

Overview of Part II

Although we will be performing a number of apparently unrelated molecular experiments in this section, in reality each experiment will be a component of a larger experiment in environmental microbiology. Thus, this section is essentially a project whose goal is the profiling of bacterial communities in the environment using both phylogenetic and metabolic genes. Community profiling is the study of bacterial diversity and population structure in the environment and is based on the molecular analysis of total environmental genomic DNA and mRNA. Several molecular techniques form the foundation on which community profiling is built including PCR, cloning, sequencing, and hybridization analysis. We will be performing most of these techniques over the course of the next several weeks, along with other standard molecular techniques such as agarose gel electrophoresis and computer database analysis.

Community metabolic profiling involves utilizing a number of key metabolic genes to assess the abundance and diversity of bacteria in an environment capable of that metabolism. For example, one can determine the number of different bacterial species capable of sulfate reduction in an environmental sample by characterizing via PCR and sequence analysis the dissimilatory sulfite reductase genes *dsrAB*. In the simplest approach, total genomic DNA is first isolated from an environmental sample such as sediment or water. All of the *dsrAB* genes in that sample are then PCR amplified, cloned and sequenced. The sequences are analyzed to determine the number of different species capable of sulfate reduction present in that environment based on the assumption that each species will have a different *dsrAB* sequence.

Phylogenetic profiling of a bacterial community utilizes the 16S rRNA gene to assess the total species diversity in any given environment. In addition to documenting the vast bacterial diversity found on our planet and examining their biotic and abiotic interactions, this approach is particularly useful when comparing similar environments that differ in one or a few key environmental parameters. For example, let's say you were interested in isolating and characterizing microbes capable of degrading benzene, a toxic contaminant, under anaerobic conditions. By comparing the bacterial 16S rDNA profiles obtained from a site contaminated with high concentrations of benzene to a neighboring site that has little or no benzene, one may be able to identify and subsequently isolate those bacterial species only present (or more abundant) in the benzene-contaminated samples.

Of course, both metabolic and 16S rDNA primer sets (for amplification) or probes (for hybridization) can also be used on individual bacterial isolates to identify the species (using the 16S rRNA gene) or determine the metabolic potential of the isolate (using a metabolic gene). Given the utility of both 16S rDNA and metabolic genes in environmental microbiological analysis, we will be focusing our molecular biological efforts of the next section of the course on these issues.

In the next section, you will learn how to:

- Isolate total genomic DNA from an environmental soil sample
- Test for the presence of different bacterial groups in your sample using PCR to amplify 16S rRNA genes and specific metabolic genes
- Clone rRNA genes
- Construct a non-radioactive probe of a metabolic gene
- Perform a DNA hybridization
- Use computer analysis to phylogenetically identify the source of a 16S rDNA sequence.

Experiment: Isolation of Total Genomic DNA

In order to analyze the different genes that are present in an environment, we must first be able to extract the genomic DNA from an environmental sample. This previously laborious process has been rendered quick and easy with the advent of specialized kits that rely on physical (not chemical) cell lysis and silica beads that trap DNA. The kit we will be using consists of three general components:

1. A lysing matrix composed of ceramic and silica particles. When used in a high-speed mechanical shaking device (called a Bead Beater), these particles lyse all microorganisms in a sample, even bacterial spores and fungi.
2. Homogenization reagents for complete sample homogenization and protein solubilization. These reagents enable the extraction of genomic DNA with very little RNA contamination (that could titrate away some of the primers in the subsequent PCR steps).
3. DNA purification and elution reagents including a silica-bead matrix that binds DNA and eliminates contaminants that may inhibit subsequent PCR reactions.

After performing this procedure, we must prove that DNA is now present in our final sample. This is done by electrophoresing our sample on an agarose gel in order to visualize the DNA. Once we have confirmed the presence of DNA in our sample, we will use this genomic DNA (gDNA) as a template for a series of PCR amplifications.

Experimental Method: Isolation of Total Genomic DNA

Materials per group or individual:

- Environmental soil sample
- Pipettors and tips
- 1.5 ml tubes
- 5 ml snap-cap tubes
- Lysing Matrix E Tube
- Sodium Phosphate Buffer
- MT Buffer
- PPS Reagent
- Binding Matrix Suspension
- SPIN Filter
- SEWS-M
- Catch tube
- DES

DAY 19: Lab Period

1. Add 978 μ l Sodium Phosphate Buffer and 122 μ l MT Buffer to a Lysing Matrix E Tube.
2. Add soil not to exceed 7/8 of the volume of the tube.
3. Give the tube to the TA to process in the Bead Beater for 30 seconds.
4. Centrifuge the tube for 1 min.
5. Transfer supernatant to a clean 1.5 ml tube.
5. Add 250 μ l PPS Reagent and mix by shaking the tube 10 times.
6. Centrifuge for 5 min to pellet the protein precipitate.
7. Transfer the supernatant to a 5 ml. snap-cap tube.
8. Add 1 ml Binding Matrix Suspension.
9. Cap the tube tightly and invert by hand for 2 min to allow binding of DNA to the matrix.
10. Let tube rest for 3 min to allow settling of matrix.
11. WITHOUT DISTURBING THE MATRIX, carefully remove and discard 600 μ l of supernatant.
12. Resuspend matrix in remaining supernatant by vortexing and transfer 650 μ l of the mixture to a SPIN filter.
13. Centrifuge the SPIN filter for 1 min and empty the catch tube.
14. Add the remaining supernatant to the SPIN filter, centrifuge for 1 min and empty the catch tube.
15. Add 500 μ l SEWS-M to the SPIN filter.
16. Centrifuge the SPIN filter for 1 min and empty the catch tube.
17. Centrifuge the SPIN filter for 2 min to "dry" the matrix.
18. Place the filter in a fresh Catch tube and air dry for 5 min.
19. Add 50 μ l DES to the filter, centrifuge for 1 min, discard filter and store the eluted DNA at -20° C.

Experiment: Agarose Gel Electrophoresis

Perhaps the most widely used physical method in all of molecular biology is gel electrophoresis. The idea behind electrophoresis is that positive charges attract negative charges and vice versa. Conversely, two charges of the same sign repel each other. To perform electrophoresis we dissolve molecules which carry electrical charges in water and stick into the solution two electrodes - one positive and the other negative. When we switch on the current, the negatively charged molecules are attracted to the positive electrode and move through the solution until they reach it. The positively charged molecules move in the opposite direction. Molecules carrying charges are known as ions.

The greater the charge, the faster an ion will swim under the influence of an electrical attraction. On the other hand, the larger the molecule the more force needed to get it moving. Molecules of nucleic acid have exactly one negative charge for each nucleotide so these two factors cancel out. Consequently all molecules of DNA or RNA will move at the same speed towards the positive electrode as long as they are free in solution.

If we want to separate our DNA or RNA on the basis of size we must bring in an extra handicap to slow down the larger molecules - the gel. The gel is a meshwork of cross-linked polymer chains - usually of agarose for nucleic acids. The molecules of DNA are slowed down as they try to wriggle through the gaps in the gel meshwork. The larger they are, the harder it is to squeeze through the holes. The result is that a mixture of DNA molecules separates according to size, the smaller molecules moving through the gel much faster than the larger ones.

The sample buffer contains a tracking dye, in our case, bromophenol blue. When the bromophenol blue band nears the end of the gel, we switch off the power supply.

Once we have run our gel, we must stain it to see the DNA bands. This is done with a chemical called ethidium bromide which intercalates in between the nucleotides of the DNA and gives off an orange glow when exposed to ultraviolet light. The major drawback to this sensitive and inexpensive staining method is that ethidium bromide is a powerful mutagen and so we must be very careful when handling gels containing ethidium bromide.

To estimate the molecular weight of a DNA fragment of interest, we must run a DNA marker containing DNA fragments of known molecular weight on the same gel. When the distance that each DNA fragment has migrated is plotted against the logarithm of its molecular weight we get a straight line. The distance migrated by the unknown DNA fragment is also plotted and the log of its molecular weight is read from the plot. That's in theory. In practice, you can easily estimate the size of your DNA fragment by simply comparing it visually to the bands in the marker lane.

(What do you expect to see on the gel when you run your environmental genomic DNA? One band? More than one? High or low molecular weight? Why?)

Experimental Method: Agarose Gel Electrophoresis

Materials per lab:

1% agarose in TBE (1 gram of agarose per 100 ml of 1x TBE) with 100 μg of ethidium bromide added per 100 ml; provided in 40 ml aliquots
Horizontal agarose gel rig
1x TBE running buffer
DNA size marker (1 kb ladder)
DNA samples to be electrophoresed
Agarose Loading Dye
Low voltage power supply
Pipettors, tips, gloves
Transilluminator or other UV light source
Protective eye goggles or shields (MUST BE UV-PROTECTIVE)
Waste container for ethidium bromide-stained gels and running buffer

CAUTION: During this and all labs involving gel electrophoresis of DNA, several potential sources of danger exist. Ethidium bromide (EtBr) is a powerful mutagen and is to be handled with extreme care. Always wear gloves when handling gels containing EtBr and be very careful not to drip any buffer on the bench. Gels containing EtBr are NOT to be thrown in the trash; instead, throw into the waste receptacles provided in class. You will be visualizing your EtBr-stained DNA fragments under short-wavelength UV light which is another mutagen and carcinogen. Always wear protective eye goggles when using the transilluminator as it is possible to damage your retinas with only a few seconds exposure to UV. In addition, always wear gloves when working on the transilluminator to prevent sunburn. Keep the transilluminator on for as short a time as possible since exposure of your skin to strong UV will cause severe sunburns. Finally, the gels are run with fairly high voltage so be sure never to touch the gel or the gel apparatus while the power supply is on.

DAYS 21, 23, 25 and 29: Lab Period

Set up your horizontal agarose gel rig with the gel tray in the pouring position and make sure you have the comb in place. With gloves on, microwave your 1% agarose/EtBr solution for about 2 minutes, until the agarose is completely dissolved. Immediately pour the agarose solution into the gel rig, pouring carefully to avoid introducing any bubbles. Allow the gel to set for 20-30 min.

During the time that your gel is solidifying, prepare your DNAs for loading onto the gel. Pipette 5 μl of your DNA sample into a clean Eppendorf tube. Add 2 μl of Agarose Loading Dye and store on ice until ready to load your gel. Also have your DNA marker on ice and ready to load.

Carefully remove the comb and set the gel tray in the running position (with the wells closer to the negative [black] electrode connection). Pour 1x TBE running buffer in the gel rig until the buffer completely covers the gel. Place the cover on the gel rig, plug the electrodes into the proper outlets in the power supply (black cord to black outlet [negative electrode] and red cord to red outlet [positive electrode]). Turn on the power supply and set the range knob to low. Turn the milliamps to approx. 45-50 mAmps and make sure that the voltage is approx. 80-100 mV. Turn off the power supply and disconnect the gel rig cover. You're now ready to load your gel.

Load 5 μl of the DNA marker in one lane and the 7 μl of your DNA sample + dye in the next lane. Cover the gel rig and turn on the power supply. Run the gel at approx. 45-50 mAmps (80-100 mV) until the dye is about 1 cm from the bottom of the gel. Wearing gloves, remove the gel from the gel rig and place it on the transilluminator. Put on UV-protective eye gear (goggles or a face shield) and turn on the transilluminator to view your DNA.

Experiment: PCR Amplification Using Metabolic and Phylogenetic Primers

The polymerase chain reaction, or **PCR**, is a technique used for amplifying a specific segment of DNA. Since the procedure essentially mimics the bacterial replication process, the reagents involved are simply DNA polymerase, all four dNTPs, primers for both DNA strands, template dsDNA, and an appropriate buffer system. There are three steps involved in each **cycle** of amplification: denaturation, annealing and elongation. During the **denaturation** step, the mixture is heated to 94°C to denature the dsDNA template. The **annealing** step is performed at a lower temperature (usually between 36°C and 60°C) and allows the primer to bind to the complementary sequence on the template strand. The DNA polymerase then extends DNA synthesis from the primer during the **elongation** step. Typically, 30-40 cycles are performed with the amount of DNA in the mixture theoretically doubled in each cycle. In this way, a large amount of target DNA can be synthesized starting from very little (even a few molecules of) template DNA. The one item that transformed PCR from a clever trick into a revolutionary technique was the use of a thermostable DNA polymerase. The one most commonly used is the polymerase isolated from *Thermus aquaticus*, an extremely thermophilic bacterium. This enzyme is stable even at 94°C and so needs to be added only once at the beginning of the cycling. (If you would like to read more about PCR and its technical applications, there are many excellent textbooks that discuss these issues in great detail.)

The power of PCR is that only a very small amount of starting material (template DNA) is needed for a successful amplification. In environmental microbiology, that means that we can theoretically amplify genes from a single bacterium out of the billions that might be present in an environmental sample. And by using primer sets specific to one gene, PCR allows us to ascertain whether or not a specific gene is present in the environment. What will this tell us? Say, for example, you want to determine whether or not there are organisms capable of sulfate reduction in a shallow lake. You could take a sample of lake water and, using PCR primers specific to a gene involved in sulfate reduction, verify the presence or absence of sulfate-reducing bacteria in the lake. That is, if a positive PCR is obtained, you can conclude that there are organisms capable of sulfate reduction in the lake. What this approach will NOT tell us is whether or not those organisms capable of sulfate reduction are actually reducing sulfate. (Using PCR technology, how do you think you might address this question?) Primer sets that are based on genes involved in a specific metabolic pathway are called metabolic primers.

Although in the previous example, a metabolic gene was used to screen an environment for the presence of a specific type of bacteria, it is also possible to use the 16S rRNA gene in this capacity. Because the 16S rRNA has regions of conserved sequence and regions of sequence variation, and because the 16S rRNA sequence tracks the phylogenetic relationships of organisms, bacteria that are closely related to one another share parts of their 16S rRNA sequences that are exclusive to that group of bacteria and are not found in any other group of bacteria. Thus, it is possible to design primer sets that can detect 16S rDNA sequences that are specific to, for example, green sulfur bacteria and won't amplify the DNA of any organisms unless they are green sulfur bacteria. Primer sets based on 16S rDNA signature sequences are called phylogenetic primers.

In this experiment, we will be examining a single environmental sample for the presence or absence of a number of specific bacterial groups using both metabolic and phylogenetic primer sets. Specifically, we will be looking for:

- **Sulfate-reducing bacteria** using a primer set specific to the *dsrA* gene that encodes dissimilatory sulfite reductase.
- **Nitrate-reducing bacteria** using a primer set specific to the *narG* gene that encodes dissimilatory nitrate reductase.
- **Nitrogen-fixing bacteria** using a primer set specific to the *nifH* gene that encodes dinitrogenase reductase.
- **Anoxygenic photosynthetic purple bacteria** using a primer set specific to the *pufM* gene that encodes an essential component of the light-harvesting mechanism.
- **Perchlorate-reducing bacteria** using a primer set specific to the *cld* gene that encodes chlorite dismutase.
- **Pathogenic bacteria** using a primer set specific to the *fur* gene that encodes the ferric uptake regulator protein that controls virulence gene expression.
- **Geobacter species** using a primer set specific to signature **16S** sequences in these organisms.
- **Green sulfur bacteria** using a primer set specific to signature **16S** sequences in these organisms.
- **Shewanella species** using a primer set specific to signature **16S** sequences in these organisms.
- **Archaea** using a primer set specific to signature **16S** sequences in these organisms.

In addition to the above primer sets, we will also be amplifying our environmental samples using a universal bacterial primer set (8F/1394R) that amplifies the 16S rRNA gene from any bacterial species. Since all of our environmental samples (presumably) contain bacteria, we are using this primer set as a positive control to ensure that amplification from our samples is possible and that there are no substances inherent in our sample that will interfere with our PCR reaction.

Experimental Method: PCR Amplification using Metabolic and Phylogenetic Primers

Materials per group or individual:

- Environmental genomic DNA isolated from previous experiment
- DNA for the positive control (see below)
- PCR reagents (water, BSA, buffer, MgCl₂, dNTP mix, Taq polymerase)
- PCR primer sets
- Pipettors and tips
- 0.2 ml tubes
- Thermal cycler

Each group will be given two primer sets. One of the primer sets is for 16S rDNA (primers 8F [forward] and 1394R [reverse]; expected product size is 1,386 bp). For the other primer set, each group will be given a different set as follows. The expected size of the amplification product is in parentheses.

- Group 1: dsr F and dsr R primers (1.9 kb)
- Group 2: narG1960F and narG 2650R (690 bp)
- Group 3: PolF.nifH and PolR.nifH (360 bp)
- Group 4: pufM F and pufM R primers (229 bp)
- Group 5: cld F and cld R primers (518 bp)
- Group 6: fur F and fur R primers (318 bp)
- Group 7: Geo F and Geo R primers (416 bp)
- Group 8: GSB F and GSB R primers (525 bp)
- Group 9: Shew F and Shew R primers (462 bp)
- Group 10: Arch F and Arch R primers (580 bp)

DAY 21: Lab Period

Thaw your frozen environmental genomic DNA. Place all reagents on ice.

Label two 0.2 ml tubes with your group number and the number 1 (for the universal 16S rDNA positive control) or 2 (for the metabolic/phylogenetic primer set). For each reaction, in the 0.2 ml tube add IN ORDER:

- 21 μ l sterile water
- 15 μ l BSA (bovine serum albumin) at 10 mg/ml
- 5 μ l 10x PCR buffer
- 3 μ l 25 mM MgCl₂
- 4 μ l dNTP mix
- 0.5 μ l Forward primer
- 0.5 μ l Reverse primer
- 0.5 μ l Taq polymerase
- 1 μ l environmental sample DNA

Mix well by tapping the tube. Spin in a microcentrifuge (with adapters) for a few seconds. Place the tubes in the thermal cycler. The TAs will start the thermal cycler this afternoon and refrigerate the completed reactions tomorrow morning.

DAY 23: Lab Period

Run your completed PCRs on an agarose gel with your positive controls in the lane right next to your metabolic PCRs. Record the results in your lab notebook.

Experiment: PCR Amplification of 16S rDNA from a Bacterial Culture

The aim of this experiment is to amplify the **16S rRNA gene** from cell pellet of a pure bacterial culture. This differs from our previous experiment in which we amplified the 16S rDNA from an environmental sample in that we only expect a single product from amplification of a pure culture. (What are your expected products when you amplify the 16S from an environmental sample? Are they all the same?) This single PCR product will then be inserted into a plasmid vector to generate a 16S clone.

In order to amplify our DNA from the cell pellet, we must first lyse the cells. This is achieved by adding chloroform to a suspension of bacterial cells in sterile water and then boiling the mixture for several minutes to burst the cells. Once the cells have been lysed, no additional purification of the DNA is necessary before we perform the PCR amplification. Thus, a small amount (1 μ l) of the lysed cells is added directly to our PCR reaction mix. The PCR reaction mix contains the following necessary components:

- Sterile water
- BSA (bovine serum albumin; 10 mg/ml)
- 10x PCR buffer (without $MgCl_2$)
- $MgCl_2$ (25 mM)
- dATP, dCTP, dGTP and dTTP (10 mM each)
- Template DNA (in this case, your lysed cell culture that contains DNA)
- Two primers (a forward primer and a reverse primer)
- Taq polymerase

The primers we will be using are universal for bacterial 16S rDNA, 8F and 1394R. Following amplification in a thermal cycler, we will be electrophoresing the PCR products on an agarose gel to make sure that the amplification worked and that the amplification products are of the expected size (1386 bp). Additionally, we will be using the results from this experiment in another experiment. Your PCR products will be used in a cloning experiment to generate clones of the 16S rRNA genes. It is important that the PCR products be fresh (recently made) in order to ensure that our ligation efficiency is high.

Experimental Method: PCR Amplification of 16S rDNA from a Bacterial Culture

Materials per group:

- Frozen bacterial cell pellet
- Sterile water
- Chloroform
- Heating block set to 95-100°C
- PCR reagents
- Primers 8.F and 1394.R
- Pipettors and tips
- 0.2 ml tubes
- Thermal cycler

DAY 23: Lab Period

Thaw your frozen cell pellet. Add 40 μ l of sterile water and vortex well to resuspend pellet. Add 5 μ l of chloroform and vortex. Heat at 95-100°C for 10 min. Place on ice.

In a separate 0.2 ml tube, add IN ORDER:

- 21 μ l sterile water
- 15 μ l 10 mg/ml BSA
- 5 μ l 10x PCR buffer
- 3 μ l 25 mM MgCl₂
- 4 μ l dNTP mix
- 0.5 μ l 8.F primer
- 0.5 μ l 1525.R primer
- 0.5 μ l Taq polymerase
- 1 μ l lysed cell culture

Mix well by tapping the tube. Spin in a microcentrifuge (with adapters) for a few seconds. Place the tube in a rack and freeze.

DAY 25: Lab Period

The TAs will cycle your PCR reactions in a thermal cycler (with a heated cover) this morning so that the amplifications are complete by the lab period this afternoon.

Transfer 5 μ l of your PCR reaction to a fresh 1.5 ml tube and refrigerate the remaining PCR product. Run the 5 μ l of your PCR reaction on an agarose gel to make sure it worked before using it in the cloning experiment (below).

Experiment: Cloning of 16S rDNA PCR Products

Cloning is the process of amplifying a select fragment of "foreign" DNA by inserting the fragment into a vector DNA molecule, transforming a host bacterium with the new recombinant DNA molecule, and allowing the host cell and, therefore, the recombinant DNA molecule, to replicate many times over. One of the critical factors in any cloning experiment is the choice of **cloning vector**. Cloning vectors are artificially constructed DNA molecules that are originally derived from naturally-occurring plasmids or bacteriophage genomes. These small DNA molecules are constructed in such a way that foreign segments of DNA can be stably and reversibly inserted.

All successful (*i.e.* truly useful) cloning vectors contain a number of components that make the cloning process faster and more convenient. These include:

- *ori*: an **origin of replication** for a specific host organism, usually *E. coli*, that allows the vector to be replicated multiple times independent of host genome replication events
- *lacZ*: a gene that encodes β -galactosidase whose expression is under control of the Lac promoter. Colonies or plaques that produce β -galactosidase appear blue on medium containing X-Gal (5-Bromo-4-chloro-3-indoyl- β -D-galactopyranoside). In recombinant vectors, the *lacZ* gene has been disrupted by insertion of foreign DNA. Colonies harboring recombinant vectors are, therefore, unable to produce β -galactosidase and appear white on X-Gal medium. The ability to visually distinguish recombinant vs. non-recombinant vectors in this way is called **blue/white color selection**.
- MCS: a **multiple cloning site** containing unique sites for a number of restriction enzymes
- Antibiotic^R: an **antibiotic resistance gene** for selection of successfully transformed host cells. Usually Amp^R or Tet^R.

In this particular experiment, we will be cloning a PCR amplification product into a special plasmid vector, a TOPO TA vector, specifically designed for rapid cloning of PCR products. Taq polymerase, the DNA polymerase we used in our PCR experiment, is not a typical bacterial DNA polymerase in that it will put a template-independent adenosine nucleotide at the 3' end of each DNA strand in the reaction. The manufacturers of the TOPO TA vector have taken advantage of this quirk of Taq polymerase by creating a linear DNA fragment with a single T overhang on the 3' end that is complementary to the A overhang on the 3' ends of the PCR products. Thus, the PCR product can be ligated with high efficiency into the TOPO TA cloning vector.

The *E. coli* host strain to be used with the TOPO TA cloning vector is TOP 10 that has the genotype: F⁻ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *deoR* *araD139* Δ (*ara-leu*)7697 *galU* *galK* *rpsL* (Str^R) *endA1* *nupG*. TOP 10 does not express the Lac repressor; therefore, your product may express from the TOPO TA cloning vector in the absence of IPTG, a gratuitous inducer of the Lac operon, because there is no Lac promoter that needs to be inactivated. Thus, blue/white color selection can be achieved with only X-Gal added to the plates.

The goal of this experiment is to clone your bacterial 16S rDNA PCR product into the TOPO TA cloning vector. This will be achieved in several steps including ligation of your insert DNA (in this case, your PCR product) into the vector DNA, transformation of competent *E. coli* cells with the recombinant vector, growing the *E. coli* cells in non-selective media to allow for phenotypic expression of the antibiotic resistance marker encoded by the vector, and plating of the transformed *E. coli* cells onto selective antibiotic plates.

We are lucky in that we will be using a vector that has been designed not only to accommodate PCR products but has also been designed to rapidly ligate the insert DNA into the vector DNA WITHOUT DNA ligase. So instead of _____ ligating overnight as is typically done with DNA

ligase, the ligation step only takes 5 minutes! This ligation step is performed by a topoisomerase that is covalently bound to the vector. Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5'-CCCTT-3' in one strand. The energy from the broken phosphodiester bond is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, reversing the reaction (that is, creating a phosphodiester bond) and releasing the topoisomerase.

Transformation is a method of genetic exchange among cells in which the recipient takes up "naked" DNA from the environment. The donor must release DNA, perhaps by lysing, or DNA may be purified by a microbial geneticist. Bacterial transformation was discovered in 1928 by Griffith using *Streptococcus pneumoniae*. It was not until the early 40's, however, that Avery and his colleagues proved that the "transforming principle" was DNA. It was shown that the recipient takes up the DNA and can subsequently undergo recombination. Bacteria that have the ability to take up DNA are said to be competent. Many species of *Streptococcus*, *Thermus*, *Bacillus*, and other genera are naturally competent. That is, in a given culture some fraction of the bacteria will be competent. This fraction can often be increased by media or temperature shifts. *Escherichia coli*, though long the bacteria of choice for most genetic studies, was never observed to be competent. This changed in 1970 when Mandel and Higa observed transformation by using a competency regime involving starvation at 0°C in the presence of CaCl₂ and then a heat pulse to induce the cells to take up DNA. Other common chemical methods of producing competent *E. coli* cells have since been developed. A relatively new procedure called electroporation involves use of an electric field to reversibly permeabilize the cell. Electroporation has been used to transform a variety of bacterial, fungal, mammalian and plant cells and many recent papers describe genetic manipulations involving electroporation to produce competent cells.

One can transform *E. coli* with naked chromosomal DNA and look for recombinants as was done in the early studies with *Streptococcus*. In *E. coli*, though, it is usually easier to use conjugation or transduction to obtain the recombinants. However, one doesn't need to use chromosomal DNA. One can use plasmid DNA, transform and select for a plasmid-encoded phenotypic trait (no recombination is necessary). This is an extremely valuable technique since in genetic engineering one often makes new plasmids *in vitro* and introduces them into host cells. Transfection is the term used when naked phage DNA is employed.

In this experiment, we will transform a specialized strain of *E. coli*, TOP10, with our recombinant TOPO TA cloning vector. The TOPO TA cloning vector contains an origin of replication, both Amp^R and Kan^R markers (present in both recombinant and nonrecombinant plasmids) and a multiple cloning site (MCS) located within the *lacZ* gene for blue/white color selection.

Any cell that has taken up the plasmid vector (either recombinant or nonrecombinant) will be resistant to ampicillin and kanamycin but only those cells that have been transformed with a recombinant plasmid will produce white colonies on X-Gal. We will transform competent cells (supplied in the TOPO TA cloning kit) and select both for kanamycin resistance and white colonies in the presence of X-Gal. Following transformation, we will need to allow the cells time to express the plasmid-encoded phenotypes before plating onto selective media.

Experimental Method: Cloning of 16S rDNA PCR Products

Materials per group:

Reagents from the TOPO TA Cloning Kit (Catalog # K4600-01, Invitrogen):
TOPO TA cloning vector DNA, salt solution, sterile water, SOC medium and chemically competent TOP10 *E. coli* cells
PCR amplification products from previous experiment
SOC medium
2 LB Kan X-Gal plates
Pipettors, tips, tubes
Ice buckets with ice
42°C water bath
37°C shaking incubator
37°C non-shaking incubator

DAY 25: Lab Period

1. In a clean Eppendorf tube, combine the following ligation mix IN ORDER:

1 μ l fresh PCR product (no more than 24 hours old)
3.5 μ l sterile water
1 μ l salt solution
0.5 μ l TOPO TA cloning vector DNA at 25 ng/ μ l

Tap the tube to mix well and spin for a few seconds. Incubate the tube at room temperature for 5 minutes then place on ice.

2. Thaw one tube of chemically competent TOP10 *E. coli* cells on ice.
3. AS SOON AS THE CELLS ARE THAWED, add 2 μ l of the ligation mix (above) and mix by stirring gently with your pipette tip.
4. Incubate on ice for 10 min.
5. Place the tubes in the 42°C water bath for exactly 30 seconds and immediately put the tube on ice.
6. Add 250 μ l of SOC medium to the tube.
7. Shake the tube horizontally 200 rpm at 37°C for one hour.
8. Plate out 50 μ l of your transformation mix on one LB Kan X-Gal plate and 5 μ l on the other plate. Incubate the plates upside down at 37°C overnight.

Experiment 13: Preparation of Plasmid DNA

Because plasmids, by definition, contain their own *ori*, they are capable of being replicated independent of the host genome. This means that for every round of genomic DNA replication, the plasmid may go through hundreds of rounds of replication. Thus, *E. coli* becomes a miniature plasmid factory and, depending on the plasmid copy number, any single host cell may contain hundreds of copies of the same plasmid. This is good news for the molecular biologist as all we have to do is get one copy of a plasmid into a single *E. coli* cell, grow up that cell to stationary phase, and the *E. coli* culture has made millions of copies of the plasmid for us to use for further analysis.

We will be purifying the plasmid DNA using a column-based kit that employs a modified alkaline-lysis approach. In this strategy, bacterial cells are harvested and resuspended in buffer containing RNase A (Buffer P1) before being lysed in alkaline conditions with NaOH and SDS (Buffer P2 in the kit). The SDS solubilizes the phospholipid and protein components of the cell membrane while the NaOH denatures the DNA and proteins. It is important that you add the Buffer N3 immediately after mixing in the lysing Buffer P2 because the short lysis time preferentially allows the release of small plasmid DNA rather than the much larger chromosomal DNA from the cell. After the addition of a neutralizing high-salt buffer (Buffer N3), the cell debris (containing the chromosomal DNA) and proteins are precipitated while the small plasmid DNA renatures under the neutral conditions and stays in solution. Following centrifugation to pellet the cell debris and proteins, the plasmid DNA is bound to a positively-charged silica matrix and purified.

In this experiment, you will be able to determine whether you have successfully cloned your 16S rDNA PCR insert into the TOPO TA cloning vector by extracting plasmid DNA from two positive colonies, digesting the DNA with a restriction enzyme that should digest the plasmid only once (in this case, HindIII), and running the digests on an agarose gel to determine the size of your plasmid. If the plasmid is 3.9 kb, you have not cloned an insert into it. However, if the plasmid is 5.4 kb, you have successfully inserted the 1.5 kb PCR amplification product into the 3.9 kb TA cloning vector.

Experimental Method: Preparation of Plasmid DNA

Materials per group:

Pipettors, tips, tubes, gloves
QIAprep Spin Miniprep Kit by Qiagen (cat. #27106)
TA prep: Add RNase to Buffer P1 and ethanol to Buffer PE.
ddH₂O
Hind III and 10x HindIII buffer
1% agarose gel in TBE with ethidium bromide
1x TBE gel running buffer
Agarose loading dye
Transilluminator

DAY 26: Unscheduled Lab

Pick two white colonies from your plates; put each into a sterile tube containing 2 ml of SOC + Kan. Incubate at 37°C with shaking overnight. The TAs will remove your tubes the next day and refrigerate them.

DAY 27: Lab Period

Today we will be preparing plasmid DNA using the QIAprep Spin Kit and digesting your plasmid with HindIII.

1. Transfer 1.5 ml of the culture to a microcentrifuge tube and spin for 5 minutes in a microcentrifuge. Pour off supernatant.
2. Resuspend pelleted bacterial cells in 250 μ l of Buffer P1 and vortex to completely resuspend the cell pellet.
3. Add 250 μ l of Buffer P2 and invert tube gently 10 times to mix. Do not vortex.
4. Immediately add 350 μ l of Buffer N3 and invert tube immediately 10 times to mix.
5. Centrifuge for 10 minutes at maximum speed in tabletop microcentrifuge.
6. Transfer the supernatant (about 850 μ l) from step 5 to a QIAprep column by pipetting.
7. Centrifuge the column for 1 minute. Discard the flow-through.
8. Wash the QIAprep column by adding 500 μ l Buffer PB. Centrifuge for one minute and discard the flow-through.
9. Wash the column again, but this time use 750 μ l Buffer PE (NOT PB). Centrifuge for one minute and completely discard the flow-through.
10. Centrifuge for an additional 5 minutes to remove residual wash buffer.
11. Place the QIAprep column in a clean 1.5ml microcentrifuge tube. To elute DNA, add 50 μ l Buffer EB to the center of the column, let stand for 2 minutes and then centrifuge for 1 minute.

Now, you will digest your clones with the restriction enzyme HindIII.

For each clone, in an Eppendorf tube add in order (total reaction volume is 20 μ l):

13.5 μ l ddH₂O
4 μ l plasmid DNA
2 μ l 10x Hind III buffer
0.5 μ l HindIII

Mix well, spin briefly, and incubate at 37°C until the next lab period.

DAY 29: Lab Period

Today we will be electrophoresing our digested plasmid DNA to see if the plasmid contains an insert. Add 4 μ l of Agarose Loading Dye to your restriction digests and load onto a 1% agarose gel. Load 5 μ l of DNA marker in the next lane. Run the gel until the dye is 1 cm from the bottom of the gel. The TAs will make a digital image of your gel.

Experiment: Nonradioactive Labeled Probe Construction

The goal of this experiment is to prepare a labeled probe for use in an upcoming dot-blot hybridization experiment. (For a discussion of the hybridization technique, please refer to the Dot Blot experiment.) Although radioactive isotopes like ^{32}P and ^{35}S are widely used for labeling hybridization probes, nonradioactive labeling techniques are rapidly gaining popularity because of their convenience, safety, and sensitivity. Thus, nonradioactive procedures are ideal for a laboratory classroom setting such as this. We will be using a steroid-based label called digoxigenin (DIG) that is supplied as DIG-11-dUTP and is stable for over a year. This experiment will entail labeling of a DNA fragment (a metabolic gene from a pure bacterial culture) with DIG. The convenience of this approach is that the DIG label is incorporated during a PCR amplification. You will be using the same primer sets as you did for the amplification of environmental genomic DNA using metabolic and phylogenetic primer sets.

Experimental Method: Nonradioactive Labeled Probe Construction

Materials per group:

Bacterial genomic DNA (gDNA)

Various metabolic gene primer sets (you will be given a list in lab)

DIG dNTP mix (13.2 μl 1 mM DIG-11-dUTP, 2.68 μl 10 mM dTTP, 4 μl 10 mM dATP, 4 μl 10 mM dGTP, 4 μl 10 mM dCTP, 12.12 μl sterile water)

10x PCR buffer (minus MgCl_2)

MgCl_2

Sterile water

Taq polymerase

0.2 ml tubes, 0.5 ml tubes, tips, pipettors

Thermal cycler

DAY 29: Lab Period

In a 0.2 ml tube, add IN ORDER:

36 μl sterile water

5 μl 10x PCR buffer

3 μl 25 mM MgCl_2

4 μl dNTP mix

1 μl of your assigned primer set

0.5 μl Taq polymerase

1 μl gDNA

Mix well by tapping the tube. Spin in a microcentrifuge (with adapters) for a few seconds. Give to the TA to cycle in the thermocycler. The TAs will remove the reactions from the thermal cycler and store them in the freezer until the next lab period.

Experiment: Dot Blot Hybridization

Although most of what we know about prokaryotic molecular biology has been learned from studying *E. coli*, many other organisms have very different metabolic capabilities than *E. coli*. Today, researchers are delving into a wide variety of organismal models and discovering metabolic pathways and regulatory mechanisms that do not exist in *E. coli*. Emerging from this work is an ever-expanding database of key metabolic genes. That is, we now have information regarding a wide variety of genes that are critical for specific metabolic pathways. For example, the *cld* gene encoding chlorite dismutase is found only in those organisms capable of (per)chlorate reduction. Along with our increasing knowledge of novel metabolisms is our emerging ability to isolate in pure culture many organisms, even those previously considered unculturable. The efficient synthesis of these two facets of microbiology (genetics and bacteriology) often requires the ability to quickly determine the metabolic potential of new bacterial isolates. This is most easily and reliably accomplished using hybridization analysis. (Why is PCR not the best approach?) In this experiment, we will be performing a **dot blot** hybridization.

A dot blot is a piece of a membrane to which various denatured DNAs have been bound. The procedure for making a dot blot is: 1). label (number) the location on the membrane where each different DNA sample will be bound, 2). heat denature a small amount ($< 1 \mu\text{g}$) of the various DNAs, and apply 1-2 μl of the denatured on the membrane beneath each number (Fig. 1A). This dot blot is then used in a **hybridization** experiment with a specific probe; the probe will only hybridize to those DNAs on the membrane that share a certain degree of sequence similarity (Fig. 1B). By examining those dots (DNAs) that hybridized to the probe, we would be able to identify those organisms that contain a gene that is similar in sequence to the probe gene sequence.

By changing the stringency of the hybridization conditions, we can distinguish between those DNAs that exhibit a low degree of sequence similarity and those that have a high degree of similarity. For example, if we hybridize in high-stringency conditions (*e.g.* higher temperature, lower salt), the DNA similarity (between the probe and the test DNA) must be high, but if we use low-stringency conditions (*e.g.* by decreasing the hybridization temperature), the DNA similarity may be relatively low. (If we used very low stringency conditions, what problem might we encounter with probes such as nitrate reductase?)

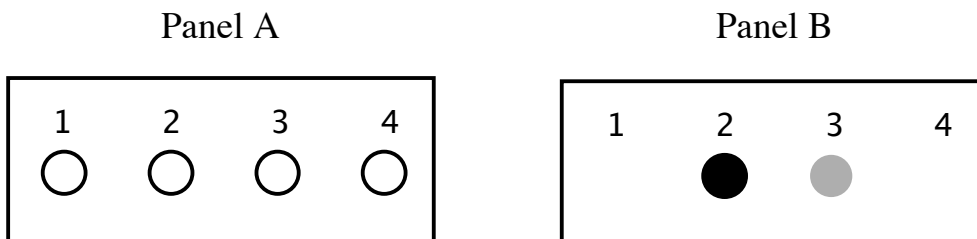


Figure 1. Panel A: Example membrane after addition of the various DNA samples. Panel B: Possible results after the hybridization step with a *cld* (chlorite dismutase) gene probe. 1) *Escherichia coli*, 2) *Dechloromonas aromatica*, 3) Strain TPB-16, 4) Strain Amp2.

One advantage of dot blots over Southern blots is speed, because there is no need to digest the DNA or run a gel. On the first day of our experiment, we will be heat denaturing the DNAs from four different bacterial species (*E. coli*, *Dechloromonas aromatica*, and two new bacterial isolates, TPB-16 and Amp2). These DNAs will then be dotted onto a piece of nylon membrane and bound to the membrane by cross-linking with UV light. On the second day of the experiment, we will be hybridizing our dot blot under relatively high-stringency conditions with the metabolic gene probe that you constructed in a previous experiment. On the third day of the experiment, we will be using a colorimetric detection procedure to identify those organisms that contain the metabolic gene of interest. Although detection using fluorescence and X-ray film is more sensitive and quantitatively accurate, colorimetric detection is suitable for our needs because it is safe, easy to perform and gives readily visible results of sufficient sensitivity. Once you have obtained your dot blot hybridization results, you should be able to speculate as to the metabolic potential of the new isolates TPB-16 and Amp2.

Experimental Method: Dot Blot

Materials per person:

Four different genomic DNAs at 1 $\mu\text{g}/\mu\text{l}$

To include: *E. coli*, *Dechloromonas aromatica*, and strains TPB-16 and Amp2

Eppendorf tubes, pipettors and tips

Nylon membrane and 3MM paper

EasyHyb solution

Digoxigenin-labeled probes

Small plastic box for color development

2x SSC, 0.1% SDS

0.1x SSC, 0.1% SDS pre-heated to 68°C

Wash Buffer (1X Maleic Acid buffer; 0.3% Tween 20)

Blocking Solution (Dilute 10X Blocking Stock with 1X Maleic Acid buffer)

Antibody Solution (Same as 1X Blocking Solution except add 1 μl of Anti-dig-AP Fab fragments per 10 ml of Blocking Solution)

Detection Buffer (Dilute 10X detection buffer to 1X with water)

Color Solution Color solution (freshly prepared): 45 μl NBT solution and 35 μl BCIP solution in 10 ml Detection Buffer

NBT solution: 75 mg/ml nitroblue tetrazolium salt in 70% dimethylformamide

BCIP solution: 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide

DAY 29: Lab Period

Heat-denature the four different DNAs in a boiling water bath for 10 minutes. While the DNAs are denaturing, mark your nylon membrane with a ball-point pen with the numbers 1 through 4 (see Fig. 1 for arrangement of marks). Spin down the DNAs briefly (1-2 seconds), then pipette 2 μl of each DNA under the appropriate number on your membrane. Be careful – don't jab the pipette tip through the membrane. Cross-link the membrane. Sandwich the membrane in between two pieces of 3MM paper and store in a ziploc bag until the next lab period.

DAY 31: Lab Period

Place the membrane in a heat-sealable bag. Place 1 ml of EasyHyb in the bag with the membrane, seal the bag and incubate for 1 hour in a 55°C water bath. Meanwhile, heat-denature 5 μl of your DIG-labeled metabolic probe in a boiling water bath for 10 min; chill immediately on ice. Add 1 ml of EasyHyb Solution to the tube with the denatured probe (this is your Hybridization Solution). After the one-hour incubation, cut a small corner off the bag containing the membrane and squeeze out the prehybridization solution into the sink. Using a pipette, add the Hybridization Solution to the membrane and seal, being careful to exclude all air bubbles. Gently squeeze the sealed bag to evenly distribute the probe over the surface of the membrane. Hybridize until the next lab period in a 55°C water bath.

DAY 33: Lab Period

Never allow the nylon membrane to dry during this detection procedure. Slit open the hybridization bag and squeeze out the hybridization solution. Use forceps to gently handle the membrane.

- Wash membrane 2x 5 min. in 2X SSC containing 0.1% SDS at room temperature with constant agitation.
- Wash 2x 15 min. in 0.1X SSC containing 0.1% SDS at 68°C. (Wash buffer must be pre-heated to 68°C.)
- Rinse membrane 1-5 min. in 50 ml 1X Wash Buffer.
- Incubate for 30 min. in 50 ml 1X Blocking Solution.
- Incubate for 30 min. in 20 ml Antibody Solution.
- Wash 2X 15 min. in 50 ml 1X Washing Buffer.
- Equilibrate 2-5 min. in 10 ml 1X Detection Buffer.
- Incubate in Color Solution in the dark; check color after first 5 min, then every 10 min.
- When dots are detected, you may stop the reaction by rinsing the membrane thoroughly in water.

Experiment: Phylogenetic Computer Analysis of 16S rRNA Sequences

Prior to the molecular revolution of the 1970s and 80s, the task of investigating bacterial evolution was a difficult one. This was the case because of the relative simplicity of the organisms. Looking at a typical mixed bacterial culture, it's easy to see how the classification of bacteria on morphological grounds alone would be a daunting task. Bacteria do not readily lend themselves to the basic taxonomic measures usually employed. Put another way, these organisms "barely got an anatomy." Thus, bacterial relationships were based on such features as gross morphology (shape) and basic metabolism. It was obvious that other criteria would have to be employed if the study of the natural relationships among bacteria was to be more than the mere classification and identification of these organisms.

The turning point in bacterial evolution came in 1965 with the publication of Zuckerkandl and Pauling's paper "Molecules as Documents of Evolutionary History." With the discovery that molecules could be used to measure evolutionary relationships came a resurgence of interest in the scientific community to uncover the natural relationships of bacteria. What was needed, however, was a suitable macromolecule and ribosomal RNA was chosen.

rRNA is a molecule which, when complexed with proteins, forms the ribosomes of the translation apparatus. In prokaryotes, the 23S and 5S are components of the large-subunit of the ribosome and the 16S in the RNA component of the small subunit. The situation is comparable in eukaryotes, with only the sedimentation coefficients of the RNAs differing. Thus, the 23S is called the large-subunit rRNA and the 16S the small-subunit rRNA.

Why was rRNA, specifically the small subunit, an appropriate molecule for addressing phylogenetic questions? Since rRNA is an integral part of the translation apparatus, it is present in every type of organism. In addition, rRNA represents 80-85% of the total RNA of a cell, so it is abundant and easily isolated or PCR amplified. And with a length of approx. 1500 nucleotides, the 16S rRNA contains a sufficiently large number of characters for analysis. But most importantly, rRNA contains some areas that are highly conserved and some that are highly variable. The conserved regions of the 16S rDNA gene are useful as priming sites for amplification and sequencing and useful for alignment purposes. However, it is the variable regions of the 16S rRNA that allow one to examine evolutionary relationships.

For all of the reasons outlined above, the 16S rRNA has been the molecule of choice for examining bacterial diversity. The Ribosomal Database Project, a database of rRNA sequences (<http://rdp.cme.msu.edu/html/>), currently has more than 17,000 prokaryotic 16S rRNA sequences. Of these, well more than 10,000 are from environmental clones of 16S genes, not the 16S genes of bacterial isolates. It's easy to see why clone sequences far outnumber sequences from actual organisms when, during a "typical" community sampling of forest soil, over 300 different rRNA genes can be isolated and sequenced. Here, then, is the way in which a 16S sequence can be analyzed to "match" the sequence to its closest known relative.

Experimental Method: Phylogenetic Computer Analysis of 16S rRNA Sequences

DAY 31: Lab Period

- 1) Open the Ribosomal Database Project II (<http://rdp8.cme.msu.edu/html/>).
- 2) Click on "Online Analyses" button.
- 3) Click the Sequence Match "Run" button (Small Subunit).
- 4) In the Sequence Upload Form, copy and paste your sequence into the appropriate box.
- 5) Type the number 5 into the box "Show the 20 most similar sequences", format the output as an HTML list (faster) in the pull down menu and click on the "Submit Sequences" button.
- 6) Record the results by cutting and pasting the 5 lines of results into a word processor (or just write them down).

Example:

```
env.Aspo5 .823 1404 clone Aspo5.  
SBAJ216 .817 1433 Shewanella baltica 16S rRNA gene.  
SPU91552 .808 1283 Shewanella putrefaciens 16S ribosomal RNA (rrs) gene, partial  
AF005252 .792 1412 Shewanella sp. MR-4 16S ribosomal RNA gene, partial sequence.  
AF011335 .785 1411 Shewanella pealeana 16S ribosomal RNA gene, complete sequence.
```

- 7) Click the "Return to main analysis page" link to return to the main analysis screen.
- 8) Click the Sequence Aligner "Run" button (Small Subunit).
- 9) In the Sequence Upload Form, copy and paste your sequence into the appropriate box.
- 10) In the Sequence Aligner Output Options, click on the HTML format and indicate the number of related sequences to include as 3.
- 11) Click the "Submit Sequences" button.
- 12) Make a note of the 3 sequences that were aligned to your sequence and look at the alignment to make sure it's well aligned.

Example:

```
env.Aspo5  
She.algaFe  
She.putre2
```

- 13) Click the Back button twice on your browser window to return to the main analysis screen.
- 14) Click the Similarity Matrix "Run" button (Small Subunit).
- 15) In the Specify a Sequence File on your Machine section, copy and paste your sequence into the appropriate box.
- 16) In the Similarity Matrix Options section, type the number 10 into the "For each of my sequences, include ___ most similar RDP sequences."
- 17) Now click the "Calculate Matrix" button.
- 18) In the next window, find the smallest number in the first column. Write down the number and the organism that it's closest to.

Example:

```
env.Aspo5 .037
```

This means that the 16S rRNA sequence from organism env.Aspo5 is only 3.7% different from my input sequence.

- 19) Now click on the name (it should be in blue letters) of the closest organism (e.g. env.Aspo5). If it's a clone (like this example), click on the next most closely related sequence to see if it's an isolated organism. (e.g. In my example, the closest one that was an organism, not a clone, was .047 She.putre3 which, in the next window, described it as *Shewanella putrefaciens* str. B.W. Hammer.) Write down the name of the organism and the percent similarity to your sequence.

Example: *Shewanella putrefaciens* str. B.W. Hammer 95.3%