Chapter 4

The Struggle for Existence: Competition Between Bacterial Strains

Beth A. D. Nichols[1], Linda B. Cholewiak[1], and Richard Condit[2]

[1]Departments of Ecology and Evolutionary Biology
and Molecular Biology
Princeton University
Princeton, New Jersey 08544

[2]Center for Tropical Forest Science
Smithsonian Tropical Research Institute
APO AA 34002-0948
Panama City, Panama

Beth Nichols is a lecturer in Molecular Biology and Ecology and Evolutionary Biology at Princeton University. She received her B.S. (1974) in Natural Resources and M.S. (1976) in Botany from the University of Michigan. She is Director of the General Biology Laboratories and editor of the laboratory manuals for the introductory biology courses at Princeton.

Linda Cholewiak is the Associate Director of the General Biology Laboratories at Princeton University. She received her B.S. from Salisbury State College (1967) and M.S. (1970) in Developmental Biology from the University of Virginia.

Richard Condit is the Coordinator of the Center for Tropical Forest Science at the Smithsonian Tropical Research Institute in Panama City, Panama. He received has B.S. (1978) from the University of Illinois, and his Ph.D. (1984) from the University of California at Santa Cruz.

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Introduction

This laboratory exercise has been used in the general biology laboratories at Princeton University for 2 years. We have approximately 250 students enrolled in the course each year; there are 20 students in each laboratory section. This exercise is appropriate for science majors and non-majors.

The first part of the laboratory can be completed in 3 hours; for a sample timetable see Appendix B. The students' plates can be incubated, refrigerated, and returned for counting at the laboratory meeting the following week. Instructors with students who have flexible schedules can have students perform their own transfers for 4 days. The laboratory can also be done as a demonstration; the sample plates can be set up and students can be asked to interpret the results.

In addition to analyzing data, students also learn basic techniques in microbiology, for example, sterile technique, serial dilutions, and plating.

Notes for the Instructor

1. Materials (plates, LB broth, and saline tubes) can be prepared weeks in advance (Appendix C).
2. Review sterile technique with the students (Appendix A).
3. Gloves should be worn when handling the antibiotic, nalidixic acid (nal). Discard according to your safety officers' instructions.
4. In our course, the students determined the growth curves for each strain of *E. coli* in LB broth only. Ideally, some of the class should examine the growth of each strain individually in high nal LB and low nal LB as indicated in the Student Outline.
5. Well-labelled charts and color-coded tubes of media help students keep track of cultures. Because the week's results depend on careful transfers and sampling, the students should be closely monitored and reminded to carefully label all tubes so that the next day's class can find the specific cultures they need to continue the serial transfers.

6. The class cultures should be checked after the laboratory and any tubes that have not been sampled and transferred should be done.

7. The following chart drawn on the board might serve as a guide to help students identify colonies on the plates (color of colony in parentheses); see Appendix A for an explanation of the abbreviations.

Strain	Growth of bacteria in broth			Growth of bacteria on plates		
	LB (no nal)	LB (low nal)	LB (high nal)	TL	TLN	ML
7+	+	+/-	-	+ (pink)	-	+ (white)
7N-	+	+	+	+ (red)	+ (red)	-

8. Students should leave the 1-ml pipets in the 10% lysol solution at the benches. The staff should discard the pipets after the laboratory.

9. For a more investigative laboratory exercise, students may want to try lower concentrations of nalidixic acid to find a point where the growth of 7+ is slightly inhibited.

Materials (Per 10 pairs of students)

Alcohol lamps (10)
Beakers, 1 litre, for pipet disposal (10)
Bins, for plates, color-coded (3)
Bin, for used saline tubes (1)
Bucket, with plastic bag, for used plates and pipets (1)
Color dots, different colors for each laboratory section
Cultures, (7+) (10)
Cultures, (7N-) (10)
EtOH, 70%, in 250-ml glass beaker with glass petri dish top (10)
EtOH, 95%, in wash bottles (10)
Gloves
Glass disposal container (1)
Glass spreaders (10)
Goggles (10)
Incubator, 37°C
Luria broth tubes, plain, 10 ml per tube (4)
Luria broth tubes, with low concentration of nalidixic acid, 10 ml per tube (4)
Luria broth tubes, with high concentration of nalidixic acid, 10 ml per tube (4)
Marking pens (10)
Matches, boxes (10)
Paper towels
Pipettors, 1 ml (10)
Pipets, 1 ml, serological (500)
Plates, ML, use red marker to label (Day 1 = 0; Days 2 to 5 = 20)
Plates, TL, use black marker to label (Day 1 = 130 ; Days 2 to 5 = 160)
Plates, TLN, use green marker to label (Day 1 = 0; Days 2 to 5 = 20)
Racks for test tubes (10)
Rubber bands (cup)
Sterile saline tubes, 10 ml per tube (Day 1 = 200; Days 2 to 5 = 270)
Vortex (2)
Water bath, shaking, 37°C (1)

Student Outline

Introduction

The purpose of this laboratory will be to use bacteria as a model organism to study competition. Because of the very rapid growth rate of some bacteria, it is possible to do an experiment lasting 30 generations in just 4 days, thus it is possible to observe competitive displacement of one strain by another. Because the bacteria we will be using are completely asexual, the two strains can easily be viewed as two different species, even though they are both *Escherichia coli*.

We will grow bacteria in liquid culture, using a medium known as Luria broth (or LB), which is rich in all the nutrients *E. coli* needs for growth. To estimate populations of bacteria, samples can be drawn from the liquid culture and spread on agar plates that contain LB media. On these plates, single bacterial cells will grow (after about 24 hours) into visible colonies, so by counting colonies it is possible to calculate how many bacterial cells were in the liquid culture.

The two strains we will be using are derivatives of Cold Spring Harbor Strain 7 (CSH7). The standard CSH7 is able to use the sugar lactose for growth, and so is called lac^+; we will abbreviate this strain as 7+. The second strain we will use is a derivative of 7+ which differs at two genes: it is missing the gene allowing the use of lactose (so it is lac^-), and it is resistant to an antibiotic called nalidixic acid. For brevity, we call this strain 7N-.

Objectives

The purpose of the experiment is to determine which strain will "win" in competition with the other strain when the two are mixed in a culture of LB. If nalidixic acid is present in the media, we expect the resistant strain 7N- to "win." But what will happen in pure LB? We might expect no difference in competitive ability since the two strains differ by only two genes. (Remember that there is no lactose in the LB, so the ability to use it should not matter). On the other hand, just keeping the antibiotic resistant machinery ticking may be costly and reduce strain 7N-'s ability to compete in the presence of strain 7+. To find out who will win, all we need to do is inoculate media (LB or LB and nalidixic acid) with both strains. But to understand why one strain is competitively superior we will first examine the population biology of each strain when grown alone and use some of its biological attributes to see if we can predict who should win. To do this, we will plug these biological characteristics into simulation programs and observe the outcomes. Only after we generate outcomes and population trajectories from realistic parameter values will we run real competitive experiments and compare the actual outcomes with those derived from the simulation.

We will measure the growth ability of the two strains in LB with and without nalidixic acid. With the help of a computer model, this will allow us to predict which strain should be the superior competitor. Then we will actually perform the competition experiment and see if our predictions are borne out.

Background: Bacterial Population Growth

To understand how to make the predictions, it is necessary to understand a little about bacterial growth. When bacteria cells are placed in a fresh, rich medium like LB, they will begin to grow very rapidly, each cell dividing every 20–30 minutes. This is known as exponential growth. The rate of growth is called r (which should be defined in your lecture); the faster growing strain will have a higher r. Resources are consumed by the growing bacteria, and eventually, one critical resource falls to such a low concentration that bacteria can no longer divide. At this point, the population has reached "carrying capacity"; different strains may be able to grow at lower concentrations, and thus would have different values for carrying capacity. Carrying capacity, or

the maximum population density achieved by a strain, is symbolized by *K*. Note that *K* depends on the initial resources available, but in our experiments, this will never change.

There is a third important growth parameter in bacterial populations, and this is called the "lag time," symbolized by *L*. When the bacteria are first given fresh LB, there is a delay (a lag) before they begin to divide. We will generally find a lag time of about 1.5 hours in LB with the strains we are using. Lag time can be critical to a strain's competitive ability: growth is so rapid in LB that a 15-minute delay for one strain can easily mean victory for the other.

Graph A in Figure 4.1 illustrates population growth of a hypothetical bacterial strain. Cell population densities are given in exponential notation: 4e+9 means 4×10^9, or 4 billion. (Bacterial populations can be very large.)

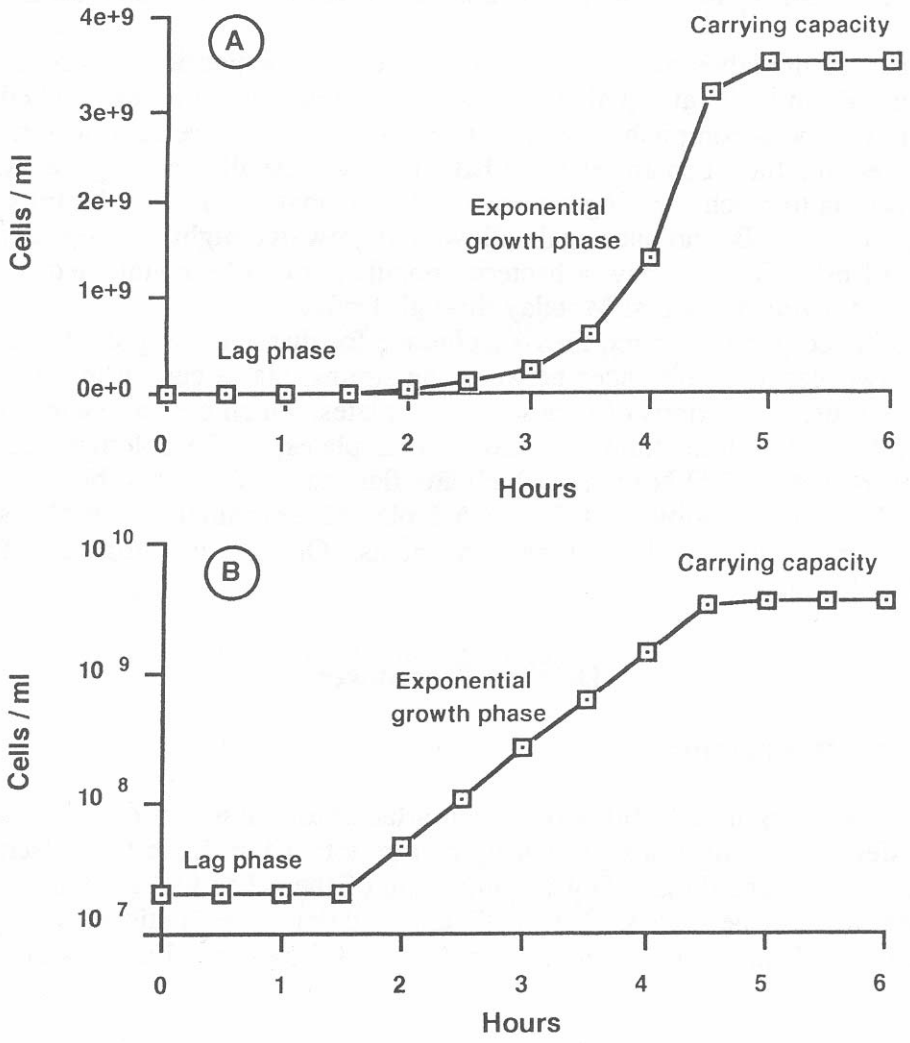

Figure 4.1. Population growth of a hypothetical bacterial strain. Graph A uses exponential notation on the *y*-axis; graph B uses logarithmic notation on the *y*-axis.

Graph B in Figure 4.1 is a logarithmic plot. When population size is plotted against time on a semi-log graph, (logarithms on the *y*-axis, arithmetic values on the *x*-axis), exponential growth appears as a straight line. This makes it clearer when the lag time is over and when the carrying capacity is reached.

Overview of the Laboratory

The first part of the laboratory will be to grow each strain separately in LB during a 1-day experiment. Each student will sample two different population (one 7+ and one 7N-) as they grow for a 3-hour period. Samples will be taken at 2:15, 3:15, and 4:15 p.m. This experiment will be terminated the following day by a sample taken by the next laboratory class. Using data from these 1-day experiments, it will be possible to calculate critical growth parameters for the two strains, and from these, to predict which strain should win out in competition. By repeating these experiments in three kinds of LB—pure, with low nalidixic acid concentration, and with high nalidixic acid concentration—we should be able to predict which strain will win under each set of environmental conditions.

The second part of the laboratory will be to carry out the competition experiment. The strains will be mixed in LB, initially at equal densities, and populations will be tracked for about 25 generations. This will be accomplished using a technique known as serial transfer. First, the two strains will be mixed in a flask containing fresh LB. They will be allowed to grow overnight, plenty of time for the bacteria to reach carrying capacity. Then a small volume of the fully-grown culture will be transferred to fresh LB, and once again allowed to grow overnight. By repeatedly transferring a small portion to fresh LB every day, a bacterial population can be maintained indefinitely. We will maintain them for four transfers: Monday through Friday.

Sampling the mixed population requires a technique for distinguishing the two strains. Taking advantage of the two genetic differences between the strains, plates can be made which allow the distinction. We will use three kinds of plates: (1) TL plates, which contain lactose and a chemical called TTC, as well as LB. Both strains can grow on TL plates, but 7+ colonies will be pink, while 7N- colonies will be red. (2) TLN plates, which are identical to TL plates, but also have nalidixic acid, so that only 7N- cells can grow. (3) Finally, ML plates are minimal lactose plates. They contain no LB, only the sugar, lactose, and a few basic nutrients. Only 7+ can grow on ML plates, since 7N- cannot utilize lactose.

Detailed Procedures

Part I: 1-Day Growth Experiment

The experiments will begin at 12:00 noon, when a laboratory assistant or TA starts a population growing by transferring 0.1 ml of a high-density culture into 10 ml fresh LB. Each lab group will take two test tubes with already-growing bacteria: one of the tubes will contain the strain 7+, the other 7N-. They will not be mixed. You will sample them three (3) times during the afternoon laboratory: at 2:15, 3:15, and 4:15. Sampling is done as follows (see Figure 4.2):

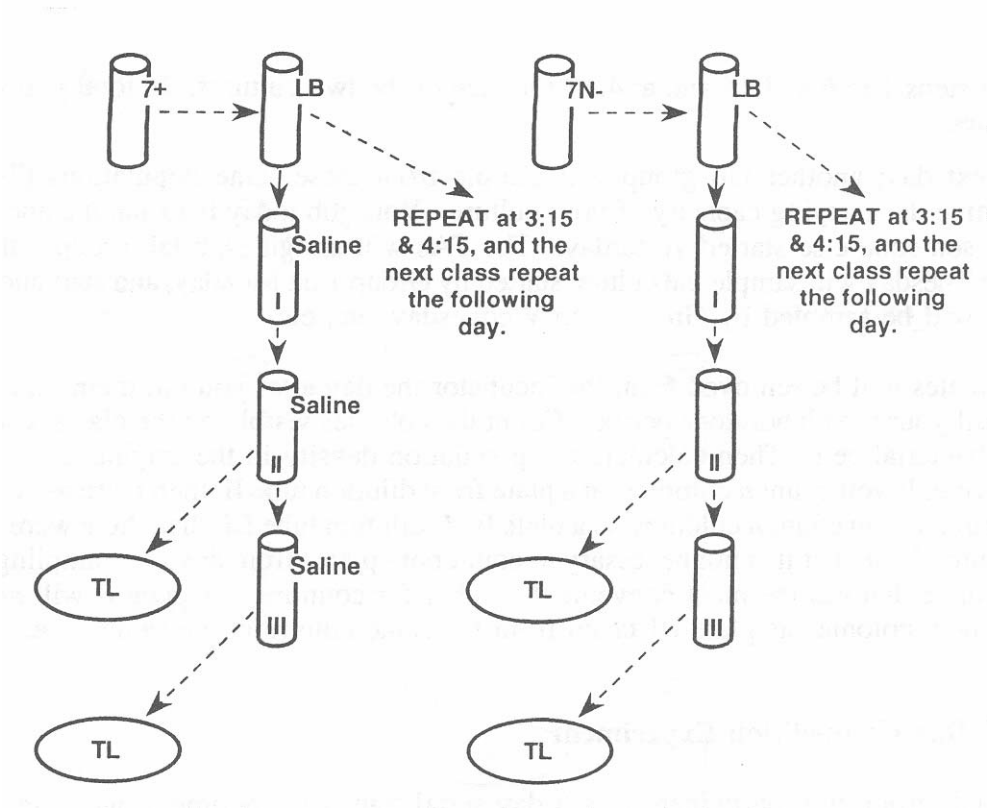

Figure 4.2. Flow chart for Part I: 1-Day Growth Experiment; 12 TL plates per pair and four plates the next day. "LB" represents Luria broth; "TL" are 2,3-5 triphenyltetrazolium chloride plus lactose plates. See Appendix C.

1. Pipette 0.1 ml of a bacterial culture into a dilution tube which has 9.9 ml of saline. The dilution tubes will be provided for you. Label this dilution tube with "2:15:I", meaning time 2:15, dilution I.
2. Mix tube I well, then pipette 0.1 ml from it into a second clean dilution tube. Label this "2:15:II".
3. Repeat once more: 0.1 ml from tube II into a third tube, and label it "2:15:III". *Note:* You *cannot* reuse tubes.

   (What you have done is diluted the culture by a precise amount. This is necessary before counting the cells because bacterial populations are so dense. You can't count 1 billion cells, so you dilute them exactly 1 million-fold, then count.)
4. Take 0.1 ml from tube II and pipette it onto one TL plate. Dip a glass "spreader" in alcohol, flame it for a moment (to kill any bacteria on it), and spread the drop of liquid all around the plate.
5. Repeat step 4 with 0.1 ml from tube III.
6. Label the bottom of the plates (*not* the tops) very carefully with your lab group name, your group number, the date, the time, and the dilution tube number. Place the plates in the incubator at 37°C.

7. Repeat steps 1 to 6 at 3:15 and at 4:15 for each of the two cultures. In total you will have made 12 plates.
8. The next day, another lab group will sample from these same populations (7+ and 7N-) to determine the carrying capacity of your culture. Your job today is to sample another population which someone else started yesterday. The TAs will assign each lab group a number. Group 1 from Tuesday will sample the culture started by Group 1 on Monday, and start another population which will be sampled by Group 1 on Wednesday, etc, etc.

Your plates will be removed from the incubator the day after you put them in, and held in cold storage until your next laboratory period. Count the colonies visible on the plates: each colony grew from one bacterial cell. Then calculate the population density in the original culture by using the following rule: If you count *n* colonies on a plate from dilution tube II, then there were $n \times 10^5$ cells/ml in the culture. If you count *n* colonies on a plate from dilution tube III, then there were $n \times 10^7$ cells/ml in the culture. Note that it is not necessary to count both plates from any one sampling time. Instead, count the plate that has the most convenient number for counting. A plate II will *always* have 100 times as many colonies as plate III taken from the same culture at the same time.

Part II: 5-Day Competition Experiment

Each lab group will participate in a 5-day serial transfer experiment, working together with a lab group from the other days of the week (see Figure 4.3). That is, Monday's Group 1 will start an experiment, Tuesday's Group 1 will continue it, etc., until Friday. This experiment requires much less sampling than the 1-day growth experiments. It should be easy to finish it between the 2:15 and 3:15 samples from the 1-day experiments.

Monday: Three (3) containers of fresh LB will be available: one with no nalidixic acid, one with a low concentration of nalidixic acid, and one with a high concentration of nal.

1. Add 10 ml of fresh LB from one of the containers to a sterile test tube. You must use a sterile technique when handling LB: flame the glass pipet just before using, and shut the container as soon as you are done. (LB is such good growth medium, that a single fungal spore or bacterial cell accidently added will rapidly grow overnight. The next day's class would find cloudy LB.) Each lab group will be told which kind of LB to start with, whether pure or with antibiotic. Each group will also get a number to facilitate sharing an experiment with lab groups from the other days of the week.
2. To the test tube with fresh LB, add 0.1 ml of the culture of 7+ bacteria *and* 0.1 ml of the culture of 7N- bacteria. Use a sterile technique when handling the bacteria. Label the test tube with your lab group number and the day.
3. After inoculating the fresh LB, sample it as described above, but making only dilution tubes I and II.
4. Spread 0.1 ml of tube II onto a TL plate (as described above). Label the plate carefully on the bottom and place it in the incubator.

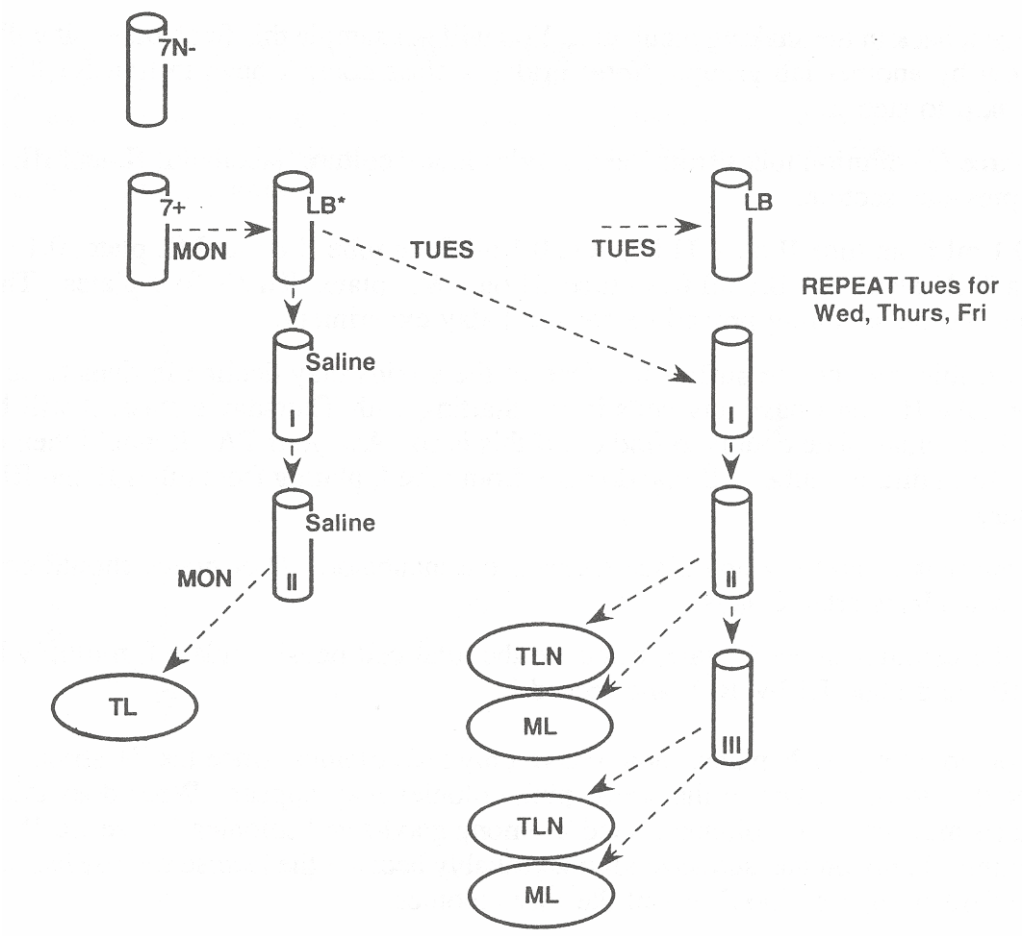

Figure 4.3. Flow chart for Part II: 5-Day Growth Experiment; Monday: one TL plate per pair; Tuesday to Friday: 2 TLN and 2 ML plates per pair. "LB" represents Luria broth;" TL" are 2,3-5 triphenyltetrazolium chloride plus lactose plates; "TLN" are 2,3-5 triphenyltetrazolium chloride plus lactose plus nalidixic acid; and "ML" are minimal media plus lactose plates. See Appendix C.

5. Place the test tube in the shaking incubator.
6. The next day, there will be a mixture of red and pink colonies on the plate. Count each separately (pink are 7+, red are 7N-) and calculate the total population density of each. Remember, counts from plate II are multiplied by 10^5 to calculate real density.

Tuesday to Friday: Each lab group must find the test tube which was started by Monday's group of the same number. There are two things to be done with it: transfer a small amount to fresh LB and sample the culture for population estimates.

1. Transfer first. Add 10 ml of fresh LB to a sterile test tube, making sure that you use the same kind of LB used in the previous culture. An experiment started in LB containing high amounts of nalidixic acid should be continued with the same amount of nalidixic acid. Take 0.1 ml of yesterday's culture (which will be fully cloudy) and add it to the fresh LB. Label the tube carefully, and place it back in the shaking incubator. You will not sample this fresh tube—it will be sampled tomorrow by another lab group. *Note:* Friday's class doesn't have to transfer the culture and so can skip to step 2.

2. Make three (3) dilution tubes from yesterday's cloudy culture, labelled I, II, and III, as described in the previous section.
3. Plate 0.1 ml from tube II on a TLN plate, 0.1 ml from tube II on an ML plate, 0.1 ml from tube III on a TLN plate, and 0.1 ml from tube III on a ML plate. That's four plates. This is exactly the same as the sampling procedure for the 1-day experiment.
4. *However*, this may not be sufficient. One of the strains may decline in density so rapidly that dilution tube II won't have any cells in it. Starting with Thursday's class, it will be necessary to check previous plate counts to find out if this is so. Ask your TA. It would then be necessary to plate from dilution tube I. If you do plate from tube I, plating from tubes II and III is no longer necessary.
5. Label the plates carefully and place them in the incubator. TLN plates should come out after 1 day, ML plates after 2 days.
6. Count the colonies on each plate, calculate the total cell density (plate I, multiply by 10^3, plate II by 10^5, and plate III by 10^7), and record it.

Notes on counting: TLN plates should have only red colonies, since the 7+ strain can't grow on nalidixic acid. However, late in the week, pink colonies may appear. What does this mean?

ML plates may have tiny colonies mixed in among good-sized colonies. These are 7N- cells which grow extremely slowly on the lactose medium, probably because the lactose we buy has contaminating quantities of other sugars. Don't count the tiny colonies.

Recording Data

There will be master sheets kept in the laboratory for recording population sizes from both the 1-day and 5-day experiments. Each will be labelled with a lab group number at the top, and will also tell how much nalidixic acid was in the medium. Each lab group should copy their population estimates onto the appropriate master sheet, so that other lab groups can get their numbers. Every group can then get all the data for each experiment they participated in.

Graph population density versus time for both experiments (1-day and 5-day), using semi-log paper. Based on the 1-day experiment, what are the lag times, growth rates, and carrying capacities of each strain when grown alone? Calculate all three parameters for each strain.

Carrying capacity is easy—it's simply the total density after one day of growth. It should be somewhere around 200×10^7 cells/ml, or about 2 billion.

Growth rate is found as follows: (1) Divide the 3:15 population by the 2:15 population. (2) Divide the 4:15 population by the 3:15 population. (3) Average the two numbers (if the cells were growing exponentially, the two numbers should be similar). (4) Find the natural logarithm (ln) of the average. This is the growth rate. It should be between 1 and 2.

Lag time is trickier. On your logarithmic plot of population density versus time, draw a line connecting the 2:15, 3:15, and 4:15 population sizes. Draw a second line, horizontal, at the starting population density. Where the two lines meet gives an approximation of the lag time. For example, the graph in Figure 4.4 shows four population estimates (large circles) on a semi-log plot of population density versus time. The intersection of the two lines is at about 1 hour, so the lag time is about 1 hour. Lag time should be between 30 minutes and 2 hours.

Now you have quantified three population features of each strain under different environmental conditions. Using these features you can now simulate how the strains will fare when competing for a common and limiting resource.

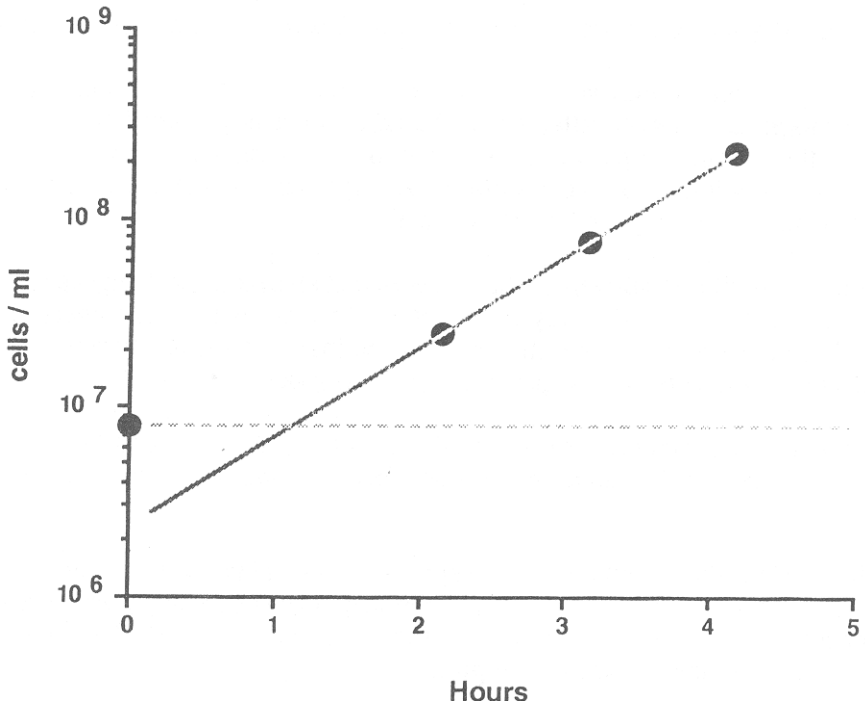

Figure 4.4. Plot of population density versus time to determine the approximate lag time of a bacterial population.

Conclusions

The final part of the laboratory is for different groups to compare results to answer the basic question: Which strain won when there was no antibiotic? When there was nalidixic acid present? Do two small genetic changes influence the competitive ability of a bacterial strain? If so, speculate as to why. *Hint:* Are there possible costs as well as benefits associated with these physiological processes?

Acknowledgements

Dr. Daniel Rubenstein and Dr. Richard Condit of the Department of Ecology and Evolutionary Biology at Princeton University developed this laboratory exercise.

APPENDIX A
Sterile Technique

Studies with bacteria are complicated by the presence of many potential contaminating microorganisms in the air and on all laboratory furniture and equipment. It is therefore necessary to use sterile containers, media, and other equipment in order to obtain satisfactory results.

Lab bench: Always wipe off your bench area with a 95% EtOH solution before you begin to work.

Petri plates: Do not open petri plates until you are ready to use them. When you open a plate, raise the lid only as high as necessary to plate or streak cells. Close the lid as soon as you are finished. When plates are placed into the incubator, they should be put in upside down; this prevents water from condensing on the lid and "raining" onto the cells growing on the agar surface. Always label your plates; write on the bottom of the plate and include your name, lab day, and room number.

Pipets: Individually wrapped, sterile, plastic pipets should be used at all times. Each pipet should be unwrapped only when it is needed. Never touch the delivery tip region of the pipet with your fingers. Never put pipets on the lab table; if they have already been used, they will contaminate the surface bacteria; if they are sterile, they will be contaminated. Containers will be provided for used pipets.

Other sterile material: You will be provided with capped tubes of sterile saline for dilution. Again, minimize the time that these tubes are open during dilution. Always flame the mouth of the tubes before making any transfers.

Spreading and streaking of bacterial cells: Cells are spread on petri plates with a triangular glass spreader. After pipetting a solution of cells onto the agar surface, the cell should be spread out over the surface with a flame sterilized spreader. (Be sure spreader is cool before touching bacteria!) The objective is to obtain an even distribution of cells over the surface of the plate.

Sterile transfer of cells and solutions:

1. Pick up the tube containing the cell suspension or solution and holding a pipet or inoculating loop in your right hand, remove the plug or cap of the tube with the fourth and fifth fingers of your right hand. Keep the tube slanted as much as possible to prevent contaminants in the air from falling into the tube.
2. Pass the open end of the tube through a flame. Any contaminating microorganism lurking on the edge ready to fall in will be destroyed by the flame and the hot air currents will carry microorganisms away from the opening.
3. Insert the pipet or inoculating loop into the solution and withdraw the sample. Replace the cap on the tube.
4. If you are transferring to another tube, remove its cap or plug, pass the open end through a flame and deliver the sample. Replace the cap. If you are transferring to a petri dish, raise the lid slightly, streak or pipet the sample and replace the lid.

APPENDIX B
Sample Timetable for a 3-hour Session

Time (approximate)	*Step*
1:30 p.m.	Sterile technique demonstration; swab benches.
1:45	Label tubes and plates for 2:15 sample of Part I.
2:00	Get two cultures from bath for Part I (7+ and 7N-). **Sign log.**
2:15	Sample, dilute, and plate out.
2:30	**Monday only**: Get one tube of broth for Part II. Inoculate with 7+ and 7N-. Sample, dilute, and plate out. Return three cultures to bath. **Sign log!** **Tuesday–Friday:** Return two cultures from Part I to bath. Get one tube from bath from Part II (7+/7N-). **Sign log!** Transfer culture to tube of the same kind of broth. Label this fresh tube and return to bath. Sample, dilute, and plate out old tube. Place in discard rack.
3:15	Re-sample Part I; return to bath.
3:45	Learn to count colonies.
4:15	Re-sample Part I; return to bath.
4:30	Place labelled, banded, stickered plates in proper bins. Place saline tubes in discard racks. Swab benches.

APPENDIX C
Supplies, Preparation of Materials, and Bacterial Cultures

Chemical supplies:

Tryptone (Difco #0123)
Yeast extract (Difco #0127)
Sodium chloride
Agar (Bactoagar; Difco #0140)
Lactose monohydrate
Ammonium sulfate
Potassium phosphate monobasic
Potassium phosphate dibasic
Sodium citrate
Magnesium sulfate
Thiamine
Triphenyltetrazolium chloride (TTC) (Sigma #T8877)
Nalidixic acid, sodium salt (Sigma #N4382)

Difco Laboratories, P.O. Box 1058, Detroit, Michigan 48232
Sigma Chemical Co., P.O. Box 14508, St. Louis, Missouri 63178

Procedure for making solutions:

1. Stock solutions:

   10% $MgSO_4$ (100 ml): Put 10 g $MgSO_4$ in a 100-ml volumetric flask. Bring volume up to 100 ml with distilled water. Filter sterilize the solution (with a Millipore filter) then pour into two sterilized Wheaton bottles. Refrigerate.

   0.2% Thiamine (100 ml): Put 0.2 g of thiamine in 100 ml of distilled water. Filter sterilize the solution (with a Millipore filter) then pour into two sterile 50-ml erlenmeyer flasks. Refrigerate.

   0.5% TTC (2, 3-5 Triphenyltetrazolium chloride): Add 5 g TTC to 100 ml of distilled water. Add stir bar; stir until TTC is dissolved. Filter sterilize the solution (with a Millipore filter). Pour approximately 50 ml of the TTC solution into each of two sterilized Wheaton bottles that have been wrapped with aluminum foil. Refrigerate.

   Nalidixic acid 20 mg/ml (100 ml): Add 2 g of nalidixic acid to 95 ml distilled water. *Caution: Wear gloves. Nalidixic acid is a possible carcinogen.* While the solution is stirring, add approximately 5 ml of 5 M NaOH drop by drop until the nalidixic acid dissolves. Filter sterilize the nalidixic acid solution and pour approximately 50 ml of the solution into each of two sterilized Wheaton bottles that have been wrapped with aluminum foil. Refrigerate.

2. Plates:

   TL plates: **T**TC plus **L**actose
   TLN plates: **T**TC plus **L**actose plus **N**alidixic acid
   ML plates: **M**inimal media plus **L**actose

TL and TLN plates		
Ingredient	Per 1 liter of media (g)	Per 3 liters of media (g)
Tryptone	10.0	30.0
Yeast extract	1.0	3.0
Sodium chloride	5.0	15.0
Agar	16.0	48.0
Lactose	10.0	30.0

Instructions: To make 3 liters of media, use a 4-liter flask. Add the above ingredients to the dry flask. Then add distilled water to the 3-liter mark. Autoclave for 30 minutes; cool to 60°C in a 60°C water bath for 1–2 hours.
TL plates: Add 3.0 ml of 5% TTC solution.
TLN plates: Add 3.0 ml of 5% TTC solution and 3.0 ml of 20 mg/ml nalidixic acid.

ML plates		
Ingredient	Per 1 liter of media (g)	Per 3 liters of media (g)
Potassium phosphate dibasic	7.0	21.0
Potassium phosphate monobasic	2.0	6.0
Ammonium sulfate	1.0	3.0
Sodium citrate	0.5	1.5
Agar	16.0	48.0
Lactose	4.0	12.0

Instructions: To make 3 liters of media, use a 4-liter flask. Add the above ingredients to the dry flask. Then add distilled water to the 3-liter mark. Autoclave for 30 minutes; cool to 60°C in a 60°C water bath for 1–2 hours. Add 3.0 ml of 10% $MgSO_4$ and 3 ml of 0.2% thiamine.

Procedure for pouring/storing plates:

Before we begin to pour plates, we wipe down the benches with a 10% Lysol solution. For each session, we autoclaved four 4-liter flasks; each with 3 liters of media. In addition we autoclaved four empty 1-liter flasks. After the media cooled to 60°C, we transferred some of the media from the 4-liter flask into the 1-liter flasks using the sterile technique. We kept the 4-liter flasks in the 60°C water bath as we poured from the 1-liter flasks. We used 100 mm × 15 mm sterile petri dishes. We poured the plates 1/2 full.

We leave plates on the benches for 2 days to dry. We marked the plates with a color code; using black marker for TL plates, green marker for TLN plates, and red marker for ML plates. We stored plates upside down in the plastic sleeves and returned the sleeves to the original boxes for storage in the cold room.

3. Luria broth:

500 ml	1000 ml	3000 ml	Final volume
5.0 g	10.0 g	30.0 g	Tryptone
2.5 g	5.0	15.0 g	Sodium chloride
2.5	5.0	15.0	Yeast extract
dH_20	dH_20	dH_20	To make final volume

To make plain LB tubes, make up the LB broth. Pipet 10 ml of the broth into test tubes. Autoclave the solution for 25 minutes. LB tubes can be stored at room temperature.

4. Luria broth with high nalidixic acid (20 ppm nal):

   For a week's supply, make up 500 ml of LB broth. Autoclave. Refrigerate. Add 0.5 ml stock nalidixic acid solution (20 mg/ml) to broth. *Caution: Wear gloves.* Aliquot 10 ml of the solution into sterile test tubes. Allow four tubes per class.

5. Luria broth with low nalidixic acid (2 ppm nal):

   Make up six 500-ml LB flasks. Autoclave. Refrigerate. Each day, add 0.05 ml stock nalidixic acid solution (20 mg/ml) to broth. *Caution: Wear gloves.* Aliquot 10 ml of the solution into sterile test tubes. Allow four tubes per class.

6. Saline (0.9% Sodium chloride):

   Add 36 g of sodium chloride to 4000 ml distilled water. Use a pipetting aid (e.g., Repipet) to add 9.9 ml of the solution to each test tubes. Autoclave. Store at room temperature.

Bacteria Cultures

1. Cultures of *E. coli* strains, 7+ and 7N-, may be obtained by writing Linda Cholewiak at Princeton University.
2. Set up the stock cultures 48 hours before the laboratory. Using the sterile technique, use an inoculating loop to transfer a single colony to 10 ml of LB in a test tube. Grow up both stocks at 37°C for 2 days. Test the stock cultures on TL, TLN, and ML plates.
3. During the laboratory week, 1 hour before each laboratory, transfer 0.1 ml of each stock culture to a fresh tube. For each laboratory section, transfer 0.1 ml of each 48-hour stock to 10 tubes of LB. Place all tubes in a shaker bath at 37°C.
4. Cultures used for the 3-hour bacterial growth curve should be kept shaking vigorously on the shaker bath during the laboratory period; they should be removed only to sample the culture.