citation
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Bacterial Pathogens of Shellfish
A Case Study

A commercial oyster grower was experiencing mortality in their larval cultures. A disease specialist working with the grower isolated several strains of bacteria from the larval cultures. He has sent us two strains of bacteria to test the pathogenicity of the isolates in larval culture. We will also do some phenotypic characterization of the bacteria. In this laboratory exercise we would like to address the following questions:

1. Do these bacteria cause mortality in oyster larvae?
2. Using phenotypic characterization, what type of bacteria do we have?
3. What should the oyster grower do about it?

To answer these questions, you will need to do some experiments. First, you will use Koch's postulates to confirm that one of the bacterial cultures is capable of reproducing the disease.

Koch's Postulates:

1. The causative agent must be present in every case of the disease and must not be present in healthy animals
2. The pathogen must be isolated from the diseased host animal and must be grown in pure culture
3. The same disease must be produced when microbes from the pure culture are inoculated into healthy susceptible animals
4. The same pathogen must be recoverable once again from this artificially infected animal and must be grown in pure culture

In this experiment you will challenge larval cultures with one of two bacterial to assess its pathogenicity. You will monitor the cultures for signs of morbidity or mortality over a 48 hour period. You will attempt to re-isolate the bacteria from any dead larvae and then conduct some preliminary phenotypic characterization on any bacteria you recover. These characterizations should give you a tentative identification of the pathogenic bacterial strains. Finally, you will be required to write a case report, which includes background, the methods you used, results, and a discussion that will outline your recommendations on how to control the problem. It would also be interesting for you to speculate on further experiments that would help you to further characterize the bacterial strains.

Lab 1. Larval challenge using unknown bacterial strains.

Serial dilutions of bacteria
Challenge of oyster larvae with serial dilution of bacteria
Plate counts of bacteria
Return at day 1 (Tuesday) and count dead larvae
Return at day 2 (Wednesday) and count the dead and live larvae
On Wednesday we will collect the larvae and freeze for next week

Lab 2. Analyze the survivorship data and attempt to recover bacteria

Compare the relative pathogenicity of the two strains
Homogenize and streak plates of larval suspension

Streak onto TCBS and Marine 2216 agar
Examine microscopically by wet mount and gram stain

Lab 3. Type the virulent bacteria with API strips.

Examine plates from previous week
API strip tests
Review data to date in preparation for writing your case report.

Laboratory Exercise 1
Larval Oyster Challenge

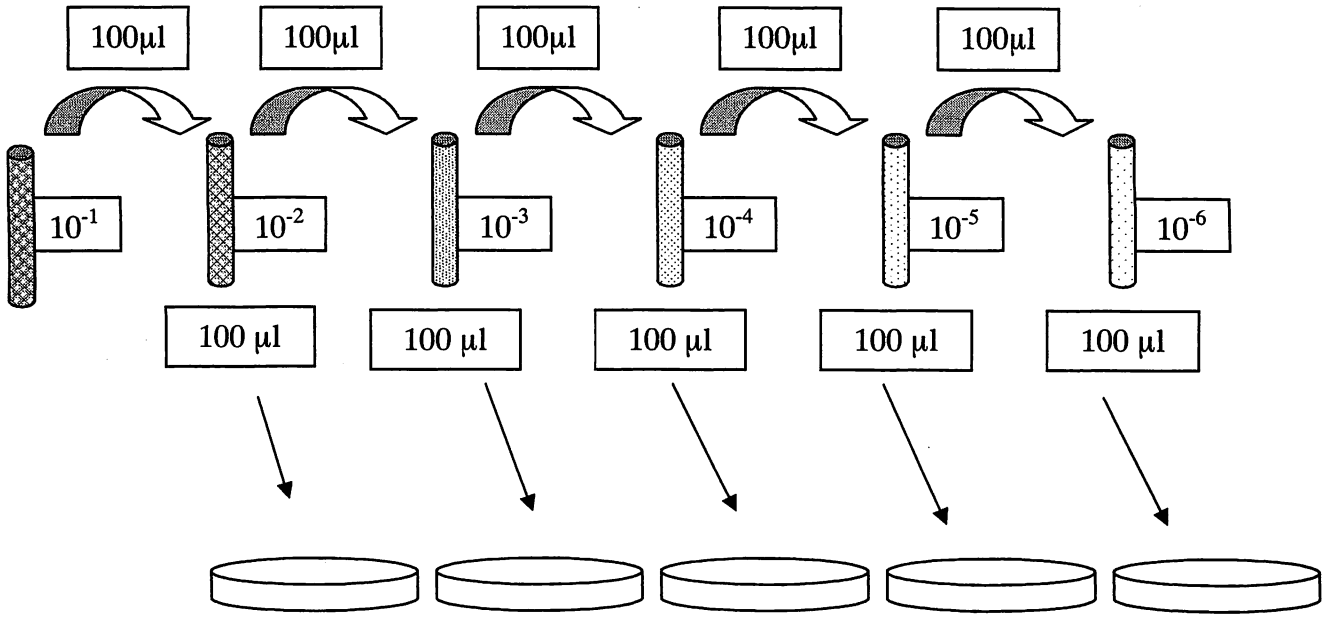
Step 1: Serial dilutions and spread plates

Supplies

One culture tube containing bacteria (pick EITHER strain A or B)
6 microfuge tubes
Seawater for dilutions
P200 Pipettor and tips
P1000 Pipettor and tips
10 marine agar plates
Hockey stick
Ethanol and sand bath
Parafilm
Sharpie Marker
Bunsen burner
Strickers

Take a look at the figure on the next page that illustrates the spread plate concept.

1. Obtain the bacterial strain you will be using and record the strain designation (A or B) in your notebook.
2. Make a serial dilution of your bacteria
    a. Start by adding 900 µl of seawater to 6 culture tubes
    b. Label each of the cultures tube with one of the following dilution labels (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6})
    c. Start dilution series by taken 100 µl of bacterial suspension and adding it to the tube labeled 10^{-1}. This is your first 10-fold dilution. Mix well.
    d. Next take 100 µl of your 10^{-1} dilution tube and add to the tube labeled 10^{-2}. This your second dilution. Mix well.
    e. Repeat the above process until you finish the dilutions out to 10^{-6}.
3. Obtain 10 marine agar 2216 plates. Label each of the plates with a dilution (10^{-2} through 10^{-6}).
4. Plate 0.1 mL (100 µl) of bacterial suspension onto the appropriate plate. If you start from the lowest dilution (10^{-6}) and then plate "upwards", then you can use the same pipet.
5. Dip hockey stick into ethanol sand bath and flame the hockey stick. Cool the hockey stick by touching to the agar and then spread the bacterial suspension.



Step 2: Larval oyster challenge

Serial dilutions of bacteria from step 1
Larvae culture
1 tissue culture plate
Seawater
Inverted or dissecting scope

1. Obtain tissue culture plate. Using relatively sterile technique, add 900 µl of larval suspension to the each of the wells of the tissue culture plate.
    a. Sterile technique includes not setting the lid on the bench but keeping it hovered over the plate.
2. Examine the plates with an inverted or dissecting scope and confirm that each well has larvae present. If not, remove the liquid from the well and try again.
3. Add 100 µL (0.1 mL) of bacteria or seawater to the appropriate wells. See the plate map below. Be cautious to not cross-contaminate between wells!!
4. Incubate in the dark at room temperature for 2 days. On day 1 post-challenge (Tuesday), record the number of dead larvae. At 2 days (Wednesday), record the number of dead AND ALIVE larvae. Average the values across the four replicates and then calculate the average percentage of larvae that survived at each time point.
5. On Thursday, you will remove the dead larvae and store for next weeks class.

	1	2	3	4	5	6
A	Seawater	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
B	Seawater	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
C	Seawater	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
D	Seawater	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}

6. Leave on the bench right-side up for about an hour. Wrap the plates in parafilm, invert, and incubate at room temperature.