Please Minimize Printing!! There’s no need to print out all the documents you download for this course! We ourselves will print out for you the protocols you’ll actually use during the lab sessions (we’ll notify you at the appropriate place the pages we’ll print).

A Fluctuation Test in Yeast:
Using math to test Darwin’s theory

When exposed to an antibiotic, microorganisms (e.g., bacteria or yeast) often evolve resistance with astonishing speed. If you spread a culture of antibiotic-sensitive cells on a Petri dish containing nutrient agar supplemented with an antibiotic, after a suitable period of growth in an incubator at a suitable temperature (e.g., overnight at 37°C for bacteria, 3–6 days at 30°C for yeast), a few colonies (clones) of survivors appear. These can be proved to be genetically resistant; they have DNA mutations that confer resistance. To many early microbiologists, this adaptive capacity of microorganisms seemed too rapid to be explained by Darwinian processes. Microbiologist Salvador Luria called bacteriology “the last stronghold of Lamarckism” (we’ll explain what he meant by “Lamarckism” below). In 1944, Luria and physicist-turned-molecular biologist Max Delbrück addressed the question of the origin of microbial mutants with a beautiful experiment, the Luria-Delbrück fluctuation test. We will perform and analyze a version of the L-D experiment in yeast.

Do microbial mutations arise adaptively or pre-adaptively?

Charles Darwin in 1854  Jean-Baptiste Lamarck (1744-1829)

Luria and Delbrück sought to distinguish two opposing theories of the origin of microbial mutations: adaptive vs pre-adaptive. We’ll explain the theories below. First, though, a warning. We’re going to adopt Luria’s and Delbrück’s rhetoric in calling the adaptive theory “Lamarckian” and the pre-adaptive theory “Darwinian.” In doing so, we’re guilty of a type of misrepresentation that historians call “Whig history.” WH is the distortion of the actual historical record (untidy in the extreme) in order to fit a tidy narrative of steady progress to our current state of enlightenment. Professor André Ariew of the Philosophy Department will lead a lecture later in the semester in which Darwin and Lamarck’s actual views are considered more
seriously. Until then, though, we’re going to accept the WH identification of the adaptive theory with Lamarck and the pre-adaptive theory with Darwin.

Darwin’s theory of evolution by natural selection differed from older theories of evolution, such as that of Lamarck, in the way it explained the origin of mutation. Both Darwin and Lamarck believed that organisms adapted to their environment by acquiring favorable characteristics. However, Lamarck thought that these favorable traits were acquired in direct response to the environment, and then inherited by subsequent generations. That is, the mutations arose as an adaptation to the challenges of the environment. Darwin proposed that mutations occurred by chance, before exposure to the selective environment. Such mutations are pre-adaptive in that they arise before, and without regard to, any adaptive advantage they may confer. Many of these chance mutations decrease the organism’s fitness, many have no effect on fitness, and some (usually a tiny minority) happen by increase to increase fitness. Those rare mutants carrying a fitness-increasing mutation naturally tend to increase in the overall population. This leads to the impression that the species as a whole progressively adapts to the environment, even though the underlying mutation process is random, not progressive.

In the case of microorganisms acquiring antibiotic resistance, “Lamarck” would have said that the fitness challenge imposed by the drug caused the adaptive mutations to occur. “Darwin” would have said that the mutations had already occurred in a minority of cells within the populations, and were subsequently selected by the antibiotic, which killed the cells that weren’t resistant (the vast majority of the cells in the population) and allowed only the rare resistant mutants to grow and form colonies.

You can see that this theory might be tricky to test. Antibiotic-resistant microorganisms can only be observed by exposing the cells to the antibiotic, so how can you tell if the mutations occurred before or after that exposure?

Canavanine resistance in yeast

Yeast are naturally sensitive to the antibiotic canavanine under some circumstances. That is, in some growth media, yeast will not grow or form colonies in the presence of canavanine. However, rare mutations can arise that allow yeast to form colonies in the presence of the antibiotic. You’ll learn more about the nature of these mutations later in the semester. For the time being, all we need to know is that for the strain of yeast we’ll use in this lab, there are two classes of canavanine-resistant mutant: one that gives red colonies and one that gives normal white colonies on a certain nutrient medium. We’ll focus on the red mutants.

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1 Darwin knew nothing about genetics, and certainly wasn’t familiar with the concept of a mutation. He talked about “variations,” and postulated that some variations were partly heritable. The identification of heritable variation with genetic mutation wouldn’t come until well into the twentieth century.

2 Lest you think this is a dusty, antique controversy it was revived in 1988 by renowned geneticist John Cairns, who claimed to have observed mutations in bacteria which were directed by the growth environment. Cairns’s paper generated lively controversy and a fruitful line of research which uncovered new genetic mechanisms. By 2001, the Cairns controversy had been resolved in favor of Darwin, but with an enriched understanding of bacterial adaptation to stress.
One more piece of information: the red mutants have a “loss-of-function” mutation in a particular gene (you’ll learn about the gene and its function later in the semester). Any mutation that disables (knocks out the function of) that gene gives rise to the red canavanine-resistant phenotype. Loss-of-functions mutations contrast with “gain-of-function” mutations, which are mutations that give rise to a new gene function.

An example of a loss-of-function mutation is the one that causes the Lesch-Nyhan syndrome in boys. These boys have a disabling (i.e., loss-of-function) mutation in their HPRT gene, so they don’t make any functional HPRT enzyme. That enzyme defect causes a heartbreaking suite of mental and behavioral malfunctions (the exact mechanisms by which the enzyme defect leads to the mental and behavioral malfunctions aren’t known). Many dozens of different HPRT mutations can cause the L-N syndrome. That’s not a surprise: there are an enormous number of ways of disabling a gene or the enzyme it’s responsible for. The multiplicity of different loss-of-function mutations also means that they’re relatively frequent.

An example of a gain-of-function mutation would be reversion of a loss-of-function HPRT mutation. Such a back-mutation would reverse or compensate for the original loss-of-function mutation, thus restoring HPRT enzyme function. It’s not a surprise that gain-of-functions mutations are generally extremely rare compared to loss-of-function mutations in the same gene. That’s because there are countless ways of disabling a functional gene, but only one or a few ways of restoring function to any particular disabled gene.

**Materials for the experiment**

- Our starting yeast strain, named SJR1921, was “streaked” on a nutrient agar petri dish without the antibiotic to isolate single colonies ([http://en.wikipedia.org/wiki/Streaking_(microbiology)]). Such media are called “non-selective” since they don’t select in favor of the mutants: both mutant and non-mutant yeast grow to create colonies.
- Non-selective liquid yeast media for growth of the yeast cells; again, both mutant and non-mutant yeast cells grow.
- Selective and non-selective nutrient agar petri dishes. The selective dishes have canavanine, so only mutants will grow to create colonies. The non-selective dishes have no canavanine, so both mutant and non-mutant cells will grow to create colonies.
- Sterile 13-ml screw-cap tubes (Sarstedt 60.541.21) labeled with team numbers (7 for each team); these will be used for small (60-µl) cultures.
- Sterile 1 × 3.5 inch culture tubes for “bulk” (2.58-ml) cultures

**Preparation for the Proseminar Lab I**

(done by the staff before the proseminar)

1. Just before the lab class, the staff used sterile disposable plastic inoculating loops (see picture below) to pick up five well-separated yeast colonies (colonies A–E) and suspend them in 100 µl of sterile non-selective liquid medium in sterile 500-µl microtubes (picture below); the microtubes were vortexed vigorously in order break up clumped cells and suspend the cells evenly in the medium; the tubes were allowed to stand for a few minutes to allow remaining
large clumps to settle while single cells remained in suspension; then 50 µl of the supernatants (the liquid above the settled clumped material) were transferred to fresh sterile microtubes.

2. A 15-µl portion of each suspension was pipetted into a counting chamber (see below); the number of cells in a 0.2 mm × 0.2 mm squares (like the one circled in blue) were counted under a microscope; from those counts the number of cells per ml of suspension was estimated for each of the five suspensions (see the problem set below); the estimated cell densities were all in the neighborhood of $5 \times 10^7$ cells/ml.

3 It would have been more accurate to have counted the cells in a number of squares and average the results, as in the problem set below; but we were in a hurry and great accuracy was not required.
PROBLEM SET 1 (DUE 8/25/2010 BEFORE THE PROSEMINAR LAB AT 4:00 PM)

In one of the pilot experiments for this lab, we counted the number of cells in a colony suspension in three 0.2 mm × 0.2 mm squares; the counts were 136, 93 and 80. On the basis of these numbers, estimate the number of cells/ml in the colony suspension. Some facts to take into consideration in making your calculation:

- The sample depth (space between the bottom of the cover glass and the ruled surface of the counting chamber) is 0.1 mm
- $1 \text{ mm}^3 = 1 \mu\text{l} = 0.001 \text{ ml}$

You might also ask yourself this question: what is the volume in mm$^3$ under one of the 0.2 mm × 0.2 mm squares?

Write your estimate of the cell concentration with a clear explanation of how you arrived at it, using Microsoft Word or any other word processor; save it as an Adobe pdf file (if you don’t know how to do this, ask Sam Richards or me (999-1829 any time)), using the following document naming convention:

```
Lastname@ProblemSet#.pdf (NO SPACES!!), where:
```

Lastname = your last name (capitalized)
@ = the initial of your first name (capitalized)
# = number of the Problem Set (1 in this case)
So let’s say your name is Aloysius McGillacuddy and you’re handing in the first problem set; the name of your document must be:

McGillacuddyAProblemSet1.pdf

with no spaces. Attach that document to an e-mail to me at smithgp@missouri.edu. Demanding that you abide by the above naming convention is not arbitrary tyranny on my part. It means I can download your documents automatically instead of having to hunt for them individually in my e-mail in-box. (The e-mails themselves will be deleted without being read, so if you need to communicate with me send another e-mail without an attached problem set document.) If you use the wrong name for your document (e.g., inserting spaces as in McGillacuddy A Problem Set 1.pdf), I won’t even know you sent it, and you’ll miss the deadline.

3. On the basis of the counts previous step, a portion of each suspension (~30 µl) was diluted in 15 ml non-selective medium to make a $10^5$-cell/ml suspension.

4. Four 3-ml portions of each diluted suspension were pipette into four sterile 1 × 3.5 inch culture tubes labeled with team numbers as in the table below; one set of ten culture tubes was used in the Proseminar Lab; the other set of ten was kept in reserve.

<table>
<thead>
<tr>
<th>Colony</th>
<th>Culture tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Two Team 1 and two Team 2 culture tubes</td>
</tr>
<tr>
<td>B</td>
<td>Two Team 3 and two Team 4 culture tubes</td>
</tr>
<tr>
<td>C</td>
<td>Two Team 5 and two Team 6 culture tubes</td>
</tr>
<tr>
<td>D</td>
<td>Two Team 7 and two Team 8 culture tubes</td>
</tr>
<tr>
<td>E</td>
<td>Two Team 9 and two Team 10 culture tubes</td>
</tr>
</tbody>
</table>
Instructions for Proseminar Lab I (125 LSC, 4:00–5:40 pm, 8/25/2010)

(THESE PAGE WILL BE PRINTED FOR YOU)

5. Each student team will be supplied with:

- A 200-µl pipetter
- Sterile yellow tips for the 200-µl pipetter
- Seven sterile 13-ml screw-cap tubes labeled with your team number in a 72-hole rack
- One sterile 1 × 3.5 inch culture tube labeled with your team number, containing 3 ml of a 10^5-cell/ml colony suspension from step 4 above; in a red rack (1 red rack for each quartet of four students)

NOTE: You’ll receive instructions on sterile pipetting and other aspects of sterile technique.

6. Remove the screw caps of your 13-ml tubes and place them facing up on the benchtop (putting them face-down on the filthy benchtop invites contamination; contamination falling into a face-up cap from the air is possible but much less likely); vortex your team’s 1 × 3.5 inch culture tube (with the colony suspension) to ensure the cells are thoroughly suspended; open the cap of the culture tube and place it facing up on the bench; immediately pipette 60 µl of the suspension steriley into each of the 7 13-ml tubes; try to deliver the 60 µl to the bottom of the tube, but don’t worry if you miss; screw the caps on tight (the goal here is to seal the tube so the tiny 60-µl cultures don’t evaporate during their 5 days in the incubator).

7. Take your 7 13-ml tubes (with the 60-µl portions of the colony suspension) and your one 1 × 3.5 inch culture tube (with the remainder—theoretically 2.58 ml—of the colony suspension) to the shaker incubator and put them in the appropriate holes. We will call the larger (2.58-ml) cultures “bulk” cultures. The incubator will shake the cultures vigorously at 30ºC (optimal growth temperature for yeast) until the proseminar lab meets again 5 days from now.

8. The staff will set up two counting chambers with yeast colony suspensions; you will examine them in microscopes at 400× magnification. Each student will count the number of cells in one of the 0.2 mm × 0.2 mm squares and write his or her count in the appropriate square in the grid pattern next to the microscope. This will give you an idea of what yeast cells look like under the microscope; you should be able to estimate the diameter of a yeast cell. Here is a table of the counts:

<table>
<thead>
<tr>
<th></th>
<th>19</th>
<th>17</th>
<th>8</th>
<th>10</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>18</td>
<td>12</td>
<td>11</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>19</td>
<td>10</td>
<td>14</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>20</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4 Sealing the tube will also cut off the supply of oxygen from the air. However, there’s enough oxygen in a sealed 13-ml tube to allow complete oxidation of all the nutrients in 60 µl of non-selective medium.
What will happen during the 5-day incubation?

Each 60-µl culture starts with 6000 yeast cells (and each bulk culture starts with 2.58 \times 10^5 yeast cells). During the 5-day incubation, the yeast cells grow by repeated cell divisions until the cell density is so high or the remaining nutrients in the medium are so depleted that the cells can’t divide any more. That state, when the cell density no longer increases, is called “stationary phase.” For our cultures, the cell density at stationary phase will be about 3 \times 10^8 cells/ml. So each 60-µl culture will have about 1.8 \times 10^7 cells (and each bulk culture about 7.74 \times 10^8 cells)—a 3000-fold increase in cell number.

Cell growth (increase in cell density) is the obvious consequence of incubation, but if “Darwin” (as defined above) is right, there’s another, hidden consequence as well: accumulation of random canavanine resistance mutations. The starting 60-µl cultures, having only ~6000 cells, have a very small chance of having any canavanine-resistant mutants; but the fully grown (stationary-phase) 60-µl cultures, having ~1.8 \times 10^7 cells, have a much higher probability of having some canavanine-resistant mutants. Of course we can’t see these mutants: they look just like the non-mutant yeast cells. But we’ll be able to count them nonetheless by spreading the cultures on an agar dish with selective (canavanine-containing) medium and incubating the petri dishes for several days at 30ºC. Although you’d be inoculating each petri dish with a vast number of cells (~1.8 \times 10^7), most of those cells will be killed by the canavanine and won’t form colonies. Only the rare canavanine-resistant mutants will grow to form colonies. By counting those colonies, you’ll in effect be counting the number of canavanine-resistant mutants in each stationary-phase culture. That’s what you’ll be doing in the next proseminar lab next Monday.

But let’s now consider the cultures from “Lamarck’s” point of view. “Lamarck” and “Darwin” agree that the yeast cells will grow in number (~3000 fold, as explained in the previous paragraph). But “Lamarck,” unlike “Darwin,” doesn’t think that any canavanine-resistance mutations will accumulate in those cultures. That’s because “Lamarck” thinks such mutations arise adaptively: that is, only when there’s a biological “need” for them. He’d predict that no canavanine-resistance mutations would arise before the cells actually encountered canavanine. It’s the yeast cells’ struggle to adapt to the new environment—an environment that includes a poison they’ve never encountered before (i.e., canavanine)—that results in the canavanine-resistance mutations. That encounter won’t occur until the cultures are spread on the selective petri dishes. Only a few of the cells will manage to adapt successfully, according to “Lamarck”: both he and “Darwin” would agree that canavanine-resistant mutants will be rare. So you may wonder: how are we going to distinguish one theory from the other?? Stay tuned!!

Preparation for Proseminar Lab II

9. Selective agar dishes (with nutrient medium containing canavanine) will be pre-labeled with small rectangular labels as follows:

- 70 dishes will be labeled (with a red vertical stripe):
  Team 1 [or 2 or 3 or…or 10]
  0.06-ml
Dish 1 [or 2 or 3 or…or 7]

➢ For each team, five dishes will be labeled (with a blue vertical stripe):

Team 1 [or 2 or 3 or…or 10]

Bulk culture

10. For each team (1–10), five sterile 13-ml screw-cap tubes will be labeled with rectangular labels as follows with a blue vertical stripe:

Team 1 [or 2 or 3 or…or 10]

Bulk culture

11. For each team, a 15-ml bottle of water will be autoclaved and labeled “Sterile water”; this will be used to dilute your cultures prior to spreading them on the selective petri dishes.

12. For each team, a glass 13×100 mm tube labeled “diluted culture” containing 3 ml medium; and another labeled “reference” containing 4.5 ml of medium; these tubes will be used to quantifying turbidity of your bulk cultures in the spectrophotometer.

13. A few hours before lab, 600-µl portions of the bulk cultures from step 7 will be pipette into labeled 1.5-ml microtubes.

14. The remainders of the bulk cultures (theoretically 2.4 ml remaining of each, but in practice probably somewhat less) are poured into a tared (pre-weighed) beaker, and the net weight in grams = volume in ml is noted (turned out to be 16.13 ml); the pooled culture is diluted with sufficient additional medium to bring the total volume to 52 ml.

15. In a 500-µl microtube, 45 µl medium and 5 µl of the pooled, diluted culture previous step are mixed, thus making a further 1/10 dilution of the pooled, diluted culture; 15 µl of the 1/10 dilution is counted in the counting chamber (see step 8); the counts per 0.2 × 0.2 mm square were 30, 33, 33, 20, 52, 32, 49 and 30.

16. Various further dilutions of the pooled, diluted culture step 14 were made in 13 × 100 mm glass tubes by mixing various volumes of step 14 with various volumes of medium as specified in the table below; these dilutions were analyzed with the spectrophotometer (as you’ll learn how in the lab), with the results shown in the table.
<table>
<thead>
<tr>
<th>Volume of medium (ml)</th>
<th>Volume of step-14 dilution (ml)</th>
<th>Cells/ml in the dilution</th>
<th>Percent transmission at 600 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.75</td>
<td>0</td>
<td>0</td>
<td>100 (by definition)</td>
</tr>
<tr>
<td>3.6</td>
<td>0.15</td>
<td></td>
<td>78.5</td>
</tr>
<tr>
<td>3.45</td>
<td>0.3</td>
<td></td>
<td>60.1</td>
</tr>
<tr>
<td>3.3</td>
<td>0.45</td>
<td></td>
<td>47.0</td>
</tr>
<tr>
<td>3.15</td>
<td>0.6</td>
<td></td>
<td>37.4</td>
</tr>
<tr>
<td>3</td>
<td>0.75</td>
<td></td>
<td>31.4</td>
</tr>
<tr>
<td>2.85</td>
<td>0.9</td>
<td></td>
<td>26.0</td>
</tr>
<tr>
<td>2.7</td>
<td>1.05</td>
<td></td>
<td>22.0</td>
</tr>
<tr>
<td>2.55</td>
<td>1.2</td>
<td></td>
<td>18.9</td>
</tr>
<tr>
<td>2.4</td>
<td>1.35</td>
<td></td>
<td>15.9</td>
</tr>
<tr>
<td>2.25</td>
<td>1.5</td>
<td></td>
<td>14.0</td>
</tr>
<tr>
<td>2.1</td>
<td>1.65</td>
<td></td>
<td>12.4</td>
</tr>
<tr>
<td>1.95</td>
<td>1.8</td>
<td></td>
<td>11.1</td>
</tr>
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<td></td>
<td>10.2</td>
</tr>
<tr>
<td>1.65</td>
<td>2.1</td>
<td></td>
<td>9.0</td>
</tr>
<tr>
<td>1.5</td>
<td>2.25</td>
<td></td>
<td>8.4</td>
</tr>
<tr>
<td>1.35</td>
<td>2.4</td>
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<td>1.2</td>
<td>2.55</td>
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<tr>
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<td>2.7</td>
<td></td>
<td>6.8</td>
</tr>
<tr>
<td>0.9</td>
<td>2.85</td>
<td></td>
<td>6.2</td>
</tr>
<tr>
<td>0.75</td>
<td>3</td>
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</tr>
<tr>
<td>0.6</td>
<td>3.15</td>
<td></td>
<td>5.2</td>
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<tr>
<td>0.45</td>
<td>3.3</td>
<td></td>
<td>5.0</td>
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<tr>
<td>0.3</td>
<td>3.45</td>
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<td>4.9</td>
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<tr>
<td>0.15</td>
<td>3.6</td>
<td></td>
<td>4.7</td>
</tr>
<tr>
<td>0</td>
<td>3.75</td>
<td></td>
<td>4.2</td>
</tr>
</tbody>
</table>

Fill in the third column of the table from the information given in this step; at step 19, you’ll use these results as a calibration standard to determine the cell density in your stationary phase bulk cultures.
Instructions for Proseminar Lab II (125 LSC, 4:20–6:00 pm, 8/30/2010)  
(PAGES 10–13 WILL BE PRINTED FOR YOU)

17. Each student team will be supplied with:

- A 100-µl pipetter
- A 1-ml pipetter
- Sterile yellow tips for the 100-µl pipetter
- Sterile blue tips for the 1-ml pipetter
- A sterile 1.5-ml microtube with a 600-µl portion of your bulk culture from step 13
- Seven sterile 13-ml screw-cap tubes with your 60-µl cultures from step 7 (labels have a red vertical stripe)
- Five empty sterile 13-ml screw-cap tubes from step 10 (labels have a blue vertical stripe)
- Two glass 13×100 mm tubes containing 3 and 4.5 ml medium
- A 15-ml bottle of sterile water from step 11
- Seven petri dishes labeled with a red vertical stripe

Team 1 [or 2 or 3 or... or 10]
0.06-ml
Dish 1 [or 2 or 3 or... or 7]

- Five petri dishes labeled with a blue vertical stripe:

Team 1 [or 2 or 3 or... or 10]
Bulk
culture

- 12 individually wrapped sterile blue plastic spreaders
- A beaker for discarding used pipette tips, tubes and spreaders

18. NOTE: This step requires sterile technique. Spread cultures on all 12 of your petri dishes as follows:

- Remove the caps from all 12 of your 13-ml screw-cap tubes and discard them in the discard beaker
- Vortex your team’s bulk culture in the 1.5-ml microtube (see previous step) and open the cap
- Without delay, use the 100-µl pipetter to steriley pipette 60 µl of the bulk culture into each of the five 13-ml bulk culture tubes (with blue vertical stripes on the label)
- Use the 1-ml pipetter to steriley pipette 160 µl of sterile water (in the 15-ml bottle) into all 12 13-ml tubes
- Spread the contents of each of the 12 13-ml tubes on the petri dishes as follows, being sure that tubes labeled with a red vertical stripe are spread on dishes labeled with a red vertical stripe, and tubes labeled with a blue vertical stripe are spread on dishes labeled with a blue vertical stripe:
  - Vortex the 13-ml tube
Pour the contents of the tube onto the dish, thumping the heel of your hand onto the bench to knock out all the liquid onto the agar surface.

Use a blue plastic spreader to spread the liquid evenly over the surface of the dish.

Leave the dishes on the bench to dry; they will be put into a 30º incubator after lab; canavanine-resistant colonies will grow and red color will develop; after three days in the 30º incubator, the dishes will be incubated at room temperature for two days in order to allow the red color to develop. Moldy contaminants typically grow on a few of the dishes; those dishes will be counted and discarded to minimize contamination of the remaining dishes; meanwhile, the uncontaminated dishes will be refrigerated upside down until they are examined in the next Proseminar Lab; ten of those dishes will be counted and set aside as the source of colonies to be sequenced starting in the next Proseminar Lab.

19. Re-vortex your team’s bulk culture in the 1.5-ml microtube; use the 1-ml pipetter to pipette 130 µl of the bulk culture into the glass 13×100 mm tube labeled “diluted culture” (already contains 3 ml medium), thus making a 1/24 dilution; if you compare the two tubes, you’ll see that the reference tube (medium with no yeast cells) is clear while the diluted culture tube is turbid (cloudy). The turbidity arises because the yeast cells suspended in the medium scatter light. If a light beam is shone through the reference tube, it passes through the empty medium largely unimpeded and emerges at almost full strength through the other side of the tube. We’ll define the intensity of light that emerges at the other side of the reference tube as 100% transmission. In contrast, if the same light beam is shone through the diluted culture tube, much of the light is lost to scattering in all different directions, and not all the light makes it all the way to the other side of the tube; the transmission through the tube is less than 100%. The higher the concentration of yeast, the lower the percent transmission; percent transmission is thus an indirect measurement of yeast cell concentration. By comparing the percent transmission for a sample with unknown cell concentration (the sample in your diluted culture tube) with the percent transmission from a graded series of samples with known cell concentrations (data at step 16), it’s possible to convert the percent transmission measurement to an estimate of actual cell concentration.

The spectrophotometer (see schematic diagram above) is designed to measure percent transmission of a light beam through samples in 13 × 100 mm glass tubes. In the diagram above, we’re looking downward into a tube that’s been inserted into the instrument’s sample well. In
our experiment, the samples in the tube will be suspensions of yeast in medium; the black specks are meant to represent the suspended yeast. A beam of light with a single wavelength (we’ll use 600 nm) is shone through the tube from one side (from the left in the diagram), and the light that emerges at the other side of the tube (the right in the diagram) hits a photodetector, which produces an electrical signal that’s proportional to the intensity of the light beam (i.e., the number of photons hitting the photodetector per second). The electrical signal is amplified, and the amplified signal drives the transmission meter on the front of the instrument. Beside the wavelength adjustment, there are two controls, controlling the “dark current” and the amplifier’s gain. Here’s how the controls are used:

- **Dark current control**: When there’s no tube in the sample well, a shutter falls into place, blocking the light beam so that no light reaches the photodetector. Theoretically the photodetector doesn’t send out any electrical signal in these circumstances, but in practice there’s a very small “dark current” even when there’s no light at all. The dark current control allows the user to offset this small dark current, so that no signal reaches the amplifier when there’s no light getting to the photodetector. How does the user accomplish this? Easy! Just turn the dark current knob (the one on the left-hand side on the front of the instrument) until the transmission meter reads 0% transmission (left-most tick on the black percent transmission scale). Don’t touch the dark current control once it has been properly adjusted.

- **Gain control**: Now put the reference tube (with medium containing no yeast) into the sample well. The shutter moves out of the way, so that the light that passes through the tube can reach the photodetector. Since there are no yeast in the reference tube to scatter light (i.e., the solution isn’t turbid), this is the maximum light that’s going to get through the tube—i.e., 100% transmission. Accordingly, adjust the gain control (the knob on the right-hand side of the front of the instrument) until the transmission meter reads 100% transmission (right-most tick on the black percent transmission scale). Don’t touch the gain control (or the dark current control) until you’ve made your measurement.

- **Measuring percent transmission**: Remove the reference tube from the sample well; vortex your diluted culture tube gently (if you vortex too vigorously, your sample will splatter out of the tube); insert the just-vortexed tube into the spectrophotometer; record the percent transmission on the transmission meter.

- By comparing your observed transmission with the table at step 16, estimate the number of cells/ml in your undiluted bulk culture.
PROBLEM SET II (DUE FRIDAY 9/3/2010 BY 5:00 PM)

A. From the information provided in steps 15–16, fill in the cell concentrations in the third column of the table at step 16, thus completing the table.

B. Copy the data in the third and fourth columns of the completed table to Excel; use Excel to graph Percent transmission (y axis; fourth column in the table) as a function of cell density (x axis; third column in the table). The best kind of Excel graph to insert for this purpose is a scatter diagram. Here’s an example of a scatter graph from last year’s MLS lab (the cell concentration numbers aren’t anything like yours, since they were analyzing a bacterial culture, not a yeast culture). If you need help with Excel, ask Sam Richards or me.

All you have to submit for this problem set is your Excel file; no need for a written explanation. Name the Excel document according to the following convention:

Lastname@ProblemSet2.xls (or .xlsx) (NO SPACES!!!), where:

Lastname = your last name (capitalized)
@ = the initial of your first name (capitalized)
Sequencing the mutant gene in red mutant clones

As you’ll see over the course of the semester, it will be instructive in many ways to sequence the mutant gene in a sampling of the red canavanine-resistant mutant colonies from the selective petri dishes from step 18. We will do so in two stages:

- First, we’ll use the polymerase chain reaction (PCR) to specifically amplify a tiny (350-bp) segment of the yeast genome (12.5 million base pairs altogether) that contains the mutant gene. The input to PCR—the “template” in PCR parlance—will be a tiny, very crude sample of genomic DNA obtained just by extracting a bit of colony. At most this template will have a million copies of the yeast genome, but in practice probably more like 10,000 or 100,000 copies; the number isn’t important, and neither is purity. The output—the “PCR product”—will be a large amount (a few µg) of nearly pure 350-bp fragment. That’s about 10 trillion copies of the fragment! So PCR amplifies one tiny 350-bp segment of the genome by a factor of at least 10 million (and probably more like 100 million or a billion), while leaving the remaining 12.5 million base pairs of the genome unamplified. You’ll learn the logic of PCR’s magic in this introduction. We’ll prepare the 350-bp PCR product in this way from 24 mutant colonies.

- Second, we will use the chain termination method to determine the nucleotide sequence of one strand of each PCR product. More accurately, we’ll prepare samples of the PCR products and submit them to Mizzou’s DNA Core facility, which will do the chain termination reactions for us and return the sequencing results as computer files.

Structure of double-stranded DNA

Please read the Wikipedia article on DNA at [http://en.wikipedia.org/wiki/DNA](http://en.wikipedia.org/wiki/DNA). As you’ll learn there (or undoubtedly know already), natural double-stranded DNA molecules contain two strands that are complementary to each other. The nucleotides in each strand are strung together like beads on a string, and are held together by very strong covalent bonds. Each DNA strand has two different kinds of ends and thus a natural “direction” or polarity. The two kinds of end are called 5´ and 3´.

In contrast to the strong covalent bonds that link the nucleotides within a single strand, the two complementary strands of double-stranded DNA are held together by weak, non-covalent bonds. The double-stranded complex is nevertheless reasonably stable because there are thousands to billions of such weak bonds in a single double-stranded molecule. The weak bonds can form only when a base in one strand is matched in a specific geometric arrangement with the complementary base in the opposite strand. The two properly-positioned bases form a non-covalent complex that is called a base pair, and are said to “base-pair” with each other. This geometry requires that the two strands lie against each other with opposite polarity and be

---

5 “bp” is the abbreviation for base pair (= nucleotide pair). Normal, double-stranded DNA contains two complementary strands with the same number of bases each. So it’s logical to gauge the length (= size) of a DNA molecule in terms of it number of base pairs. Somewhat illogically, the bp abbreviation is extended to mean just base when we’re considering the length (size) of a single-stranded DNA molecule. Kb or Kbp means kilobase (pair), Mb or Mbp means megabase (pair), Gb or Gbp means gigabase (pair), etc. The human genome has about 3 Gb of DNA; the yeast genome has 12.5 Mb (= 0.0125 Gb).
twisted around each other to form a right-handed double-helix. The two strands in the helical complex, like the individual base-pairs, are said to “base-pair” with each other. By extension, formation of the double helix from two complementary DNA strands is called base-pairing; it’s also called “annealing” and “hybridization.” The excellent animation in the Wikipedia article shows the basic double helix structure very clearly.

Two complementary strands that are base paired with each other can be separated by “denaturing” the double helix. Denaturation breaks the non-covalent base-pairing bonds that hold the helix together, but doesn’t break the covalent bonds that keep the single strands intact. Denaturation is most often accomplished experimentally simply by heating a solution of DNA to 94–98°C. Because heat is so often used to denature DNA, denaturation is often called “melting”—even when denaturation is not accomplished by heating. The two DNA strands that are separated by denaturation are “separated” only at the molecular level. They remain dissolved together in a single solution. If, say, a solution of a million identical double-stranded DNA molecules is denatured, the result is a solution with a million identical “plus” strands and a million identical “minus” strands all mixed together randomly in a single solution. Under denaturing conditions (e.g., at 94–98°C), the two strands can’t base-pair with each other to make a stable double helix. If the solution is then returned to non-denaturing conditions (e.g., if the temperature is cooled to, say, 65°C), complementary “plus” and “minus” strands can now form stable helices, and eventually the solution consists entirely of re-formed double-stranded helices.

**Representing the DNA structure**

In most cases, it is the nucleotide sequence of DNA molecules that’s of primary importance. It’s in the order of the As, Cs, Gs and Ts that its information content lies. So consider the following three representations of a simple 6-bp DNA sequence

\[
\begin{align*}
5' & \rightarrow ACTTGA \rightarrow 3' \\
\text{ACTTGA} \\
5' & \rightarrow ACTTGA \rightarrow 3' \\
3' & \rightarrow TGAACT \rightarrow 5'
\end{align*}
\]

In the first, just one strand is represented, the other being implied in context. The 5’→3’ polarity of the strand is explicitly represented. The second representation is like the first except that polarity is understood, not explicitly represented. Unless otherwise specified, the polarity of a string of letters representing nucleotides is assumed to be written with the 5’→3’ polarity going from left to right. In the third representation, the two strands of the double helix are explicitly represented. Polarity must be explicitly represented here, since the “bottom” strand is written with the 5’→3’ going from right to left.
If we’re talking about DNA structure generically without regard to its information content, we often use an arrowed line to represent each single strand. The arrowhead end of the line is the 3´ end, while the feather end (the feathers almost never being included) is the 5´ end. The arrowed line “points” in the 5´→3´ direction. The following represent single strands and double-stranded helices, either with or without explicit designation of 5´→3´ polarity:

\[ \text{DNA polymerases} \]

Both PCR and DNA sequencing depend critically on the properties of DNA polymerases. All known DNA polymerases have a single function: they add a new nucleotide to the 3´ end of a “primer” strand that’s base-paired with a template strand:

A simple job, but many DNA polymerases, especially those that are responsible for replicating our chromosomes, perform it extraordinarily well: they make a phenomenally small number of mistakes. A mistake would be when a polymerase adds a nucleotide that is not complementary to the opposite base on the template strand.

DNA polymerases use deoxynucleoside triphosphates (dNTPs) as the nucleotide monomers. As each new nucleotide is added to the growing primer strand, two of the phosphates are cleaved from the triphosphate, the remaining one becoming part of the growing primer strand. The bonds that link the chain of three phosphates together are high-energy bonds; it is the breaking of these high-energy bonds that provides the chemical energy that drives DNA polymerization.

When a DNA polymerase completes its one job, it can’t just go home and watch TV. That’s because the structure it thus creates is again a primed template (that’s what we call a primer strand base-paired with a template strand). So it goes ahead and adds another nucleotide. Then another. Then another. Etc. Only when it gets to the end of the template can it stop:
At that point, the enzyme has “copied” all the information in the template strand into the newly-synthesized primer strand. The DNA sequence in the primer-strand “copy” is the complement of the DNA sequence in the template-strand “original,” but the information content is the same.

Many DNA polymerases can be tricked into incorporating fake nucleotides into growing primer strands, a feature that’s exploited in DNA sequencing by the chain termination method (see below). Chain terminators are artificial nucleotides that can be added to the 3’ end of the primer strand, but that don’t themselves have an intact 3’ end that a new nucleotide can be added to. When DNA polymerase adds an artificial chain-terminating nucleotide to the 3’ end of a primer strand, therefore, all further elongation of that primer strand ceases.

In some primed templates, each strand acts both as primer and as template. The following primed template would be copied by two DNA polymerase molecules working in opposite directions:

![Diagram of DNA replication]

**Thermophilic DNA polymerases and thermocycling**

The DNA polymerases used in PCR and sequencing are “thermophilic” enzymes obtained from bacteria or archaeae that live in very hot environments. The “extremophile” *Pyrococcus furiosus*, for example, grows optimally at boiling temperature. It was first isolated from a geothermally heated marine sediment. Use of such DNA polymerases permits thermocycling, in which template strands, a large excess of primer strands, the DNA polymerase and deoxynucleoside triphosphate monomers are subject to dozens of three-stage temperature cycles:

- Denaturation stage: mixture is heated to 94–98°C to denature all double helices, so that all the single strands are unpaired; ordinary (mesophilic) DNA polymerases don’t survive such temperatures, but thermophilic DNA polymerases do, especially polymerases from extremophiles like *P. furiosus*.
- Priming stage: mixture is cooled to a temperature that’s optimal for primer strands to base-pair with template strands.
- Extension stage: mixture is heated to the optimal temperature for DNA polymerase to do its thing: add nucleotides to the 3’ end of template-paired primer strands until the ends of the template strands are reached.
PCR

Please read the excellent Wikipedia article at http://en.wikipedia.org/wiki/Polymerase_chain_reaction. Here is the sequence of the unmutated form of the 350-bp PCR fragment we’ll amplify (only one strand is shown):

5’-gggaatgcagctgcgtacgcttggaatgctgctttcagttacctttgagatccggacgggataattatttgaaatctctttttcaattgtatatgtatgtatctctctttcttttcacacaatataatctcctcggtatccaaatggccggttttatggtttaagggcagacttttaattatcaactacaagatcgcgggttcgactcgccccggagaatttttttgtttttttatgtctccatctcactctccccagacttgcaagttggaattttttttttttttcaagaaattgggctcatcctttgtgactgaagcagggcctcttttgaccggcagggctttctatagcc-3’

The PCR primers will 20 bases long. If you understand PCR, you’ll be able to write the sequences of the two 20-base primers, including their 5’→3’ polarity.

DNA sequencing by the chain-termination method

Please read the Chain-termination methods section of the Wikipedia article at http://en.wikipedia.org/wiki/DNA_sequencing. The DNA Core will sequence your 24 samples using the Dye-terminator sequencing method, using one of the 20-base PCR primers as the sequencing primer. They will return colored traces like the one pictured in the figure titled “View of the start of an example dye-terminator read.”

Preparation for Proseminar Lab III

20. Label two sets of 24 color-coded 200-µl PCR tubes with Team and Dish numbers (see below)

Team@/Dish#

One set of labeled PCR tubes will be left empty for use in the PCR amplifications themselves. Into the other set of PCR tubes pipette 30 µl of 0.2% SDS. SDS is a powerful detergent; heating a suspended yeast colony to 94ºC in this detergent will break open the yeast cells and the nuclei inside them, thus allowing some of their genomic DNA to leak out; small portions of those extracts will serve as the templates for the PCR amplifications. The dishes will be chosen as described at step 18:

- The 8 blue tubes will be for 8 colonies from one “jackpot” dish (Team 1 Dish 6)
- The 8 red tubes will be for 8 colonies from another “jackpot” dish (Team 3 Dish 5)
- The 8 green tubes will be for single colonies from each of 8 “non-jackpot” dishes:
  - Team 2 Dish 5
  - Team 3 Dish 3
  - Team 4 Dish 3
  - Team 5 Dish 5
  - Team 6 Dish 3
  - Team 7 Dish 4
  - Team 8 Dish 2
21. Label 24 color-coded 500-µl microtubes

Team @ Dish & gDNA

Into each pipette 90 µl water; these tubes will contain 1/10 dilutions of genomic DNA (“gDNA” stands for genomic DNA) prepared by centrifuging the heated extracts in the 200-µl PCR tubes (previous step) and transferring 10-µl portions of the supernatants into the 90 µl of water that’s already in the 500-µl microtubes; that genomic DNA is heavily contaminated with countless other molecules extracted from the yeast colonies, but those contaminants won’t interfere with subsequent PCR amplification.

Also label 24 color-coded 1.5-ml microtubes

Team @ Dish & Colony # (# = 1, 2, 3,…, 7 or 8 for blue and red tubes; # = 1 for green tubes) PCR product

These will hold the final PCR products at step 31.

22. Into a glass 4-ml vial on ice pipette the ingredients (other than the templates) for the PCR amplifications:

- 1.8 ml water
- 500 µl 5-fold concentrated PCR buffer
- 50 µl 10-mM dNTPs (an equimolar mixture of the four deoxyribonucleoside triphosphate monomers—dTTP, dATP, dCTP and dGTP—that DNA polymerase adds to the growing primer strand)
- 1.25 µl 1-mM left-hand primer
- 1.25 µl 1-mM right-hand primer
- 25 µl DNA polymerase from an extremophile (2 enzyme units/µl; the enzyme is stored in the deepfreeze, and will be added to the vial just before use)
23. Students will be supplied with:

- Selective petri dishes from step 18 (a few will have already been counted and either discarded because they were contaminated or set aside as explained at step 18)
- Sharpies for counting colonies
- Color-coded, labeled 200-μl PCR tubes, each containing 30 μl 0.2% SDS (step 20)
- Color-coded, labeled 500-μl microtubes, each containing 90 μl water (step 21)
- Color-coded, labeled empty 200-μl PCR tubes for the PCR amplifications (step 21)
- Color-coded, labeled empty 1.5-ml microtubes for storing the final PCR products (step 21; these will be filled by the staff after the Proseminar Lab, not by the students)
- Sterile, disposable white plastic inoculating loops; individually wrapped
- 10-μl pipetters
- Tips for 10-μl pipetters
- Beakers for discarding used inoculating loops, tubes and tips
- Microcentrifuge with adaptors for centrifuging 200-μl PCR tubes
- The pre-mixed PCR ingredients from step 22
- A thermocycler

24. Count the red and white colonies on your petri dishes, using the “Sharpie” method that will be demonstrated to you. The red colonies will be relatively uniform in size and easy to count, while the white colonies will often differ greatly in size and be difficult to count unambiguously. Don’t worry about the size variation. It’s the red colonies that are important in the experiment; it’s OK if a few of your white colony counts are only approximate because there are so many white colonies of widely varying sizes on those dishes. Write the counts in the table below, being careful to get the dish numbers correct; some of the spaces will be blank because the dishes have already been counted and either discarded or set aside, as explained at step 18; at the end of the lab, one table from each team will be handed in so the results from the entire class can be entered in a single spreadsheet to be posted on Blackboard.

<table>
<thead>
<tr>
<th>Team to enter this!!!</th>
<th>Dish</th>
<th>Number of red colonies</th>
<th>Number of white colonies</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
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<td>Be</td>
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<td>Dish 7</td>
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</tbody>
</table>
25. Each team will be assigned particular petri dish(es) and a particular number of clones from each dish. These are the clones whose genes will be sequenced. Use the small (1-µl) loop of a plastic inoculating loop to pick up a bit of red colony (about this much: ●), avoiding picking up any of the agar (if you do accidentally pick up agar, discard the inoculating loop and try with another colony); immerse the loop in the 30 µl of detergent solution in a properly color-coded 200-µl PCR tube; if the colony material sticks to the loop, vortex the tube with the loop held immersed in it; once the colony material is off the loop, discard the loop and close the cap of the PCR tube; vortex the PCR tube vigorously until the cells in the colony are completely suspended.

26. Put the PCR tubes in the Thermocycler; run Program 10, which will heat the tubes to 90º for 4 min.

27. Using the special adaptors (step 21), microfuge the heated PCR tubes for 5 min to pellet insoluble material.

28. Use the 10-µl pipetter with a 10-µl pipette tip to draw up 10 µl of supernatant from each centrifuged PCR tube, being very careful to avoid letting the pipette tip reach the pellet; deliver the 10 µl of supernatant into a properly labeled and color-coded 500-µl microtube (already contains 90 µl water); close the cap of the microtube; vortex it to mix the supernatant in with the water. After removing 5-µl portions of the supernatants for PCR amplifications next step, the 500-µl microtubes will be stored in the deepfreeze as a backup.

29. Pipette 5 µl of each supernatant into the corresponding empty labeled color-coded 200-µl PCR tube, using a different tip for each tube.

30. When all 24 PCR tubes are ready, a 95-µl portion of the PCR ingredient premix (the 4-ml glass vial step 22) will be pipetted into each PCR tube, changing tips with each addition; close the caps of the PCR tubes securely; vortex the PCR tubes; if necessary, shake droplets down to the bottoms of the tubes; put the tubes in the thermocycler and run Program 7, which will commence the repeated cycles of temperature:

- Program 7: 98°C for 30 sec (initial denaturation of DNA)
- Links to Program 8: 35 cycles of
  - 10 sec 98°C melting (denaturation)
  - 30 sec 61°C annealing (primers base-pair with template strands)
  - 20 sec 72°C polymerizing (optimum temperature for DNA polymerase)
- Links to Program 9: 5 min extension at 72°C (any incomplete primer strands are completed)
- Links to Program 5: indefinite soak at 4ºC (final product kept at refrigerator temperature until the tubes are removed)

31. When the temperature cycles are complete a few hours after the Proseminar Lab, the PCR reaction mixtures will be transferred to the corresponding color-coded labeled 1.5-ml microtubes step 21; these will be stored in the deepfreeze for analysis in the next Proseminar Lab.