

Microbial Diversity in Virginia Old Hardwood Forest Soil

Week #1

Bio 203 laboratory module
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Introduction

Microbes (bacteria, archaea, fungi, and protozoans) are crucial to all processes related to soil function. These include soil formation, soil structure, cycling of carbon, nitrogen, phosphorous, and sulfur. Additionally the microbial constituents of soil are entirely responsible for the breakdown of organic matter and the degradation of toxic molecules. Certain soil microbes cause plant disease while others may suppress soil borne plant diseases and even promote plant growth.

An example of one of these critical soil processes mediated by soil microbes is the process of nitrogen fixation (turning atmospheric N₂ into a biologically usable form such as NH₃). The ability to perform this critical function is found in no other domain of life other than among the bacteria. Without this bacterial contribution, global nitrogen cycling would falter and life could not continue (more on nitrogen fixation later!).

There is currently a renewed interest in the effects of soil microbial diversity on terrestrial ecological functioning and the ability of soils to resist environmental stresses. This renewed interest is partially fueled by the pressures placed upon our soils by the increasing agriculturally intense land management practices used to feed our growing population as well as those of our food and fiber animals. In addition, the effect of increased rates of contamination of soils with various chemicals such as fertilizers, insecticides, herbicides, and industrial wastes constitutes another challenge to the microbes existing there.

Another important aspect of soil microbial diversity relates to our continuing search for novel antibiotics for use in clinical medicine. Virtually all our currently used antibiotics have their origins in the metabolites of soil bacteria, especially within a family known as the Actinomycetes (e.g. members a genus in that family, *Streptomyces*, produce the antibiotic Streptomycin). It is hypothesized that antibiotic production by soil bacteria helps in competition with other species of bacteria that may utilize the same resources in this complex niche.

Not only must we continue to search for new drugs in this ecosystem, but we must also look to the bacteria in this environment as a means to study antibiotic resistance. While the advent of antibiotic resistant bacteria is a relatively new phenomenon in human medicine (first seen in the late 1940's, but the rate of isolation of resistant bacteria has accelerated in the past 20 years), resistance is likely not new in the soil. Bacteria exposed over their long evolutionary histories to these antibiotics must have developed means to resist these chemicals lethal activities. At a minimum, any bacterium synthesizing a compound that is inhibitory to bacteria, must itself have a mechanism that renders it "immune" to that antibiotic.

How is microbial diversity studied ?

From a methodological point of view, two principal means have been used to assess microbial diversity. Historically, cultivation of microbes in the laboratory using numerous media formulations has been widely used. This culture method suffers greatly due to the fact that **>98% of environmental bacteria cannot be cultivated in the laboratory**. This has led to a gross underestimation of microbial diversity in all environments.

The second major means to assess microbial diversity in the soil is based upon non-culture dependent means such as the **Polymerase Chain Reaction (PCR)**. This method allows the amplification of DNA sequences from all organisms in any environment regardless of their ability to grow in a laboratory.

How and why are we going to study microbial diversity?

In this four-week laboratory exercise, you will examine soil bacterial diversity by means of both the culture dependent and amplification dependent means. Your target in this exercise will be soil from old Virginia hardwood forest found here in our own College Woods. Although little, if any, virgin forest remains in Virginia, our College Woods contains one of the longest standing non-harvested forests in Virginia (over 150 years old). Over the next four weeks, you, your colleagues, TA's and I will initiate a long-term, student driven research project to experimentally determine and quantify the bacterial diversity of the soil that perhaps reflects the pristine Virginia soils encountered by the Jamestown settlers! Future studies in this lab will expand to include a area of the College Wood damaged extensively by Hurricane Isabel in 2003 and a commercially ranched, Loblolly Pine Forest soil to potentially examine differences in the microbial ecosystems of these systems dominated by different plant flora.

Week #1 Protocols

A. Bacterial and Fungal Cultivation and Quantification.

1. Each lab section will be given a 10 gram soil sample from the College Woods. Your TA will add this to 100 ml of sterile water and shake until quite well dispersed.
2. In groups of 4-5 students, you will perform a serial 10-fold dilution series as follows: Label 5 sterile 1.5 ml capacity microcentrifuge tubes on their caps 1 through 5. To each of these tubes, pipette in 900 ul (0.9 ml) sterile water.
3. You will then pipette 100 ul (0.1ml) of the soil:water suspension into tube #1 and mix well. **Discard the pipette tip and get a new sterile one.**

Transfer 100 ul from tube 1 to tube 2 and mix well. Again, using a new, sterile tip, transfer 100 ul from tube 2 to tube 3. Repeat all the way through tube 5. Be certain to discard 100 ul from tube 5 after putting in 100 ul from tube 4. You should then have 5 tubes, each with 900 ul of fluid, each tube should have 10-fold fewer microbes than the previous tube.

4. Label 5 petri dishes (always on the bottom or smaller side) 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} .
5. add 100 ul of tube #1 to a molten agar pour in the 45°C water bath. Pour into Petri dish labeled 10^{-3} . Gently mix this by carefully sliding the plate slowly in a figure 8 pattern on the bench for a few seconds. Then allow the plate to stand undisturbed for 15 minutes to harden. Repeat this procedure with tubes 2-5 placing the bacteria/agar mixture in plates labeled 10^{-4} through 10^{-7} respectively. Your TA will incubate the plates appropriately and you will examine and count colony forming units (CFUs) on the plates next week

Amplification of an internal section of bacterial 16S rrnA

1. Your TA has a total DNA preparation made from this same soil sample. It has been quantified and diluted to a concentration of 200 ng DNA/ul. It is labeled “Soil DNA” It is on ice.
2. Your TA has also created a Polymerase Chain Reaction (PCR) Master mix containing the appropriate concentrations of buffer, $MgCl_2$, deoxyribonucleotides (dATP, dTTP, dGTP, and dCTP) as well as the thermostable DNA polymerase known as Taq polymerase. This reagent is labeled “PCR Master Mix”. It is on ice and important that it remain on ice as much as possible. There are also 2 additional reagents in the master PCR mix. These are the oligonucleotide primers specific for all bacterial 16S *rrnA* genes. These artificially synthesized single stranded pieces of DNA match perfectly with two sections of all bacterial 16S *rrnA* genes. These primers are referred to as “27F” (sequence is 5' AGA GTT TGA TCM TGG CTC AG 3') and “519R” (5' GWA TTA CCG CGG CKG CTG 3').
3. Each person should label a PCR tube note, these are different than the 1.5 ml capacity microcentrifuge tubes) with the lab section and a single letter designation given uniquely to each student. In this lab, you will be student A or B or C etc. Make a note of this in your lab notebook. For example if you are in section 5 and your TA tells you that you are K, your tube will thus be labeled “5K”. Each of you will assemble a DNA amplification reaction (i.e., a PCR) in your labeled PCR tube as follows:
 - in a PCR tube (add the following reagents in this precise order:
 1. 34.5 ul dH₂O,
 2. 13.5 ul Master Mix.
 3. 2.0 ul Soil DNA preparation. (final volume should be 50 ul).

4. Mix briefly and “snap down” to ensure entire reaction volume is at the bottom of the PCR tube.

A 529 base pair internal section of the bacterial gene 16S *rrnA* from every species present in this DNA preparation will be amplified as a mixture of identically sized pieces of DNA. Amplification will be accomplished on a thermal cycling machine using the following thermal parameters:

94° C for 30 seconds followed quickly by 55°C for 1 minute, then 72°C for 1 minute. These 3 temperatures, in this order, will be repeated for 35 cycles. TA's will store your reactions frozen until next week.

An important recent paper if you're interested in more in depth learning

D'Costa VM, McGrann KM, Hughes DW, Wright GD. Sampling the antibiotic resistome. **Science**. 2006 **311**(5759):374-7.

Abstract:

Microbial resistance to antibiotics currently spans all known classes of natural and synthetic compounds. It has not only hindered our treatment of infections but also dramatically reshaped drug discovery, yet its origins have not been systematically studied. Soil-dwelling bacteria produce and encounter a myriad of antibiotics, evolving corresponding sensing and evading strategies. They are a reservoir of resistance determinants that can be mobilized into the microbial community. Study of this reservoir could provide an early warning system for future clinically relevant antibiotic resistance mechanisms.

An important recent if you're interested in more in depth learning.

Garbeva P, van Veen JA, van Elsas JD. Microbial diversity in soil: selection microbial populations by plant and soil type and implications for disease suppressiveness. 2004. **Annu Rev Phytopathol**. **42**:243-70.

Abstract:

An increasing interest has emerged with respect to the importance of microbial diversity in soil habitats. The extent of the diversity of microorganisms in soil is seen to be critical to the maintenance of soil health and quality, as a wide range of microorganisms is involved in important soil functions. This review focuses on recent data relating how plant type, soil type, and soil management regime affect the microbial diversity of soil and the implication for the soil's disease suppressiveness. The two main drivers of soil microbial community structure, i.e., plant type and soil type, are thought to exert their function in a complex manner. We propose that the fact that in some situations the soil and in others the plant type is the key factor determining soil microbial diversity is related to the complexity of the microbial interactions in soil, including interactions between microorganisms and soil and microorganisms and plants. A conceptual framework, based on the relative strengths of the shaping forces exerted by plant and soil versus the ecological behavior of microorganisms, is proposed.

Week 2 Student Protocols – Microbial Diversity in Virginia Forest Soils

Overview: This week you will 1) assess the success of your PCR amplification of 16 S *rrnA* and 2) Clone the PCR amplicon into the plasmid vector. PGemT-Easy.

For reasons of time efficiency, the enzymatic reactions that ligate your PCR amplicons into the plasmid vector. PGEM T Easy, have been done for you. They were accomplished as follows:

-PCR Amplicons were purified via a commercially available affinity column matrix– (QIAquick PCR Purification Kit – Qiagen Inc.).

Ligation reactions: (done for you – *you're welcome!!*)

-3 μ l purified PCR amplicon

-5 μ l 2X Ligase Buffer (Promega Inc) – includes salts and ATP as energy source for T4 DNA Ligase enzyme.

-1 μ l T4 DNA Ligase (3 Units enzyme) – Promega Inc.

-1 μ l pGEM T-Easy (50ng) Promega Inc.

10 μ l – incubated at 20°C over night.

Each of you will use 5 μ l of one of these DNA Ligation reactions and transform a “**competent**” preparation of the *Escherichia coli* strain, DH5 α . Competent refers to the ability of a bacterium to take up free DNA from the environment. While many species of bacteria are “**naturally competent**” to take up external DNA, *E. coli* is not. Therefore, we have had to rough up *E. coli* a bit in order to convince it that it is in its best interest to cooperate and take up DNA!! (In this case, if they don't take up the DNA we feed them, we will kill them!!).

Add 5 μ l of ligation reaction to a labeled 15 ml Falcon tube that is on ice (make certain to place the ligation mix at the very bottom of the tube).

Next add 50 μ l of competent *E. coli*, DH5 α to the bottom of the tube, cap it, flick gently to mix and place on ice for 30 minutes. This incubation allows the plasmids to adhere to the surface of the Ca⁺⁺ treated, competent *E. coli* cells.

After 30 minutes on ice, heat shock the mix by incubating in a 42°C water bath for 90 seconds. This helps drive the plasmids across the cell envelope into the cytoplasm where the plasmid can replicate and begin to express its genes.

Outgrowth

Next, you will perform an **outgrowth step**. Here you will add 1 ml of Luria-Bertani (LB) broth ***with no antibiotics***. This mix will be allowed to grow at 37°C for 30 minutes. Here, all DH5 α cells, whether they were transformed with a plasmid or not, will grow. The point here is to allow those cells that did take up a plasmid during the transformation step, to express the gene whose product makes them resistant to the antibiotic, ampicillin. This is important as the next step places these cells onto a growth medium that contains ampicillin. Without this outgrowth step, it is possible that the ampicillin may kill the cells even if they have successfully taken up the plasmid, because until the β lactamase gene (*bla*) is expressed, the cells are sensitive to the effects of this antibiotic.

After the 30 minute outgrowth, you will plate 200 μ l of this culture onto Luria Bertani Agar plates that contain 100 μ g Ampicillin/ ml of medium. This will kill the *E. coli* cells that have not taken up the plasmid, but allow those with plasmid pGEM T Easy to grow.

There is another important chemical that has been added to this medium, 5-Bromo-4-Chloro-3-Indoyl- β -D galactopyranoside. Thankfully, this reagent is more frequently referred to as **X-GAL**. This reagent allows the differentiation of transformed DH5 α cells that have either **1)** taken up a pGEM T-Easy plasmid possessing an inserted PCR amplicon (**resulting in a clear colony**) or **2)** have taken up a pGEM T-Easy plasmid that does not have a cloned PCR amplicon (**resulting in a Blue colony**).

The reason for this is that *E. coli* DH5 α possesses a defective copy of the gene *lacZ* that encodes the enzyme β Galactosidase that breaks down the sugar lactose (and the artificial substrate, X-GAL). The pGEM T-Easy plasmid encodes a portion of this enzyme (the β peptide). It is within this β peptide-encoding gene that you are cloning your PCR amplicons. If an amplicon inserts here, it disrupts the β peptide and the resulting *E. coli* will not be able to break down X-GAL from its colorless form ***so the colony remains clear – this is a good result!!*** If, on the other hand, the pGEM T-Easy plasmid fails to ligate in a PCR amplicon, the β peptide-encoding region is restored and can “***complement***” the defective enzyme of DH5 α . This functional enzyme (β galactosidase) breaks down X-GAL to a blue product and the colony turns brilliant blue. ***As pretty as these colonies are, these are not the colonies you want!!***

You will sterilely spread this mixture over the surface of the plate, allow it to dry and your TA will incubate this plate overnight in a 37°C incubator.

You will have to come back to the lab on your own time to pick a single clear colony into a 5 ml broth tube containing LB broth with ampicillin. This will only take 5 minutes of your time. Your TA will tell you the times available for you to return to pick your clear colony. Once you have inoculated this colony into the 5 ml of LB/amp broth, it will be grown overnight at 37°C with rapid shaking (for adequate aeration of culture). Your TA will then harvest the *E. coli* cells by centrifuging the culture at $\sim 5000 \times g$ for 15 minutes, discarding the culture supernatant and will freeze your pellet of *E. coli* cells. Next week,

you will purify the plasmid from these cells and perform Dye-Terminator DNA Sequencing protocol.

Transformation (short form)

1. in a 15 ml Falcon tube on ice, add 5 ml of ligation reaction
2. add 50 μ l of competent E. coli DH5 α
3. incubate on ice 30 minutes
4. heat shock at 42°C 90 seconds.
5. add 1 ml LB broth and incubate at 37°C 30 minutes (Outgrowth)
6. place 200 μ l of outgrowth in center of LB/ampicillin/X-GAL plate and spread evenly using sterile spreader.
7. plate is incubated @37°C overnight.
8. comeback and pick a single clear colony using disposable sterile loop and use this to inoculate a tube of LB/Ampicillin broth – this will grow over night and be harvested for you by centrifugation.

Checking the success of your PCR: Amplicon present in your sample?

While your transformation is proceeding (during one of the 30 minute incubations above), you should assess the success of your PCR amplification of the 16S *rrnA* gene fragment from the soil DNA.

-on a piece of Parafilm, spot 5 μ l of a DNA Agarose Gel Loading Buffer (a blue buffer containing a blue dye to track the progress of your electrophoresis and 40% sucrose to add density).

-to that 5 μ l spot of Loading buffer, pipette in 10 μ l of your PCR reaction and mix by gently pipetting back and forth a few times.

-load all 15 μ l of this mix into one lane on the agarose gel your TA has provided. Be certain to write down your sample number by which lane you've loaded your sample in on the sign-up sheet as well as in your lab notebook.

-Once all lanes have been loaded, electrophoresis will be done at 110 Volts for 30 minutes. Your TA will take a digital image of the gel and it will be made available via BlackBoard. Your TA will notify you when it is available.

Analysis:

Print the gel picture out and paste it into your lab notebook and label it appropriately.

Plasmid Purification & DNA Sequencing

Bio 203 Forest Soils Microbial Diversity Research Project – Week 3

Purpose: To extract and purify the plasmids from each of your *E. coli* clones containing (hopefully!) a portion of the 16S *rrnA* gene of one of the soil dwelling bacteria as well as to determine the precise DNA sequence of the cloned PCR amplicon.

PART 1 – Plasmid Extraction and Purification (Caution, this requires that you kill millions of bacteria! Not for the faint of heart!!)

- a. your bacteria have been harvested by centrifugation by your TA in a tabletop clinical centrifuge. They were centrifuged at ~ 5,000 x g for 10 minutes.
- b. the clear supernatant was discarded leaving behind the *E. coli* cells, now incredibly dizzy, at the bottom of the tube. The cell pellet has been frozen at -20°C until today. Using a P 1000 pipette, add 250 ul of Buffer P1 (Qiagen Inc.). Resuspend the cell pellet by trituration and then remove the full volume and place into a 1.5 ml epi tube that was previously labeled with your initials
- c. add 250 ul of Buffer P2. This is a lysis buffer and contains NaOH and a detergent, sodium dodecyl sulfate (SDS) that lyses the *E. coli*. Mix by closing the cap on the epi tube and gently inverting 4-6 times. The SDS/ NaOH combination dissolves the membrane and denatures bacterial proteins.
- d. Next, add 350 ul of Buffer N3, close the cap and mix by inversion 4-6 times. A white precipitate will form. N3 contains potassium acetate which neutralizes the alkaline pH generated by the NaOH of the lysis buffer, but also substitutes K⁺ for Na⁺ in the SDS detergent. Potassium salts of this detergent (potassium dodecyl sulfate) are insoluble and this is the precipitate you see. It is also precipitating the cell wall, protein and genomic DNA of the *E. coli*. The plasmids are unaffected.

Now spin in a microcentrifuge at maximum speed for 10 minutes (note that this is not the same centrifuge used to harvest the bacteria in step a).

- e. While spinning, retrieve and label a Qiagen Mini Prep Spin Column along with an accompanying collection tube. At this point you need to adopt a uniform plasmid labeling protocol to avoid future confusion. Each of you is preparing a plasmid and should label them using your lab section # and your letter. Plasmid designations always begin with a lower case “p”. For instance, p5H would be the designation of the person given the letter H in lab section #5.

After microcentrifuge is done, carefully decant (pour) your supernatant into the appropriate spin column and place back in the microcentrifuge. Spin at max speed 1 minute. Discard the flow through in the bottom collection tube.

- f. You will use the same collection tube for this next step. Add 750 ul of Buffer PE into the columns. This is the column wash buffer and will remove bacterial products left in the column, but will leave your plasmid bound to the column matrix. Spin at max speed, 1 minute. Then discard the flow through. Place the column back in the same collection vial and spin an additional minute without having added anything else. This ensures that the PE buffer has been removed completely. At this point (after this 1 minute spin), save the columns, and discard the collection tubes.
- g. Place each of your columns into a 1.5 ml epi tube (REMEMBER TO LABEL THIS TUBE WITH YOUR PLASMID DESIGNATION) and add 50 ul of Buffer EB, the Elution Buffer, to the center of the column matrix. Let stand for 1 minute and then centrifuge at max speed for 1 min. The flow-through this time is your purified plasmid, ready for DNA sequencing. ***Label well!!***

PART 2 - only 4 plasmids per lab section will be sequenced. Your TA will determine which plasmids get sequenced. Eventually all plasmids will be sequenced, but you'll be gone for the summer by the time they are finished!

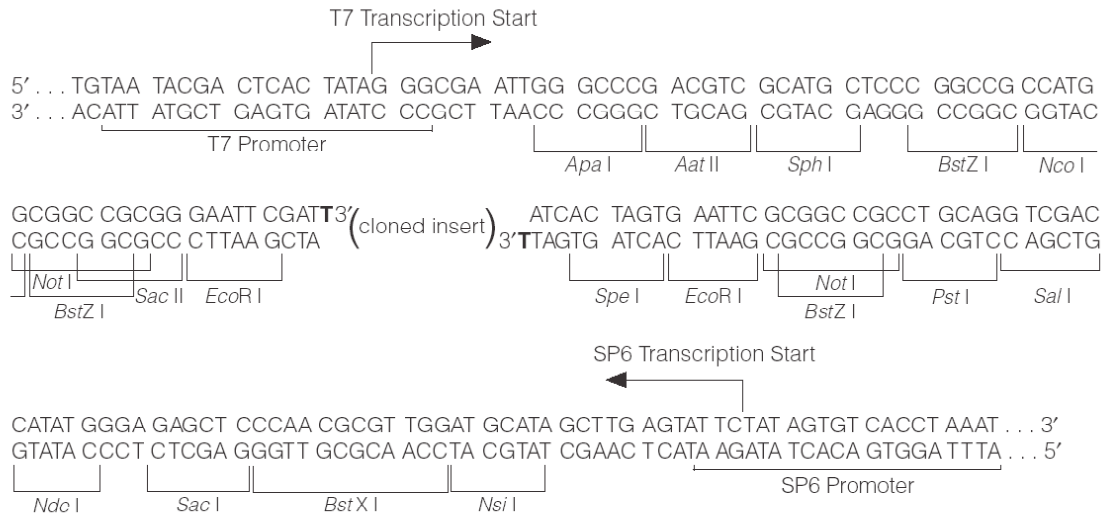
Sequencing reaction Protocol

3 ul plasmid (should be ~ 200-300 ng of plasmid)
2 ul sequencing primer (SP6 **OR** T7)
2 ul Big Dye Reactant
4 ul 5X Big Dye Terminator Reaction Buffer
9 ul sdH2O

20ul Total Volume

- mix gently and pulse down briefly in a microcentrifuge to ensure all reactants are at bottom of tube.

pGEM[®]-T Easy Vector



T7 primer -- 5' GTAATACGACTCACTATAGGGC 3'

SP6 primer - 5' ATTTAGGTGACACTATAG 3'

Cycle Sequencing Program

Hot Start: 94⁰C for 5 min

26 cycles of: 94⁰C/45 sec, 50⁰C/30 sec, 60⁰C/60 sec

Then; hold on 10⁰C.

The way cycle sequencing works:

When your plasmid is heated to 94⁰C, the double stranded DNA “melts” into two ssDNA circles.

Each ssDNA molecule is now available to base pair with one of the sequencing primers, T7 or SP6. Note that each of these primers will hybridize (base pair) with a different single strand of the melted (denatured) plasmid. This base pairing of primer and ssDNA plasmid occurs during the 50⁰C annealing step.

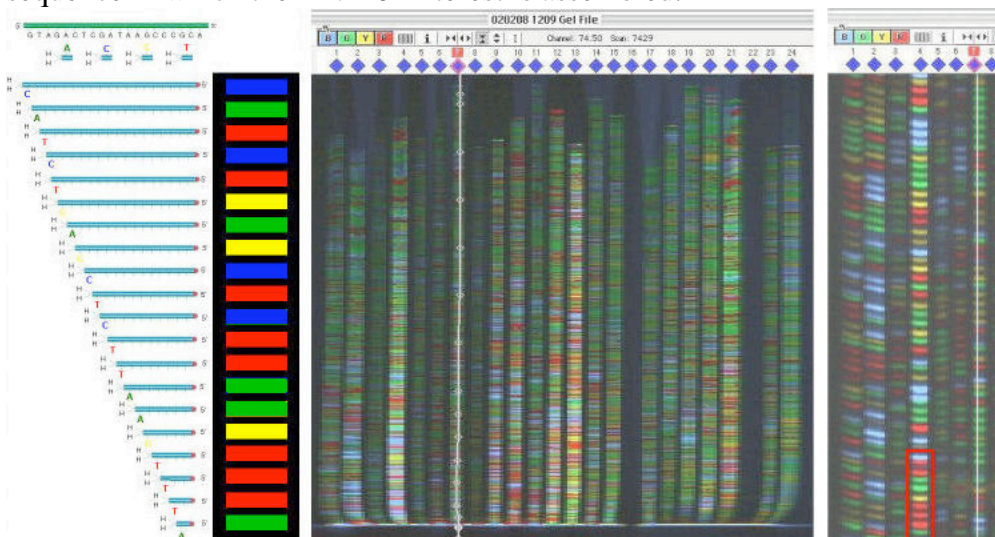
The third temperature is the extension temperature. At this temperature, the thermostable DNA polymerase in the reaction will extend either T7 or SP6 creating a complementary copy of that strand.

This cycle of 3 temperatures is repeated 25 more times.

If this sounds a lot like the PCR reactions you already did, you're right. But there are some critical differences between this cycle sequencing reaction and your earlier PCR. Here in cycle sequencing, you are using only a single primer, not two as in a typical PCR and this sequencing primer (either T7 or SP6) hybridizes to the vector (plasmid) sequence, not your cloned gene,

The second major difference between the cycle sequencing reaction you're performing today and the PCR done previously lies in the nature of the nucleotides in the reaction. In a **PCR** reaction, all four deoxyribonucleotides (dNTPs – dATP, dTTP, dGTP, and dCTP) exist in their natural state. In the Big Dye Cycle Sequencing reagent from Applied Biosystems, there exist these same dNTPs, but also smaller quantities of dideoxynucleotides (ddNTPs – ddATP, ddTTP, ddGTP, and ddCTP). These nucleotides are **lacking the 3'OH group** that is critical to formation of the next phosphodiester bond. So when one of these ddNTPs is incorporated into the growing chain of DNA being synthesized, the chain is terminated because of the absence of a 3' OH group.

These chain terminating ddNTPs have another characteristic besides the lack of 3' OH's. Each of the four different species of ddNTP possesses a different fluorescent dye. In this way, when a DNA chain that has been terminated with a ddTTP is hit with a laser, it will fluoresce red, while a DNA chain terminated due to the incorporation of ddGTP will fluoresce yellow (chains terminated by ddATP and ddCTP fluoresce green and blue, respectively). In this way a capillary electrophoresis apparatus (located on the 3rd floor of Millington – **an Applied Biosystems Avant 3100 Automated Fluorescent DNA Sequencer**) can distinguish DNA chains based on their size as well as the color with which they fluoresce. This can be used to assemble a chromatogram showing the sequence in which the DNA of interest is assembled.





We (Penny, myself, your TA's) will perform the purification of your reactions when they are completed because the excess dye terminator ddNTPs must be removed prior to loading your sequencing reactions on the ABI 3100 Automated Fluorescent DNA Sequencer.

Purification of Sequencing Reactions

Instructions for using DTR (Dye Terminator Removal) Gel Filtration Cartridges:

1. Spin cartridge for 2 minutes at 3000 RPM in micro-centrifuge
2. Transfer cartridge to a new, sterile 1.5 ml ependorf tube and add your 20 ul of sequencing reaction to the center of the column bed
3. Close the cap and centrifuge for 2 minutes at 3000 RPM.
4. Remove cartridges and discard
5. Dry eluate your samples in the Speed-Vac, 20-25 minutes using lowest heat setting (slowest drying rate).

Samples will then be resuspended in dimethylformamide (DMF) and applied to the ABI Avant 3100 Fluorescent Sequencer. Your TA will provide you with the sequences of the DNA from your lab section for web-based database searching in the lab next week.

DNA Sequence Analysis – Week 4

Objective: To use web-based database search algorithms to determine the identity of the sequences of your PCR amplicons cloned into the plasmid clones.

The identity here will be used as our initial assessment of what bacteria are present in the relatively undisturbed portion of the oldest growth portion of the college woods.

Once you have received your DNA sequence that you generated, you need to proceed to the following website:

<http://www.ncbi.nlm.nih.gov/BLAST/>

Once there, under the “Special” subheading, click “Screen for vector Contamination (VecScreen)”

This site will determine which portion(s) of the sequence you generated is from the plasmid and which portion is from the DNA you cloned in (*H. pylori*, strain J68).

Copy and paste your entire sequence into the search window and click “Run VecScreen”

The next screen that appears, requires you to click on the button that says “Format”. What comes back is a reasonable estimation of where your cloned fragment begins. You can disregard that portion of the sequence that the program tells you is part of the plasmid. Actually, discard those sequences (it is usually the first 20-50 bases). If your cloned fragment is small, you may find vector (plasmid) sequence at the far end as well. This indicates you may have sequenced all the way through your cloned insert. You can discard that plasmid sequence as well. If you have cloned the targeted full length PCR amplicon of 529 bp, it is doubtful that your DNA sequencing reaction will have proceeded through the entire cloned amplicon, but it is possible.

Go back to the original web page and now, under the “Nucleotide” heading, click on “Nucleotide-nucleotide BLAST (blastn) – BLAST stands for Basic Local Alignment Search Tool.

Copy and paste your that portion of your DNA sequence that is not part of the vector (plasmid) in the search window. This sequence should now NOT contain any vector (plasmid) sequence. Leave the database selection as “nr”; this means non-redundant. When you now click BLAST button, it will search your sequence against every DNA sequence in the non-redundant DNA database, which is every known sequence from every known organism to date. Again, the first screen returned to you will require you to click the “format” button.

It may take several minutes depending on user traffic at that moment, but what is returned to you is a hierarchal list of sequences that match your sequence to varying degrees. If you scroll down, it will show you the actual alignments and you can see how closely the sequence you generated is to any of the related sequences found in the database. Remember, your sequence is referred to as “Query”. Each match contains the corresponding numbers and a hypertext link that will take you to the database entry that matches your sequence. You will there, by matching up numbers, be able to determine the name (or more often, the number), of you homologous gene. That’s it!!