Of Mudwatts and Microbiomes

Phylogenetic structure of the prokaryotic domain: The primary kingdoms

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PNAS November 1, 1977. 74 (11) 5088-5090; https://doi.org/10.1073/pnas.74.11.5088

16S rRNA (1.5 kb)

Part of the ribosomal, a structural scaffold organizing ribosomal proteins of the small subunit of the ribosome. Ubiquitous – all living organisms have this. Composed of very well conserved (green) sequence and hypervariable regions (blue).
Traditional method to quantify is based on 16S rRNA (bacterial) or 18S rRNA (eukaryotic).

16/18S rRNA is ubiquitous – highly conserved and variable regions

- good primers designed; decades of analysis
- good reference databases of 16S rRNA sequences (RDP, GreenGenes)
- culture independent
- established bioinformatics toolkits available

Microbiome
Collection of all the micro-organisms that exist in an environment of interest

Some bacteria can be cultured, some are hard/impossible to culture or conditions are not known

Need analysis to be culture independent to be comprehensive

Use DNA primers to well conserved regions
Designed to amplify 16S in all/most bacteria

Sequence across variable regions
variable regions are genus or species specific
act as a barcode defining a species
Ribosome

Translates mRNAs to proteins

Bacterial Ribosome (70S)
Two subunits – 50S and 30S

50S subunit – 31 proteins
30S subunit – 21 proteins

S stands for Svedberg coefficient, an old way of measuring size of a protein or complex based on how far it sediments in an ultracentrifuge. Non-linear, depends on size and conformation.

Each are composed of several rRNAs and many proteins

23S (large subunit), 16S (small subunit), and 5S (large subunit) rRNAs

Eukaryotic Ribosome (80S)

60S subunit – 49 proteins
40S subunit – 33 proteins

28S (large subunit), 18S (small subunit), and 5.8S (large subunit) and 5S (large subunit) rRNAs
1 cycle
Denaturing 95C
Annealing 55-60C
Extenion 72C
Metagenomics based on whole genome sequencing

Metagenome: a collection of assembled genomes of different organisms present in an environment of interest

Advantages

- not limited to one specific locus (16S or 18S)
- major gaps in eukaryotic reference genomes (skewed to model systems, easily culturable/maintainable organisms and warm and fuzzy creatures)
- missing a lot of simpler eukaryotes (protists)
- possibility of assembling entirely unknown genomes
- not biased towards bacteria
- discovery – novel metabolic pathways/natural products

Figure 2

Science, 2004

Genome Res 28: 569
### Table 1. Experimental design for comparison of soil DNA extraction methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Soil pretreatment</th>
<th>Estimated time</th>
<th>DNA extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>0.5 g glass beads 0.5-mm diameter</td>
<td>4h</td>
<td>Modified by Sagova et al. (2008) from Miller et al. (1999)</td>
</tr>
<tr>
<td>SP</td>
<td>0.5 g glass beads 0.5-mm diameter</td>
<td>5h</td>
<td>Modified of Sagova et al. (2008)</td>
</tr>
<tr>
<td>S-CTAB</td>
<td>0.5 g glass beads 0.5-mm diameter and 0.5 g glass beads of 0.1mm diameter</td>
<td>5h</td>
<td>Modified of Sagova et al. (2008)</td>
</tr>
<tr>
<td>Gns-GII</td>
<td>0.5 g glass beads 0.5-mm diameter and 0.5 g glass beads of 0.1mm diameter</td>
<td>5h</td>
<td>Modified of Plassart et al. (2012)</td>
</tr>
<tr>
<td>N</td>
<td>Glass beads provided in the isolation kit</td>
<td>2h</td>
<td>Soil DNA Isolation Kit NorgenBiotech</td>
</tr>
</tbody>
</table>
PCR amplification of the 16S rRNA genes was successful with all DNA extracts, except SP protocol, but resulted in different relative intensity of PCR products (38 successful PCRs out of 50). The intensity of the amplification was higher when 3mg/ml BSA was added to PCR reactions (Figure 3).

![Agarose gel of 16S rDNA amplicon obtained using various procedures from soil samples (b lanes = +3mg/ml BSA): DNA Ladder λ/ EcoRI and HindIII (M); Soil DNA Isolation Kit NorgenBiotek (1, 1b); S (2, 2b); SP(3, 3b); S-CTAB (4, 4b); GnS-GII (5, 5b); P. aeruginosa PAO1 (6,6b); No DNA control (7, 7b).](image_url)

Fig. 3. Agarose gel of 16S rDNA amplicon obtained using various procedures from soil samples (b lanes = +3mg/ml BSA): DNA Ladder λ/ EcoRI and HindIII (M); Soil DNA Isolation Kit NorgenBiotek (1, 1b); S (2, 2b); SP(3, 3b); S-CTAB (4, 4b); GnS-GII (5, 5b); P. aeruginosa PAO1 (6,6b); No DNA control (7, 7b).

No PCR product of the negative control was detected and the intensity of PCR product for P. aeruginosa
DNA purification

Soil (0.5 g) was homogenized on a vortex mixer (S8A Stuart) at 2200 rpm for 5 min with 600 μl of extraction buffer (50 mM Na-phosphate buffer [pH 8], 50 mM NaCl, 500 mM Tris-HCl [pH 8], and 5% SDS) and 300 μl of phenol/chloroform–isoamyl alcohol (25:24:1 v/v) and 0.5 g sterile glass beads 0.5-mm diameter, for S and SP protocol.

Resulted lysate was centrifuged at 16000g for 2 minutes. The supernatant was mixed with the same volume of phenol-chloroform-isooamyl alcohol (25:24:1 v/v) and centrifuged at 6,000g for 5 min. The upper layer was transferred and mixed with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) and centrifuged at 16,000g for 5 min for S protocol, and prior to this step at SP protocol, we added a 100 μl proteinase K stock solution, and incubated for 30 min at 37°C. The supernatant was then precipitated with 1 volume of ice-cold isopropanol, and incubated at -20°C for 20 min.

S protocol worked best, and has the simplest components

Simple extraction buffer:
50 mM Na-phosphate buffer, pH 8
50 mM NaCl
500 mM Tris-HCl, pH 8
5% SDS

Modifications for us:
Use chloroform-isoamyl (24:1) only
The quality of DNA isolates was estimated by quantifying amplification of 16S rRNA genes using the universal primers specific for Eubacteria: GM3F (5’-AGAGTTTGATCMTGGC-3’) and GM4R reverse (5’-TACCTTGTTACGACTT-3’) according to Muyzer et al. (1995), using a 50°C annealing temperature on a Mini Cycler MJ Research.

PCR assays were carried out in a 30 µl reaction volume containing PCR Master Mix 2X (ThermoScientific), with and without 3 mg/ml Bovine Serum Albumin (BSA) and 50 µM of each primer, including a no template control and a positive control represented by chromosomal DNA from P. aeruginosa PAO1.

Template DNA concentration was determined and we used 25ng of crude DNA extract for each PCR reaction. Amplification products from 10 reactions for each procedure were checked for correct size (1500 bp) by 1% agarose gel electrophoresis.
Ribosomal Database Project

RDP Release 11, Update 5 :: September 30, 2016
3,356,809 16S rRNAs :: 125,325 Fungal 28S rRNAs
Find out what's new in RDP Release 11.5 here.

Cite RDP's latest tool articles.

RDP provides quality-controlled, aligned and annotated Bacterial and Archaeal 16S rRNA sequences, and Fungal 28S rRNA sequences, and a suite of analysis tools to the scientific community. New to RDP release 11:

- RDP tools have been updated to work with the new fungal 28S rRNA sequence collection.
- A new fungal 28S Aligner and updated Bacterial and Archaeal 16S Aligner. We optimized the parameters for these secondary-structure based homology aligners to provide improved handling for partial sequences.
- Updated RDPpipeline offers extended processing and analysis tools to process high-throughput sequencing data, including single-strand and paired-end reads.
- Most of the RDP tools are now available as open source packages for users to incorporate in their local workflow.

https://rdp.cme.msu.edu/index.jsp

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http://greengenes.secondgenome.com

https://github.com/rdpstaff

https://www.mothur.org

http://qiime.org
Considerations for Experimental Design

Where to sample?
Top
Bottom
Single or multiple locations

How, and how much to sample
Need to take a sterile core sample
Replicates and store one at -20°C
Multiple locations
Separate sequencing runs or pool samples

Eubacteria vs archaea primers

Time course
Initial establishment, every three months for a year?

Others???