Molecular Methods in Microbial Ecology

Contact Info: Julie Huber, jhuber@mbl.edu

Schedule:

Thursday  11/5/15  Introduction, Extraction of DNA from Winogradsky columns
Run DNA products on gel

Tuesday  11/10/15  Lecture on PCR, Prepare PCR reactions

Wednesday  11/11/15  Analyze PCR results, Lecture and Tour Bay Paul Center

Day 1

- Introduction to molecular methods in microbial ecology
- Extract DNA from Winogradsky Columns
- Run DNA on agarose gel
The Challenge for Microbial Ecology

<table>
<thead>
<tr>
<th>Habitat</th>
<th>Culturability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seawater</td>
<td>0.001-0.1</td>
</tr>
<tr>
<td>Freshwater</td>
<td>0.25</td>
</tr>
<tr>
<td>Sediments</td>
<td>0.25</td>
</tr>
<tr>
<td>Soil</td>
<td>0.3</td>
</tr>
</tbody>
</table>

How do you study something you can’t grow in the lab?

From Amann et al. 1995 Microbiological Reviews
Community sampling approach

Extract total community DNA

Amplify single gene, for example, gene encoding 16S rRNA

Sequence and generate tree

Outcomes

1. Phylogenetic snapshot of most members of the community
2. Identification of novel phylotypes

Single-gene phylogenetic tree

DNA

Environmental genomics approach

Restriction digest total DNA and then shotgun sequence, OR sequence directly (without cloning) using a high throughput DNA sequencer

Assembly and annotation

Partial genomes

Total gene pool of the community

1. Identification of all gene categories
2. Discovery of new genes
3. Linking of genes to phylotypes
DNA Extraction Overview

1. **Cell Lysis**
   - Centrifuge
   - Add Solution C2
   - Incubate at 4°C

2. **Inhibitor Removal Technology®**
   - Centrifuge
   - Add Solution C3
   - Incubate at 4°C

3. **Bind DNA**
   - Centrifuge
   - Add Solution C4
   - Load into Spin Filter

4. **Wash**
   - Centrifuge
   - Wash with Solution C5

5. **Elute**
   - Centrifuge
   - Elute with Solution C6

6. **Vacuum**
   - Alternate Protocol for PowerVac™ Mini Spin Filter Adapter

7. **Elute**
   - Centrifuge
   - Elute with Solution C6
**DNA Extraction**

1. Lyse cell membrane
   a. Chemically → detergent
   b. Physically → bead beating

2. Pellet cell membrane, proteins and other cell parts while DNA stays in solution

3. Remove other inhibitors from DNA

4. Mix DNA with acid and salt → stick to filter

5. Wash filter-bound DNA several times with alcohol

6. Elute DNA off membrane with pH 8, low-salt buffer
Choosing a Depth Horizon

- 16S rRNA Bacteria
- 16S rRNA Archaea
- mcrA Methanogens
  - Methyl coenzyme M reductase
- dsrB Sulfate reducers
  - Dissimilatory bisulfite reductase

[Diagram of depth horizons with layers for oxygen concentration, air, water aerobic zone, microaerophilic zone, and anaerobic zone.]
Day 1, Part II

• Run an electrophoresis gel of the DNA products extracted from your columns
Genomic DNA

The sum total of all DNA from an organism or a community of organisms
Basics of Gel Electrophoresis

- The gel is a matrix (like jello with holes)
- DNA is negatively charged- will run to positive
- Smaller fragments run faster than larger ones
- Gel contains SYBR SAFE, which binds to DNA and fluoresces when hit with UV light (WEAR GLOVES!!!)
Genomic DNA

The sum total of all DNA from an organism or a community of organisms.
Day 2

• Learn about PCR

• Set up PCR reactions using the DNA from your extractions and an assortment of primers
Environmental sample

- Fix cells
- Extract nucleic acids

- Electrophoretic analysis
- Standard analysis
- DGGE

- Probe with specific oligonucleotide

Amplify rRNA genes by PCR

- Design specific probes from cloned sequences or sequences from cultured organisms

Ligate PCR products and cloning vector

- Excise bands, reamplify and sequence

Transform E. coli

- Sequence and characterize individual clones

- rRNA sequence determination

- Cultured isolates

Targeted enrichment and isolation

Whole cell in situ hybridization

Enrichment and isolation

Head et al. 1998
The Nobel Prize in Chemistry 1993 was awarded "for contributions to the developments of methods within DNA-based chemistry" jointly with one half to Kary B. Mullis "for his invention of the polymerase chain reaction (PCR) method" and with one half to Michael Smith "for his fundamental contributions to the establishment of oligonucleotide-based, site-directed mutagenesis and its development for protein studies".

Photos: Copyright © The Nobel Foundation
Polymerase Chain Reaction (PCR)

- Rapid, inexpensive and simple way of making millions of copies of a gene starting with very few copies

- Does not require the use of isotopes or toxic chemicals

- It involves preparing the sample DNA and a master mix with primers, followed by detecting reaction products
Polymerase Chain Reaction (PCR)

- Takes advantage of properties of Taq DNA polymerase to amplify (make copies of) a selected gene region

- Requirements
  - You must know the sequence flanking the region to be amplified
DNA Structure

• Directionality
  – each strand has 5’-3’ direction
  – Always grows from 3’ end
  – Never from 5’

• Antiparallel 2’ structure
  – Purines hybridize with pyrimidines

• Canonical base pairs
  – A-T, G-C
Every PCR contains:

- A DNA Polymerase (most common, Taq)
- Deoxynucleotide Triphosphates (A, C, T, G)
- Buffer (salt, MgCl$_2$, etc)
- A set of primers, one Forward, one Reverse
- Various chemicals to minimize inhibition
- Template DNA
Polymerase chain reaction - PCR

1. **Denaturation** at 94-96°C
2. **Annealing** at ~68°C
3. **Elongation** at ca. 72 °C
# Typical PCR Profile

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>95ºC</td>
<td>5 minutes</td>
<td>DNA Taq polymerase activation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35 cycles of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95ºC</td>
<td>1 minute</td>
<td>DNA denaturization</td>
</tr>
<tr>
<td>54ºC</td>
<td>1 minute</td>
<td>Primer annealing</td>
</tr>
<tr>
<td>72ºC</td>
<td>1 minute</td>
<td>Extension creation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72ºC</td>
<td>10 minutes</td>
<td>Final extension created</td>
</tr>
</tbody>
</table>
https://www.youtube.com/watch?v=hyDLYLGmBr8
Things you can optimize

• Temperature and time to activate Taq polymerase
• Temperature and time to allow primer annealing
• Temperature and time for extension
• Concentration of reagents, especially primers, dNTPs, and MgCl₂
• Concentration of template DNA
• Number of replication cycles
• Etc…
The Star of the Show: SSU rRNA

• Everybody has it

• Contains both highly conserved and variable regions
  - allows making comparisons between different organisms over long periods of time (evolutionary history)

• Not laterally transferred between organisms

• HUGE and growing database
Ribosomes

- Make proteins
- rRNA is transcribed from rDNA genes
SSU rRNA

Escherichia coli

Domain: Bacteria
Kingdom: Purple Bacteria
Order: gamma

July 3, 1995 v4.0 (JD1695)

http://www.rna.icmb.utexas.edu
Universal Tree of Life

BACTERIA

ARCHAEA

EUKARYA

You Are Here

Modified from Norman Pace
Beyond 16S rRNA: What is their functional potential?
Our 4 Targets

• 16S rRNA Bacteria

• 16S rRNA Archaea

• mcrA Methanogens
  – Methyl coenzyme M reductase

• dsrB Sulfate reducers
  – Dissimilatory bisulfite reductase
### What To Do

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1X (25 µl rxn)</th>
<th>X 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>5.5 µl</td>
<td></td>
</tr>
<tr>
<td>OneTaq 2X</td>
<td>12.5 µl</td>
<td></td>
</tr>
<tr>
<td>Master Mix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4% BSA</td>
<td>4 µl</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>22 µl</td>
<td></td>
</tr>
</tbody>
</table>
### What To Do

<table>
<thead>
<tr>
<th>Tube</th>
<th>Master mix</th>
<th>Target</th>
<th>Template</th>
<th>Vol</th>
<th>F primer</th>
<th>Vol</th>
<th>R primer</th>
<th>Vol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>Sulfate reducers</td>
<td>Sediment DNA</td>
<td>1</td>
<td>dsr1F</td>
<td>1</td>
<td>dsr4R</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>Methanogens</td>
<td>Sediment DNA</td>
<td>1</td>
<td>ME1</td>
<td>1</td>
<td>ME2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
<td>Bacteria</td>
<td>Sediment DNA</td>
<td>1</td>
<td>8F</td>
<td>1</td>
<td>1492R</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>22</td>
<td>Archaea</td>
<td>Sediment DNA</td>
<td>1</td>
<td>21F</td>
<td>1</td>
<td>958R</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>Archaea</td>
<td>+ control</td>
<td>1</td>
<td>21F</td>
<td>1</td>
<td>958R</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>22</td>
<td>Water</td>
<td>- control (water)</td>
<td>1</td>
<td>21F</td>
<td>1</td>
<td>958R</td>
<td>1</td>
</tr>
</tbody>
</table>