak. These will be named: '*P. acnes*-your group's name, #' (1 – 3). Note the morphology of each colony you choose.

5. Obtain 3 fresh blood agar plates; label these with your group name, the date, and the isolate name.

6. Flame your loop until it glows orange, and then cool it on an area of the plates with no bacterial growth. Touch the sterilized loop to the center of your first colony; you should see a small amount of bacterial growth stick to the end of the loop. Streak this using the three-streak method onto your first blood agar plate (make sure that the plate is labeled correctly), using the same method employed previously (remember to flame and cool loop each time you streak).

7. Repeat **Step #6** for the remaining 2 colonies that you have chosen. Invert plates and incubate in sealed box with a GasPak 37°.

DAY 3: PHAGE ISOLATION, PURIFICATION, OR PLAQUE SCREENING

Inspect plates to determine whether or not you obtained plaques via the spot test or from your enrichment culture.

- If at least one of your samples from the spot test is confirmed to contain phage as evidenced by clear areas on the plate, proceed to **Plaque Purification** (pages 18-21). You will choose ONE of your phage samples, and re-plate this in order to purify phage from the plaque to ensure a homogeneous population of phages. The other phages that you find either will be stored as 'back-ups' or shared with students who have not isolated phages by any method.

- If you have phage from your enrichment culture or RCM culture, you will pick plaques according to steps 1-7 of the **Screen Putative Plaques Using The Spot Test** procedure (pages 12-13). Once you have picked the plaques, proceed to the **Plaque Purification** (pages 18-21). You will choose one of the plaques to purify. You will skip the screening using the spot test procedure.

- If you do not have plaques by any of the methods, talk to an instructor. We will arrange for you to adopt a phage from a classmate.

PLAQUE PURIFICATIONS

The purpose of this procedure is to re-plate one of the plaques you picked previously, which you have confirmed to contain infectious phage by spot titer. This protocol will enable you to plaque purify your samples to ensure that the population of phage you are working with is pure and homogeneous; that is, all the phages are genetically identical to one another. You will perform two rounds of plaque purification (time permitting) to obtain a pure phage preparation.

Round One:

Materials (per student)
Sterile microcentrifuge tubes
Sterile test tube (4)
Fresh *P. acnes* culture
A Media plates (4)
0.5% A Media Top Agar
phage buffer

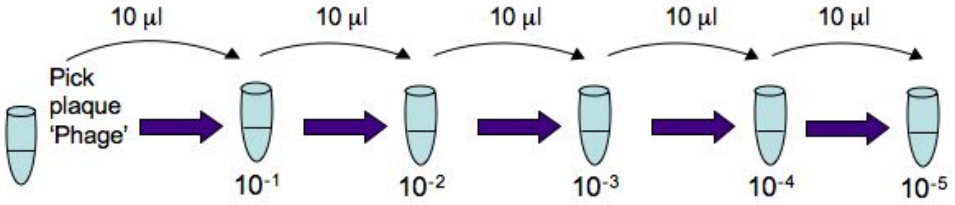
Procedure

1. Wash your hands thoroughly with antibacterial soap and warm water, and put on a fresh pair of laboratory gloves.

2. Pick ONE phage to continue purifying. Obtain the lysate for this phage (the tube with the plaque that you picked).

3. Obtain 5 sterile microcentrifuge tubes; label these -1, -2, -3, -4, -5. Using your P100 micropipettor, aseptically aliquot 90 μl of phage buffer into each tube.

4. Prepare serial 10-fold dilutions of this confirmed phage lysate as shown.



Dilution scheme for plaque purifications.

Flick the tube containing the plaque a few times to mix, and remove 10 μl from using your P20 micropipettor. Pipette this into a tube labeled '-1'. Flick this tube to mix; using a fresh tip, remove 10 μl, and pipette this into the tube labeled '-2'.

Repeat and continue (using a fresh tip each time) until you reach the tube labeled '-5'. Discard the tube labeled '-1', and place the remaining tubes on ice.

5. Obtain 4 sterile tubes, and label these $10^{-2,}$ 10^{-3}, 10^{-4}, and 10^{-5}, respectively. Aseptically aliquot 500 μl of *P. acnes* into each tube. You also will need to label four A Media plates with the date, your group name, and $10^{-2,}$ 10^{-3}, 10^{-4}, and 10^{-5}, respectively.

6. Using your P20, add 10 μl of 10^{-2} dilution to the *P. acnes* bacteria in the test tube labeled '10^{-2}'. Similarly, add 10 μl of the 10^{-3} dilution to the *P. acnes* bacteria in the test tube labeled '10^{-3}'. Continue with the remaining dilutions and tubes.

7. Incubate tubes on your bench for 30 minutes.

8. Retrieve a tube of 0.5% A Media Top Agar from the water bath. Working quickly but carefully, add the contents of the tube labeled 10^{-2} (510 μl) to the tube of molten A Media Top Agar. Then rotate it rapidly between the palms of your hands to mix the contents (do not shake so that air bubbles are introduced). Immediately pour the entire contents of the tube onto the surface of an A Media Hard Agar plate. While rocking the plate gently but rapidly, spread the melted A Media Top Agar over the entire surface of the plate before it has time to solidify. Avoid splashing it onto the sides of the Petri dish.

9. Place the plate on a level surface and allow it to stand undisturbed until the soft agar is solidified (at least 20 minutes).

10. Next add the contents of the tube labeled 10^{-3} (510 μl) to another tube of molten A Media Top Agar. Rotate it rapidly between the palms of your hands to mix the contents, and immediately pour the entire contents of the tube onto the surface of an A Media Hard Agar plate. While rocking the plate gently but rapidly, spread the melted A Media Top Agar over the entire surface of the plate before it has time to solidify. Avoid splashing it onto the sides of the Petri dish. Repeat this step for tubes labeled 10^{-4} and 10^{-5}.

11. Once all four plates have had time to solidify, they may be stacked, taped together, and inverted for incubation at 37°C in a sealable pouch with a fresh GasPak.

12. Remember to SAVE the new undiluted phage sample, storing it at 4°C until the phage titer is calculated. Make sure the tube is well labeled and easy to identify.

13. Record the following information in your notebook:
    - ✓ problems with the protocol, additional observations

Round Two

1. Count the number of plaques on a plate with a range of 20 to 200 plaques.

2. Record in your lab notebook the designation of the putative phage, the dilution plate, the volume tested (in this case, 10 μl), and the number of plaques on the plate.

3. Calculate the titer (pfu/ml).

4. **Iteration #2:** REPEAT the plaque assay with a fresh phage lysate prepared as follows:

   - ✓ Pick a single plaque using the plate from the dilution series that gave discrete plaques
   - ✓ Place a **sterile** tip onto a P100, depress the plunger to the first stop, and hold it there.
   - ✓ Open the lid of the Petri dish and keep it in your hand (do not invert – remember the sterile field!).
   - ✓ Carefully push the tip into the center of the plaque.
   - ✓ Slowly pull the tip out of the agar, and as you do so, slowly release the plunger of the micropipettor. This should pull an 'agar plug' into the tip.
   - ✓ Return the cover to the Petri dish.
   - ✓ Pipette the agar plug into 100 µl of phage buffer (PB) in a microcentrifuge tube. You may have to pipette up and down a few times to get it out. It will look like a tiny, clear worm in your tube.
   - ✓ Close the cap and place the tube on ice. Discard the tip.

   Follow the protocol for the plaque purification assay (pages 18-19). Once again plate 10 µl of the 10^{-2} to 10^{-5} serial dilutions of your new phage sample.

   Remember to SAVE the new undiluted phage sample, storing it at 4°C until the phage titer is calculated. Make sure the tube is well labeled, dated, and easy to identify.

OPTIONAL: You may want to adjust the dilutions plated depending on the concentration of your phage lysates (i.e., plate smaller dilution series, or plate alternating dilutions from series). Talk to an instructor about your plan.

Remember to SAVE the new undiluted phage sample, storing it at 4 °C. Make sure the tube is well labeled and easy to identify.

After completion of Round Two: Check for plaques. Did the plaque morphologies and other characteristics become more consistent with the second round of isolation,

infection, and plating (i.e., breed true)? Count the number of plaques on a plate with a range of 20 to 200 plaques, and calculate the titer (pfu/ml). Record this information in your lab notebook.

- SAVE the plate in which the bacterial lawn is nearly cleared and has a webbed pattern of bacterial growth and lysis. This plate will be used to make a minilysate.

All other plates may be discarded.

DAY 4: Phage Purification Round 2, Restreak *P. acnes*

Phage:
• Pick a plaque and repeat the **Plaque Purification** for Round 2 (page 20).

P. acnes:
P. acnes are Gram-positive bacteria that are generally rod shaped, but occasionally can be branched or irregularly shaped (see figure below). The most common contaminants isolated from faces are Gram-positive, spherical *Staphylococcal* species.

***P. acnes* isolation - Round 2**:

1. Use **microscopy** and **Gram staining** to determine if any of the 3 bacterial isolates that you restruck onto blood agar plates on Day 2 are likely to be *P. acnes*.

2. Restreak 1 or 2 of the isolates that appear to be *P. acnes* onto fresh blood agar plates following the **ISOLATING CANDIDATE COLONIES OF *P. ACNES*** protocol on page 16.

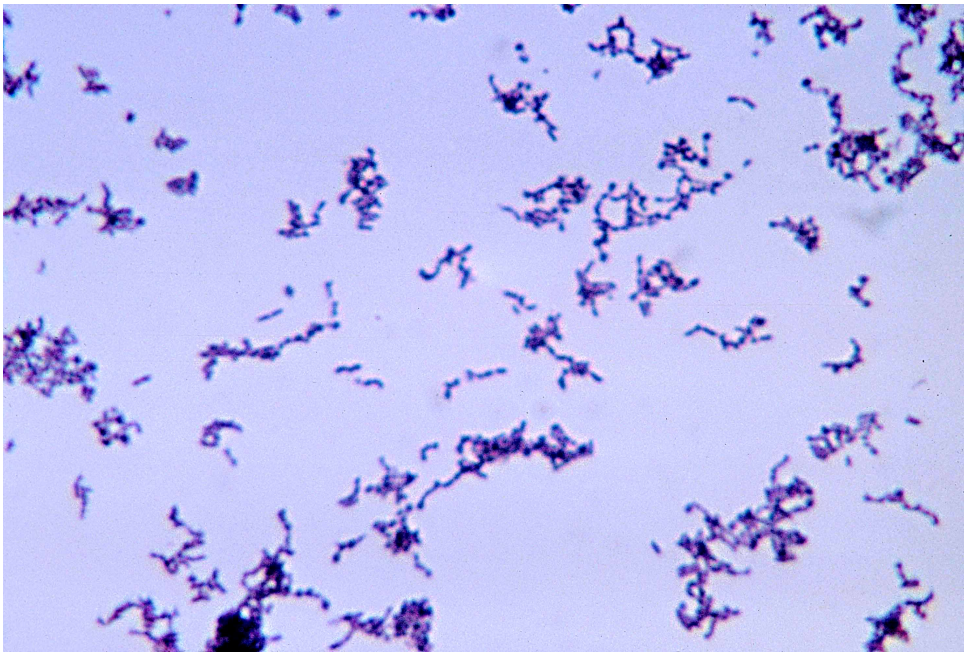


Image from Center for Disease Control Image Gallery, ID # 3083 http://phil.cdc.gov/phil/details.asp

DAY 5: Prepare and titer a phage mini-lysate

Prepare a mini-lysate and titer it following the **Preparation of a Filter-Sterilized Phage Mini-Lysate** procedure.

PREPARATION OF A FILTER-STERILIZED PHAGE MINI-LYSATE

The purpose of this procedure is to prepare a high-titer mini-lysate then plate it on *P. acnes* to obtain the titer.

Materials

Phage buffer
10 ml plastic syringe
0.22 μm syringe filter
Sterile tubes
Fresh *P. acnes* culture
A Media plate
0.5% A Media Top Agar
Sterile microcentrifuge tubes
P200 & P20 micropipettors

Procedure

1. Wash your hands thoroughly with antibacterial soap and warm water, and put on a fresh pair of laboratory gloves.

2. Retrieve the plate that you saved with a 'webbed' pattern of bacterial growth and lysis.

3. Using a sterile pipette, flood the surface of this plate with about 4 ml of phage buffer.

4. Incubate at room temperature for 1 hour. While the plate is incubating, obtain a sterile tube and label it with your team phage name and today's date.

5. Using a fresh pipette, collect the buffer from your plates. Carefully tilt to collect as much as possible. Transfer the lysate to the sterile tube.

6. Attach a 0.22 μm syringe filter to a 10 ml syringe, and filter the lysate into a fresh sterile 15 ml conical tube (again labeled with your team phage name and date). **Store this sample at 4°C.** This is your **filter-sterilized phage MINI-lysate**.

7. Discard the syringe and the filter into the proper waste containers.

8. Follow the procedure for the spot test (page 13-14) to titer your mini-lysate. Briefly, make a series of serial dilutions of your mini-lysate from 10^{-1} to 10^{-8}. Prepare a plate with *P. acnes* in A medium top agar on A medium hard agar. Spot 5 μl of each dilution onto the plate.

Day 6: Broth Culture of P. acnes and EM sample prep

1. Inoculate liquid culture of *P. acnes* in RCM using the protocol **Broth Culture of *P. acnes* in RCM**.

2. Determine the titer (pfu/ml) of your phage minilysate and prep phage for EM.

BROTH CULTURES OF *P. ACNES* IN RCM

Examine your blood agar streak plates and record your results.
Record the following information in your notebook:

- ✓ Overall appearance of colonies on plates (e.g. clear areas, changes in color, contamination, etc…)
- ✓ Were you able to obtain single colonies?
- ✓ Describe the appearance of the colonies (size, color, etc…). Look back at your notes from the previous lab period. Is the colony morphology observed for each streak similar to that which you described for the parent colony?
- ✓ Other: any additional observations, problems that may have occurred.

For those strains in which you obtained single colonies, you may set up liquid cultures of putative *P. acnes* isolates by picking single colonies from your *P. acnes* streak plates and inoculating Reinforced Clostridial Media (RCM). These cultures will be used to assess host range and test for antibiotic resistance.

Materials
Sterile test tubes
Reinforced Clostridial Media (RCM)
Metal inoculating loop

Procedure

1. Wash your hands thoroughly with antibacterial soap and warm water, and put on a fresh pair of laboratory gloves.

2. Retrieve your blood agar streak plates, which were streaked previously with your *P. acnes* isolates.

3. Obtain sterile test tubes containing RCM; label these with your group name, the date, and the isolate name.

4. Flame your loop until it glows orange, and then cool it on an area of the plates with no bacterial growth. Touch the sterilized loop to the center of a colony from your first plate; you should see a small amount of bacterial growth stick to the end of the loop. Inoculate this into the media in the first tube (make sure the strain name on the tube is the SAME as the plate from which you took the colony).

5. Incubate tubes in a sealed box with GasPak at 37°C.

Day 7: Experiment Planning and Electron Microscopy

1) View EM images of your phage.

2) Design your **host-pathogen specificity** experiment.

Each group will design an experiment to evaluate the specificity of the pathogen (phage) and host (*P. acnes*) relationship. You can use isolates from other groups in your experiment. For example, you could test the ability of your phage to infect different *P. acnes* isolates or the resistance of your *P. acnes* to different phage isolates. Formulate a hypothesis, make predictions, and design the experiment. Don't forget controls! You can use any of the top agar plating methods outlined in this lab manual.

3) Design your **antibiotic resistance** experiment – see procedure below.

The skin microbiome is a potential reservoir for bacteria resistant to certain antibiotics such as those used to treat acne or even those an individual has taken to treat other infections. Each group will design an experiment to test the antibiotic resistance profile of its *P. acnes* strain. The most frequently used antibiotics to treat acne are tetracycline, erythromycin, minocycline (a tetracycline derivative), doxycycline, and clindamycin. The other antibiotics used for the pet experiment will be also available. If there are additional antibiotics you would like to use, ask. We will try to get them for you.

SCREEN YOUR *P. ACNES* STRAINS FOR ANTIBIOTIC RESISTANCE

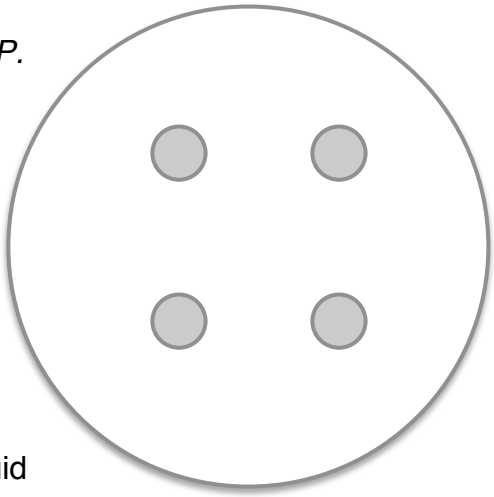
PART I:

1. Obtain a sterile cotton swab and dip it into one of the *P. acnes* culture tubes, thoroughly moistening the cotton swab with the cell suspension.

2. Roll the swab against the sides of the tube to squeeze out excess liquid, although you do not want to let the swab go dry.

3. Use the swab to paint the entire surface of a blood agar plate.

4. Let dry 15 minutes with the lid slightly ajar until no liquid can be seen.



5. Place antibiotic discs on the plate. Discs should be at least 10 mm from the edge of plate and 30 mm from the center of each other.

6. Incubate for up to 7 days at 37°C in sealed box with GasPak.

7. Store the original *P. acnes* culture in a sealed tube at 4°C.

PART II: Inspect plates from antibiotic-resistance screen for zones of growth inhibition around the discs. Measure the **diameter** (not radius) of each clearing (in **cm**) and record in your lab notebook.

Plot the antibiotic resistance your *P. acnes* strain. To represent those strains that are *completely* resistant to a given antibiotic (e.g., no clearing visible around disc), use the diameter of a single disc - 0.5 cm

Day 8: Perform antibiotic resistance experiment.

Day 9: Perform host-pathogen specificity experiment.

APPENDIX I
COMPOSITION OF MEDIA

* Distilled water (dH_2O) is used for all media.

Phage buffer (PB; 1 Liter)

Ingredient	Amount	Final Concentration
1 M Tris stock (pH 7.5)	10 ml	10 mM
1 M $MgSO_4$ stock	10 ml	10 mM
NaCl	4 g	68 mM
ddH_2O	970 ml	
100 mM $CaCl_2$ stock	10 ml	1 mM

Liquid A Medium

Ingredient	Amount
Casitone	12.0 g
Yeast extract	12.0 g
D + Glucose	4.0 g
KH_2PO_4	4.0 g
$MgSO_4.7H_2O$	1.0 g
ddH_2O	up to 1.0 L

A Medium Hard Agar (2.8% w/v)

Ingredient	Amount
Casitone	12.0 g
Yeast extract	12.0 g
D + Glucose	4.0 g
KH_2PO_4	4.0 g
$MgSO_4.7H_2O$	1.0 g
Agar	28.0 g
ddH_2O	up to 1.0 L

A Medium Top Agar (0.5% w/v)

Ingredient	Amount
Casitone	12.0 g
Yeast extract	12.0 g
D + Glucose	4.0 g
KH_2PO_4	4.0 g
$MgSO_4.7H_2O$	1.0 g
Agar	5.0 g
ddH_2O	up to 1.0 L

Reinforced Clostridial Media (RCM)

Ingredient	Amount
RCM	38.0 g
Agar	28.0 g
ddH_2O	up to 1.0 L

Brucella Blood Agar

(available commercially)