Induction of Phage Using Mitomycin C to Check for Phage Presence

Purpose

To induce phage using mitomycin C in Staphylococcus aureus strains ATCC25923 and S. aureus Btn1260 (aka DAR31) and then plate the induced phage lysate and check for plaques. S. aureus DAR31 is aka Btn1260 from the article "*Staphylococcus aureus* temperate bacteriophage:carriage and horizontal gene transfer is lineage associated" by Alex J. McCarthy, Adam A. Witney, and Jodi A. Lindsay". The latter bacteria has one prophage in its genome so it was used as the donor and induced for phage. This prophage is in the psi2 family and does not have any reported virulence factors, making it a good possible prophage to use in our experiments. It has a genome size of about 45-49 kb.

Preparation of overnight cultures of bacterial isolates

1. Pipette 5 ml of sterile TSB into a sterile glass tube
2. Take an inoculating loop, flame it, and touch a bacterial colony
3. Mix the bacterial colony into the broth.
4. Incubate overnight at 37ºC on the shaker incubator (150-200 rpm)

Mitomycin C Induction Protocol

1. Turn on spectrophotometer (switch is located on back of device). Load a cuvette with 1.0 mL of fresh TSB broth to be used as a blank for zeroing the device. Label this cuvette on the top of it with a mark so as to not mix it with test samples.
2. Set the device's wavelength to 600 nm. Load the cuvette blank into the spectrophotometer and zero the device. Keep the blank cuvette in a cuvette rack for future use.
3. Dilute overnight culture 1:100 in fresh TSB media (200 ml). Mix by swirling the flask, remove 1 ml using pipette-aid, place it into a disposable cuvette. Incubate the culture at 37oC in shaker incubator at 190 rpm. Be careful to make sure the flask does not spill when shaking. Record the time.
4. Zero the spectrophotometer with blank cuvette. Take OD600 reading of the culture.
5. At 30 minute-1 hour intervals, remove 1 ml of the culture and read the optical density (OD_{600})
6. When the OD_{600} is at 0.2 , the culture is ready to be induced.
7. Add mitomycin C to a final concentration of 0.5 ug/ml (add 50 ul of 2 mg/ml mitomycin C stock to 200 ml culture).
8. The mitomycin C stock is located in the -20oC freezer in a box in the door.
9. **Optional**: Added phleomycin (in -20ºC freezer) - added 10 ul of the 1 mg/ml phleomycin stock to 50 ml.
10. Mix by swirling the flask and place in incubator/shaker. Reduce shaker speed to about 150 rpm and continue incubation at 37oC.

11. At 1 hour intervals, read the OD600 as described above, record it, and remove 4 mLs of culture from the flask and put it into 3 sterile 1.5 mL microfuge tubes. Continue incubating the flask and take readings and samples every hour.
12. While the bacteria is incubating, take the 4 ml of bacterial culture and dispense into 3 x 1.5 ml microfuge tubes. Centrifuge at to speed for 2 minutes, room temperature. The bacteria will be pelleted to the bottom of the tube.
13. While the bacteria is being centrifuged, prepare for filtration. Remove the barrel of a 5 ml syringe and attach a 0.2 micron filter onto the syringe.
14. Remove the supernatant fluid (liquid separated from the bacterial cell pellet), put it into a syringe (with a 0.2 micron syringe filter attached) and filter it by pushing on the syringe plunger. Collect the filtrate into a 15 ml conical tube.
15. Label the tube with your initials, the date, time of collection, what you have collected (phage from induction of bacteria X). Make sure you record everything on Benchling. Store the phage filtrate at 4oC (refrigerator) or continue with the protocol.
16. Continue this process for 6 hours.
17. After 6 hours, the induced bacteria can be left overnight. The following day, take another sample and keep your induced culture (store induced bacteria in the refrigerator or cold room).

Soft Agar Overlay Method for Observing Phage Plaques

1. Prepare a 5-10 ml overnight culture of the bacterial host that you will use to infect and observe plaques. Each plaque represents a single phage particle.
2. You will need to melt your soft agar (top agar) ahead of time to make the soft agar overlays and prewarm your bottom agar, so put your tubes containing soft agar in a rack in the autoclave for 4 minutes (liquid setting) to allow the autoclave to melt the soft agar.
3. Turn the dry incubator bath on to let it warm up to 55-60oC. Make sure that your soft agar tubes will fit in the placeholders in this bath.
4. Meanwhile, prepare serial dilutions of your phage filtrates. Take a 96 well plate and add 180 ul of broth media to each well.
5. Take your first filtrate and prepare serial dilutions across the 96 well plate. To do this, add 20 ul of the phage filtrate to the 1st well, and mix up and down with your pipetteman.
6. Discard the pipette tip.
7. Place a new tip on the pipetteman. Remove 20 ul from the first well and add to the second well. Pipette up and down and discard the tip.
8. Complete this process until you have made dilutions across the top row of the 96 well plate. Each row will represent a different time point for your induction.
9. Once the soft agar is out of the autoclave, place the tubes in the dry bath incubator.
10. Add 50 ul Staph host (the bacteria that will allow the phage to replicate in it and make plaques) to a tube of soft agar, mix, and quickly pour this over a prewarmed TSA plate (bottom agar). Allow it to solidify (~20 minutes).
11. Once solidified, take 10 ul from each well and pipette (spot it) onto the soldified soft agar). Make sure you follow a specific pattern and mark the back to the plate so you will know which dilution is being put in each spot. Allow it to absorb.
12. Keep the plates upright (do not invert) and incubate at 37oC overnight.
13. The next day you should see plaques (clearing zones) in the agar.