

The Use of DNASTAR Lasergene Educational Software with Molecular Techniques to Support Bacterial Identification

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Physiologic tests in microbiology are typically performed to aid in differentiating and identifying bacterial isolates. With the increasing utilization of molecular techniques in both research and clinical communities, it is beneficial to improve classroom activities through supplementing physiologic methods with molecular techniques and computer analysis. DNASTAR, Inc. has made available free Lasergene software programs for educational purposes. This article discusses multiple pedagogical applications of this software and includes an inexpensive, classroom-friendly protocol for amplifying and performing restriction enzyme digestion on bacterial 16S to acquire a DNA fingerprint which will be used to support the identification of an unknown bacterial isolate.

Keywords: 16S RFLP, phylogenetic trees, PCRs

Introduction

In 1977, Carl Woese proposed a new phylogenetic taxonomy based on the nucleotide sequences of ribosomal RNA due to its requirement in all self-replicating systems and slow mutation rate over time (Woese and Fox, 1977). Amongst prokaryotes, the phylogenetic taxonomy was based on their 16S ribosomal RNA sequence, thus resulting in major subdivisions into genera and species. Almost all microbiology textbooks discuss Woese's phylogenetic tree, but now with a plethora of nucleotide sequences available through the NCBI website students can recreate their own phylogenetic tree using nucleotide analytic software. Additionally, due to affordable reagents and technologies amplifying DNA has become feasible within the classroom, and when combined with analytical computer software programs, can provide multiple pedagogical tools. This activity was designed to incorporate a molecular component into a microbiology laboratory exercise for biology majors. Specifically, the design primarily expanded an existing laboratory exercise, "Determining an Unknown Bacterial Isolate" to apply molecular techniques discussed in lecture. Laboratory modifications familiarized students with utilizing the NCBI website to locate and download DNA sequences with subsequent analysis using DNASTAR Lasergene software programs. These DNA and protein analytical tools can be used for pedagogical applications in microbiology, genetics and molecular biology courses. However, this workshop focuses on applying these technologies within a microbiology laboratory course, while some alternative computational uses are discussed in the notes for the instructor.

The design of the following laboratory exercises was to help support or disprove a student's identification of their unknown bacterial isolate, which was based on physiologic tests and differentiating stains. In these exercises, students will amplify 16S rRNA from their unknown isolates using polymerase chain reaction (PCR), a molecular technique used to amplify a segment of DNA, and digest it with restriction enzymes. Successful amplification is evident with the appearance of a band at ~ 1550bps after agarose gel electrophoresis. Due to time restraints, it is suggested to start the restriction digestion of the PCR product before the electrophoresis is finished. Typically one would wait to see the presence of an amplicon of the correct size before proceeding to restriction enzyme digestion.

Restriction fragment length polymorphism (RFLP) analysis has been used in a plethora of experiments elucidating bacterial diversity in a variety of environments. In this class activity, it will use RFLP analysis to provide molecular support to confirm the results of previous physiologic tests on an unknown bacterium. RFLP analysis is a molecular technique that utilizes variability between DNA sequences in restriction sites to differentiate between the DNA sequences (see Fig. 2 for an example). Restriction enzymes recognize specific, short nucleotide sequences (~4-8 nucleotides) and cleave the DNA at the defined location. This results in varying DNA fragment sizes, which can be separated using agarose gel electrophoresis. The banding pattern represented on the agarose after digestion is known as a molecular DNA fingerprint.

The following laboratory experiments were designed for a microbiology lab (BIOL 332) with a two hour and a one hour lab session every week. The addition of molecular protocols and usage of the DNASTAR Lasergene computer software to already existing physiologic assays, provides a research element of experience to the classroom.

In order to determine if the 16S RFLP molecular fingerprint matches the predicted unknown fingerprint, the student will utilize NCBI to acquire the 16S rRNA sequence of the suspected bacterial genus and species. They will then use DNASTAR Lasergene software to determine the molecular fingerprint of 16S after double digestion with two restriction enzymes, AluI and MboI. This hypothetical molecular fingerprint will be used to compare to the molecular fingerprint obtained in class after digestion of the 16S PCR reaction with AluI and MboI.

Student Objectives:

1. To understand the principles and applications of the following molecular techniques: polymerase chain reaction (PCR), agarose gel electrophoresis, and restriction fragment length polymorphism (RFLP) analysis
2. To learn how to acquire nucleotide sequences of individual genes using NCBI website
3. To acquire multiple DNA sequences from different genera and species of bacteria and determine their DNA fingerprint and homology using DNASTar Lasergene SeqBuilder and MegAlign.
4. To be able to create a phylogenetic tree from the above analysis

Student Outline

Part I (one hour)

Obtain bacterial DNA

1. Obtain an overnight bacterial culture of an isolated colony from your unknown sample plate.
2. Vortex overnight culture and transfer 1 μl to a 1.6 ml microcentrifuge tube with 20 μl of **room-temperature** Buffer BR-A.
3. Set the pipette at 10 μl and gently pipette up and down ten times making sure NOT to cause bubbles. Place the tube on ice to minimize nuclease activity (nucleases are enzymes that digest nucleic acid, DNA and RNA). Store in a -20°C freezer until you are ready for PCR (up to one week with good results).

Perform polymerase chain (PCR) reaction

1. Add the indicated volumes of the following reagents to the bottom of an empty sterile PCR epitube in the order listed.
2. Place PCR tubes in a quick spin centrifuge to ensure all fluid are at the bottom. (This is extremely important!)
3. Now place PCR tubes in the thermocycler and run a program with the following steps: 94°C for 3 minutes, followed by 35 cycles (94°C for 1 minute, 50°C for 30 seconds, and 72°C for 1 minute), followed by a final elongation step at 72°C for 7 minutes followed by 4°C hold.

Table 1. Reagents and volumes for PCR reaction.

Reagent	Volume
2X GoTaq Green Master Mix	10 μl (bottom of tube)
Master 16S Primer Mix (Fwd and Rev with water)	8 μl
DNA from step 3 above	2 μl
Total volume	20 μl

Primers sequences (melting temperature= T_m):

Forward primer (27FACA) 5' - AGAGTTTGATCATGGCTCAG - 3' $T_m=55$ (20bps)

Reverse primer (1492RT) 5' - GGTTACCTTGTTACGACTT - 3' $T_m=49$ (19bps)

Part II (two hours)

Agarose gel electrophoresis

1. Load 5 μl of your PCR reaction into an open well in a 1.2% agarose gel. This step is to ensure you have amplified the 16S gene from your unknown isolate.
2. Run the gel at 80-100 millivolts until the loading dye has run $\frac{3}{4}$ of the way down the gel.
3. Analyze under UV lamp to ensure presence of an amplicon ~ 1.5 kbps

Restriction digestion

1. Add 5 μl of the restriction enzyme master mix directly to the bottom of your PCR tube containing the remaining 15 μl .
2. Vortex and quick spin your tubes to ensure all of the fluid is at the bottom.
3. Incubate at 37°C overnight.

Part III (one hour)

Determine your unknown bacterial 16S sequence from NCBI.

1. Go to the NCBI PubMed home page (www.ncbi.nlm.nih.gov/PubMed)
2. Under the search drop down menu at the top, select **gene**
3. Type in the full name of your predicted bacterial unknown in the search box, followed by *16S ribosomal RNA* (i.e. *Staphylococcus aureus 16S ribosomal RNA*).
4. Search for a result that states 16S ribosomal RNA **only** (should be ~1.55 kb). Click on the entry.
5. Scroll down until you see the heading: **genomic regions, transcripts, and products**
6. Click on **FASTA**
7. Highlight all of the nucleotides **only** and click on copy
8. Open up SeqBuilder in DNStar Lasergene
9. Click on **File** then **New DNA**
10. **Paste** your sequence into the screen
11. In the left margin, **click on the '+'** next to **"Enzymes displayed"**
12. Click on "All Enzymes", then AarI, etc, until you find **AluI and MboI. Check both of these boxes.**
13. Because our primers are most likely amplified **within** the complete 16S rRNA sequence you just obtained, you need to modify the size by excluding all nucleotides upstream (5'-end) of the forward and downstream (3'-end) of the reverse primer.
 - a. To do this click on **"edit"** menu. Then click on **"Find"**
 - b. Type the first six nucleotides of the forward primer (listed above).
 - c. Under search options, click on **"literal"** and **"Find Next"**. This should identify a segment within the first 50 nucleotides.
 - d. Delete all nucleotides upstream (towards the 5'- end) of the forward primer.
 - e. Repeat steps 13. a-c for the reverse primer, but instead type CCTTGTT. The reverse complement should be towards the very end > 1450bps. When identified, count **five nucleotides** towards the 3'-end and delete everything **downstream**. You should be left with ~ 1480-1520bps.
14. Scroll towards the top again and click on **"Site Summary"**. This will show you where each restriction enzyme cuts and its predicted size. Next, click on the heading bar **"Position"**. This will show you all the fragments you should expect to see depending on the nucleotide length. The size (bps) of the fragments are in parentheses preceded by the position of the cut.
15. Look at the class 4% Metaphor agarose gel results and determine if the 16S molecular fingerprint in your lane matches what you predicted. If you narrowed your unknown bacteria to a couple different species, repeat this process for each possibility to aid in proper identification.

Notes for the Instructor

First lab session (1 hr)

1. I used this time to discuss different reagents included in a PCR reaction and the effects of altering their concentrations. It complements lecture material where I typically focus on the steps involved in PCR and the enzymatic reaction.
2. During this session, students will set up PCR reactions (~20 min) and run them in the thermocycler (~3 hours). Depending on your schedule lab time, the reaction will likely be finished after class, so it may be helpful to make arrangements for the PCR tubes to be placed in a freezer (-20°C) until the next lab period.

Second lab session (2 hr)

1. A 1.2% (w/v) agarose gel should be made before class. Depending on your comfort level, ethidium bromide, which is a mutagen, can either be added into gel or after gel is done running, though the latter would take more time. Less toxic compounds, such as GelRed™ (Biotium) and Fast Blast DNA Stain (Bio-Rad), are available and can be used in place of the ethidium bromide as well. Students should begin lab by loading their wells with 5 µl of their PCR reaction (10-20 min) and perform gel electrophoresis (run gel at 80-100 millivolts until samples have ran ¾ of the way down the agarose gel).
2. At this point, it can be advantageous to use the downtime (30-45 min) during the gel electrophoresis to hold a short lecture discussing agarose gel electrophoresis and RFLP analysis techniques (30-45 min).
3. Also during the downtime, students can perform the restriction digestion on the remaining 15 µl of PCR reaction (10-15 min).

Third lab session (1 hr)

1. To maximize the efficiency of lab, the instructor or lab assistant can run RFLP samples on 4% Metaphor agarose gel following manufacturer's instructions before class and send the students a digital image of their RFLP results.
2. Students will use NCBI PubMed to find the 16S sequence of their suspected unknown bacteria, as described above.
3. Students will use SeqBuilder to determine their 16S molecular DNA fingerprint after AluI and MboI double digest. See Fig. 1 for an example using *Micrococcus luteus* 16S nucleotide sequence.
4. Student will determine if predicted 16S molecular fingerprint agrees with what was obtained in the Meta-

phor gel. This process will most likely not be perfect, but the majority of bands should agree. Remember, this part is like a puzzle; addressing any discrepancies will enhance student understanding and application of the material. In the case it disagrees to a large extent, have the student ascertain that they have analyzed **all** 16S sequences on PubMed for their genus and species and propose reasons for the differences. Some bacteria contain multiple copies of 16S and have microheterogeneities within *AluI* and *MboI* restriction sites (Alperi *et al.* 2008) resulting in varying banding patterns.

Materials

- Buffer BR-A (Genscript, Cat#L00202, \$111, 100 preps; ask for extra BR-A instead of PCR reagents)
- GoTaq Green Master Mix (Promega, Cat#M7122, \$40, 100 rxns)
- AluI (Promega, Cat#R6281, \$73/500U (10U/ul))
- MboI (Promega, Cat#R6711, \$69/200U (10U/ul))
- MetaPhor Agarose (Fisher, Cat#50181, 25g, \$160.94)
- Universal Primers (make a 0.5 µM working stock of both primers diluted in DNase free water)
 - 27FACA
5'- AGAGTTTGATCATGGCTCAG – 3'
Tm=55 (20bps)
 - 1492RT
5' - GGTTACCTTGTTACGACTT – 3'
Tm=49 (19bps)
- DNASTar Lasergene 8 software (free for educational use. Go to their website: www.dnastar.com and find your local sales representative for more information).

Additional Instructor Notes

Here are the reagents and volumes needed for the restriction digestion master mix

Table 2. Preparation of the restriction digest master mix.

Reagents	Reaction	Master Mix (multiply by # of students + 4 µl)
Buffer 4 10X Mix	2.0 µl	
AluI (10U/ µl)	0.25 µl	
MboI (10U/ µl)	0.25 µl	
Water	2.5 µl	

Instructions for Other Applications using DNASTar Largegene Software

Determining variation in restriction sites for AluI and MboI between different bacterial genera and species.

1. Download 16S ribosomal sequences from NCBI website for a variety of Gram positive and Gram negative bacteria following student instruction above (Third lab period).
2. Open **MegAlign** program from DNASTAR software.
3. Click on “**Enter Sequences**” from the “File” pull-down menu
4. Find your sequences and click “Add →”. All of the sequences should be in the “Selected Sequences” box. Click “Done” button.
5. Click on “**By Clustal W Method**” under the “Align” pull-down menu and something like *Figure 2* should appear. The degree of homology between bacterial strains is color-coded, where red is the most homologous. Also in *Figure 2*, a black box is drawn around one conserved *AluI* restriction site (-AGCT-). Conversely, strains that no longer have a *MboI* restriction site (-GATC-) are highlighted in black. Together, this alignment helps students understand how this method can distinguish between multiple bacterial strains.

Constructing a phylogenetic tree.

1. Follow instructions above for determining variation in restriction sites for *AluI* and *MboI*.
2. After you have performed the Clustal W alignment, click on “**Phylogenetic Tree**” under the “View” drop-down menu. Additionally, if you want to go one step further, click on “**Performing Bootstrapping**” under the “Align” drop-down menu. It should look like Fig. 3.
3. More information on bootstrapping and phylogenetic trees can be found by Efron *et al.* (1996). Patterns that exist among clusters can also be determined (i.e. genus and species groupings, Gram positive versus negative, etc.).

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About the Author

Much of the work for this paper was developed while Troy Skwor was an Assistant Professor at Gannon University. The above labs were modifications from his research experiments identifying potential human pathogens and antibiotic resistance among *Aeromonas* bacterial strains isolated from Lake Erie. This research expands his previous graduate and postdoctoral research projects elucidating the host immune response to medically relevant bacteria at Texas A&M University College of Medicine and Children’s Hospital Oakland Research Institute in Oakland, CA respectively. Currently, Troy is an Assistant Biology Professor at Rockford College and has taught Microbiology, Virology, Genetics, Molecular Biology, Immunology, Medical Microbial Pathogenesis, and Principles of Biology.

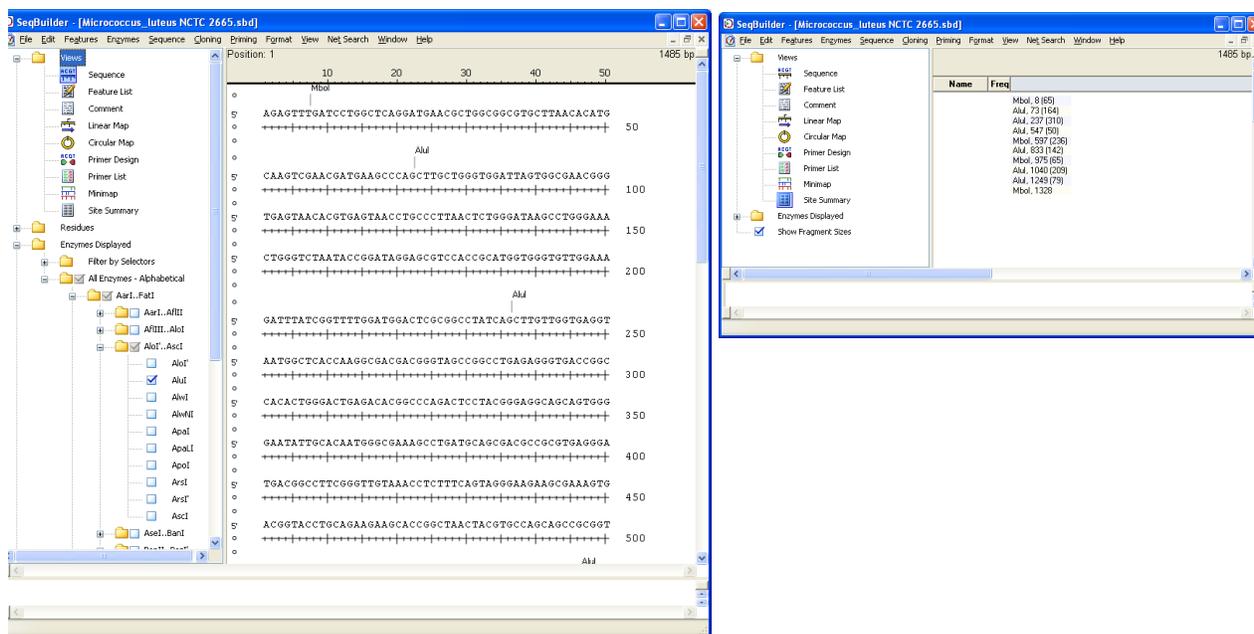


Figure 1. Predicting the banding sizes of *Micrococcus luteus* 16S after double digestion using SeqBuilder software. In Fig. 1A the basic outline of SeqBuilder is shown, as well as where you click on the desired restriction enzyme in the left-most column. Notice that the enzyme name appears above its associated restriction site. After ‘checking’ both restriction enzyme (*AluI* and *MboI*) boxes, ‘click’ on “Site Summary,” which is represented in Figure 1B. The nucleotide location of the cut site is displayed first, followed by the resulting base pair size in parentheses.



Figure 2. Determining 16S sequence variation among different genera and species of bacteria using MegAlign. Multiple 16S sequences were downloaded from NCBI and aligned using the Clustal W method. An *AluI* restriction site (-AGCT-) is encompassed by a red box to demonstrate the strong degree of homology at this location. However, the *MboI* site (-GATC-) at Pos +296 demonstrated heterogeneity between genera and species in some cases. This variability is one example responsible for differentiating bacterial 16S RFLP patterns.

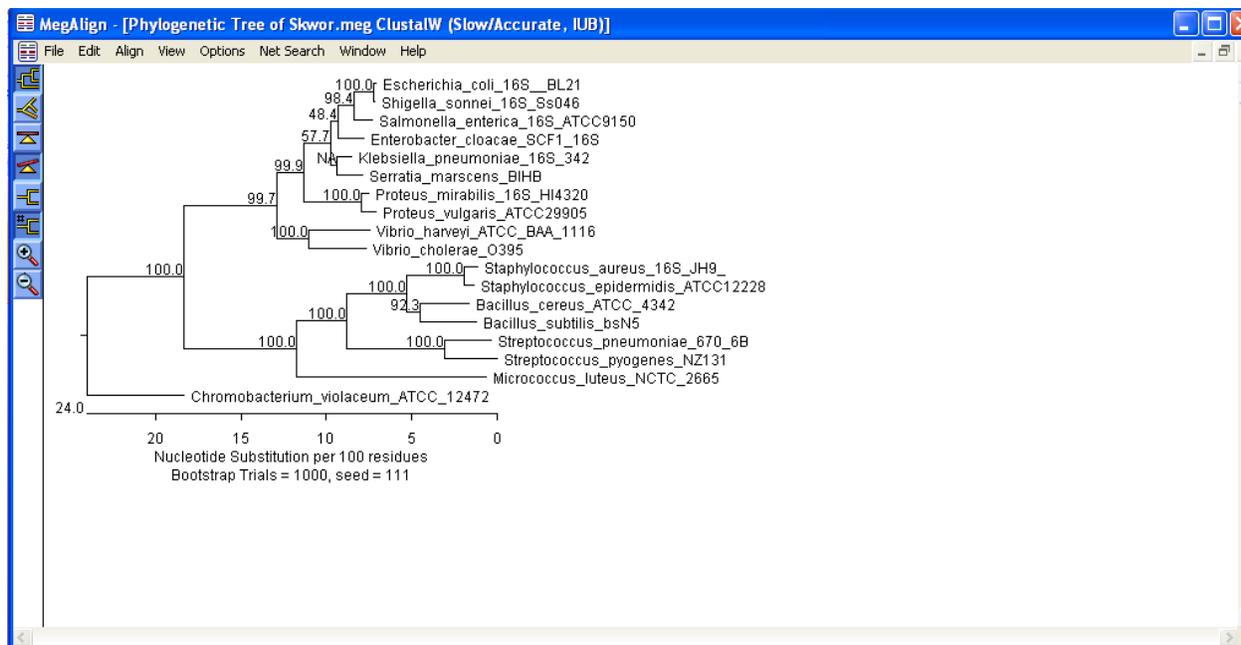


Figure 3. A phylogenetic tree constructed from 16S rRNA sequences using bootstrapping in MegAlign program. Notice that the majority of Gram positive and Gram negative bacteria are clustered together, as well as bacteria within the same genus.

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