

Microbiology Laboratory: MOLECULAR IDENTIFICATION OF UNKNOWN BACTERIA

Classical Microbiology courses are typically structured to introduce the identification of bacterial species using a series of biochemical tests. Indeed, for introductory microbiology courses, the American Society for Microbiology stipulates that essential laboratory skills include the following: bright-field microscope use, slide preparation, aseptic technique, use of media and biochemical tests, microbial quantity estimation, and use of standard lab equipment (<http://www.asm.org/Education/index.asp?bid=10051>). However, concepts recommended by the ASM within the same document list microbial genetics as well as genetic engineering and biotechnology as “Core Themes and Concepts for an Introductory Microbiology Course,” an aspect omitted from the recommended laboratory skills. Certainly, most current microbiological endeavors are geared toward modern genetic techniques and studies; biochemical tests that identify species based on metabolic differences are used less frequently outside the classroom. More recently, industries have arisen to identify microbes based primarily on genetic testing, typically involving comparison of 16S ribosomal RNA.

With this current reality in mind, we decided to update our Microbiology course curriculum to include more contemporary practices. In the past we have required students to learn basic methods for culturing bacteria and use of aseptic technique, followed by collection and identification of an unknown bacteria from the local environment. They learn biochemical techniques in the course of this identification. In order to introduce modern microbiological procedure, we propose to have them take this a step further by performing genetic tests on their unknown to determine whether their biochemical identification was correct.

There is a plethora of Microbiology Lab Manuals that detail the biochemical tests. We recommend that students begin this laboratory unit by following a standard unknown collection and biochemical identification. Once this laboratory exercise is complete, followed by a presentation in which the students present their findings, we have them begin a lab section in which they will confirm the identification using molecular techniques presented below.

Protocol as presented to students:

Microbiology Lab: Identification of Bacteria Using Genetic Sequence Comparison

You have tentatively identified an unknown bacterial isolate by using a series of biochemical tests. We will now use a molecular technique to confirm your identification by comparison of a part of the DNA sequence of your unknown with a national database of established genetic sequences. We will copy a part of the bacterial genome that encodes conserved sequences of ribosomal RNA by using

polymerase chain reaction (PCR). The small ribosomal subunit (SSU) or 16S rRNA has been frequently used for such comparisons.

We will begin by preparing genomic DNA from our bacterial cultures to use as a template for the PCR. We will use primers that are complementary to highly conserved areas of the sequence so that we get copies of part of the rRNA gene sequence. We will send the DNA produced to a sequencing facility to determine the exact sequence and will then compare that to the NCBI and RDP databases for identification.

Procedure

Select an isolated colony of your unknown and subculture it, shaking, overnight in Trypticase Soy Agar at the temperature most appropriate for the bacteria you have identified. If possible, use an instructor-provided broth containing a positive control for your bacteria.

Preparation of bacterial genomic DNA using Qiagen DNeasy Tissue Kit

Collecting and Resuspending Cells for Gram Negative Bacteria

1. Put on **gloves**.
2. Label 2-- 1.5 ml microcentrifuge tubes and pipet **1 ml** of your fresh bacterial culture into one tube and 1 ml of the control culture into the other.
3. Centrifuge (balanced) for **5 min.** at **7,500 rpm**.
4. Remove and discard the supernatant.
5. Resuspend cells in **180 µl Buffer ATL**.

Cell lysis and removal of RNA

6. Add **20 µl Proteinase K**. Mix by vortexing.
7. Incubate in **56°C** water bath for **1 hour**, **vortexing** every **20 min**.
8. Briefly centrifuge to remove drops from the lid.
9. Add **10 µl RNase A** and mix by **pulse-vortexing** for **15 sec**.
10. Incubate at **room temperature** for **5 min**.
11. Briefly centrifuge to remove drops from the lid.
12. Add **200 µl Buffer AL** to the sample and mix **well** by **pulse-vortexing** for **15 s**.
13. Incubate at **70°C** for **10 min**.
14. Add **200 µl 100% ethanol** and mix by **pulse-vortexing** for **15 s**.
15. Briefly centrifuge to remove drops from the lid. Continue by skipping to step 16 below.

Collecting and Resuspending Cells for Gram Positive Bacteria with Qiagen DNeasy Tissue Kit

1. Put on **gloves**.
2. Label 2-- 1.5 ml microcentrifuge tubes and pipet **1 ml** of your fresh bacterial culture into one tube and 1 ml of the control culture into the other.

3. Centrifuge (balanced) for **5 min.** at **7,500 rpm.**
4. Remove and discard the supernatant.
5. Resuspend pellet in **180 µl Enzymatic Lysis Buffer** (20mM TrisCl pH 8.0, 2mM NaEDTA, 1.2% Triton X-100)
6. **Incubate 30 min. at 37°C.**

Cell lysis and removal of RNA

7. Add **20 µl Proteinase K** and **200 µl Buffer AL (separately).** Mix by vortexing.
8. Incubate in **70°C** water bath for **30 min.**
9. Briefly centrifuge to remove drops from the lid.
10. Add **10 µl RNase A** and mix by **pulse-vortexing** for **15 s.**
11. Incubate at **room temperature** for **2 min.**
12. Briefly centrifuge to remove drops from the lid.
13. Add **200 µl 100% ethanol** and mix by **pulse-vortexing** for **15 s.**
14. Briefly centrifuge to remove drops from the lid.

Purification of genomic DNA

16. **CAREFULLY** apply the **ENTIRE mixture** (including any precipitate) to a **QIAamp spin column** (in a 2 ml collection tube).
When using spin columns:
Do NOT touch the membrane with the pipet tip.
Keep rim dry.
Close the cap tightly before centrifuging.
17. Close the cap and centrifuge (balanced) for **1 min.** at **8,000 rpm** (or until all liquid flows into the collection tube).
18. Empty tube with liquid and place the **spin column** back into **collection tube.**
19. **CAREFULLY** open the spin column and add **500 µl Buffer AW1** without wetting the rim.
20. Close the cap and centrifuge (balanced) for **1 min.** at **8,000 rpm** (or until all liquid flows into the collection tube).
21. Empty tube with liquid and place the spin column back into collection tube.
22. **CAREFULLY** open the spin column and add **500 µl Buffer AW2** without wetting the rim.
23. Close the cap and centrifuge (balanced) at **full speed (14,000 rpm) for 3 min.**
24. Discard tube with liquid and place the spin column in a fresh collection tube.
25. Close the cap and centrifuge (balanced) at **full speed (14,000 rpm) for 1 min.**

Elution of genomic DNA from column

26. Discard tube with liquid and place the spin column in a **new, labeled 1.5 ml microfuge tube** (one with a snap cap, not another collection tube).

27. **CAREFULLY** open the spin column and add **50 µl Buffer AE** without wetting the rim.
28. Incubate at **room temperature** for **5 min.**
29. Close the cap and centrifuge (balanced) for **1 min.** at **8,000 rpm**
30. Remove column from tube and store genomic DNA in tube on ice or at -20°C until use.

Amplification of bacterial ribosomal DNA by PCR (set up on ice)

1. Label 2 -- 0.5 ml special PCR tubes with the reaction (one for your sample DNA, the other for control) **and your initials.**
2. **Dilute each** genomic DNA by adding **1 µl to 4 µl water** (1:5 dilution)
3. Add **1 µl genomic DNA** or **1 µl diluted Buffer AE** (for the no template control) to the appropriate tube.
4. Add **49 µl PCR mix** to each tube and mix **gently** by pipetting (no bubbles!).
5. Place **tubes on ice** until everyone is ready to put their reactions in the **DNA thermocycler** (aka PCR machine).
6. Instructor to store finished PCR reactions at -20°C until next week.

PCR reaction mix

200 µM dNTPs (dATP, dGTP, dTTP, dCTP)
 50 pmol 16S rRNA forward primer
 50 pmol 16S rRNA reverse primer
 2 U Taq polymerase
 1xThermapol reaction buffer (10 mM KCl, 20 mM Tris-HCl (pH 8.8),
 10 mM ammonium sulfate, 2 mM magnesium sulfate,
 0.1% TritonX-100)
 1 µl DMSO

PCR reaction conditions

1. 95°C, 2 min. (Denature)
2. 92°C, 1 min. (Denature)
3. 33°C, 2.5min. (Anneal)
4. 75°C, 4 min. (Extend)
5. Repeat steps 2-4, 34 more times (Cycle)
6. 4°C, until taken off (Final storage)

Visualization of amplified bacterial ribosomal DNA (Week 2)

1. Thaw PCR reactions.
2. For each tube, remove **10 µl** into a fresh tube and mix with **2 µl gel-loading dye**. Give remaining reaction mix to instructor for storage at -20°C.
3. Load each 12 µl sample onto the agarose gel.
4. **Write down order of samples** on paper next to the gel.
5. Electrophorese at **100 V** for **40-50 min.**

6. **Photograph gel** with documentation system; include **picture in your notebook**.
7. Instructor will purify DNA from bands and have them sequenced.
8. When you receive the sequence of your DNA, **run a BLAST search** (NCBI website: When you receive the sequence of your DNA, **run a BLAST search** (NCBI website: <http://www.ncbi.nlm.nih.gov/BLAST>) to identify your sequence.
9. Go to <http://rdp.cme.msu.edu/> , the website of the Ribosomal Database Project, and compare your sequence to their database. Try using some of their features to look at where your sequence places your bacteria on a phylogenetic tree or taxonomic structure.

Post-lab assignment

The European ribosomal RNA database, run by a group in Belgium, is an excellent resource for information about rRNA.

- a. Go to their site: <http://oberon.fvms.ugent.be:8080/rRNA/>. What do SSU and LSU stand for and which one are you interested in?
- b. To determine the location of the primers we used to use to amplify the 16S rDNA, select "Primers" on the left.
- c. Look at the superposition of the primers on the *E. coli* 16S sequence. Locate sites that have been shown to be variable among bacterial strains.

Instructor Notes for Molecular ID of Unknown

For this study students subcultured an isolated colony of their unknown into Trypticase Soy Broth, grown overnight. Template DNA was then extracted from the culture broth.

The following products were used:

Template DNA: Qiagen DNeasy Blood and Tissue Extraction Kit
(www.qiagen.com) # 69504

PCR: Qiagen TaqPCR Master Mix #201443 (contains all necessary ingredients for PCR except template DNA and primers; if it is desirable to introduce PCR by having students add each ingredient, the instructor can order the Taq polymerase, dNTPs, and buffer separately)

Primers: In the PCR we used 10mM of primers PC5 (5'-TACCTTGTTACGACTT-3') and P3mod (5'-ATTAGATACCCTGTAGTCC-3'), based on a previously published study (Wilson, Kenneth, Blitchington, R, and R. Greene, 1990. Amplification of Bacterial 16S Ribosomal DNA with Polymerase Chain Reaction. *Journal of Clinical Microbiology*, 28 (9), pp. 1942-6). Primers were ordered from Integrated DNA Technologies (www.idtdna.com) but could be obtained from a number of oligo synthesis companies.

The PCR reactions were stored at -20°C until the following week's lab. Students then set up a gel electrophoresis in a 1% agarose gel to determine the size of their PCR product and to separate the gene product expected for 16S rRNA from any extraneous products of the reaction. As a time saving measure, instructors ran 75% of the PCR reaction in a gel with loading dye prior to class, cut the gel slices containing the gene under study, extracted the DNA from the gel with a Qiagen QIAquick Gel Extraction Kit #28704, and sent it off for sequencing. There are numerous DNA sequencing facilities available.

NOTE: If a class has a lot of time to perform this, the students can run most of their PCR reaction in the gel (one must use 3 lanes of a gel to fit all of the product in, including the loading dye), cut their slices, and use the Qiagen kit to extract the DNA and prepare it for sending according to the parameters of the sequencing facility.

Once the sequences were returned from the sequencing facility, students were instructed and assisted in use of the NCBI BLAST and RDP websites to identify their sequence.