Lab: Colony PCR amplification of the 16S ribosomal RNA gene

I. OBJECTIVES
(1) Learn how to use the polymerase chain reaction (PCR) to amplify the small subunit ribosomal RNA (SSU rRNA) gene from a bacterial colony; (2) Learn how to run an agarose gel to visualize the resulting PCR amplicons and extract the amplified DNA from the agarose gel.

II. INTRODUCTION
Microbial community structure is a critical determinant of the biogeochemical processes occurring within the pelagic marine ecosystem. Due to the difficulties of cultivating many marine microbes, microbial ecologists frequently employ molecular biology-based techniques to reveal the complexity of aquatic microbial communities.

Culture-independent techniques for characterizing microbial biodiversity are primarily based on the analysis of small subunit ribosomal RNA (SSU rRNA) genes from environmental samples (e.g., Giovannoni et al. 1990). This gene is an excellent phylogenetic marker for Bacteria and Archaea. With the aid of the polymerase chain reaction (PCR), these strategies have greatly enhanced our ability to describe the genetic diversity of microorganisms in the natural environment without the need for cultivation.

The cloning and sequencing of PCR-amplified SSU rRNA genes is now a routine procedure. An overview of the typical steps is as follows: 1) SSU rDNAs (rDNA = rRNA gene) are amplified via the polymerase chain reaction using conserved oligonucleotide primers, 2) amplified products are cloned into a plasmid vector, 3) E. coli cell are transformed with the recombinant vectors 4) individual colonies of transformed E. coli are picked and the plasmids are purified 5) the purified plasmids are used as a template for the sequencing reaction.

In some cases, it is possible to simplify the procedure by eliminating the cloning step. In this case the PCR product is used directly as the template for the sequencing reaction. This requires that the amplified product be homogenous (not a mixture of fragments having different sequences).

In either case, the resulting sequences are compared to all of the known sequences that have been deposited in public nucleotide sequence databases such as GenBank (a general sequence database) or the Ribosomal Database Project (a specialized database containing only ribosomal RNA gene sequences).

In this laboratory exercise and the next, we will (1) use the polymerase chain reaction to amplify SSU rRNA gene fragments directly from a colony, (2) run an agarose gel in order to visualize the resulting PCR amplicons, (3) purify the PCR amplicons using a commercially available kit, and (4) sequence the amplified SSU rRNA gene fragments.

II. DETAILED PROCEDURE

A. PCR AMPLIFICATION OF BACTERIOPLANKTON SSU rRNA GENES DIRECTLY FROM A COLONY
Notes: This reaction employs conservative oligonucleotide primers that target members of the domain *Bacteria*.

**Equipment**
- Vortex mixer
- Thermal cycler
- 1-10 µl and 10-100 µl adjustable pipettors with tips

**Materials**
- PCR reagents
- Microcentrifuge tubes
- Thin wall PCR amplification tubes
- Ice and ice bucket

**Detailed Protocol**
1. You will amplify 2 colonies from your agar plates. In addition, you will want to run a PCR reaction that does not contain a colony or template DNA—this reaction is called the No Template Control (NTC). Thus everyone will run 3 PCR reactions in total. For each PCR reaction you will need:
   - 43 µl ultrapure water
   - 1.0 µl dNTP’s (10 mM)
   - 5 µl 10X PCR buffer
   - 0.3 µl Primer 27F-B (10 µM)
   - 0.3 µl Primer 1492R (10 µM)
   - 0.75 µl Polymerase enzyme (2.5 U/µl)

2. Place ultrapure water, dNTP’s (10 mM), 10X buffer, primer 27F-B (10 µM), and primer 1492R (10 µM) on ice to thaw.

3. Pipet the appropriate amount (according to your calculations) of the above reagents into a sterile microcentrifuge tube to make a “master mix”.

4. Making sure to keep the enzyme at -20°C as much as possible, add the appropriate volume of the polymerase enzyme (2.5 U/µl) to the master mix and vortex.

5. Aliquot 50 µl of master mix into each PCR tube and label appropriately.

6. From the agar plates you streaked from Queen’s Beach and the Ala Wai, touch one of the colonies of interest with a toothpick. Place the end of the toothpick into the appropriate PCR reaction tube that contains the PCR reagents. Remove toothpick and cap tube.

7. Place the PCR tubes into the thermocycler. Run the specific program, making sure conditions are set as desired. Upon completion, transfer PCR tubes to 4 °C.
   - Sample Program for the above primers:
     - 95°C for 5 min (1 cycle)
     - 95°C for 0.5 min, 51°C for 0.5 min, 72°C for 2 min (30 cycles)
     - 72°C for 10 min (1 cycle)
     - 4 °C hold
B. AGAROSE GEL ELECTROPHORESIS

**Equipment**
- Electrophoresis unit and power supply
- 250 ml Erlenmeyer flask
- 100 ml Graduated cylinder
- UV box and camera or other gel visualization and documentation method
- 1-10 µl, 10-100 µl, and 100-1000 µl adjustable pipettors with tips

**Materials**
- Agarose powder
- 10 mM Lithium acetate (Running buffer)
- 6X gel loading buffer
- ethidium bromide stock (10 mg/ml)
- parafilm
- DNA Mass Ladder

**Detailed Protocol**
1. Determine the volume of gel your gel form requires. Some common sizes of gel forms are: 6x7 cm, 15x7 cm, 15x10 cm and 25x10 cm. For ~5 mm thick gels these forms will contain respectively the following volumes: 25 ml, 55 ml, 75 ml and 125 ml. If you are unsure of the volume, set up the gel form completely, add water to an appropriate level (ca. 5 mm thick), and measure the amount of water required.
2. Clean the gel form and comb with water. Dry with Kimwipes.
3. Seal the ends of the gel form to prepare it for pouring. In some cases, the gel forms are sealed by their gasketed ends in the gel bed; in others, they have an external device for sealing the ends of the form. It is unique for each gel setup, so if you are unsure ask for assistance. Once the gel form is in place, insert the comb.
4. In a 250 ml Erlenmeyer flask, weigh out enough agarose to yield a 1% (w/v) gel based on the volume of gel you determined in step 1. For example, for a 100 ml gel, 1 g of agarose would be required.
5. Add enough running buffer to the flask to yield a 1% (w/v) gel.
6. Microwave the flask uncovered on high until it just begins to boil.
7. Stop the microwave and, using a protective glove, swirl the gel solution. Inspect the gel solution for any undissolved agarose granules; re-microwave until all of the agarose has melted. Set aside to cool.
8. Once the gel solution has cooled enough to where no steam is escaping from the top of the flask (ca. 5 min), add ethidium bromide to a dilution of 1:20,000 of stock to the flask and swirl to mix. Note that ethidium bromide is a mutagen and should not come in contact with the skin.
9. Carefully place the gel form in its proper orientation in the gel electrophoresis rig, and pull out the comb(s).
10. Gently add enough running buffer to the gel electrophoresis rig to just cover the gel itself.

11. Cut a small piece of Parafilm (ca. 2 x 4 inches) and lay it paper-side down on the counter. Onto the Parafilm surface, aliquot out 1 µl of 6X gel loading buffer per PCR sample to be run, including at least one ladder per comb. Be sure to vortex the loading buffer prior to aliquoting. Space all of the aliquots out so that they will not cross-contaminate.

12. For each PCR reaction, mix 5 µl of PCR product with a 1 µl aliquot of loading buffer on the Parafilm, and load the entire 6 µl into a well on the gel. Be sure to note the order and location of all of the PCR reactions. For the DNA Mass Ladder, load 5 µl as well.

13. Replace the cover on the gel electrophoresis rig and plug in the electrical leads. Check that the leads are plugged in to the power source with the correct polarity, and that your gel is placed in the proper orientation (DNA runs toward the red, positive, electrode).

14. Set the power supply on “constant voltage” and run at high voltage (250V). Set the timer for 30 minutes.

15. Periodically check your gel to determine if it has run a sufficient amount of time. A variety of factors can influence this decision; most notably are the size of your expected products and your analytical objectives. Our objective is to detect the presence of PCR products in the size range of 500 and 1500 base pairs. The progress of the migration can be monitored by observing the dyes in the loading buffer. On a 1% gel, bromophenol blue (the purple dye) runs at about the same speed as a 300 base pair double-stranded DNA fragment, and xylene cyanol (the blue band) runs at about the same speed as a 4000 base pair double-stranded DNA fragment. When in doubt you can always examine the gel on the transilluminator and return it to the gel box to run longer if it isn’t finished.

16. Remove the gel from the gel box, keeping it in its form. Be sure to turn off the power supply first, and wear gloves. Also, carefully drain off any excess running buffer. Place the gel on the transilluminator. Wear a clean pair of gloves when handling any gel documentation equipment.

17. Capture an image of your gel, and turn off the transilluminator.

18. Dispose of gel and gloves into special ethidium bromide waste containers. Rinse the gel tray and combs with water and dry before putting away.

F. PCR PRODUCT PURIFICATION

Equipment
- Forceps
- Microcentrifuge
- Vortex mixer
- 20, 200, and 1000 µl pipettes with tips

Materials
- Qiagen QIAquick PCR Purification kit
- Molecular biology grade ethanol
- 1.5 ml microcentrifuge tubes
Detailed Protocol

1. This protocol utilizes the Qiagen QIAquick PCR Purification kit, with instructions slightly modified from that of the manufacturer. All reagents are supplied with kit except for molecular biology grade ethanol and ultrapure water.

2. Add 5 volumes of buffer PB to 1 volume of PCR product (i.e. 225 µl buffer PB to 45 µl PCR product) and vortex.

3. Transfer samples to a labeled QIAquick spin column and centrifuge at 13,300 rpm for 1 min and discard flow-thru. Column can be placed back in the same collection tube.

4. Add 750 µl buffer PE (with ethanol already added per kit instructions) and centrifuge at 13,300 rpm for 1 min. Discard flow-thru and place column in the same collection tube.

5. Repeat the above centrifugation (13,300 rpm) for 1.5 min to dry column completely.

6. Place each column in a sterile, labeled 1.5 ml tube and add 30 µl ultrapure water to the center of each column. Let stand 1 min at room temp., then centrifuge at 13,300 rpm for 1.5 min.

7. Discard the columns, cap the 1.5 ml tubes, and store the eluted DNA at -20 °C.

IV. REFERENCES


V. APPENDICES

Appendix 1. Recipe for 6X gel loading dye (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF)

To make 10 ml total add the following:

- 3 ml glycerol
- 2.5 ml of 1% stock bromophenol blue
- 2.5 ml of 1% stock xylene cyanol FF
- Ultrapure water to 10 ml (ca. 2 ml)

Appendix 2. Oligonucleotide primer sequences

27F-B: AGRGTTYGATYMTGGGCTCAG
1492R: GGYTACCTTTGTTACGACTT
519R: GWATTACCGCGCGCKGCTG

All are listed 5' to 3'.
The 27F-B is 5'-end labeled with a fluorescent moiety for T-RFLP analysis.