# You and your oral microflora

The human body is comprised of approximately 75 trillion eukaryotic cells. As you've already learned, eukaryotic cells contain membrane-bound organelles and have a nucleus containing DNA that is separate from the cytoplasm. Throughout the course of the semester you will be learning a lot about how these cells function individually and, when grouped together, how they function as complex tissues. However, these are not the only cells that are present in and on our bodies. Our bodies are also home to prokaryotic cells, cells outnumber our human cells by a factor of 10 [rev. in [1]], and these cells will be the focus of this lab module. Look up further characteristics of prokaryotic and eukaryotic cells and provide at least four similarities and four differences between these two cell types and list them below. Do this before coming to lab so you are prepared for the first lab!

| | Eukaryotic | Prokaryotic |
|---|---|---|
| Similarities | _____ | _____ |
| | _____ | _____ |
| | _____ | _____ |
| | _____ | _____ |
| Differences | _____ | _____ |
| | _____ | _____ |
| | _____ | _____ |
| | _____ | _____ |

Microorganisms were first observed under the microscope by Antony van Leeuwenhoek in 1676 [2]. Using samples of dental plaque from his mouth and the mouths of others, he identified what he called "animalcules", which have since been classified as bacteria [rev. in [3]]. Scientists have determined that the human mouth is home to over 1 million bacteria, comprising at least 600 different species of bacteria [4] [5]. Most of these are part of the "normal flora", and serve a protective function, helping to initiate the digestive process, although some of these have been proposed to play a role in systemic diseases such as cardiovascular disease [6]. Likewise, some of the bacteria that have been shown to cause dental plaque may be present in the human mouth without causing disease. Figure 1 gives an overview of how the bacteria in your mouth may attach to the surface of a tooth, and the order in which they will appear on a recently cleaned tooth. Bacteria referred to as "early colonizers" will attach first to the tooth surface soon after cleaning (within minutes). The presence of these first "early colonizers" will influence the types of bacteria that attach later (hours to days- "late colonizers") [7].

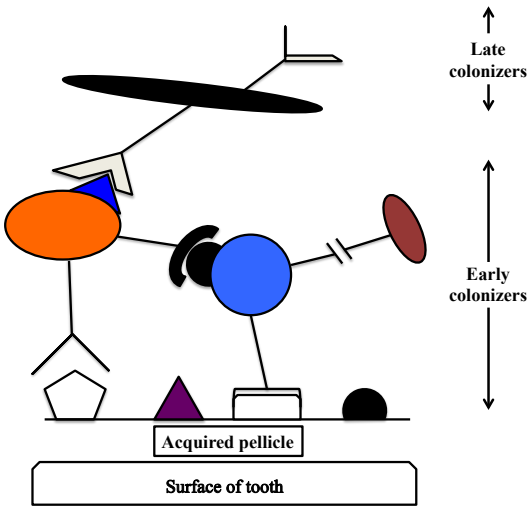



**Figure 1.** Bacteria in the mouth attach to the tooth surface in an ordered manner. Early colonizers (orange, blue and brown shapes) effect which microbes can adhere at later time points. The different shapes represent different bacteria with their unique receptors. The "acquired pellicle" refers to proteins produced in the mouth that attach to the surface of the tooth and allow attachment of bacteria. [adapted from [7]].

To more closely study the microorganisms identified in the mouth (and from other sources), culture-based methods were developed soon after Leeuwenhoek first described his oral flora. "Culture-based" means using a growth medium that allows scientists to grow an organism in the laboratory, typically something that uses agar and nutrient sources to support growth of the organisms. In this lab, we are going to sample our oral microflora to identify differences between bacteria that are "culturable", and to begin to appreciate the diversity of microorganisms present on the surfaces of our bodies.

## SOME IMPORTANT SAFETY INFORMATION BEFORE YOU BEGIN

You will be working with microorganisms in this module. This work should not be harmful to you, but it does require you to use some basic common sense, as well as basic sterile techniques. Some important things to remember:

1) You MUST handle your own specimens at all times! After day 1, you should wear gloves every time you work on this project.

2) Whenever you are opening your plates to work with the bacterial colonies, you will work on a blue laboratory "chuck" (a disposable paper covering). These will be placed into the appropriate containers for proper disposal. Laboratory coats will be available for your use.

3) When extracting DNA, you will need to wear gloves as well as eye protection.

4) At the end of each day, you will need to:
   a) clean up your work space by spraying a 10% bleach solution on your work surface AND
   b) wash your hands well before leaving the lab area.

5) Remember: If at any time you have questions, ask before disposing of anything.

## I. Lab Day One- What's in your mouth?

Have you ever wondered what might be living in your mouth? Have you wondered if there are differences between the different surfaces of your mouth (the tooth enamel versus under the tongue)? Today you will be choosing one or more of the surfaces of your mouth as a source of bacteria to inoculate agar plates. These agar plates are called Luria-Bertani (LB) and they contain a variety of nutrients and minerals to help support the growth of bacteria.

Before you begin, here are some questions for you to think about. Write your answers in the space provided:

1) How many bacteria do you think may be present on your own mouth surfaces?

2) Could you alter the number or types of bacteria present in your mouth? If so, how?

**General protocol for sampling your oral microflora:**

You will be working in pairs throughout this experiment and sharing supplies provided on a tray for two people. However, remember that you will be handling your own specimens.

To sample your oral flora, you will need the following for each student:

4 LB plates
Sharpie
4 sterile swabs (in packets of 2)
1sterile drape

**Safety precaution:**
**ALWAYS REMEMBER TO ONLY HANDLE YOUR OWN SPECIMENS!**

1. Obtain a tray for each pair of students. From the tray, find the blue sterile drape and place on the benchtop surface.

2. Select a sampling site. This could be your teeth, your tongue, your throat (though be careful not to gag), the roof of your mouth; it could be a combination of the two, or it could be one site sampled before and after some manipulation (for example: brushing, flossing, eating, rinsing with mouthwash, drinking a beverage-though not in the lab). Please take 1-2 minutes to think about what sampling sites you want to swab. Record here what surfaces you are sampling, and if you are sampling the same location twice after some manipulation (i.e. your teeth before and after you rinse with mouthwash).

Sampling site 1:_____

Sampling site 2:_____

Sampling site 3:_____

Sampling site 4: _____

3. Obtain four LB plates. Label these plates with your initials, today's date, and the sampling location and treatment strategy (if applicable). It is best to write this information on the outside edge of the bottom of the plate (the part that contains the agar). This is because the lid can easily be moved between plates, but the bottom always stays with the agar. See Figure 2 for details. After labeling the plates, place them lid-side down on the bench in front of you, within easy reach for swabbing. You will be doing much of the work coming up one-handed, so you don't want to have to reach far or fumble around for materials.

4. Obtain four sterile cotton swabs for sampling. These should be individually wrapped, or wrapped in a package of two. Open the "stick" end, the end that does not have the cotton swab on it. If there are two sticks in the package, this allows you to place the unused swab back on the benchtop without losing the sterility of the swab, so long as the swab end stays inside the container. Remember, you'll be putting this swab into your mouth- you don't want it to touch the surface of the lab bench first. Using one swab, sample the area of interest by swiping/rubbing over the surface for at least 15 seconds. Consider the sample site: for hard surfaces you can rub hard, for soft surfaces (such as your tongue or cheeks) you will want to be a bit gentler.

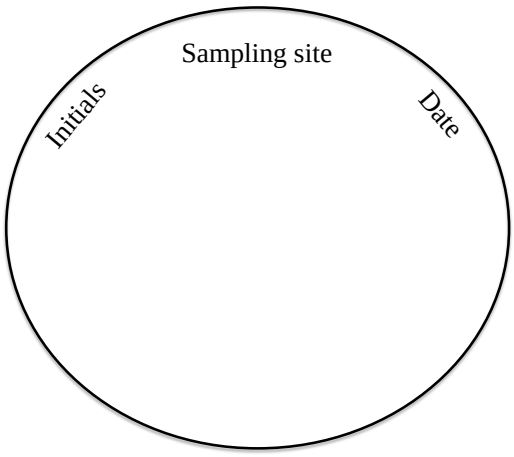
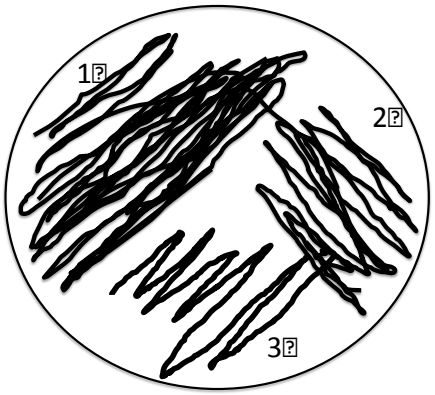



Figure 2: The bottom of your plates (containing the agar) should be labeled with your initials, sampling site and date.

5. Immediately after swabbing, locate the appropriately labeled plate. If you have placed them lid-side down on the lab bench surface, it should be easy to read the writing on the plate and identify your plate. Pick up the bottom of the plate, flip it over to access the agar surface, and inoculate your plate by rubbing your swab gently over the entire surface of the plate. See Figure 3 for further instructions. Your goal is to try to spread out the bacteria that you swabbed from your mouth so that you will have well-separated colonies on your plate after they have been incubated. This will allow you to analyze individual

colonies later, as well as isolate DNA from individual colonies. When finished, flip the plate back over onto the cover that is still sitting on your bench top, and then set this inoculated plate aside. Discard the swab into the biohazard container as directed by your TA.




6. Repeat this procedure with the remaining swabs and plates. Be careful to match the swab that you are inoculating with the appropriately labeled plate. Using tape, tape all your plates in one stack and proceed to the next step.

7. Put labeled plates into the bins for your TAs to collect. These plates will be incubated overnight at 37ºC and then held at 4ºC until next week.

Figure 3: You should swab your plates in this order. In section 1, rub your swab back and forth across 1/3 of the surface area of the plate. In section 2, using the same fluid motion, drag your swab once through area 1, and then repeat the back and forth swabbing across another 1/3 of the plate as shown in the figure. In section 3, repeat the process of dragging once through section 2, and swab back and forth to cover the last portion of the plate. NOTE: Do not cross back over to the other sections more than once after you start covering a new section of the plate!

8. There are two instructions for the blue drape:
    a. If you did not touch your used swab to the surface of the drape, your drape may go in the trash.
    b. If you set your used swab down on the blue drape, then the drape must go into the autoclave waste beaker.

9. Your gloves (if used) may go into the trash.

10. Please spray down your benchtop surface with the 10% bleach before you leave the workbench. Finally, be sure to wash your hands before you leave the lab.

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## II. Lab Day Two- Making observations

Your plates were incubated at 37ºC for 24-48 hours in order to allow bacteria to grow. They were then stored at 4ºC in order to keep the bacteria from overgrowing on the plate. It is important to note that only bacteria that can grow at 37ºC in the presence of oxygen, and can utilize LB agar as a nutrient source, will be able to grow on your plates.

Question: Why do you think this might be important to remember? Please write your responses in the space provided:

Bacterial cells "grow" by binary fission. This means that after a single cell has completed the cell cycle, it will divide itself approximately in half, resulting in two (basically) identical cells. On average, bacterial cells can complete a single cell cycle in 20 minutes (some do take longer than this, some less). This means that after 24 hours a single cell has replicated itself 72 times. Of course, with each division, there are additional cells that can now replicate and divide, so after 24 hours a single bacterial cell can result in $2x10^{21}$cells. What you will be looking at on your plates is the result of all of these divisions, for potentially many different types of bacteria. The result of all of these divisions is a pile of cells called a "colony". Each colony arose from a single "colony-forming-unit", indicating that there was a live bacterial cell that was deposited at that spot on your plate, and that the appropriate conditions were provided for it to grow.

There are many different ways to classify and identify bacteria, but one standard technique is to look at the morphology (shape) of the colony that forms when one bacterial cell has divided enough times to be visualized as growth on a plate. Today you will describe your bacteria by answering the following questions:

1) What **color** are your colonies?
2) How **high** above the surface of the plate are your colonies?
3) What is the general **shape** of the entire colony?
4) What does the **edge** of the colony look like?
5) What does the **surface** of the colony look like? (For more information, see the descriptions in Figure 4 below).

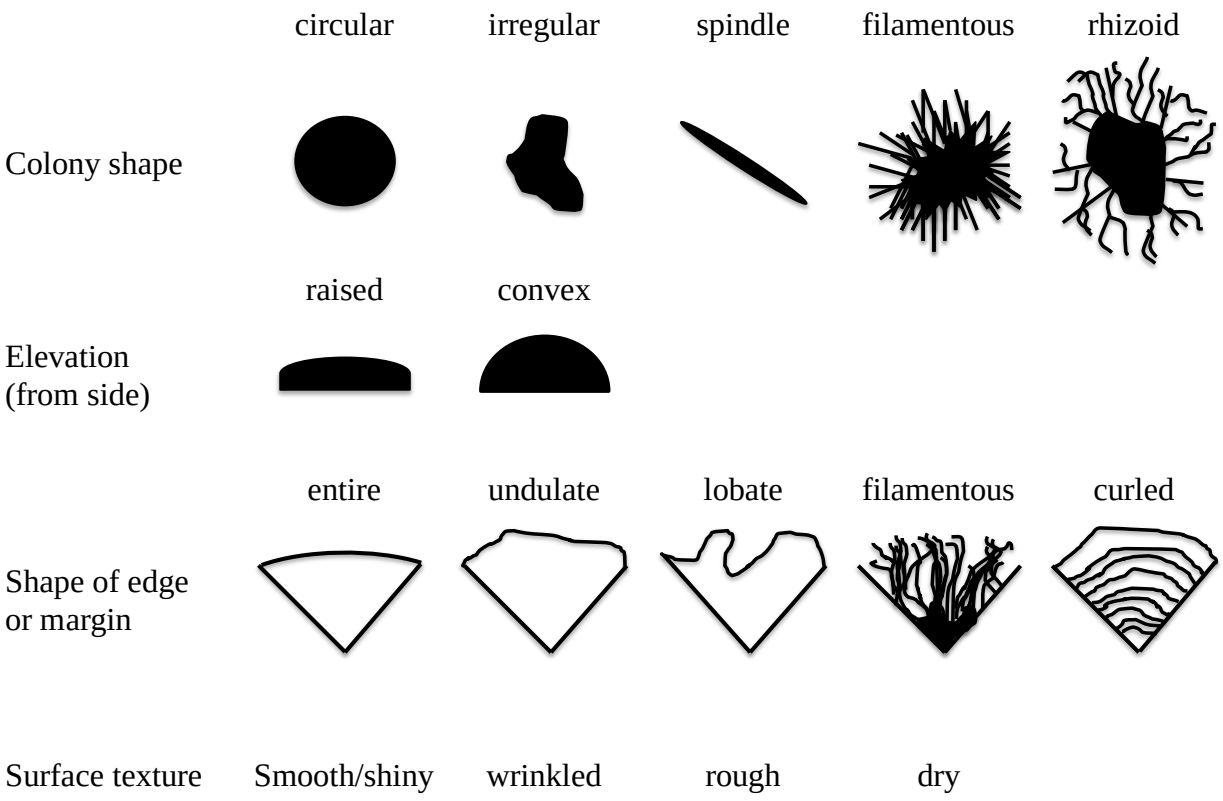


**Figure 4**. Colony morphology descriptions, adapted from [8]. Use these images to help you describe your bacterial colonies.

**General protocol for analyzing your plates and preparing wet mount slides:**

What you will need:

Your plates from last time
Colony morphology worksheet (at end of packet)
Glass microscope slides
Coverslips
Sterile toothpicks
Gloves
Water
Water dropper for preparing slides
Microscopes
Sterile drape for benchtop.

1. Procure a tray with the additional materials needed (again, provided for teams of two). REMINDER: YOU ARE WORKING ONLY WITH YOUR OWN SPECIMENS! Place the blue drape down on the bench surface prior to opening any of your plates from last time.

2. Find your plates from the bin from last week. If you need to open your plates for any reason, be certain to put on gloves prior to taking the lid off of your plate. Otherwise, if the lids remain on your plates, you will not need to wear gloves for this lab day.

3. Analyze your plates from last week. On your colony morphology worksheet (see end of the laboratory manual), indicate the following:
    a. Approximately how many total bacterial colonies do you have on your plate? Record this information for each sampling site.
    b. Different bacteria form different types of colonies when grown on LB. They are judged based on their colony color, the shape of the colony, the height of the colony on the plate, if they look wet or dry, if they look like they are embedded into the plate, etc. Using the worksheet provided and the colony morphology descriptions above, classify the number of different types of colony morphologies observed on your oral swab plates from last week.

        i. How many colonies (approximately) do you have on each plate?
        ii. How many different kinds of bacterial colonies do you have on each plate?
        iii. Does one colony morphology seem to predominate?
        iv. Take pictures of your plates to keep for your records. This data gives you some preliminary information about your isolated organisms, and provides you with some of the details that would be used to identify and classify which organisms are present within your mouth.

4. Make a wet mount of some of your colonies so that you can look at them under the microscope to compare them to the eukaryotic cells you will also be looking at this week. To make a wet mount side, follow the directions below.

**REMINDER: Wear gloves, work only on the bench paper, work with your OWN plates.**

5. To make a wet mount, find the sterile toothpicks, a glass microscope slide, a cover slip, a pipette and pipette tips, and a pair of gloves. Put on your gloves! Look at your plates and decide which colony you want to pick cells from. It might be useful to draw a circle on the bottom of the plate around the colony chosen using a Sharpie, so that you know which colony you sampled from. You may want to use this same colony later for the DNA analysis. Prepare a wet mount by placing a drop of water (no more than 20 microliters) on the microscope slide. Dispose of the pipette tip in the tip bucket. Pick up a sterile toothpick (touching only one end) with one hand, and carefully lift the lid off one of your plates with the other. Using the sterile toothpick, pick up a small portion of one well-isolated colony with the tip of your toothpick and spread it around in the drop of water. Replace the lid of your plate. Dispose of the used toothpick in the beaker with the autoclave bag, as directed by your TA. Cover your sample on the microscope slide with a cover slip, being careful not to get any bubbles trapped under the cover slip.

6. Observe your cells under the compound microscope. Keep in mind that prokaryotic cells range in size from 0.2-5 microns, while eukaryotic cells can be 10-100 micrometers in

size. If you need help finding bacterial cells, don't hesitate to ask for help. Use the space below to draw what you see:

7. When done, return your plates to the bins for your TAs to put back into storage until next week. Be sure to dispose of your slide(s) in the appropriate waste container for glass waste. Throw your gloves and the blue drape into the autoclave bag for disposal, and spray down your bench top with the 10% bleach solution.

8. Turn in your colony morphology worksheet to your TA for review, and wash your hands before you leave the lab area.

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## III. Lab Day Three- Extracting DNA

Now that you have determined the number and kinds of colony morphologies formed by bacteria that can be cultured from your mouth, we are going to attempt to identify some of these microbes. There are many different ways that microbiologists have used in the past to identify and classify microbes, including biochemical, molecular and physical tests. These typically require additional time for growth, as well as special media and reagents. The molecular technique that we are going to use to classify our oral microbes also requires special reagents but does not rely on growth. Instead, it relies on the ability to obtain an adequate source of DNA. Since you've isolated single colonies, you should have an ample source of genetic material to utilize! The problem will be deciding what part of that genetic material to analyze.

Bacterial genomes can be as small as 500,000 base pairs or as large as 5 million base pairs, which is a lot of genomic material to cover. What is needed in order to classify microbes is a gene that is found in all bacterial cells and that has a conserved function so that it will not change (evolve) quickly. One gene that serves this purpose quite nicely for bacteria is the sequence that codes for the 16S ribosomal RNA (16S rRNA; the gene sequence encoding this molecule is referred to as the 16S rDNA). This molecule is part of the 30S ribosomal molecule in prokaryotes, and is required for the production of proteins.

Because the ribosome serves an essential role in the cell, its structure and function must be maintained. Any mutation that significantly alters the ability of the ribosome to bind to the mRNA or to the incoming tRNA could be lethal. That being said, the 16S rRNA has conserved and variable regions, based on the portion of the molecule that is important for function. When the 16S rRNA molecule folds and becomes a part of the ribosome (RNA plus protein, see Figure 5 below), the conserved regions play a role in maintaining functionality (ribosomal structure and translation initiation), while the variable regions are not as important for these functions. The variable regions are useful for classifying, because they generally differ between species. You will be using a portion of the 16S rDNA sequence within the V5-V6 hypervariable region to classify your oral microflora by amplifying this region using the polymerase chain reaction (PCR). This procedure will be described in more detail later; for now, you need to obtain the genetic material that will be used in this analysis using the Chelex protocol described below.

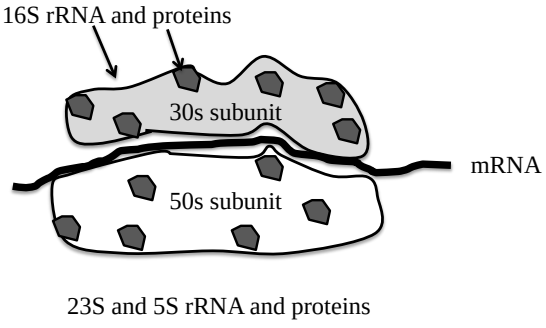


**Figure 5.** The 30S ribosomal subunit of prokaryotes is made up of the 16S rRNA and proteins. The 16S rRNA gene has both conserved and variable regions, which can be useful for identifying and classifying prokaryotes.

**REMINDER: Even though you are sharing a bench space and equipment with your benchmate, you must continue to work with YOUR OWN bacterial plates.**

**General Protocol for the isolation of bacterial DNA, Chelex procedure [adapted from** [9]**]**

Materials needed:

Chelex solution (prepared)
Pipettes (P1000, P100, P10)
Pipette tips for the above pipettes
Sterile water (one bottle for each of group of 4 students)
Microcentrifuge tubes
Sterile toothpicks
Microcentrifuge tube rack
Gloves
Eye protection
Ice bucket
Heat block
Sharpies (extra-fine or Ultrafine tips, for writing on caps)

1. Place your blue drape on the benchtop surface. Put on your gloves and eye protection. Place your plates from Day 1 onto the disposable lab drape, along with the other materials you need for steps 2-5.

2. Choose three different colony morphologies (total) from your four different plates. You will be taking a small (toothpick-tip sized) sample of each colony to analyze by PCR. Obtain three sterile microcentrifuge tubes and label the caps with your initials. Also indicate somehow what the sample is that you will be placing in each tube. Keep a record of which colonies you are sampling! Again, it may help to circle the colony on the bottom of the plate in order to be able to visualize which colony you're working with. After choosing your colonies, place the plates back on your lab drape, lid side down.

    a. Colony 1: (sampling site, morphology)

    b. Colony 2: (sampling site, morphology)

    c. Colony 3: (sampling site, morphology)

3. Using the p1000 micropipettor set at 1 ml (1000), with a 1ml size tip, fill each sterile microcentrifuge tube with 1 ml of sterile water. Close the caps securely and set these tubes aside for now in a tube rack.

4. Obtain a sterile toothpick with one hand, being careful not to touch the end of the toothpick that will be going into your colony. Pick up your plate from the bottom, so that you can flip it over and pick up a small portion of your first selected colony. Carefully pick up a small amount of a selected colony from your plate (enough to cover the end of the toothpick). After getting your cells, return your plate to its lid, which should still be sitting on the benchtop.

5. Open the appropriately labeled microcentrifuge tube with your free hand. Insert the inoculated toothpick into the tube. Swish the toothpick around until the water looks cloudy. Close the tube, dispose of the toothpick in the appropriate waste container indicated by your TA, and set the tube into a tube rack on your bench while you inoculate the remaining samples.

6. Repeat steps 4-5 for the remaining two tubes and chosen colonies, using a fresh toothpick each time. Be sure to put the appropriate colony sample into the appropriate tube.

7. Place your tubes in the microcentrifuge on your bench, making sure that your tubes are balanced against other tubes in the opposite positions in the rotor. Centrifuge for 1 minute to pellet the cells to the bottom of the tubes. Wait 30 seconds before opening the microcentrifuge to allow any microaerosols to settle before you open the lid.

8. Gently pour off the liquid into a paper cup. Be careful not to disturb the cell pellet at the bottom. Do not shake the tubes. A small amount of liquid will remain in the tube.

9. You will use the p1000 micropipettor (set at 200) and an appropriate tip to transfer 200 µl Chelex solution to the cell pellet. Chelex is a chelator, which is a compound that will grab up any metal ions in a solution that might interfere with the PCR reaction you will be performing in the next lab period. To use the Chelex, shake the Chelex solution to suspend the Chelex beads. Chelex settles out very quickly. Before the Chelex has had a chance to settle, transfer 200 µl to the tube containing your cell pellet. Look at the tip to make sure you have gotten at least a few Chelex beads before transferring to your tube. Use the pipet tip to break up the cell pellet. Dispose of the pipet tip after a single use, into the appropriate waste container indicated by the TA.

10. Repeat step 9 for the remaining two tubes.

11. Mix the cells with Chelex by vortexing or by flicking the end of the tube with your finger (your TA will show you how to do this).

12. Place the tubes in the 100°C heat block for 10 minutes. This will burst open the cells, releasingthe DNA.

13. After heating, place the tubes on ice for approximately 2 minutes to stabilize the DNA.

14. Mix the cells and Chelex by flicking the tube. Place the tubes in a balanced configuration in the microcentrifuge rotor, and spin for 1 minute to pellet the Chelex beads at the bottom of the tube. Wait 30 seconds before opening the microcentrifuge lid to allow any microaerosols to settle. After centrifuging, the supernatant will contain your DNA. DNA is very light, so the cell debris and Chelex will fall to the bottom and the DNA will remain suspended in the liquid.

15. Obtain an additional 3 sterile microcentrifuge tubes and label them as before. Place them into the tube rack. You will be transferring the DNA from step 14 into these new tubes

for storage until next time. Be sure not to mix your DNA samples together, and make sure you transfer the DNA from step 14 into a new tube labeled with the same information. You've worked hard to get your DNA; you don't want to mix up your samples at this stage! Use the p100 micropipettor (set at 100) and an sterile tip to transfer 100 µl of your DNA from step 14 into your new sterile microcentrifuge tube. Be careful not to transfer the Chelex beads or any of the cell debris from the bottom of the tube. Dispose of the used tip in the used tip beaker, as indicated by your TA. Place the labeled tubes containing your transferred samples into the rack indicated by your TA for storage until next time.

16. Dispose of your used microcentrifuge tubes into the autoclave waste as directed by your TA, along with your used gloves and lab drape. Spray down your benchtop with 10% bleach solution. Be sure to wash your hands before you leave the lab area today.

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## IV. Lab Day Four- Amplifying your DNA

During the previous lab period you extracted DNA from three different colonies, which could be from three different species of bacteria, if they each had distinct colony morphologies. The extraction protocol used was fairly simplified, but has proven to be very effective at obtaining enough genetic material to amplify a selected region of the bacterial genome. In order to increase the chance we'll be able to identify the organism present in your sample, we will first perform a polymerase chain reaction (PCR) to selectively amplify the piece of DNA that we are interested in (the 16S rDNA).

The polymerase chain reaction (PCR) was developed by Kary Mullis in the 1980s, for which he won the 1993 Nobel Prize in Chemistry [10]. The basic process is similar to the process used by cells to replicate DNA, although there are a few key differences. PCR requires the user to supply all of the necessary reagents, which are found within the cell. These include the template DNA, two primers, deoxynucleotides (dATP, dCTP, dGTP, dTTP), a thermostable polymerase, water and some buffer (to keep everything working).

DNA replication begins with the separation of the DNA template strands; this is done by enzymes within the cell, while PCR uses a high heat step to break the hydrogen bonds that hold the two strands together. All of the steps for PCR happen inside a machine called a thermocycler, which will go through multiple heating/cooling steps in order to amplify a given template.

During cellular DNA replication, enzymes are used to produce an RNA primer to provide the free 3'OH group to initiate replication at the origin of replication. In PCR, the user provides two DNA primers that are complementary to either end of the region of the template that they wish to copy. This is an important difference, as only the region between where the primers are complementary will be replicated during a PCR reaction, while in a cell DNA replication results in the complete replication of the entire genome.

For our reactions, we will be using primers that should allow us to make millions of copies of approximately 280 basepairs of the V5/V6 region of the 16S rDNA. One of the primers is complementary to nucleotides 785 to 805 on one strand of the 16S rDNA template, while the other primer is complementary to nucleotides 1041-1061 on the opposite strand of the 16S rDNA template (remember that the two strands of DNA are paired together by complementary base pairing). When the two primers are used together in a PCR reaction, only the region between those two primers will be copied, resulting in millions of copies of a piece of DNA that is ~280 base pairs in length.

After amplification, it is easy to determine whether your PCR reaction worked by running your samples on an ethidium-bromide stained gel. After separating the DNA by size and charge through an agarose gel, a positive PCR reaction should result in a single band in the gel, indicating that only the portion of DNA that you wanted to copy was actually copied. See the diagram below (Figure 6) for a general outline of how PCR can selectively copy a single region of DNA.

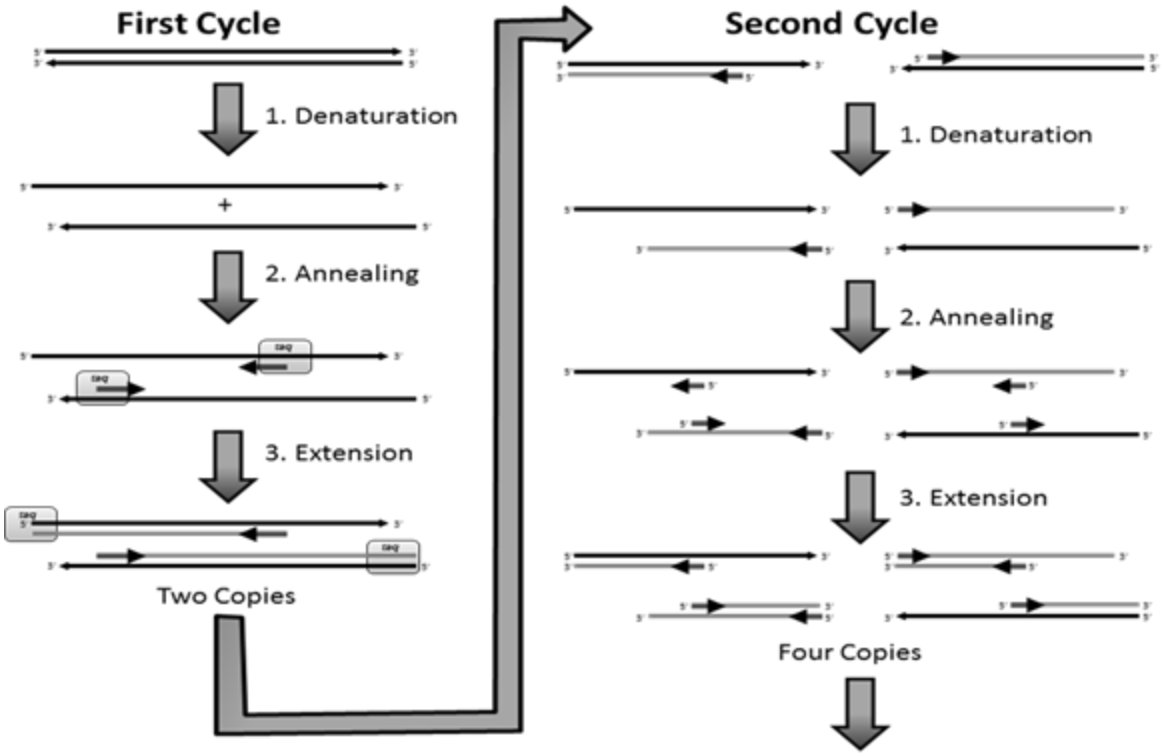



**Figure 6.** PCR consists of three basic steps: 1) Denaturation (of the hydrogen bonds holding the template strands together) by heating to 95ºC. 2) Annealing of the primer to the template by cooling to the "melting temperature" of the primer set, which allows the primers to "sit down" on the template, also bringing in the thermostable polymerase to begin replicating the DNA. 3) Extension, which allows the DNA polymerase to add on nucleotides to the initial primer, resulting in two copies of the initial piece of DNA. This process is then repeated 30-40 times, resulting in exponential copying of the selected portion of DNA. Figure provided by Brian Gibbens, University of Minnesota.

## General protocol for PCR amplification of the 16S rDNA sequence

Materials you will need:
3 tubes containing colony DNA isolated during the previous lab meeting
1 sterile microcentrifuge tube
3 PCR tubes
Sterile water (one bottle per group of 4 students)
Pipet tips (P200, P20)
Pipettes (P200, P20, P10)
PCR tube rack
Ice bucket
PCR Master Mix
Primer mix; Primers 1 and 2 pre-mixed, sequence from [4]
Gloves
Microcentrifuge tube rack
Ultra-fine point sharpie

1. Obtain the reagents listed above. Put on your gloves; these are being worn today in order to keep "you" from getting into your PCR tubes. Label your PCR tubes with your initials and some identifying mark that will let you know which tube contains the DNA from

which of your original DNA samples. For example, C1 for "Colony 1". Place these tubes into your PCR tube rack, and put the rack into the ice bucket. Put your tubes containing your DNA from last time into your ice bucket.

2. Label your empty microcentrifuge tube as "MasterMix" and place into your ice bucket. Add the following items to this tube, **using a fresh tip each time** (dispose of each used tip in the appropriate tip container):
    a. 50 microliters of the Promega Master Mix (this contains buffer, dNTPs and the polymerase)
    b. 35 microliters of sterile water (keep this sterile- you never know when you might need it again!)
    c. 3.2 microliters of the primer mix (this contains a mix of the forward and reverse primers).

3. Mix these together well by briefly vortexing or flicking the bottom of the tube. Place the tube back into the ice bucket.

4. Reset the P100 tip to 22 microliters. Carefully open each of the PCR tubes in your rack and add 22 microliters of your "Master Mix" to the bottom of each tube. You may use the same tip each time for this process, so long as you do not touch any outside surfaces. After adding your "Master Mix" to each of the three tubes, discard the used pipette tip.

5. Your PCR tubes should now contain everything needed for the PCR reaction except for the DNA, all in 22 microliters. They should also be in a PCR rack in an ice bucket. To finish prepping your tubes for PCR, you need to add your template DNA. You will add 3 microliters of template DNA to each tube. Set your P10 pipette to 3 microliters. Gently vortex one of your DNA tubes prepared last week. Obtain a pipette tip and carefully draw up 3 microliters of template DNA. Check the end of your tip to be sure that you have 3 microliters of liquid in the end of your tip. Carefully dispense these three microliters into the bottom of the appropriately labeled PCR tube, trying not to get any bubbles in the tube. There should now be 25 microliters of liquid in your PCR tube. Discard the pipette tip.

6. Repeat step 3 for the remaining two PCR tubes, disposing of the pipette tip between each sample.

7. Once you have all three tubes prepared, bring your sample (in your ice bucket) to the community ice bucket and PCR rack. Place your PCR tubes into the community PCR rack.

**IMPORTANT:** Be sure to note in your notebook and MOST IMPORTANTLY on the 96 well grid sheet provided the location of your PCR tubes so that you can get your samples back next week.

Even though you've labeled your tubes, that labeling may not stand up to the rigors of the

thermocycler, so we need to have an additional means of identifying your samples. The grid sheet is laid out identical to the PCR rack, with letters going down the side of the rack from A-H and numbers across the top from 1-12. Your samples will be in (for example) A1, A2, A3, and you would write this information down on the grid for your TA, who will be placing your samples into the thermocycler using the program listed below.

a. Thermocycler program: name Bio1010A
    i. First denaturing step: 94ºC for 2 minutes
    ii. Denaturing step: 94ºC for 30 sec
    iii. Annealing step: 50ºC for 40 sec
    iv. Extension step: 72ºC for 80 sec
    v. Repeat steps ii-iv 30times
    vi. Final extension: 72ºC for 3 minutes
    vii. Hold: 4ºC indefinitely
b. The expected product should be about 280 bp

8. When finished setting up your tubes, dispose of your gloves in the appropriate waste container indicated by your TA. Place your DNA tubes back into the tube rack for your TA to store.

NOTES:

| Please record<br>Label of sample | Location of sample in 96-well grid |
|---|---|
| 1._____ | _____ |
| 2._____ | _____ |
| 3._____ | _____ |

## V. Lab Day Five- Looking at your DNA

The PCR reactions run last time should have resulted in numerous copies of the 16S rDNA gene from your bacterial cells. To verify that everything worked as expected it to before you send samples out for sequencing, you will be running your samples on an agarose gel to separate out the bands of DNA based on size and charge. The agarose gel is run in a liquid buffer that has an electrical current running through it. The DNA will travel through the gel from the negatively charged anode to the positively charged anode, because DNA carries a net negative charge. The agarose in the gel functions as a sieve to additionally sort the fragments based on size. We are looking for a single band on the gel, indicating that the primers were specific for only one region of DNA and that they made copies of the appropriate piece of DNA.

Materials needed:
Your PCR reactions from last time
Pipette tips for P10 pipette
P10 pipette
1% Agarose gel (prepared for you)
1Kb ladder
Loading dye
Parafilm square
Clean-up pipette tips (for use after you see the gel results)
P200 pipette and tips

1. Find the following items: your PCR reactions in a PCR tube rack, the P10 pipette and pipette tips, loading dye and a piece of parafilm. Take these items over to one of the gel loading areas. Place the piece of parafilm down in front of the gel box and pipet 2 microliters of loading dye onto one spot on the parafilm. Make two more 2 microliter spots of loading dye on the parafilm. Discard this used tip.
2. Gently mix your PCR reactions either by flicking the tubes or vortexing. Set your pipet to 5 microliters, obtain a new tip, and draw up 5 microliters of one of your PCR reactions. Mix this into one of the spots of loading dye on the parafilm by pipetting up and down several times. Carefully pull up all of the liquid from this one spot and load it into a single lane on the agarose gel as you did in the Genetics lab earlier this semester. Carefully note which gel and which lane you have loaded your sample into.
3. Repeat this process for your remaining two samples. Take your PCR reactions back to your bench. We will run these gels during the lab practical and will look at the results at the end of exam so that we can determine which samples will be used for sequencing.
4. Each gel should have a ladder run on it, for determining the size of the fragments that are amplified. In addition, at least one gel will have the following control samples run, to ensure that the PCR reactions worked appropriately:
    a. *E. coli* DNA
    b. *Staphylococcus epidermidis* DNA
    c. *Candida albicans* DNA

In the space provided answer the following question:

When using the primers for 16S rDNA, two of these samples should produce a specific band, while the third should not. Which one and why not?

5. Run the gel at 100V for 30 minutes and check to see what the sample looks like using a UV lightbox. Be sure to wear protective eyewear when visualizing bands on a UV lightbox! Take a picture of the gel in order to facilitate quantifying the amount of DNA present in your samples. This is important when we set up the sequencing reactions.

6. If the samples are clean (single bands), clean up the reactions using the PCR tips from Midwest Scientific. To do this, a separate tip must be used for each PCR reaction, and you will need to obtain a new, sterile PCR tube to transfer your sample into. Set the P20 to 30 microliters. Attach a CleanUp tip to the end of the pipette. Carefully draw up your PCR reaction into the tip. Slowly pipet up and down for 60 seconds. On the last mix, transfer the suspension to a fresh tube, labeled with the appropriate information (Remember that you don't want to mix up your samples now!). Place your "clean" DNA into the community PCR tube rack, again indicating where your samples are in the new 96-well grid. This will ensure you get your clean samples back next time.

NOTES:

## VI. Lab Day Six- Sequencing your DNA

The bands that you saw on the gel at the end of day five were the result of multiple rounds of replication of a single portion of the DNA template that was present in the original sample. The presence or absence of a band can provide you with some information about your sample. If a single band of the appropriate size is present in the gel, this suggests that there was an appropriate DNA template present in your sample, one that contained sequences that were complementary to the *E.coli*-based 16S rDNA primers that were used in the PCR reaction. This would suggest that perhaps you amplified bacterial 16S rDNA, although it doesn't guarantee that is what was amplified. If you ran a sample of known *E. coli* 16S rDNA in the PCR reaction and then on the gel, this would provide further evidence that you amplified the appropriate DNA fragment. If you also ran a PCR reaction using those primers and *S. aureus* DNA and obtained a band it would suggest that the primers work on more than just one type of bacteria, which could be helpful if you didn't know exactly what type of bacteria you were working with. Finally, if you used a sample containing eukaryotic DNA and the 16S rDNA primers and didn't see a band, you could feel fairly confident that the primers were specific for prokaryotic DNA and not eukaryotic DNA. All of this information still doesn't tell you exactly what type of DNA you've amplified. For this, you will need to determine the sequence of the DNA that you amplified, to see 1) if your primers amplified the 16S rDNA gene and if so, 2) what species of bacteria was the sequence amplified from? Reading the sequence of 16S rDNA can help in identifying an unknown sample because there are enough 16S rDNA sequences available in the database to allow for comparison across thousands of species to see how closely related different sequences are.

In the early days of DNA sequencing the samples you obtained last time would have been run on a very large sequencing gel using radioactive di-deoxynucleotides to determine the sequence of the DNA we had amplified. Luckily, we no longer need to run these reactions ourselves but can simply supply our template DNA, one primer and a little water in tube and send the samples out to be sequenced for us. This process again is similar to the process that occurs during DNA replication in the cell. This time, however, in order to identify the nucleotide sequence special nucleotide analogs called di-deoxynucleotides are used that will terminate replication after they are incorporated into the sequence. This is because they do not have a free 3' hydroxyl group to allow the addition of the next nucleotide. In addition, they are labeled with a fluorescent dye that can be detected and read by the sequencer. Each di-deoxynucleotide (ddATP, ddCTP, ddGTP, ddTTP) has its own color, so they can be readily distinguished from one another. The results that are sent back from sequencing facilities will include the raw data obtained from the sequencing read and the sequence itself. You will use these results to identify at least one of the bacteria present in your mouth.

Materials you will need:
Your clean PCR reactions from last time, those identified as having worked well
Sterile water
P10 pipette and pipette tips
Primer 1
Two clean PCR tubes

1. Obtain one PCR tube, and label with your initials and your sample ID information.

2. Choose one of your samples that gave a nice band on the gels run last time. Using the P10 pipette set to 2 microliters, transfer 2 microliters of your sample to your tube.

3. Set the P10 pipet to 7 microliters. Obtain a fresh pipette tip. Transfer 7 microliters of sterile water to your PCR sequencing tube. Discard the used tip in the appropriate container. There should now be 9 microliters of liquid in your sequencing tube.

4. Gently mix the tube containing Primer 1. Set the P10 pipette to 3.1 microliters. Obtain a new tip and transfer 3.1 microliters of Primer 1 to your sequencing tube marked "P1". Discard the used tip.

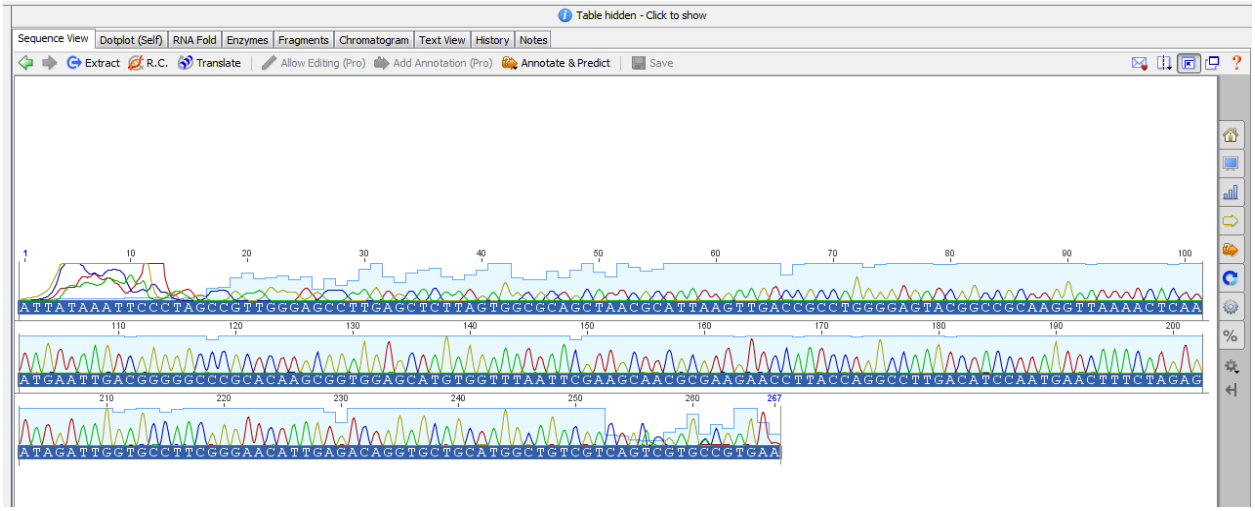
5. Take your labeled sequencing PCR tube to the community PCR rack and ice bucket. Place your tube into the PCR rack, and indicate in the 96-well gridsheet where your sample is located in the rack. This is again going to be important for identifying which sequence belongs to you when it comes back from BMGC. The TAs will transfer a portion of your sample into a 96-well plate format for sequencing, as this is less expensive than running individual tubes. The results should be available within 72 hours of submitting the sequences.

NOTES:

## VII. Lab Day Seven- Identifying your bacteria

At last we reach the culmination of all of our work of swabbing cheeks, plating, waiting for growth, analyzing colonies, extracting DNA, running PCR, cleaning up reactions and sending the samples out for sequencing. You should have received your sequencing results via the Moodle site by now, and you should be ready to take the next step to determine the possible identity of at least one member of your oral microflora.

The typical process for this step would be to analyze your sequence traces, to verify that the sequences that the computer automatically "called" for you were actually what they should be. An example of a sequence trace is shown below, using a program called "Geneious". Note that the first 30 or so peaks on the chromatogram are "junky", and often overlap. These peaks correspond to the nucleotides that were incorporated into the growing DNA chain during the sequencing reaction. However, even though the machine suggests that it could distinguish which




nucleotide was added to the growing chain, it is clear from thechromatogram that some of the "calls" that the computer made could not possibly be accurate (look at the first 15 peaks in particular). Because it takes a bit of time and experience to be able to read a chromatogram, you are not going to do that step of the analysis. The results that were sent to you were the text files indicating the bases that the computer determined for your sequences, with the "junky" ends trimmed off. This information is sufficient to move on to the next phase in the analysis, which is to perform a BLAST search.

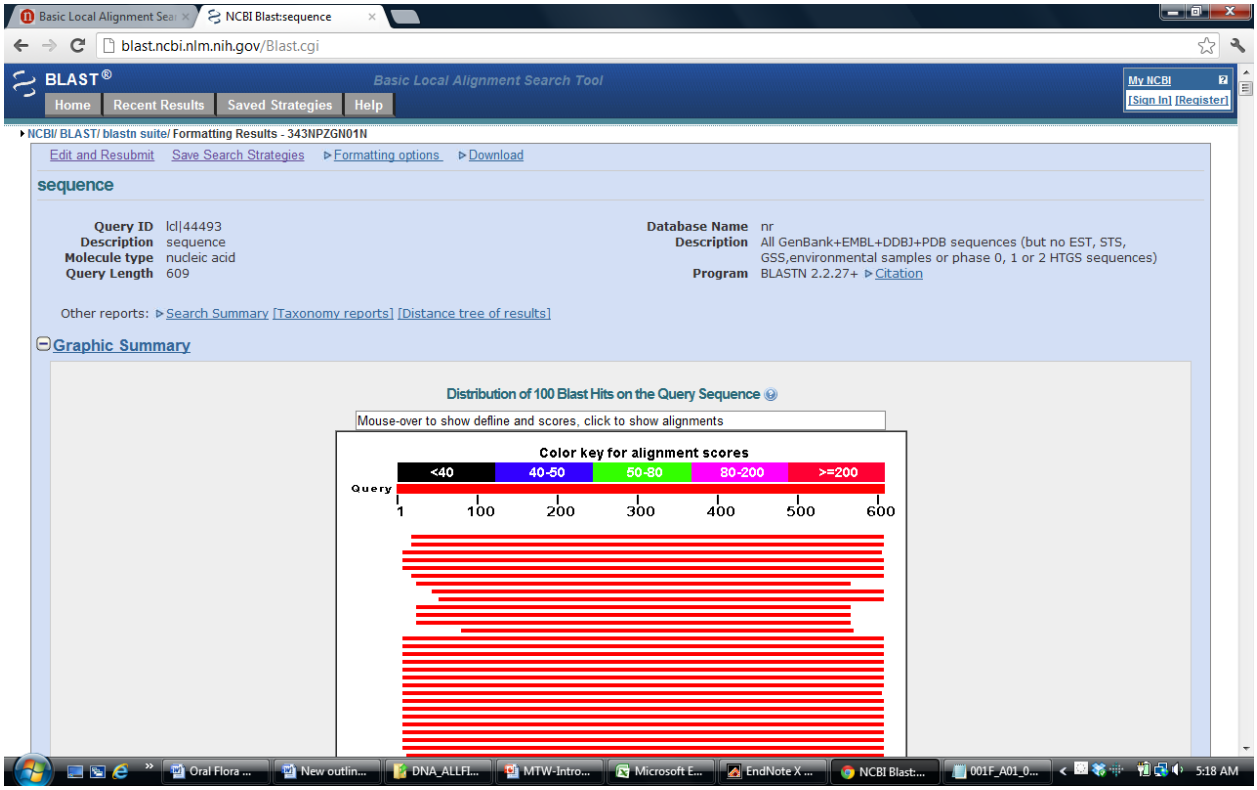
What is a BLAST search? BLAST stands for Basic Local Alignment Search Tool, and it is one of the most-used tools in bioinformatics. It can be found on the National Center for Biotechnology Information website at http://blast.ncbi.nlm.nih.gov/. The BLAST search tool has been around since 1990 [11], as a way to search through, organize and compare the millions of sequence records (DNA and protein) that have accumulated (and continue to accumulate) at an exponential rate. The BLAST tool allows you to enter your query sequence on the database server and it then uses an algorithm to compare it to all the sequences present in the database, depending on your search parameters. The final output is then an alignment of your sequence with all the sequences that the search program decided that it is similar to, along with some numbers to help you determine how well those sequences match one another. How to perform a

BLAST search has already been discussed in class, and a tutorial video will be available on the course Moodle website, so we won't go into detail here, other than to explain what to do with your sequences results

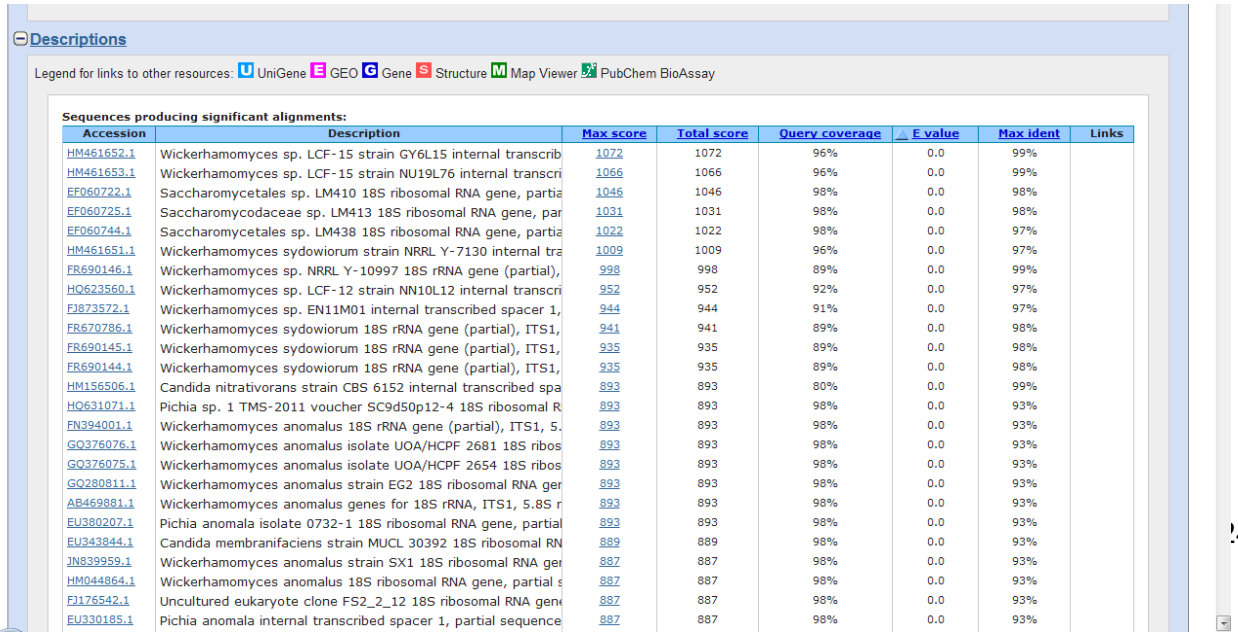
**General protocol for performing a BLAST search.**
Materials you need: your sequence text files, access to a computer with internet access

**The assignment:** identify the top hit for your sequence using the BLAST algorithm, which will provide you with the identity of one of the organisms found in your mouth. After identifying this organism, find out a bit more information about it, such as whether it is a gram positive or gram negative microorganism, is it something that would normally be found in a human mouth, is there anything else interesting about this microbe? This will be turned in at the next lab period.

1. Go to the ncbi BLAST webpage at http://blast.ncbi.nlm.nih.gov/.
2. Click on the link for "nucleotide blast"
3. In the box that says "Enter Query Sequence", do the following. On the first line, it is helpful to include some information about your sequence. This should be entered exactly as shown below, replacing the phrase "My tooth sequence" with your name and however you identified your specimen throughout this experiment.
   > My tooth sequence
4. After entering that information, you need to add a carriage return by pushing the "enter" button to move the cursor to the next line. On this next line, you should copy and paste the text for one of your DNA sequence files. Only enter one of the sequences at this time.
5. Under the "Choose Search Set" section, be sure to check the "Others (nr etc)" choice instead of the default "Human genomic+transcript". If you leave it on the default, it will only be searching for similarities among human sequences, and hopefully you have amplified prokaryotic sequences. "nr" stands for non-redundant, which means that it will not search through sequences that are incomplete or partial reads.
6. You may leave all the other parameters on the default settings initially. After making the indicated changes above, feel free to click the "BLAST" button.
7. After a period of time, you will be taken to your results page. It should look something like the screen shots below. Reading the color coded heat map will give you some pretty good information as to how well your sequence aligned to the sequences in the database. Each of colored lines corresponds to a single alignment between your sequence and a sequence in the database. You can point your mouse over the top of each one to see what each sequence is, or you can scroll further down the page to see those alignments for yourself.




8. The next screen shot shows additional details about your sequence alignment, providing you with the hard numbers such as "Max score", "E-value", "Query coverage" and "Max. Ident."
    a. The information that is most useful is E-value, which tells you the likelihood that this match happened by chance. In general, the lower the e-value (the closer it is to zero), the better (although this is not always true- read on).
    b. The "Query coverage" statistic tells you how much of the length of your sequence matches the sequence in the database. This is important, because you can have a very low E-value, but if only 10 nucleotides out of 200 nucleotides in your original query are all that is included in that match, then the E-value doesn't really matter here and you don't have a good match.
    c. Another statistic that is important is the "Max. Ident." which stands for maximum




| Accession | Description | Max score | Total score | Query coverage | E value | Max ident | Links |
|---|---|---|---|---|---|---|---|
| HM461652.1 | Wickerhamomyces sp. LCF-15 strain GY6L15 internal transcrib | 1072 | 1072 | 96% | 0.0 | 99% | |
| HM461653.1 | Wickerhamomyces sp. LCF-15 strain NU19L76 internal transcri | 1066 | 1066 | 96% | 0.0 | 99% | |
| EF060722.1 | Saccharomycetales sp. LM410 18S ribosomal RNA gene, partia | 1046 | 1046 | 98% | 0.0 | 98% | |
| EF060725.1 | Saccharomycodaceae sp. LM413 18S ribosomal RNA gene, par | 1031 | 1031 | 98% | 0.0 | 98% | |
| EF060744.1 | Saccharomycetales sp. LM438 18S ribosomal RNA gene, partia | 1022 | 1022 | 98% | 0.0 | 97% | |
| HM461651.1 | Wickerhamomyces sydowiorum strain NRRL Y-7130 internal tra | 1009 | 1009 | 96% | 0.0 | 97% | |
| FR690146.1 | Wickerhamomyces sp. NRRL Y-10997 18S rRNA gene (partial), | 998 | 998 | 89% | 0.0 | 99% | |
| HQ623560.1 | Wickerhamomyces sp. LCF-12 strain NN10L12 internal transcri | 952 | 952 | 92% | 0.0 | 97% | |
| FJ873572.1 | Wickerhamomyces sp. EN11M01 internal transcribed spacer 1, | 944 | 944 | 91% | 0.0 | 97% | |
| FR670786.1 | Wickerhamomyces sydowiorum 18S rRNA gene (partial), ITS1, | 941 | 941 | 89% | 0.0 | 98% | |
| FR690145.1 | Wickerhamomyces sydowiorum 18S rRNA gene (partial), ITS1, | 935 | 935 | 89% | 0.0 | 98% | |
| FR690144.1 | Wickerhamomyces sydowiorum 18S rRNA gene (partial), ITS1, | 935 | 935 | 89% | 0.0 | 98% | |
| HM156506.1 | Candida nitrativorans strain CBS 6152 internal transcribed spa | 893 | 893 | 80% | 0.0 | 99% | |
| HQ631071.1 | Pichia sp. 1 TMS-2011 voucher SC9d50p12-4 18S ribosomal R | 893 | 893 | 98% | 0.0 | 93% | |
| FN394001.1 | Wickerhamomyces anomalus 18S rRNA gene (partial), ITS1, 5. | 893 | 893 | 98% | 0.0 | 93% | |
| GQ376076.1 | Wickerhamomyces anomalus isolate UOA/HCPF 2681 18S ribos | 893 | 893 | 98% | 0.0 | 93% | |
| GQ376075.1 | Wickerhamomyces anomalus isolate UOA/HCPF 2654 18S ribos | 893 | 893 | 98% | 0.0 | 93% | |
| GQ280811.1 | Wickerhamomyces anomalus strain EG2 18S ribosomal RNA ger | 893 | 893 | 98% | 0.0 | 93% | |
| AB469881.1 | Wickerhamomyces anomalus genes for 18S rRNA, ITS1, 5.8S r | 893 | 893 | 98% | 0.0 | 93% | |
| EU380207.1 | Pichia anomala isolate 0732-1 18S ribosomal RNA gene, partial | 893 | 893 | 98% | 0.0 | 93% | |
| EU343844.1 | Candida membranifaciens strain MUCL 30392 18S ribosomal RN | 889 | 889 | 98% | 0.0 | 93% | |
| JN839959.1 | Wickerhamomyces anomalus strain SX1 18S ribosomal RNA ger | 887 | 887 | 98% | 0.0 | 93% | |
| HM044864.1 | Wickerhamomyces anomalus 18S ribosomal RNA gene, partial s | 887 | 887 | 98% | 0.0 | 93% | |
| FJ176542.1 | Uncultured eukaryote clone FS2_2_12 18S ribosomal RNA gene | 887 | 887 | 98% | 0.0 | 93% | |
| EU330185.1 | Pichia anomala internal transcribed spacer 1, partial sequence | 887 | 887 | 98% | 0.0 | 93% | |

identity. This tells you how many of the nucleotides in your sequence are identical to the given sequence from the database.

9. Finally, if you click on the hyperlink listed under "Max score", it will take you to the actual sequence alignment of your sequence with the sequence from the database. This will show you exactly how well the two sequences match. Use this information to complete the homework assignment listed above.

**What happens next?**

After all of this work, you are perhaps wondering if this is really how microbiologists identify the bacteria living in a given environment. This is the way that well-known microbiologists such as Robert Koch and Louis Pasteur first identified various microorganisms, especially those that are involved in disease in humans and animals. Robert Koch, a German scientist, developed a set of postulates (rules) that needed to be observed in order to link a particular microorganism to a particular disease. His work proved that specific bacteria cause anthrax, tuberculosis and cholera, diseases that have played important roles in human health. In order to apply Koch's postulates to an organism, however, microbiologists must be able to do one very important thing. Think back to day one of this experiment, when you swabbed your mouth and used that swab to inoculate an agar plate. What would have happened if the bacteria in your mouth were unable to grow on the plate? What if the nutrients were not available in the media for the bacteria to grow? What if oxygen was actually deadly to the bacterial cells that you swabbed on your plate? Nothing would grow on your plate! BUT: does that mean that there are no bacteria present?

With the advent of DNA sequencing technology and techniques for obtaining DNA from many different types of environments, it has become clear that less than 1% of the microbes present in a given environment are able to grow in the lab and that many more species are present than were originally thought [1]. To identify the missing 99%, microbiologists have begun to sequence the 16S rDNA gene from non-cultured samples to identify all of the microbes present in a given sample site. This field of science, called "metagenomics", allows scientists to study all of the DNA present in a given site, whether it's bacterial, viral, or eukaryotic.

Metagenomics is a fast-growing field and is being used to answer questions about the populations of microbes present in environments as diverse as the human mouth, the human gut, deep-sea thermal vents, and hyena scent glands. The most important thing required, after a source of DNA, is some sequence within all those many DNA sources that will be able to represent all of a given type of organisms present in a sample, whether bacterial or fungal, for example, and that can differentiate between the types of organisms present. As we talked about on day 4, this is why bacteriologists choose to use the 16S rDNA sequence to identify the types of bacteria present in a sample. Metagenomics allows microbiologists to study and identify microbes that can't be grown in the laboratory, because the conditions needed for growth just aren't known. Metagenomics comes with its own downsides, as sequencing a metagenome results in tens of thousands (or more) sequences that need to be analyzed from a single sampling site, and this sequencing is not cheap. However, it does open up entirely new areas for research, and is allowing scientists to ask questions about how different microbes and microbial populations may play a role in health and disease.

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## Colony Morphology Worksheet

**(fill in as many as you can)**

| | Shape of entire colony | Elevation (seen from side) | Shape of edge or colony margin | Surface texture |
|---|---|---|---|---|
| Colony type 1 | | | | |
| Colony type 2 | | | | |
| Colony type 3 | | | | |
| Colony type 4 | | | | |
| Colony type 5 | | | | |
| Colony type 6 | | | | |
| Colony type 7 | | | | |
| Colony type 8 | | | | |
| Colony type 9 | | | | |
| Colony type 10 | | | | |
| Colony type 11 | | | | |

Overall Number of colonies:
Plate 1 _____
Plate 2 _____
Plate 3 _____
Plate 4 _____

Overall number of different colony types:
Plate 1 _____
Plate 2 _____
Plate 3 _____
Plate 4 _____