Bi 1x
E. coli Growth Curves

1 Overview

In this lab, you will investigate growth of the bacterium *E. coli*, watching the growth in two ways. First, you will determine growth rate in a solution of media by measuring optical density over time. The optical density is proportional to the mass of bacteria in the solution, within a certain dynamic range. You will measure the growth rate of *E. coli* using different sugars to compare growth rates. You will also repeat and expand upon a foundational experiment in molecular biology by growing *E. coli* in media with different sugars and observing the characteristics of growth curves.

Second, you will watch individual bacteria grow and divide using fluorescence microscopy. In this case, the mass of bacteria is proportional to the fraction of your images occupied by bacteria.

Through these two different approaches, you will learn about the rates that bacterial populations convert media into new life. You will also learn about the ability of bacteria to respond to changes in external conditions by turning on a suite of diverse catabolic enzymes.

2 Background

2.1 Bacterial growth

As you have already seen in our introductory experiments, bacteria can grow and divide very rapidly. A solution containing approximately 100 bacteria can grow to well over 10^9 bacteria—roughly equal to the entire human population—overnight. As with human or animal populations growing under optimal conditions, the rate of population increase is proportional to the current population. This means that

$$\frac{dN}{dt} = rN, \tag{1}$$

$$N(t) = N_0\, \mathrm{e}^{rt}, \tag{2}$$

where $N(t)$ is the number of bacteria in your sample at time t and $N_0 = N(0)$. The growth rate r depends on many features of the environment, such as the growth medium, temperature, and presence of antibiotics. We will investigate how the sugar present in the media changes characteristics of bacterial growth.

2.2 The Beer-Lambert Law and optical density

To determine the density (concentration) of bacteria in liquid media, we load a plate reader with the suspension of cells. The plate reader is a parallel spectrophotometer, in which light with a wavelength of 600 nm enters the sample at an initial intensity (I_0). Cells, having a different index of refraction as the surrounding medium, randomly reflect and scatter light out of the incident light path. The amount of scattering is proportional to the mass of cells at any given point in the sample. (Caveat: to get an accurate reading, cell density must be low enough that light is only scattered once in its journey through the sample. Often, this means that cells must be diluted to get an accurate reading.) Upon exiting the sample, the light intensity is reduced by the cells from I_0 to $I = I_0 \mathrm{e}^{-\varepsilon l c}$, where ε is the wavelength-dependent molar absorption coefficient of the species in question, l is the path length (that is, the total length of the trajectory of a light beam through the sample), and c is the concentration. This is known as the Beer-Lambert Law. The transmission efficiency of the sample is the ratio of the final and initial light intensity, $T = I/I_0$, and the reported quantity is called the absorbance,

$$A = -\ln T = \ln \frac{I_0}{I} = \varepsilon l c. \tag{3}$$

Note that in order to compute the absorbance, I_0 must be measured. This is done by measuring a **blank** sample, which contains only the media in which the bacteria are grown, but no bacteria. Internally, the instrument has a value for I_0, which we will call I_0^{inst}. So, the absorbance value given by the instrument for the measured absorbance is

$$A^{\text{inst}} = \ln \frac{I_0^{\text{inst}}}{I}. \tag{4}$$

Measurement of the blank gives

$$A_{\text{blank}}^{\text{inst}} = \ln \frac{I_0^{\text{inst}}}{I_0}, \tag{5}$$

since $I_0 = I_{\text{blank}}$ because there are no bacteria to scatter light. Therefore, the absorbance is

$$A = A^{\text{inst}} - A_{\text{blank}}^{\text{inst}} = \ln \frac{I_0^{\text{inst}}}{I} - \ln \frac{I_0^{\text{inst}}}{I_0} = \ln \frac{I_0}{I}. \tag{6}$$

The general rule of thumb is that the accurate absorbance readings lie in the range of $0.01 < A < 1$. (Serial dilutions of your sample may be necessary to bring the absorbance into this range.) In the context of bacterial growth, the absorbance A is often called **optical density**, abbreviated OD_{600} for absorbance of light at a wavelength of 600 nm.

2.3 Calibration of optical density

In the previous section, we established that the optical density is proportional to cell concentration. To determine the constant of proportionality (the product εl), we dilute a sample of known OD to a very low concentration of bacteria. We then spread a small volume of this diluted sample onto a plate with a good growth medium. We incubate the plate and count the number of bacterial colonies present. Provided the initial solution was dilute enough, each colony should correspond to a single bacterium. Since we know how much we diluted the original sample, we can then back-calculate its concentration. This gives us the constant or proportionality between OD_{600} and cell concentration. A good rule of thumb is that an OD_{600} of 0.1 corresponds to about 10^8 cells/mL.

2.4 Measuring growth at single-cell level

While the scattering experiments provide *bulk* information of growth rates, we can also look at small colonies under a microscope and investigate growth rates of *individual cells*. Here, we simply mount a freshly inoculated agar pad containing LB medium on a microscope slide and capture images over time. If our initial concentration of bacteria is sufficiently low, we can watch isolated, individual bacteria proliferate into massive colonies of cells over the course of just a few hours. Though we could do this with phase contrast microscopy, this module will introduce you to modern fluorescence microscopy to acquire time lapse movies.

2.5 Your Mission

- Determine bulk growth rates and diauxic transitions using different carbon sources.
- Determine the correspondence between OD_{600} and cell concentration in liquid medium.
- Determine the growth rate of cells on an agar pad with LB medium by acquiring images of individual cell colonies.

3 Protocol

3.1 Bulk Assay

Prior to your involvement, overnight cultures of wild type *E. coli* will be inoculated in both LB medium and M9 minimal medium with glucose in a shaker at 37°C. These cultures will be completely saturated and at stationary phase.

3.1.1 Bulk Assay OD Calibration

You will measure OD_{600} using the lab plate reader.

1. Pipette 1 mL of overnight culture into an Eppendorf tube.
2. Prepare a series of eight ten-fold dilutions of your culture (i.e., 10^0 to 10^{-7}) in 500 µL volumes using the provided Eppendorf tubes. Note that because you may transfer some of one dilution to the next, your final volume may not be exactly 500 µL for some of the dilutions. For example, say you have made 500 µL of a 10^{-3} dilution and you choose to make your 10^{-4} dilution by mixing 50 µL of your 10^{-3} dilution with 450 µL of media, then you would end up with 450 µL of your 10^{-3} dilution.
3. Add 300 µL of each dilution to the wells assigned to you in the 96 well plate.
4. Once all groups have loaded their samples, measure the OD in the plate reader and write down the values you measured for your samples.
5. Using the multichannel pipette, prepare a drip plate using the dilutions from the well plate.
6. To do this, use a multichannel pipette to remove 10 µL from each of your dilutions from the 96-well plate. Dispense onto the top of agar plate, fully expelling the volume.
7. Gently tilt the plate until vertical, allowing the droplets to streak to the opposite edge of the plate. Avoid mixing the droplets.
8. Place the plate back flat onto the bench top until the liquid dries.

This will be done one group at a time, so while you are waiting, prepare the media as described in the next section.

3.1.2 Bulk Assay Growth Preparation

1. Each group will be working with glucose and one additional sugar, chosen from xylose, sorbitol, galactose, rhamnose, ribose, and arabinose. You will be given 50 µL of a sugar at 20% concentration, 10 µL of glucose at 20% concentration, and 5 mL of M9 minimal media. In one 1.5 mL Eppendorf tube, make 1 mL of M9 minimal media with 0.2% glucose. In a 15 mL Falcon tube, make 5 mL of M9 minimal media at 0.2% of your other provided carbon source.
2. In three 1.5 mL Eppendorf tubes, prepare 1 mL of the sugar:glucose solutions as worked out in the prelab. Make sure to label your tubes.
3. Pipette 300 µL of each of your 4 solutions (sugar alone and 3 sugar:glucose mixes) into 3 wells of your assigned row (so you have 3 replicates for each). Note down the order in your lab notebook.

4. Using a multichannel pipette, pipette 3 µL of the 1:1000 M9 minimal media culture dilution that your TAs have prepared from a clean well boat into your assigned row. Make sure to fully expel the cells.

After all groups have filled their respective row, you will discuss and decide together how to fill the remaining row. Here you can get creative! Would you like to mix two different carbon sources other than glucose? Mix at different ratios? Use lower or higher sugar concentrations?

Your TAs will prepare 3 replicate wells of *E. coli* in glucose and M9 minimal media the same way in which you prepared your assigned sugar. They will also prepare 3 replicate wells of *E. coli* in LB (a media rich with nutrients for bacteria). You will use these results to compare to the growth characteristics in your assigned sugar.

Also note, as mentioned above, that having a blank is essential for OD_{600} calculation. Your TAs will also include 3 minimal media blanks and 3 LB blanks in the top row of the 96-well plate.

The 96-well plate will then be placed in the plate read, kept at a constant temperature of 37°C, and OD_{600} measurements will be made for the next 24 hours as bacteria grows.

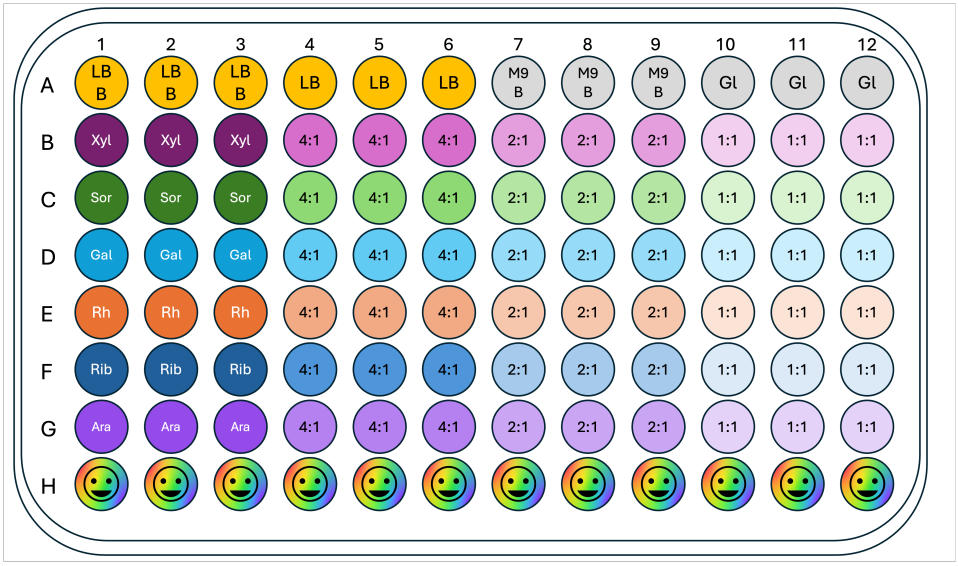

Figure 1: Layout of samples in the 96-well plate. Ratios in row B through G are all sugar:glucose. In row H, you are encouraged to try any sugar combination that you fancy.

3.2 Growth Movie

As a complement to the bulk measurements you did, you will measure cell division at the single cell level under the microscope. The idea is to compute the growth

rate based on the movie you will obtain. This serves also to introduce the single cell measurements that can be done using an automated microscope.

3.2.1 Preparing the agar pads

The agar pads will be prepared by your TAs, but the protocol for making them is displayed below. Each group should have two or three pads, just in case there is a problem with one.

1. Prepare LB with 2% agarose, keep in waterbath to prevent it from solidifying (temperature depends on agarose used; for ours, this is 42 C).
2. Align three microscope slides in parallel on the benchtop.
3. On each of the outer two slides, place two coverslips on top of each other at the center of the slide. These will determine the height of your agarose pad.
4. Pipette 250 µL of LB with 2% agarose onto the middle slide.
5. Lower another slide across the agarose and coverslip spacers, creating an even pad.
6. When the agarose solidfies, split the slides, revealing the pad on one of them. Use a coverslip to cut the pads into approx 3x3 mm squares.

3.2.2 Mounting the sample

1. Your TAs will provide you with two samples of cells: one at an appropriate concentration for our growth movies (about 10^6 cells/mL), and another at a higher concentration (about 10-100x more concentrated). While not strictly necessary, the latter is used to make finding the correct focal plane easier (see below).
2. Spot 1 µL of cells on each pad. Remember that you should NOT touch the pad with the pipette tip! Two pads should get cells from the low concentration and one pad from the high concentration cultures.
3. Cover the pads with a lid of a plate or a tip box lid and let them dry for a few minutes until the water spot is no longer visible. Depending on how dry the pad is, we might put it in the 37°C incubator to speed up drying.
4. Gently transfer the pads to a glass-bottomed dish as demonstrated by your TA. Remember that the side where you spotted the cells should face the bottom of the petri dish. Be sure to mark the edge of the dish with a Sharpie and note in your notebook the orientation of the pads in the dish so you know which is which when you go under the microscope (remember to account for the mirror image flip when inverting the dish!).

5. Seal the dish using parafilm. Make sure the parafilm does not interfere with the placement of the dish on the stage. The parafilm is meant to reduce evaporation from the pad which would result in drying.
6. You will need to you a petri dish adapter for your microscope's stage. Begin by finding your focal plane using 10x and 40x objectives. When you are ready to move to 100×, remove your plate from the stage, make sure that there is a TA supervising, and rotate in the 100× objective.
7. Put a very small drop of oil on the 100× objective and on the bottom of the dish where the pads are located. Load the dish onto the stage with a petri dish adapter.

3.2.3 Setting up the movie

1. Use the high-concentration "helper" pad to find a bacteria and get a rough estimate of the focal plane for your more dilute pad that you will use for imaging. You will need to apply oil to the glass bottomed dish, since you will be using a 100× oil objective. You should use brightfield illumination for phase contrast microscopy while doing this. The TAs can help you with this, and also setting up Köhler illumination.
2. While still on the concentrated pad, determine a good exposure time for the fluorescence channel. This is done by trial and error. Different scopes will vary, but most will have reasonable times are between 100 ms and 2000 ms. Begin with shorter exposures.
3. Move over to a more dilute pad, again imaging using phase contrast (not fluorescence). You should now only need to make fine adjustments to the focus to find the bacteria. You want to find one to four bacteria that are far from others for your growth movie, since you do not want other colonies encroaching.
4. When you have found the cells you want to watch grow, you will acquire images over time. Set up an acquisition where you take a brightfield (phase) image and a fluorescent image for each time point. We will be manually focusing and acquiring the images, so set a timer to remind you when to take the images. Think carefully about how often you want to take pictures (see prelab question 3).

4 Assignment

Problem 0 (Summary)**.**
Write a summary of this experiment, its objectives, and conclusions between one paragraph and one page in length.

Problem 1 (Serial dilution (prelab for plate reader growth curves)).
Write a sensible protocol for the serial dilutions for the OD calibration.

Problem 2 (Bulk growth recipes (prelab for plate reader growth curves)).
You will make two M9 miminal media solutions, one with 0.2% glucose and another with 0.2% of an assigned sugar. Fill out the table below with volumes to bring solutions to desired ratios.

Table 1: Media recipes.

sugar:glucose	0.2% sugar in M9	0.2% glucose in M9	Total volume
4:1			300 µL
2:1			300 µL
1:1			300 µL

Problem 3 (Microscope frame rate (prelab for growth movie)).
When acquiring your growth movies, how long should you wait between frame acquisitions? What is your reasoning?

Problem 4 (OD calibration).
Count the colonies on your plates and use this value to determine a calibration factor between OD_{600} and absolute number of bacteria. You should also assess for what range of concentrations is the OD linear in the number of cells. How does this calibration factor compare to our rule of thumb that $OD_{600} = 0.1$ corresponds to 10^8 cells/mL?

Problem 5 (Plots of bulk growth curves: All the sugars).
In one plot, plot all growth curves obtained from pure sugar sources. That is, plot all curves obtained from your session's plate in wells containing glucose + minimal media, xylose + minimal media, galactose + minimal media, etc. Additionally, plot curves obtained from LB. Comment on what you see.

Problem 6 (Plots of bulk growth curves: Glucose and assigned sugar).

a) For each of the three glucose + minimal media wells, plot OD_600 versus time. All three curves should be on the same plot. (You already did this in the previous problem, but make a fresh plot.)

b) Perform a regression to obtain growth rates for each of the three replicates. Report the growth rates and add the best fit lines to the plot.

c) Repeat parts (a) and (b) the three wells containing your sugar + minimal media and also for the LB wells. All nine curves should be on the same plot for ease of comparison.

d) How do the growth rates compare? Why do you think this is?

Problem 7 (Bulk growth: measuring growth rates before and after diauxic shifts)**.**
For your three ratios (glucose:assigned sugar as 1:1, 1:2, and 1:4), plot your OD_{600} measurements versus time. First, note what you see qualitatively: where does the shift in growth rates occur for each ratio, and why do you think that is?

Next, for each ratio, select regions of the growth curve before and after the diauxic shift. Perform a regression to get the growth rates separately for each region. How do these growth rates compare with the ones you fit in the previous problem for *E. coli* grown in glucose and your assigned sugar alone? Is this what you expected? Explain what may be happening in the cells to allow for the observed shift in growth rates.

Problem 8 (Doubling time from movies)**.**
Estimate the cell doubling time from your movies acquired on the microscope by using the segmentation techniques we discussed in class and again performing a regression. Do you get the same growth rate that you got for the bulk measurements in LB?

Problem 9 (Data and code)**.**
Attach all code used for data analysis. Also, include the following:

a) Write down the exact serial dilution protocols you did to calibrate your OD_{600}. This will likely be what you did in prelab question 1, but make sure you note what you actually did in lab.

b) Show the initial and final frames of your movie. Be sure that physical distances are clear in the display of the images.

c) Show segmented (binary black-and-white) images for the images you showed in (b).